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Phytochrome-Specific Type 5 Phosphatase Controls Light Signal Flux by Enhancing Phytochrome Stability and Affinity for a Signal Transducer

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Summary

Environmental light information such as quality, intensity, and duration in red (∼660 nm) and far-red (∼730 nm) wavelengths is perceived by phytochrome photoreceptors in plants, critically influencing almost all developmental strategies from germination to flowering. Phytochromes interconvert between red light-absorbing Pr and biologically functional far-red light-absorbing Pfr forms. To ensure optimal photoresponses in plants, the flux of light signal from Pfr-phytochromes should be tightly controlled. Phytochromes are phosphorylated at specific serine residues. We found that a type 5 protein phosphatase (PAPP5) specifically dephosphorylates biologically active Pfr-phytochromes and enhances phytochrome-mediated photoresponses. Depending on the specific serine residues dephosphorylated by PAPP5, phytochrome stability and affinity for a downstream signal transducer, NDPK2, were enhanced. Thus, phytochrome photoreceptors have developed an elaborate biochemical tuning mechanism for modulating the flux of light signal, employing variable phosphorylation states controlled by phosphorylation and PAPP5-mediated dephosphorylation as a mean to control phytochrome stability and affinity for downstream transducers.

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Introduction

Many organisms have developed various strategies for perceiving and processing environmental light information to optimize their growth, development, and behavior (Sullivan and Deng, 2003). Plants, being photosynthetic and sessile, exhibit particularly plastic development and growth, depending on the environmental light information. The quality, intensity, duration, and direction of the environmental light provide plants with information not only on the ambient light condition but also on other elements in their environments such as neighboring plants and seasonal changes (Neff et al., 2000; Quail, 2002). Thus, plants possess a sophisticated light sensing and signaling system that ensures optimal photoperception and responses to their ever-changing environmental conditions.

The light-sensing system in plants includes multiple photoreceptors; the UV-B receptor, the UV-A/blue light receptors, and phytochromes (Gyula et al., 2003). Phytochrome molecules that sense light information in red (R) and far-red (FR) light consist of an N-terminal photosensory domain and a C-terminal protein kinase domain (see Supplemental Figure S1 in the Supplemental Data available with this article online). The photosensory domain contains a chromophore, phytochromobilin, which allows phytochromes to adapt the two interconvertible spectral forms, Pr and Pfr (Neff et al., 2000). The Pfr form is biologically active in mediating photoresponses. The known substrates of phytochrome kinase activity include PKS1, Aux/IAAs, cryptochromes, and phytochromes themselves (Fankhauser et al., 1999; Colon-Carmona et al., 2000; Ahmad et al., 1998; Yeh and Lagarias, 1998). Phytochromes are phosphorylated at specific serine residues by their autophosphorylation activity as well as by yet-unknown phytochromes-associated kinase(s) (Lapko et al., 1999; Kim et al., 2004). Phosphorylation plays fundamental roles in the functional regulation of receptor molecules in many organisms. For example, photoinduced phosphorylation of rhodopsin, the mammalian photoreceptor, by rhodopsin kinase desensitizes the light-excited receptor by enhancing its binding to a capping protein, arrestin (Maeda et al., 2003). However, for phytochromes, the biological role and the regulatory mechanisms that govern the phosphorylation state are largely unknown.

Phytochrome signaling pathway in *Arabidopsis* is composed of an intricate network of numerous downstream signaling components (Quail, 2002). However, for the proper regulation of biological responses to light, biological light signaling system, in addition to having the information flow pathways to downstream components, should tightly control the flux of information through the pathways (Neff et al., 2000; Nagy and Schäfer, 2002; Maeda et al., 2003). Although control of the information flux in phytochrome-mediated pho-

toperception may occur at various steps in the signaling pathway, the earliest control appears to occur at the photoreceptor level. Considering the unique nature of phytochromes, light-dependent nucleocytoplasmic partitioning (Fankhauser et al., 1999) and light regulation of protein stability (Clough and Vierstra, 1997; Seo et al., 2004) are possible mechanisms to control the light information flux. Furthermore, phosphorylation of phytochromes has been suggested to be a signal attenuation mechanism (Casal et al., 2002; Kim et al., 2004). Although the regulatory mechanisms of the phosphorylation status of phytochromes are still poorly understood, modulation of the phosphorylation state can certainly provide a way of tuning the light information flux at the earliest step of light signaling.

In this report, we investigate the regulation of the phosphorylation status of phytochromes and its role in controlling light signal flux through functional analyses of a type 5 serine/threonine protein phosphatase, PAPP5, which preferentially binds to and specifically dephosphorylates the biologically functional Pfr-phytochromes.

Results and Discussion

PAPP5 Physically Interacts with Phytochromes in a Spectral Form-Dependent Manner

PAPP5 was identified by yeast two-hybrid screening of phytochrome-interacting proteins, employing the full-length *Arabidopsis* phytochrome A (phyA) as a "bait." Among 78 positive cDNA clones, the deduced amino acid sequence of one cDNA clone showed significant similarity to the type 5 serine/threonine protein phosphatases and was designated PAPP5 (*P*hytochrome-*A*ssociated *P*rotein *P*hosphatase *5*). The interaction between PAPP5 and *Arabidopsis* phyA was confirmed by reciprocally using each molecule as the "bait" and as the "prey" in a yeast two-hybrid assay (Figure 1A). The interaction between PAPP5 and *Arabidopsis* phyA was further confirmed by in vitro binding assay (Figure 1B). *Arabidopsis* has five phytochromes (phyA–phyE) that are classified into two groups (Sullivan and Deng, 2003): photo-labile type I (phyA) and photo-stable type II (phyB–phyE). While PAPP5 was initially isolated as a phyA-interacting protein, it also bound to *Arabidopsis* phyB, a representative member of type II phytochromes, in an in vitro binding assay (Figure 1B). The region in *Arabidopsis* phyA that is responsible for binding to PAPP5 was mapped within the C-terminal 253 amino acids (Supplemental Figure S1).

A test for functional importance of phytochrome-interacting proteins is to determine their relative binding affinity for the two photoconvertible forms of phytochromes, Pr and Pfr (Ni et al., 1999; Fankhauser et al., 1999; Choi et al., 1999). We employed a native oat phyA preparation (Lapko et al., 1999) for this test since the photoconvertible holoprotein is prepared facilely from dark-grown oat seedlings and is known to interact with several *Arabidopsis* photo-signaling components in vitro (Fankhauser et al., 1999; Choi et al., 1999). Moreover, oat phyA is biologically active as a photoreceptor in transgenic *Arabidopsis* (Boylan and Quail, 1991). As shown in Figure 1C, oat phyA treated with an R light

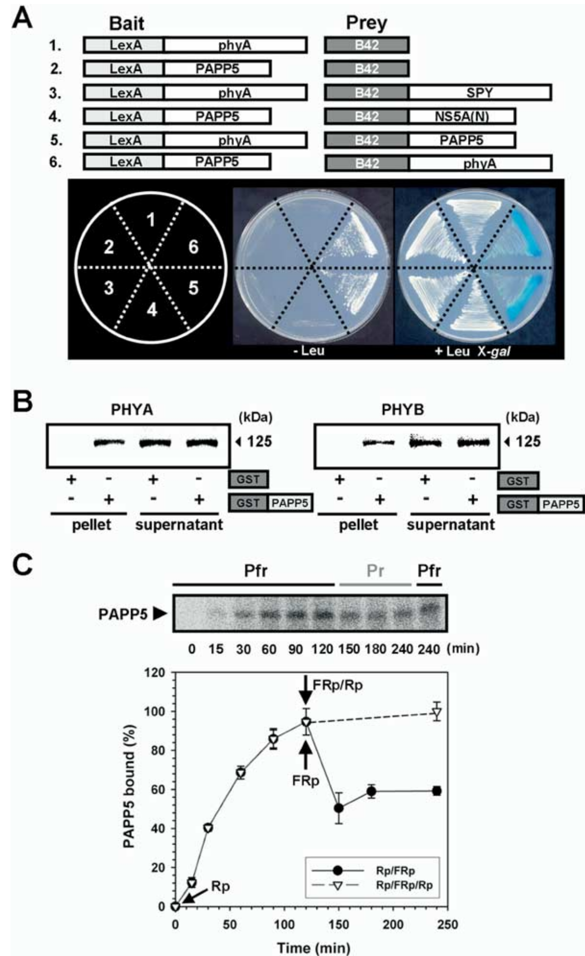


Figure 1. PAPP5 Physically Interacts with Phytochromes in a Spectral Form-Dependent Manner

(A) Yeast two-hybrid analysis. "Bait" and "prey" pairs (upper panel) and the growth of the yeast strains on selective (–Leu) and β-galactosidase assay (+Leu, X-*gal*) media (lower panel). SPY (*Arabidopsis* SPINDLY) and NS5A(N) (the N-terminal region of HCV NS5A protein) were included as negative controls for binding specificity.

(B) In vitro binding pull-down assay. ^{35}S-labeled *Arabidopsis* PHYA or PHYB was incubated with GST-PAPP5 or GST alone. The reaction complexes were pulled down with glutathione Sepharose 4B beads. ^{35}S-labeled phytochromes in the pellet and supernatant fractions were visualized by autoradiography.

(C) Binding of Pr- or Pfr-phyA to ^{35}S-labeled PAPP5. The reaction mixture containing oat phyA was treated with 5 min of R light pulse (Rp). After incubation with ^{35}S-labeled PAPP5 for 2 hr, the reaction mixture was irradiated with either 5 min of FR light pulse (Rp/FRp) or 5 min of FR light followed by 5 min of R light pulse (Rp/FRp/Rp). At the indicated time points, the PAPP5/oat phyA complex was immunoprecipitated with the oat phyA-specific antibody (oat22). Coimmunoprecipitated PAPP5 was visualized by autoradiography (upper panel) and was quantified as values (mean ± SD; n = 3) relative to that of the Pfr form at the 240 min point (lower panel).

pulse (Rp), which converts Pr-oat phyA to the Pfr form, showed increasing binding to PAPP5 in a time-dependent manner. A subsequent FR light pulse treatment (Rp/FRp), which converts the Pfr form to the Pr form, resulted in approximately 40% less binding. In comparison, treatment of the same sample with a following R

light pulse (Rp/FRp/Rp), which converts Pr-oat phyA to the Pfr form, resulted in recovery of binding affinity of PAPP5 to oat phyA. These results show that binding of PAPP5 to oat phyA is differential for the two different spectral forms with a preferential binding to the biologically active Pfr form.

PAPP5 Is a Type 5 Serine/Threonine Protein Phosphatase

The deduced amino acid sequence of the *PAPP5* gene has two distinctively noticeable domains, the N-terminal domain containing the three tetratricopeptide repeats (TPRs) and the C-terminal domain containing the highly conserved signature motifs of a type 2A serine/threonine protein phosphatase (PP2Ac) (Figure 2A). This domain structure is a characteristic feature of members of the type 5 serine/threonine protein phosphatase (PP5) subfamily (Chinkers, 2001). The amino acid sequence of PAPP5 also shows high similarity to other known PP5s (Supplemental Figure S2).

The TPR motifs of the PP5 subfamily are known to form a bundle of antiparallel, amphipathic α helices to mediate protein-protein interactions (Chinkers, 2001). We thus investigated if the TPR domain of PAPP5 mediates the interaction of PAPP5 with phytochromes. As shown in Figure 2B, the TPR domain alone is both necessary and sufficient for interaction with phytochromes. The interaction with phytochromes is specific for the TPR domain of PAPP5; SPINDLY (SPY), which is involved in gibberellic acid signaling in plants (Jacobsen et al., 1996), bears 10 TPR motifs in its N terminus but did not interact with *Arabidopsis* phyA (Figure 1A).

We further confirmed the enzymatic properties of PAPP5 as a PP5, utilizing the glutathione S-transferase (GST)-fused recombinant PAPP5 (GST-PAPP5). In the presence of 100 μM arachidonic acid (AA), GST-PAPP5 exhibited a phosphatase activity with the usual Michaelis-Menten kinetics with a Km value of 160 mM *p*NPP and a Vmax values of 22 μmol Pi released/min/mg of protein (Figure 2C).

A distinguishing enzymatic characteristic of PP5s is their activation by polyunsaturated long-chain fatty acids such as AA (Chinkers, 2001). In mammalian PP5s, phosphatase activity is autoinhibited by the N-terminal TPR domain, and allosteric conformational change induced by AA binding to the TPR domain relieves this inhibition. The phosphatase activity of the GST-PAPP5 was stimulated by AA in a concentration-dependent manner (Figure 2D). The TPR domain of PAPP5 autoinhibited the phosphatase activity of PAPP5; the PP2Ac domain alone, which lacks the TPR domain, exhibited AA-independent phosphatase activity at a level comparable to that of the full-length PAPP5 in the presence of AA (Figure 2E). On the other hand, the phosphatase activity of PP5s derived from a C-terminal PP2Ac domain is known to be inhibited by okadaic acid (OA) (Chinkers, 2001). The phosphatase activity of recombinant PAPP5 was also inhibited by OA with an IC_{50} value of 5 nM (data not shown). Taken together, these results show that PAPP5 is a member of the PP5 subfamily of protein phosphatases.

Phytochromes Are Substrates for PAPP5 Phosphatase Activity

Phytochromes are phosphoproteins and physically interact with PAPP5. We thus tested if phosphorylated phytochromes are substrates of PAPP5 phosphatase activity, utilizing a native oat phyA preparation. Oat phyA was effectively phosphorylated in this preparation without addition of exogenous protein kinases (Yeh and Lagarias, 1998; Fankhauser, et al., 1999), the Pfr form being slightly more phosphorylated (Figure 2F). The phosphorylated Pfr-oat phyA was effectively dephosphorylated upon incubation with PAPP5. In contrast, Pr-oat phyA was only slightly dephosphorylated by PAPP5. The results show that phytochromes are substrates for PAPP5 phosphatase activity and that the phosphatase activity is rather specific to the Pfr form of phytochromes. The specificity of PAPP5 phosphatase activity toward the Pfr form is likely due, at least partly, to preferential binding of PAPP5 to the Pfr form (Figure 1C). The PP2Ac domain alone showed lower phosphatase activity toward Pfr-phytochromes than full-length PAPP5. Since phytochrome binding to PAPP5 is mediated by the TPR domain of PAPP5 (Figure 2B), this finding suggests that recruitment of phytochromes to PAPP5 through the TPR domain is necessary for their effective dephosphorylation.

PAPP5 Colocalizes with and Binds to Phytochromes In Vivo

For PAPP5 to function as a phosphatase for phytochromes in vivo, subcellular localization of PAPP5 should be at least partly coincided with phytochromes. We thus examined intracellular localization of PAPP5 and phytochromes by coexpressing cyan fluorescent protein (CFP)-tagged PAPP5 (PAPP5-CFP) and yellow fluorescent protein (YFP)-tagged phyB (phyB-YFP) in etiolated mustard seedlings. As reported previously, phyB-YFP was mainly localized in the cytoplasm in darkness and was translocated into the nucleus in light (Nagy and Schäfer, 2002). The subcellular localization of PAPP5-CFP followed that of phyB-YFP, being localized in the cytoplasm in darkness and in the nucleus in light (Figure 3A). In the nucleus, some of the phyB-YFP formed distinctive nuclear speckles (Figure 3A); the nucleoplasmic speckles of phytochromes observed in our experimental condition appear to be the late nuclear speckles (Bauer et al., 2004) since they are stable over 4 hr (data not shown). In addition, some of the nuclear PAPP5-CFP was also found as speckled form throughout the nucleoplasm (Figure 3A). Analysis of the fluorescence images of phyB-YFP and PAPP5-CFP showed that some portions of the two images are detected in the same nuclear space, especially in the speckled regions. The results indicated that at least a certain fraction of the two proteins are colocalized in the nucleus (Figure 3A). This is supported by confocal image analyses (Supplemental Figure S3B), in which the confocal fluorescence images from the two proteins were simultaneously detected in some of the nuclear speckles. Interestingly, when PAPP5-CFP alone was expressed in etiolated mustard seedlings, fluorescence was observed in both cytoplasm and nucleus regardless of the light conditions (Supplemental Figure S3A). Further-

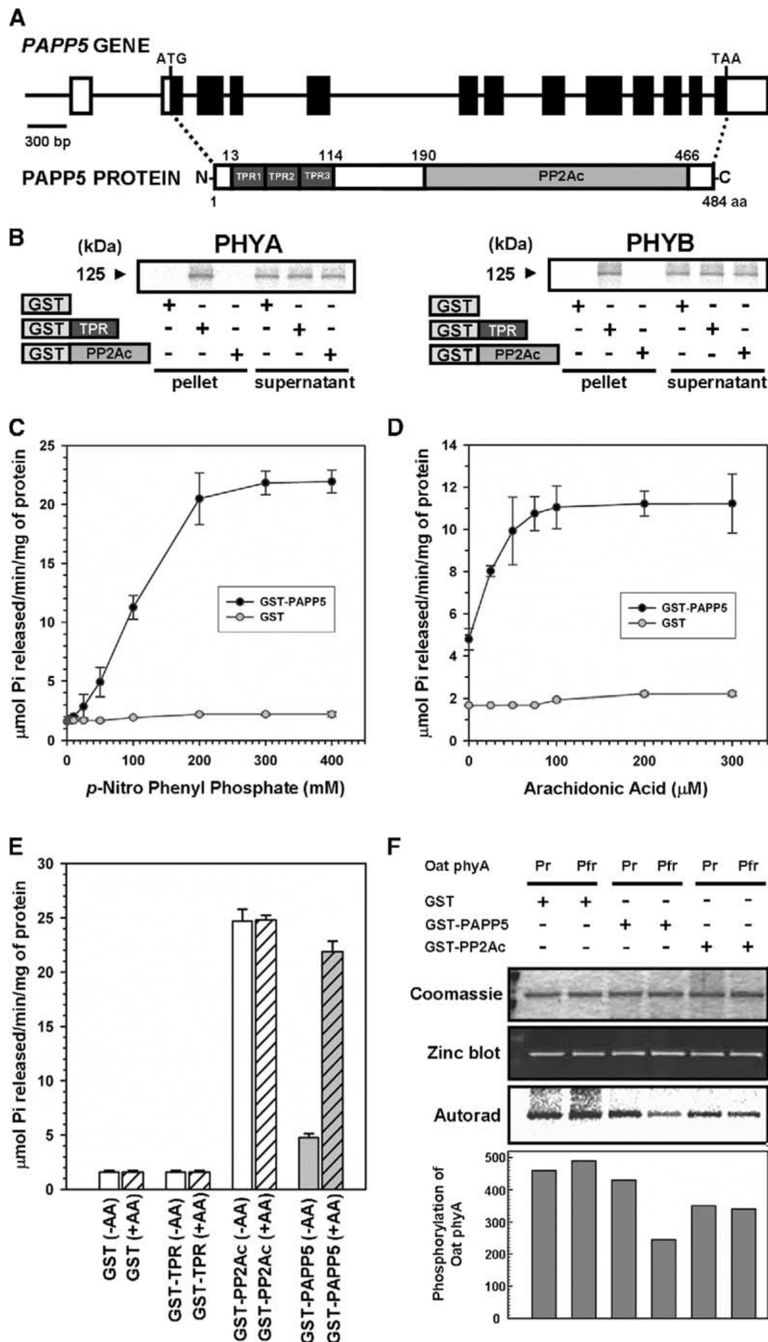


Figure 2. PAPP5 Is a Type 5 Protein Phosphatase and Dephosphorylates Specifically the Pfr form of Phytochromes

(A) The *PAPP5* gene and the encoded protein. *PAPP5* encodes an open reading frame of 484 amino acids. Black and white boxes, protein-coding and noncoding exons, respectively; TPR, tetratricopeptide repeat; PP2Ac, type 2A protein phosphatase catalytic domain. Note that the first exon is located within the 5′ untranslated region.

(B) In vitro interaction of the TPR domain of PAPP5 with phytochromes. ^{35}S-labeled *Arabidopsis* PHYA or PHYB was incubated with GST-TPR, GST-PP2Ac, or GST. The ^{35}S-labeled phytochromes in the pellet and supernatant fractions were visualized by autoradiography.

(C) Phosphatase activity (mean ± SD; n = 3) of PAPP5 assayed with GST-PAPP5 or GST in the presence of 100 μM AA and various concentrations of the artificial substrate, *p*-nitrophenyl phosphate (*p*NPP).

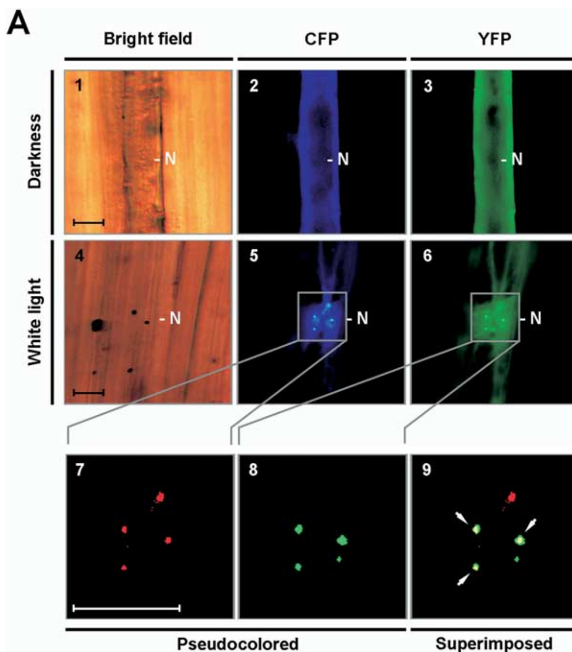
(D) Phosphatase activity (mean ± SD; n = 3) of PAPP5 measured in the presence of 100 mM *p*NPP and various concentrations of AA.

(E) Roles of the TPR and PP2Ac domains in PAPP5 phosphatase activity. The phosphatase activity (mean ± SD; n = 3) of GST-TPR, GST-PP2Ac, GST-PAPP5, and GST was measured in the presence of 400 mM *p*NPP with (+AA) or without (−AA) 100 μM AA.

(F) PAPP5 differentially dephosphorylates the two spectral forms, Pr and Pfr, of oat phyA. ^{32}P-oat phyA labeled in R (Pfr) or R/FR (Pr) light was incubated with GST-PAPP5, GST-PP2Ac, or GST in the absence of AA. The phosphorylation state of oat phyA was visualized by autoradiography. The amount of oat phyA was examined by Coomassie staining and zinc blot. Shown at the bottom is the phosphorylation degree of oat phyA quantified by Image Gauge Software version 3.12 (Fuji Photo Film).

more, in this case, we did not observe formation of the nuclear speckles. This result suggests that light-dependent nuclear localization and nuclear speckle formation of PAPP5 observed upon coexpression of PAPP5-CFP and phyB-YFP (Figure 3A and Supplemental Figure S3B) depended on the presence of phyB, supporting that PAPP5 interacts with phyB in vivo.

We further examined an in vivo physical interaction between PAPP5 and phytochromes by conducting in vivo pull-down assay. For this purpose, we transiently expressed a hemagglutinin (HA)-tagged PAPP5 (PAPP5-HA) or PP2Ac (PP2Ac-HA) in mesophyll cell protoplasts of the PBG-5 transgenic *Arabidopsis* (Yamaguchi et al., 1999) that overexpresses a biologically active and GFP-fused phyB (phyB-GFP). When the whole lysate of the protoplast cells was coimmunoprecipitated with the monoclonal anti-GFP antibody (α_mGFP), PAPP5-HA but not PP2Ac-HA was pulled down together with phyB-GFP (Figure 3B). This result shows that PAPP5 is physically associated with phytochromes in vivo. Furthermore, R light irradiation to the protoplast cells, which converts the Pr form of phyB-GFP to the Pfr conformer, significantly increased the amount of PAPP5-HA coimmunoprecipitated with phyB-GFP. In contrast, in dark-



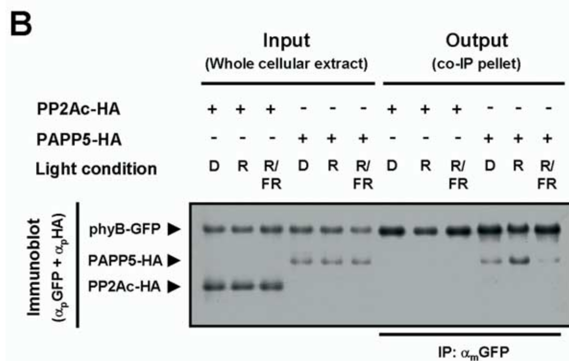


Figure 3. PAPP5 Is Colocalized and Coimmunoprecipitated with *Arabidopsis* phyB In Vivo.

(A) Subcellular colocalization of PAPP5-CFP and phyB-YFP. The hypocotyl cells of mustard seedlings were cotransfected with *PAPP5-CFP* and *PHYB-YFP* and incubated in darkness or in white light for 15 hr. Fluorescence images of PAPP5-CFP and phyB-YFP expressed in the hypocotyl cells are shown in blue and green colors, respectively. 1 and 4, bright field images; 2 and 5, PAPP5-CFP images; 3 and 6, phyB-YFP images. The superimposed fluorescence images of PAPP5-CFP and phyB-YFP in the nucleus were obtained by analysis of the pseudocolored images of PAPP5-CFP (red) and phyB-YFP (green). Arrowheads indicate nuclear speckles where the spatially overlapping images (yellow) of the two signals are clearly noticeable. N, the nuclei; Scale bars, 10 μm.

(B) Coimmnuoprecipitation of PAPP5 with phyB in vivo. Protoplast cells of the *Arabidopsis* PBG-5 line expressing phyB-GFP were transfected with *PP2Ac-HA* or *PAPP5-HA*. After incubation in darkness for 22 hr, the cells were kept in darkness (D) for 2 hr, in 2 hr of R light (R), or in 2hr of R light followed by 2 hr of FR light irradiation (R/FR) before preparation of whole-cell lysates. PhyB-GFP and HA fusion proteins expressed in the protoplasts are detected in the whole-cell lysates (Input). Coimmunoprecipitation was performed with an agarose-conjugated anti-GFP monoclonal antibody (α_mGFP). PhyB-GFP, PAPP5-HA, and PP2Ac-HA in the pellet fraction (Output) were detected by immunoblot analysis with a mixture of the polyclonal anti-GFP (α_pGFP) and anti-HA (α_pHA) antibodies.

ness or upon FR light irradiation followed by R light irradiation, where the Pr form of phyB-GFP is prevalent, a lower level of coimmunoprecipitated PAPP5-HA was detected (Figure 3B). This result supports that in vivo interaction between PAPP5 and phyB is spectral form dependent, consistent with the spectral form-dependant interaction between PAPP5 and oat phyA observed in vitro (Figure 1C). The result also suggests that phyB-dependent nuclear localization and speckle formation of PAPP5 observed in Figure 3A may be due in part to the physical interaction between PAPP5 and phyB.

PAPP5 Positively Regulates Photoresponses Mediated by Both phyA and phyB

The finding that phosphorylated phytochromes are substrates of PAPP5 in a spectral form-dependent manner suggested that PAPP5 should play a functional role in photosignaling. To test this idea, we analyzed various photoresponses in two *Arabidopsis* loss-of-function mutants, *papp5-1* and *papp5-2*, and two transgenic *Arabidopsis* lines, *PAPP5-OX1* and *PAPP5-OX2*, that overexpress the *PAPP5* mRNA under the control of the constitutive CaMV35S promoter (Supplemental Figure S4). PAPP5 binds to both phyA and phyB as shown in Figure 1B. PhyB is responsible for the photophysiological responses to R light with a classical R/FR light reversibility. Recombinant and native preparation of phyA shows the reversible R/FR light photoconversion typical to phytochromes (Lapko et al., 1999; Kim et al., 2004; Figure 1C and Figure 5C). Physiologically, however, phyA mediates so-called high irradiance responses in FR light and very low fluence responses in a wide spectral range in an irreversible manner by a yet-uncovered mechanism, while the Pfr form of phyA is still believed to be biologically active (Neff et al., 2000). Irradiation of R and FR light upregulates expression of various light-inducible genes such as *CHS*, *CAB2*, and *RBCS*. The extent of induction of these genes was clearly reduced and increased in the loss-of-function mutants and in the overexpressing lines, respectively, under both R and FR light irradiation (Figure 4A). The so-called end-of-day far-red (EODFR) light response (Okamoto et al., 2001) mediated by phyB was more strongly reduced in the loss-of-function mutants (Figure 4B) than in the wild-type. Additionally, the loss-of-function mutants and the overexpressing lines exhibited a lower and a higher level of phyA-mediated anthocyanin accumulation (Kim et al., 2003) in FR light, respectively (Figure 4C). Fluence-rate response curves for inhibition of hypocotyl growth (Neff et al., 2000) further revealed that the loss-of-function mutants and the overexpressing lines were hyposensitive and hypersensitive, respectively, to both continuous R (Rc) and continuous FR (FRc) light (Supplemental Figure S5A). We further examined the degree of amplification of responsiveness to R light pulses by FR light preirradiation (Hennig et al., 1999), which is mediated by a functional interaction between phyA and phyB. When an R light pulse was applied immediately after FR light preirradiation, the degree of inhibition of hypocotyl elongation was significantly higher and lower in the overexpressing lines (36%) and in the loss-of-function mutants

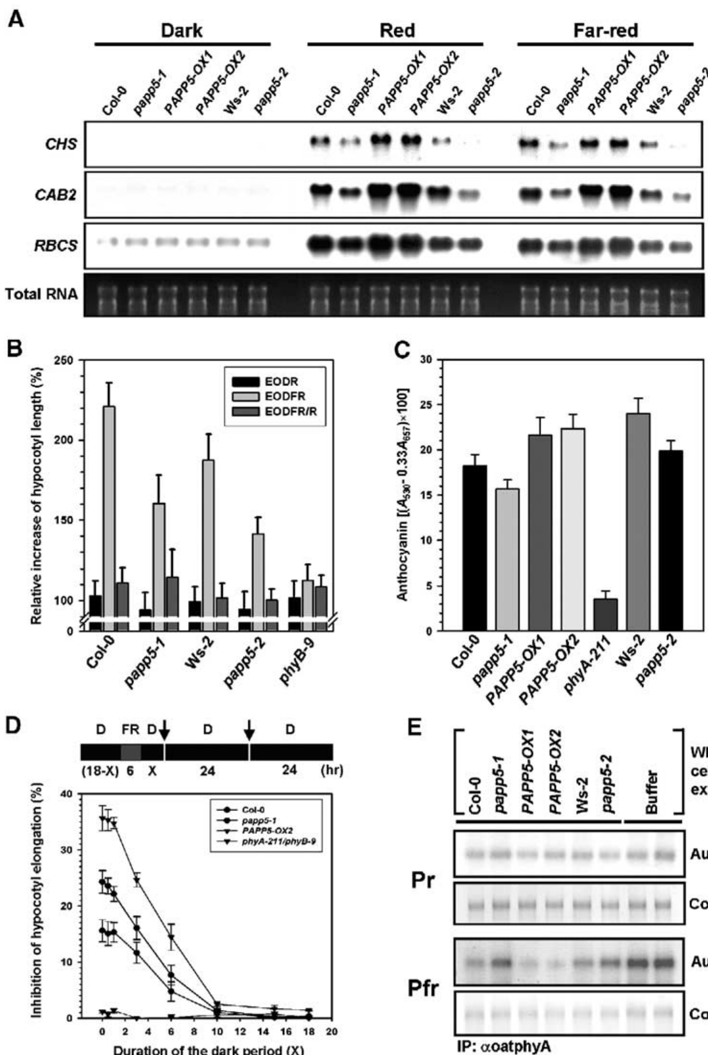


Figure 4. Photoresponses of *Arabidopsis* Seedlings and Dephosphorylation Degree of Oat phyA in the Loss-of-Function Mutants (*papp5-1*, *papp5-2*) and *PAPP5*-Overexpressing Lines (*PAPP5-OX1*, *PAPP5-OX2*).

(A) RNA gel blot analysis of expression of the light-inducible genes, *CHS*, *CAB2*, and *RBCS* in darkness, R light, or FR light irradiation.

(B) Hypocotyl growth response to EODFR light treatment. EODR, EODFR, and EODFR/R denote R, FR, and FR/R light pulse treatment, respectively. The hypocotyl length in each condition is noted as a value relative that of control plants with no EOD light treatment.

(C) Anthocyanin accumulation response of seedlings in FRc light. The amount of anthocyanin was calculated as $A_{530} - 0.33A_{657}$. Mean ± SD; n = 3 (200 seedlings per n).

(D) Measurement of responsiveness amplification in inhibition of hypocotyl elongation. Amplification of R light pulse response by FR light preirradiation was measured. After induction of germination, seedlings of Col-0 (wild-type), *papp5-1*, *PAPP5-OX2*, or *phyA-211/phyB-9* were grown in darkness (D) for 0 to 18 hr (18–X hr), irradiated with FR light for 6 hr, and then transferred into darkness for various periods (X hr). R light pulses (arrows) were given twice at the indicated time points. Inhibition of hypocotyl elongation was measured as values relative to that of seedlings without R light pulse. Mean ± SD; n = 3 (50 seedlings per n).

(E) Dephosphorylation of Pr- or Pfr-oat phyA by whole-cellular protein extracted from *papp5* mutants or *PAPP5*-overexpressing seedlings. ^{32}P-oat phyA labeled in R (Pfr) or R/FR (Pr) light was incubated with whole-cellular extracts. Oat phyA was then immunoprecipitated with the oat22 (IP: αoatphyA) and visualized by autoradiography and Coomassie staining. The last two lanes are buffer controls.

(16%), respectively, than in the wild-type (24%) (Figure 4D). We also noticed that the loss-of-function mutants have a flowering time earlier than that of wild-type in a long-day condition (Supplemental Figure S5B), which is a characteristic of phyB-defective mutants. These results show that PAPP5 positively modulates various light-dependent processes mediated by both phyA and phyB. It should be noted that the degree of modulation of the light responsiveness by PAPP5 is variable depending on light regimes. The response modulated by PAPP5 is more pronounced with pulsed light responses (Figures 4A, 4B, and 4D) than with prolonged light responses (Figure 4C and Supplemental Figure S5).

PAPP5-Mediated Dephosphorylation of Phytochromes Correlates with Modulation of the Photoresponsiveness

The phosphatase activity of PAPP5 toward phytochromes in the loss-of-function mutants and the overexpressing lines is differential for the two different spectral forms (Figure 4E), in accordance with in vitro dephosphorylation assay (Figure 2F). When whole-cell extracts from the loss-of-function mutants and the overexpressing lines were assayed for phosphatase activity toward phosphorylated oat phyA, the loss-of-function mutants and the overexpressing lines exhibited decreased and increased phosphatase activity, respectively, toward the Pfr form (Figure 4E). In contrast, the phosphorylation state of the Pr form was only minimally affected. Thus, the phosphatase activity of PAPP5 toward phytochromes in these plants was positively correlated with the phytochrome-mediated photoresponsiveness. This result suggests that changes in PAPP5-mediated dephosphorylation of biologically active Pfr-phytochromes are responsible for alterations in phyA- and phyB-mediated photoresponsiveness in these plants.

Since phytochrome phosphorylation was suggested to play a role in attenuating light signal (Neff et al., 2000; Kim et al., 2004), the above results led us to the idea that control of phosphorylation status of the Pfr-phytochromes through a process coupled by phosphoryla-

tion and PAPP5-mediated dephosphorylation is a plausible molecular mechanism for fine-tuning of the light responsiveness mediated by phytochromes.

PAPP5-Mediated Dephosphorylation of Pfr-Phytochromes Enhances Their Binding Affinity for the Light Signal Transducer, NDPK2

How does the phosphorylation status of Pfr-phytochromes lead to control of light responsiveness of plants? Light signaling initiated by light-triggered photoconversion of phytochromes from the Pr form to the Pfr form is transmitted to the early signal transducers such as NDPK2 and PIF3 through preferential binding of these molecules to the Pfr-phytochromes (Ni et al., 1998; Choi et al., 1999). We tested a possibility that PAPP5-mediated dephosphorylation of Pfr-phytochromes affects binding of phytochromes to NDPK2 to control the light responsiveness. When the phosphorylated Pfr-oat phyA were incubated with PAPP5, its binding affinity for NDPK2 was significantly (>6-fold) increased, whereas no noticeable effect was detected for the Pr form (Figure 5A). Addition of OA, an inhibitor of PAPP5 phosphatase activity, negated the PAPP5-mediated effect on NDPK2 binding to phytochromes (Figure 5A), indicating that PAPP5 phosphatase activity is required for the enhanced binding affinity. Furthermore, cellular extracts from the loss-of-function mutants and the *PAPP5*-overexpressing lines showed markedly diminished and increased binding of Pfr-oat phyA to NDPK2, respectively (Figure 5B). PAPP5 phosphatase activity was decreased and increased in the loss-of-function mutants and the overexpressing lines, respectively, as shown in Figure 4E. These observations together suggest that changes in PAPP5 phosphatase activity regulate the binding affinity of the Pfr-phytochromes for the signal transducer, NDPK2. NDPK2 activity is known to be enhanced by binding to Pfr-phytochromes to positively regulate photoresponses (Choi et al., 1999). Thus, our results further suggest that the altered photoresponses observed in these plants (Figure 4) are at least partly due to changes of NDPK2 activity that results from the effect of PAPP5 phosphatase activity on NDPK2 binding to Pfr-phytochromes.

The function of PAPP5 in photosignaling is derived from its phosphatase activity specific to phytochromes and is not due to a nonspecific phosphatase activity. Transgenic lines that overexpress the PP2Ac domain only show no noticeable alteration in photoresponsiveness in either Rc or FRc light (Supplemental Figure S6). Furthermore, the PP2Ac domain peptide and the cellular extracts of *PP2Ac*-overexpressing lines did not significantly affect phytochromes/NDPK2 interaction (Figures 5A and 5B). These observations are correlated with the result that the PP2Ac domain alone showed lower phosphatase activity toward Pfr-phytochromes than PAPP5 (Figure 2F), although it can still efficiently dephosphorylate an artificial substrate (Figure 2E). These results thus suggest that PAPP5 positively functions in phytochrome-mediated photoresponses by enhancing NDPK2 binding to Pfr-phytochromes through its phosphatase activity that is specific to Pfr-phytochromes due to the presence of the TPR domain.

PAPP5 Dephosphorylates the Phospho-Serine Residues Located in the Hinge and N-Terminal Extension of Phytochromes

The in vivo and in vitro phosphorylation sites of phytochromes have been identified; the three serine residues, Ser7, Ser17, and Ser598, are phosphorylated in oat phyA (Yeh and Lagarias, 1998; Lapko et al., 1999). A recent biochemical study showed that phosphorylation of oat phyA on Ser598 in the hinge region inhibits the interaction of oat phyA with its downstream signal transducers, NDPK2 and PIF3 (Kim et al., 2004). Since PAPP5 affects binding of NDPK2 to oat phyA, we tested if PAPP5 can dephosphorylate the Ser598 residue of oat phyA. For this test, we utilized recombinant wild-type oat phyA phosphorylated by protein kinase A (PKA) in vitro, which results in phosphorylation mostly on Ser17 and Ser598 (Kim et al., 2004). We also utilized a deletion mutant (Δ65) of oat phyA that lacks the N-terminal 65 amino acids and thus is phosphorylated only on Ser598 by PKA (Kim et al., 2004). As shown in Figure 5C, PAPP5 can clearly dephosphorylate both recombinant wild-type and Δ65 mutant oat phyA when they were in their Pfr forms. In contrast, dephosphorylation of the Pr forms in both wild-type and the Δ65 mutant oat phyA was affected minimally. Thus, PAPP5 dephosphorylates Ser598 of oat phyA in a spectral form-dependent manner. In accordance with a previous report that phosphorylation of Ser598 reduces binding of NDPK2 to oat phyA (Kim et al., 2004), our results indicate that dephosphorylation of Ser598 by PAPP5 results in enhanced affinity of NDPK2 for phytochromes. Thus, we suggest that phosphorylation and the PAPP5-mediated dephosphorylation of the serine residue in the hinge region of phytochromes in a light-dependent manner provide a mechanism to attenuate and enhance, respectively, the flux of light information from the photoreceptor to downstream photoresponses by controlling phytochromes/NDPK2 binding affinity.

Native oat phyA preparation is phosphorylated in vivo mostly on Ser7 in the N-terminal extension and on Ser598 in the hinge region (Lapko et al., 1999). Phosphorylation of Ser7 is mediated by intrinsic autophosphorylation activity of phytochromes and is spectral form independent. In contrast, Ser598 is not autophosphorylated to a significant extent and is phosphorylated mostly by yet-unknown phytochrome-associated kinase(s) in a Pfr-preferential manner in vivo (Lapko et al., 1999; Kim et al., 2004). We observed that phosphorylation degree of the Pfr form of native oat phyA preparations was only slightly higher than that of Pr form in our experimental condition (Figure 2F), showing mostly spectral form-independent phosphorylation. Thus, most of the phosphoryl group we detected in Figure 2F is on Ser7 in this experimental condition. The result that PAPP5 can dephosphorylate Pfr-oat phyA to a significantly greater extent than Pr form (Figure 2F) then indicates that PAPP5 should be able to dephosphorylate the phosphoryl group on Ser7. In addition, PAPP5 effectively dephosphorylated the Pfr-form of S598A mutant oat phyA (Figure 5C), in which Ser598 was substituted to alanine and thus mainly Ser17 is phosphorylated. Thus, PAPP5 can also dephosphorylate phospho-Ser17. These results show that PAPP5 can

Figure 5. PAPP5 Enhances the Binding Affinity of Oat phyA for NDPK2 and Dephosphorylates Specific Phospho-Serine Residues of Oat phyA

(A) Effect of PAPP5 on the binding affinity of oat phyA for NDPK2. Oat phyA (Oat phyA*) phosphorylated in R (Pfr) or R/FR (Pr) light was incubated with GST, GST-PP2Ac, or GST-PAPP5 in the presence or absence of 100 nM OA. The recombinant NDPK2 (rNDPK2) was then added to the reaction mixture (Input) and subjected to immunoprecipitation with the oat22 (IP: αoatphyA). The coimmunoprecipitated oat phyA and rNDPK2 molecules were detected by Coomassie staining and by immunoblot analysis with anti-NDPK2 antibody (αNDPK2), respectively (Output).

(B) Effect of whole-cellular extracts of *papp5* mutants (*papp5-1*, *papp5-2*), *PP2Ac*-overexpressing (*PP2Ac-OX1*, *PP2Ac-OX2*), or *PAPP5*-overexpressing (*PAPP5-OX1*, *PAPP5-OX2*) seedlings on binding affinity of NDPK2 for Pfr-oat phyA. Oat phyA phosphorylated in R light (Pfr-oat phyA*) was incubated with whole-cellular extracts from the various seedlings. rNDPK2 was then added to the reaction mixture (Input), followed by immunoprecipitation with the oat22 (IP: αoatphyA). The coimmunoprecipitated oat phyA and rNDPK2 (black triangle) molecules were detected by Coomassie staining and by immunoblot analysis with anti-NDPK2 antibody (αNDPK2), respectively (Output). The endogenous NDPK2 (white triangle) protein in the cellular extracts was hardly detectable in this assay. Note that the molecular weights of rNDPK2 and the endogenous NDPK2 are approximately 21 and 26 kDa, respectively (Choi et al., 1999).

(C) PAPP5 dephosphorylates Ser598 and Ser17 residues of recombinant oat phyA. PKA-mediated phosphorylation sites (black circle) in the recombinant wild-type (wt), Δ65, and S598A mutant oat phyA are noted at the top. After phosphorylation by PKA, the Pr and Pfr forms of the recombinant oat phyAs were incubated with GST-PAPP5. The phosphorylation state and the amount of the recombinant oat phyAs were visualized by autoradiography and by Coomassie staining and zinc blot, respectively. Shown at the bottom is quantification of phosphorylation degree of recombinant oat phyAs.

dephosphorylate all of the three phospho-serine residues of oat phyA.

It is notable that, while phosphorylation at the N-terminal extension is spectral form independent, dephosphorylation of this region by PAPP5 is spectral form dependent. This provides a mean by which the Pfr form is biologically more active, as phosphorylation at the N-terminal extension was suggested to be involved in light signal attenuation (Jordan et al., 1997; Casal et al., 2002); by being a better substrate for PAPP5, the Pfr form overcomes the signal attenuation caused by phosphorylation at the N-terminal region.

Phytochrome Stability Is Increased by PAPP5

It was previously suggested that phyA in plant cells undergoes phosphorylation in the N-terminal extension to regulate the light-dependent stability of phytochromes (Clough and Vierstra, 1997). Since PAPP5 can dephosphorylate the phospho-serine residues in the N-terminal region, we tested if PAPP5 can modify phytochrome stability in vivo utilizing the spectrophotometrical measurement. PhyA is most prevalent in etiolated seedlings among the five phytochromes (phyA–E) and rapidly degraded upon light treatment. When dark-grown *Arabidopsis* seedlings were transferred to Rc light, a loss-of-function mutant (*papp5-1*) showed almost no spectrophotometrically measurable phytochromes after 2 hr, showing that PAPP5 is critically necessary for phyA stability in Rc light (Figure 6A). In accordance, a *PAPP5*-overexpressing line (*PAPP5-OX1*) retained a higher amount of phytochromes after 2hr in Rc light compared to wild-type (Col-0). The *PHYA* transcript level was similar in dark-grown and R light-irradiated seedlings of Col-0, *papp5-1*, and *PAPP5-OX1* (Figure 6B), suggesting that the differences in the amount of phyA were not due to differences in *PHYA* mRNA expression in these plants. We further examined the stability of Pfr-phyA by measuring the amount of phyA species at various time points after irradiation with an R light pulse. As shown in Figure 6C, the Pfr species of phyA persisted much longer in *PAPP5-OX1* than in Col-0, showing that PAPP5 enhances stability

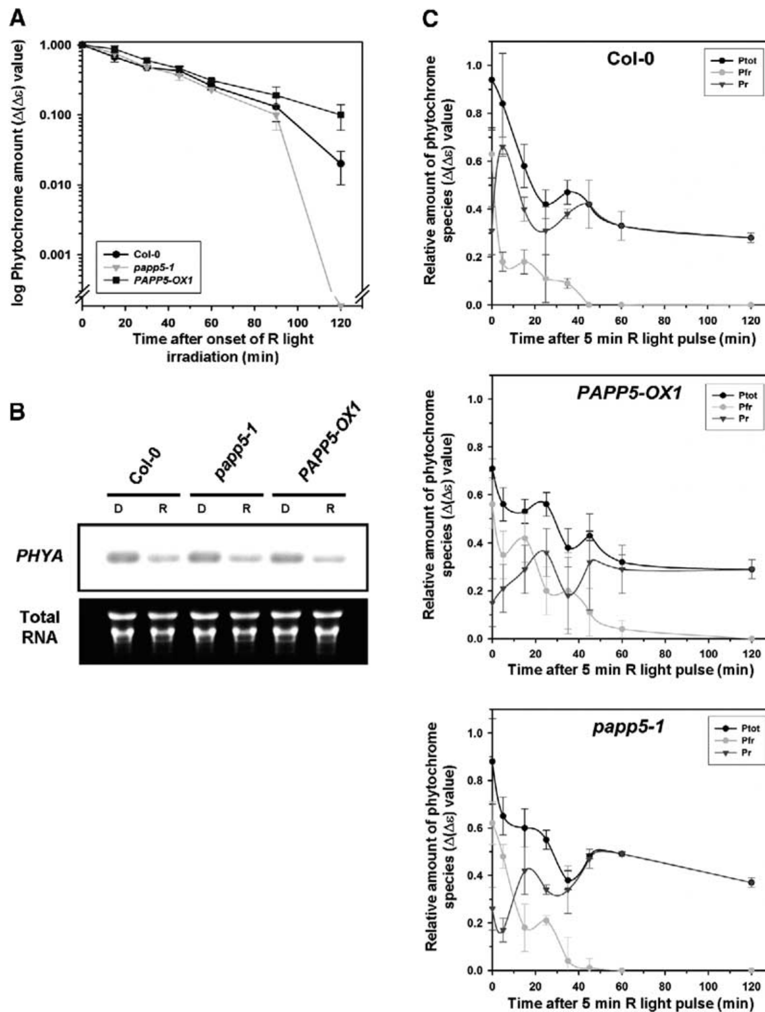


Figure 6. Effect of PAPP5 on In Vivo Stability of Phytochromes

(A) Depletion of phyA after transfer of 3-day-old etiolated seedlings to Rc light (30 μmol m^{-2} s^{-1}). Total phytochrome amount ($\Delta(\Delta\epsilon)$) was determined by in vivo spectroscopy, using approximately 100 mg of seedlings. $\Delta(\Delta\epsilon)$ values were normalized to the fresh weight of seedlings. Mean ± SE; n = 5.

(B) RNA gel blot analysis of *PHYA* transcript levels of 3-day-old etiolated seedlings before (D) and after (R) irradiation with R light (30 μmol m^{-2} s^{-1}) for 2 hr.

(C) Depletion of phyA by R light pulse treatment. Three-day-old etiolated seedlings were subjected to a 5 min pulse of R light (30 μmol m^{-2} s^{-1}). $\Delta(\Delta\epsilon)$ values of total (Ptot), Pfr-, and Pr-phytochromes were determined at various time points after the pulse treatment by in vivo spectroscopy. $\Delta(\Delta\epsilon)$ values were normalized to the fresh weight of seedlings. Mean ± SE; n = 3.

of the Pfr species in vivo. In contrast, life span of the Pfr species in *papp5-1* was shorter than that in Col-0. The results suggest that altered photoresponses observed in the loss-of-function mutants and overexpressing lines (Figure 4) are at least partly due to the changes in phytochrome stability conferred by the altered PAPP5 phosphatase activity in these plants.

Since the phosphorylation of Ser598 of oat phyA does not significantly affect phytochrome stability (Kim et al., 2004), we suggest that the observed effect of PAPP5 on phytochrome stability in *Arabidopsis* is mainly due to dephosphorylation of serine residue(s) other than the residue(s) corresponding to Ser598 of oat phyA and is thus possibly due to dephosphorylation at the N-terminal extension.

Reversible Phosphorylation of Pfr-Phytochromes Modulates the Flux of Light Information by Altering Phytochrome Stability and Phytochrome/Transducer Binding Affinity

PP5 is a subfamily lately added to a growing protein phosphatase family and is present in various species from yeast to human (Chinkers, 2001). They function in diverse cellular signaling processes such as cell cycle regulation, nuclear receptor signaling, and regulation of hormone receptors and ion channels. In this report, we have shown that a higher plant, *Arabidopsis*, also possesses a biologically functional PP5 that plays an important role as a regulator of phytochrome-mediated photoresponses.

The results described here provide important implications for the role and regulation of the phosphorylation status of the plant photoreceptors, phytochromes. First, the phosphorylation state of phytochromes is reduced by a type 5 protein phosphatase (PAPP5) that specifically dephosphorylates the biologically active Pfr form. Second, photoresponsiveness is correlated with the phosphatase activity of PAPP5 that is a positive regulatory component in plant photosignaling. Thus, phosphorylation of Pfr-phytochromes by autophosphorylation and phytochrome-associated kinase(s) is a signal attenuation mechanism that is counteracted specifically by the phosphatase activity of PAPP5. Third, dephosphorylation of the serine residue(s) in the hinge region of Pfr-phytochromes by PAPP5 results in enhanced affinity of phytochromes for the positive signal transducer, NDPK2, which in turn is positively correlated with photoresponses. Fourth, dephosphorylation

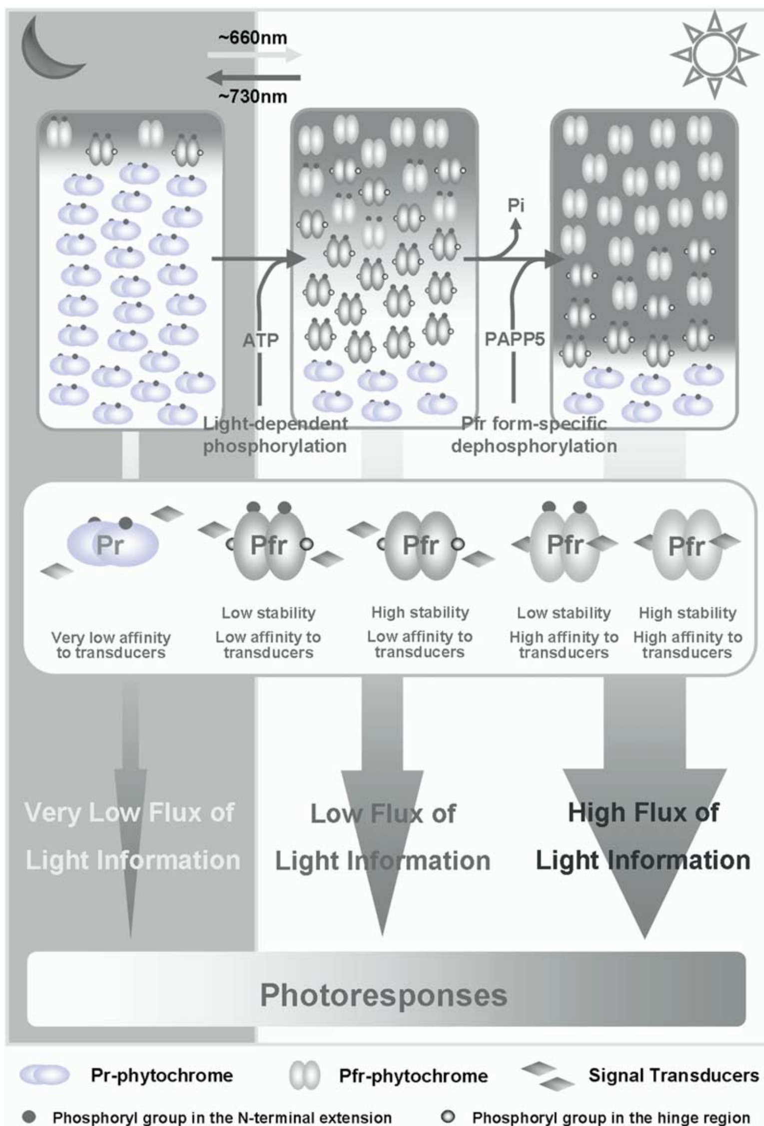


Figure 7. The Proposed Mechanism for the Modulation of Light Signal Flux by Variable Phosphorylation States of Pfr-Phytochromes

The photon energy at ~660 nm wavelength triggers photoconversion of the Pr-phytochromes to the Pfr-phytochromes, which initiates the phytochrome-mediated photosignaling. Pfr-phytochromes are phosphorylated by their intrinsic kinase activity as well as by phytochromes-associated kinase(s) and are reversibly dephosphorylated by a Pfr-specific phosphatase, PAPP5. The Pfr-phytochromes dephosphorylated in the N-terminal extension are relieved from phosphorylation-mediated destabilization, transmitting a more flux of light signal. Furthermore, the phosphorylated and dephosphorylated Pfr-phytochromes possess a lower and a higher affinity, respectively, toward signal transducers such as NDPK2. NDPK2 bound to Pfr-phytochromes has an activity higher than free NDPK2 (Choi et al., 1999) to transmit a more flux of light information. Thus, the higher proportion of the dephosphorylated Pfr-phytochromes is in a cell, the more of light information is transmitted to downstream photoresponses. This provides plants with a mechanism for finely tuning the flux of light signal to downstream photoresponses.

of the serine residue(s) in the N-terminal extension by PAPP5 results in enhanced stability of phytochromes in a Pfr-form preferential manner, leading to enhanced photoresponses.

Light signaling through phytochromes is initiated by a conformational change of phytochromes triggered by incoming photon energy. The light information decoded by the conformational change is translated into cellular responses through the action of downstream signal transducers that constitute an intricate signaling network. Photoresponsiveness of plants to phytochrome signals is, then, regulated by how much the signal flows through the given signaling network. The results presented here provide clear evidences that photoresponsiveness in phytochrome signaling is negatively correlated with the phosphorylation state of phytochromes. Namely, dephosphorylation of the biologically active Pfr-phytochromes leads to increased flux of light signal to downstream components in the signaling cascades and to enhanced photoresponsiveness, while phosphorylation of phytochromes by autophosphorylation and phytochrome-associated kinase(s) leads to decreased flux of light signal. We thus propose a model in which the counteraction of the pair of phytochrome autophosphorylation/phytochrome-associated kinase(s) activity and Pfr form-specific phosphatase activity of PAPP5 provides a tuning mechanism that finely controls the flux of light information to downstream photoresponses (Figure 7). As reported here, this control is mediated through regulation of phytochrome stability and affinity for downstream signal transducers such as NDPK2.

Although light-dependent phosphorylation also occurs in rhodopsin-mediated vision signaling in animals, neither the photoreceptor stability nor affinity for downstream transducers has been reported to be directly regulated by the phosphorylation status of the rhodopsin photoreceptor. In animals, the rhodopsin photoreceptor is phosphorylated by a separate kinase and is then capped by binding of arrestin for signal desensiti-

zation (Maeda et al., 2003). In plants, the phytochrome photoreceptor appears to have developed a unique signal tuning mechanism, employing variable phosphorylation states according to their ambient light condition as a mean to ensure their optimal photoperception and responses.

Experimental Procedures

Coimmunoprecipitation of PAPP5 and Oat phyA Holoprotein

Oat (*Avena sativa* L.) phyA holoprotein was prepared as described (Lapko et al., 1999). The reaction mixture containing 1 μg of oat phyA holoprotein in 290 μl of binding buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM dithiothreitol, 0.1% Tween 20) was subjected to a 5 min pulse of R light (50 μmol m^{-2} s^{-1} at 660 nm) on iced water. Ten microliters of TnT (Quick Coupled Transcription/Translation Systems; Promega, Madison, Wisconsin)-produced, ^{35}S-labeled PAPP5 was then added to the reaction mixture under dim green safe light. The mixture was incubated at 4°C in darkness for 2 hr before treatment with a pulse of R or FR light at the intensity of 50 μmol m^{-2} s^{-1}. Oat phyA/PAPP5 complex was immunoprecipitated by incubating the reaction mixture with an oat phyA antibody (oat22) attached to protein A/G-Sepharose (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom) at 4°C for 1 hr. The immunoprecipitated proteins were subjected to 10% SDS-PAGE.

In Vitro Phosphorylation and Dephosphorylation Assay of Native Oat phyA

Phosphorylation reaction was performed in 50 μl kinase/phosphatase (KP) buffer (25 mM Tris-HCl [pH 7.5], 5 mM $MgCl_2$, 20 mM $Mg(CH_3COO)_2$, 0.2 mM EDTA, 0.2 mM EGTA) containing 3 μCi of [γ-^{32}P]ATP (PerkinElmer Life Sciences, Boston, Massachusetts) and 1 μg of oat phyA holoprotein preparation. Oat phyA in KP buffer was first subjected to a 5 min irradiation of R (50 μmol m^{-2} s^{-1}) light alone or 5 min of R light followed by 5 min of FR (50 μmol m^{-2} s^{-1}) light irradiation on iced water. [γ-^{32}P]ATP was then added for phosphorylation reaction mixture, which proceeded for 30 min at 30°C in darkness. For dephosphorylation assay, 1 μg of recombinant proteins (GST, GST-PAPP5, and GST-PP2Ac) was added to the above mixture and the reaction proceeded for 1 hr at 30°C in darkness prior to addition of Tris-glycine SDS sample buffer to terminate the reaction.

Semi-In Vivo Assay of Oat phyA Dephosphorylation

Whole-cell protein extract was prepared from seedlings grown for 10 days in white light as described (Lagarias et al., 1997), except that KP buffer was used in protein extraction. A reaction mixture containing 1 mg of cellular protein extract and 1 μg of phosphorylated oat phyA in 200 μl of KP buffer was incubated for 1 hr at 30°C in darkness. Oat22 antibody bound to protein A/G-Sepharose was added to the reaction mixture to a final volume of 300 μl for immunoprecipitation. The antibody bound phyA was subjected to 10% SDS-PAGE.

Analyses of Subcellular Localization of Fluorescent Reporter Proteins

Images of the fluorescent reporter proteins were obtained with an Axioscope 2 plus microscope equipped with an AxioCam color camera (Zeiss, Germany) and specific fluorescent filter sets for CFP (exc. D 436/20; beam splitter 455 DCLP; em. D 480/40) and YFP (HQ 500/20; beam splitter Q 515 LP; em. HQ 535/30) (AHF Analysentechnik, Tübingen, Germany). Photographs were assembled and processed using the Photoshop 7.0 (Adobe Systems Europe, Edinburgh, United Kingdom). To visualize CFP and YFP fluorescence in the superimposed images, each fluorescence image was binarized and pseudocolored into either red (CFP) or green (YFP). The resulting red and green layers were subtracted from each other. In case of colocalization of the two images, the resulting color is yellow. Confocal analyses were performed with the confocal laser scanning microscope LSM 510 (Zeiss, Germany) as described (Bauer et al., 2004).

In Vitro Assay of Interaction between Oat phyA and NDPK2

Phosphorylation of oat phyA was performed as described above, except that 1 μM of unlabeled ATP was used instead of [γ-^{32}P]ATP. One microgram of phosphorylated oat phyA was incubated with 1 μg of recombinant GST-PAPP5, GST-PP2Ac, or GST in 50 μl KP buffer for 1 hr at 30°C in the presence or absence of 100 nM OA. After adding 2 μg of recombinant NDPK2 (Choi et al., 1999) to the reaction mixture, final volume was adjusted to 300 μl with KP buffer. The binding reaction was performed at 4°C for 3 hr. The oat phyA/NDPK2 complex was then immunoprecipitated with the oat22 antibody and subjected to 10% SDS-PAGE followed by electroblotting onto PVDF membranes. NDPK2 and oat phyA was detected with an anti-NDPK2 antibody (Moon et al., 2003) and Coomassie brilliant blue staining, respectively.

Semi In Vivo Assay of Interaction between Oat phyA and NDPK2

Whole-cell protein extract and phosphorylated oat phyA were prepared as described above. One μg of phosphorylated oat phyA was added in 200 μl reaction mixture containing 1 mg of cellular extracts. After incubation for 1 hr at 30°C, 2 μg of recombinant NDPK2 was added to the reaction mixture and final volume was adjusted to 300 μl with KP buffer. Immunoprecipitation of oat phyA/NDPK2 complex was performed as described above.

Supplemental Data

Supplemental Data include six figures and Supplemental Experimental Procedures and can be found with this article online at http://www.cell.com/cgi/content/full/120/3/395/DC1/.

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