A Circadian Clock- and PIF4-Mediated Double Coincidence Mechanism is Implicated in the Thermosensitive Photoperiodic Control of Plant Architectures in *Arabidopsis thaliana*

Yuichi Nomoto, Saori Kubozono, Miki Miyachi, Takafumi Yamashino*, Norihito Nakamichi and Takeshi Mizuno

Laboratory of Molecular and Functional Genomics, School of Agriculture, Nagoya University, Chikusa-ku, Nagoya, 464-8601 Japan

*Corresponding author: E-mail, yamasino@agr.nagoya-u.ac.jp; Fax, +81-52-789-4091.

(Received August 9, 2012; Accepted September 27, 2012)

In *Arabidopsis thaliana*, the circadian clock regulates diurnal and photoperiodic plant growth including the elongation of hypocotyls in a time-of-day-specific and short-day (SD)-specific manner. The clock-controlled PHYTOCHROME-INTERACTING FACTOR 4 (PIF4) encoding a basic helix–loop–helix (bHLH) transcription factor plays crucial roles in this regulation. PIF4 is transcribed precociously at the end of the night in SDs, under which conditions the protein product is stably accumulated, while PIF4 is expressed exclusively during the daytime in long days (LDs), under which conditions the protein product is degraded by light-activated phytochrome B. The dawn- and SD-specific elongation of hypocotyls is best explained by the coincident accumulation of the active PIF4 protein during the night-time before dawn specifically in SDs. However, this coincidence model was challenged with the recent finding that the elongation of hypocotyls is markedly promoted at high growth temperature (i.e. 28°C) even under LDs in a PIF4-dependent manner. Here, we reconciled these apparently conflicting facts by showing that the transcription of PIF4 occurs precociously at the end of the night-time at 28°C in LDs, similarly to in SDs. Both the events resulted in the same consequence, i.e. that a set of differential transcription of PIF4 target genes (ATHB2, GH3.5, IAA19, IAA29, BRox2, GAI, ACS8 and CKX5) was induced accordingly in a time-of-day-specific manner. Taken together, we propose an extended double coincidence mechanism, by which the two environmental cues (i.e. photoperiods and temperatures), both of which vary on a season to season basis, are integrated into the same clock- and PIF4-mediated output pathway and regulate a hormone signaling network to fit plant architectures properly to domestic habitats.

**Keywords:** *Arabidopsis thaliana* • Circadian clock • External coincidence • Hormone signaling • Photomorphogenesis • Temperature response.

Abbreviations: bHLH, basic helix–loop–helix; LDs, long day; phyB, phyB, phytochrome B; PIF4, PHYTOCHROME-INTERACTING FACTOR 4; qRT-PCR, quantitative real-time PCR; SD, short day; ZT, Zeitgeber time.

**Introduction**

The plant circadian clock generates rhythms with a period close to 24 h, and it controls a wide variety of physiological and developmental events (Hotta et al. 2007, de Montaigu et al. 2010). In *Arabidopsis thaliana*, the best-characterized clock-controlled output pathway is the so-called photoperiodic control of flowering time, in which the clock regulates the long-day (LD)-specific promotion of flowering (or transition from vegetative to reproductive growth) (Fornara et al. 2010, Imaizumi 2010). The second one is the diurnal and photoperiodic control of vegetative growth, including the short-day (SD)-specific promotion of the elongation of hypocotyls and petioles (Breton and Kay 2007, Nozue et al. 2007, Niwa et al. 2009, Kuniihro et al. 2011, Parre 2012). The underlying molecular mechanisms of these clock-controlled (or photoperiod-dependent) complementary outputs are explained by the ‘external coincidence model’ (Salome and McClung 2004, Imaizumi and Kay 2006).

Our series of recent studies made it possible to understand the photoperiodic control of vegetative plant growth, based on the following external coincidence model (Niwa et al. 2009, Kuniihro et al. 2011, Nomoto et al. 2012). Briefly, the phytochrome-interacting basic helix–loop–helix (bHLH) transcription factor PHYTOCHROME-INTERACTING FACTOR 4 (PIF4) plays a prominent role in the regulation of hypocotyl elongation during seedling growth (Quail 2002, Leivar and Quail 2011). The light-activated form of phytochrome B (phyB) interacts directly with PIF4 (Hug and Quail 2002, Khanna et al. 2004).
and this event results in rapid degradation of PIF4 in the light (Lorrain et al. 2008). The residual PIF4 proteins in the light are inactivated through the direct binding with DELLA proteins, which serve as repressors in the light–gibberellin signaling pathway (de Lucas et al. 2008). On the other hand, the transcription of PIF4 is under the control of the circadian clock so as to be expressed in the daytime and also at the end of night-time in SDs (Niwa et al. 2009), while its night-time expression tends to disappear in LDs. Through these phyB- and clock-dependent mechanisms, PIF4 is stably activated at the end of the night only in SDs. The stabilized PIF4 in SDs induces the transcription of a set of target genes to promote the elongation of hypocotyls in a time-of-day- (dawn) and photoperiod (SD)-dependent manner (Breton and Kay 2007, Nozue et al. 2007, Niwa et al. 2009). The model was further extended by identifying a set of hormone-associated genes specifically as the downstream targets of PIF4 and PIF5 (Nomoto et al. 2012).

Since plants are sessile, they must be able to sense changes in environmental conditions and adapt to them properly. Light and temperature are two particularly important environmental cues for plant growth on both a day to day and a season to season basis. In this respect, the results from a series of recent studies indicated that PIF4 is able to integrate both light and temperature cues to regulate a wide variety of physiological processes, including (i) skotomorphogenesis/photomorphogenesis during etiolation/de-etiolation (Lorrain et al. 2009, Leivar and Quail 2011); (ii) shade-appropriate photomorphogenesis (Lorrain et al. 2009, Hornitschek et al. 2012); (iii) photomorphogenesis in response to a low blue light intensity (Keller et al. 2011); (iv) morphogenesis in response to high temperatures (e.g. 28°C) (Koini et al. 2009, Franklin et al. 2011); and, surprisingly, (v) control of flowering in SDs at high temperatures (Kumar et al. 2012).

Considering these interesting facts (i–v) with regard to PIF4 functions, we needed to reassess the previously proposed PIF4-mediated coincidence mechanism underlying the photoperiodic control of plant growth. In particular, finding (iv) indicated that the elongation of hypocotyls and petioles is markedly promoted at high temperature (e.g. 28°C) even in LDs. This raised a serious objection against the principle of the external coincidence model, because the model claims that the activity and stability of PIF4 are regulated to be very low throughout daytime and night-time in LDs.

To reconcile the conflict, here we carried out a series of critical experiments to reassess the coincidence mechanism underlying the photoperiodic control of plant growth. Taking the results of this study together, we successfully refined and extended the clock- and PIF4-mediated external coincidence mechanism. We provide evidence to support a double coincidence model, by which two seasonally varying environmental cues (i.e. photoperiods and temperatures) are integrated into the same clock- and PIF4-mediated coincidence mechanism, which in turn coordinately regulates a hormone signaling network to fit plant morphologies properly to domestic habitats.

Results and Discussion

High temperature-regulated plant growth

Plant growth is regulated in response to photoperiod through the clock- and PIF4-mediated external coincidence mechanism in Arabidopsis (Nomoto et al. 2012). PIF5, the closest homolog of PIF4, is also involved in the control of hypocotyl elongation. However, it was shown that PIF4 plays a more prominent role than PIF5 in diurnal growth of hypocotyls in SDs (Nozue et al. 2007). Briefly, young seedlings grown in SDs (8 h light/16 h dark) at 22°C in white light show longer hypocotyls than those grown in LDs (16 h light/8 h dark), and this event is attenuated in the pif4 pif5 mutant seedlings (Fig. 1A). Interestingly, aberrantly high temperature (e.g. 28°C) affects this photoperiodic control of plant growth. When wild-type seedlings grown at 22°C for 3 d in LDs were transferred at Zeitgeber time (ZT) = 0 (dawn) to 28°C and grown for another 4 d in the same LD photoperiod conditions, the resulting seedlings showed longer hypocotyls than those grown continuously at 22°C, and this event was also dependent on PIF4/PIF5 (Fig. 1A). These responses to

Fig. 1 Photoperiodic control and thermoregulation of the elongation of hypocotyls and petioles. (A) Wild-type (WT) and pif4 pif5 mutant seedlings were grown in white light at 22°C in either LDs or SDs for 8 d for comparison between growth under LD and SD conditions. They were also grown in white light in LDs either at 22°C for 7 d, or at 22°C for 3 d followed by incubation at 28°C for 4 d for comparison between growth under 22 and 28°C conditions. Pictures were taken of representative plants to compare the lengths of hypocotyls with each other. (B) The length of hypocotyls in A was examined quantitatively (n > 9). (C) Phenotype of 20-day-old plants grown as in A. Pictures were taken to compare their morphologies, particularly the lengths of petioles, indicated by arrows.
photoperiods and temperatures were confirmed quantitatively (Fig. 1B). The above temperature-induced phenomenon is referred to as ‘thermoregulation of plant growth’ hereafter in order to distinguish it from ‘photoperiodic control of plant growth’. When the seedlings were grown at 28°C for a prolonged time, the resulting adult plants showed longer petioles than those grown continuously at 22°C (Fig. 1C). The observed phenotype of the thermoregulation of leaf morphology was also similar to those seen in photoperiodic control (Fig. 1C). The high temperature-induced elongation of hypocotyls and petioles was consistent with that reported previously (Koini et al. 2009, Stavang et al. 2009). These findings raised a serious objection against the previously proposed coincidence model underlying the photoperiodic control of plant growth, as pointed out earlier (see the Introduction). In this respect, one may argue that the previously proposed coincidence model holds only within a narrow ambient temperature range (or around 22°C). Hence, we reassessed the external coincidence model underlying the photoperiodic control of plant growth in this study.

High temperature cue affects the clock-controlled diurnal oscillation profile of PIF4 at the level of transcription

To gain an insight directly into this problem, the diurnal transcriptional oscillation profile of PIF4 was compared between wild-type seedlings grown at 22 and 28°C in LDs. As described above, seedlings were grown either at 22°C for 7 d, or at 22°C for 3 d followed by incubation at 28°C for 4 d. RNA samples were prepared at 3 h intervals, and subjected to quantitative real-time PCR (qRT-PCR) quantification. The diurnal oscillation profile of PIF4 was clearly changed when seedlings were grown at 28°C, as compared with that of seedlings grown continuously at 22°C (Fig. 2A). Namely, the PIF4 transcripts were accumulated precociously at the end of the night at 28°C. This phenomenon was less evident in the case of the PIF5 transcripts (Fig. 2B). We examined the diurnal expression profiles of PIF7 because PIF7 together with PIF4 plays crucial roles in shade avoidance, and both of them appear to regulate an overlapped set of target genes (Li et al. 2012). It was found that the expression of PIF7 also oscillated with a peak at the same phase as that of PIF4, but the diurnal expression profile of PIF7 was not affected under high temperature conditions of 28°C (Supplementary Fig. S1).

Because the new finding that PIF4 exhibited a precocious expression at the end of the night under high temperature conditions in LDs is critical for the proposed external coincidence mechanism, we confirmed the result by replicating the same experiment with biologically independent samples (Fig. 2C). These consistent results led us to remember the effect of SD photoperiods on the diurnal oscillation profile of PIF4 (Kunihiro et al. 2011), as shown here reproducibly (Fig. 2D). Namely, the treatment of seedlings with high temperature (e.g. 28°C) in LDs causes the same effect on the expression profile of PIF4 in SDs. When seedlings were grown under either SDs/22°C or LDs/28°C, the PIF4 transcripts were precociously accumulated at the end of the night in a similar manner (compare Fig. 2C with D). These results suggest that the thermoregulation of plant growth is also explained on the...
basis of the clock- and PIF4-mediated external coincidence mechanism.

The clock- and PIF4-mediated external coincidence model also holds in the case of thermoregulation of plant growth

The principal hallmark of the coincidence model for the photoperiodic control of plant growth is the concomitant induction of the PIF4 target genes (e.g. ATHB2) in the end of the night. If the high temperature-induced precocious expression of PIF4 at the end of the night was a main cause of the thermoregulation of plant growth, the ATHB2 gene should be expressed concomitantly in a time-of-day-specific manner at 28°C even in LDs in a PIF4-dependent manner. This was indeed shown in Fig. 2E and G. These results indicate the following scenario: high temperature affects the phase of PIF4 transcript rhythm so that the PIF4 expression (internal cue) could be coincident with the dark period (the external cue) even in LDs. As a result, the PIF4 protein is accumulated at the end of the night to induce the downstream target genes including ATHB2, which promote the elongation of hypocotyls. Therefore, the thermoregulation of plant growth could also be explained on the basis of the clock- and PIF4-mediated external coincidence mechanism, for which the coincidence between the internal cue (PIF4 rhythm) and the double external cues (temperature and photoperiod) is crucial.

A hormone signaling network is implicated in the thermoregulation of plant growth

It was previously demonstrated that a global regulation of the hormone signaling network through the PIF4-mediated coincidence mechanism is important for the photoperiodic control of plant growth. These PIF4 target and hormone-associated genes are auxin-associated genes (GH3.5, IAA19 and IAA29), in addition to BR6ox2 (brassinosteroids), GAI (gibberellic acids), ACS8 (ethylene) and CKX5 (cytokinins) (Nomoto et al. 2012). If the coincidence model is applicable to the thermoregulation of plant growth, these hormone-associated genes should also be expressed under high temperature conditions in LDs in a time-of-day-specific manner. To address this issue, the auxin-associated IAA29 gene was tested first, by employing the appropriate negative reference TAA1 gene, which is responsible for the biosynthesis of auxin. Indeed, the auxin-associated IAA29 gene was induced significantly at both 22 and 28°C in a PIF4-independent manner (Fig. 3A, C), but TAA1 was expressed constantly at both 22 and 28°C in a PIF4-dependent manner (Fig. 3B, D). Other hormone-associated genes were also induced at the end of the night at 28°C, although the characteristic was less clear in the case of BR6ox2 (Supplementary Fig. S2). These results were consistent with the idea that the thermoregulation of plant growth is explained by extending the clock- and PIF4-mediated coincidence model, which has been proposed to understand the mechanism underlying the photoperiodic control of plant growth.

An additional PIF4 target implicated in the thermoregulation of plant growth

It was found that XTH15 (also known as XTR7) encoding a xyloglucan endotransglycosylase is also a downstream target of the PIF4-mediated photoperiodic control of plant growth (Nomoto et al. 2012). If the pathway of thermoregulation of plant growth overlaps with that of photoperiodic control of plant growth, XTH15 must also be induced at the end of the night under the high temperature conditions in LDs in a PIF4-dependent manner. The expected results were obtained for XTH15 (Supplementary Fig. S3).

phyB and thermoregulation of plant growth

The phyB photoreceptor plays a crucial role in the clock- and PIF4-mediated external coincidence mechanism (Breton and Kay 2007). Hence, the thermoregulation of hypocotyl elongation was examined for phyB mutant seedlings, considering the fact that the light-activated phyB leads to a rapid degradation of PIF4 at the protein level. It was found that the phyB mutant phenotype of long hypocotyls was further exaggerated at 28°C (Fig. 4). This suggests that the phyB photoreceptor is not directly implicated in the integration of the temperature cue. If this view is correct, the markedly enhanced elongation of
hypocotyls in the phyB mutant seedlings at 28°C should be explained by an enhanced transcription of PIF4 and its target genes at the end of the night. To address this issue at the molecular level, the diurnal expression profile of PIF4 was examined for phyB mutant seedlings grown at 28°C in LDs. The precocious expression of PIF4 in the night-time observed in the phyB mutant was further enhanced under high temperature conditions, indicating that high temperature affects the circadian rhythm of PIF4 mRNA independently of phyB signaling (Fig. 5A). Concomitantly, the expression of PIF4 target genes (e.g. ATHB2, IAA19 and IAA29) was accelerated at the end of the night at 28°C (Fig. 5B–5D). Therefore, we concluded that the photoreceptor (at least phyB) is not responsible for the perception of the temperature cue. Here, it should also be noted that the stability of PIF4 in seedlings grown at 28°C is the same as that grown at 22°C (Stavang et al. 2009), and that PIF4 is rapidly degraded in the light to the same extent at both 22 and 28°C (Kumar et al. 2012). Taken together, it was suggested that the circadian clock, rather than the phyB photoreceptor, is responsible for the integration of the temperature cue that results in the coincident expression of PIF4 and its target genes in the dark at 28°C, as further addressed below.

**Circadian clock and thermoregulation of plant growth**

In the above section, we provided evidence that the thermoregulation of plant growth is mediated through the effect of high temperature mainly on the clock-controlled diurnal oscillation of PIF4 at the level of transcription. To gain further insight into this view, we examined the thermoregulation of hypocotyl elongation by employing a set of clock-defective mutants, including a prr9 prr7 prr5 triple mutant and a mutant of ELF3, the product of which is a component of the transcriptional repressor complex for PIF4 (Nusinow et al. 2011). It was observed that the mutant phenotypes of long hypocotyls were somewhat exaggerated at 28°C. However, the responsiveness of the clock mutants to high temperature was lower than that of the phyB mutant (Fig. 6; Supplementary Fig. S4). This is compatible with the idea that the thermoregulation of hypocotyl elongation is mediated mainly through the clock-controlled regulation of PIF4 expression.

For an examination at the molecular level, the thermoregulation of PIF4 was examined by employing the elf3 clock mutant (Fig. 6). In contrast to the wild type, PIF4 was expressed mainly during the night at both 22 and 28°C in elf3. Consequently, the PIF4 target genes (e.g. ATHB2, IAA29, GAI, ACS8 and CKX5) were expressed during the night, regardless of the ambient temperatures. In other words, a temperature-independent expression of the PIF4 targets in the dark was observed in elf3. These findings at the molecular level are consistent with the observed phenotype of elf3, in which the thermoregulation of hypocotyl elongation was attenuated, if not completely (Fig. 4). These results further supported the idea that the thermoregulation of plant growth is explained on the basis of the clock- and PIF4-mediated coincidence model.
Effect of high temperature on the clock function

In general, the effect of high temperature on the clock-controlled diurnal oscillation of a given gene has not been elucidated. The findings of this study pointed out that high temperature affects the clock-regulated oscillation profile of PIF4. To gain an insight into this issue, we designed the following experiments. Considering the fact that the PIF4 transcripts show a robust peak at noon in LDs, wild-type seedlings were entrained at 22°C in LDs for 7 d, and mRNA samples were prepared at 3 h intervals. The diurnal expression profiles of the PIF4 (A, D), ATHB2 (B, E), IAA29 (C, F), GAI (G), ACS8 (H) and CKX5 (I) genes were examined by means of qRT-PCR analyses. Relative expression levels are shown as mean values ± SD (n = 3), for which the maximum value was taken as 1.0. The dark periods are indicated by shading.

Effect of high temperature on the clock function

In general, the effect of high temperature on the clock-controlled diurnal oscillation of a given gene has not been elucidated. The findings of this study pointed out that high temperature affects the clock-regulated oscillation profile of PIF4. To gain an insight into this issue, we designed the following experiments. Considering the fact that the PIF4 transcripts show a robust peak at noon in LDs, wild-type seedlings were entrained at 22°C in LDs for 7 d, and then, 3 h after the dark period had started (i.e. at ZT19) on the eighth day, the growth temperature was suddenly increased to 28°C. The same treatment was also done 3 h after light (i.e. at ZT = 3, the 15 h time point), and the expression profiles of the gene indicated were followed at 1 h intervals. (B–G) The response of PIF4 and the PIF4 target genes to high temperature. The transcriptional responses of the PIF4 (B), ATHB2 (C), IAA29 (D), GAI (E), ACS8 (F) and CKX5 (G) genes to temperature shift from 22 to 28°C were examined by means of qRT-PCR analyses. Relative expression levels are shown as mean values ± SD (n = 3), for which the value at the time 7 h was taken as 1.0. The dark periods are indicated by shading.

Proposition of a double coincidence mechanism in response to distinct external cues, photoperiod and high temperature

Based on the experimental evidence presented above, here we would like tentatively to propose an extended external
accumulate stably in the dark to induce its downstream targets including a set of hormone-associated genes that regulate plant growth in response to varied photoperiods. This is the original external coincidence model to explain SD-specific induction of the PIF4 target genes. In this study, it was demonstrated that high temperature (e.g. 28°C) causes the same effect on PIF4 expression through affecting the clock function even in LDs (Fig. 8C). This is the newly proposed external coincidence model to explain high temperature-dependent induction of the PIF4 target genes. As a consequence, the seedlings grown in LDs at 28°C show similar morphologies with elongated hypocotyls and petioles to those grown in SDs at 22°C (Fig. 1).

**Evidence for a double coincidence mechanism in response to distinct external cues: photoperiod and high temperature**

If these coincidence models were both correct, and photoperiod and high temperature affect the diurnal expression profile of PIF4 independently through acting on the circadian clock, one would expect to see an additive effect on the diurnal expression profile of PIF4 transcripts when seedlings were grown at 28°C in SDs. This was indeed the case. Expression of PIF4 in the dark period was additively enhanced by both high temperature and SD conditions, as shown in Fig. 8D. Also, one would expect to see an additive morphological effect on the length of hypocotyls when seedlings were grown under the same condition. Consistently, the seedlings grown at 28°C in SDs showed much longer hypocotyls than those grown at 22°C in SDs in a PIF4-dependent manner (Fig. 8E). At the molecular level, the enhanced PIF4 expression during the night at 28°C in SDs should also result in the coincident expression of the PIF4 target genes. This was shown for CKX5 (Fig. 8D), ATHB2, and IAA29 (Supplementary Fig. S5). The enhanced expression of these genes at 28°C in SDs was abolished in pif4 pif5 mutant seedlings (Supplementary Fig. S6). These results nicely fit the double coincidence model, in which two distinct external cues (photoperiod and ambient temperature) independently affect the internal clock-controlled oscillation of PIF4, thereby resulting in the orchestrated regulation of a set of hormone-associated genes, which coordinately control plant growth in response to both photoperiod and ambient temperature. The concept newly proposed in this study is schematically illustrated in Fig. 9).

**Implication**

It was suggested previously that high temperature regulates the elongation of hypocotyls through PIF4 by somehow affecting a network of signaling pathways involving auxin, brassinosteroids and gibberellic acids (Stavang et al. 2009). It was also suggested recently that there is a direct molecular link among PIF4–auxin–hypocotyl elongation at high temperatures (Franklin et al. 2011). However, the underlying molecular mechanism remained to be clarified in detail. In this study, a novel double coincidence model was proposed, by which two
seasonally varied environmental cues (i.e. photoperiods and temperatures) are integrated through the same clock-regulated PIF4 (and/or PIF5)-mediated output pathway, which in turn coordinately regulates a hormone signaling network to regulate the elongation of hypocotyls and petioles (Fig. 9). Furthermore, it was demonstrated that the clock-controlled PIF4 transcription factor orchestrates not only an auxin signaling pathway but also a variety of hormone signaling pathways to regulate the morphogenesis in *A. thaliana*.

Since plants are sessile, they must be able to sense changes in environmental conditions and adapt their development accordingly. In this respect, light and temperature are two particularly important environmental cues for plant growth. However, the characteristics of ambient temperature are principally different from those of photoperiod, because the former varies on a time to time, a day to day, a season to season, whereas the latter is much more constant in the sense that photoperiod varies only a season to season and a latitude to latitude basis. During plant evolution, they would need to acquire sophisticated mechanisms to adapt to ever-changing ambient temperature to accomplish the lateral and/or horizontal drift of domestication at the same latitude, where temperature keeps changing more radically than does photoperiod. Theoretically, one can envisage at least two ways for such an adaptive evolution. One is the invention of two completely independent mechanisms to adapt to the changes in photoperiod and temperature, respectively, in order to regulate the same biological events including the elongation of hypocotyls and petioles. The other is the so-called co-optional evolution, which adopts the same principle of the molecular mechanism for an apparently distinct but closely related purpose. In general, we know that life frequently has adopted such co-optional evolution. The double coincidence mechanism proposed in this study might be a good example of co-optional evolution. Through perceiving two distinct environmental cues (i.e. photoperiod and temperature) by the circadian clock, they are integrated into the same clock-regulated PIF4 pathway, which in turn coordinately regulates a hormone signaling network to fit plant morphologies to natural domestic habitats properly.

In this study, we investigated whether the external coincidence mechanism underlying SD-specific plant growth is applicable to the adaptive response to high ambient temperature. It should thus be noted that we do not know the molecular mechanism by which plant morphologies are modified in response to low temperature, although it is known that moderate decreases in ambient temperature (e.g. from 22 to 15°C) modulate plant morphologies during vegetative growth (Sidaway-Lee et al. 2010). It should also be mentioned that we do not know how high temperature affects the clock function per se so as to affect the rhythmic profile of PIF4, although we demonstrated that high temperature and photoperiod independently affect the clock function (Figs. 8, 9). These will be the interesting future subjects from the viewpoint of molecular biology of the plant clock.

### Materials and Methods

**Plant lines and growth conditions**

*Arabidopsis thaliana* Col-0 was used as the wild type in this study. The *prr9-10 prr7-11 prr5-11 phyB-9* and *pif4-101 pif5-1* mutants were described previously (Niwa et al. 2009). The *elf3-8* mutant (Hicks et al. 2001) was obtained from the Arabidopsis Biological Resource Center (ABRC). The growth conditions of these plants were also described previously (Niwa et al. 2009). Briefly, seeds were sown on gellan gum plates containing Murashige and Skoog (MS) salts with sucrose (1%) and kept at 4°C for 48 h in the dark. After the seeds were exposed to white light for 3 h to enhance germination, they were kept at 22°C for 21 h in the dark. They were then incubated in a growth chamber in white fluorescent light (70 μmol m–2 s–1) at 22 or 28°C under LD (16 h light/8 h dark) or SD (8 h light/16 h dark) conditions.

**Preparation of RNA and qRT-PCR.**

Total RNA was purified from frost plant materials (the aerial part of 7-day-old seedlings) with an RNeasy plant mini kit (Qiagen). To synthesize cDNA, RNA (1 μg of each) was converted to 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, analyzed by using a Stepone Plus™ Real-Time PCR System (Life Technologies). The primer sets used are described 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.

Supplementary data

Supplementary data are available at PCP online.

Funding

This study was supported by the Japan Society of the Promotion of Science [No. 23580133 and No. 23012018 to T.Y., No. 20370018 to T.M.]; a GCOE program in Nagoya University [Advanced Systems-Biology].

Acknowledgments

We thank Dr. S. Prat (Campus University, Spain) for providing Arabidopsis pif4 pif5 double mutant seeds.

References


