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Functional Characterization of Phytochrome Interacting Factor 3 for the *Arabidopsis thaliana* Circadian Clockwork

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Light, in a quality- and quantity-dependent fashion, induces nuclear import of the plant photoreceptors phytochromes and promotes interaction of these receptors with transcription factors including PHYTOCHROME INTERACTING FACTOR 3 (PIF3). PIF3 was shown to form in vitro a ternary complex with the G-box element of the promoters of LATE ELONGATED HYPOCOTYL (LHY) and CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) and the Pfr conformer of phytochromes. CCA1 and LHY together with TIMING OF CAB EXPRESSION 1 (TOC1) constitute a transcriptional feed-back loop that is essential for a functional circadian clock in *Arabidopsis*. These findings led to the hypothesis that the PIF3-containing ternary complex regulates transcription of light-responsive genes and is involved in phototransduction to the central circadian clockwork. Here we report that (i) overexpression or lack of biologically functional PIF3 does not affect period length of rhythmic gene expression or red-light-induced resetting of the circadian clock and (ii) the transcription of PIF3 displays a low-amplitude circadian rhythm. We demonstrated previously that irradiation of etiolated seedlings induces rapid, phytochrome-controlled degradation of PIF3. Here we show that nuclear-localized PIF3 accumulates to relatively high levels by the end of the light phase in seedlings grown under diurnal conditions. Taken together, we show that (i) PIF3 does not play a significant role in controlling light input to and function of the circadian clockwork and (ii) a yet unknown mechanism limits phytochrome-induced degradation of PIF3 at the end of the day under diurnal conditions.

Keywords: Circadian clock — Light — Phototransduction — Phytochrome — Protein degradation.

Introduction

Plants as photoautotroph organisms use light not only as a primary energy source but also as a key developmental regulatory signal. In higher plants light promotes seed germination, initiates photomorphogenesis and phototropism, regulates flowering time and shade avoidance responses. To monitor the intensity, wavelength, direction and timing of light and sense changes in their ambient light environment plants have evolved diverse photoreceptor systems. These sensory systems include the blue light absorbing cryptochromes and phototropins (for reviews see Lin and Shalitin 2003, Briggs and Christie 2002, respectively) and the red/far-red light absorbing phytochromes (for a review see Nagy and Schäfer 2002). The prominent feature of natural light environment is the cyclic changes of light/dark (LD) periods. Thus plants, like other eukaryotes and some prokaryotes, have adapted to the day/night cycle by evolving the circadian system, which drives matching rhythms of metabolism, physiology and behaviour (Harmer et al. 2000). These rhythms are generated by the circadian clockwork, which is maintained by the reciprocal negative feedback regulation of three genes in *Arabidopsis*: CIRCADIAN CLOCK ASSOCIATED 1 (*CCA1*) (Wang and Tobin 1998), LATE ELONGATED HYPOCOTYL (*LHY*) (Schaffer et al. 1998) and TIMING OF CAB EXPRESSION 1 (*TOC1*) (Strayer et al. 2000). According to the current model, the MYB-related transcription factors CCA1 and LHY negatively regulate the expression of *TOC1* by directly binding to its promoter region. Conversely, the pseudo response regulator TOC1 positively regulates the expression of the *CCA1*/*LHY* genes, probably by interacting with specific transcription factors (Alabadi et al. 2001). These elements appear to be essential for oscillator function (Mizoguchi et al. 2002, Mas et al. 2003). Although the approximately 24 h period length oscillations, generated by the core clockwork, are substantially self-sustained, their phase can be reset by light/temperature signals. This process, called entrainment, is crucial to synchronize the clock with the actual environment ensuring that clock-driven rhythmic changes occur at the appropriate time of the day. To be entrainable, the circadian clockwork must be connected to sensors monitoring the ambient light and temperature. It has been established that light-signalling pathways initiated by both phytochromes and cryptochromes regulate clock components to achieve entrainment (for a review see Fankhauser and Staiger 2002). Thus,

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light, photoreceptors and photoreceptor-initiated signalling play a dual role in plant development: they regulate photomorphogenesis and entrainment of the circadian system, which in turn makes most of the photomorphogenic responses rhythmic. The intimate relationship between light signalling for photomorphogenesis and phototransduction to the central clockwork is underlined by the fact that all components of the circadian clockwork display aberrant circadian and photomorphogenic phenotypes when mutated.

In contrast to the photoreceptors, the signalling (phototransduction) pathways and their components that mediate entrainment of the circadian clock (often called also light input pathways) are poorly understood. As for the phytochrome-controlled signalling to the circadian clockwork, discovery of a phytochrome A (phyA) and phytochrome B (phyB) interacting transcription factor, designated PIF3 (Ni et al. 1998) drew much attention for the following reasons. PIF3 was found to interact with the light-activated conformers (Pfr) of phyA and phyB (Ni et al. 1999) and form a ternary complex in vitro with the G-box elements of promoters of genes encoding key regulators of photomorphogenesis and essential components of the circadian clock, namely *CCA1* and *LHY* (Martinez-Garcia et al. 2000). These data, together with results of micro-array analysis of phytochrome modulated transcription (Tepperman et al. 1998) and observations indicating that overexpression or lack of PIF3 protein in transgenic plants (Ni et al. 1998) and in a genetic mutant (*photocurrent 1*, *poc1*; Halliday et al. 1999) indeed altered a variety of photomorphogenic responses, led to a very attractive model to explain the molecular mechanism of light signalling. Accordingly, it was postulated that phytochromes, through PIF3 and other yet unidentified factors, launch a transcriptional cascade by regulating transcription of a master set of regulators such as *CCA1* and *LHY* and then these regulators control the transcription of those later genes that represent more terminal steps of signalling. Moreover, it has been demonstrated that TOC1 interacts with PIF3 (and other PIF3-like bHLH factors) in the yeast two-hybrid assay, which could explain the molecular mechanism by which TOC1 regulates *CCA1*/*LHY* expression and suggests an important role for PIF3 not only in the light input pathway, but also in the core oscillator mechanism itself (Yamashino et al. 2003).

However, the postulated central role of PIF3 acting as a key positive regulator in phytochrome-mediated signalling and as an important TOC1 co-factor has been recently challenged. A series of articles reported that (i) *PIF3* acts as a negative regulator of phyB signalling, (ii) it degrades rapidly after irradiation of dark-grown seedlings, (iii) its light-induced degradation is controlled by phytochromes which process is (iv) mediated by the 26S proteosome (Kim et al. 2003, Bauer et al. 2004, Park et al. 2004). These experiments showed that somewhat unexpectedly, phytochrome-initiated degradation of negative regulatory factors represents an important step in light signalling. However, the apparent transient nature of PIF3 raised an intriguing question concerning the in vivo significance of the phyB Pfr–PIF3–DNA ternary complex in mediating phototransduction to the circadian clock and of the TOC1–PIF3 complex in maintaining the oscillator mechanism, especially in constant light conditions.

To ultimately clarify the function of PIF3 in the circadian system, we raised transgenic plants expressing the *CHLOROPHYLL A/B BINDING PROTEIN:LUCIFERASE*$^+$ (*CAB:LUC*$^+$), *COLD-CIRCADIAN RHYTHM-RNA BINDING 2* (*CCR2*)*:LUC*$^+$ and *CCA1:LUC*$^+$ circadian reporters in a PIF3 overexpressor background or in mutants lacking a detectable amount of PIF3. Luciferase imaging allowed collection of data with exceptional time resolution in vivo from a large population of seedlings. We monitored period length and phase of the rhythmic expression of these genes under diurnal and free running conditions, assessed the effect of various PIF3 levels on light-induced resetting of the clock by constructing phase response curves (PRCs) and characterized the transcription profile of *CCA1* and *LHY*. Data obtained were analysed by appropriate mathematical/statistical methods. In addition, we complemented a *pif3* null mutant by expressing a *PIF3* promoter-driven *PIF3–YFP* transgene and monitored localization and abundance of the PIF3–YFP fusion protein under diurnal and free running conditions. These results were further supported by Western analysis and measuring the abundance of an ectopically expressed PIF3–LUC$^+$ fusion protein via luciferase enzyme assays. Our data suggest that PIF3 is not required to mediate phototransduction (input signalling) to the central oscillator or for the oscillator mechanism itself. Moreover, we show that in seedlings grown under LD cycles, light-driven phytochrome-mediated degradation of PIF3 differs from that found in etiolated seedlings.

Results

Experiments to define function for PIF3 within the circadian system

To test the postulated functions of *PIF3* for the *Arabidopsis* circadian system we used several complementing experimental approaches. First, we monitored the rhythmic expression of well-characterized clock-controlled genes in transgenic lines overexpressing, and in a mutant lacking a detectable amount of, the PIF3 protein. To this end we introduced the *CCR2:LUC*$^+$ and *CAB:LUC*$^+$ standard circadian reporters into the *poc1* mutant or in transgenic plants expressing the PIF3–rsGFP fusion protein under the control of the viral *35S* promoter in the wild-type (WT) background (PIF3-OX, Bauer et al. 2004). We note that expression of either the *35S:PIF3–rsGFP* or the *PIF3:PIF3–YFP* transgene complemented the *poc1* mutant phenotype. Fig. 1 shows that red-light-induced inhibition of hypocotyl elongation of the complemented lines is nearly identical to that of the wild type. These data indicate that the PIF3–rsGFP and PIF3–YFP fusion proteins are biologically functional both in these complemented and the overexpressing lines described by Bauer et al. (2004).

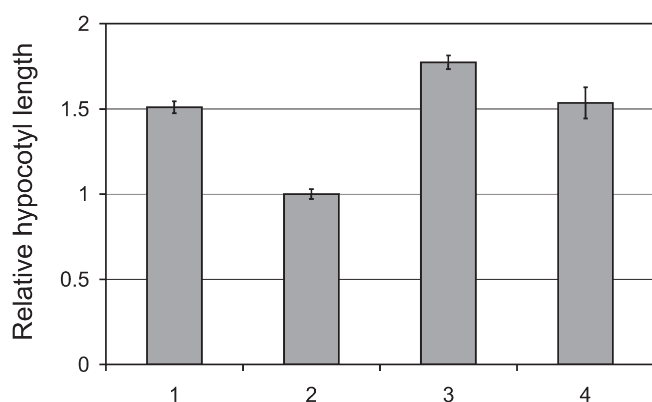


Fig. 1 Expression of the PIF3–rsGFP and PIF3–YFP chimeric proteins complements the *poc1* mutant. Hypocotyl length of 4-day-old *Arabidopsis* seedlings grown in 10 µmol m^{-2} s^{-1} cR was measured. PIF3–rsGFP or PIF3–YFP proteins were expressed under the control of the *35S* or the *PIF3* promoter (columns 3 and 4, respectively) in *poc1* mutant (column 2). The value corresponding to the Wassilewskija background of the *poc1* mutant is also shown (column 1). Hypocotyl length values were normalized to the value corresponding to *poc1*.

The expression pattern of *CCR2:LUC*$^+$ and *CAB:LUC*$^+$ expression was recorded by in vivo luciferase imaging in plants grown under 12 h light/12 h dark cycles (LD) or released to free running conditions [constant dark (DD) and constant light (LL)]. This method provided excellent time resolution and simultaneous measurement of a large number of individual seedlings required for reliable statistical analysis. Period lengths were estimated by the Fast Fourier Transform-Non-Linear Least Squares (FFT-NLLS) analysis by using the Biological Rhythm Analysis Software System (BRASS, Southern et al. 2005). Fig. 2A, C, D, F convincingly documents that period lengths of the *CCR2:LUC*$^+$ and *CAB:LUC*$^+$ reporters under any light conditions do not differ significantly in the various lines, i.e. they are not affected by the varying levels of the PIF3 protein.

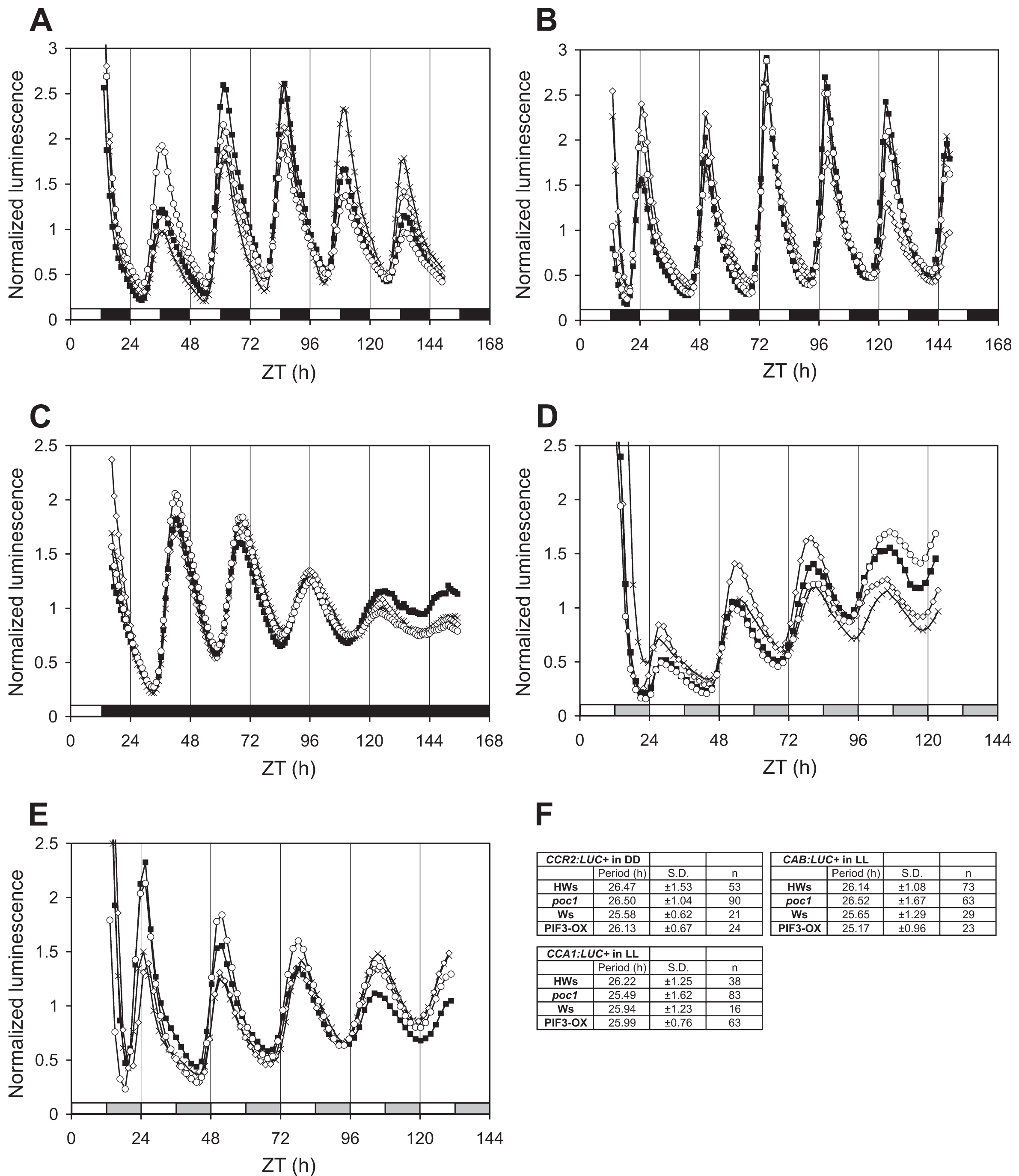
These data indicate that PIF3 does not play a role in the central clockwork or output pathways mediating oscillating expression of these reporters. These data are corroborated by the fact that the period length of rhythmic leaf movement in the *poc1* mutant is also undistinguishable from that of the wild type (K. Halliday, personal communication). To test whether abundance of the PIF3 protein does affect light-induced and/or rhythmic expression of *CCA1* and *LHY*, we also determined the period length of the *CCA1:LUC*$^+$ reporter and light inducibility of the *CCA1* and *LHY* mRNA in etiolated seedlings representing these genetic backgrounds. Fig. 2B, E, F documents that the period length of the *CCA1:LUC*$^+$ reporter, similarly to those of *CCR2:LUC*$^+$ and *CAB:LUC*$^+$ reporters, is identical in all lines studied. As for light inducibility, Fig. 3A, B shows that red-light-induced transcript accumulation of *CCA1* and *LHY* mRNA, respectively, measured for up to 18 h, follows the expected pattern. They display the so-called acute response mediated by clock-independent, phytochrome-controlled signalling (Anderson et al. 1997) in about 1 h after the onset of red light treatment. Afterwards the levels decrease first and rise again after 12 h, culminating in the appearance of the first circadian maximum later.

Fig. 3, however, also shows that not only the patterns but also the steady-state levels of *CCA1* and *LHY* mRNAs are identical in the *poc1* mutant and corresponding wild type. These data demonstrate that neither the light-inducible nor the circadian-responsive transcription of *CCA1* and *LHY* genes is compromised by the lack of PIF3.

PIF3 was hypothesized to mediate phototransduction to the circadian clock by directly interacting with the Pfr conformers of phyA and phyB and the promoter of the *CCA1* and *LHY* genes (Martinez-Garcia et al. 2000). We showed above that the absence of PIF3 does not significantly change light-induced and rhythmic expression of these genes and the period length of various circadian reporters. To investigate the role of PIF3 specifically in the light input pathway, we measured red-light-induced phase shifts of the *CCR2:LUC*$^+$ rhythms free running in DD in *poc1*, PIF3-OX and wild-type backgrounds and constructed PRCs. PRCs are useful and sensitive tests to detect slight alterations in the activity of the light input pathway (Johnson 1992). PRCs shown in Fig. 4. display the expected shape: light pulses applied during the early subjective night-induced phase delays, while pulses during the late subjective night/early subjective day caused phase advances. However, the magnitude of the phase changes was not significantly affected by the lack or overexpression of PIF3 (Fig. 4A, B, respectively), demonstrating that PIF3 plays a marginal if any role in mediating red-light-induced resetting of the *Arabidopsis* circadian system.

Taken together these data indicate that PIF3 is not involved in the signalling pathways mediating (i) light- and circadian clock-responsive expression of *CCA1* and *LHY*, (ii) red-light-induced phototransduction to the central clockwork, and (iii) PIF3 is dispensable for the function of the circadian system that mediates rhythmic expression of the circadian reporters used in this study. To test whether expression of *PIF3* itself is induced by light or subjected to regulation by the circadian system we monitored the expression pattern of a chimeric gene containing the *PIF3* promoter fused to the *LUC*$^+$ reporter in transgenic lines. Fig. 5A, B shows that expression of the *PIF3:LUC*$^+$ reporter is not regulated by light but displays a characteristic, low-amplitude oscillation, reminiscent of regulation by the circadian clock. On the one hand these data corroborate and extend our earlier findings (Bauer et al. 2004) and indicate that the trancription of *PIF3* is not regulated by light. On the other hand, these data suggest that at least the activity of the *PIF3* promoter is influenced by the circadian clock.

Finally, we determined whether the lack or overexpression of PIF3 impairs signalling for flowering time in *Arabidopsis*. These experiments were initiated by a recently



CCR2:LUC+ in DD			
	Period (h)	S.D.	n
HWs	26.47	±1.53	53
poc1	26.50	±1.04	90
Ws	25.58	±0.62	21
PIF3-OX	26.13	±0.67	24

CAB:LUC+ in LL			
	Period (h)	S.D.	n
HWs	26.14	±1.08	73
poc1	26.52	±1.67	63
Ws	25.65	±1.29	29
PIF3-OX	25.17	±0.96	23

CCA1:LUC+ in LL			
	Period (h)	S.D.	n
HWs	26.22	±1.25	38
poc1	25.49	±1.62	83
Ws	25.94	±1.23	16
PIF3-OX	25.99	±0.76	63

Fig. 2 PIF3 does not affect the period length of free running rhythms. Seven-day-old LD-grown seedlings carrying *CCR2:LUC*$^+$ (A, C), *CCA1:LUC*$^+$ (B, E) and *CAB:LUC*$^+$ (D) were transferred to LD (A, B), DD (C) and LL (D, E) light regimes and the emitted luminescence was monitored for 5–6 d. Normalized luminescence values are plotted against ZT (Zeitgeber Time, ZT zero corresponds to the time of the last dark to light transition before the onset of the constant conditions). HWs (crosses), *poc1* (open diamonds), Ws, (squares) and PIF3-OX (open circles) genetic backgrounds were examined. White, black and grey rectangles on the horizontal axis represent light, dark and subjective night periods, respectively. (F) Period length values were calculated as the variance-weighted means (Period) with variance-weighed standard deviations (SD). The numbers of seedlings analysed are also shown (*n*).

published report (Oda et al. 2004). These authors used in their experiments a PIF3 antisense transgenic line (Ni et al. 1998) reported to have a possible second site mutation (Monte et al. 2004). To determine whether data reported by Oda et al. could be recapitulated in a true *pif3* loss-of-function background, or could be supported by the flowering phenotype of PIF3 over-

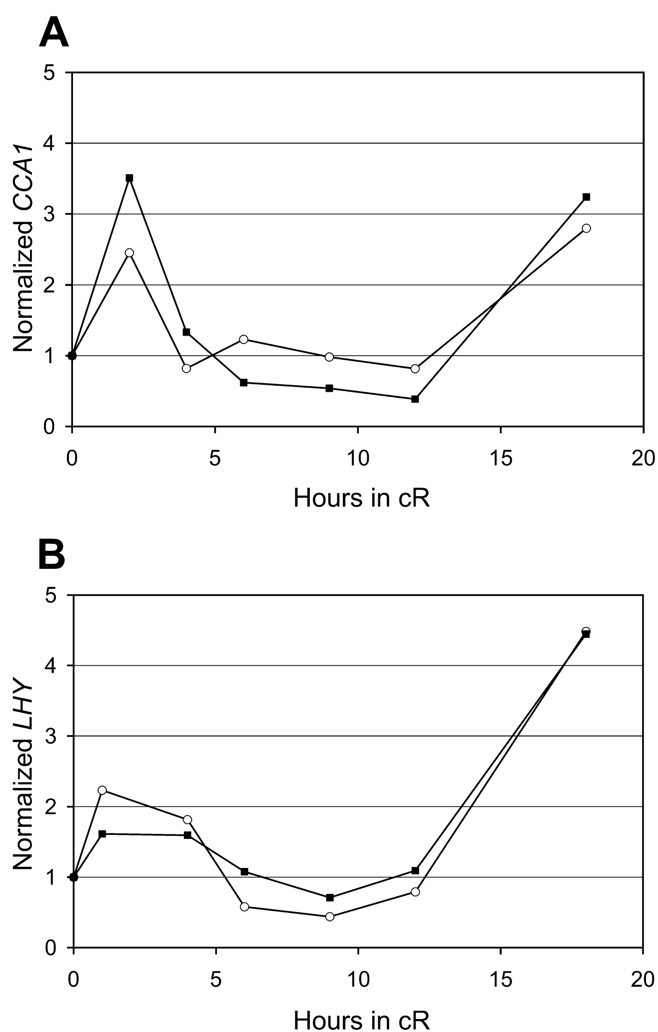


Fig. 3 Red-light-induced expression of CCA1 and LHY is independent of PIF3. four-day-old etiolated WT and *poc1* seedlings were illuminated with red light and tissue samples were collected at the times indicated. *CCA1* (A) and *LHY* (B) transcript levels were determined by Northern blot. Quantified and normalized values are presented on the graphs: open circles represent wild-type (WT), and filled squares denote *poc1* genetic background, respectively.

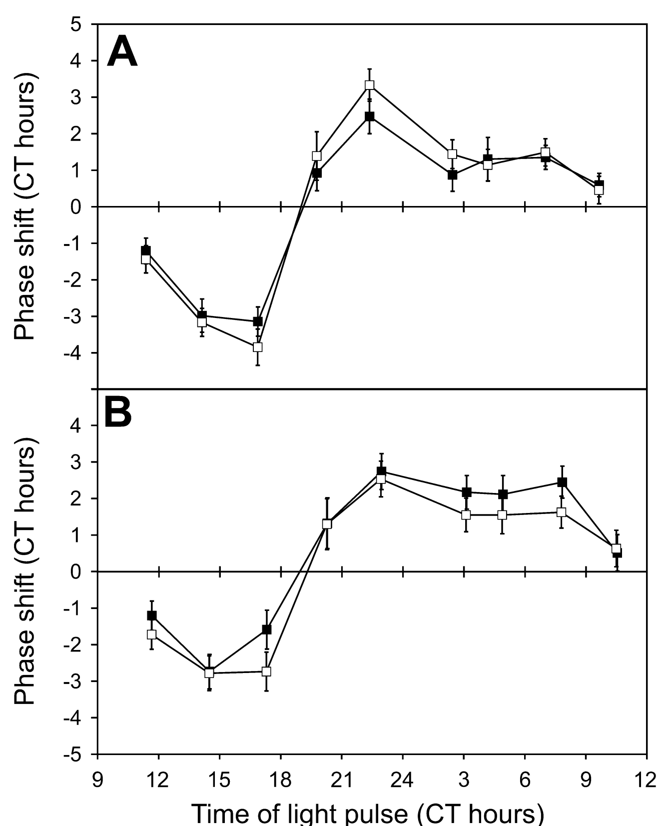


Fig. 4 PIF3 does not play a role in the light-induced resetting of the circadian clock. PRCs for *poc1* (A, open squares), PIF3-OX (B, open triangles) and the corresponding wild-type plants (filled symbols) were constructed. Phase shifts of the rhythm of *CCR2:LUC*$^{+}$ expression triggered by red light pulses (15 μmol m^{-2} s^{-1} for 1 h) are plotted against the circadian time of the light pulse was given. Phase advances are shown as positive values, while phase delays are shown as negative values. Error bars represent ± SE values.

expression, we tested the *poc1* mutant and the PIF3-OX lines for flowering time phenotypes. Fig. 6 shows that the lack or overexpression of PIF3 does not affect flowering time in the mutant plants. No significant difference was observed between *poc1*, PIF3-OX and wild-type plants growing either under short day (8 h light/16 h dark) or long day (16 h light/8 h dark) conditions regarding the number of rosette leaves at the time of bolting or the number of days passed from germination to flowering (data not shown).

Localization and abundance of the PIF3 protein displays an unexpectedly complex pattern in plants growing under diurnal conditions

Our data show that (i) *PIF3* promoter activity does not display a light responsiveness whereas (ii) abundance of the PIF3 protein had been shown to be controlled by light. In particular, PIF3 levels were shown to decrease below the detection limit in immunoblot analysis in a few hours after onset of the photomorphogenic developmental programme in young seedlings (Bauer et al. 2004). Other studies have shown that this process is accompanied by polyubiquitination of PIF3 and is dependent on a functional proteasome pathway (Park et al. 2004). To test whether PIF3 levels are permanently reduced in light-grown plants, seedlings expressing PIF3–YFP under the control of the *PIF3* promoter were entrained to LD cycles and localization of the fusion protein was analysed by microscopy. As shown in Fig. 7 we monitored the localization of the PIF3–YFP fusion protein at the middle of the day phase (MOD –7A), 1 h before the end of the day phase (EOD –7B), at the middle

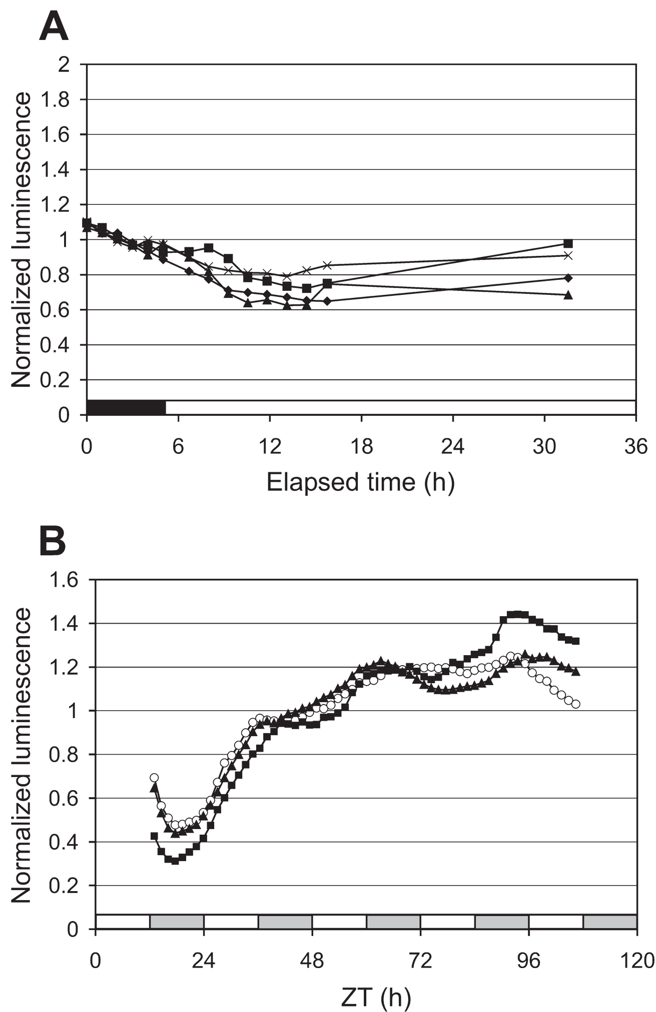


Fig. 5 *PIF3:LUC*+ is not light induced but displays a low amplitude circadian rhythm. (A) 4-day-old etiolated seedlings expressing the *PIF3:LUC*+ transgene were illuminated with different wavelength of light: 10 µmol m^{-2} s^{-1} R (diamonds), 1 µmol m^{-2} s^{-1} FR (squares), 70 µmol m^{-2} s^{-1} WL (triangles) or were kept in darkness (crosses). Normalized luminescence values are plotted against the elapsed time. Rectangles on the horizontal axis symbolize dark and light conditions (black and white, respectively). (B) 7-day-old LD-grown seedlings expressing the *PIF3:LUC*+ transgene were transferred to red LL conditions and the emitted luminescence was normalized and plotted against ZT. Values from three independent transgenic lines (#1, #2, #3) are presented (symbolized as squares, triangles and open circles, respectively). Rectangles on the horizontal axis symbolize subjective light and subjective dark conditions (white and grey, respectively).

of the night phase (MON –7C) and at the end of the night phase (EON –7D). The PIF3–YFP fusion protein is not detectable at MOD, but it accumulates to surprisingly high levels in hypocotyl cells near to the hook region of the hypocotyl at EOD. In contrast, the fusion protein is not detectable in nuclei of these plants at MON and only occasionally visible in a few nuclei at EON. These data indicate that (i) light-induced, phytochrome-mediated degradation of PIF3 is completed by the middle of the day (ii) then PIF3 accumulates to detectable levels by the end of the day, presumably because during this period light-induced degradation of PIF3 is inhibited; (iii) degradation of PIF3 apparently occurs in the first part of the night and (iv) reaccumulation of PIF3 does not reach significant levels in the remaining hours of the night.

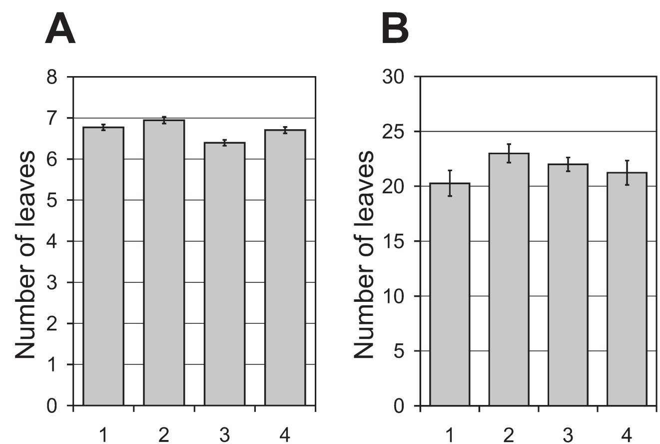


Fig. 6 The effect of PIF3 on flowering time. The number of rosette leaves at the time of bolting under long day (A) and short day (B) conditions are shown. 1: HWs, 2: *poc1*, 3: Ws, 4. PIF3-OX. Error bars represent ± SE values.

To test whether this surprisingly complex pattern is maintained under constant conditions, 12L/12D entrained plants were transferred to DD or LL at the end of the day phase and localization of the fusion protein was monitored during the next subjective day and night. In plants transferred to DD accumulation of PIF3–YFP reached high levels after 18 h darkness (Fig. 8C) and kept rising afterwards. Fig. 8D illustrates PIF3–YFP levels after 30 h incubation in dark. Fig. 8 also shows that in control plants grown under LD cycles no YFP signals were detectable at the same time points (Fig. 8A, B, respectively). In contrast, in plants transferred to LL accumulation of the fusion protein was not detectable at any time points tested at 24, 30, 36, 42 and 48 h after the light-on signal (data not shown).

Taken together, these data indicate that in plants grown under 12L/12D cycles (i) light-driven degradation of PIF3 is inhibited in the later part of the light phase, (ii) degradation of PIF3 is completed in the first hours of the dark phase and (iii) that 12 h dark incubation is not sufficient to induce strong reaccumulation of PIF3. However, our data show that in extended darkness (re-etiolation) accumulation of PIF3 reaches high levels, similar to that found in etiolated seedlings (Bauer et al. 2004). This finding indicates that the inhibitory signal blocking degradation of PIF3 around the end of day in LD-grown plant material is transient. This conclusion is further supported by the fact that abundance of PIF3 decreases below detection levels in plants kept longer than 16 h in continuous light (data not shown).

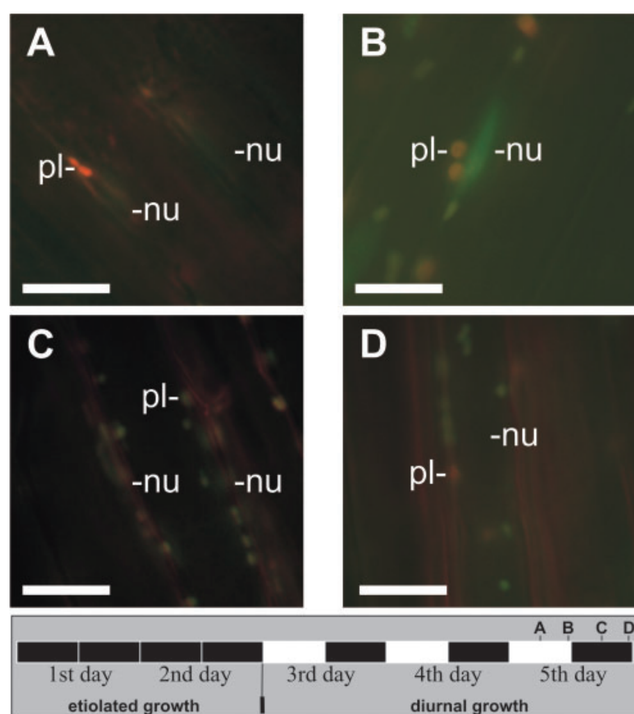


Fig. 7 Intracellular localization of the PIF3–YFP fusion protein under diurnal conditions. *Arabidopsis* seedlings expressing PIF3–YFP fusion protein under the control of the *PIF3* promoter were grown for 2 d in darkness before transfer to 12 h WL/12 h darkness cycles. On the third cycle hypocotyl cells near to the hook region were analysed by epifluorescence and bright-field microscopy at middle of day (A, 6 h light, MOD), end of day (B, 11 h light, EOD), middle of night (C, 6 h darkness, MON) and end of night (D, 11 h darkness, EON) time points. YFP fluorescence (green channel) and chlorophyll fluorescence (red channel) of each cell were collected sequentially by specific filter sets and a Coolsnap HQ camera (Roper scientific) and afterwards combined into overlaid images. Nu points to nuclei, scale bars indicate 10 μm.

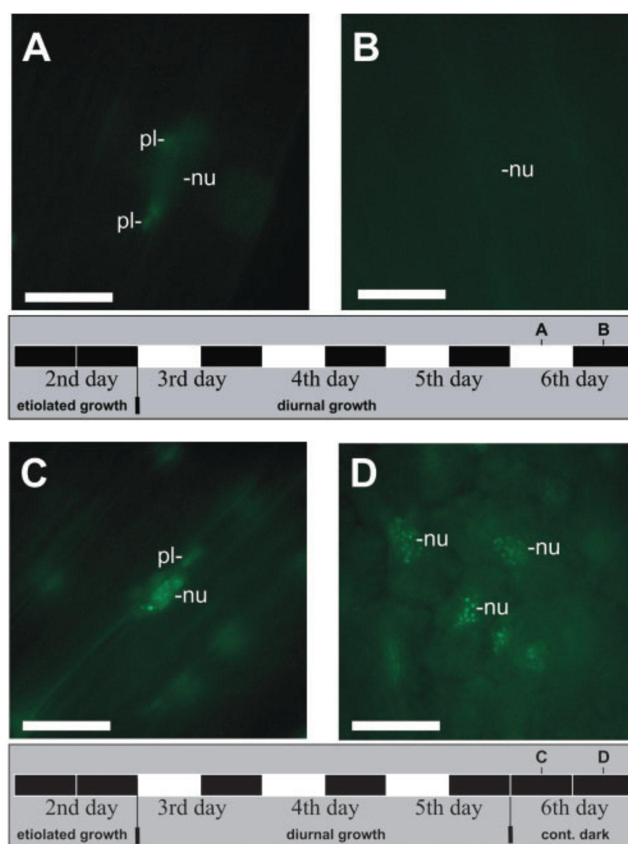


Fig. 8 Intracellular localization of the PIF3–YFP fusion protein after transfer of seedlings to constant darkness. *Arabidopsis* seedlings expressing PIF3–YFP fusion protein under the control of the *PIF3* promoter were grown for 2 d in darkness before transfer to 12 h WL/12 h darkness cycles. After the third cycle the seedlings were either kept in cycling conditions (A, B) or transferred to continuous darkness (C, D). Hypocotyl cells near to the hook region (A, B, C) or cotyledon cells (D) were analysed by epifluorescence microscopy. The time points analysed were middle of day (A) and middle of night (B) or middle of subjective day (C) and middle of subjective night (B) time points as outlined in the graphs. YFP fluorescence was detected by a specific filter set and an Axiocam camera (Zeiss). Nu points to nuclei, scale bars indicate 10 μm.

Although we demonstrated earlier (Bauer et al. 2004) that the fluorescence intensity reflects perfectly the amount of the PIF3–rsGFP fusion protein, we attempted to support the present observations by Western analysis. PIF3:PIF3–YFP expressing plants were grown as for microscopy and samples were harvested on the fifth day of growth at MOD, EOD, MON and EON (according to Fig. 7). A sample from 5-day-old etiolated plants was also included as a control. The YFP antibody detected high levels of the PIF3–YFP fusion protein in the extract from etiolated plants, but very low amounts in samples from the LD-grown plants (Fig. 9A). Using a dilution series from the etiolated sample on a separate Western blot, it was demonstrated that in LD-grown plants PIF3–YFP levels decrease to about 10% of its level in etiolated seedlings (data not shown). However, the very low signals from the LD samples prevented the precise quantitative comparison of PIF3–YFP levels among these extracts. To overcome the problem with the low sensitivity and resolution of the Western analysis, we generated transgenic plants expressing the PIF3–LUC^+ fusion protein. The plants were grown and harvested under the same conditions and at the same time as for the Western analysis and the relative amount of the fusion protein was determined by in vitro luciferase assay. Similarly to the immunoblot results, the data in Fig. 9B clearly show an approximately 10-fold difference in luciferase activity between extracts from etiolated vs. LD-grown plants; but more importantly, similarly to the microscopy results, demonstrate the transient increase in luciferase activity in the EOD samples. Fig. 9C shows that the PIF3–LUC^+ fusion protein is functional since its expression in the *poc1* mutant complements the mutant photomorphogenic phenotype. This result validates the data obtained by the luciferase assays.

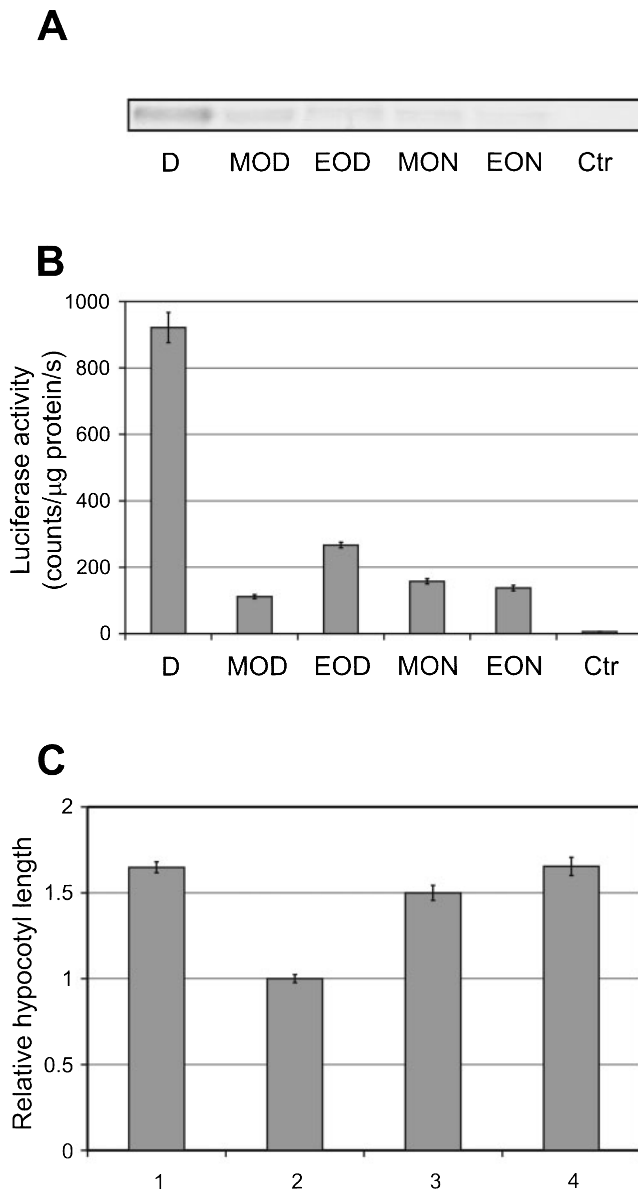


Fig. 9 Diurnal oscillations in the abundance of PIF3–YFP and PIF3–LUC$^+$ fusion proteins. (A) Detection of the PIF3–YFP fusion protein by YFP antibody. MOD, EOD, MON and EON denote the time of harvesting of the LD-grown plants (see legend for Fig. 7). D: 5-day-old etiolated plants, Ctr: non-transformed wild-type plants (control). Total proteins (20 µg) from each extract were separated on the gel. (B) Detection of the PIF3–LUC$^+$ fusion protein by in vitro luciferase assays. Transgenic seedlings expressing *35S:PIF3–LUC*$^+$ were grown under the same conditions and harvested at the same times as in (A). Total proteins (20 µg) from each extract were subjected to luciferase measurements. Error bars represent ± SE values. (C) Expression of the PIF3–LUC$^+$ fusion protein complements the *poc1* mutant. Hypocotyl length of 4-day-old *Arabidopsis* seedlings grown in 10 µmol m^{-2} s^{-1} cR was measured. Columns 3 and 4 correspond to two independent transformant lines expressing the PIF3–LUC$^+$ protein in *poc1* mutant (column 1). The value corresponding to the Wassilewskija background of the *poc1* mutant is also shown (column 2). Hypocotyl length values were normalized to the value corresponding to *poc1*.

Taken together, we used three independent methods to analyse changes in PIF3 protein abundance in 6-day-old seedlings grown in 12L/12D cycles. The results clearly demonstrate that (i) PIF3, albeit at a very low level, is present during the LD cycle; (ii) its level does not significantly increase during the night, but shows a characteristic diurnal rhythm with maxima around the end of the day.

Discussion

The biological function, organization and molecular structure of the plant circadian system has been extensively studied in recent years. These experiments revealed that (i) the *Arabidopsis* circadian clock modulates transcription of genes representing about 10% of the genome (Harmer et al. 2000) (ii) the molecular principles of the plant clockwork are similar to that described in *Drosophila* and human cells thus a negative regulatory feedback loop consisting of the *CCA1*/*LHY* and *TOC1* genes is absolutely required for a functional clockwork (Alabadi et al. 2001), (iii) phototransduction to the plant clock is mediated by a set of defined photoreceptors (Somers et al. 1998). A series of recent papers implied that one of the identified phyB interacting proteins, namely PIF3, is likely to play a role in a functional circadian system for the following reasons: (i) PIF3 could act directly in light signalling to the clock by forming a ternary complex with the G-box element of the *CCA1* and *LHY* genes and the Pfr conformer of phyA/B (Martinez-Garcia et al. 2000), (ii) PIF3 could mediate the positive effect of TOC1 on *CCA1*/*LHY* transcription by interacting simultaneously with the *CCA1*/*LHY* promoters and the TOC1 protein. These hypotheses had been challenged recently by reports documenting that the PIF3 protein degrades rapidly in a light-induced fashion in etiolated seedlings exposed to light of various wavelengths (Bauer et al. 2004, Park et al. 2004). These results indicated that the PIF3 protein is light labile, thus its most likely function is to mediate light signalling at an early phase of development and/or dark/light transition. To clarify the function of PIF3 protein for a functional circadian system in *Arabidopsis* we determined whether *pif3* mutants affect phototransduction to the clock, function of the central clockwork and other clock-regulated physiological responses such as flowering time. In order to provide additional support to conclusions drawn from these experiments we also determined the accumulation level and pattern of the PIF3 protein in young seedlings grown under LD cycles.

Here we provide conclusive evidence that PIF3 does not play a significant role in mediating light input to the central clockwork. Altering the activity of the light input is expected to affect the phase and/or the period length of the overt rhythms in a light-dependent manner and definitely would result in a change of magnitude of the PRC reflecting the efficiency of light-induced resetting. We show that (i) the manipulation of PIF3 levels (use of PIF3-OX and *pif3* null lines) had no effect on the phase or period length of several circadian markers

including the circadian output *CAB* and *CCR2* reporters and the clock component *CCA1* itself (Fig. 2) under any light conditions and (ii) the PRCs established in wild type and *pif3* mutants were nearly identical (Fig. 4). In addition we demonstrate that the transcription profile of *CCA1* and *LHY*, including the acute response and the appearance of the first circadian peak, was also insensitive to varying PIF3 levels. These data clearly indicate that light-induced expression of *CCA1* and *LHY* is not mediated by PIF3, thus the postulated ternary complex consisting of phyB, PIF3 and the G-box element of the *CCA1*/*LHY* promoters does not play a significant role in planta in regulating expression of these genes or in the light input pathway to the oscillator. Taken together, these data corroborate and extend results published recently by Monte et al. (2004). These authors showed that neither the rhythmic oscillation of *LHY* mRNA under free running conditions nor its light-induced transcription is impaired in a *pif3* null mutant.

It has been suggested previously that the apparent lack of a robust circadian phenotype of *pif3* mutants could be explained by the potential functional redundancy of the numerous bHLH type transcription factors (Oda et al. 2004). This hypothesis was based on the high-level functional redundancy found between *CCA1* and *LHY*. In this case the single *cca1* loss-of-function mutant shows a relatively weak short period phenotype (Green and Tobin 1999), in contrast to the *cca1*/*lhy1* double mutant (Mizoguchi et al. 2002), whereas overexpression of CCA1 resulted in an arrhythmic phenotype (Wang and Tobin 1998). Thus our results with PIF3-OX plants indicate that this explanation is unlikely since the PIF3-OX plants used in this study showed at least a 3- to 4-fold overexpression of the PIF3–rsGFP protein compared with the endogenous PIF3 protein and displayed a clear photomorphogenic phenotype (Bauer et al. 2004).

To further substantiate our conclusions we provide evidence that the PIF3–rsGFP, PIF3–LUC$^+$ and PIF3–YFP fusion proteins are biologically functional by showing that the *poc1* mutant is efficiently complemented by expressing them under the control of the constitutive *35S* or the endogenous *PIF3* promoter, respectively (Fig. 1, 9). These data are in good agreement with results published by Kim et al. (2003) and Bauer et al. (2004). Although PIF3 is not required for a functional circadian clock we determined whether its expression is light induced or is subject to regulation by the circadian clock. We found as shown in Fig. 5 that the *PIF3* promoter confers circadian responsiveness but not light inducibility to the *LUC*$^+$ reporter. We note, however, that this low amplitude oscillation can only be measured by the highly sensitive luciferase imaging method and we failed to detect oscillation by monitoring changes in *PIF3* mRNA (data not shown).

We and others showed that light induces rapid degradation of PIF3 (Bauer et al. 2004, Park et al. 2004). In good agreement with these results Monte et al. (2004) reported that the GUS–PIF3 fusion protein indeed turns over rapidly when etiolated seedlings are exposed to light. However, these authors found that the GUS–PIF3 fusion protein was also detectable, although at very low levels in plants grown under continuous light or during the day phase in plants grown under diurnal conditions, but it is reaccumulated to high levels by the end of the night phase. Thus Monte et al. (2004) concluded that PIF3 might have a broader function in phytochrome signalling rather than only transiently functioning during the early stage of seedling development. Our data obtained by analysing localization and accumulation of the biologically functional PIF3–YFP and PIF3–LUC$^+$ fusion proteins in seedlings grown under diurnal cycles confirm and extend these results. Fig. 7, 9 illustrate that the PIF3–YFP and PIF3–LUC$^+$ fusion proteins are indeed detectable and their abundance changes during a 24 h period in plants grown under 12L/12D cycles. However, in contrast to Monte et al. (2004), we detect (i) only low reaccumulation during the night phase of the cycles, which is enhanced under extended dark conditions but (ii) a pronounced increase in the level of the PIF3–YFP/LUC$^+$ fusion proteins at the end of the light phase. We emphasize the fact that we obtained the same accumulation patterns in plants expressing the PIF3–YFP fusion protein under the control of the constitutive *35S* promoter (data not shown), indicating that the endogenous *PIF3* promoter does not contribute to the observed changes in abundance of the PIF3 fusion proteins. The discrepancy between the two transgenic studies may be better explained by (i) the different structure and stability of the GUS–PIF3 versus PIF3–YFP/LUC$^+$ fusion proteins and/or (ii) the different monitoring methods. At present we know that (i) accumulation of the inhibitory signal/factor reaches significant levels only after exposing the seedlings to at least 6–8 h continuous light and (ii) it seems to be transient since we failed to detect rhythmic reaccumulation of PIF3 in continuous light or darkness. In our assays accumulation of nuclear-localized PIF3 drops below detection level after extended illumination or keeps rising in extended darkness. Thus our data and those reported by Monte et al. (2004) indicate that the circadian system does not play a major role in controlling degradation/accumulation of PIF3; it is driven by a diurnal rhythm. Independent of the differences regarding the reaccumulation pattern of PIF3 in LD-grown plants, both of these studies point to a potentially broader physiological function of PIF3 in photomorphogenic development. bHLH proteins function as homo- and heterodimers, so it is interesting to speculate whether the low PIF3 levels detected in LD cycles are sufficient to modify the stoichiometry of homo- and heterodimerization of related bHLHs, thus regulating specific aspects of light response. The mechanism by which PIF3 is targeted to the proteosome pathway is still unknown. It is generally accepted that modification of the target protein is required for recognition by the 26S proteasome. In plants, phosphorylation has been demonstrated to mediate degradation of proteins involved in light signalling (Hardtke and Deng 2000, Duek et al. 2004). Phytochromes have been shown to posses serine/threonine kinase activity (Yeh and Lagarias 1998). Light-induced degradation of PIF3 is mediated by the con-

certed action of phyA, phyB and phyD (Bauer et al. 2004), and Ryu et al. (2005) have suggested that fine tuning of phy signalling requires kinase activity. Thus it is possible that degradation of PIF3 in response to light requires phosphorylation by the Pfr forms of these phytochromes.

Since PIF3–YFP and PIF3–LUC^+ are functional and show the same kinetics of light-induced degradation, we plan to use these reporters in specific mutants to unravel (i) the yet unknown mechanism which mediates gating of the light-induced degradation of PIF3 at the end of the day and (ii) the additional physiological role of PIF3 in plant growth and development.

Materials and Methods

Molecular cloning and generation of transgenic plants

Creation of *CAB:LUC*$^+$, *CCR2:LUC*$^+$ and *CCA1:LUC*$^+$ binary vector constructions were described previously (Hall et al. 2001, Doyle et al. 2002). The 2.5 kb *PIF3* promoter was amplified from genomic DNA template isolated from wild-type Columbia ecotype with the ProofSprinter polymerase system (AGS, Heidelberg, Germany). The promoter was cloned as a *Hin*dIII–*Bam*HI fragment into a pPCVB812 binary vector (Bauer et al. 2004), which contained the *PIF3* coding region fused to the coding region of *YFP* (Clontech, Palo Alto, CA, USA) and to pPCV812 vector containing the *LUC*$^+$ reporter gene (Tóth et al. 2001). The *35S:LUC*$^+$ pPCV812 vector (Bognar et al. 1999) was used to create the *35S:PIF3–LUC* construct by inserting the *PIF3* coding region as a *Bam*HI–*Xho*I fragment. All DNA manipulation techniques were performed according to standard protocols (Sambrook and Russell 2001). The *Agrobacterium*-mediated floral dip transformation method of *Arabidopsis* was performed according to Clough and Bent (1998). The method of selection of transformants was also described earlier (Bauer et al. 2004). The *CAB:LUC*$^+$, *CCR2:LUC*$^+$ and *CCA1:LUC*$^+$ reporter constructs were transformed to *poc1* (Halliday et al. 1999) and PIF3-OX (Bauer et al. 2004) and the corresponding Wassilewskija controls (marked as HWs and Ws, respectively, in the figures). The *PIF3:PIF3–YFP* construct was transformed to *poc1* and Ws backgrounds, while *PIF3:LUC*$^+$ transgene was expressed in Ws. For each construct, we generated and examined at least 15–20 independent homozygous lines.

Plant RNA isolation and Northern blot

Seeds were sown on four layers of wet filter paper and kept for 2 d in darkness at 5°C before germination induction with white light at 25°C for 8 h. Seedlings were grown for 72 h in subsequent darkness and transferred to Rc (LED sources, λ_{max} = 670 nm, 8 µmol m^{-2} s^{-1}). Samples were collected at the indicated timepoints and total RNA was extracted as described (Ádám et al. 1996) from whole seedlings. Ten micrograms of RNA was blotted and probed according to Ulm et al. (2004). Probes for *LHY* and *CCA1* were described earlier by Martinez-Garcia et al. (2000). Radioactive signals were visualized in a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) and were quantified using the ImageQuant 1.1 software. Gene-specific signals were normalized to the corresponding 18S rRNA signals. To aid comparison, values at time point 0 h were set to 1 for each transcript.

Protein assays

Protein isolation and Western analysis was performed according to Bauer et al. (2004).

LUC assays were performed as described (Frohnmeyer et al. 1999). The samples were normalized to the amount of soluble protein present, as determined by the Bradford assay (Bio-RAD, Hercules, CA, USA).

Measurement of flowering time

Seeds were sown on soil and incubated for 2 d in darkness at 4°C. They were subsequently transferred to short-day [8 h white light (WL)/16 h D] or long-day (16 h WL/8 h D) conditions. Light sources were fluorescent (cool white) tubes producing light at a fluence rate of ~60 µmol m^{-2} s^{-1}. Flowering time was recorded as the number of rosette leaves at the time when inflorescences reached a height of 1 cm. The experiment was repeated twice using 40–100 plants in each experiment from each genotype.

Hypocotyl measurement

Seeds were sown on filter paper, stratified and germination was induced as described above. After 16 h of dark treatment seeds were placed under 10 µmol m^{-2} s^{-1} cR and hypocotyls (at least 50 seedlings per different samples) were measured as described by Bauer et al. (2004).

Light induction of PIF3:LUC$^+$

After stratification and germination induction, etiolated seedlings were grown in groups of 25–30 plants on Murashige and Skoog plates. The seedlings were sprayed with sterile 2.5 mM luciferin (Biosynth Ag, Staad, Switzerland) solution 20 h before the onset of different wavelengths of light. The emitted luminescence was perceived with a liquid nitrogen-cooled CCD camera (Princeton Instruments, Trenton, NJ, USA). Processing of data by using MetaView software was performed as described previously by Tóth et al. (2001).

Measurement of circadian rhythm

Transgenic plants expressing the *CAB:LUC*$^+$ or *CCR2:LUC*$^+$ or *CCA1:LUC*$^+$ or *PIF3:LUC*$^+$ reporter after stratification and light induction were grown under 12 h light (fluorescent cool white, ~60 µmol m^{-2} s^{-1}) 12 h dark cycles (LD) for 7 d on MS medium then transferred to 96-well microtitre plates and 15 µl 5 mM luciferin was added to each well. Three types of measurement (DD, LL, LD) were performed in a Topcount NXT luminometer (Packard Instruments) according to Tóth et al. (2001).

Phase response curves

poc1, PIF3-OX and the corresponding wild-type seedlings expressing the *CCR2:LUC*$^+$ reporter were grown under 12 h LD cycles for 5 d, then transferred to the luminometer at ZT12 (lights off) and luminescence was detected for 5 d in constant darkness (DD). After 24 h in DD, individual plates were removed from the luminometer at 3 h intervals, irradiated with 15 µmol m^{-2} s^{-1} red light (LED light sources, λ_{max} = 670 nm) for 1 h and returned to the instrument to resume the measurement. Phase shifts were calculated by comparing the phase values of the pulsed plants with those of the non-pulsed controls. The free running period lengths of the *CCR2:LUC*$^+$ rhythms of the non-pulsed plants were estimated by FFT-NLLS analysis and were used to calculate the circadian time of the light pulses and to convert the magnitude of the phase shifts to circadian hours. The standard error values for the phase shifts were calculated by using the following formula:

$$SE = [(SEp)^2 + (SEnp)^2]^{0.5}$$

where SEp and SEnp are the standard errors for phase values of the pulsed and non-pulsed plants, respectively.

Arabidopsis growth for microscopic analysis and microscopic techniques

Arabidopsis seeds containing the *PIF3:PIF3–YFP* transgene were stratified and germination was induced as described above. Seedlings were grown for 2 d in darkness before transfer to cycles of 12 h WL/12 h darkness in growth cabinets (Ehret GmbH, Emmendingen/Reute, Germany). On the third cycle microscopic analysis of PIF3–YFP localization took place. For epifluorescence and light microscopy, *Arabidopsis* seedlings were handled under dim, green safelight until analysis with an Plan Apochromat Objective (63× and N.A. 1,4) of an Axioplan II microscope (Zeiss, Oberkochem, Germany). Excitation and detection of the YFP fluorophore was performed with a specific YFP filter set (excitation HQ 500/20; beam splitter Q 515 LP; emission HQ 535/30; AHF Analysentechnik Tübingen, Germany). False positive signals derived from chlorophyll fluorescence were excluded by a subsequent analysis using a second filter set (excitation 546/12; beam splitter 585; emission LP 590; Z15, Zeiss, Obercochem, Germany). Representative cells were recorded with a digital CoolSnap HQ camera system (Roper Scientific, by Visitron Systems, Munich, Germany) controlled by Metamorph software (Universal Imaging, Dowingtown, PA, USA). Photographs were processed and overlayed for optimal presentation using ImageJ (NIH, Bethesda, MA, USA) and MS Office 97 (Microsoft, Redmond, WA, USA) software packages. Green channel represents YFP, red channel chlorophyll fluorescence.

On request, all the biological and chemical materials not commercially available that are used for the experiments reported here, will be available in a timely manner for non-profit research.

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