Phytochrome regulates translation of mRNA in the cytosol

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An array of photoreceptors including crytochomos, phototropin, and phytochromes regulates various light responses in plants. Among these photoreceptors, phytochromes perceive red and far-red light by switching between two interconvertible spectral forms (Pr and Pfr). The Pfr form promotes light responses partly by destabilizing negatively acting, phytochrome-interacting basic helix-loop-helix transcription factors (PIFs), thus modulating transcription in the nucleus. The Pfr form is also present in the cytosol. However, the role of phytochromes in the cytosol is not well understood. Here we show that the Pfr form interacts with the cytosolic protein PENTA1 (PNT1) and inhibits the translation of protochlorophyllide reductase (PORα) mRNA. PNT1 possesses five C3H-type zinc finger domains and displays similarity to various RNA binding proteins including Tristetraprolin, which regulates stabilities of mRNAs such as TFNα mRNA in humans. Consistent with its function as an RNA binding protein, PNT1 directly binds to mRNAs of a key chlorophyll biosynthetic gene, protochlorophyllide reductase in vivo and inhibits the translation of PORα mRNA in the presence of phytochromes. The present results demonstrate that phytochromes transmit light signals to regulate not only transcription in the nucleus through PIFs, but also translation in the cytosol through PNT1.

light signaling | protein–protein interaction | post transcriptional regulation | translational regulation | nucleocytoplasmic shuttling

Phytochromes are dimeric proteins consisting of an apoprotein and a chromophore. They are synthesized in the cytosol in the Pr form, which can be converted into the Pfr form by red light and back into Pr by far-red light (1). Pfr translocates into the nucleus and inhibits a set of negatively acting phytochrome-interacting basic helix-loop-helix transcription factors (PIFs), partly by destabilizing them (2–7). Among PIFs, four PIFs (PIF1, PIF3, PIF4, and PIF5) play key negative roles to repress light responses in the dark. A pif quadruple mutant (pif1/pif3/pif4/pif5, collectively referred as quadruple mutants pifq) is therefore constitutively photomorphogenic and expresses many light responsive genes, even in the dark (8–10). However, dark-grown quadruple mutant seedlings still have longer hypocotyls than light-grown wild-type seedlings and the correlation coefficient of gene expression is only 0.7 between dark-grown pifq mutant seedlings and red light-grown wild-type seedlings (9, 10). This implies that phytochromes transmit light signals not only through these four PIFs, but also through other phytochrome-interacting proteins such as PIF7 and PHYTOCHROME KINASE SUBSTRATE 1 (PKS1) (8, 11, 12).

Ever since researchers discovered the nuclear translocation of Pfr and identified the nuclear-localized PIFs, the nucleus has been considered the focal site of phytochrome signaling (13). Mutant or transgenic phytochromes that are localized to the cytosol are unable to induce the majority of light responses, including hypocotyl elongation (14–16), supporting the notion that the phytochromes mainly function in the nucleus. The PIFs, which are transcription factors, also function in the nucleus. A genome-wide chromatin immunoprecipitation (ChIP-chip) study coupled with microarray analysis showed that one of the PIFs (PIF1) bound to 748 sites in vivo and regulated the expression of 166 target genes (105 positively and 61 negatively) in imbibed seeds (17). Among PIF1 binding sites, 59% were found to possess G-box elements (CACGTG). Other PIFs have also been shown to bind to individual G-box elements both in vitro and in vivo and to regulate the expression of target genes (18–25). Collectively, these previous results indicate that phytochromes regulate light responses through PIFs in the nucleus.

However, a few lines of evidence suggest that the cytosol should also be considered as a site of phytochrome action and processes occurring in the cytosol as a target of phytochrome signaling. First, cytoplasmic motility is accelerated by red light within a few seconds (26). Second, ion flux is also changed very rapidly by red light (27, 28). Third, hypocotyl negative gravitropism and red-enhanced phototropism are partly regulated by cytoplasmic phytochrome A (phyA) (29). Consistent with the evidence of cytoplasmic phytochrome signaling, phytochromes are present in the cytosol. The Pr form is abundantly present in the cytosol and the presence of the Pfr form of phytochrome is also present in the cytosol, as indicated by the sequestering of the Pr of phyA in the cytosol and the slow accumulation of phytochrome B (phyB) nuclear speckles after red light irradiation (30–32). In addition, PKS1, which negatively regulates phytochrome signaling, was shown to interact with both phytochrome and phototropin 1 in the cytosol (11, 33). The molecular function of PKS1 has not been fully elucidated.

Translation is one of the cytosolic processes that could be regulated by phytochromes. To investigate this possibility, we sought to identify phytochrome-interacting proteins that might control translation in the cytosol. The translation of most eukaryotic mRNAs is initiated by attachment of the 43S preinitiation complex (comprising the 40S subunit, the eIF2 complex, eIF3, eIF1, and eIF1A) to the capped 5′ end region of the mRNA (34, 35). This attachment is facilitated by the eIF4F complex, which binds to the cap. Once the 43S complex is attached, it scans downward to find the initiation codon, where it is subsequently joined by the 60S subunit to form the elongation-competent 80S ribosome. In animals, translation is extensively controlled by different mechanisms, including protein modification of eIFs and the selection of specific mRNAs by RNA-binding proteins. Translational control also occurs in plants, where it plays important roles in various plant processes, including the responses to environmental stress, pathogens, and hormonal signaling. However, the role of phytochromes in translational control is not yet known (36, 37). Here, we report that the Pfr forms of phyB can interact with a cytosolic zinc finger protein that binds...
to the 5′-UTR of the protochlorophyllide reductase (PORA) mRNA to light-dependently inhibit translation of the PORA mRNA. Our results demonstrate that phytochromes can control gene expression not only through transcriptional regulation in the nucleus, but also through translational control in the cytosol.

**Results**

PNTA1 Is a Cytosolic Phytochrome-Binding Protein That Promotes Far-Red (FR) Block of Greening. In the present study, a putative cytosolic phytochrome-interacting protein was identified through yeast two-hybrid screening and named PNTA1 (PNT1) on the basis of its structure, which includes five C3H-type zinc finger motifs (Fig. 1A). The five CXXC3H motifs are present in two clusters that consist of three N-terminal zinc finger motif clusters and two C-terminal zinc finger motif clusters. Proteins with multiple C3H-type zinc finger motifs are found in various eukaryotic proteins ranging from yeast and rice to human proteins, and some of which have been shown to be RNA binding proteins (38, 39). A database search indicated that *Arabidopsis* contains 10 proteins with similar multiple zinc finger motifs and rice contains 7 proteins. (Fig. S1). One of them, known as ENHANCER OF AG-4-1 (HUA1), was shown to bind an intron of *AGAMOUS* mRNA to regulate the splicing (40).

The interaction between PNT1 and phytochrome was further probed by in vivo communoprecipitation assay using transgenic plants expressing either GFP-tagged PNT1 for phyA or both GFP-tagged PNT1 and myc-tagged phyB for phyB. PNT1-GFP was functional as its phenotype is similar to transgenic lines expressing PNT1 without any tag (PNT1-OXI). PhyB-myc was also functional (Fig. S2). PNT1-GFP was immunoprecipitated with anti-GFP antibody after either red or far-red light pulse and the precipitated phyA was detected by anti-phyA antibody, whereas the precipitated phyB-myc was detected by anti-myc antibody. The assay showed that PNT1 binds preferentially to the Pfr form of both phyA and phyB (Fig. 1B). A subcellular localization of PNT1 was determined by transgenic plants expressing PNT1-GFP. Similar to PKS1, PNT1 is localized in the cytosol rather than in the nucleus and red or far-red light did not change its subcellular localization (Fig. 1C). Taken together, these results indicate that PNT1 is a cytosolic C3H-type zinc finger protein that interacts preferentially with the Pfr form of both phyA and phyB.

Mutation and overexpression of PNT1 alter a subset of phytochrome-mediated light responses. Among these light responses, FR block of greening was strongly enhanced in PNT1 overexpressing lines, whereas it was mildly suppressed in two pnt1 mutant alleles with different ecotype backgrounds (Col-0 for pnt1-1 and WS-2 for pnt1-2) (Fig. 2A). The FR block of greening response is caused by photobleaching when FR-grown seedlings are transferred to white light (41). The FR block of greening was quantified by measuring the amount of produced chlorophyll after transferring to white light. *PNT1-OX1* (*PNT1-OXI, PNT1-GFP1*) accumulated chlorophyll at approximately half the rate of wild type when FR-grown seedlings older than 3 d were transferred to white light, whereas the *pnt1* mutants accumulated more chlorophyll than wild-type seedlings (Fig. 2B). A previous study showed that gibberellins (GA) promote photobleaching by repressing the expression of all POR mRNAs (42). GA promoted photobleaching both in wild type and the *pnt1* mutant (Fig. S3), suggesting that they function independently. Unlike the FR block of greening, light-dependent hypocotyl elongations were not affected by PNT1 (Fig. S4).

**Phytochrome and PNTA1 Inhibit the Translation of PORA mRNA Light Dependently.** In previous studies, FR-grown seedlings were shown to photobleach when transferred to white light, partly because phyA represses the expression of *PORA* mRNA (41), which leads to an imbalance between chlorophyll intermediates and protochlorophyllide reductase (POR) under FR conditions. Irrespective of PNT1 mutation, all plants expressed similar levels of two key chlorophyll biosynthetic genes, *HEMA* and *GUN5* (Fig. 3A). PORA mRNA levels were also either similar in the *pnt1* mutant or slightly higher in the *PNT1-OX1* under far-red light conditions (Fig. 3B). These results suggest that PNT1 does not promote the FR block of greening through transcriptional repression of *PORA* gene or activation of other chlorophyll biosynthetic genes.

Instead, PNT1 was found to inhibit the translation of *PORA* mRNA in the light. Western blot analysis indicated that PORA protein levels were similar or slightly higher in the *pnt1* mutant, but much lower in the *PNT1-OX1* than in the wild type, both under red and far-red light conditions (Fig. 3B). The decrease in PORA protein levels could be caused by the repression of *PORA* mRNA translation. The effect of PNT1 on the inhibition of
the *PORA* mRNA translation was further investigated by constructing a luciferase reporter gene with or without the 5′- and 3′-UTRs of the *PORA* gene under the constitutive 35S promoter and generating transgenic *Arabidopsis* plants. Compared with transgenic plants harboring a luciferase reporter gene without UTRs, transgenic plants harboring the luciferase gene with *PORA* UTRs showed decreased luciferase activities both under red and far-red light conditions but not in the dark (Fig. 3C). This light-induced decrease of luciferase activity was not due to a decreased stability of luciferase mRNA with UTRs, as levels of luciferase mRNA were similar irrespective of the UTRs (Fig. 3D). The role of PNT1 was also investigated by generating transgenic *pnt1* mutants harboring the reporter gene with *PORA* UTRs. Luciferase activities were decreased less by red and far-red light in the *pnt1* mutant than in wild-type plants, indicating that PNT1 is partially responsible for the translational repression in red and far-red light (Fig. 3E). The partial release of the repression in the *pnt1* mutant may be caused by the presence of additively acting PNT1 homologs (Fig. S1).

To further determine which UTR is necessary for the translational repression of *PORA* mRNA, constructs containing the luciferase reporter gene with the 5′-UTR alone or the 3′-UTR alone were generated and a transient expression assay using protoplasts was performed (Fig. 3F). In constructs containing no UTRs or only the 3′-UTR fused to the reporter gene, luciferase activities were not decreased by light. However, when the 5′-UTR or both the 5′-UTR and 3′-UTR were fused, luciferase activities were strongly decreased by light. The correct sequence of the 5′-UTR was required to repress the translation of the reporter mRNA, as insertion of the 5′-UTR in the reverse direction had no effect. These results suggest that PNT1 represses the translation of *PORA* mRNA through its 5′-UTR.

The role of phytochromes in light signaling-mediated inhibition of the translation of the reporter gene with *PORA* UTRs was investigated using a transient expression assay. Red light reduced luciferase activity only partially in the *phyB* mutant (Fig. 3G), suggesting that *phyB* is not the only phytochrome-mediating red light signaling. A *phyA phyB* double mutant did not show any inhibition by red light, suggesting that both *phyA* and *phyB* additively mediate red light signaling to inhibit the translation of *PORA* mRNA. Continuous far-red light also reduced luciferase activity in wild type but not in *phyA* mutants, supporting the role of *phyA* in mediating far-red light signaling. Consistent with the inhibitory role of phytochromes on the translation of *PORA* mRNA, the overexpression of *phyB* caused more severe photobleaching in a PNT1-dependent manner (Fig. S5). Taken together, the present results indicate that phytochromes mediate red and far-red light signaling to inhibit the translation of *PORA* mRNA in the cytosol.

To demonstrate that cytosolic phytochromes are capable of inhibiting the translation, we introduced the same luciferase reporter gene with the *PORA* UTRs into *phyA* mutant and *fhy1/fhl* double mutant to generate stable transgenic lines. Due to the lack of carrier proteins, phyA protein is localized in the cytosol of the *fhy1/fhl* double mutant (29, 43). Compared with the *phyA* mutant, luciferase activities were decreased in the *fhy1/fhl* double mutant both under red and far-red light conditions but not in the dark (Fig. 3H). Because the inhibition of translation under far-red light condition is solely caused by phyA (Fig. 3G), the results further support that cytosolic phyA can inhibit the translation.

**PENTA1 Binds to 5′-UTR of PORA mRNA and Recruits Phytochrome to Inhibit the Translation.** PNT1 possesses a putative RNA binding domain, suggesting that PNT1 binds to *PORA* mRNA in vivo. RNA immunoprecipitation (RIP) analysis was performed using transgenic plants expressing PNT1-GFP and the luciferase reporter gene sandwiched between the 5′-UTR and 3′-UTR of the *PORA* gene. Immunoprecipitation of PNT1-GFP by an anti-GFP antibody precipitated high levels of endogenous *PORA* mRNA compared with levels of *PP2A* mRNA (Fig. 4A), indicating that PNT1 binds to *PORA* mRNA in vivo. To further investigate the light dependency of the association between PNT1 and *PORA*
mRNA, transgenic plants harboring a luciferase reporter gene with the PORA UTRs were used. The RNA-IP assay showed that PNT1 precipitated high levels of the luciferase reporter mRNA irrespective of the light conditions, whereas it did not enrich the mRNA of the HPTII transgene (present in the same T-DNA with the luciferase reporter gene) (Fig. 4B). These results indicate that PNT1 is a phytochrome-interacting protein that binds to PORA mRNA through its UTR in vivo irrespective of light conditions. Phytochromes do not regulate the binding of PNT1 to its target RNA as evidenced by the light-independent binding of PNT1 to the reporter gene containing the UTRs of PORA. Phytochromes also do not regulate the protein stability of PNT1, which is in contrast with the degradation of PIF3 under light conditions (Fig. 4C).

Fig. 3. Phytochromes inhibit the translation of PORA mRNA through PNT1. (A) Expression levels of chlorophyll biosynthetic gene mRNAs in pnt1 and PNT1-OXs. Results are expressed as relative levels of PP2A mRNA. Four-day-old red light-grown (Rc) or FR-grown seedlings were used for the expression analysis. (B) Altered POR protein levels in pnt1 and PNT1-OX. Tubulin was used as a loading control. (C) Light-dependent decrease of luciferase activity in transgenic Arabidopsis harboring a luciferase reporter gene with 5′- and 3′-UTRs of PORA (5L3) in comparison with a luciferase gene without any UTRs (L). Upper diagrams describe the 5L3 and L constructs. Relative luciferase activities of L samples were taken as one for each light condition. (D) Similar levels of luciferase reporter mRNAs irrespective of UTRs in transgenic Arabidopsis. (E) Reduced light-dependent decrease of luciferase activity in the pnt1 mutant harboring a luciferase reporter gene with 5′- and 3′-UTRs of PORA (5L3). (F) Sufficiency of 5′-UTR of PORA for the translational inhibition of luciferase reporter gene in protoplasts. Various reporter constructs are shown. Relative luciferase activities of dark samples were taken as one for each construct. (G) Abolishment of light-dependent translational inhibition of the luciferase reporter with UTRs of PORA in protoplasts of phytochrome mutants. Relative luciferase activities of dark samples were taken as one for each phytochrome mutant. (H) Light-dependent decrease of luciferase activity in transgenic phy1/fhl double mutant harboring a luciferase reporter gene with PORA UTRs. Relative luciferase activities of phyA mutant samples were taken as one for each light condition (SD, n = 3).

Discussion

The present results uniquely show that phytochrome directly inhibits translation of mRNA in the cytosol. We show that PNT1, possessing multiple C3H-type zinc finger motifs, binds to PORA mRNA through its UTR in vivo irrespective of light conditions. Phytochromes do not regulate the binding of PNT1 to its target RNA as evidenced by the light-independent binding of PNT1 to the reporter gene containing the UTRs of PORA. Phytochromes also do not regulate the protein stability of PNT1, which is in contrast with the degradation of PIF3 under light conditions (Fig. 4C). Instead, phyB was found to be recruited to PORA mRNA through PNT1. The RNA-IP of phyB-myc with an anti-myc antibody enriched the PORA mRNA fraction at much higher levels in PNT1-OX than in the pnt1 mutant (Fig. 4D). Consistent with the preferential binding of the Pfr form to PNT1, the binding of phyB-myc to PORA mRNA was increased by red light (Fig. 4E). Taken together, these results indicate that the Pfr form of phyB inhibits the translation of PORA mRNA after recruited to the 5′-UTR of PORA mRNA through PNT1.
of both transcription and translation is likely to rapidly reduce the level of PORA protein during the dark-light transition. Finally, it is noteworthy that Prt is the functional form of phytochromes in both the nucleus and the cytosol.

Different explanations may account for how phytochromes and PNT1 inhibit the translation of the *PORA* mRNA. The recruitment of phytochromes to the 5′-UTR of the *PORA* mRNA may inhibit the activity of one or more of the eIFs required for loading of the 43S preinitiation complex onto the 5′ cap and the subsequent formation of the 48S initiation complex (34, 35). In mammals, the phosphorylation of various eIFs (e.g., eIF1, eIF2, eIF3, eIF4E, and eIF4G) by mammalian kinases can affect the activities of these eIFs. Because phytochromes were shown to have protein kinase activity in vitro (46), the recruited phytochrome may phosphorylate some of the eIFs, thereby inhibiting their activities. Alternatively, the phy-PNT1 complex on the 5′-UTR may inhibit the loading of the 43S preinitiation complex or other proteins onto the 5′-UTR by steric hindrance. A well-known example of this process is the interaction between the iron response element (IRE) in the 5′-UTR of ferritin mRNA and the iron response protein (IRP) (47). When the concentration of iron is low, IRP binds to the IRE and blocks loading of the 43S preinitiation complex onto the ferritin mRNA (48), thereby inhibiting translation of the ferritin mRNA. Analogous to IRP, the phy-PNT1 complex could inhibit translation by blocking the loading of the 43S preinitiation complex or other protein components. However, future work will be required to unravel the detailed molecular mechanism(s) of translational inhibition by PNT1 and phytochromes.

Our results further suggest that there may be additional cytosolic phytochrome-mediated signaling events. The translational regulation of the *PORA* mRNA does not seem to account for the classical cytoplasmic light responses (e.g., rapid changes in cytoplasmic motility and ion fluxes) (26–28), indicating that there is likely to be cytosolic phytochrome-mediated signaling other than the PNT1-mediated translational control described herein. In addition, although PNT1 was identified as a *PORA*-binding protein, PNT1 may also bind and regulate other mRNAs. PNT1 itself belongs to a small protein family that has 10 members in *Arabidopsis* and 7 members in rice (Fig. S1). It is somewhat analogous to the PIFs, which belong to a subgroup of the bHLH transcription factors having 15 members in *Arabidopsis* and at least 6 members in rice (49, 50). As many PIF family members play key roles in nuclear phytochrome signaling, it would be interesting to determine the potential roles of other PNT1 family members in cytosolic phytochrome signaling.

**Methods**

**In Vivo Pull Down Assay.** In vivo pull down assay was performed with 4-d-old dark-grown seedlings. Total proteins were solubilized in extraction buffer (50 mM Tris·Cl, pH 7.5, 150 mM NaCl, 10% glycerol, 0.1% Nonidet P-40, 1× complete mini protease inhibitor, 100 μM MG132) and filtered through a QIAshredder. The filter through was divided into two and anti-GFP antibody was added. Each sample was irradiated with either red (4.4 μmol/m²s) or far-red (2.4 μmol/m²s) light for 15 min and incubated in the dark for 1 h with gentle rotation at 4 °C. Antibody-bound protein complexes were precipitated by protein A agarose. Beads were recovered and washed three times with 500 μL binding buffer in spin columns (Pierce). Proteins were eluted and analyzed by Western blot using anti-phyA (Agrisera) and anti-myc (Santa Cruz) antibody. All procedures were performed in the dark or under safety green light.

**RIP.** All procedures were basically performed as described previously, which was reported in the mammalian cell (51). To optimize RIP in *Arabidopsis* we introduced minor changes. In brief, 2 g of long-day grown *PNT1-GFP1* plants were dark adapted for 24 h before sampling and grinding in liquid nitrogen. Alternatively, 2 g of 4- to 6-d-old dark-grown seedlings were used in the experiments. Total proteins were solubilized in the same volume of polysome lysis buffer (100 mM KCl, 5 mM MgCl2, 10 mM Hepes pH 7.0, 0.5% Nonidet P-40, 1× complete mini protease inhibitor, 100 μM MG132) and filtered through a QIAshredder. The filter through was divided into two and anti-GFP antibody was added. Each sample was irradiated with either red (4.4 μmol/m²s) or far-red (2.4 μmol/m²s) light for 15 min and incubated in the dark for 1 h with gentle rotation at 4 °C. Antibody-bound protein complexes were precipitated by protein A agarose. Beads were recovered and washed three times with 500 μL binding buffer in spin columns (Pierce). Proteins were eluted and analyzed by Western blot using anti-phyA (Agrisera) and anti-myc (Santa Cruz) antibody. All procedures were performed in the dark or under safety green light.

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1. Rockwell NC, Su YS, Lagarias JC (2006) Phytochrome structure and signaling mecha-

2. Bauer D, et al. (2004) Constitutive photomorphogenesis 1 and multiple photo-


5. Oh E, et al. (2006) Light activates the degradation of PILS protein to promote seed
germination through gibberellin in Arabidopsis. *Plant J* 47:124–139.

6. Nozue K, et al. (2007) Rhythmic growth explained by coincidence between internal and

ated inhibition of shade avoidance involves degradation of growth-promoting bHLH

with PIF3 and PIF4, regulates responses to prolonged red light by modulating phyB


negatively-acting phytochrome-interacting factors. *Proc Natl Acad Sci USA* 106:
7660–7665.

11. Fankhauser C, et al. (1999) PKS1, a substrate phosphorylated by phytochrome that


15. Toledo- Ortiz G, et al. (2010) Subcellular sites of the signal transduction and degra-

16. Huq E, Al-Sady B, Quail PH (2003) Nuclear translocation of the photoreceptor phy-
tochoke B is necessary for its biological function in seedling photomorphogenesis.

17. Oh E, et al. (2009) Genome-wide analysis of genes targeted by PHYTOCHROME IN-
TERACTING FACTOR 3-LIKEs during seed germination in Arabidopsis. *Plant Cell* 21:
403–419.


19. Martinez-Garcia JF, Huq E, Quail PH (2000) Direct targeting of light signals to a pro-


21. Hentze MW, Gray NK, Hentze MW (1998) IPR-1 binding to ferritin mRNA prevents
the recruitment of the small ribosomal subunit by the cap-binding complex elf4F. *Mol Cell* 2:383–388.

photoactivated phytochrome signaling to specific helix-loop-helix transcription factors.

a set of phytochrome-interacting factor-like bHLH proteins in Orzya sativa. *Biosci

24. Keene JD, et al. (2006) RIP-Chip: The isolation and identification of mRNAs, micro-

25. Brownlee C, Kendrik RE (1979) Ion fluxes and phytochrome proteins in mung bean
hypocotyl segments: II. Fluxes of chloride, protons, and orthophosphate in apical and


27. Shen H, Moon J, Huq E (2005) PIF1 is a negative regulator of phytochrome B signaling

28. Lariguet P, et al. (2006) PHYTOCHROME KINASE SUBSTRATE 1 is a phototropin


30. Ollenberg N, Hinnenbusch AG (2009) Regulation of translation initiation in eukar-


34. Cheminant S, et al. (2011) DELLAS regulate chlorophyll and carotenoid biosynthesis
to prevent photooxidative damage during seedling deetiolation in Arabidopsis. *Plant

35. Palmbrunner A, et al. (2006) FHY1 and FHL act together to mediate nuclear accumu-


38. Cheminant S, et al. (2011) DELLAS regulate chlorophyll and carotenoid biosynthesis
to prevent photooxidative damage during seedling deetiolation in Arabidopsis. *Plant


Supporting Information

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SI Methods

Plant Material and Growth Condition. Arabidopsis thaliana plants were grown in a growth room with a 16-h light/8-h dark cycle at 22–24 °C for phenotypic analysis and seed harvest.

For the generation of PNT1 overexpression lines, full-length PNT1 coding sequence with (PNT1-OXI) or without (PNT1-GFP) a stop codon was amplified with specific primer sets (Table S1) and cloned into binary vectors. Two independent homozygous lines that express transgenes were established for each construct. The pnt1-1 allele was isolated from GABI_676A09, which has a T-DNA insertion at the sixth exon (GATTTCATTA-(T-DNA)-CTTCTCATTG) and the pnt1-2 allele was isolated from T-DNA insertions lines onWs-2 background, which has a T-DNA at the fourth intron (GATTTCATTA-(T-DNA)-CTTCTCATTG). For PHYB-myc lines, the full-length PHYB was amplified with a specific primer set and cloned into pHTM, which expresses a protein tagged with 9 myc epitopes (1). PIF3-myc and GFP-myc were described previously (1, 2). Other mutants used in this work are phyA-211, phyB-9, phyA-211/phyB-9 double mutant, and fhy1/fhl double mutant, all of which are Col-0 background. For transgenic Arabidopsis harboring luciferase reporter gene, a firefly luciferase gene or a firefly luciferase gene with 5′-UTR (and 3′-UTR of POR4 gene were introduced to wild type (Col-0) or mutants (pnt1-1, phyA-211, fhy1/fhl) and homozygous transgenic lines were selected and used for the assay.

Luciferase Reporter Assay. Protoplast transient reporter assay was performed as described previously (3). Leaf number 7–9 of 3.5–4 wk old plants were used as material. 3 μg of UTR containing luciferase reporters were transfected with 0.3 μg of Renilla luciferase. After PEG transfection samples were irradiated with far-red light for 30 mins and incubated 15 h in the dark, red and far-red at 22 °C. For the luciferase reporter assay using transgenic plants, 40 seeds were grown on 1/2 MS agar plates without sucrose for 3 d under various light conditions before sampling. Sample seedlings were ground in the liquid nitrogen and total proteins were solubilized in 50 μL of PLB (supplied in Promega’s dual luciferase assay kit) supplemented with 1× complete mini protease inhibitor mixture (Roche). The luciferase activities were measured by dual-luciferase assay kit (Promega).

Photobleaching Assay. For FR-block of greening assay, 50 seeds were surface sterilized and plated on ½ MS agar media without sucrose. Germination was synchronized by 4 d of cold treatment followed by 6 h of white light treatment. After indicated days of far-red light (2.4 μmol/m²/s) treatments or in the dark, the plates were transferred to white light for 4 d. The degree of photobleaching was determined either by measuring chlorophyll levels by spectrophotometer or by counting number of seedlings with green cotyledons. For gibberellins (GA) treatment, 50 seeds were plated and grown 1 d on the 3M filter paper on ½ MS agar media in far-red light, and germinating seedlings on the filter paper were transferred to the same media containing 5 μM GA4+7.

Protein and mRNA Analysis. A total of 50 seedlings were grown in the red and far-red light for 4 d. Collected seedlings were ground in liquid nitrogen and total proteins were solubilized by protein extraction buffer (100 mM NaH2PO4, 10 mM Tris-Cl pH 7.5, 8 M urea, 1× complete mini protease inhibitor). Cell debris were precipitated by centrifugation for 10 min in 4 °C and supernatant was boiled with SDS sample buffer. The same amount of total proteins were separated by 10–17% SDS/PAGE gel. Anti-POR antibody (Agrisera) and anti-α-tubulin antibody (Sigma) were used for the blotting. Total RNAs were isolated with plant total RNA kit (Sigma) according to the manufacturer’s instruction. A total of 2 μg of total RNAs were reverse transcribed and real-time PCR was performed to quantify the levels of each gene with specific primers.

Yeast Two-Hybrid Screening. A C-terminal domain of Arabidopsis phyA (514–1122 aa) cloned in pGBT9 vector was used to screen interacting proteins from 7-d-old, light-grown seedling cDNA library cloned in pGAD424 vector (Clontech).

**Fig. S1.** A neighbor joining tree showing the relationships between PNT1 family members of *Arabidopsis* and rice. The tree was drawn by the MEGA4 program.

**Fig. S2.** Hypocotyl elongation of *PHYB-myc* transgenic *Arabidopsis* under red light condition.

**Fig. S3.** Quantification of chlorophyll after transferring FR-grown seedlings to white light in the presence of gibberellins (GA$_4+7$). For GA treatment, 1 d-old FR-grown seedlings were transferred to 5 μM GA$_4+7$ and they grew 4 more days under far-red light (2.4 μmol/m$^2$/s) before transfer to white light (100 μmol/m$^2$/s) (SD, n = 3).
Fig. S4. PNT1 does not play a major role in light-dependent hypocotyl elongation. Seeds were imbibed 3 d at 4 °C in the dark, irradiated with white light for 6 h for the induction of germination, and grown for 4 d under various fluence rates of red and far-red light. Thirty seedlings were randomly chosen for the measurement for each fluence rate (SD, \( n = 30 \)).

Fig. S5. Greening rate of etiolated PHYB-myc and PHYB-myc/pnt1-1 lines. Five day-old etiolated seedlings were transferred to white light for 2 d and seedlings with green cotyledons were counted (SD, \( n = 3 \)).

Fig. S6. Expression level of PNT1 mRNA in two PNT1 overexpression lines (PNT1-OX1 and PNT1-GFP).

Table S1. Primer sequences used in this study

<table>
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<th>Primer name</th>
<th>Primer sequence</th>
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<tbody>
<tr>
<td>PNT1 LP</td>
<td>GATCCCTAGG ATGGATTTTA ATGCCGGAGT TC</td>
</tr>
<tr>
<td>PNT1 RP1 (PNT1-GFP)</td>
<td>CCGAAGATCTCCACCTGCTGTGATATCAAATG</td>
</tr>
<tr>
<td>PNT1 RP2 (PNT1-OX1)</td>
<td>CCGAAGATCTTCAGTGTGATATCAAATG</td>
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<tr>
<td>PNT1 real time LP</td>
<td>GATGGAAACC CTCACGGTGG TGAG</td>
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<tr>
<td>PNT1 real time RP</td>
<td>TCCCGGATTG TACCCCGAGA ATGAT</td>
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<td>HEMA real time LP</td>
<td>AGCGGGTGAAGCATGCTAAAAGCGAGGT</td>
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<tr>
<td>HEMA real time RP</td>
<td>GGTCGCTACGGTGTCTAAGT</td>
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<td>GUN5 real time LP</td>
<td>GAGCTGTGAAGCGCAGTGAACCTGTGAAGTCCATGT</td>
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<td>GTCCCTACATTACCTTTATACGTTGGAACAT</td>
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<tr>
<td>PORA real time LP</td>
<td>TTA CCC CGG TGT TAT TGC AAC GAC T</td>
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<tr>
<td>PORA real time RP</td>
<td>GAG CCT CTT CAG CAT CGC TAG CTT</td>
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<tr>
<td>HPTII real time LP</td>
<td>CGGTGCGTC ATCACAGTT TGCCAGT</td>
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<tr>
<td>PP2A real time LP</td>
<td>TTTGCGATGACGTATTCTCGGC</td>
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<td>PP2A real time RP</td>
<td>GCTTGCTGACTATCGGAAGTGGG</td>
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<tr>
<td>Luciferase real time LP</td>
<td>GAATGGAAAT CCTCTTGCT CCAAC</td>
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<tr>
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<td>GACGTAATCC AGCATCCTTT TTTCC</td>
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<tr>
<td>phyB LP (phyB-myc)</td>
<td>AGACGGATCCAAATAGTTTCCCGGAGTGCAGGGT</td>
</tr>
<tr>
<td>phyB RP (phyB-myc)</td>
<td>AGACGGATCCAAATAGTTTCCGGAGTGCAGGGT</td>
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LP, left primer; RP, right primer.