PHYTOCHROME-INTERACTING FACTOR 4 and 5 (PIF4 and PIF5) Activate the Homeobox ATHB2 and Auxin-Inducible IAA29 Genes in the Coincidence Mechanism Underlying Photoperiodic Control of Plant Growth of Arabidopsis thaliana

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The plant circadian clock generates rhythms with a period close to 24 h, and it controls a wide variety of physiological and developmental events. Among clock-controlled developmental events, the best characterized is the photoperiodic control of flowering time, which is mediated through the CONSTANS (CO)–FLOWERING LOCUS T (FT) pathway in Arabidopsis thaliana. The clock also regulates the diurnal plant growth including the elongation of hypocotyls in a short day (SDs)-specific manner. In this mechanism, phytochromes (mainly phyB) and the PHYTOCHROME-INTERACTING FACTOR 4 (PIF4) and PIF5, encoding phytochrome-interacting basic helix–loop–helix (bHLH) transcription factors, play crucial roles. The time of day-specific and photoperiodic control of hypocotyl elongation is best explained by the accumulation of the PIF4 and PIF5 proteins during night-time before dawn, especially under SDs, due to coincidence between the internal (circadian rhythm) and external (photoperiod) time cues. However, the PIF4- and/or PIF5-controlled downstream factors have not yet been identified. Here, we provide evidence that ARABIDOPSIS THALIANA HOMEOBOX PROTEIN2 (ATHB2), together with auxin-inducible IAA29, is diurnally expressed with a peak at dawn under the control of PIF4 and PIF5 specifically in SDs. This coincidently expressed transcription factor serves as a positive regulator for the elongation of hypocotyls. The expression profiles of ATHB2 were markedly altered in certain clock and phytochrome mutants, all of which show anomalous phenotypes with regard to the photoperiodic control of hypocotyl elongation. Taken together, we propose that an external coincidence model involving the clock-controlled PIF4/PIF5–ATHB2 pathway is crucial for the diurnal and photoperiodic control of plant growth in A. thaliana.

Keywords: Arabidopsis thaliana • Circadian clock • Critical day length • External coincidence • Light signaling • Photomorphogenesis.

Abbreviations: ARF, auxin-responsive transcription factor; ATHB2, ARABIDOPSIS THALIANA HOMEOBOX PROTEIN 2; bHLH, basic helix–loop–helix; CCA1, CIRCADIAN CLOCK-ASSOCIATED 1; CO, CONSTANS; COP1, CONSTITUTIVE PHOTOMORPHOGENESIS 1; FT, FLOWERING LOCUS T; HA, hemagglutinin; HD-ZIP, homeodomain leucine-zipper; LD, long day; LHY, LATE ELONGATED HYPOCOTYL; phyB, phytochrome B; PIF, PHYTOCHROME-INTERACTING FACTOR; PMSF, phenylmethylsulfonyl fluoride; PRR, PSEUDO-RESPONSE REGULATOR; qRT-PCR, quantitative real-time PCR; SD, short day

Introduction

The plant circadian clock generates daily rhythms in the activity of many biological processes (Hotta et al. 2007, de Montaigu et al. 2010). During the last decade, significant progress has been made in Arabidopsis thaliana in defining the mechanisms by which clock-controlled output pathways are activated at specific seasons or times of the day or night. We know in detail how the circadian clock generates circadian rhythms (McClung 2006, McClung 2008); how the clock regulates long day (LD)-specific promotion of flowering (Yanovsky and Kay 2003, Imaizumi and Kay 2006, Fornara et al. 2010); and how the clock modulates night-enhanced rhythmic growth, including the diurnal elongation of hypocotyls in a short day (SD)-specific manner (Breton and Kay 2007, Nozue et al. 2007, Plant Physiol. 52(8): 1315–1329 (2011) doi:10.1093/pcp/pcr076, available online at www.pcp.oxfordjournals.org © The Author 2011. Published by Oxford University Press on behalf of Japanese Society of Plant Physiologists. All rights reserved. For permissions, please email: journals.permissions@oup.com
Niwa et al. 2009). These two examples of clock-controlled output pathways are conceptually termed ‘photoperiodic control’, and the underlying molecular mechanism is referred to as the ‘external coincidence model’ (de Montaigu et al. 2010).

The circadian clock can measure the daylength or photoperiodicity, and determines the flowering time (Imaizumi and Kay 2006, Fornara et al. 2010, Imaizumi 2010). The key player in the photoperiodic induction of flowering is the flowering time gene CONSTANS (CO) (Putterill et al. 1995, Wenkel et al. 2006). Transcription of CO is under the control of the circadian clock; it is expressed in both the daytime and night-time in LDs, while the daytime expression of CO tends to disappear in SDs (Yanovsky and Kay 2003, Sawa et al. 2007). Furthermore, the CO protein is stabilized particularly in the evening, since it is degraded through a phytochrome B (phyB)-dependent mechanism early in the day and a mechanism involving CONSTITUTIVE PHOTOMORPHOGENESIS1 (COP1) during the night. Consequently, CO promotes transcription of FLOWERING LOCUS T (FT) in the evening only in LDs (Jang et al. 2008). The resultant FT gene product moves from the leaf phloem to the shoot apical meristem to induce development of the floral meristem (Corbesier and Coupland 2006). As a result, the clock-controlled CO–FT pathway is activated due to coincidence between internal (circadian rhythm) and external (photoperiod) time cues to promote flowering specifically in LDs.

The circadian clock regulates not only the photoperiodic flowering time but also light-responsive seedling growth (Vandenbussche et al. 2005, Nozue and Maloof 2006, Alabadi and Blázquez 2009). During seedling growth, the red light photoreceptor phytochromes (mainly phyB) play a prominent role in the regulation of hypocotyl elongation (Quail 2002). The light-activated form of phyB interacts directly with the downstream target proteins PHYTOCHROME-INTERACTING FACTOR4 (PIF4) and PIF5 (Huq and Quail 2002, Khanna et al. 2004), which are homologous basic helix–loop–helix (bHLH) transcription factors that redundantly promote the elongation of hypocotyls (Fujimori et al. 2004, Duek and Fankhauser 2005, Monte et al. 2007). The interaction with phyB results in the degradation of PIF4 and PIF5 in light (Lorrain et al. 2008). On the other hand, transcription of PIF4 and PIF5 is under the control of the circadian clock so as to be expressed in both the daytime and night-time in SDs, while the light-time expression of PIF4 and PIF5 tends to disappear in LDs (Niwa et al. 2009). Through these phyB- and clock-dependent mechanisms, PIF4 and PIF5 are activated at the end of the night only in SDs, as schematically shown in Fig. 1, and the elongation of hypocotyls occurs accordingly (Breton and Kay 2007, Nozue et al. 2007). This regulation makes it possible for seedlings to grow in such a way that elongation of hypocotyls is accelerated especially in SDs due to coincidence between circadian clock-dependent internal and photoperiod-dependent external time cues (see the photograph denoted by wild-type (Col) in Fig. 1). This phenomenon is referred to as the photoperiodic control of hypocotyl elongation, in which PIF4 and PIF5 play crucial roles as positive regulators (see the photograph denoted by pif4 pif5 in Fig. 1). As explained above, the functions of PIF4 and PIF5 are regulated by the coordinate interplay between photoreceptor phyB and the circadian clock. Accordingly, certain loss-of-function mutants in clock-associated genes show an altered phenotype with regard to the elongation of hypocotyls (see the photograph denoted by prr9/7/5 in Fig. 1) as well as in phyB loss-of-function mutants (see the photograph denoted by phyb in Fig. 1).

In the model (Fig. 1) proposed previously, however, PIF4- and PIF5-activated downstream factors (Xs) that are involved in photoperiodic control of plant growth remain to be identified (Niwa et al. 2007). In this study, we attempt to identify such hypothetical factors, which positively regulate the elongation of hypocotyls in a manner dependent on photoperiod. Here, we provide evidence that ARABIDOPSIS THALIANA HOMEobox PROTEIN2 (ATHB2, also known as HAT4) serves as one of such coincidence-dependent factors that correspond to FT involved in the photoperiodic control of flowering time. Based on the results of this study, we propose that the clock–PIF4/5–ATHB2 pathway is crucial for the external coincidence model underlying the photoperiodic control of plant growth. This mechanism is similar to the clock–CO–FT photoperiod-dependent flowering pathway, as will be discussed. Also, evidence is presented that the auxin-responsive factor IAA29 plays a role in concert with ATHB2.
Results

Inspection of microarray databases to uncover factors involved in the photoperiodic control of hypocotyl elongation

According to the model in Fig. 1, transcription of the candidates for X should be activated directly or indirectly by PIF4 and/or PIF5 in the dark, and also they should be rapidly repressed in the light. Leivar et al. (2009) reported a set of data from their extensive microarray analyses on red light responses in gene expression by employing a quadruple pif1 pif3 pif4 pif5 mutant. They identified and classified many genes whose expression is regulated upon exposure to red light. Among those publicly available data sets, we particularly selected a family of Class 7 consisting of 56 genes, which are most probably positively regulated by PIFs in the dark, and rapidly down-regulated in the light (Leivar et al. 2009). In other words, they fulfill the minimal requirement for candidates for X. Among these 56 genes, we focused in particular on 18 genes, each of which encodes a putative transcription factor (Supplementary Table S1).

Identification of PIF4- and PIF5-dependent genes

It was inferred that the 18 selected transcription factors were induced at least either by PIF1, PIF3, PIF4 or PIF5. As noted above, PIF4 and PIF5 are critically relevant to the photoperiodic control of hypocotyl elongation, but PIF1 and PIF3 are not. Furthermore, the transcription of PIF4 and PIF5 is under the control of the circadian clock, but that of PIF1 and PIF3 is not (Yamashino et al. 2003). Hence, we examined these 18 candidates to determine whether their expression is predominantly dependent on PIF4 and PIF5. For that purpose, pif4 pif5 double loss-of-function mutant plants, together with wild-type (Col) plants, were grown for 14 d under 12 h light and 12 h dark cycles, and RNA samples were prepared at the end of the night, and also at 1 h after lights on. In most cases, their expression was dependent on PIF4 and/or PIF5 at least in part, and they were rapidly repressed in the light, as expected (Fig. 2).

Calculation of the percentage of light and PIF4/5 dependency revealed that these characteristics were particularly striking in the genes encoding bZIP52 (AT1G06850), IAA19 (AT3G15540), BBX23/DBB24 (AT4G10240), ATHB2 (AT4G16780), IAA29 (AT4G32280) and ATHB52 (AT5G53980), as compared with the others examined (both >70%; see Supplementary Table S1). Hence, we focused on these six proteins as candidates for X (the genes are highlighted by red hexagons in Fig. 2).

Identification of a gene whose expression is induced diurnally at the end of the night

All of these six selected candidates for X might be the one implicated in the photoperiodic control of hypocotyl elongation. According to the external coincidence model, however, another criterion is that the transcription of X should be induced diurnally at the end of the night specifically in SDs. The diurnal transcription profiles of these six candidates were thus examined (Fig. 3). The results revealed that the transcripts of IAA29 and ATHB2 are sharply and markedly induced at the end of the night, but those of the others were less evident. Therefore, hereafter attention was focused on the IAA29 and ATHB2 genes.

These experiments were repeated using biologically independent samples, and it was confirmed that the end of the night-specific expression of IAA29 and ATHB2 is indeed dependent on PIF4 and PIF5 (Fig. 4A, B). IAA29 encodes a member of the well-known auxin-inducible Aux/IAA proteins, whereas ATHB2 encodes a class II homeodomain-leucine zipper (HD-Zip) protein (Reed 2001, Elhiti and Stasolla 2009). To examine whether the expression of ATHB2 also responds to auxin, young seedlings of Col were grown in continuous light, and then they were externally treated with auxin. The results showed that the expression of ATHB2 was not significantly induced in response to auxin, whereas the expression of IAA29 was markedly induced in both the Col and pif4 pif5 seedlings (Fig. 4C, D). It was also found that only IAA19 was induced by auxin among the other four candidate genes, i.e. bZIP52, IAA19, BBX23/DBB24, and ATHB52 (Supplementary Fig. S1).

ATHB2 was selected as a prime candidate for X

One of the candidates for X, IAA29, functions as a negative repressor of auxin-responsive transcription factors (ARFs). It was known auxin-dependent degradation of Aux/IAA proteins and the resulting activation of ARFs are the early steps of auxin signal transduction (Mockaitis and Estelle, 2008). We showed that the diurnal expression of IAA29 is dependent on PIF4 and PIF5, whereas induction of IAA29 by auxin is not (Fig. 4). Here, we took the following facts into consideration. (i) Auxin together with light is a global growth regulator (Alabadi and Blázquez 2009, Robertson et al. 2009, Cole et al. 2010). (ii) Auxin in particular controls plant growth including the elongation of hypocotyls in a time of day-specific fashion (Covington and Harmer 2007, Covington et al. 2008, Loudet et al. 2008, Michael et al. 2008). (iii) Furthermore, the relevant issue has recently been addressed extensively (Nouze et al. 2011). Taken these together, we assumed that some of the candidates for X (i.e. IAA29, and perhaps IAA19) play complex roles by serving as an integrator of both the auxin and light signals immediately downstream of PIF4 and PIF5 (see the Discussion). Hence, we selected ATHB2 instead as a prime candidate to investigate the circadian clock–PIF4/PIF5-dependent pathway underlying the photoperiodic control of plant growth.

ATHB2 might be a direct target of PIF5

To extend our approach, we verified that the expression of ATHB2 is PIF4/PIF5 dependent by different means. Here, a transgenic line overexpressing hemagglutinin (HA)-tagged
Fig. 2 Characterization of a set of Class 7 genes (Leivar et al. 2009) in terms of light repression and PIF4/5 dependency. The levels of transcripts in dark-grown seedlings of wild-type (Col) and pif4 pif5 mutants before and 1 h after light exposure are shown by shaded bars and open bars, respectively. The names of genes whose expression was both significantly repressed by light and dependent on PIF4 and PIF5 are highlighted by red hexagons (see also Supplementary Table S1). Expression levels relative to Col under darkness (set to 1.0) are presented as mean values ± SD (n = 3).
PIF5 protein (PIF5-ox) was employed. Wild-type (Col), pif4 pif5 mutant and PIF5-ox plants were grown under 12 h light and 12 h dark cycles, and the expression levels of ATHB2 at the end of the night was compared with each other. A markedly elevated expression of ATHB2 was observed for PIF5-ox (Fig. 5A). The results indicated that ATHB2 is positively regulated at the level of transcription in a manner dependent on the cellular content of PIF5. We then examined whether ATHB2 is a direct target of PIF5 by chromatin immunoprecipitation analyses (Fig. 5B). It is known that the members of the PIF family bind to the G-box sequence (CACGTG) (de Lucas et al. 2008, Hornitschek et al. 2009). There is a long non-coding intervening region (C24 kb) between ATHB2 and its upstream gene (AT4G16770) on chromosome 4 (Fig. 5B) and many G-box sequences were found within this intervening region. By employing the PIF5-ox line, 13 amplicons (labeled A–M in Fig. 5B) were examined to determine whether PIF5 binds to the 5′-proximal upstream region of ATHB2. The results showed that PIF5 is capable of binding to the G-box-rich regions upstream of ATHB2. Hence, it is speculated that the ATHB2 gene is a direct target of PIF5.

ATHB2 serves as a positive regulator for hypocotyl elongation

Another question is whether ATHB2 is a positive regulator for the elongation of hypocotyls, as hypothesized in Fig. 1. In this respect, two independent groups have already characterized transgenic lines overexpressing ATHB2 (ATHB2-ox), and they consistently showed that the resultant transgenic young seedlings display a phenotype of long hypocotyls (Schena et al. 1993, Steindler et al. 1999). These results clearly indicate that ATHB2 is a positive regulator of the elongation of hypocotyls. Although the previous results of other groups are highly consistent with each other and thus reliable, we wanted to confirm this point ourselves. We constructed a few independent transgenic lines overexpressing ATHB2, and examined the positive effect with regard to the elongation of hypocotyls (Fig. 5C). Transgenic lines (T 2) carrying a 35S promoter–ATHB2 fusion gene showed the consistent phenotype of long hypocotyls in both continuous light and LDs. The results were in good agreement with those of Schena et al. (1993) and Steindler et al. (1999), supporting the idea that ATHB2 is implicated as a positive regulator in the photoperiodic control of hypocotyl elongation.
Expression of ATHB2 is not directly regulated through the circadian clock

According to the model (Fig. 1), it was predicted that ATHB2 is regulated by the circadian clock indirectly through clock-controlled PIF4 and PIF5. In other words, the transcription of ATHB2 itself most probably does not show a free-running rhythm in continuous light. Thus we clarified this point by examining whether or not the transcription of ATHB2 is under the direct control of the circadian clock (Fig. 6A). Wild-type seedlings were grown under 12 h light and 12 h dark cycles, and they were released into continuous light. mRNA samples were prepared at 3 h intervals, and the free-running circadian rhythm of ATHB2 was analyzed by quantitative real-time PCR (qRT-PCR). As expected, the transcription of PIF4 showed a robust free-running rhythm. However, the expression of ATHB2 in the seedlings grown in continuous light was very low without oscillating. This is consistent with the view that the expression of ATHB2 is severely repressed in the light (see Fig. 2). These results suggested that the diurnal expression of ATHB2 is not attributed to the direct control of the circadian clock but to the function of activated PIF4 and PIF5.

Expression of ATHB2 diurnally oscillates in a short day-specific manner

If ATHB2 is indeed a candidate for factor X, the transcription of the ATHB2 gene should be induced in a strictly SD-specific manner. Furthermore, the oscillation profile of ATHB2 should have a peak at the end of the night, the timing of which should overlap with the expression profile of PIF4 in the dark. These critical issues were addressed by examining the diurnal oscillation profiles of ATHB2 under three different photoperiodic conditions (18 h light/6 h dark cycles, 10 h light/14 h dark cycles and 6 h light/18 h dark cycles). mRNA samples were prepared at 1.5 or 3 h intervals, and the diurnal expression of ATHB2 was analyzed by qRT-PCR. The transcriptional induction of ATHB2 was not observed under LD conditions (Fig. 6B). Expression of ATHB2 was observed only when PIF4 was expressed at the end of the dark period (Fig. 6C). As the length of the dark period became longer, higher levels of ATHB2 were observed (Fig. 6D). Importantly, the extent of hypocotyl elongation under these different photoperiodic conditions was correlated with the expression level of the ATHB2 gene (see Fig. 1). These expression profiles of ATHB2 under these different photoperiods were exactly what we expected for X. These observations are in good agreement with the hypothesis that PIF4/PIF5 promote the elongation of hypocotyls at the end of the night by positively regulating ATHB2 coincidentally in SDs.

Phenotype of phyB and changes in expression profiles of ATHB2

The photoperiodic control of hypocotyl elongation is impaired in phyB loss-of-function mutants (e.g. phyB-9) (Fig. 1). If ATHB2...
is involved in the photoperiodic control of hypocotyl elongation, the phenotype of phyB-9 would be explained by the changes in the expression profile of ATHB2. Indeed, we observed that the expression of ATHB2 was markedly up-regulated in phyB-9 (Fig. 7B). This is consistent with the fact that phyB-9 seedlings show a phenotype of long hypocotyls (Fig. 7A). However, it may be worth mentioning that the expression of ATHB2 was still low during daytime even in the phyB-9 mutant. This suggests that phytochromes other than phyB (e.g. phyD) are also effective for the degradation of PIF4 and PIF5.

**Phenotypes of circadian clock mutants and changes in expression profiles of ATHB2**

**Background.** The photoperiodic control of hypocotyl elongation is severely impaired in certain clock mutants (see Fig. 1).
Elongation (Niwa et al. 2009). prr9 prr7 prr5 mutant seedlings showed a phenotype of long hypocotyls even in LDs (Fig. 8A), whereas cca1 lhy mutant seedlings showed a phenotype of short hypocotyls even in SDs (Fig. 9A). With these clock-defective mutants, we asked the question as to whether the altered phenotype of these mutants with regard to the elongation of hypocotyls was reasonably explained by the expression levels of ATHB2.

Clock mutants with long hypocotyls. In the prr9 prr7 prr5 mutant with long hypocotyls, the transcription of PIF4 during the night was high even in 12 h light and 12 h dark cycles, and coincidentally the transcription of ATHB2 during the night was also high, as compared with Col (Fig. 8B, C). At the onset of lights on, however, transcripts of ATHB2 disappeared rapidly. Importantly, this light-dependent repression of ATHB2 was
observed in the daytime under the conditions where PIF4 was still highly transcribed. This event is consistent with the proposed model, in which the PIF4 proteins are degraded in the light (see Fig. 1). Taken together, the long hypocotyl phenotype of prr9 prr7 prr5 is consistently explained by the coordinately altered expression profiles of PIF4 and ATHB2.

Clock mutants with short hypocotyls. In the cca1 lhy mutant with short hypocotyls, the transcription of PIF4 during the night was low even in 5 h light/19 h dark cycles, and coincidentally the transcription of ATHB2 during the night was also low, as compared with Col (Fig. 9). Hence, the short hypocotyl phenotype of cca1 lhy is also consistently explained by the altered expression profiles of PIF4 and ATHB2. Taken together (Figs. 7–9), it was strongly suggested that ATHB2 is the clock-, phyB- and PIF4-controlled factor which is implicated in the photoperiodic control of hypocotyl elongation.

ATHB2 is also involved in photoperiodic control of adult plant growth
As previously reported, photoperiod affects not only the length of hypocotyls of young seedlings, but also the development of leaves of adult plants in a manner dependent on the clock–PIF4/5 pathway (Niwa et al. 2009). Namely, leaves of SD-grown
plants show the morphologies of long petioles and small leaf blades, as compared with those grown in LDs (Supplementary Fig. S2). Therefore, the final question is whether AHTB2 is also involved in the photoperiod control of adult plant growth. To gain an insight into this issue, both the wild-type (Col) and 

\( \text{prr9 prr7 prr5} \) mutant plants were grown in 16 h light and 8 h dark cycles for 14 d, and then they were further grown for 7 d in varied photoperiod conditions. Photoperiod conditions of 21 : 3, 18 : 6, 15 : 9, 12 : 12, 9 : 15, 6 : 18 and 3 : 21 [light (h) : dark (h)] were adopted. LL denotes constant light conditions; DD denotes constant darkness. The expression levels of \( \text{PIF4} \) (B) and \( \text{AHTB2} \) (C) at the end of the night were analyzed by qRT-PCR. The length of the dark period is schematically shown by shading. Expression levels relative to the samples under a 21 h light / 3 h dark photoperiod condition in Col (set to 1.0) are presented as mean values \( \pm \) SD \( (n = 3) \).

Fig. 10 Correlation between \( \text{PIF4} \) and \( \text{AHTB2} \) expression levels at the end of the night under different photoperiodic conditions. (A and B) Examination of expression levels of \( \text{PIF4} \) and \( \text{AHTB2} \). Wild-type (Col) and \( \text{prr9 prr7 prr5} \) plants were grown in 16 h light/8 h dark cycles for 14 d, and then they were further grown for 7 d in varied photoperiod conditions. Photoperiod conditions of 21 : 3, 18 : 6 : 15 : 9, 12 : 12, 9 : 15, 6 : 18 and 3 : 21 [light (h) : dark (h)] were adopted. LL denotes constant light conditions; DD denotes constant darkness. The expression levels of \( \text{PIF4} \) (B) and \( \text{AHTB2} \) (C) at the end of the night were analyzed by qRT-PCR. The length of the dark period is schematically shown by shading. Expression levels relative to the samples under a 21 h light / 3 h dark photoperiod condition in Col (set to 1.0) are presented as mean values \( \pm \) SD \( (n = 3) \).

Discussion

Clock-controlled external coincidence mechanisms regulating plant growth including flowering and photomorphogenesis

Among clock-controlled developmental events in plants, the best characterized is the photoperiodic control of flowering time in \( \text{A. thaliana} \) (Yanovsky and Kay 2003, Imaizumi and Kay 2006, Fornara et al. 2010). The light- and clock-controlled CO–FT pathway provides a signal to promote flowering only in LDs (Fig. 11A; for details see the Introduction). This is the best-characterized example of an external coincidence model, in which coincidence between the internal (circadian rhythm) and external (photoperiod) time cues is crucial. The promotion of hypocotyl elongation is also dependent on changes in photoperiods in such a way that an accelerated hypocotyl elongation occurs diurnally at the end of every night specifically in SDs (Fig. 1). In this study, we propose another external coincidence model, by which the SD-specific activation of the \( \text{PIF4/5–AHTB2} \) pathway is responsible for the photoperiodic control of vegetative plant growth, at least in part (Fig. 11B).

AHTB2 is involved in the photoperiodic control of plant growth

At the molecular level, the following two types of transcriptional and post-transcriptional regulation make it possible to modulate the \( \text{PIF4/5–AHTB2} \) pathway. (i) The circadian clock regulates the expression of \( \text{PIF4} \) and \( \text{PIF5} \) so that these genes should be expressed in both the daytime and night-time in SDs, but expressed predominantly during the daytime in LDs (Fig. 11B) (Nozue et al. 2007, Niwa et al. 2009). (ii) The \( \text{PIF4} \) and \( \text{PIF5} \) proteins are degraded in the light in a phytochrome (mainly phyB)-dependent manner. Taken together, \( \text{PIF4} \) and \( \text{PIF5} \) would be active as positive regulators for the elongation of hypocotyls only at the end of the night by inducing the downstream factors (Xs) specifically in SDs. This coincidence...
We do not intend to propose that ATHB2 is the sole candidate for X. There might be a few ATHB2 homologs which could be redundantly implicated in the elongation of hypocotyls (Ciarbelli et al. 2008). It was reported that an athb2 loss-of-function mutant shows no detectable phenotype with regard to early photomorphogenesis, suggesting that there are other redundant genes that could mask the defect of ATHB2 function (Schena et al. 1993). However, three independent groups including ours characterized transgenic lines overexpressing ATHB2 by showing that the transgenic young seedlings display the phenotype of long hypocotyls. More intriguingly, a transgenic line carrying an antisense transgene of ATHB2 shows a phenotype of short hypocotyls (Schena et al. 1993, Steindler et al. 1999). This fact suggests that the antisense ATHB2 transgene might co-suppress the expression of not only ATHB2, but also the putative redundant genes. In fact, A. thaliana has several genes that encode proteins quite similar to ATHB2 in terms of their amino acid sequences (e.g. HAT1–HAT3 and ATHB4) (Ciarbelli et al. 2008). For instance, a transgenic line overexpressing HAT2 displays a phenotype of long hypocotyls (Sawa et al. 2002). Therefore, it is possible that these ATHB2 homologs play redundant roles in the photoperiodic control of hypocotyl elongation. It remains to characterize these putative ATHB2 paralogs in terms of whether they are under the control of PIF4 and PIF5. It might also be necessary to characterize other different mutant alleles of athb2 to assess its physiological function in depth.

The function of the ATHB2 family is not sufficient for the photoperiodic response of plant growth

Although ATHB2 plays a crucial role in the photoperiodic control of plant growth, we do not think that ATHB2 is sufficient for the elongation of hypocotyls, as suggested by the observation that the effect of ATHB2 overexpression on hypocotyl lengths was modest (Schena et al. 1993, Steindler et al. 1999), when compared with those of PIF4- or PIF5-overexpressing transgenic lines (Fujimori et al. 2004). Therefore, it is highly probable that PIF4 and PIF5 control the expression of other downstream factors (Ys), which are quite distinct from ATHB2 in their molecular nature. The results of this study suggested that the auxin signaling regulator IAA29 is most likely to be such a candidate for Y (Figs. 3, 4). Furthermore, the diurnal expression profiles of IAA29 were markedly altered in both the phyB-9 and prr9 prr7 prr5 clock mutants in a manner very similar to those of ATHB2 (Supplementary Fig. S3). Involvement of IAA29 in the photoperiodic control of plant growth is intriguing in that there is abundant evidence for cross-talk between light and auxin signaling pathways (Alabadí and Blázquez 2009, Robertson et al. 2009). Indeed, the importance of the cross-talk between light and auxin signaling pathways for the time of day-specific regulation of plant growth is highlighted extensively through bioinformatics (Michael et al. 2008, Nozue et al. 2011).

In this context, we should specifically mention a series of reports from Chory's group, because they shed light on the auxin pathway with special reference to the regulation of...
hypocotyl elongation in response to diurnal light conditions (Michael et al. 2008). They proposed the general concept that a group of plant hormone-associated genes are co-expressed at the end of the night when the hypocotyl growth rate is maximal. They proposed a coincidence model, by which the circadian clock together with phyB controls diurnal plant growth in a time of day-specific fashion by permissive gating of light-mediated phytohormone transcript levels to the proper time of day. A similar idea was proposed recently by Maloof’s group with special emphasis on a link between PIF4/PIF5 and the auxin pathway (Nozue et al. 2011). The auxin-mediated mechanisms appear to be closely relevant to our coincidence model. However, they are distinct from each other in detail at the molecular level. However, they are highly compatible with, or complementary to, each other in the sense that they together nicely explain the complex mechanisms underlying the photoperiodic control of plant growth. Here we propose that the clock-controlled PIF4 and PIF5 directly activate ATHB2, which then promotes the elongation of hypocotyls in a photoperiod-dependent manner (or at the end of the night in SDs). This pathway is merged into the auxin pathway, in which IAA29 serves as a hub to integrate both the auxin and light stimuli to regulate the elongation of hypocotyls properly at the end of every night (Fig. 1C).

ATHB2 and shade avoidance responses

PIF4 and PIF5 also play important roles in shade avoidance responses in the canopy (or under low red/far-red light conditions) (Lorrain et al. 2008, Koini et al. 2009). There are many previous reports which suggest that ATHB2 is implicated in the shade avoidance response (Sessa et al. 2005, Roig-Villanova et al. 2006). ATHB4 (a homolog of ATHB2) is also involved in the shade avoidance response (Sorin et al. 2009). Syndromes caused by the photoperiodic control of plant growth in extreme SD conditions are seemingly similar to those of shade avoidance syndromes (Niwa et al. 2009; see also Supplementary Fig. S2). Therefore, it is tempting to speculate that there is a close linkage between the molecular mechanisms underlying the PIF4/5–ATHB2-mediated photoperiodic control of plant growth and shade avoidance responses. It was recently reported that an extreme ectopic expression of ATHB2 results in severe defects in growth, including poor development of inflorescence stems and also poor elongation and branching of roots (Kollmer et al. 2011). This suggests that ATHB2 is involved in not only the regulation of hypocotyl elongation, but also the regulation of other developmental processes.

It is also known that auxin plays primary roles in shade avoidance (Cole et al. 2011). Specifically, Chory’s group identified the novel gene, named TAA1, the product of which catalyzes the formation of indole-3-pyruvic acid from L-tryptophan. Certain taa1 loss-of-function mutants show an overt phenotype of short hypocotyls in the canopy. This is partly due to the fact that taa1 mutants are unable to induce some auxin-responsive genes including IAA29 in the canopy. Taken together, it is conceivable that ATHB2 plays a crucial role in shade avoidance, in concert with IAA29, which serves as an auxin signal integrator immediately downstream of PIF4 and PIF5 (Fig. 11C). In other words, the molecular mechanism underlying shade avoidance appears to overlap with that underlying the photoperiodic control of plant growth.

Other possible factors that regulate ATHB2 in the photoperiodic control of hypocotyl elongation

The photoperiodic control of hypocotyl elongation is still seen in the pif4 pif5 double loss-of-function mutant (Fig. 1). This suggests that there might be a factor(s), other than PIF4 and PIF5, which also regulates the expression of ATHB2 in response to certain light conditions. In this respect also, Chory’s group identified the putative transcription factor, named TZP, containing tandem zinc knuckle/PLU3 domains (Loudet et al. 2008). TZP acts downstream of the circadian clock and blue light signaling pathways, and directly regulates expression of genes responsible for the elongation of hypocotyls. The expression of ATHB2 together with some auxin-responsive genes was markedly up-regulated in a TZP-overexpressing (TZP-ox) transgenic line, which shows a phenotype of long hypocotyls. However, PIF4 and PIF5 were expressed at control levels in TZP-ox seedlings, suggesting that TZP acts in parallel with PIF4 and PIF5 in the photoperiodic control of hypocotyl elongation. Therefore, it is conceivable that the clock/red light–PIF4/5–ATHB2 pathway plays roles in concert with the clock/blue light–TZP–ATHB2 pathway to regulate the elongation of hypocotyls properly in response to ever-changing light conditions.

Ecological advantage of the photoperiodic response of plant growth

In this study, we also showed that the clock–PIF4/5–ATHB2 pathway plays important roles in not only the regulation of hypocotyl elongation in young seedlings, but also the photoperiodic control of overall plant growth (Supplementary Fig. S2). In this connection, it is of interest to discuss the ecological advantage of the photoperiodic response of plant growth. In wild-type plants, morphological changes of both the young seedlings and adult plants are less sensitive to changes in photoperiods, as compared with those of the ppr9 ppr7 ppr5 mutant (Fig. 1 and Supplementary Fig. S2). In this severe clock-defective mutant, its overall growth including leaf development was attenuated more rapidly and severely in response to the length of dark periods (Niwa et al. 2009). Hence, it is conceivable that the circadian clock modulates plant growth by functioning as a buffer against changes in photoperiods. In this context, both the coincidence mechanisms underlying the photoperiodic regulations of plant growth and flowering time (Fig. 11A, B) might be ecologically advantageous for seasonal and shade-appropriate growth regulation and proper seasonal switching of vegetative growth to reproductive development, respectively (Hotta et al. 2007, Pouteau et al. 2008). One can see the physiological
importance of the plant circadian clock in these two coincidence mechanisms.

Materials and Methods

Plant lines and growth conditions

The A. thaliana Col-0 ecotype was used in this study. The cca1-1 lhy-11, pfr9-10 pfr7-11 pfr5-11, phyB-9, pif4-101 pif5-1 mutants, and a transgenic line overexpressing PIF5-HA, were described previously (Niwa et al. 2009). The transgenic lines of ATHB2-ox were constructed in this study. The growth conditions of these plants were also described previously (Niwa et al. 2009).

Preparation of RNA and qRT-PCR

The procedures for qRT-PCR, including mRNA preparation, were the same as those described previously (Niwa et al. 2009). The primer sets used in this study are summarized in Supplementary Table S2. APX3, encoding an ascorbate peroxidase isozyme, was used as an internal reference (Hazen et al. 2005).

Construction of transgenic lines of ATHB2-ox

To construct the cauliflower mosaic virus 3SS promoter::ATHB2 fusion gene, the entire coding sequence of the ATHB2 gene was amplified using the primers, 5'-CCGAATTCAGAAGATGACGTTCAAGAAGGACG-3' (italics indicate the EcoRI site) and 5'-CCGGAATTCCTTAACTAGGAGCTAGGACGAAGACG-3' (italics indicate the BamHI site), digested by EcoRI and BamHI, and then subcloned into the EcoRI–BamHI cloning site of the cloning vector pBluescript SK(+) (Stratagene). After confirmation by sequencing that intact ATHB2 was cloned, the 0.9 kbp EcoRI–BamHI DNA fragment was blunt-ended and cloned into the blunt-ended XbaI site of the binary vector pSK1 (Kojima et al. 1999) to make the ATHB2 gene under a control of the constitutive 3SS promoter. The constructs were transformed into Agrobacterium tumefaciens strain EHA101, and then wild-type Arabidopsis plants (Col) were transformed by vacuum infiltration procedures (Bechtold and Pelletier 1998).

Chromatin immunoprecipitation

A transgenic line overexpressing PIF5-HA was 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. After plants had been kept in extended darkness for 6 h to allow stable accumulation of PIF5-HA, about 1 g of fresh weight aerial parts of the seedlings were harvested in a dark room and cross-linked for 15 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× Tris-buffered saline (TBS), 125 mM glycerine], 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 300 μl 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. and 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 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 three times to achieve an average fragment size of 0.3–1.0 kb. The sonicated 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), then, 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) was added to 1 ml of chromatin solution and incubated overnight at 4°C. Another 1 ml of chromatin solution without any antibody was also incubated for the mock control. Each solution was incubated with 100 μl of 50% protein A–Sepharose beads 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 protease K (Invitrogen) at 65°C to remove cross-links and all proteins, and then treated with 2 μg of RNase A for 30 min at 37°C. DNA was purified by NucleoSpin (Macherey-Nagel) according to the manufacturer’s protocol. The amount of each precipitated DNA was determined by real-time PCR using specific primers (Supplementary Table S3). The fold enrichment of the specific PIF5–HA-immunoprecipitated chromatin was calculated for each amplicon using the following equation: \[\frac{2^{Ct(\text{MOCK})}}{2^{Ct(\text{ChIP})}}.\]

Supplementary data

Supplementary data are available at PCP online.
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References


