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# Phosphorylation by CK2 enhances the rapid light-induced degradation of PHYTOCHROME INTERACTING FACTOR 1 in Arabidopsis*

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**The phytochrome family of sensory photoreceptors interacts with Phytochrome Interacting Factors (PIFs), repressors of photomorphogenesis, in response to environmental light signals and induces rapid phosphorylation and degradation of PIFs to promote photomorphogenesis. However, the kinase that phosphorylates PIFs is still unknown. Here we show that CK2 directly phosphorylates PIF1 at multiple sites. [illegible]1 and [illegible]2 subunits individually phosphorylated PIF1 weakly *in vitro*. However, each of four [illegible] subunits strongly stimulated phosphorylation of PIF1 by [illegible]1 or [illegible]2. Mapping of the phosphorylation sites identified 7 Ser/Thr residues scattered throughout PIF1. Ser/Thr to Ala scanning mutations at all 7 sites eliminated CK2-mediated phosphorylation of PIF1 *in vitro*. Moreover, the rate of degradation of the Ser/Thr to Ala mutant PIF1 was significantly reduced compared to wild type PIF1 in transgenic plants. In addition, hypocotyl lengths of the mutant PIF1 transgenic plants were much longer than the wild type PIF1 transgenic plants under light, suggesting that the mutant PIF1 is suppressing photomorphogenesis. Taken together, these data suggest that CK2-mediated phosphorylation enhances the light-induced degradation of PIF1 to promote photomorphogenesis.**

Because light is an essential environmental signal for modulating plant growth and development throughout the life cycle, plants have several different classes of photoreceptors sensing and responding to major bandwidths of the light spectrum (1,2). Of these photoreceptors, the phytochrome (phy) family (which consists of five members, PHYA to PHYE, in Arabidopsis) senses and responds to a broad spectrum (red, far-red and blue) of light signals and pleiotropically regulates plant growth from seed germination to flowering time (3-5). Within a cell, phys are synthesized as an inactive Pr conformer that resides in the cytosol. Exposure to red (R) light triggers a conformation shift to a biologically active Pfr form, which migrates into the nucleus and initiates phy signaling (6). The active Pfr form can be reverted back to inactive Pr form by exposure to far-red (FR) light. The active Pfr form interacts with multiple signaling partners within the nucleus (7), and controls the expression of a large number (10-30% of the genome) of genes to regulate photomorphogenesis (8-10).

One of the ways phys exert this strong effect on gene expression is by differentially regulating the stability of positively and negatively acting transcription factors (TFs) functioning in light signaling pathways (11,12). For example, the positively acting transcription factors (e.g., HY5, HFR1, LAF1 and possibly others) are degraded in the dark and stabilized under red, far-red and blue light conditions. By contrast, the negatively acting transcription factors (e.g., the Phytochrome Interacting Factors, PIFs) are stable in the dark and degraded in response to light. The net effect of this stabilization and destabilization of TFs shapes the transcriptome that regulates photomorphogenesis.

PIFs have been shown to play central roles in phy signaling pathways (13-15). They belong to the bHLH class of transcription factors and are encoded by six family members in Arabidopsis

(PIF1 and PIF3-7) (13,14,16). PIFs physically interact with the Pfr forms of phys with differential affinity for various phys. PIFs bind to G-box DNA sequence motifs and regulate gene expression (17-22). Genetic and photobiological experiments showed that PIFs function as negative regulators of photomorphogenesis largely by modulating the level of phyB under red light (23-25). Moreover, quadruple (*pifQ*) mutant seedlings of *pif1*, *pif3*, *pif4* and *pif5* are constitutively photomorphogenic, suggesting that PIFs repress photomorphogenesis in the dark (24,26,27). This morphological phenotype is reflected at the gene expression level where a majority of the light regulated genes are constitutively expressed in the *pifQ* mutant in the dark (27,28). In wild type seedlings, light signals perceived by phys promote degradation of PIFs through the ubiquitin (ubi)/26S proteasomal pathway to derepress gene expression and promote photomorphogenesis (13,15,29).

Although the mechanisms of dark-induced degradation of positively acting TFs are well understood (12), the light-induced degradation of PIFs are only beginning to be understood. All PIFs except PIF7 are rapidly phosphorylated and ubiquitinated in response to light *in vivo* prior to their degradation (26,30,31). The mutant PIF1 and PIF3 that fail to interact with the Pfr forms of phyA and phyB, do not undergo light-dependent phosphorylation, suggesting that phy-interaction is necessary for the light-dependent phosphorylation and subsequent degradation of PIFs. However, the putative kinase(s) and the putative E3 ligase(s) responsible for phosphorylation of PIFs and recognition of the phosphorylated forms for subsequent ubiquitination and degradation are still unknown.

CK2 (formerly known as Casein Kinase II), a ubiquitious Ser/Thr kinase, has been implicated in regulating light signaling and circadian rhythms in Arabidopsis (32-38). The CK2 holoenzyme consists of two catalytic α and two regulatory β subunits. The Arabidopsis genome has four α subunit and four β subunit genes (39). Various members of both the α and β subunit families have been shown to be localized in the cytoplasm, nucleus and also in chloroplasts (39). A CK2 like activity has been shown to phosphorylate HY5, a positively acting component in a phy signaling pathway (34). In addition, CK2 phosphorylates HFR1, a positively acting HLH transcription factor functioning under FR and blue light pathways (35). Phosphorylation of both HY5 and HFR1 has been shown to stabilize these transcription factors (34,35). Because CK2-mediated phosphorylation stabilizes the positively acting transcription factors involved in light signaling in Arabidopsis, we reasoned that CK2 might also phosphorylate the negatively acting transcription factors (e.g., PIFs) and regulate their stability/function in Arabidopsis. Here we show that CK2 phosphorylates PIF1 at multiple sites *in vitro*. In addition, CK2-mediated phosphorylation promotes rapid light-induced degradation of PIF1 to fine tune photomorphogenesis.

## EXPERIMENTAL PROCEDURES

*Protein Expression and Kinase Assays*- PIF1 open reading frame (ORF) was cloned in frame with the 6x his-tag in pET21d vector and verified by sequencing. The vector was transformed into Arctic DE3 cell for protein expression and purification using his-tag as described (40,41). CK2 phosphorylation assays were performed as described (40,41). Briefly, 20 µl kinase assay mixtures contained 50 mM Hepes-KOH, pH 7.6, 5 mM $MgCl_2$, 2.4 mM DTT, 0.2 mM γ-[$^{32}$P]ATP (~250 cpm/pmol), 100 mM KCl, ~1 pmol CK2 and ~10-20 pmol PIF1. The reaction was incubated at 30$^{o}$C for 30 min and terminated by the addition of 4x SDS loading buffer. Samples were boiled 3 min and separated on a 10% SDS-PAGE gel. The gels were dried and exposed to a phosphorImager.

*Mapping CK2 Phosphorylation Sites in PIF1*- The identification of CK2 phosphorylation sites in PIF1 was performed essentially as described (41). Briefly, the phosphopeptides were enriched using an iron based affinity resin (Phos-Select, Sigma-Aldrich Co., St. Louis, MO) followed by analysis on a MALDI-TOF/TOF mass spectrometer (4700 Proteomics Analyzer, ABSciex, Foster City, CA). Phosphopeptides were identified in the MS by mass shift using the MASCOT (Matrix Science) search algorithm and the MS/MS were acquired for potential phosphopeptides. These were manually interpreted to confirm the presence of

strong neutral loss of phosphoric acid ions (MH+ - 98) and to assign the most probable location of the phosphorylation site based on shifts in b and y fragment ions.

*Plant Growth Conditions and Phenotypic Analyses*- Plants were grown in Metro-Mix 200 soil (Sun Gro Horticulture, Bellevue, WA) under constant light at 24 °C ± 0.5°C. Monochromatic R and FR light sources and the spectroradiometer (Model EPP2000, StellarNet Inc., Tampa, FL) used to measure fluence rates are as described (42). Seeds were surface sterilized and plated on Murashige-Skoog (MS) growth medium (GM) containing 0.9% agar without sucrose (GM-Suc) as described (42). After stratification at 4ºC in the dark, seeds were exposed to 3 hours white light at room temperature to induce germination before placing them in the dark for additional 21 hours. The plates were then either placed in the dark or under specific wavelengths of light for additional 3 days. For quantitation of hypocotyl lengths, a digital photograph was taken and at least 30 seedlings were measured using the publicly available software ImageJ. The experiments were repeated at least three times.

*Construction of Plasmids and Generation of Transgenic Plants*- PIF1 ORF was amplified by PCR and cloned into pENTRY vector (Invitrogen, Inc., Carlsbad, CA), and recombined with pNTAPa (43) to produce 35S:TAP-PIF1. A 1.6 kb *PIF1* promoter fragment was amplified with primers containing restriction sites (Supplementary Table S1) and was cloned into pPZP121 vector. A 3.5 kb fragment containing the TAP-PIF1 from the 35S:TAP-PIF1 construct was cloned into the pPZP121-pPIF1 to generate *pPIF1:TAP-PIF1* in pPZP121 background. The specific amino acid mutations in full-length PIF1 were introduced using a site-directed mutagensis kit in pENTRY vector background (Stratagene, La Jolla, CA). The 35S:LUC-PIF1 (LP) line is as described (42). The S464-466 to Ala and S459-461 to Ala mutations were created in the LUC-PIF1 background using the site directed mutagenesis kit (Stratagene, La Jolla, CA), and transformed into *pif1* mutant. The CK2 β1 and β2 subunits were amplified by PCR and cloned into pENTRY vector, and subsequently recombined with pB7FWG2 vector to produce 35S:CK2β1/2-GFP fusion constructs (44). All the constructs were verified by sequencing. The resulting vectors were transformed into Agrobacterium strain GV3101 by electroporation and transformed into wild type Col-0 background using the floral dip method. Transgenic plants were selected on appropriate antibiotics and homozygous lines were selected from single insert lines.

*Protein Extraction and Western Blotting*- Protein extraction and Western blotting were performed essentially as described (4,26). Briefly, four day-old dark-grown seedlings were either kept in dark or exposed to pulses of R or FR light followed by incubation in the dark for various times as indicated on each figure before protein extraction. To detect TAP-PIF1 and LUC-PIF1 proteins in transgenic plants, boiling denaturing buffer (100 mM MOPS, pH 7.6, 5% SDS, 10% Glycerol, 4 mM EDTA, 40 mM β -mercaptoethanol, 1X protease inhibitor cocktail [F. Hoffmann-La Roche Ltd, Basel, Switzerland]) was added at a 1:3 (w/v) ratio before grinding. PMSF (2 mM) was also added during extraction. To detect native PIF1 in wild type plants, about 0.2 g of tissue were ground in 1 mL of extraction buffer (100 mM Tris-HCl pH 6.8, 20% glycerol, 5% SDS, 80 µM MG132, 20 mM DTT, 1 mM bromophenol blue, 2 mM PMSF, and 1X protease inhibitor cocktail [F. Hoffmann-La Roche Ltd, Basel, Switzerland]) and boiled for 2 min. Total proteins were separated on 8% SDS-PAGE gels, blotted onto PVDF membrane and probed with anti-PIF1, anti-myc or anti-RPT5 antibodies. Membranes were developed with SuperSignal West Pico Chemiluminescent substrate kit (Pierce Biotechnology Inc., Rockford, IL), and visualized on an X-ray film. The intensity of the PIF1 and the control bands from each blot was quantified using ImageJ software and the PIF1 values were divided by the control values to make a ratio for each sample. The dark control for each sample is set to 1 from these ratios and the relative values of the other samples are calculated based on dark values. These relative values are shown in each figure under the blots.

## RESULTS

*PIF1 is Phosphorylated by CK2* in vitro. Sequence analyses using three software packages (Scansite 2.0, NetPhosK 1.0 and KinasPhos 2.0) revealed that PIF1 has multiple putative CK2

phosphorylation sites (Supplementary Table S2). We purified recombinant his-tagged PIF1, two CK2 α (α1 and α2) subunits and four β (β1–4) subunits and performed *in vitro* kinase assays as described (40). Results show that PIF1 is weakly phosphorylated by the α1 subunit of CK2. However, addition of the β1 subunit strongly stimulated phosphorylation of PIF1 (Fig. 1A). Figure 1B shows that phosphorylation of PIF1 was inhibited in proportion to the concentration of heparin, a specific inhibitor of CK2 activity (35). This experiment showed that CK2, and not a contaminating kinase, phosphorylates PIF1 in vitro. Taken together, these data strongly suggest that PIF1 is a *bona fide* substrate for Arabidopsis CK2 kinase *in vitro*.

*Effect of Different Subunit Composition on the Phosphorylation of PIF1*. Because the Arabidopsis genome encodes four α and four β subunits of CK2 (39), we investigated whether different CK2 α and β subunit combinations have differential phosphorylation activity toward PIF1 as described (40,41). Results show that all the αβ holoenzyme combinations had stronger activity than either of the two α subunits alone. However, among all the holoenzyme combinations, α1β2, α2β3 and α2β4 combinations showed stronger activities toward PIF1 compared to other combinations (Fig. 1C; Supplementary Table S3), suggesting that various holoenzyme combinations have differential activities toward PIF1. Relatively weak phosphorylation by the catalytic α subunit and strong stimulation by the regulatory β subunit might provide plants with enhanced regulatory power to modulate PIF1 activity *in vivo*. Moreover, CK2 subunit genes are differentially expressed in different tissues (39), suggesting that PIF1 might also be regulated in a tissue-specific manner.

*Mapping of the Phosphorylation Sites in PIF1*. To map the CK2 phosphorylation sites in PIF1, we performed *in vitro* kinase assays using cold ATP and α1β1 holoenzyme. We then mapped the phosphorylation sites using mass-spectrometry as described (41), and identified six (T10, T197, S202, S464, S465 and S469) phosphorylation sites scattered throughout the PIF1 polypeptide with a cluster at the C-terminal end (Fig. 2A, top). Some of the sites were identified with higher confidence than others. For example, the MS/MS of 1-27 and 463-489 are the best, with 196-213 moderate to weak, and 468-489 weak (Fig. 2A, bottom; Supplementary Figure S1). Because we wanted to eliminate all the CK2 phosphorylation sites, we performed site-directed mutagenesis to change the six Ser/Thr sites to Ala including the high and low confidence ones for investigating the biological functions of PIF1. We then purified recombinant mutant PIF1 named PIF1-6M. *In vitro* kinase assays using either α1 or α1β1 holoenzyme showed that a majority of the phosphorylation of the mutant PIF1-6M is eliminated, while the wild type PIF1 is strongly phosphorylated by the holoenzyme (Fig. 2B). Because PIF1-6M is still phosphorylated by CK2, and S466A mutation in S464-465A (two CK2 sites mapped in this study, Fig. 2A) background displayed a strong reduction in the light-induced degradation of PIF1 (Fig. 4), we reasoned that S466 might also be a CK2 site as predicted by the ScanSite 2.0 and http://kinasephos.mbc.nctu.edu.tw/index.php software packages (Supplementary Table S2). We created S466A mutation in the PIF1-6M background and named PIF1-7M. *In vitro* kinase assay showed that phosphorylation by CK2 is completely eliminated in PIF1-7M, while PIF1-6M is weakly phosphorylated by CK2 (Fig. 2C). As expected, wt PIF1 showed strong phosphorylation by CK2. Taken together, these data suggest that we have successfully mapped all the CK2 phosphorylation sites in PIF1.

*Reduced Degradation of TAP-PIF1-6M Compared to Wild Type TAP-PIF1 under R Light*. Previously, phosphorylation of HFR1 and HY5 by CK2 has been shown to stabilize these transcription factors from ubiquitin-mediated degradation (34,35). To investigate the effect of CK2-mediated phosphorylation on PIF1's stability and *in vivo* function, we generated homozygous transgenic plants expressing four single mutants (T10A, S202A, S464A and S465A), and the PIF1-6M as TAP fusion proteins using the endogenous *PIF1* promoter (Supplementary Figure S2). All four single mutants were phosphorylated and degraded in response to a pulse of R light (data not shown). However, the rate of degradation of TAP-PIF1-6M is significantly slower than that of wild type TAP-PIF1 after a pulse of R light (Fig. 3A; two independent transgenic lines are shown at the top and bottom). Moreover, the light-induced

phosphorylation is still present in the TAP-PIF1-6M similar to the wild type TAP-PIF1 as indicated by the characteristic band-shift under light conditions observed previously (26). To experimentally verify that these band-shifts represent phosphorylated forms of PIF1, we performed alkaline phosphatase (CIAP) treatment as performed previously (26). Results show that the slow-mobility forms of PIF1 under light is largely converted to fast-mobility forms of PIF1 similar to the dark samples after CIAP treatment (Supplementary Figure S3). These data suggest that, in response to light, either CK2 phosphorylates PIF1 at different sites than the ones mutated or another light-specific kinase may phosphorylate PIF1 at different locations than the CK2 phosphorylation sites.

To determine the roles of TAP-PIF1-6M in phy signaling, we investigated their photomorphogenic phenotypes under R light. Results show that the hypocotyl lengths of all the transgenic TAP-PIF1-6M seedlings were longer compared to that of the wild type TAP-PIF1 transgenic seedlings under R light conditions (Figs. 3B, C), suggesting that TAP-PIF1-6M is functioning as a negative regulator of phy signaling similar to the wild type TAP-PIF1. The increased stability of TAP-PIF1-6M under light promotes hypocotyl elongation.

*S464-466 are Necessary for Rapid Light-Induced Degradation of PIF1.* Previously, we have shown that both the N- and C-terminal regions of PIF1 are necessary for light-induced degradation of PIF1 (26). The N-terminal region contains the active phyA (APA) and active phyB (APB) binding domains necessary for phytochrome interactions. However, the molecular determinants present at the C-terminal region in PIF1 is still unknown. To map the C-terminal amino acids necessary for light-induced phosphorylation and degradation, we mutated two clusters of three serine residues at the C-terminal region of PIF1 (S459-461 and S464-466) to Ala separately. The mutant PIF1s were fused to luciferase (LUC) and expressed using a constitutive (CaMV35S) promoter in the *pif1* background. Homozygous transgenic plants were selected and assayed for PIF1 stability and photomorphogenic phenotypes. Results show that the rate of degradation of the mutant LUC-PIF1(S459-461A) was similar to that of wild type LUC-PIF1 under R light (Fig. 4A, top). Strikingly, the rate of degradation of the mutant LUC-PIF1(S464-466A) was strongly reduced compared to wild type LUC-PIF1, despite the presence of the light-induced phosphorylation in this mutant PIF1 (Fig. 4A, bottom). Consistent with these data, the hypocotyl lengths of the mutant LUC-PIF1(S464-466A) were much longer than the wild type LUC-PIF1, while the hypocotyl lengths of the mutant LUC-PIF1(S459-461A) were largely similar to that of the wild type LUC-PIF1 (Fig. 4B, C, Supplementary figure S4). We have also included LUC-PIF1-3M transgenic line as a control. LUC-PIF1-3M has reduced affinity for both phyA and phyB due to three mutations (G47A, L95A, N144A) at the APA and APB domains (26). LUC-PIF1 (S464-466A) transgenic plants showed similar phenotype compared to that of the LUC-PIF1-3M transgenic lines (Fig. 4B, C). These data strongly suggest that S464-466 are necessary for the rapid light-induced degradation of PIF1.

S464 and S465 were identified as CK2 phosphorylation sites by MS/MS mapping (Fig. 2A). Site-directed mutagenesis showed that S466 is also a CK2 site, as the residual phosphorylation observed in the PIF1-6M is eliminated in PIF1-7M (Fig. 2C). Because S464-466 to Ala scanning mutations showed similar but stronger phenotypes compared to PIF1-6M (Figs. 3, 4), these data suggest that S464-466 play major role in regulating PIF1 stability. Conversely, it is likely that the other CK2 sites (e.g., T10, T197, S202 and S469) play minor roles, if any, in regulating PIF1 stability.

*The Light-Induced Degradation of PIF1 is Enhanced in CK2 β Subunit Overexpression Lines*. The α and *β* subunits of CK2 are each encoded by four genes in Arabidopsis (39), and single and higher order mutants in all the subunits are not available. In addition, dominant-negative CK2 α mutant showed severe developmental defects (45). PIF1 degradation is unaffected in the *ck2β3* single mutant background under both R and FR light conditions (Supplementary Figure 5A, B). We overexpressed CK2 β1 and β2 subunits and selected homozygous transgenic plants (Supplementary Figure S6). We then examined the rate of degradation of native PIF1 in these lines

under R and FR light conditions. Results show that the rate of degradation of native PIF1 is enhanced under both R and FR light conditions in the CK2 β1 and β2 overexpression lines compared to wild type seedlings (Fig. 5A, top and bottom).

We examined seedling deetiolation phenotypes for these transgenic plants compared to wild type. Results showed that the CK2 β1 and β2 overexpression lines were modestly hypersensitive to FR light compared to wild type seedlings (Figs. 5B, C). Because CK2 regulates circadian clock (33,36,37), we also examined the circadian clock phenotypes by performing leaf movement assays. Results showed that only CK2 β1 overexpression line #20 has shorter period compared to wild type and the other transgenic plants (Supplementary Figure S7). CK2 β1 line#20 has the strongest overexpression of the β1 subunit compared to other transgenic plants (Supplementary Figure S6). It is possible that strong overexpression is necessary to robustly regulate seedling deetiolation and circadian clock phenotypes. Taken together, these data suggest that CK2 may regulate the stability/activity of PIF1 and other factors *in vivo* and modulate deetiolation and circadian clock in Arabidopsis.

## DISCUSSION

Previously, it was shown that PIF1 and PIF3-6 were phosphorylated in response to light before being degraded through the 26S proteasomal pathway (13,15,26). Interactions with both phyA and phyB are necessary for the light-induced phosphorylation and degradation under all three light conditions (4,13,15,26). This study provides biochemical evidence that PIF1 is a substrate for Arabidopsis CK2, a ubiquitious Ser/Thr kinase present in all organisms. Phosphorylation by CK2 appears to be necessary for the rapid light-induced degradation of PIF1.

Several lines of evidence suggest that PIF1 is a *bona fide* substrate for Arabidopsis CK2. First, *in silico* studies predicted that PIF1 has multiple CK2 phosphorylation sites (Supplementary table S2). Second, *in vitro* kinase assays demonstrated that PIF1 is strongly phosphorylated by purified Arabidopsis CK2 α and β subunit holoenzyme combinations (Fig. 1A). Third, CK2 mediated phosphorylation of PIF1 is inhibited by a specific inhibitor, heparin in a concentration-dependent manner (Fig. 1B). Fourth, mapping of the phosphorylation sites identified seven phosphorylation sites scattered throughout PIF1 polypeptide with a cluster of sites at the C-terminal end (Fig. 2A, B, C; Supplementary Figure S1). Fifth, CK2-mediated phosphorylation enhances the rapid light-induced degradation of PIF1 (Figs. 3, 4). Sixth, overexpression of CK2 β subunits accelerates the light-induced degradation of native PIF1 *in vivo* (Fig. 5A). These data provide strong evidence that PIF1 is a substrate for Arabidopsis CK2, and that CK2 phosphorylation specifically plays a role in the light-induced degradation of PIF1 to promote photomorphogenesis. However, the above data do not show that PIF1 is a substrate for CK2 *in vivo*. Further experiments are necessary to demonstrate whether CK2 phosphorylates PIF1 *in vivo*.

Previously, CK2 substrates have been identified in plants. These include translation initiation factors (e.g., eIF2α, eIF2β, eIF3c, eIF4B, eIF5) (40,41), chromatin remodeling enzyme (histone deacetylase 2B) (40), circadian clock components (e.g., CCA1 and LHY) (33,36,37,46), HMBG proteins from maize and Arabidopsis (47), abscisic acid responsive protein Rab17 in maize (48), and positively acting transcription factors (e.g., HY5 and HFR1) involved in light signaling pathways (34,35). However, phosphorylation by CK2 has been shown to either stabilize or modulate the activity of these factors (34,35,40). By contrast, our data show that phosphorylation by CK2 promotes the light-induced degradation of PIF1 through ubi/26S proteasomal pathway (Fig. 3A). This is similar to the posttranslational regulation of mammalian IκBα and promyelocytic leukemia (PML) proteins, where CK2 mediated phosphorylation enhanced their polyubiquitination and proteasomal degradation (49,50). Similar to PIF1, CK2 phosphorylates a cluster of sites at the C-terminus of IκBα, and this phosphorylation is UV light inducible (50). Therefore, CK2 mediated stabilization and destabilization of proteins might represent an evolutionarily conserved mechanism.

Although, our data provide strong evidence that CK2 promotes the light-induced degradation

of PIF1 *in vivo*, PIF1-6M (that lacks majority of the CK2 phosphorylation sites) and PIF1 (S464-466A) mutants are still robustly phoshorylated in response to light *in vivo* as previously observed for wild type PIF1 (Figs. 3A, 4A, Supplementary figure S3) (26). These data suggest that either CK2 phosphorylates PIF1 in response to light at different sites than the ones identified and mutated or a separate light-specific kinase may phosphorylate PIF1 at different Ser/Thr residues under light. Because phy-interaction is necessary for the light-induced phosphorylation and degradation of PIFs (12,13,26), and because phyA has been shown to have Ser/Thr kinase activity (51), it is possible that phys might directly phosphoryate PIFs in response to light. However, convincing *in vivo* evidence for the phyA kinase hypothesis is still lacking. Therefore, these data suggest that phosphorylation of PIF1 by CK2 and phosphorylation by either phys directly or a phy-associated kinase is necessary for the rapid light-induced degradation of PIF1. Further work is needed to identify the additional phosphorylation sites by either phytochromes or a phytochrome-associated kinase.

In addition, the mechanism of CK2-mediated enhanced degradation of PIF1 is still unknown. It is possible that phosphorylation of PIF1 by CK2 enhances PIF1's affinity for phys, as phy-interaction has been shown to be necessary for rapid degradation of PIF1 (26). However, this is unlikely as the isolated APA and APB domains, which are located within the first 150 amino acids, are both necessary and sufficient for physical interaction with phyA and phyB, respectively (Fig. 2A) (26,52). Although PIF1 has one CK2 phosphorylation site near the APB sequence (T10) (Fig. 2A), the results show that the CK2 sites at the C-terminal end (S464, S465 and S466) play the major role in PIF1 degradation compared to other CK2 sites (Figs. 2, 3A, 4A). An alternative hypothesis is that CK2-mediated phosphorylation of PIF1 at the C-terminus enhances PIF1's interaction with substrate recognition factors responsible for polyubiquitination and subsequent degradation (e.g., F-box proteins in SCF complex). This is more likely because enhancement of proteasomal degradation of multiple factors by signal-induced phosphorylation has been demonstrated (50,53). This possibility is also consistent with our previous data that isolated N- (1-150 amino acids) and C-terminal (151-478 amino acids) regions are not phosphorylated and degraded in response to light (26). Conversely, both N- and C-terminal regions are necessary for the light-induced degradation of PIF1 *in vivo* (26). Identification of factors responsible for recognition and polyubiquitination of PIF1 will help distinguish these possibilities.

In summary, our data and those of others show that light stabilizes positively acting transcription factors (e.g., HY5, LAF1 and HFR1) (34,35,54), and induces proteasomal degradation of negatively acting transcription factors (e.g., PIFs) to promote photomorphogenesis (Fig. 6). CK2 phosphorylates both positively and negatively acting transcription factors involved in light signaling. However, the significance of CK2 phosphorylation is opposite for these two classes of transcription factors. CK2-mediated phosphorylation enhances the stability of the positively acting factors, while it decreases the stability of the negatively acting factors (e.g., PIF1 and possibly other PIFs). This contrasting, but apparently synergistic, effect is expected to promote robust photomorphogenesis. However, our phenotypic analyses showed that CK2 β subunit overexpression lines have modest seedling deetiolation and circadian phenotypes (Figs. 5B, C, Supplementary Figure S7). Moreover, a stable PIF1 is expected to promote hypocotyl growth as observed (Figs. 3, 4) (26), while a CK2 dominant negative mutant displayed a short hypocotyl phenotype in the dark (45). These apparent contradictions might be explained, in part, by the fact that CK2 mediated phosphorylation of HY5 reduces the DNA binding ability of HY5 (34). In addition, all organisms have multiple CK2 substrates *in vivo* that may function in multiple pathways. More than 300 substrates have been identified for CK2 in animal system (55). Therefore, the phenotypes of the CK2 mutants and overexpression lines will reflect the net effect of CK2 on these diverse pathways that regulate plant growth and development. Identification and characterization of these substrates will shed light on how CK2 optimizes photomorphogenesis.

## REFERENCES


1. Rockwell, N. C., Su, Y.-S., and Lagarias, J. C. (2006) *Annu. Rev. Plant Biol.* 57, 837–858
2. Bae, G., and Choi, G. (2008) *Annu Rev Plant Biol* 59, 281-311
3. Franklin, K. A., and Quail, P. H. (2010) *Journal of Experimental Botany* 61, 11-24
4. Castillon, A., Shen, H., and Huq, E. (2009) *Genetics* 182, 161-171
5. Schaefer, E., and Nagy, F. (2006) *Photomorphogenesis in plants and bacteria*, 3rd ed., Springer, Dordrecht, The Netherlands
6. Fankhauser, C., and Chen, M. (2008) *Trends Plant Sci* 13, 596-601
7. Huq, E., and Quail, P. H. (2005) Phytochrome signaling. in *Handbook of Photosensory Receptors* (Briggs, W. R., and Spudich, J. L. eds.), Wiley-VCH, Weinheim, Germany. pp 151-170
8. Ma, L., Li, J., Qu, L., Hager, J., Chen, Z., Zhao, H., and Deng, X.-W. (2001) *Plant Cell* 13, 2589-2607
9. Quail, P. H. (2007) *J. Integr. Plant Biol.* 49, 11-20
10. Jiao, Y., Lau, O. S., and Deng, X. W. (2007) *Nature Rev|Genetics* 8, 217-230
11. Huq, E. (2006) *Trends Plant Sci* 11, 4-7
12. Henriques, R., Jang, I.-C., and Chua, N.-H. (2009) *Curr Opin Plant Biol* 12, 49-56
13. Castillon, A., Shen, H., and Huq, E. (2007) *Trends Plant Sci* 12, 514-521
14. Duek, P. D., and Fankhauser, C. (2005) *Trends Plant Sci* 10, 51-54
15. Leivar, P., and Quail, P. H. (2011) *Trends Plant Sci* 16, 19-28
16. Toledo-Ortiz, G., Huq, E., and Quail, P. H. (2003) *Plant Cell* 15, 1749-1770
17. Martinez-Garcia, J. F., Huq, E., and Quail, P. H. (2000) *Science* 288, 859-863
18. Oh, E., Kang, H., Yamaguchi, S., Park, J., Lee, D., Kamiya, Y., and Choi, G. (2009) *Plant Cell* 21, 403-419
19. Moon, J., Zhu, L., Shen, H., and Huq, E. (2008) *Proc Natl Acad Sci U S A* 105, 9433-9438
20. Oh, E., Yamaguchi, S., Huc, J., Yusukeb, J., Jung, B., Paik, I., Leed, H.-S., Sun, T.-P., Kamiya, Y., and Choi, G. (2007) *Plant Cell* 19, 1192-1208
21. Hornitschek, P., Lorrain, S., Zoete, V., Michielin, O., and Fankhauser, C. (2009) *Embo Journal* 28, 3893-3902
22. Toledo-Ortíz, G., Huq, E., and Rodríguez-Concepción, M. (2010) *Proc Natl Acad Sci U S A* 107, 11626-11631
23. Leivar, P., Monte, E., Al-Sady, B., Carle, C., Storer, A., Alonso, J. M., Ecker, J. R., and Quail, P. H. (2008) *Plant Cell* 20, 337-352
24. Leivar, P., Monte, E., Oka, Y., Liu, T., Carle, C., Castillon, A., Huq, E., and Quail, P. H. (2008) *Curr Biol* 18, 1815-1823
25. Jang, I.-C., Henriques, R., Seo, H. S., Nagatani, A., and Chua, N.-H. (2010) *Plant Cell* 22, 2370-2383

26. Shen, H., Ling, Z., Castillon, A., Majee, M., Downie, B., and Huq, E. (2008) *Plant Cell* 20, 1586-1602

27. Shin, J., Kim, K., Kang, H., Zulfugarov, I. S., Bae, G., Lee, C. H., Lee, D., and Choi, G. (2009) *Proc Nat Acad Sci USA* 106, 7660-7665

28. Leivar, P., Tepperman, J. M., Monte, E., Calderon, R. H., Liu, T. L., and Quail, P. H. (2009) *Plant Cell* 21, 3535-3553

29. Chen, M., Galvão, R. M., Li, M., Burger, B., Bugea, J., Bolado, J., and Chory, J. (2010) *Cell* 141, 1230-1240

30. Al-Sady, B., Ni, W., Kircher, S., Schafer, E., and Quail, P. H. (2006) *Mol Cell* 23, 439-446

31. Shen, Y., Khanna, R., Carle, C. M., and Quail, P. H. (2007) *Plant Physiol* 145, 1043-1051

32. Lee, Y., Lloyd, A. M., and Roux, S. J. (1999) *Plant Physiol* 119, 989-1000

33. Sugano, S., Andronis, C., Ong, M. S., Green, R. M., and Tobin, E. M. (1999) *Proc Natl Acad Sci U S A* 96, 12362-12366

34. Hardtke, C. S., Gohda, K., Osterlund, M. T., Oyama, T., Okada, K., and Deng, X. W. (2000) *Embo J* 19, 4997-5006

35. Park, H.-J., Ding, L., Dai, M., Lin, R., and Wang, H. (2008) *J. Biol. Chem.* 283, 23264-23273

36. Daniel, X., Sugano, S., and Tobin, E. M. (2004) *Proc Natl Acad Sci U S A* 101, 3292-3297

37. Portolés, S., and Más, P. (2007) *Plant J* 51, 966–977

38. Andronis, C., Barak, S., Knowles, S. M., Sugano, S., and Tobin, E. M. (2008) *Mol Plant* 1, 58-67

39. Salinas, P., Fuentes, D., Vidal, E., Jordana, X., Echeverria, M., and Holuigue, L. (2006) *Plant Cell Physiol.* 47, 1295-1308

40. Dennis, M. D., and Browning, K. S. (2009) *J. Biol. Chem.* 284, 20602-20614

41. Dennis, M. D., Person, M. D., and Browning, K. S. (2009) *J. Biol. Chem.* 284, 20615-20628

42. Shen, H., Moon, J., and Huq, E. (2005) *Plant J* 44, 1023-1035

43. Rubio, V., Shen, Y., Saijo, Y., Liu, Y., Giuliana Gusmaroli, Dinesh-Kumar, S. P., and Deng, X. W. (2005) *Plant J.* 41, 767-778

44. Karimi, M., Meyer, B. D., and Hilson, P. (2005) *Trends Plant Sci* 10, 103-105

45. Moreno-Romero, J., Espunya, C., Platara, M., Ariño, J., and Martínez, M. C. (2008) *Plant J* 55, 118-130

46. Sugano, S., Andronis, C., Green, R. M., Wang, Z.-Y., and Tobin, E. M. (1998) *Proc Natl Acad Sci U S A* 95, 11020-11025

47. Stemmer, C., Leeming, D. J., Franssen, L., Grimm, R., and Grasser, K. D. (2003) *Biochem.* 42, 3503-3508

48. Riera, M., Figueras, M., López, C., Goday, A., and Pagès, M. (2004) *Proc Nat Acad Sci USA* 101, 9879-9884

49. Scaglioni, P. P., Yung, T. M., Cai, L. F., Erdjument-Bromage, H., Kaufman, A. J., Singh, B., Teruya-Feldstein, J., Tempst, P., and Pandolfi, P. P. (2006) *Cell* 126, 269-283

50. Kato, T., Delhase, M., Hoffmann, A., and Karin, M. (2003) *Mol Cell* 12, 829-839

51. Yeh, K. C., and Lagarias, J. C. (1998) *Proc Natl Acad Sci U S A* 95, 13976-13981

52. Khanna, R., Huq, E., Kikis, E. A., Al-Sady, B., Lanzatella, C., and Quail, P. H. (2004) *Plant Cell* 16, 3033-3044

53. Karin, M., and Ben-Neriah, Y. (2000) *Annu Rev Immunol* 18, 621-663

54. Seo, H. S., Yang, J. Y., Ishikawa, M., Bolle, C., Ballesteros, M. L., and Chua, N. H. (2003) *Nature* 423, 995-999

55. Meggio, F., and Pinna, L. A. (2003) *Faseb J* 17, 349-368

## *FOOTNOTES

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**The abbreviations used are:** APA, active phyA binding domain; APB, active phyB binding domain; CK2, Casein Kinase 2; PIF1, Phytochrome Interacting Factor 1; phy, phytochrome.

## FIGURE LEGENDS

**Figure 1:** CK2 Phosphorylates PIF1 *in vitro*. A) Phosphorylation of PIF1 by CK2 is enhanced by the β subunit. Autoradiogram showing that HIS-PIF1 was strongly phosphorylated by recombinant CK2 holoenzyme *in vitro*. CK2 phosphorylation assays were performed in 20 μl kinase assay mixtures that contained 50 mM Hepes-KOH, pH 7.6, 5 mM $MgCl_2$, 2.4 mM DTT, 100 mM KCl, 0.2 mM γ-[$^{32}$P]ATP (~250 cpm/pmol), ~1 pmol CK2 α or αβ holoenzyme and ~10-20 pmol PIF1. The reaction was incubated at 30°C for 30 min and terminated by the addition of 4x SDS loading buffer. Samples were boiled 3 min and separated on a 10% SDS-PAGE gel. The gels were dried and exposed to a phosphorImager. B) Autoradiogram showing that heparin effectively inhibited HIS-PIF1 phosphorylation by CK2 in a dosage-dependent manner. The kinase assays were performed as described in A. *, indicates a non-specific band. C) Different subunit combinations of CK2 differentially phosphorylates PIF1 *in vitro*. The kinase assays were performed as described in A. Statistical analyses for significant differences are shown in supplementary table S3.

**Figure 2:** PIF1 is Phosphorylated by CK2 at Multiple Sites. A) (Top) Diagram showing the CK2 phosphorylation sites identified in PIF1. (Bottom) Identification of phosphorylation sites in PIF1 by MALDI mass spectrometry. MS/MS from the phosphopeptide 463 VSSSKESEDHGNHTTGAAALEHHHHHH 489. The spectrum is dominated by the neutral loss of 98 Da phosphoric acid that is characteristic of phosphopeptides. Fragments are observed from the C-terminal charge retention y ions up to y24, all without phosphorylation, indicating that the phosphorylation site is localized onto S464 and/or S465. B) Autoradiogram showing phosphorylation of wild-type HIS-PIF1 and HIS-PIF1 6M mutant proteins by purified recombinant CK2. In vitro phosphorylation of mutant PIF1 by CK2 is severely reduced. C) Autoradiogram showing phosphorylation of wild-type HIS-PIF1, HIS-PIF1 6M and HIS-PIF1 7M mutant proteins by recombinant CK2 α1β1 holoenzyme. In vitro phosphorylation of HIS-PIF1-7M by CK2 is completely eliminated.

**Figure 3:** CK2-Mediated Phosphorylation of PIF1 is Necessary for Rapid Light-Induced Degradation of PIF1 *in vivo.* A) Light-induced phosphorylation and degradation of a PIF1 containing six CK2 phosphorylation sites mutated (TAP-PIF1-6M) compared to wild type TAP-PIF1. Two independent transgenic lines were compared with the corresponding wild type TAP-PIF1 controls (top and bottom). α-RPT5 blots are shown as loading control. Numbers under the protein gel blots show relative PIF1 level in wild type TAP-PIF1 and TAP-PIF1-6M transgenic lines. PIF1 level in each dark samples is set as 1. B) TAP-PIF1-6M promotes hypocotyl growth under red light. Photographs of seedlings of various genotypes

grown in the dark or under R light (7 μmol$m^{-2}s^{-1}$) for four days. Bar = 5 mm. Protein levels for various independent transgenic lines are shown (Supplementary figure S2). C) Bar graph showing the mean hypocotyl lengths for various genotypes as indicated. Seedlings were grown as described in B. Error bars represent standard error of mean ($n \geq 30$). *, indicates significant difference ($p<0.05$).

**Figure 4:** S464-466 are Necessary for the Rapid Light-Induced Degradation of PIF1. A) Light-induced phosphorylation and degradation of a PIF1 containing either S459-461 to Ala (top) or S464-466 to Ala (bottom) compared to wild type LUC-PIF1. The rate of light-induced degradation of LUC-PIF1(S464-466A) is strongly reduced compared to wild type LUC-PIF1 (Bottom). *, indicates cross-reacting band. Numbers under the protein gel blots show relative PIF1 level in wild type LUC-PIF1, LUC-PIF1(S459-461A) and LUC-PIF1(S464-466A) transgenic lines. PIF1 level in each dark samples is set as 1. B) LUC-PIF1(S464-466A) promotes hypocotyl growth under red light. Photographs of seedlings of various genotypes grown in the dark or under R light (7 μmol$m^{-2}s^{-1}$) for four days. Bar = 5 mm. A second allele of LUC-PIF1(S464-466A)#33 displaying similar long hypocotyl phenotype under red light is shown in supplementary figure S4. C) Bar graph showing the mean hypocotyl lengths of various genotypes as indicated. Seedlings were grown as described in B. Error bars represent standard error of mean ($n \geq 30$). *, indicates significant difference ($p<0.05$).

**Figure 5:** Degradation of PIF1 is Faster in *CK2 β1* and *CK2 β2* Overexpression Lines under Red Light and Far red light. A) Protein gel blots showing native PIF1 levels in wild-type and two independent CK2 β subunit overexpression lines. Four day-old dark-grown seedlings were exposed to red pulse (Rp) (top) or far-red pulse (FRp) (bottom) at the indicated fluences and then incubated in the dark for the times indicated before harvesting for protein extraction. Numbers under the protein gel blots show relative PIF1 level in wild type and CK2 β subunit overexpression lines. PIF1 level in the wild type Col-O dark sample is set as 1. α-RPT5 blots are shown as loading control. B) *CK2 β1* and *CK2 β2* overexpression lines are hypersensitive to FR light compared to wild type control. Photographs of seedlings of various genotypes grown in the dark or under FR light (0.4 μmol$m^{-2}s^{-1}$) for four days. Bar = 5 mm. C) Bar graph showing the mean hypocotyl lengths of various genotypes as indicated. Seedlings were grown as described in C. Error bars represent standard error of mean ($n \geq 30$). *, indicates significant difference ($p<0.05$).

**Figure 6:** Model of CK2 Function in Regulating Photomorphogenesis. Light induces rapid degradation of the negative regulators (e.g., PIFs) and stabilization of the positive regulators (e.g., HY5, HFR1 and LAF1) to promote photomorphogenesis. CK2 phosphorylates both the negative regulators and the positive regulators. However, CK2-mediated phosphorylation stabilizes the positive regulators and destabilizes the negative regulators in response to light to promote photomorphogenesis.

Figure 1

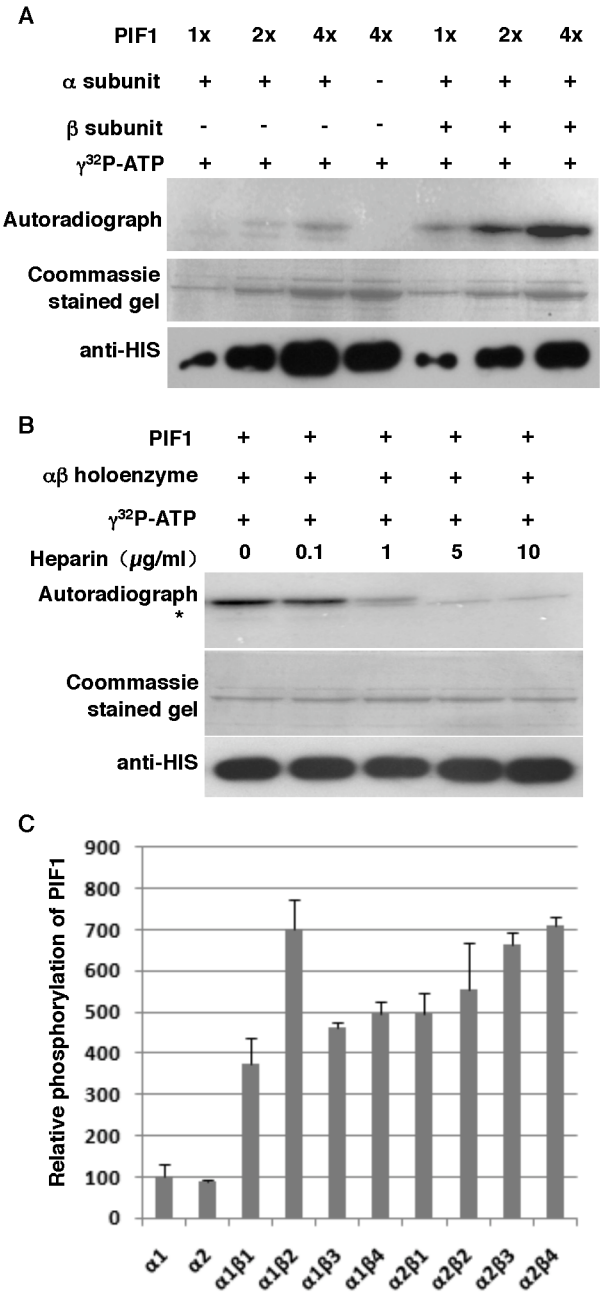


Figure 2

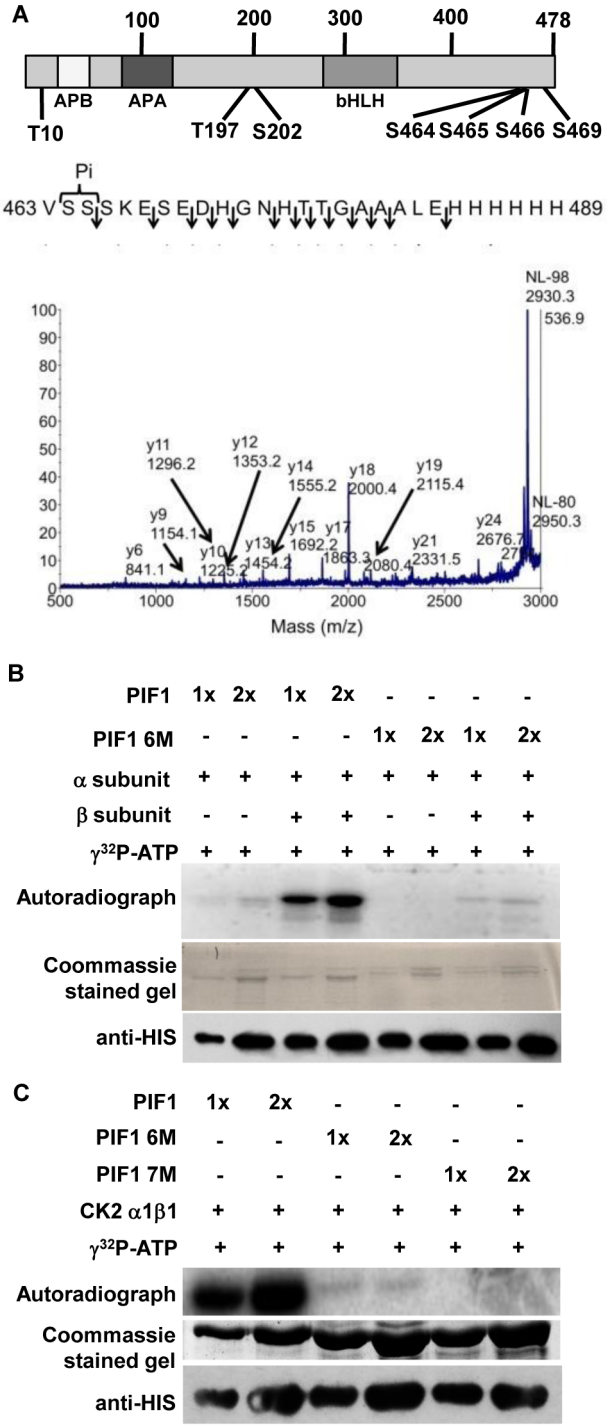


Figure 3

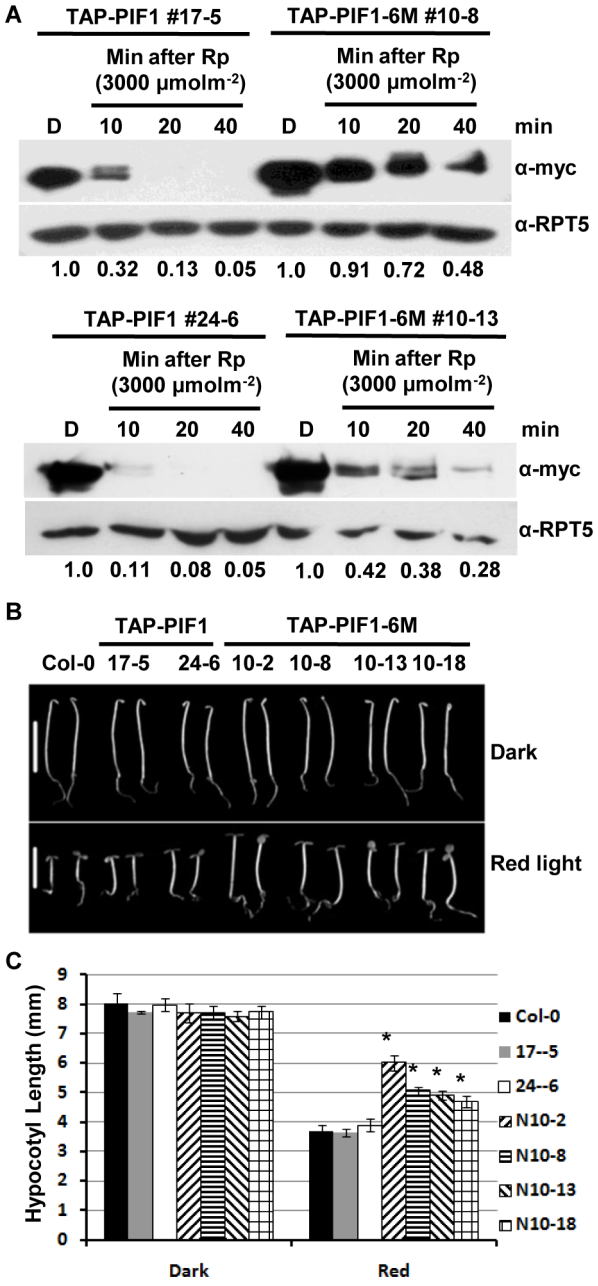


Figure 4

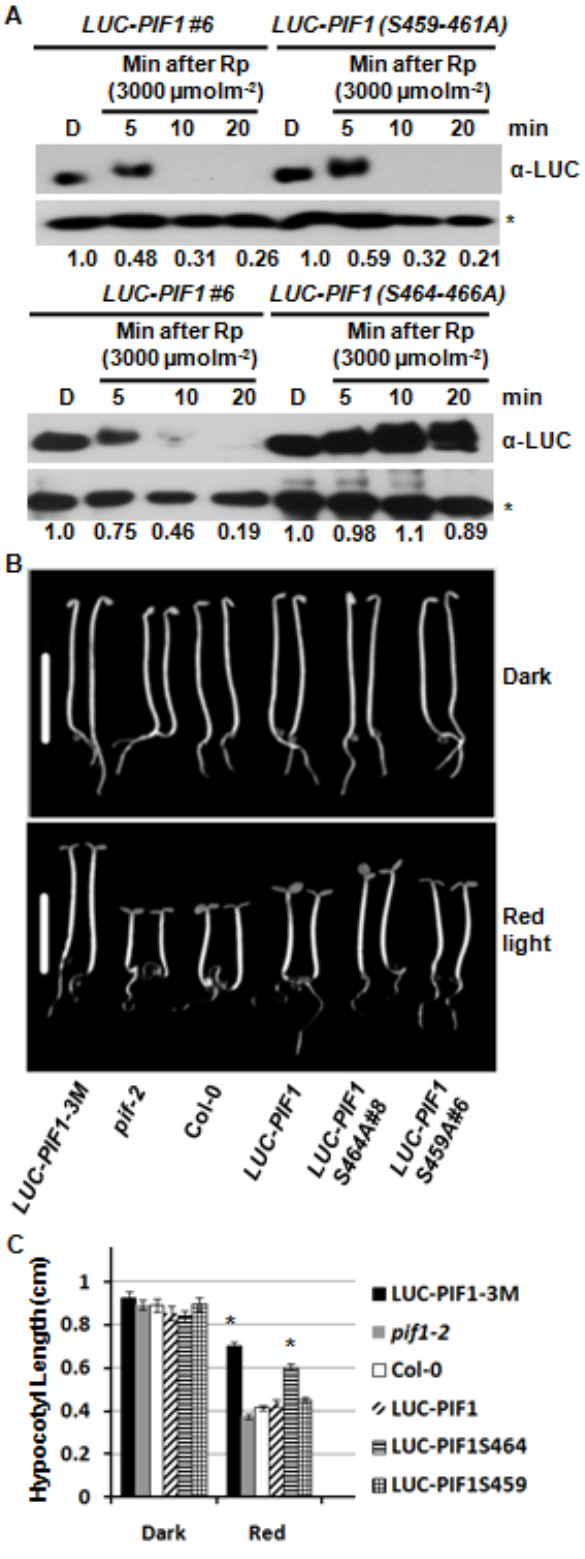


Figure 5

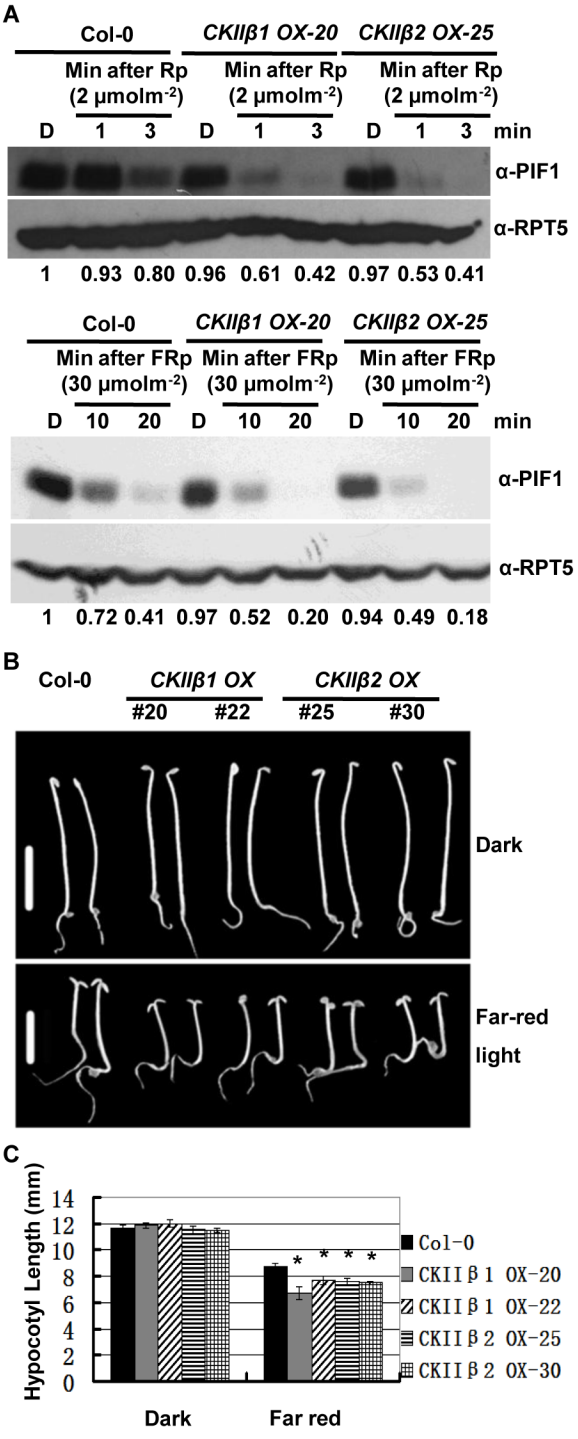
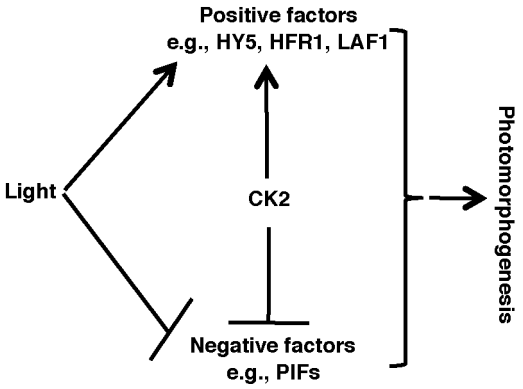


**Figure 6**



**Phosphorylation by CK2 enhances the rapid light-induced degradation of PHYTOCHROME INTERACTING FACTOR 1 in Arabidopsis**

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**Supplementary Data**

The following materials are available in the online version of this article:

## EXPERIMENTAL PROCEDURES

*Immunoprecipitation and Alkaline Phosphatase Treatment*- Immunoprecipitation followed by Calf Intestine Alkaline Phosphatase (CIAP) assay was performed as described (Shen et al., 2008). Briefly, four day-old dark-grown *pPIF1:TAP-PIF1-6M* (line #10-8) seedlings were pretreated with 40 μM MG132 for four hours in the dark to reduce ubi-mediated protein degradation. Total proteins were extracted from ~0.4 g seedlings (grown in darkness or dark-grown seedlings treated with 3000 μmolm$^{-2}$ of Rp followed by dark incubation for 30 minutes) with 1 mL denaturing buffer (100 mM $NaH_2PO_4$, 10 mM Tris [pH 8.0], 100 mM NaCl, 8 M urea, 0.05% Tween-20, 1X Protease inhibitor cocktail [F. Hoffmann-La Roche Ltd, Basel, Switzerland], 2 mM PMSF, 40 μM MG132, 25 mM β-GP, 10 mM NaF, 2 mM Na-orthovanadate and 100 nM calyculin A) and cleared by centrifugation at 16,000 g for 15 min at 4ºC. TAP-PIF1-6M was immunoprecipitated from supernatants with Ni-NTA magnetic agarose beads (Qiagen Inc., Valencia, CA) as described (Al-Sady et al., 2006; Shen et al., 2008). The pellet was washed with PBS buffer, resuspended in 100 μL CIAP reaction buffer and then incubated without (-) or with (+) native CIAP or with boiled CIAP (+B) for 30 min at 37ºC. The samples were heated at 65ºC in 1X SDS-Laemmli buffer for 5 min and then subjected to Western blot analysis with anti-c-MYC antibody as described above.

*Leaf Movement Rhythm Assays*- For leaf movement rhythm assays, seedlings were germinated on MS media and entrained for 10 d under a 12L/12D cycle and then transferred to continuous white light (50 $\mu$molm$^{-2}$s$^{-1}$). Seedlings were individually transferred to the wells of upright 24-well tissue culture plates and the positions of the primary leaves were recorded every 20 min for 7d using a CCD camera system (Model: LTC 0335, Bosch, Farmington Hills, MI). Leaf movement was assessed by measuring the vertical position of the primary leaves using the publicly available software ImageJ. Rhythmic period was measured using the freeware program PEANUTS (http://www.vu-wien.ac.at/i128/Peanuts.htm) that calculates Lomb-Scargle periodograms.

## REFERENCES:

Shen, H., Ling, Z., Castillon, A., Majee, M., Downie, B., and Huq, E. (2008) *Plant Cell* 20, 1586-1602

Al-Sady, B., Ni, W., Kircher, S., Schafer, E., and Quail, P. H. (2006) *Mol Cell* 23, 439-446

**Supplementary Table S1**: Primer sequences used for various experiments.

**Supplementary Table S2**: PIF1 has multiple predicted CK2 phosporylation sites. I*n silico* predicted phosphorylation sites were obtained from SacnSite 2.0 (http://scansite.mit.edu), NetPhosK 1.0 (http://www.cbs.dtu.dk/services/NetPhosK/) and KinasePhos 2.0 (http://kinasephos.mbc.nctu.edu/) using three cut-off values.

**Supplementary Table S3:** Statistical analyses for the differential phosphorylation activities of different CK2 subunits on PIF1. Students t-test was performed for different combinations (n=3) and statistically significant differences are shown at the bottom table.

**Supplementary Figure S1:** MS of PIF1 and MS/MS of different phosphopeptides from PIF1. MS of phosphopeptide IMAC enriched fraction of PIF1 (A) and MS/MS of observed phosphopeptides (B-H) are shown. The phosphorylation sites in each peptide are indicated with (Pi) next to the Ser/Thr residue that is phosphorylated. Strong neutral loss 98 (loss of $H_3PO_4$) is observed in all MS/MS.

**Supplementary Figure S2:** Protein levels of various homozygous independent transgenic lines of TAP-PIF1 and TAP-PIF1-6M used for phenotypic analyses. TAP-PIF1 containing six mutations at all the CK2 phosphorylation sites as well as wild type TAP-PIF1 were expressed using endogenous *PIF1* promoter in *pif1* background. Single-insert homozygous transgenic plants were selected based on antibiotic selection. Total protein was extracted from four day-old dark-grown seedlings, separated on 6.25% SDS-PAGE gel and immunoblotted with anti-myc antibody. Anti-RPT5 antibody was used as control.

**Supplementary Figure S3**: The red (R) light-induced slow migrating band of TAP-PIF1-6M is a phosphorylated form of PIF1. TAP-PIF1 was immunoprecipitated from protein extracts prepared using four day-old dark-grown *pPIF1:TAP-PIF1-6M* (line #10-8) seedlings kept in the dark or exposed to Rp (3000 $\mu molm^{-2}$) followed by dark incubation for 30 min. The immunoprecipitated pellets from the Rp-exposed samples were dissolved in Calf Intestinal Alkaline Phosphatase (CIAP) buffer and incubated without (-) or with (+) native CIAP or with boiled CIAP (+B). Samples were then separated on 6.5% SDS-PAGE gels and probed with anti-MYC antibody.

**Supplementary Figure S4:** LUC-PIF1(S464-466A) mutant promotes hypocotyl elongation under red light. Photographs showing hypocotyl lengths of various genotypes including two independent alleles of LUC-PIF1(S464-466A) transgenic plants. Seedlings were grown for four days in the dark or one day in the dark followed by three days under red light (7 $\mu molm^{-2}s^{-1}$). Bar = 5 mm.

**Supplementary Figure S5**: The light-induced degradation of PIF1 is unaffected in *ck2β3* mutant compared to wild type seedlings. Four day-old dark-grown wild type and mutant seedlings were either kept in the dark or exposed to R (A) or FR (B) light followed by incubation in the dark (1 and 3 min for R light, and 10 and 20 min for FR light) before protein extraction. Proteins were separated on 8% SDS-PAGE gel and immunoblotted with anti-PIF1 antibody. Anti-RPT5 antibody was used as control.

**Supplementary Figure S6:** Characterization of the CK2 β subunit overexpression lines. Anti-GFP Western blots showing the level of CK2 β1–GFP (A) and β2–GFP proteins (B) in various independent transgenic lines. Anti-RPT5 antibody was used as control. C) RT-PCR showing the mRNA levels of two selected CK2 β1–GFP and β2–GFP transgenic lines compared to wild type background. *ACTIN* was used as a control.

**Supplementary Figure S7:** Leaf movement rhythms under continuous white light. Seedlings were entrained for 10 d under a 12L/12D cycle and then transferred to LL. Subjective day and subjective night are denoted by white and hatched bars, respectively. Results of a representative leaf movement rhythms assay for COL-0 (n=8), CK2β1ox-20 (n=11), CK2β1ox-22 (n=6), CK2β2ox-25 (n=8) and CK2β2ox-33 (n=8) are shown. The experiment was repeated three times and an average period length was calculated.

**Supplementary Table S1: Primer sequences used in experiments described in the text**

| Gene | Forward | Reverse |
|---|---|---|
| **Cloning** | | |
| PIF1 promoter | GACCTGCAGCACTTTGTTCACTAACTTGACTAC | GTCCTGCAGCTCTCTCTACAAAGATGATGATAATG |
| PIF1-TAP tag | CACCTCATGCATCATTTTGTCCCTGAC | TTAACCTGTTGTGTGGTTTCCGTG |
| PIF1-pET21d | CGACCATGGCAATGCATCATTTTGTCCCTGAC | GTTGCGGCCGCACCTGTTGTGTGGTTTCCGTG |
| CK2β1 OX | CACCATGTATAGAGACAGAGGAACG | CGGTTTGTGTAATTTGAACCC |
| CK2 β2 OX | CACCATGTATAGGGAGAGAGGTATG | CGGCTTGTGTAGCTTGAACCC |
| **Site-directed mutagenesis** | | |
| PIF1T10A | TCCCTGACTTCGATGCCGATGATGATTATGT | ACATAATCATCATCGGCATCGAAGTCAGGGA |
| PIF1T197A | GTTTAACACGGCGGGCGGATGGTACTGACAG | CTGTCAGTACCATCCGCCCGCCGTGTTAAAC |
| PIF1S202A | CGGATGGTACTGACGCTTCCGCCGTAGCTGG | CCAGCTACGGCGGAAGCGTCAGTACCATCCG |
| PIF1S464A | GTTCGAGCAGGGTGGCTAGTAGTAAGGAATC | GATTCCTTACTACTAGCCACCCTGCTCGAAC |
| PIF1S465A | CGAGCAGGGTGAGTGCTAGTAAGGAATCTGA | TCAGATTCCTTACTAGCACTCACCCTGCTCG |
| PIF1S469A | GTAGTAGTAAGGAAGCTGAGGATCACGGAAA | TTTCCGTGATCCTCAGCTTCCTTACTACTAC |
| PIF1S464-465A | GTTCGAGCAGGGTGGCTGCTAGTAAGGAAGCTGA | TCAGCTTCCTTACTAGCAGCCACCCTGCTCGAAC |
| PIF1S464-466A | CCCAAGTTCGAGCAGGGTGGCTGCTGCTAAGGAA TCTGAGG | CCTCAGATTCCTTAGCAGCAGCCACCCTGCTCGA ACTTGGG |
| PIF1S459-461A | GCAACATCGTACCCAGCTGCGGCCAGGGTGAGTA GTAG | CTACTACTCACCCTGGCCGCAGCTGGGTACGATG TTGC |
| PIF1S466A | GCAGGGTGGCTGCTGCTAAGGAAGCTGAGG | CCTCAGCTTCCTTAGCAGCAGCCACCCTGC |

Supplementary Table S2: PIF1 has multiple predicted CK2 phosporylation sites.

| http://scansite.mit.edu | | |
|---|---|---|
| Stringency | # of sites | position |
| High | 1 | S265 |
| Medium | 5 | T10, T258, T260, T263, S265 |
| Low | 11 | T10, T245, T258, T259, T260, T263, S265, S267, S321, S466, S469 |

| http://www.cbs.dtu.dk/services/NetPhosK/) | | |
|---|---|---|
| Stringency | # of sites | position |
| 0.7 | 0 | |
| 0.6 | 6 | T10, T32, S238, T259, T260, S265 |
| 0.5 | 13 | T10, T32, S106, S236, S238, T259, T260, T263, S265, S267, S321, S465, S469 |

| http://kinasephos.mbc.nctu.edu.tw/index.php | | |
|---|---|---|
| Stringency | # of sites | position |
| 100% | 1 | S80 |
| 95% | 8 | T10, T258, T260, T263, S265 |
| 90% | 12 | S63, S80, S189, S236, T259, S265, S267, S321, S423, S464, S466, S469 |

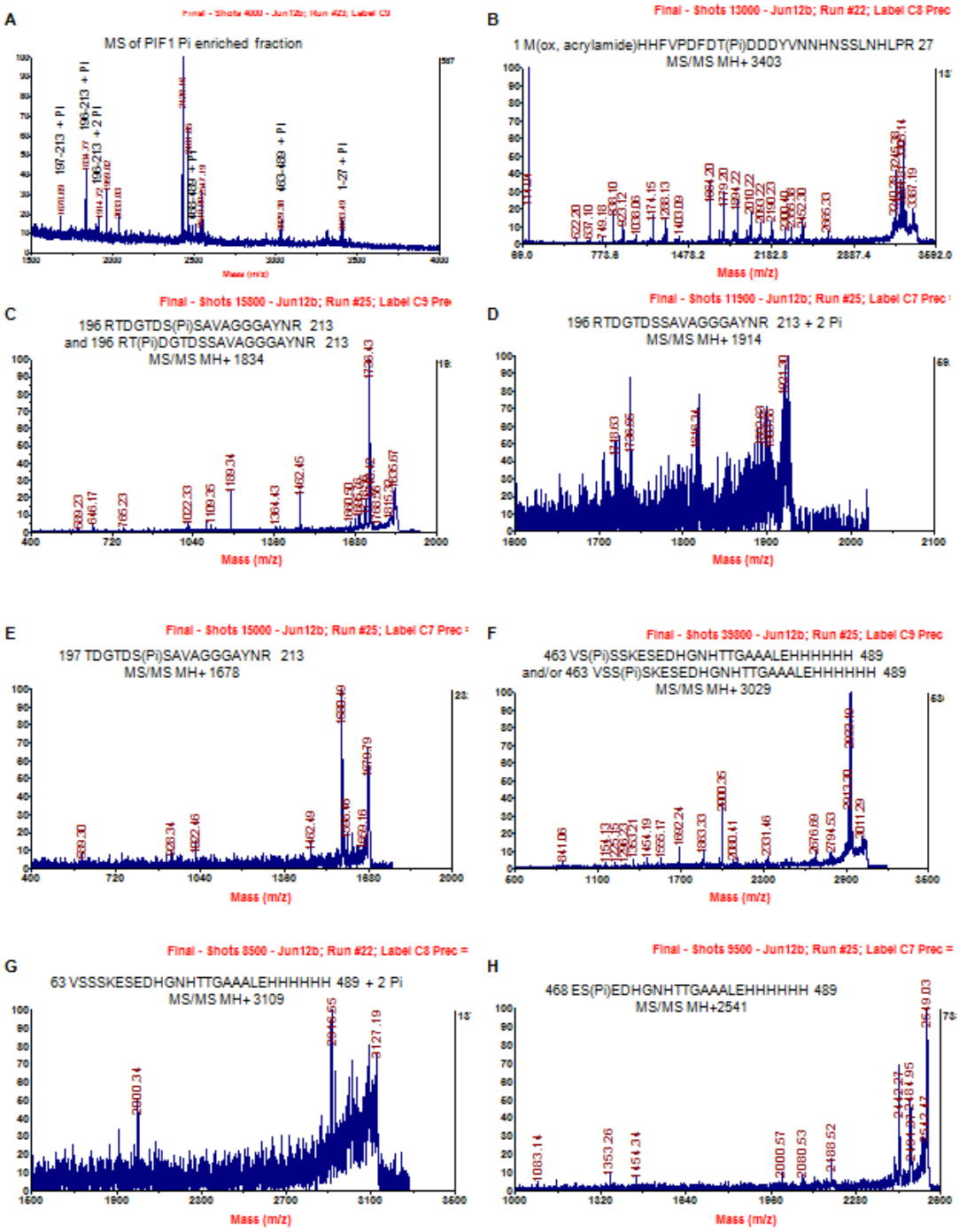
Supplementary Table S3: Statistical analyses for the differential phosphorylation activities of different CK2 subunits on PIF1.

**Raw T-test p values**

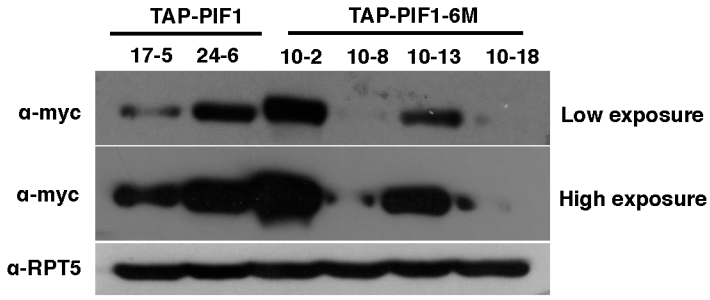
| p values | α1 | α2 | α1β1 | α1β2 | α1β3 | α1β4 | α2β1 | α2β2 | α2β3 | α2β4 |
|---|---|---|---|---|---|---|---|---|---|---|
| **α1** | *x* | | | | | | | | | |
| **α2** | 0.5210 | *x* | | | | | | | | |
| **α1β1** | 0.0030 | 0.0015 | *x* | | | | | | | |
| **α1β2** | 0.0002 | 0.0001 | 0.0041 | *x* | | | | | | |
| **α1β3** | 0.0001 | 0.0001 | 0.0777 | 0.0048 | *x* | | | | | |
| **α1β4** | 0.0001 | 0.0001 | 0.0479 | 0.0067 | 0.2225 | *x* | | | | |
| **α2β1** | 0.0003 | 0.0002 | 0.0593 | 0.0157 | 0.3253 | 0.6459 | *x* | | | |
| **α2β2** | 0.0026 | 0.0021 | 0.0754 | 0.1305 | 0.2408 | 0.4501 | 0.4717 | *x* | | |
| **α2β3** | 0.0001 | 0.0001 | 0.0019 | 0.4626 | 0.0003 | 0.0018 | 0.0071 | 0.1742 | *x* | |
| **α2β4** | 0.0001 | 0.0001 | 0.0009 | 0.8339 | 0.0001 | 0.0004 | 0.0024 | 0.3385 | 0.0763 | *x* |

**Is significance indicated by $p < 0.05$?**

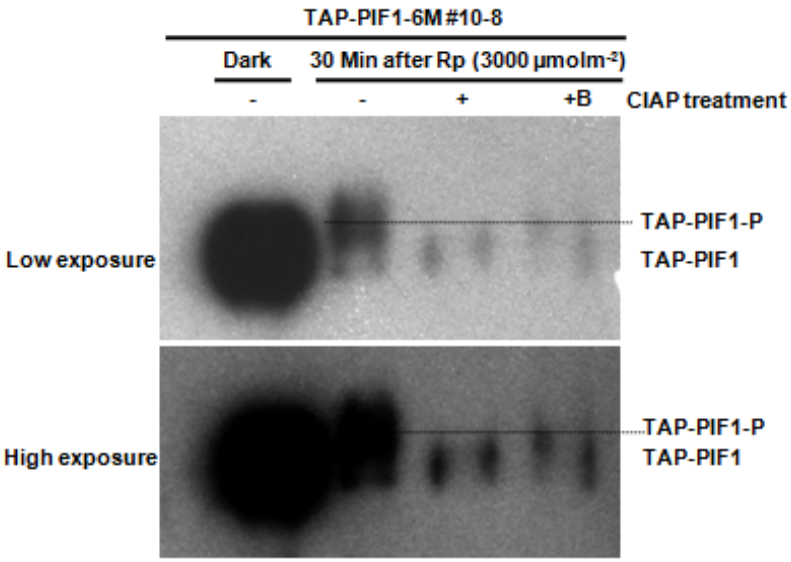
| | α1 | α2 | α1β1 | α1β2 | α1β3 | α1β4 | α2β1 | α2β2 | α2β3 | α2β4 |
|---|---|---|---|---|---|---|---|---|---|---|
| **α1** | *x* | | | | | | | | | |
| **α2** | no | *x* | | | | | | | | |
| **α1β1** | yes | yes | *x* | | | | | | | |
| **α1β2** | yes | yes | yes | *x* | | | | | | |
| **α1β3** | yes | yes | no | yes | *x* | | | | | |
| **α1β4** | yes | yes | yes | yes | no | *x* | | | | |
| **α2β1** | yes | yes | no | yes | no | no | *x* | | | |
| **α2β2** | yes | yes | no | no | no | no | no | *x* | | |
| **α2β3** | yes | yes | yes | no | yes | yes | yes | no | *x* | |
| **α2β4** | yes | yes | yes | no | yes | yes | yes | no | no | *x* |



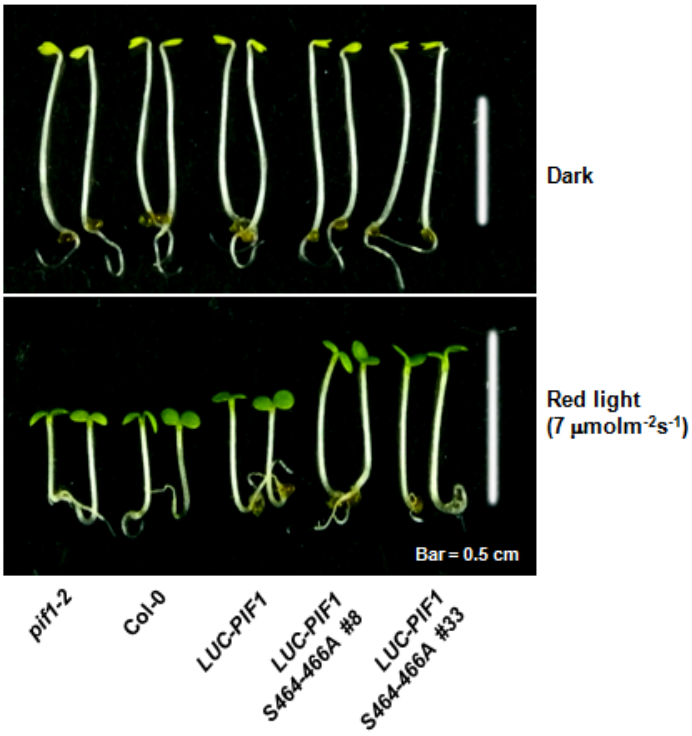

**Supplementary Figure S1:** MS of PIF1 and MS/MS of different phosphopeptides from PIF1. MS of phosphopeptide IMAC enriched fraction of PIF1 (A) and MS/MS of observed phosphopeptides (B-H) are shown. The phosphorylation sites in each peptide are indicated with (Pi) next to the Ser/Thr residue that is phosphorylated. Strong neutral loss 98 (loss of $H_3PO_4$) is observed in all MS/MS.



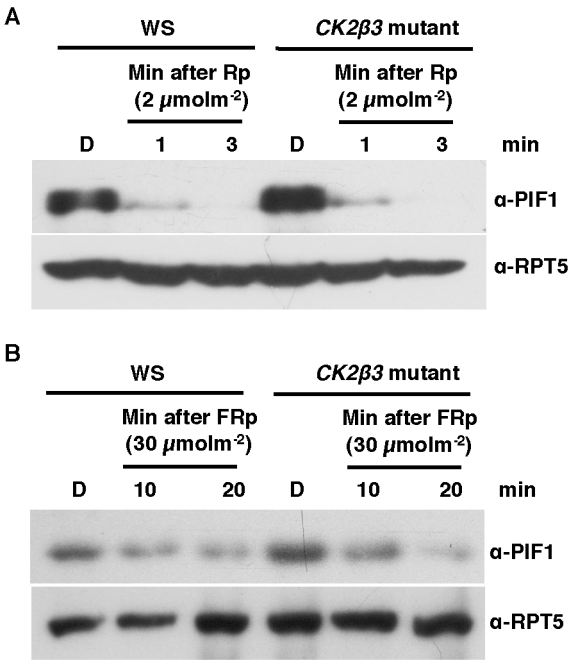

Supplementary Figure S2: Protein levels of various homozygous independent transgenic lines of TAP-PIF1 and TAP-PIF1-6M used for phenotypic analyses. TAP-PIF1 containing six mutations at all the CK2 phosphorylation sites as well as wild type TAP-PIF1 were expressed using endogenous *PIF1* promoter in *pif1* background. Single-insert homozygous transgenic plants were selected based on antibiotic selection. Total protein was extracted from four day-old dark-grown seedlings, separated on 6.25% SDS-PAGE gel and immunoblotted with anti-myc antibody. Anti-RPT5 antibody was used as control.



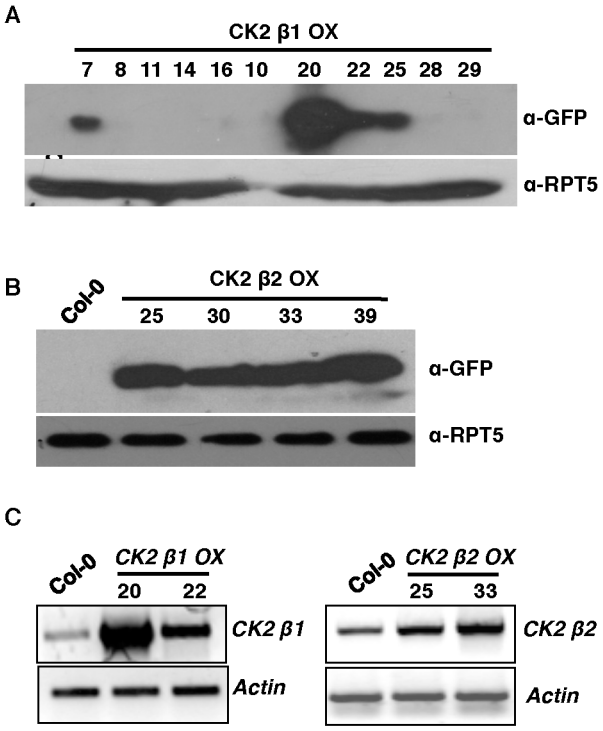

Supplementary Figure S3: The red (R) light-induced slow migrating band of TAP-PIF1-6M is a phosphorylated form of PIF1. TAP-PIF1 was immunoprecipitated from protein extracts prepared using four day-old dark-grown *pPIF1:TAP-PIF1-6M* (line #10-8) seedlings kept in the dark or exposed to Rp (3000 $\mu molm^{-2}$) followed by dark incubation for 30 min. The immunoprecipitated pellets from the Rp-exposed samples were dissolved in Calf Intestinal Alkaline Phosphatase (CIAP) buffer and incubated without (-) or with (+) native CIAP or with boiled CIAP (+B). Samples were then separated on 6.5% SDS-PAGE gels and probed with anti-MYC antibody.



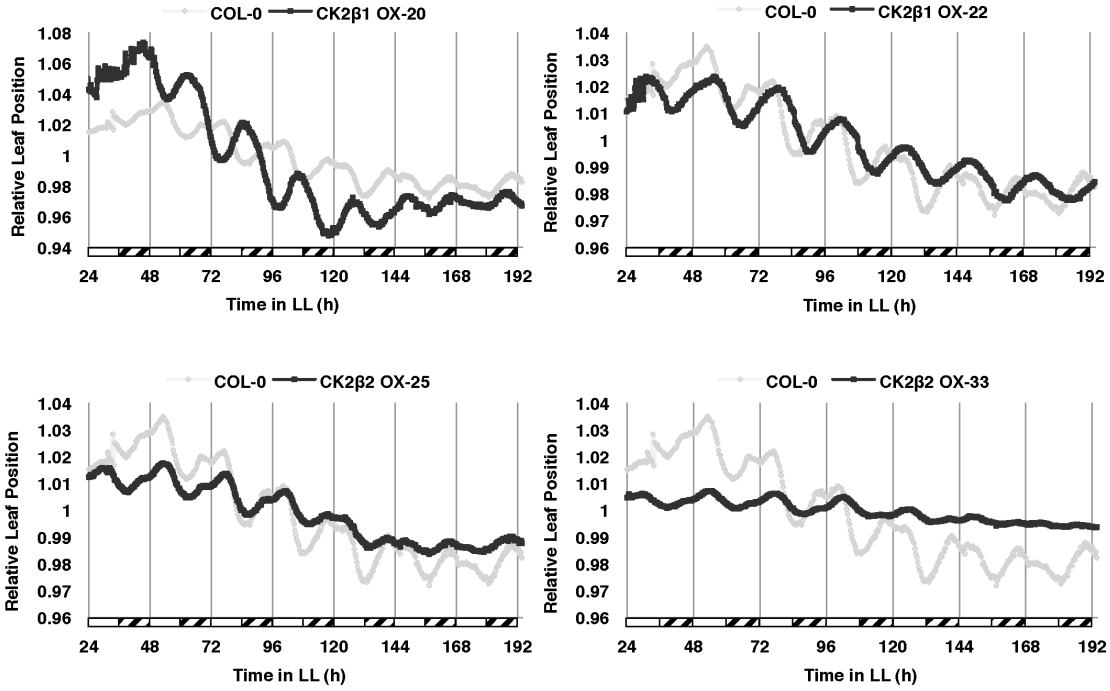

Supplementary Figure S4: LUC-PIF1(S464-466A) mutant promotes hypocotyl elongation under red light. Photographs showing hypocotyl lengths of various genotypes including two independent alleles of LUC-PIF1(S464-466A) transgenic plants. Seedlings were grown for four days in the dark or one day in the dark followed by three days under red light (7 µmolm$^{-2}$s$^{-1}$). Bar = 5 mm.




Supplementary Figure S5: The light-induced degradation of PIF1 is unaffected in *ck2β3* mutant compared to wild type seedlings. Four day-old dark-grown wild type and mutant seedlings were either kept in the dark or exposed to R (A) or FR (B) light followed by incubation in the dark (1 and 3 min for R light, and 10 and 20 min for FR light) before protein extraction. Proteins were separated on 8% SDS-PAGE gel and immunoblotted with anti-PIF1 antibody. Anti-RPT5 antibody was used as control.




Supplementary Figure S6: Characterization of the CK2 β subunit overexpression lines. Anti-GFP Western blots showing the level of CK2 β1–GFP (A) and β2–GFP proteins (B) in various independent transgenic lines. Anti-RPT5 antibody was used as control. C) RT-PCR showing the mRNA levels of two selected CK2 β1–GFP and β2–GFP transgenic lines compared to wild type background. *ACTIN* was used as a control.




| | COL-0 | CK2β1 OX-20 | CK2β1 OX-22 | CK2β2 OX-25 | CK2β2 OX-33 |
|---|---|---|---|---|---|
| Period (h) | 24.10 | 21.52 | 24.09 | 24.23 | 24.49 |
| Stdev (h) | 0.39 | 0.59 | 0.58 | 0.37 | 0.20 |

Supplementary Figure S7: Leaf movement rhythms under continuous white light. Seedlings were entrained for 10 d under a 12L/12D cycle and then transferred to LL. Subjective day and subjective night are denoted by white and hatched bars, respectively. Results of a representative leaf movement rhythms assay for COL-0 (n=8), CK2β1ox-20 (n=11), CK2β1ox-22 (n=6), CK2β2ox-25 (n=8) and CK2β2ox-33 (n=8) are shown. The experiment was repeated three times and an average period length was calculated.