A Link between Circadian-Controlled bHLH Factors and the APRR1/TOC1 Quintet in Arabidopsis thaliana

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APRR1 (ARABIDOPSIS PSEUDO-RESPONSE REGULATOR 1) (or TOC1, TIMING OF CAB EXPRESSION 1) is believed to be a crucial component of biological clocks of Arabidopsis thaliana. Nevertheless, its molecular function remains to be fully elucidated. Based on the results of yeast two-hybrid and in vitro binding assays, we previously showed that APRR1/TOC1 interacts with certain bHLH factors (i.e. PIF3 and PIL1, which are PHYTOCHROME INTERACTING FACTOR 3 and its homolog (PIF3-LIKE 1), respectively). To critically examine the relevance of PIL1 with reference to the function of APRR1/TOC1, T-DNA insertion mutants were isolated for PIL1. No phenotype was observed for such homozygous pil1 mutants, in terms of circadian-associated events in plants. We then examined more extensively a certain set of bHLH factors, which are considerably similar to PIL1 in their structural designs. The results of extensive analyses of such bHLH factors (namely, HFR1, PIL2, PIF4, PIL5 and PIL6) in wild-type and APRR1-overexpressing (APRR1-ox) transgenic lines provided us with several new insights into a link between APRR1/TOC1 and these bHLH factors. In yeast two-hybrid assays, APRR1/TOC1 showed the ability to interact with these proteins (except for HFR1), as well as PIL1 and PIF3. Among them, it was found that the expressions of PIF4 and PIL6 were regulated in a circadian-dependent manner, exhibiting free-running robust rhythms. The expressions of PIF4 and PIL6 were regulated also by light in a manner that their transcripts were rapidly accumulated upon exposure of etiolated seedlings to light. The light-induced expressions of PIF4 and PIL6 were severely impaired in APRR1-ox transgenic lines. Taken together, here we propose the novel view that these bHLH factors (PIF4 and PIL6) might play roles, in concert with APRR1/TOC1, in the integration of light-signals to control both circadian and photomorphogenic processes.

Keywords: Arabidopsis thaliana — Circadian rhythm — bHLH factors — Light signalling.

Introduction

Circadian rhythms are driven by an endogenous biological clock(s) that regulates many biochemical, physiological and behavioral processes in a wide variety of organisms (for reviews, see Dunlap 1999, Young and Kay 2001). In higher plants too, there is a wide range of biological processes that are presumably controlled through such circadian clocks (Kreps and Kay 1997, Koornneef et al. 1998). They include movement of organs such as leaves and petals, opening of stomata, daily fluctuations of metabolic activities such as respiration and photosynthesis. One of the well-characterized circadian-regulated events is the control of flowering time through the photoperiodic long-day pathway (for reviews, see Simpson and Dean 2002, Mouradov et al. 2002). Furthermore, several circadian clock-associated components are involved in light-dependent signal transduction, for instance, through which elongation of hypocotyls is regulated (for a recent review, see Quail 2002). In these respects, recent intensive studies on the model plant, Arabidopsis thaliana, have begun to shed light on the molecular mechanisms underlying such circadian-related biological events (for recent reviews, see Barak et al. 2000, Murtas and Millar 2000, McClung 2000, Samach and Coupland 2000, Putterill 2001, Somers 2001, Mouradov et al. 2002).

In A. thaliana, a certain number of circadian-associated proteins have been identified, including the best candidates of clock components, CCA1 (CIRCADIAN CLOCK-ASSOCIATED 1) and LHY (LATE ELONGATED HYPOCOTYL) (Wang and Tobin 1998, Schaffer et al. 1998, Green and Tobin 1999, Alabadi et al. 2002, Mizoguchi et al. 2002). CCA1 and LHY are homologous Myb-related DNA-binding transcription factors. Another protein, TOC1 (TIMING OF CAB EXPRESSION 1), is also believed to be a component of the central oscillator (Somers et al. 1998, Strayer et al. 2000, Mas et al. 2003). Furthermore, reciprocal and intimate interactions were observed between the functions of CCA1/LHY and TOC1 (Alabadi et al. 2001, Makino et al. 2002, Matsushika et al. 2003).
2002a, Mizoguchi et al. 2002). In this connection, we have been characterizing a small family of proteins, designated as \textit{ARABIDOPSIS PSEUDO-RESPONSE REGULATORS} (APRR1, APRR3, APRR5, APRR7 and APRR9) (Makino et al. 2000, Makino et al. 2001, Makino et al. 2002, Matsushika et al. 2000, Matsushika et al. 2002a, Matsushika et al. 2002b, Murakami-Kojima et al. 2002, Sato et al. 2002, Nakamichi et al. 2003). These homologous APRRs are nuclear proteins with a common domain similar to the phospho-accepting receiver of response regulators (Mizuno 1998), together with an additional (CCT) motif common in the CO (CONSTANS) family of proteins (Makino et al. 2000, Strayer et al. 2000). In fact, APRR1 turned out to be identical to TOC1 (Matsushika et al. 2000, Strayer et al. 2000). More interestingly, not only the \textit{APRR1}/\textit{TOC1} gene, but also other \textit{APRRs} are all subjected to circadian rhythms (Matsushika et al. 2000). We previously speculated that such rhythmic events, termed “circadian waves of the \textit{APRR1}/\textit{TOC1} quintet”, might be the basis of a biological clock. Several lines of evidence have already been provided to support the view that APRR9 and APRR5 (together with \textit{APRR1}/\textit{TOC1}) must be taken into consideration for a better understanding of the molecular links between circadian rhythms, control of flowering time through the photoperiodic long-day pathway, and also light-signal-controlled hypocotyl elongation (Matsushika et al. 2002b, Sato et al. 2002). This view is based on the findings that both APRR9-overexpressing (APRR9-ox) and APRR5-ox transgenic lines flowered much earlier than wild-type plants in a manner independent of photoperiodicity, and also that both APRR1-ox and APRR5-ox transgenic lines showed an SRL (short-hypocotyls under red light) phenotype that is indicative of hypersensitivity to light during early photomorphogenesis (or de-etiolation of seedlings) (for a review, see Quail 2002).

Nonetheless, little is known about the molecular mechanisms, by which the \textit{APRR1}/\textit{TOC1} quintet plays a circadian-associated role(s) in plants. Furthermore, the functional interactions among the \textit{APRR} members have also remained to be entirely elusive. In this respect, several intriguing (but fragmental) observations as to the \textit{APRR1}/\textit{TOC1} quintet have been reported. (i) The circadian-controlled \textit{APRR9} gene is induced by white light (or red light pulse) (Makino et al. 2001). (ii) Such circadian-controlled and light-induced expressions of \textit{APRR9} were severely impaired in \textit{APRR1}-ox transgenic lines (Makino et al. 2002). (iii) The results of yeast two-hybrid and in vitro binding assays showed that \textit{APRR1}/\textit{TOC1} interacts with a putative basic helix-loop-helix (bHLH) transcription factor, named PIL1 (PIF3-LIKE 1), which is very similar to PIF3 (PHOTOCROME INTERACTING FACTOR 3). PIF3 has been identified as the protein that specifically associates with red light-activated phyB, and consequently, it functions as a DNA-binding transcriptional activator (Ni et al. 1998, Ni et al. 1999). Taken together, it was tempting to speculate that \textit{APRR1}/\textit{TOC1} might play a role in transcriptional regulation of certain genes (e.g. \textit{APRR9} and \textit{CCA1}) in response to light, through interacting with certain bHLH transcription factors (e.g. PIL1 and PIF3). To gain further insight into this hypothetical scenario, here we characterized a subset of bHLH factors that are very similar to PIL1 and PIF3 in their amino acid structures. We found that the expressions of certain bHLH genes (namely, PIF4 and PIL6) are under the control of circadian rhythms, and also that they are induced by light. These bHLH factors were further characterized with special reference to the function of \textit{APRR1}/\textit{TOC1}.

\section{Results}

\subsection*{Characterization of T-DNA insertion mutants of the PIL1 gene}

As mentioned above, the \textit{PIL1} gene encodes a putative bHLH transcription factor (MIPS code At2g46970), the amino acid sequence of which is highly similar to that of PIF3. The previous results of yeast two-hybrid and in vitro binding assays showed that PIL1 is capable of physically associating with both PIL1 and PIF3 (Makino et al. 2002). Thus, one can assume that PIL1 might play a role, in concert with APRR1, in a mechanism underlying circadian rhythms and/or light-dependent signal transduction. To directly assess this hypothetical view, here we isolated two independent \textit{Arabidopsis} mutant derivatives of the Columbia ecotype (Col-0), for each of which a T-DNA insertion was assumed to be located in the third and last exons of the \textit{PIL1}-coding sequence, respectively (Fig. 1A). The mutant seeds (labeled as KE1026 and KG22509) were obtained from The Kazusa DNA Research Institute (Chiba, Japan), and then each corresponding homozygous \textit{PIL1}::T-DNA transgenic line was established. One of the mutant alleles was designated as \textit{pil1-1}, which carries a T-DNA insertion at the position of 916/917 (the first nucleotide of the inferred initiation codon of \textit{PIL1} was taken as 1). The other was \textit{pil1-2}, which carries a T-DNA insertion at the position of 1684/1685.

Together with wild-type (Col-0) plants, these homozygous \textit{pil1} mutants were grown under the standard growth conditions with light, and then RNA samples were prepared from leaves. However, the transcript of \textit{PIL1} was hardly detected even in the RNA samples from the wild type (data not shown). We then grew these plants in the dark, and then RNA samples were prepared from the etiolated seedlings. A considerable amount of the \textit{PIL1} transcript was detected in the wild-type etiolated seedlings (Fig. 1A). The corresponding transcript of \textit{PIL1} was not detected in the RNA samples from the \textit{pil1-1} and \textit{pil1-2} mutant seedlings, although truncated transcripts of \textit{PIL1} were seen, particularly in the case of \textit{pil1-2} (Fig. 1A). These mutant plants were extensively examined in terms of their circadian-associated phenotypes, including flowering time and red light sensitivity during early photomorphogenesis (i.e. elongation of hypocotyls). Nonetheless, we could so far observe no apparent phenotype for these mutants (data not shown).

To more critically examine the phenotypes of these \textit{pil1} mutants, the hallmark circadian rhythms of \textit{CCA1}, \textit{LHY} and...
APRR9 were examined under both the light/dark (LD) cycle and continuous dark (DD) by Northern blot hybridization. The circadian profiles observed for CCA1, LHY and APRR9 in the pil1-1 mutant were indistinguishable from those seen in the wild-type (Col-0) (Fig. 1B). As another critical experiment, the light-induced expression of APRR9 was also examined, because it was previously demonstrated that the expression of APRR9 is severely repressed in an APRR1-overexpressing (APRR1-ox) transgenic line (Makino et al. 2002). Etiolated seedlings (6 d old) were exposed to white light, and then the expression of APRR9 was followed by Northern blot hybridization. The profiles of the light-induced expression of APRR9 were not affected in the pil1-1 and pil1-2 mutants (data not shown). These results did not support the idea that PIL1 plays a circadian-associated role through interacting with APRR1. Alternatively, there might be an as yet unidentified PIL1-related bHLH factor(s) that presumably plays a redundant role.

Re-investigation of a set of bHLH factors with special reference to APRR1

Faced with the negative results (Fig. 1), we needed to revise our previous view with respect to a link between APRR1 and PIL1. Thus, we decided to characterize PIL1-related bHLH factors of A. thaliana more intensively, considering the fact that this higher plant has a large number (ca. 140) of transcription factors that are basically classified into the bHLH family (Riechmann et al. 2000). Among these bHLH family members, we first selected about 50 members by comparing the amino acid sequences of the bHLH regions with each other (see MIPS codes listed in Fig. 2). Each of these selected bHLH members are relatively more similar to PIL1, as compared with those excluded from the list. With use of the selected bHLH sequences (about 60 amino acids), a phylogenetic tree was constructed by means of the neighbor-joining method (Fig. 2). The results revealed several intriguing views as to the PIL1-related bHLH factors. First, PIL1 and PIF3 showed a very close relationship in the phylogenetic tree, as pointed out previously (Makino et al. 2002). Second, two other phytochrome interacting factors (HFR1 and PIF4), which have already been characterized, also appeared to be very close to PIL1 and PIF3. HFR1 (LONG HYPOCOTYL IN FAR-RED 1) was suggested to act through interacting with phyB in a red light responsive signal transduction pathway (Fairchild et al. 2000, Kim et al. 2002), whereas PIF4 (PHYTOCHROME INTERACTING FACTOR 4) was suggested to act through interacting with phyB in a red light responsive signal transduction pathway that is involved in early photomorphogenesis (Huq and Quail 2002). Beside these known bHLH factors, three other uncharacterized bHLH factors appeared to be very similar to each other. They were tentatively designated as PHYTOCHROME INTERACTING FACTOR-LIKE 2, 5 and 6 (PIL2, PIL5 and PIL6), respectively. PIL2 (At3g62090) is very similar to PIL1, whereas PIL5 (At2g01800) and PIL6 (At3g59060) are each relatively similar to PIF3 and PIF4, respectively. When the entire amino acid sequences of these seven bHLH factors were aligned (Fig. 2), it was found that they share not only the highly conserved bHLH motif, but also another conserved motif that is commonly located at their relatively amino-terminal portions, although HFR1 exceptionally lacks this motif. This sequence was referred to as “PIL-motif”, which was not found in any other bHLH factors, and which is different a PAS (Period-Aryl hydrocarbon receptor-Single-
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The bHLH (basic helix-loop-helix) domain is found in PIF3. In any case, considering the fact that three (HFR1, FIP3 and PIF4) out of these seven bHLH members have already been implicated in phytochrome-mediated signal transduction pathways, it would be of interest to focus on these closely-related bHLH factors. In particular, it would be of interest to examine these bHLH factors in terms of circadian rhythms and light-mediated signal transduction, with special reference to the APRR1/TOC1 quintet.

**APRR1 interacts with a set of bHLH factors in yeast two-hybrid assays**

Through yeast two-hybrid and in vitro binding assays, we previously showed that PIL1 and PIF3 are capable of physically interacting with APRR1 (Makino et al. 2002). It was also shown that the bHLH region of PIL1 is responsible for this interaction. Thus, one can assume that the selected set of bHLH factors might also interact with APRR1. Based on this reasonable assumption, we extensively conducted yeast two-hybrid assays, in order to examine a possible interaction of these seven members of bHLH factors with APRR1 (Fig. 3). As reported previously, both PIL1 and PIF3 showed the abilities to interact with APRR1. HFR1 did not show such ability. Otherwise, the others (PIL2, PIF4, PIL5 and PIL6) were suggested to interact with APRR1 in the yeast two-hybrid assays. This finding did not necessarily mean that every bHLH factor could interact with APRR1, because the RAP1 bait (an appropriate negative reference) did not interact with APRR1. Rather, this alternative bHLH factor (see Fig. 2) showed the ability to interact...
act with an Arabidopsis histidine-containing phosphotransfer (HPt) factor, namely, AHP5 (Suzuki et al. 2000). From these results, it was suggested that a certain set of homologous bHLH factors (PIL2, PIF4, PIL5 and PIL6, in addition to PIL1 and PIF3) physically interact with APRR1. These bHLH factors contain a common motif (i.e. PIL-motif), as mentioned above. It thus remains to examine whether or not the common PIL motif is involved in the interaction with APRR1. These results were further confirmed by assaying β-galactosidase activities, as also shown.

We then examined the expressions of these bHLH genes at the level of transcription in response to light. As described above, 6-day-old etiolated seedlings (Col-0) were exposed to white light, and then changes in the levels of transcripts were examined by Northern blot hybridization (Fig. 4). The transcript of PIL1 disappeared very rapidly upon the exposure of etiolated seedlings to light. This is consistent with our earlier observation that the transcript of PIL1 was hardly detected in leaves prepared from the light-grown plants (see Fig. 1). The levels of the transcripts of HFR1, PIL2 and PIF3 did not significantly fluctuate after the light treatment. The transcript of PIL5 was hardly detected under both the dark and light conditions. Most interestingly, both the transcripts of PIF4 and PIL6
Circadian rhythms in *Arabidopsis* were markedly and rapidly increased by the light treatment. These critical events were demonstrated by the quantitative analyses (Fig. 4B), which showed that *PIL1* is a light-repressed gene, whereas *PIF4* and *PIL6* are light-induced ones. When each putative promoter region of the *PIF4* and *PIL6* genes was inspected, it was revealed that both the promoters contain two \"G-box\" sequences (CACGTG) (data not shown), which are often found in *Arabidopsis* light-induced promoters (e.g. in the *PIF3*-dependent and light-regulated *CCA1* and *LHY* genes, for a review see Quail 2000). We do not know whether or not these G-box sequences in the *PIF4* and *PIL6* promoters are indeed involved in the observed light-dependent expressions of these genes.

**PIF4 and PIL6 are circadian-controlled genes**

We next asked the more critical question of whether the expressions of these bHLH genes are regulated in a circadian-dependent manner. To this end, wild-type (Col-0) plants were grown under the LD cycle for 20 d. Then, diurnal oscillations of their transcripts were examined by preparing RNA samples from leaves at 3-h intervals, followed by Northern blot hybridization with each specific probe (Fig. 5A). It was shown that the level of each transcript of *PIF4* and *PIL6* oscillated robustly with a peak after dawn, and with a period of about 24 h, in a similar manner to that observed for *CCA1*, which was examined as an appropriate positive reference. Such robust diurnal oscillations were not observed for the others (*HFR1*, *PIL1*, *PIL2*, *PIF3* and *PIL5*) (data not shown). Considering the fact that both *PIF4* and *PIL6* are light-induced genes (Fig. 4), the results suggested either that the expressions of *PIF4* and *PIL6* may be diurnally oscillated in a LD cycle-dependent manner, or that they may be circadian-controlled genes. To critically discriminate these possibilities, free-running circadian rhythms were examined for *PIF4* and *PIL6* with the plants grown under continuous light (LL) (Fig. 5B). The well-marked free-running rhythms of *APRR9* and *APRR1* were also probed as appropriate positive references. The results showed that a robust free-running rhythm was seen for each transcript of *PIF4* and *PIL6* in LL. With these circadian profiles, however, the timings (or phases) of periodical peaks were different from each other. In other words, the transcript of *PIL6* first appeared at dawn, slightly earlier than that of *APRR9*, which was followed by the expression of *PIF4*. We thus concluded that *PIF4* and *PIL6* are not only light-induced genes, but also circadian-controlled ones.

**A link between the circadian-controlled and light-inducible bHLH factors and APRR1**

As addressed in the first step of this study, no evidence was provided for the existence of any functional linkage between *PIL1* and *APRR1*. However, here the relevant issue should be re-addressed with regard to a set of *PIL1*-related bHLH factors (particularly, *PIF4* and *PIL6*). To this end, we

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**Fig. 5** Northern blot hybridization analyses with regard to rhythmic expressions of certain genes. (A) Wild-type (Col-0) plants were grown under LD of 12 h light/12 h dark for 20 d. They were further grown under the same LD conditions (panel A), or the plants were transferred to continuous light (LL) (panel B), as schematically shown (the filled and shaded bars indicate objective and subjective night, respectively). RNA samples were prepared from leaves at the intervals indicated. They were analyzed by Northern blot hybridization with each specific probe, as indicated (*PIF4*, *PIL6*, *CCA1*, *APRR9*, *APRR1* and *UBQ10*). (B) The raw data (lower panel) were each quantified (by normalizing with the *UBQ10*-value). The quantified data are shown schematically as the relative amounts of mRNA (upper panel), in which the maximum level of each transcript (*PIF4* and *PIL6*) was taken arbitrarily as 10, in order to clarify the profiles.
employed APRR1-ox transgenic lines (Makino et al. 2002). In particular, the circadian profiles of PIF4 and PIL6 were examined in the APRR1-ox background. Wild-type (Col-0) and APRR1-ox plants were grown for 22 d in LD, and then they were transferred to the continuous dark (DD). RNA samples were prepared from leaves at 3-h intervals (1 d in LD and 1.5 d in DD), and they were subjected to Northern blot hybridization, with probes for PIF4 and PIL6, as well as APRR1. The rhythms of APRR9 and PIF3 were also examined as appropriate references (Fig. 6A). As expected, a high and constitutive expression of APRR1 was observed in APRR1-ox (Fig. 6A, upper panel). As emphasized earlier (see the first section), the expression of APRR9 was severely repressed in APRR1-ox in both LD and DD (Fig. 6A, middle panel). However, the expression level of PIF3 did not significantly fluctuate in the wild type, and such an expression profile of PIF3 was not affected in APRR1-ox (Fig. 6A, lower panel). On the bases of these appropriate references, the expression profiles were examined for PIF4 and PIL6 in APRR1-ox (Fig. 6B and Fig. 6C, respectively). The expression level (or amplitude) of PIF4 in LD was considerably lowered in APRR1-ox, as compared with in the wild type. The same event was also seen in DD, although a persisted rhythm of PIF4 was observed even in APRR1-ox. Essentially the same events were seen for PIL6. It was thus suggested that the rhythmic expression profiles of both PIF4 and PIL6 were somehow affected in APRR1-ox transgenic lines.

To more critically examine a link between APRR1 and PIF4 (and/or PIL6), it was of interest to examine the light-induced expressions of PIF4 and PIL6 in the APRR1-ox background. Indeed, the light-induced accumulations of both the transcripts of PIF4 and PIL6 were severely impaired in APRR1-ox, while the expression of PIF3 was not affected at all (Fig. 7). As demonstrated earlier, the transcript of PIL1 disappeared very rapidly when the etiolated seedlings were exposed to light (see Fig. 4). The kinetics of this particular event of PIL1 was also not altered in APRR1-ox (Fig. 7). In short, together with the results of Fig. 6, the results of Fig. 7 supported the view that there is a link between these bHLH factors and APRR1 in a manner that the expressions of PIF4 and PIL6 are regulated by APRR1, directly or indirectly.

Discussion

The result of this study showed that APRR1/TOC1 interacts with not only the previously reported bHLH factors (PIL1 and PIF3), but also some other closely related bHLH factors (Fig. 3). In particular, PIF4 and PIL6 were found to be very intriguing, because the expressions of their structural genes were regulated in a light- and circadian-dependent manner (Fig. 4, 5). These findings of this study provided new insight into the molecular mechanisms underlying circadian rhythms and light-dependent signal transduction in A. thaliana, as summarized in Fig. 8. Based on the results of recent studies on cir-
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Among the selected set of bHLH proteins, PIF3 has already been integrated into the framework (see Fig. 8). This phytochrome-interacting bHLH protein was proposed to be involved in the regulation of CCA1 and LHY through binding to the G-box sequences in their promoter regions (Martinez-Garcia et al. 2000, and for a review see Quail 2000). At present, the importance of others (HFR1, PIL1, PIL2 and PIL5) is less clear. They may play some other irrelevant roles. However, it should be noted that HFR1 is known to act in a phyA-mediated signaling pathway (Fairchild et al. 2000, Kim et al. 2002). Our results did not support the idea that PIL1 is involved in the framework. However, it was found in this study that the expression of PIL1 is expressed preferentially in the dark-grown etiolated seedlings, and it is regulated by light in such a manner that the transcript of PIL1 disappears very rapidly under light (Fig. 4). Thus, HFR1 and PIL1 remain to be examined with reference to light-dependent signaling pathways in plants. In this respect, it will be of interest to characterize the phenotypes of the established pil1 mutants under certain monochromatic light conditions (e.g. far-red light). Meanwhile, we mainly considered PIF4 and PIL6, as addressed above.
With regard to the hypothetical framework (Fig. 8), the results of this study suggested that PIF4 and PIL6 must be taken into consideration for a better understanding of the molecular links between APRR1/TOC1 and circadian-associated events, including light-dependent signal transduction. These bHLH factors are highly homologous to PIF3 (Fig. 2). Therefore, they are most likely DNA-binding transcriptional regulators. In sharp contrast to PIF3, the expressions of PIF4 and PIL6 are controlled in a circadian-dependent manner (Fig. 5). Also, they were controlled in such a way that the transcriptions are rapidly induced by light (Fig. 4). Furthermore, evidence was provided to suggest a molecular link between APRR1/TOC1 and these bHLH factors: (i) APRR1/TOC1 has the ability to interact with these homologous bHLH factors in yeast two-hybrid assays (Fig. 3), and (ii) the light-induced expressions of PIF4 and PIL6 are severely repressed in APRR1-ox transgenic lines (Fig. 7). Taken together, it is tempting to speculate that these bHLH factors might play roles, in concert with APRR1/TOC1, in the mechanisms underlying circadian rhythms and light-dependent signal transduction, as further addressed below.

The recently identified SRL2 (SHORT UNDER RED-LIGHT 2) gene of *A. thaliana* is critically relevant to the results of this study, because this is the structural gene for PIF4 (Huq and Quail 2002). In fact, it has already been reported that a presumed loss-of-function (sr2-1) mutant confers hyperrightivity to continuous red light, displaying short hypocotyls in detiolated seedlings, as compared with the wild type. The results of genetic studies showed that PIF4-antisense transgenic lines also display short hypocotyls, whereas PIF4-overexpressing transgenic lines display long hypocotyls under red light. The results of biochemical studies showed that PIF4 interacts physically with phyB. This genetic, photobiological and molecular evidence led Huq and Quail (2002) to propose that PIF4 functions as a nuclear localized, negatively acting transcription factor that is involved in a phyB-mediated signaling pathway, through which a light-regulated hypocotyl-elongation (or cell-expansion) is somehow regulated. The results of this study are fully consistent with this scenario as to the function of PIF4. Our results further extended the scenario by providing new insights: (i) the expression of PIF4 is controlled in a circadian-dependent manner, and (ii) there is a physical interaction between PIF4 and APRR1/TOC1, also (iii) the light-induced expression of PIF4 is severely repressed in APRR1-ox transgenic lines. These results are particularly intriguing, when considering the current view that APRR1/TOC1 is an important component of the circadian clock with a crucial function in the integration of light signals to control both circadian and photomorphogenic responses (Sato et al. 2002, Mas et al. 2003). It is thus tempting to speculate that PIF4 might act as a crucial integrator that connects circadian rhythms and photomorphogenic responses through interacting with both phytochromes and APRR1/TOC1. In this hypothetical view, APRR1/TOC1 might act as a negative regulator of PIF4 (either at protein or transcriptional level). This idea explains the striking fact that both APRR1-ox and PIF4-antisense transgenic lines display the same characteristic phenotype, namely, very short hypocotyls under red light (Sato et al. 2002, Huq and Quail 2002, Mas et al. 2003).

The results of this study further pointed out that not only PIF4, but also another homologous bHLH factor (PIL6) appears to be important in the scenario described above. PIL6 showed properties very similar to those of PIF4 in several facets, as described above. PIL6 might play an overlapping role with PIF4, for instance, through forming a PIL4/PIL6 heterodimer. Alternatively, PIL6 may play a role similar to, but distinct from, that of PIF4. The following observations are consistent with the latter view: (i) the presumed loss-of-function srl2-1 mutant singly results in the severe phenotype of short hypocotyls under red light (Huq and Quail 2002), and (ii) the circadian profile of PIL6 is considerably different from that of PIF4 in that they have the circadian peaks at the different timings from each other (Fig. 6). In any case, clarification of these interesting issues requires extensive genetic analyses with PIL6, as has already been done for PIF4.

In summary, the circadian-controlled bHLH factors (PIF4 and PIL6) might play important roles, in concert with APRR1/TOC1, in the integration of light signals to control both circadian and photomorphogenic processes. These are reminiscent of the circadian-associated bHLH factors in other organisms (CLOCK and BMAL1 bHLH factors in mouse, CLOCK and CYCLE bHLH factors in *Drosophila melanogaster* (Devlin and Kay 2001, Young and Kay 2001). Clarification of the molecular mechanisms by which these putative plant bHLH transcriptional regulators participate in the circadian-associated processes must await further investigations, which should include characterizations of *pif4* and *pil6* mutants and/or characterizations of transgenic plants overexpressing PIF4 and PIL6. Such results will shed light on the longstanding problems of circadian rhythms in plants, including the molecular function of APRR1/TOC1.

### Materials and Methods

**Plant growth conditions and related materials**

*A. thaliana* (Columbia ecotype, Col-0) was mainly used as wild-type plants. Seeds were imbibed and cold treated at 4°C for 3 d in the dark before germination under light, and then plants were grown at 22°C. Note that the imbibed seeds were exposed to white light for 30 min before incubation in the dark. Plants were grown in a chamber with light from fluorescent lights (70-90 µmol m⁻² s⁻¹) at 22°C on a compost-vermiculite mixture and/or on a gelrite-plate containing MS salts and 1% sucrose. Light/dark conditions, mainly used, were 16 h light/8 h dark (LD, long-day conditions).

**Identification of pil1-1 and pil1-2 alleles**

Screening of T-DNA tagged mutation of the *PIL1* gene was carried out by genomic PCR reactions using an appropriate combination of the following primers: LB (5'-AAGAAAAAATGCAGCATAATTCATTG-GGC) and RB (5'-TTCCCTTAATTCTCCTGATCGTC) for primers...
specific for T-DNA; PIL1-F (5'-GGTGGGATCCGAATTCCTCCTG) and PIL1-R (5'-AAAGAAGATTCGCTAGCTGAG) for primers specific for the PIL1 coding-sequence. As the result, two independent mutant lines were obtained, which were designated as pill-1 and pil1-2, respectively. Note that these mutant alleles are in the Columbia ecotype background.

RNA preparation and Northern blotting hybridization

Total RNA was isolated from appropriate organs (mainly leaves) of plants by the acid phenol method, as described previously (Sato et al. 2002). For Northern blot hybridization, RNA was separated in agarose gels (1.2%) containing 0.6 M formaldehyde, then transferred to Hybond-XL membranes. The fixed membranes were hybridized with 32P-labeled DNA fragments in 6x standard saline phosphate and EDTA (1x SSPE = 0.18 M NaCl, 10 mM phosphate buffer, 1 mM EDTA, pH 7.4), 5x Denhardt’s solution, and 0.5% SDS containing 10% dextran sulfate and 100 μg ml−1 salmon sperm DNA, at 65°C for 18 h. The membranes were washed once with 2x SSPE and 0.5% SDS for 15 min at room temperature, once with 2x SSPE and 0.5% SDS for 30 min at 65°C, and then with 0.2x SSPE and 0.5% SDS for 15 min at 65°C. The washed membranes were exposed and analyzed on a phosphoimage analyzer (BAS-2500II) (Fuji Film, Tokyo, Japan).

Probes for Northern blot hybridization

Appropriate double-stranded 32P-labeled DNA probes were used to detect each specific mRNA. The probes used were amplified by PCR with the following sets of primers, which were designed appropriately. They included CCA1 (5'-GGCTTAAGGTAAACAAACCATCC and 5'-GGGACCTTCTGTCCACATGAAT), LHY (5'-TGGACATAGAATCCGCTGCTCAGATC and 5'-GACGAAGTCCCTGTC), PIL1 (5'-AGAAGTAGCCTGAGTCTGAG), APRR9 (5'-TATGCTGAGGGTGCTGCT and 5'-CTCTGGCTCTACGTTCACAC), APRR1 (5'-TGGACGAGAAGTCTCAGC and 5'-CTCTTCTGCAGATTATTTACC), HFR1 (5'-AAGTGAATATCAAGGAGTCG and 5'-TCATGTGTTCTGCCGATG), PIL1 (5'-TAGTCTGCACTCAGGAACCA and 5'-AGAAGTAGCCTGAGTCTGAG), PIL2 (5'-ATGATTCTTTACAAACCGATATG and 5'-TCATCTGTAGTTCCTTCTG), PIL5 (5'-GACAGTATTCGCTGAGAT and 5'-GATGAGTTCTGCTGAGAT), PIL4 (5'-ATGGAACACCAAGGTGAGG and 5'-CTAGTGGCTCAGAAAGACC), PIL6 (5'-ATGGAACACCAAGGTGAGG and 5'-CTAGTGGCTCAGAAAGACC), PIL6 (5'-GACAGTATTCGCTGAGAT and 5'-GATGAGTTCTGCTGAGAT), PIL4 (5'-ATGGAACACCAAGGTGAGG and 5'-CTAGTGGCTCAGAAAGACC), PIL6 (5'-ATGGAACACCAAGGTGAGG and 5'-CTAGTGGCTCAGAAAGACC), PIL6 (5'-GACAGTATTCGCTGAGAT and 5'-GATGAGTTCTGCTGAGAT). For detecting PILF3-specific mRNA, we used the purified 1.58 kbp EcoRI-SalI DNA fragments from pGAD424-PILF3 (Makino et al. 2002). Each 32P-labeled probe was prepared with the Megaprime DNA Labeling System (Amersham Bioscience, U.S.A.).


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