Plant Signaling & Behavior 8:3, e23390; March 2013; © 2013 Landes Bioscience

Verification at the protein level of the PIF4-mediated external coincidence model for the temperature-adaptive photoperiodic control of plant growth in *Arabidopsis thaliana*

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Keywords: *arabidopsis thaliana*, circadian clock, external coincidence model, light signaling, photomorphogenesis

Abbreviations: Arabidopsis thaliana homeobox protein 2; bHLH, basic helix-loop-helix; GA, gibberellin; HA, Human influenza hemagglutinin epitope-tag; IAA29, Indole-3-Acetic acid inducible 29; LDs, long days; PIF4, Phytochrom-interchanging factor 4; qRT-PCR, quantitative real-time PCR; SDs, short days

Plant circadian clock controls a wide variety of physiological and developmental events, which include the short-days (SDs)-specific promotion of the elongation of hypocotyls during de-etiolation and also the elongation of petioles during vegetative growth. In *A. thaliana*, the *PIF4* gene encoding a phytochrome-interacting basic helix-loop-helix (bHLH) transcription factor plays crucial roles in this photoperiodic control of plant growth. According to the proposed external coincidence model, the *PIF4* gene is transcribed precociously at the end of night specifically 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 the light-activated phyB and also the residual proteins are inactivated by the DELLA family of proteins. A number of previous reports provided solid evidence to support this coincidence model mainly at the transcriptional level of the *PIF4* and PIF4-traget genes. Nevertheless, the diurnal oscillation profiles of PIF4 proteins, which were postulated to be dependent on photoperiod and ambient temperature, have not yet been demonstrated. Here we present such crucial evidence on PIF4 protein level to further support the external coincidence model underlying the temperature-adaptive photoperiodic control of plant growth in *A. thaliana*.

Plant circadian clock generates biological rhythms with a period close to 24 h, and it controls a wide variety of physiological and developmental events.[1,2] In *Arabidopsis thaliana*, the best-characterized clock-controlled output pathway is the photoperiodic control of flowering time, in which the clock regulates the long-days (LDs)-specific promotion of reproductive transition.[3,4] The second one is the photoperiodic control of vegetative growth, including the short-days (SDs)-specific promotion of the elongation of hypocotyls during de-ethiolation.[5,6] The underlying molecular mechanisms of these cock-controlled (or photoperiod-dependent) output pathways are commonly explained by external coincidence models. With regard to the photoperiodic control of plant growth, we recently proposed the clock and PHYTOCHROME-INTERACTING FACTOR 4 (PIF4)-mediated external coincidence model, as briefly introduced below.[6-10]

The circadian clock regulates the photoperiodic plant growth in an SDs-specific manner, in which the clock-controlled *PIF4* gene encoding a phyB-interacting basic helix-loop-helix (bHLH) transcription factor plays crucial roles (**Fig. 1A**).[11-16] As schematically shown in **Figure 1B**, *PIF4* is transcribed precociously at the end of night in SDs, under which conditions the active protein product is stably accumulated,[6,8] while *PIF4* is expressed exclusively during daytime in long days (LDs), under which conditions the protein product is degraded by the light-activated phyB,[15] and also the residual proteins are inactivated through binding with the DELLA family of proteins,[16] which redundantly serve as repressors in the gibberellin (GA) signaling pathway.[17] Based on the fact that PIF4 functions as a positive regulator for the elongation of hypocotyls, the SDs-specific elongation of hypocotyls is explained by the coincident accumulation of the active

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Submitted: 12/06/12; Revised: 12/21/12; Accepted: 12/21/12
http://dx.doi.org/10.4161/psb.23390
Citation: Yamashiro T, Nomoto Y, Lorrain S, Miyachi M, Ito S, Nakamichi N, Fankhauser C, Mizuno T. Verification at the protein level of the PIF4-mediated external coincidence model for the temperature-adaptive photoperiodic control of plant growth in *Arabidopsis thaliana*. Plant Signal Behav 2013; 8: e23390.

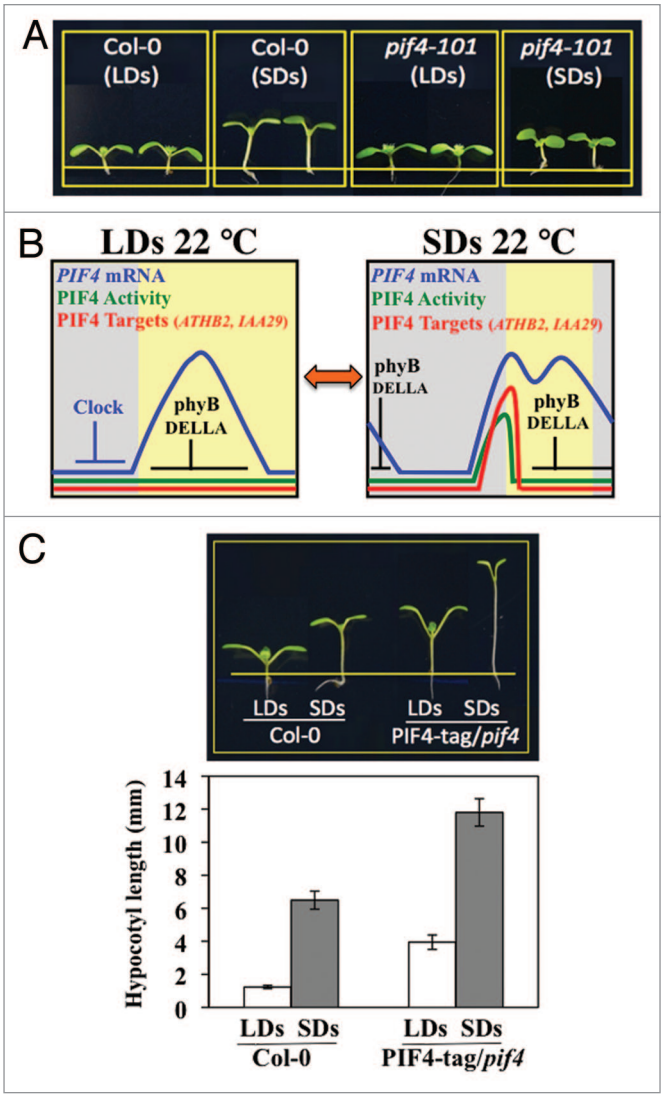

Figure 1. Essence of the external coincidence model underlying the clock and PIF4-mediated photoperiodic control of the elongation of hypocotyls. (**A**) PIF4-dependent and SDs-specific elongation of hypocotyls. Wild-type (Col-0) and *pif4–101* mutant seedlings were grown for 8 d in either LDs (16 h light/8 h dark) or SDs (8 h light/16h dark) on gellan gum plates containing MS salts with 1% sucrose, as described previously.[6] Pictures were taken for representatives to compare the lengths of hypocotyls, as indicated. (**B**) A schematic representation of the proposed external coincidence model, by which the mechanism underlying the clock and PIF4-mediated photoperiodic control of the elongation of hypocotyls is explained. Details were given in the text.[6-8] (**C**) Evaluation of the PIF4-tag/*pif4* transgenic line with special reference to the photoperiodic control of hypocotyl elongation. The experiments were performed, as described in (**A**). Pictures were taken for representatives to compare the lengths of hypocotyls, as indicated. Length of hypocotyls was also shown as mean values ± SD (n ≥ 10).

PIF4 proteins before dawn specifically in SDs.[6,7] This event is followed by the coordinate induction of a set of PIF4-target genes, such as *ARABIDOPSIS THALIANA HOMEOBOX PROTEIN 2* (*ATHB2*) encoding a homeodomain-leucine zipper protein, and *INDOLE-3-ACETIC ACID INDUCIBLE 29* (*IAA29*) encoding an auxin signaling-associated factor.[7,8] As will be discussed later, the circadian clock and PIF4-mediated external coincidence mechanism coordinately integrates both of the cues from seasonal changes in photoperiod and temperature to regulate plant growth in *Arabidopsis thaliana*.[9,10]

The Issue Addressed in this Study

A number of reports from our and other groups have provided evidence to support this coincidence model mainly at the transcriptional level of the *PIF4* and PIF4-target genes.[5-10,18-20] Nevertheless, the postulated photoperiod-dependent alteration of the PIF4 protein-profile has not yet been demonstrated, because of the lack of proper means to detect the photoperiod-dependent diurnal oscillation profile of PIF4 proteins. Here we employed an Arabidopsis transgenic line, which carries a transgene consisting of the *PIF4* promoter followed by the *PIF4*-citrine-hemagglutinin epitope tag (HA) coding sequence. The transgenic line was established by introducing the *PIF4* promoter-*PIF4*-citrine-HA composite gene into *pif4-101* null mutant background (the detailed procedures of construction will be described elsewhere, Lorrain and Fankhauser), and was named PIF4pro::PIF4-citrine-HA/*pif4-101* (line #2). Hereafter, the name will be abbreviated as PIF4-tag/*pif4* for clarity in this text. The new transgenic line was designed to properly detect cellular content of PIF4 proteins. Taking advantage of this material, here we would like to verify the proposed circadian clock and PIF4-dependent external coincidence model at the level of PIF4 proteins.

Phenotypic Evaluation of the Transgenic Line with Regard to the Photoperiodic Control of Plant Growth

For the first step, the phenotype of the transgenic line was examined with reference to the elongation of hypocotyls to make sure that the transgenic plants display the proper nature with regard to the photoperiodic control of plant growth (**Fig. 1C**). Seedlings (Col-0 and PIF4-tag/*pif4*) were grown under both the LDs and SDs conditions, as described previously.[6-8] The transgenic seedlings displayed the SDs-promoted elongation of hypocotyls, although the transgene appeared to over-complement the *pif4* mutation, suggesting that the transgenic line might produce a slightly excess amount of PIF4-tag proteins as compared with the reference strain (Col-0). However, the transgenic line is suitable enough for our purpose of this study in the sense that the phenomenon of the photoperiodic control of hypocotyls elongation is clearly reproduced (**Fig. 1C**).

Molecular Essence of the Clock and PIF4-Mediated Photoperiodic Control of Plant Growth and Critical Preconditioning of this Study

As the next step, the transgenic line was examined more critically with regard to the external coincidence model at the molecular level. The PIF4-tag/*pif4* transgenic plants together with Col-0 were grown in LDs and SDs for 8 d, and RNA samples were prepared at every 3 h interval, as described previously.[6-8] The diurnal expression profiles were examined for *PIF4*, together

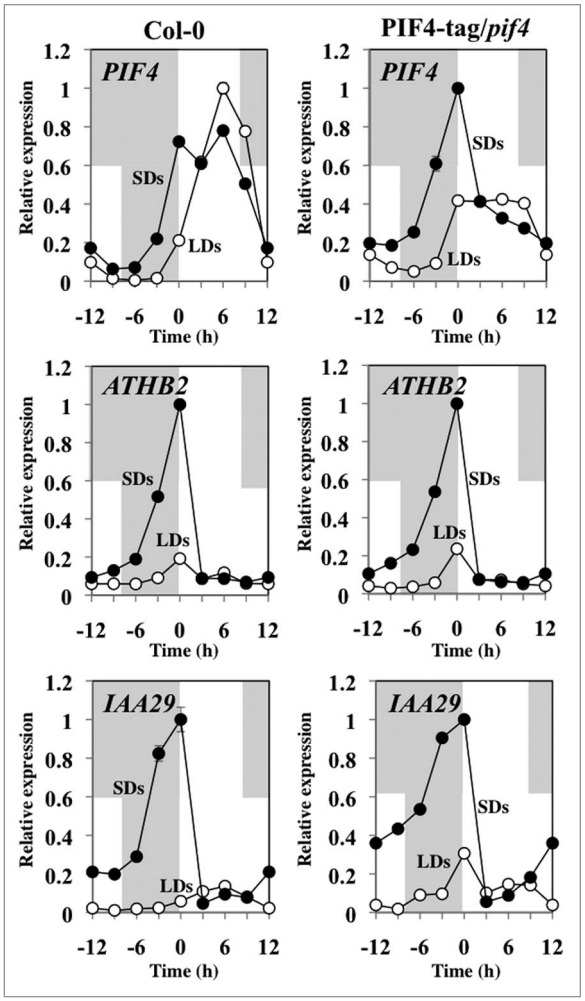

Figure 2. Characterization of diurnal expression profiles of *PIF4*, *ATHB2* and *IAA29*. Wild-type (Col-0) and PIF4-tag/*pif4* transgenic seedlings were grown in LDs and SDs for 8 d, and mRNA samples were prepared at every 3 h interval, as described previously.[6-8] The resultant diurnal expression profiles were examined by means of quantitative real-time PCR (qRT-PCR) analyses. The experimental conditions and primers used were described in the previous papers.[6-8] Note that we could not detect any *PIF4*-derived PCR products in *pif4–101* plants with the primers used, indicating that the *PIF4*-tag-derived PCR products were specifically detected. Relative expression levels were shown as mean values ± SD (n = 3), for which the maximum value was taken as 1.0. The dark periods were indicated with shadings.

with *ATHB2* and *IAA29* (*i.e.*, the representatives of PIF4-targets) (**Fig. 2**). As introduced in the earlier section (see **Fig. 1B**), the SDs-specific precocious expression of *PIF4* at the end of night was observed for Col-0 (**Fig. 2**, left upper panel). This resulted in the SDs- and dawn-specific inductions of *ATHB2* and *IAA29* (**Fig. 2**, middle and lower panels, respectively). The transcriptions of *ATHB2* and *IAA29* were hardly detected in the daytime, albeit a significant amount of *PIF4* transcripts were present. These are consistent with the proposed scenario that the PIF4 protein-products are degraded by the light-activated phyB, and also the remaining PIF4 proteins are inactivated by the DELLA family of proteins. These are the molecular essence

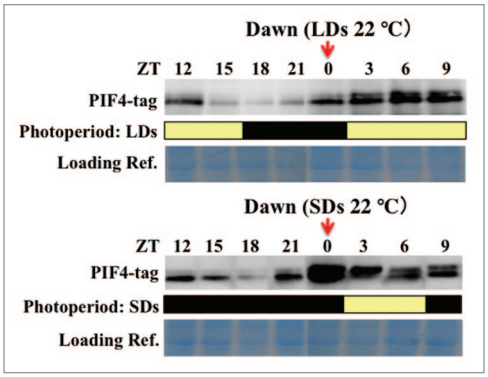

Figure 3. Characterization of diurnal expression profiles of PIF4-tag proteins. PIF4-tag/*pif4* transgenic seedlings were grown in LDs and SDs for 10 d, and whole plant samples (50 mg) were prepared at every 3 h interval, as indicated. Proteins were extracted from homogenized frozen powder of each sample with 100 mM TRIS-HCl (pH 8), 50 mM EDTA, 0.25 M NaCl, 0.7% SDS and 1mM DTT and immediately heated at 65°C for 10 min. 15 μl of the extract was added to SDS-PAGE sample buffer, and the total proteins were separated on 10% SDS-PAGE gels and transferred onto nitrocellulose membrane. The PIF4-tag products were detected by the Anti-HA monoclonal antibody (3F10) conjugated with peroxidase (Roche). The yellow and black rectangles correspond to light and dark periods, respectively, indicating the photoperiodic conditions schematically. Red arrows point out samples of ZT = 0 in order to demonstrate that a large amount of PIF4-tag proteins was detected at the end of night (denoted by Dawn) in the seedlings grown in SDs, as compared with those grown in LDs.

of the external coincidence model, which explain the mechanism underlying the photoperiodic control of plant growth (see **Fig. 1B**). Essentially the same molecular events were observed for PIF4-tag/*pif4* (**Fig. 2**, right panels). A greatly enhanced expression of *PIF4*, together with those of *ATHB2* and *IAA29*, was observed before dawn in an SDs-specific manner. In PIF4-tag/*pif4* plants, however, a significant level of *PIF4* expression was observed at the end of night even in LDs, most likely due to a slightly enhanced basal level expression of the transgene, which is consistent with the phenotype of the transgenic line (**Fig. 1C**). Taken these results together (**Figs. 1 and 2**), we concluded that the transgenic line is proper enough to study the diurnal expression level of PIF4 proteins with reference to the external coincidence mechanism, although we need to pay attention to the fact that the transgenic line tends to produce a slightly excess amount of PIF4-tag proteins as compared with the wild-type.

Verification of the Proposed External Coincidence Model at the Level of PIF4 Proteins

Then, we examined the diurnal expression profiles of PIF4-tag proteins in the transgenic plants grown in both LDs and SDs (**Fig. 3**). The results pointed out at least two events; (i) a large amount of PIF4-tag proteins was stably accumulated predominantly at the end of night in SDs, as compared with LDs (see the red arrows); (ii) a significant amount of PIF4-tag proteins was detected even in daytime in both SDs and LDs, although they were degraded along with the decrease in the PIF4 transcripts.

These events at the level of PIF4-tag proteins were essentially consistent with the proposed external coincidence model. However, they also pointed out the following new aspect. The light-activated phyB could not completely wipe off the PIF4-tag proteins during the daytime, although the proteins were eventually degraded to a basal level. This might be partly due to the fact that basal level expression of the *PIF4*-tag transgene is enhanced slightly, or might be due to the possibility that the addition of the citrine protein to the C-terminus of PIF4 results in stabilization of proteins. In any case, it was revealed that the remaining PIF4-tag proteins in daytime are largely inactivated so that they are unable to induce the transcriptions of the target-genes *ATHB2* and *IAA29* throughout daytime (see **Fig. 2**). The fact is indeed in good agreement with the idea in the proposed model that the DELLA family proteins do play a prominent role in the inactivation of PIF4 proteins during daytime, under which conditions the level of GA (antagonist of DELLA) is greatly reduced.[16,17]

Further Verification of the Proposed External Coincidence Model Underlying the PIF4-Mediated Temperature-Adaptive Photoperiodic Control of Plant Growth

The original 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 in LDs in a PIF4-dependent manner (**Fig. 4A**).[21,22] As reported previously,[9,10] however, we found that the clock and PIF4-mediated external coincidence mechanism integrates external cues not only from photoperiod but also from a wide range of ambient temperature (16°C to 28°C) into the regulation of plant architecture.[9,10] In other words, we have extended the model by showing that the transcription of *PIF4* occurs precociously at the end of nighttime at 28°C even in LDs, similarly at 22°C in SDs, as schematically shown in **Figure 4B** (compare with **Fig. 1B**). Furthermore, we showed that both the conditions (i.e., at 22°C in SDs and at 28°C in LDs) result in the same consequence that a set of PIF4-target genes (*i.e.*, *ATHB2* and *IAA29*) is induced accordingly in a time-of-day-specific manner.[9,10] Taken together, we previously proposed an extended double coincidence mechanism, by which the two environmental cues (*i.e.*, photoperiod and temperature) are integrated into the common clock and PIF4-mediated output pathway that regulates a hormone-signaling network to fit plant architectures properly to domestic habitats, where both photoperiod and temperature are ever-changing. To further address the issue with special reference to this temperature-adaptive external coincidence model, the PIF4-tag/*pif4* seedlings were grown at both 22°C and 28°C in LDs, as described previously (**Fig. 4A**).[9] Phenotypic observation showed that the transgenic line displayed high temperature induced elongation of hypocotyls in LDs. Then, we examined the diurnal expression profiles of PIF4-tag proteins in the transgenic plants grown under the same conditions (**Fig. 4C**). As observed at 22°C in SDs (**Fig. 3C**), a large amount of PIF4-tag proteins was stably accumulated predominantly at the end of night specifically at 28°C in LDs, but not at 22°C in LDs (see the red arrows). The results also strongly support the external coincidence mechanism underlying the temperature-adaptive photoperiodic control of plant growth.

Implication and Future Problems

As emphasized above, we had not pursued one of the crucial experiments in the series of our earlier studies on the clock and PIF4-medited photoperiodic control of plant growth, mainly because of lack of an appropriate way to detect the diurnal oscillation profile of PIF4 proteins. A widely-used transgenic line carrying a *35S-promoter::PIF4-tag* coding sequence is not proper for the evaluation of the diurnal expression profile of PIF4 proteins. Taking advantage of a new transgenic line carrying a *PIF4 native promoter::PIF4-tag* coding sequence, here we provided experimental evidence of photoperiod-dependent accumulation of PIF4 proteins at the end of night, which is postulated in the proposed external coincidence mechanism underlying the photoperiodic control of plant growth (**Fig. 3**). We also previously showed that this model holds under a wide range of ambient temperature conditions (16°C to 28°C).[9,10] To support this notion, we provided further evidence for the high temperature-dependent accumulation of PIF4 proteins at the end of night even in LDs (**Fig. 4**). Taken together, the results of this study strongly support that the circadian clock and PIF4-mediated external coincidence mechanism coordinately integrates both of the cues from seasonal changes in photoperiod and temperature to regulate plant growth in natural habitats.

The results of this study in turn raised a number of future problems. How is the diurnal oscillation profile of the *PIF4*-transcription regulated in a manner dependent on photoperiod? Is any known clock component (*e.g.*, the ELF4-ELF3-LUX evening complex that functions as a transcriptional repressor for *PIF4*) implicated in this regulation?[23] Similarly, how is the diurnal oscillation profile of the *PIF4*-transcription regulated in a manner dependent on ambient temperature? Can the circadian clock integrate both the cues of photoperiod and temperature coordinately in order to regulate *PIF4* at the level of transcription? The answers for these questions should shed new light on not only the specific aspect of photoperiodic control of plant growth, but also on the general aspects of plant circadian clock that has the fundamental abilities (referred to as "entrainment on temperature cycles" and "temperature compensation of period") to integrate both the environmental cues from light and temperature.

Disclosure of Potential Conflicts of Interests

There were no potential conflicts of interests to disclose.

Acknowledgments

This work was supported by Japan Society of the Promotion of Science (no. 23580133 and no. 23012018 to T.Y., no. 20370018 to T.M.).

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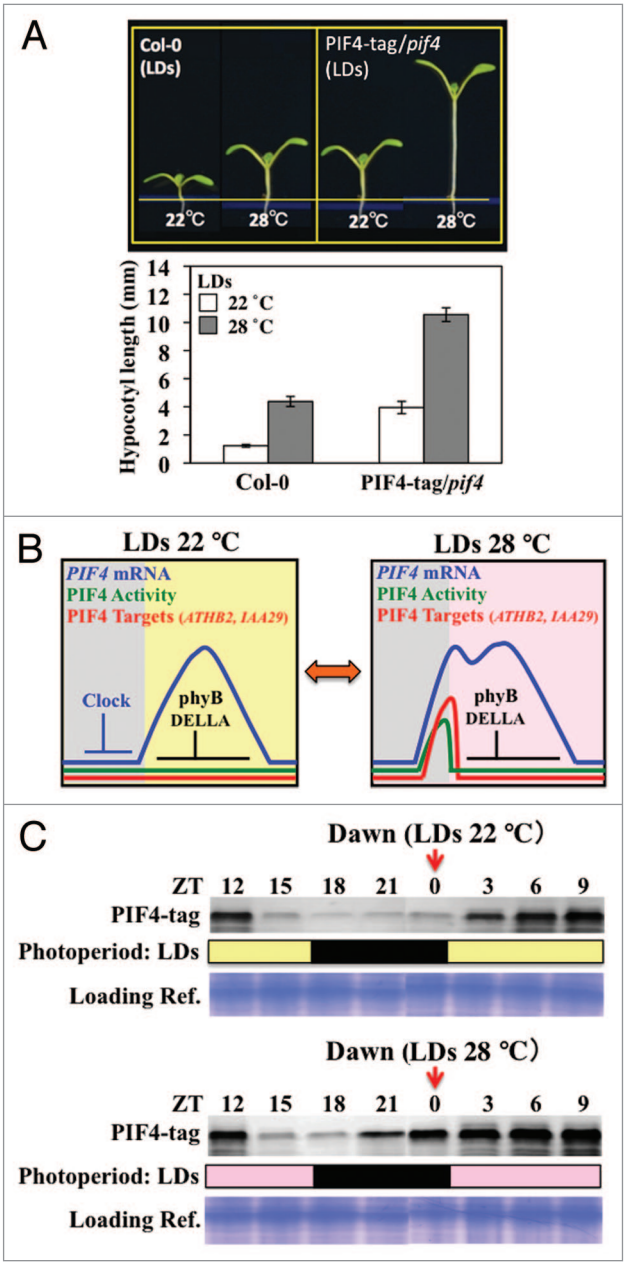

Figure 4. Characterization of diurnal expression profiles of PIF4-tag proteins with special reference to the temperature-adaptive photoperiodic control of hypocotyl elongation. (**A**) Wild-type (Col-0) and PIF4-tag/*pif4* transgenic seedlings were grown respectively at either 22°C or 28°C in LDs for 8 d. Pictures were taken for representatives to compare the lengths of hypocotyls, as indicated. Length of hypocotyls was also shown as mean values ± SD (n ≥ 10). (**B**) A schematic representation of the proposed external coincidence model, by which the mechanism underlying the temperature-adaptive photoperiodic control of the elongation of hypocotyls is explained. Details were given in the text.[6-8] (**C**) PIF4-tag/*pif4* transgenic seedlings were grown at either 22°C or 28°C in LDs for 10 d, respectively, and protein samples were prepared at every 3 h interval, as indicated. The PIF4-tag products were detected as **Figure 3**. Diurnal PIF4-tag protein expressions at 22°C (upper) and at 28°C (lower) were shown. Note that PIF4-tag proteins were detected on the same membrane for the samples of both 22°C and 28°C, and thus the detected amounts are compared relatively. Note also that the samples prepared at 22°C were biologically independent ones from those analyzed in **Figure 3**. The colored and black rectangles correspond to light and dark periods, respectively, indicating the photoperiodic conditions schematically. Red arrows point out samples of ZT = 0 in order to demonstrate that a large amount of PIF4-tag proteins was detected at the end of night (denoted by Dawn) in the seedlings grown at 28°C, as compared with those grown at 22°C.

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