Phytochrome-Regulated PIL1 Derepression is Developmentally Modulated

Yong-sic Hwang 1, 2, 3 and Peter H. Quail 1, 2, *

1 Department of Plant and Microbial Biology, University of California, Berkeley, CA 94720, USA
2 USDA/ARS-Plant Gene Expression Center, 800 Buchanan Street, Albany, CA 94710, USA
3 Department of Bioscience and Biotechnology, Konkuk university, 1 Hwayang-dong, Gwangjin-gu, Seoul 143-701, Korea

We define the photoresponsiveness, during seedling de-etiolation, of PHYTOCHROME-INTERACTING FACTOR 3-LIKE 1 (PIL1), initially identified by microarray analysis as an early-response gene that is robustly repressed by first exposure to light. We show that PIL1 mRNA abundance declines rapidly, with a half-time of 15 min, to a new steady-state level, 10-fold below the initial dark level, within 45 min of first exposure to red light. Analysis of phy-null mutants indicates that multiple phytochromes, including phyA and phyB, impose this repression. Conversely, PIL1 expression is rapidly derepressed by subsequent far-red irradiation of previously red light-exposed seedlings. However, the magnitude of this derepression is modulated over time, in a biphasic manner, in response to increasing duration of pre-exposure to continuous red light; (i) an early phase (up to about 6 h) of relatively rapidly increasing effectiveness of far-red reversal of repression, as declining phyA levels relieve initial very low fluence suppression of this response; and (ii) a second phase (beyond 6 h) of gradually declining effectiveness of far-red reversal, to only 20% of maximal derepression, within 36 h of continuous red light exposure, with no evidence of circadian modulation of this responsiveness, an observation in striking contrast to a previous report for entrained, green seedlings exposed to vegetative shade. These data, together with analysis of phytochrome signaling mutants and overexpressors with aberrant de-etiolation phenotypes, suggest that the second-phase decline in robustness of PIL1 derepression is an indirect consequence of the global developmental transition from the etiolated to the de-etiolated state, and that circadian coupling of derepression requires entrainment.

Keywords: De-etiolation — Derepression — Early response gene — Phytochrome — PHYTOCHROME-INTERACTING FACTOR 3-LIKE 1 (PIL1).

Abbreviations: bHLH, basic helix–loop–helix; FRc, continuous far-red light; FRp, far-red light pulse; phy, phytochrome; PIF, PHYTOCHROME-INTERACTING FACTOR; PIL1, PHYTOCHROME-INTERACTING FACTOR 3-LIKE 1; Rc, continuous red light; Rp, red light pulse; VLFR, very low fluence response.

*Corresponding author: E-mail, quail@nature.berkeley.edu; Fax, +1-510-559-5678.
phy-induced phosphorylation and degradation have more recently been made for the related phy-interacting bHLH factor, PIF5 (Shen et al. 2007). The data suggest that phosphorylation of these factors may flag them for degradation via the ubiquitin–proteosome system. Although the question of the identity of the responsible kinase remains open, the data suggest that phy-induced phosphorylation may represent the primary biochemical intracellular signaling transaction from the photoactivated photoreceptor molecule to its signaling partners.

Microarray analysis indicates that the expression of about 10% of the genes in the Arabidopsis genome is altered by light during the seedling de-etiolation process (Tepperman et al. 2001, Monte et al. 2004, Tepperman et al. 2004, Jiao et al. 2005, Ma et al. 2005, Tepperman et al. 2006, Jiao et al. 2007). Of these, about 10% (~250 genes) are altered within 1 h of initial exposure of dark-grown seedlings to an R signal (Tepperman et al. 2006, Quail 2007). These ‘early response’ genes are of particular interest because they are the ones that are most likely to be direct targets of the phy signaling system. PIL1, a member of the bHLH gene family, also in the same subfamily as PIF3 (Toledo-Ortiz et al. 2003), was initially identified as a rapidly and robustly phy-repressed gene in microarray analysis of dark-grown seedlings exposed to either Rc or FRc (Tepperman et al. 2001, Tepperman et al. 2004), as well as white light (Yamashino et al. 2003). This gene was also subsequently identified as a gene that is rapidly and robustly derepressed in seedlings grown under light–dark diurnal cycles, and then exposed to FR enrichment (simulated vegetative shade) during the light phase, indicating that the initial repression of PIL1 expression by Pfr formation is reversible by subsequent reduction of Pfr levels by FR light enrichment (Salter et al. 2003). Interestingly, the magnitude of the derepression response was also shown to vary systematically throughout the daily cycle in a manner indicating that it is gated by the circadian clock (Salter et al. 2003), and, conversely, PIL1 was found to be required for normal light induction of the clock-related gene PRR9, suggesting that it may have a role in regulating clock function (Mizuno and Nakamichi 2005, Khanna et al. 2006). Here we have examined the early dynamics of PIL1 expression in dark-grown seedlings upon first exposure to light to define more precisely the kinetics of initial repression, to identify the phy family members responsible for this regulation and to examine the basis for a decline in the magnitude of FR-induced PIL1 derepression observed with prolonged Rc irradiation.

Results and Discussion

PIL1 gene expression is repressed rapidly in response to very low fluence light signals

Previous microarray analysis showed that PIL1 mRNA levels declined 5- to 10-fold within 1 h of exposure of dark-grown seedlings to Rc or FRc (Tepperman et al. 2001, Tepperman et al. 2004). To define the rate of this decline more closely, we performed a time-course analysis over the first 1 h following initial exposure to Rc. The data reveal a very rapid decline, with a t1/2 of 15 min, resulting in an approximately 10-fold drop in abundance within 30–45 min (Fig. 1). To examine whether and how rapidly this repression of expression might be reversible, we determined PIL1 transcript levels in seedlings initially irradiated for 1 h in Rc, and then returned to darkness, following a terminal FRp to reconvert existing Pfr molecules to the Pr form for the duration of the subsequent dark incubation period. The results show that the PIL1 transcript rapidly re-accumulates 10-fold, to the original dark seedling level, within 60 min of dark incubation, after a lag of about 15 min (Fig. 1). These data establish that PIL1 transcript levels are rapidly and reversibly repressed and derepressed in response to phy-activating and -deactivating wavelengths of light. This observation implies that both transcription and turnover of the PIL1 mRNA are very rapid.
Developmental modulation of photoresponsiveness of PIL1

To begin to characterize this response in more detail, photobiologically, we performed an R fluence response analysis and tested the dependence of the capacity for FR reversal on the duration of prior Rc irradiation. The data show that initial PIL1 repression is triggered by a short (2 min) Rp of as little as 0.72 μmol m\(^{-2}\) (Fig. 2A). This low level of R is considered to be in the so-called VLFR range of light treatments (Wang and Deng 2003). Consistent with this observation, an FRp given immediately after a brief Rp is unable to reverse the repressive effect of the Rp, and an FRp given directly to unirradiated, dark-grown seedlings is alone capable of near full repression of PIL1 expression (Fig. 2B). This pattern of VLFR behavior is well established in the literature as being due to the action of the high levels of phyA initially present in dark-grown seedlings (Wang and Deng 2003). Consistent with this interpretation, the capacity of a subsequent FRp to potentiate reversal of the initial repression of PIL1 expression is gradually increased with increasing time of pre-irradiation with Rc, and is readily apparent by 30–60 min of Rc (Fig. 2B). This result is postulated to be due to the well-established rapid decline in phyA levels known to be induced by Rc exposure (Somers et al. 1991).

The data in Fig. 2B also demonstrate the necessity for a terminal FRp to elicit derepression of the initially R-imposed repression of PIL1. In contrast to the terminally FRp-irradiated seedlings, plants pre-irradiated with Rc for 30 or 60 min and returned directly to 1 h of darkness without the FRp display little if any reaccumulation of PIL1 mRNA (Fig. 2B). These data indicate that residual Pfr from the Rc pre-irradiation sustains PIL1 repression during this subsequent dark period.

Multiple phys repress PIL1 gene expression

To begin to define more precisely which members of the phy family are responsible for the light-induced repression of PIL1 expression, we examined the responsiveness of a series of Arabidopsis lines mutant in several phys that are considered to be involved in regulating seedling de-etiolation. The evidence indicates that phyA and phyB are strongly redundant in mediating a significant proportion of the R-imposed repression (Fig. 3). However, the phyA-phyB double mutant still exhibits considerable responsiveness to the initial Rc treatment, indicating that one or more of the remaining three phys (phyC, phyD and/or phyE) are also active in regulating this response. Additional apparently moderate reductions in responsiveness in each of the phyA-phyB-phyC and phyA-phyB-phyD triple mutants compared with the phyA-phyB double mutant (Fig. 3) suggest that both phyC and phyD also contribute, although apparently quantitatively less than phyA or phyB.

Fig. 3 also shows that all mutants lacking phyA exhibit no detectable responsiveness to FR alone, whereas all other lines lacking phyB and/or phyC display apparently wild-type responsiveness to this treatment. This result is consistent with the earlier microarray data (Tepperman et al. 2001), and verifies that phyA is exclusively responsible for mediating this FR-induced response, as mentioned above.

Re-induced declines in phyA and phyB levels result in accelerated appearance of the capacity for rapid FRp-induced derepression of PIL1 gene expression

To examine more closely the roles of phyA and phyB levels in regulating the temporal pattern of the FR-induced reversal of the R-imposed repression of PIL1 expression,
we monitored the levels of the phyA and phyB proteins in response to Rc exposure, and compared the time-courses of PIL1 derepression in wild-type seedlings pre-exposed to either 1 or 6 h of Rc before a terminal FRp and further dark incubation. The data show that, as expected, phyA levels decline rapidly upon exposure of dark-grown seedlings to Rc (Fig. 5A). In contrast, phyB levels remain relatively stable over the first 1–3 h of Rc, but do show an apparent decline down to about 60% of the initial dark level by 6 h of Rc (Fig. 5A). These data are in good agreement with the careful quantitative study of phy family levels by Sharrock and Clack (2002). In parallel, the time-course of PIL1 expression shows that FRp-induced derepression is significantly more rapid in seedlings pre-irradiated for 6 h (R6) than for 1 h (R1) in Rc (Fig. 5B). The progression of the R6 curve appears to be more similar to the phyAphyB double-mutant curve, than to the phyA curve (Fig. 4), suggesting that the effects of the longer term Rc may result from the observed combined reductions in both phyA and phyB levels, not just phyA levels (Fig. 5A). Consistent with this suggestion, phyB-overexpressor (ABO) seedlings, conversely, display a delayed capacity for FRp-induced derepression compared with the wild type (Fig. 5C), indicating that the elevated residual levels of phyB-Pfr in these seedlings compared with the wild type following the terminal FRp sustain repression longer in the subsequent dark period than in the wild type.

Prolonged Rc elicits an apparent developmental transition that results in a steady decline in the magnitude of the capacity for rapid FRp-induced derepression of PIL1 gene expression

Given the effects of the duration of Rc pre-irradiation on subsequent FRp-induced PIL1 derepression during the

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Fig. 3 The light-imposed repression of PIL1 expression is mediated by multiple phytochromes. Four-day dark-grown (D) seedlings were irradiated with either Rc (15 μmol m⁻² s⁻¹) or Fr (8 μmol m⁻² s⁻¹) for 1 h. One set of such seedlings (R60 and FR60) was then extracted immediately. Another set were instead given either a terminal 10 min Frp (8 μmol m⁻² s⁻¹) for R60-preirradiated seedlings or a terminal 10 min Rp (15 μmol m⁻² s⁻¹) for FR60-pre-irradiated seedlings and returned to the dark for 1 h (D60) before extraction (R60/Frp/D60 and FR60/Rp/D60, respectively). PIL1 mRNA levels were determined by Northern blot analysis. The single, double and triple phy mutants examined are indicated. Ler is the corresponding wild type for the phyABD triple mutant.

Fig. 4 Early FRp-induced derepression of PIL1 expression is rapid in the absence of phyA and B. (A) Northern blot, time-course analysis of PIL1 mRNA levels. (B) Quantification of PIL1 mRNA levels. Four-day dark-grown (D), wild-type (Wt), phyA, phyB and phyAphyB mutant seedlings were irradiated with Rc (15 μmol m⁻² s⁻¹) for 1 h (R60), followed by an FRp (8 μmol m⁻² s⁻¹) of 10 min, and returned to continuous darkness (Dc) for the period indicated. PIL1 levels were quantified by phosphorimagery analysis of the blots and normalized to 18S RNA. Values are normalized to the starting dark control level and represent the mean of three biological replicates. Error bars are the standard error.

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initiation of de-etiolation described above, we examined the effects of longer term Rc, sufficient to approach the fully deetiolated state (Fig. 6A). Strikingly, we found in initial experiments that PIL1 expression was repressed to a similar, constant basal level, regardless of the duration of Rc irradiation, but that the capacity for rapid derepression declined markedly with increasing duration of Rc pre-irradiation beyond about 6 h (Fig. 6B). More careful
Prolonged Rc irradiation (beyond about 6 h) causes a steady decline in the magnitude of rapid FRp-induced derepression of PIL1 expression. (A) Time-course of the visible phenotype of seedlings exposed to increasing Rc pre-irradiation times. (B) Rc-imposed repression of PIL1 is sustained unaltered throughout a prolonged irradiation period, but rapid FRp-induced derepression declines during this period. Top: Northern blot, time-course analysis of PIL1 mRNA levels in response to increasing periods of Rc pre-irradiation prior to FRp-triggered derepression. Bottom: quantification of the PIL1 mRNA levels shown above. Four-day dark-grown wild-type seedlings (D) were irradiated with Rc (15 μmol m⁻² s⁻¹) only for 6–72 h (Rc), or irradiated with Rc for the period indicated followed by a terminal FR light pulse (8 μmol m⁻² s⁻¹) of 10 min, and returned to the dark for 1 h (Rc/FRp/D1). PIL1 mRNA levels were quantified by phosphorimager analysis of the blots. Values are normalized to the starting dark control (D). (C) Top: representative Northern blot, time-course analysis of PIL1 and ATHB2 mRNA levels in response to increasing periods of Rc pre-irradiation prior to FRp-triggered derepression. Bottom: quantification of PIL1 mRNA levels from Northern blots of three biological replicates. Four-day dark-grown wild-type seedlings (D) were irradiated with Rc (15 μmol m⁻² s⁻¹) for 6–36 h, followed by an FR light pulse (8 μmol m⁻² s⁻¹) of 10 min (FRp), and returned to the dark for 1 h (D1). PIL1 mRNA levels were quantified by phosphorimager analysis of the blots. Values are normalized to the starting dark control level and represent the mean of the three biological replicates. Error bars are the standard error.

Magnitude of FRp-induced PIL1 derepression correlates inversely with the degree of de-etiolation in prolonged Rc

To investigate the potential role of proposed phy signaling intermediates in modulating the magnitude of PIL1 derepression, we examined the responsiveness of Arabidopsis seedlings perturbed in expression of the phy-interacting bHLH factors, PIF4 and PIF5, as well as the phyA, phyB and phyAphyB mutants. Although the loss of PIF5 (pif5-2 mutant) had little or no effect compared with the wild type, the overexpression of either PIF4 or PIF5 caused a striking retention of a robust capacity for FRp-induced PIL1 derepression, up to at least the longest period of Rc pre-irradiation of 48 h tested here (Fig. 7A).

Quantitative analysis showed that this decline in derepression capacity reaches a factor of 5-fold at the maximum duration of 36 h Rc examined (Fig. 6C). Given the report that this capacity for derepression in response to FR enrichment under shade avoidance conditions appears to be modulated by the circadian clock in entrained seedlings (Salter et al. 2003), it is notable that we observe no evidence of circadian oscillations under these constant Rc irradiation conditions. This is particularly remarkable given that we have shown that the central oscillator components CCA1, LHY and TOC1 are induced to oscillate robustly under these constant conditions over this period (Kikis et al. 2005). The data suggest, therefore, that additional mechanism(s) are activated under diurnal, light–dark cycling conditions that couple PIL1 derepression to the clock. It is also notable that another shade avoidance marker gene, ATHB2, does not parallel the behavior of PIL1 here, exhibiting if anything a moderate increase in capacity for FRp-induced derepression (Fig. 6C). Collectively, these data suggest that prolonged Rc induces a change in the developmental state of the responsive tissue that attenuates the transcriptional capacity of the PIL1 gene upon release from phy-imposed repression.
The time-course of this response shows that both PIF-overexpressors retain this capacity, more or less unreduced, regardless of the duration of Rc pre-irradiation throughout this period (Fig. 7B). Interestingly, the phyAphyB double mutant also retains this capacity in response to prolonged Rc pre-irradiation (Fig. 7A, B). That this rapid derepression requires a terminal FRp prior to the 1 h of darkness following termination of the Rc
Conclusions

mediated light signals.

although phyA and phyB appear to have major redundant roles in repressing PIL1 expression, phyC, phyD and/or phyE also have a significant role in regulating this response. This observation is consistent with the behavior of repressed, early-response genes in general observed by microarray analysis (Tepperman et al. 2006). It should be noted that PIL1 was not included in this earlier analysis because it is not represented on the Affymetrix ATH1 microarray.

The biphasic pattern of FR-induced PIL1 derepression observed here appears to reflect two sequential processes, illustrated in the hypothetical model in Fig. 9: (i) an initial phase, where the amplitude of the capacity for derepression is at first small or absent, but increases rapidly back to, or above, the original dark control levels, as the suppressive VLFR activity, imposed by the initially high levels of phyA, subsides due to phyA degradation (Figs. 4, 5); and (ii) a later phase, where the capacity for derepression steadily, but more slowly, declines again, apparently as a consequence of the more global developmental transition from the etiolated to the de-etiolated state (Figs. 6–8). Because light itself regulates de-etiolation and, therefore, the developmental state of the cell, it can be envisaged, in a formal sense, that the phy system functions at two levels: one controlling a more global developmental transition from the etiolated-like phenotype in prolonged Rc (Fig. 7C).

PIL1 expression is partially repressed in the dark in constitutively photomorphogenic mutants

One possible explanation for these observations is that the capacity for FRp-induced PIL1 derepression is a function of the de-etiolation state of the responsive cell, regardless of how that is achieved. If this is so, it might be predicted that seedlings exhibiting a de-etiolated state in the absence of light might also exhibit a constitutively low PIL1 mRNA level, without Rc pre-irradiation, as well as a reduced capacity to support full, FRp-induced derepression of PIL1 expression compared with the wild type. To investigate this prediction, we examined PIL1 expression in the constitutively photomorphogenic mutants cop1-4 and cop1-6. The data show that both mutants have lower PIL1 transcript levels than the wild type in dark-grown seedlings, and a reduced capacity for FRp-induced PIL1 derepression compared with the wild type in Rc-pre-irradiated seedlings (Fig. 8). The observed converse behavior of the cop mutant and PIF-overexpressing seedlings is consistent with the possibility that the developmental state of the cell at least partly regulates the responsiveness of the PIL1 gene to phy-mediated light signals.

Fig. 8 cop1 mutants displaying constitutive de-etiolation in the dark exhibit partial repression of PIL1 expression in darkness. (A) Northern blot analysis of PIL1 expression in cop1-4 and cop1-6 mutants. Two-day dark-grown (D) seedlings of each genotype were irradiated with Rc (15 μmol m⁻² s⁻¹) for 1 h (R60), followed by an FR light pulse (8 μmol m⁻² s⁻¹) for 10 min (FRp), and returned to the dark for 1 h (D60). (B) Quantification of PIL1 mRNA levels. PIL1 mRNA levels were quantified by phosphorimager analysis of the blots and normalized to 18S RNA. Values are normalized to the dark control level and represent the mean of three biological replicates. Error bars are the standard error.
exceptions of the phyAphyBphyD triple mutant of Landsberg erecta (Ler) and the PIF4 overexpressor of Wassilewskija (Ws). In cases with a different genetic background, the respective wild-type seedling was used as control. We used all the phy mutants as described in Reed et al. (1993, 1994) and Monte et al. (2003), and the PIF4- and PIF5-overexpressors and the pif5 knockout mutant used in our experiments are as described in Huq et al. (2002), Khanna et al. (2006) and Shen et al. (2007), respectively. cop1-4 and cop1-6 were described in McNellis et al. (1994).

Seeds were sterilized with 20% bleach solution with 0.1% Tween-X20, thoroughly washed with sterile water and plated on GM growth medium plates without sucrose (Valvekens et al. 1988), stratified for 3 d at 4°C in the dark and treated with white light for 3 h at room temperature to induce synchronized germination. Unless indicated specifically, seedlings were placed in a growth chamber at 21°C in darkness for 4 d and then subjected to R or FR treatment.

For R or FR light treatment, 4-day-old etiolated seedlings were irrigated with R or FR light by placing seedlings in Enconair plant growth chambers of appropriate light conditions for the indicated time. The fluence rates were monitored using a spectroradiometer (model LI-1800; LI-COR, Lincoln, NE, USA).

**Photography**

The photographs of seedlings were taken with either an Olympus digital camera C-4000 (Olympus America, Inc., Melville, NY, USA) for the light grown seedlings or a Canon G6 powershot digital camera (Canon USA, Inc., Lake Success, NY, USA) connected to Axioskop, a transmitted-light microscope of Zeiss (Carl Zeiss Inc., Thornwood, NY, USA) for magnified images of the hook opening process.

**RNA preparation and analysis**

Tissues were harvested at the indicated time and immediately frozen by liquid nitrogen. Total RNA was isolated from seedlings using an RNA miniprep kit (Qiagen, Valencia, CA, USA). A 10 µg aliquot of total RNA was separated on a 1.2% agarose gel containing formaldehyde and blotted onto a nylon membrane (Magna, Osmonics Inc., Minnetonka, MN, USA). RNA was fixed to the membrane by a UV-autolinker 1800 (Stratagene, La Jolla, CA, USA).

The PIL1 gene-specific probe (520 bp) was designed using sequences contained in the 3’ end of the gene. A fragment was amplified from the first-strand cDNA using T61GSP/FW (5’-CAACCTCAATCTCAGATGATGAG-3’) and T61GSP/RV (5’-TCAAGGCCCCAAAGGCCCAT-AGAC-3’). The PCR conditions were after 2 min of pre-denaturation, 35 cycles of denaturing at 95°C for 45 s, annealing at 55°C for 45 s, and extension at 72°C for 1 min, and, after the last cycle, 5 min of final extension at 72°C. The PCR was carried out using a thermal controller (PTC-100, MJ Research, Waterdown, MA, USA). The PCR DNA fragment was cloned into a TA cloning vector (Invitrogen, Carlsbad, CA, USA) and the probe sequence was confirmed by DNA sequencing.

The Athb-2 gene-specific probe was also PCR amplified from the primer sets, Athb-2FW (5’-TCACTCCAGTGTTGATGATGAG-3’) and Athb-2RV (5’-GCCGTGAGATATCTCGTG CG-3’). PCR was performed as described above.

The probe was excised from the pCR2.1 vector by digesting with EcoRI and purified using a PCR purification kit (Qiagen). The probe was labeled with [32P]dCTP by random priming methods (PrimeIt®II, Random primer labeling kit, Stratagene). After labeling, unincorporated nucleotides were removed by spin

**Materials and Methods**

**Plant materials**

Wild type and various mutants of Arabidopsis used in our experiments were Columbia (Col-0) ecotypic background, with the
column (Micro Bio-Spin columns P30) from BioRad (Hercules, CA, USA). Hybridization and washings were performed as suggested in the NorthernMax protocol of Ambion (Austin, TX, USA), with modification of washing at 50°C for 20 min twice.

The nylon membrane was exposed to a phosphorimager screen and visualized by a phosphorimager scanner, Storm 860 (Molecular Dynamics, Sunnyvale, CA, USA). Expression levels were quantified by ImageQuant software (Molecular Dynamics). All the experiments were repeated with at least three biological replicates. All blots were re-probed with 18S as a loading control. Expression levels were normalized with 18S, and the resulting value in the dark for each genotype was used as a reference and set to equal 100. Mean values for each treatment were plotted with the standard error.

Protein extraction and immunoblotting

Crude total protein extracts were prepared from 4-day-old etiolated seedlings of the indicated treatment by brief grinding about 150 seedlings. The plant extracts and immunoblotting analyses were prepared and performed as described in Martinez-Garcia et al. (1999). The protein concentration in the supernatant was determined by the method of Bradford (1976). For detecting phyA and phyB, 073D and B1-B7 monoclonal antibodies, respectively, were used (Somers et al. 1991, Hirschfeld et al. 1998). An anti-mouse antibody (Promega, Madison, WI, USA) conjugated with horseradish peroxidase was used as the secondary antibody and was detected with a chemiluminescent system (SuperSignal, Pierce, Rockfield, IL, USA). All procedures were performed according to the manufacturer’s instructions.

Scion Image software (Scion Corporation, Frederick, MD, USA) was employed to quantify the signal intensities of Western blots for phyB, and of protein bands on the Coomassie-stained gels. One Coomassie gel band was selected as a loading control, and its signal intensity was quantified in each lane and used to normalize the signal intensity of the phyB band to calculate the relative phyB level.

Supplementary material

Supplementary material mentioned in the article is available to online subscribers at the journal website www.pcp.oxfordjournals.org.

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References


Kikis, E.A., Khanema, R. and Quail, P.H. (2005) ELF4 is a phytochrome-regulated component of a negative-feedback loop involving the central oscillator components CCA1 and LHY. Plant J. 44: 300–313.


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