# A Phytochrome-Associated Protein Phosphatase 2A Modulates Light Signals in Flowering Time Control in Arabidopsis

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Reversible protein phosphorylation, which is catalyzed by functionally coupled protein kinases and protein phosphatases, is a major signaling mechanism in eukaryotic cellular functions. The red and far-red light–absorbing phytochrome photoreceptors are light-regulated Ser/Thr-specific protein kinases that regulate diverse photomorphogenic processes in plants. Here, we demonstrate that the phytochromes functionally interact with the catalytic subunit of a Ser/Thr-specific protein phosphatase 2A designated FyPP. The interactions were influenced by phosphorylation status and spectral conformation of the phytochromes. Recombinant FyPP efficiently dephosphorylated oat phytochrome A in the presence of $Fe^{2+}$ or $Zn^{2+}$ in a spectral form–dependent manner. FyPP was expressed predominantly in floral organs. Transgenic Arabidopsis plants with overexpressed or suppressed FyPP levels exhibited delayed or accelerated flowering, respectively, indicating that FyPP modulates phytochrome-mediated light signals in the timing of flowering. Accordingly, expression patterns of the clock genes in the long-day flowering pathway were altered greatly. These results indicate that a self-regulatory phytochrome kinase-phosphatase coupling is a key signaling component in the photoperiodic control of flowering.

## INTRODUCTION

Plant growth and development are not only regulated by intrinsic developmental programs but also are affected greatly by various environmental signals (Fankhauser and Chory, 1997). Light is the most critical environmental factor, and it plays two major roles in plants. Plants are photosynthetic and acquire virtually all of the biochemical energy required for survival and propagation solely from light energy. In addition, they constantly monitor the intensity, wavelength, direction, and duration of environmental light. The light signals are integrated subsequently into diverse growth and developmental processes throughout the whole life span, from seed germination to flowering, to achieve optimized growth under a given light condition (Neff et al., 2000; Ma et al., 2001). The light-regulated plant growth and developmental processes collectively are called photomorphogenesis.

Light signaling cascades that govern plant photomorphogenesis have been investigated widely by molecular biological and genetic analyses of various photomorphogenic mutants with altered light responses, mostly in Arabidopsis. A variety of light-signaling mediators, including the photoreceptors, have been identified, and their physiological roles have been elucidated in detail. A currently accepted scheme for plant photomorphogenesis suggests that light signals perceived by the photoreceptors are transmitted through a series of signaling mediators, such as phytochrome-interacting factors (Ni et al., 1998; Choi et al., 1999; Fankhauser et al., 1999), heterotrimeric G-proteins (Okamoto et al., 2001), Ras-like low molecular weight G-proteins (Kang et al., 2001), $Ca^{2+}$/calmodulin (Neuhaus et al., 1993; Guo et al., 2001), and protein kinases/phosphatases (reviewed by Fankhauser and Chory, 1999), and finally regulate genes involved in photomorphogenic growth and development.

The red and far-red light–sensing phytochromes and the blue light–sensing cryptochromes are two principal photoreceptors in plant photomorphogenesis. In some cases, an individual photoreceptor is sufficient to trigger a specific light response. However, recent genetic analyses of double and triple photoreceptor mutants have shown that the photoreceptors function in a mode of finely tuned coactions (Neff

and Chory, 1998; Mockler et al., 1999; Guo et al., 2001; Reeves and Coupland, 2001). The light responses mediated by the phytochromes are influenced significantly by the cryptochromes and vice versa. Moreover, physical and functional interactions between the two photoreceptors have been confirmed biochemically and genetically (Ahmad et al., 1998; Más et al., 2000; Martinez-Hernandez et al., 2002).

The phytochrome photoreceptors are light-regulated Ser/Thr-specific protein kinases (Yeh and Lagarias, 1998; Fankhauser, 2000). Although it is still a matter of some debate, two recent observations strongly support the nature of the phytochrome kinase. A prokaryotic phytochrome, Cph1, from the cyanobacterium *Synechocystis* sp PCC6803 has structural and photochemical properties very similar to those of the eukaryotic phytochromes and exhibits light-regulated His kinase activity (Yeh and Lagarias, 1998). The kinase activity of the higher plant phytochromes also has been demonstrated in vitro using highly purified native and recombinant proteins (Yeh and Lagarias, 1998). Besides the phytochrome that is autophosphorylated (Lapko et al., 1999), additional phosphorylation substrates have been identified, including PKS1 (Fankhauser et al., 1999), Aux/IAA (Colon-Carmona et al., 2000), and the cryptochromes (Ahmad et al., 1998). Furthermore, the phytochrome-cryptochrome coactions have been confirmed functionally, especially in flowering time control (Mockler et al., 1999; Más et al., 2000; Guo et al., 2001), suggesting that protein phosphorylation plays a critical role in plant photomorphogenesis. These observations, along with the reversible protein phosphorylations that are well known in many eukaryotic kinase signaling cascades (Stone et al., 1994; Keyse, 2000), necessitate the involvement of protein phosphatases in the phytochrome kinase–mediated light signal transduction (Sheen, 1993; Chandok and Sopory, 1996; Fankhauser, 2000). However, no such protein phosphatases have been identified, and no physiological roles of protein phosphorylation have been elucidated unequivocally at the molecular level.

In this work, we used a series of molecular biological, genetic, and biochemical approaches to demonstrate that reversible protein phosphorylation is a key component of phytochrome-mediated light signal transduction. A Ser/Thr-specific protein phosphatase 2A (designated FyPP for flower-specific, phytochrome-associated protein phosphatase) associates with and dephosphorylates the phytochromes in a light wavelength–dependent manner. Arabidopsis plants with reduced FyPP levels exhibited early flowering, whereas those with increased FyPP levels flowered later than control plants. The expression patterns of the circadian clock genes were altered accordingly. In agreement with these findings, the *FyPP* gene was expressed predominantly in floral organs and influenced significantly by daylength. These results indicate that phytochrome-mediated light signals are further modulated by protein phosphorylation/dephosphorylation in flowering time control, providing direct molecular

evidence for the function of protein phosphorylation in phytochrome kinase signaling.

## RESULTS

### Phytochromes Associate with a Protein Phosphatase 2A

To search for the protein phosphatase that interacts specifically with the phytochrome kinases, we conducted yeast two-hybrid screens using the C-terminal half (residues 667 to 1122) of the Arabidopsis phytochrome A as bait. We first screened cDNA libraries constructed from Arabidopsis plants but failed to isolate a gene encoding such a phytochrome-interacting protein phosphatase, possibly because its transcript is rare, if it exists, in the mRNA pools used to construct the cDNA libraries. As an alternative, we chose a cDNA library constructed from dark-grown pea seedlings (Kang et al., 2001).

Sequence analysis of the positive cDNA clones ($his3^+$ and $lacZ^+$) identified a group of cDNA clones that contained an uninterrupted open reading frame encoding the catalytic subunit of a Ser/Thr-specific protein phosphatase 2A (PP2A). The deduced polypeptide consists of 303 residues with an estimated molecular mass of 34.7 kD (Figure 1). It contains all of the structural elements (blocks I to IV) highly conserved among PP2A family members (Virshup, 2000). However, it is somewhat different from other PP2A members, such as PP2A and PPX subfamily members, as indicated by relative sequence similarities, and apparently it belongs to a distinct subfamily. The phytochrome-associated PP2A was designated FyPP.

A putative *FyPP* gene ortholog also was isolated from Arabidopsis by reverse transcription (RT)–PCR amplification using degenerate primers and poly(A) mRNA (AtFyPP3; Figure 1). It was located on chromosome 3 by sequence comparison with the Arabidopsis genome sequence. Interestingly, further database searches identified one additional Arabidopsis gene on chromosome 1 encoding a polypeptide (designated AtFyPP1) with a sequence identity of >98% to FyPP and AtFyPP3, even with most of the substitutions conserved biochemically among the three FyPP members, such as E69D, I167V, and S219T. The two Arabidopsis polypeptides differ only at two residues, Glu or Asp at position 70 and His or Tyr at position 100 in AtFyPP1 and AtFyPP3, respectively (Figure 1), suggesting that they are FyPP orthologs in Arabidopsis.

The phytochrome–FyPP interaction was examined further by yeast coexpression (Figure 2A) and by in vitro pulldown assays using a recombinant FyPP–glutathione *S*-transferase (GST) fusion protein and purified native oat phytochrome A (Figure 2B) or recombinant Arabidopsis phytochrome B (Figure 2C). Phytochrome B was expressed in *Escherichia coli* cells and reconstituted in vitro with phycocyanobilin for the assays. AtFyPP3 also was included in the assays to exam-
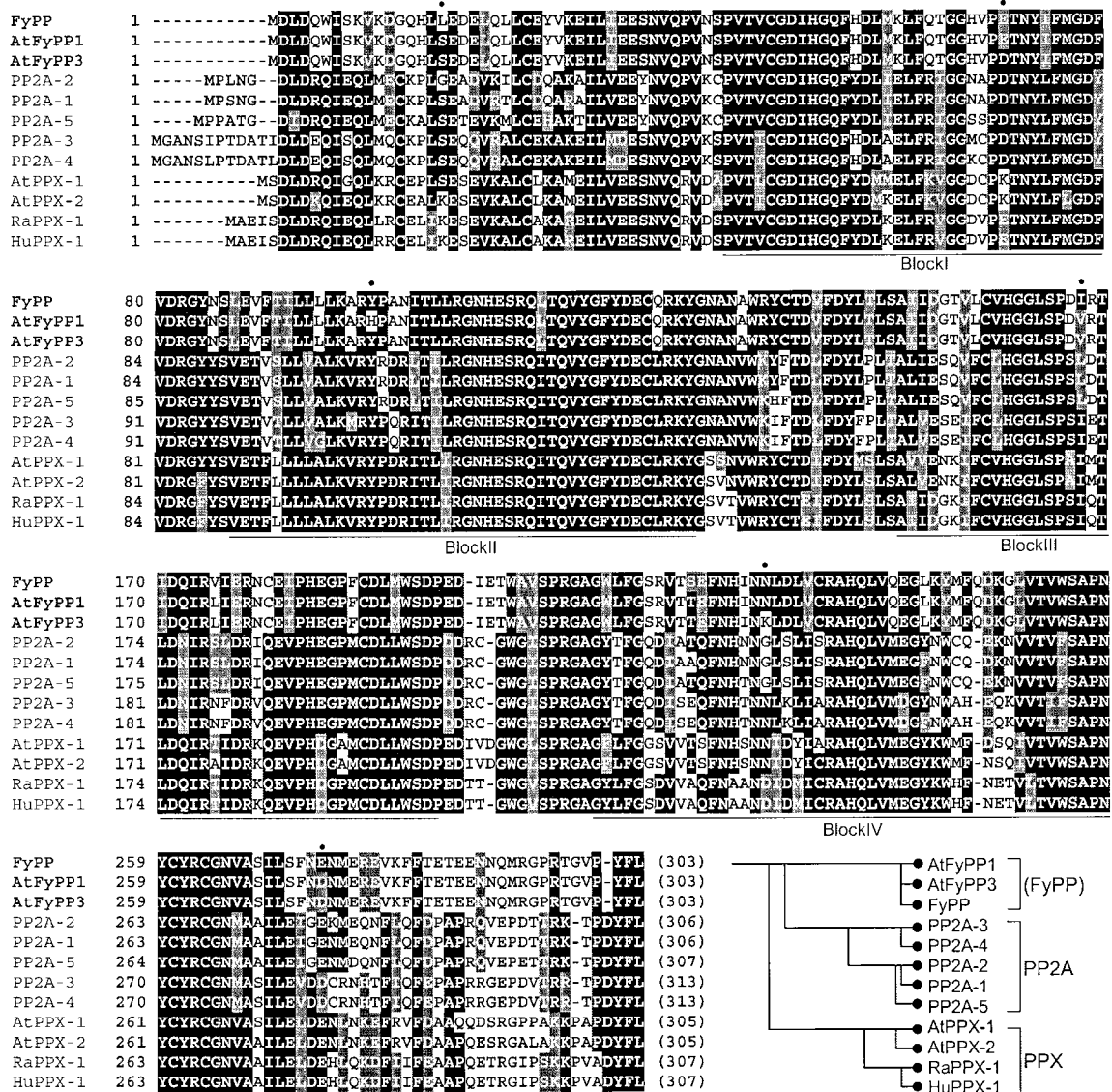
**Figure 1.** Multiple Sequence Alignment of FyPP and Related PP2A Members.

Four protein blocks highly conserved among the PP2A members are underlined (blocks I to IV). Amino acid residues that differ among FyPP, AtFyPP1, and AtFyPP3 are indicated by dots above the FyPP sequence. A phylogenetic tree, showing the interrelationships among the PP2A members, is displayed at bottom right. The PP2A members aligned are Arabidopsis PP2A-1 to PP2A-5, Arabidopsis PPX-1 and PPX-2, human and rat PPX-1s (RaPPX-1 and HuPPX-1), AtFyPP1, AtFyPP3, and FyPP. The sequences were aligned using CLUSTAL W version 1.7 (Thompson et al., 1994).

ine the molecular equivalence between FyPP and AtFyPPs. FyPP associated with both phytochromes A and B, but it associated 1.4 times more efficiently with the latter (Figure 2A). AtFyPP3 also associated with the phytochromes in patterns identical to that of FyPP. For in vitro pulldown assays, a FyPP-GST fusion protein, in which the GST was fused to the C terminus of the full-size FyPP, was generated and purified in *E. coli* cells. The FyPP-GST fusion protein efficiently bound oat phytochrome A (Figure 2B) and phytochrome B (Figure 2C). The AtFyPP3-GST fusion protein also showed identical results (data not shown). Therefore, it is evident that FyPP and its putative Arabidopsis orthologs (AtFyPPs) bind both phytochromes A and B, but with an obvious preference for the latter.
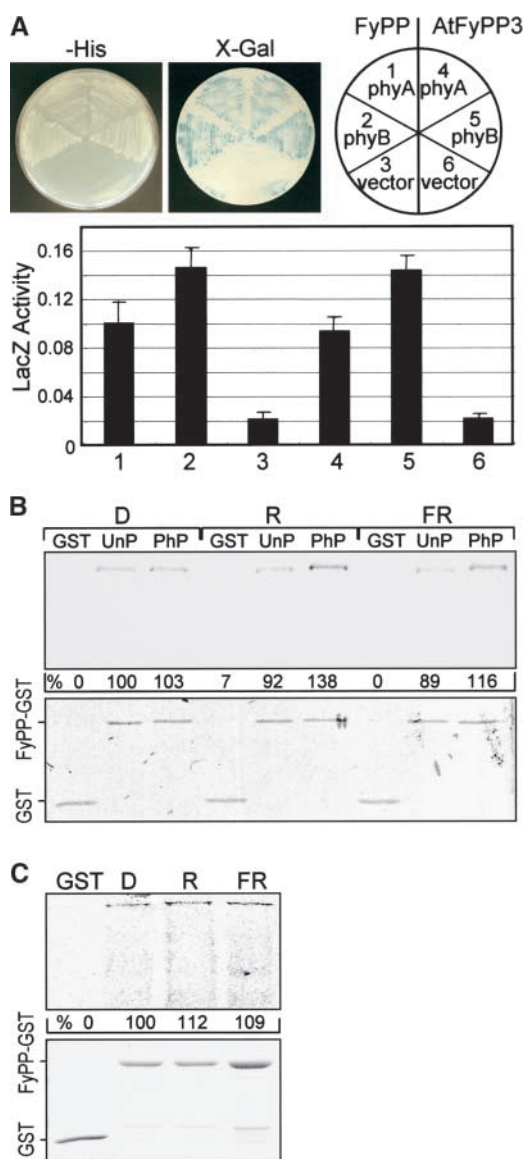
## A



## B



## C



**Figure 2.** Interactions of FyPP and AtFyPP3 with Phytochromes A and B.

The interactions were examined by yeast coexpression and in vitro pulldown assays.

**(A)** Yeast coexpression.

**(B)** Phytochrome A–FyPP interactions. The phytochromes prepared in the dark (D) were treated with either red (R) or far-red (FR) light before use. Bound phytochromes were detected immunologically using a polyclonal anti-phyA antibody. Binding activities were expressed relative to that (100%) of the unphosphorylated phytochrome A in the dark. PhP and UnP, phosphorylated and unphosphorylated oat phytochrome A, respectively.

**(C)** Phytochrome B–FyPP interactions. A full-size recombinant Arabidopsis phytochrome B from *E. coli* was reconstituted in vitro with PCB. In vitro pulldown assays were performed as described in **(B)**.

The bottom panels in **(B)** and **(C)** show Coomassie brilliant blue–stained SDS-polyacrylamide gels.

We then examined whether the phytochrome–FyPP interaction was affected by the spectral conformation and phosphorylation status of the phytochromes. FyPP bound the Pfr phytochrome ∼30% more efficiently than the Pr form. Phytochrome phosphorylation status also influenced the interactions. FyPP associated more efficiently with the phosphorylated phytochromes than with the unphosphorylated forms. Phytochrome B also bound FyPP in similar patterns (Figure 2C). These observations indicate that the phytochrome–FyPP interaction is modulated by light wavelength, signifying a role for FyPP in phytochrome-mediated light signaling.

## FyPP Is Localized in the Cytoplasm in Floral Organs

The interactions of FyPP with the phytochromes suggested that *FyPP* gene expression would be regulated by light wavelength. However, RNA gel blot hybridization analysis showed that it was not affected notably by light (Figure 3A). It was expressed to an equal level in all light conditions tested, indicating that the phytochrome–FyPP interaction is regulated by light primarily via the Pr↔Pfr phototransformation of the phytochromes. To obtain clues regarding FyPP's function, *FyPP* gene expression was examined in different plant organs. It was expressed predominantly in flowers and flowering stems, but the transcript level was relatively very low in other organs (Figure 3B), suggesting a flowering-specific role for FyPP. *AtFyPP* genes also exhibited identical light-independent (Figure 3C) but floral organ–specific (Figure 3D) expression patterns, supporting the notion that they are *FyPP* gene orthologs in Arabidopsis. As anticipated from the yeast two-hybrid screens (see above), *AtFyPP* transcript levels were very low compared with those of *FyPP*, and they were detected by RT-PCR but not by ordinary RNA gel blot analysis. This distinction may reflect a physiological difference between pea, a short-day plant, and Arabidopsis, a long-day plant.

Another clue to FyPP's function was provided by its subcellular localization. Nucleocytoplasmic translocation of the phytochromes is a critical step in phytochrome function. Phytochromes A and B are localized in the cytoplasm under far-red light but translocated into the nucleus under red light (Kircher et al., 1999; Nagy et al., 2000). To examine the subcellular localization of FyPP in plant cells, a green fluorescent protein (GFP) coding sequence was fused in frame to the 3′ end of the *FyPP* gene. The FyPP-GFP fusion protein was expressed transiently in Arabidopsis protoplasts. Bright-field and fluorescence microscopy images revealed that FyPP is localized constitutively in the cytoplasm, unlike the phytochromes (Figure 4). The GFP-FyPP fusion protein, in which the GFP was fused to the N terminus of the full-size FyPP, and the FyPP-GUS fusion protein also were localized exclusively in the cytoplasm of onion epidermal cells when expressed transiently (data not shown). These observations indicate that FyPP regulates phytochrome phosphorylation in
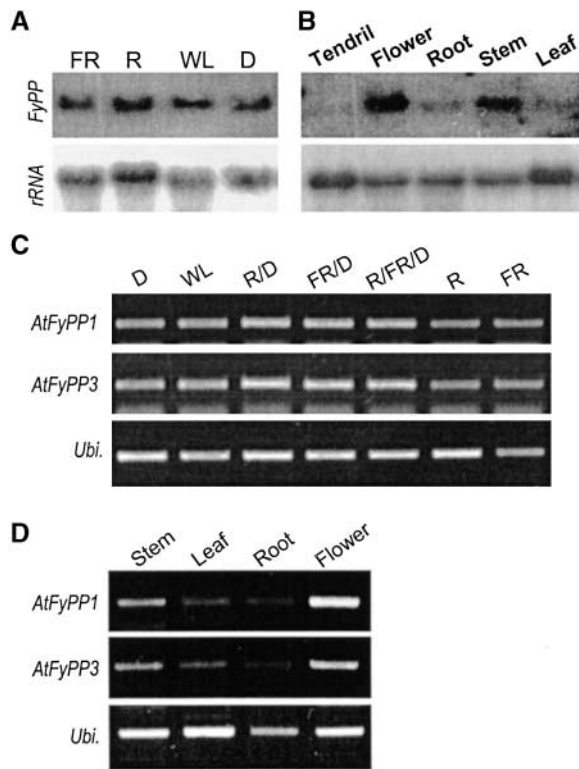
**Figure 3.** Expression Patterns of the *FyPP* and *AtFyPP* Genes.

**(A)** Light effects on *FyPP* gene expression. Pea plants were grown in far-red light (FR), red light (R), white light (WL), or in the dark (D).
**(B)** Organ-specific expression of the *FyPP* gene.
18S rRNA (18S) was probed with $^{32}$P-labeled rDNA as a control for constitutive expression in **(A)** and **(B)**.
**(C)** *AtFyPP* gene expression under various light wavelengths.
**(D)** Organ-specific expression of the *AtFyPP* genes.
An Arabidopsis ubiquitin gene (*Ubi.*) was used as a control for constitutive expression in **(C)** and **(D)**.

sic kinase activity (Yeh and Lagarias, 1998; Lapko et al., 1999). FyPP is a PP2A that interacts directly with the phytochromes (Figures 1 and 2). We tried to determine whether FyPP dephosphorylates the phytochromes. We also reasoned that phytochrome dephosphorylation by FyPP would be modulated by light wavelength if the phytochrome–FyPP interaction is functionally important. $^{32}$P-labeled oat phytochrome A was used as a dephosphorylation substrate. FyPP activity was examined using a recombinant FyPP expressed in *E. coli* cells. Various cations also were included in the assays because PP2A members require diverse cations for full enzymatic activities.

FyPP efficiently dephosphorylated the phosphorylated oat phytochrome A, but only in the presence of $Fe^{2+}$ or $Zn^{2+}$ (Figure 5A). $Zn^{2+}$ was more catalytic than $Fe^{2+}$ on FyPP activity. By contrast, other cations showed only marginal effects. These results indicate that FyPP is biochemically unique among PP2A family members (Virshup, 2000), which also is consistent with the sequence diversities observed among PP2A members (Figure 1). A divalent cation chelator, EDTA, abolished the dephosphorylation reactions completely, confirming the absolute cation requirement for FyPP activity (Figure 5B).

We then tried to determine whether FyPP differentially dephosphorylated the Pfr and Pr phytochromes. The Pfr phytochrome was dephosphorylated more readily than the Pr form by FyPP (Figures 5C and 5D). These findings are related to the facts that the Pfr phytochrome is a physiologically active form in most photomorphogenic responses (Roux, 1994) and that phosphorylation status is important for phytochrome function (Fankhauser, 2000). AtFyPPs also exhibited the same enzymatic activity and cation requirement as FyPP (data not shown). The oat phytochrome A and FyPP proteins themselves were unaffected under the assay conditions used (Figures 6A and 6B, bar graphs at bottom).
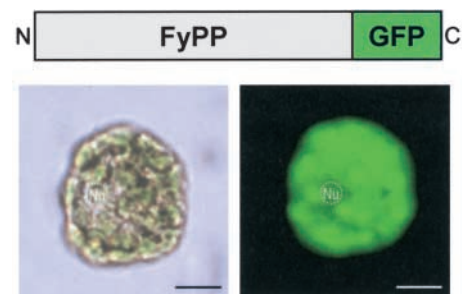
the cytoplasm, which may be important for the nuclear translocation and/or cytoplasmic function of the phytochromes.

Additionally, we observed that the FyPP-GFP and FyPP-GUS fusion proteins were unusually unstable. The fusions were visible for only a short time (<1 h) at 7 to 8 h after particle bombardment, and the fluorescent images disappeared completely after that. These findings suggest that FyPP could become stable as it binds the phytochromes or that the phytochrome–FyPP interaction is a subtle step during phytochrome signaling.

## Phytochrome Dephosphorylation by FyPP Is Regulated by Light Wavelength

Oat phytochrome A is autophosphorylated at distinct Ser residues, such as Ser-7, Ser-17, and Ser-598, by the intrin-



**Figure 4.** Subcellular Localization of FyPP.

Bright-field (bottom left) and fluorescent (bottom right) images of Arabidopsis protoplasts expressing the FyPP-GFP fusion protein (top) are shown. Nuclei (Nu) are indicated. Bars = 10 μm.
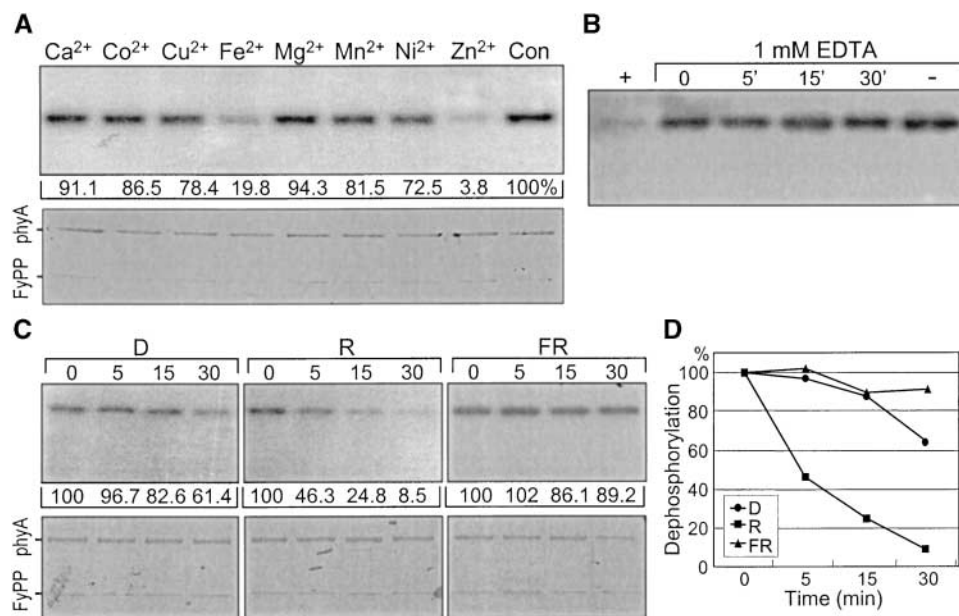
**Figure 5.** Protein Phosphatase Activity of FyPP.

$^{32}$P-labeled oat phytochrome A was used as a substrate. Dephosphorylation reactions were performed in the dark (D), in red light (R), or in far-red light (FR).

**(A)** Divalent cation requirements. Dephosphorylation rates are displayed as the radioactivity remaining after dephosphorylation reactions relative to that (100%) of a control reaction without cation (Con).

**(B)** EDTA effect. One millimolar EDTA was included as indicated. +, a control reaction without EDTA but with FyPP; −, a control reaction without FyPP but with EDTA.

**(C)** Spectral form dependence. One millimolar $Zn^{2+}$ was included in all reactions. The bottom panels show Coomassie brilliant blue–stained SDS polyacrylamide gels.

**(D)** Time course of the dephosphorylation reactions.

## FyPP Modulates Phytochrome Signals in Flowering Time Control

To explore the physiological role of the phytochrome–FyPP interaction, a full-size *FyPP* gene was introduced into Arabidopsis plants in both the sense and antisense orientations, and homozygotic transgenic lines were obtained. *FyPP* transgenic plants did not show any discernible phenotypic alterations during the seed germination and seedling growth stages. However, adult plants exhibited a marked phenotypic change. Sense transgenic plants displayed delayed flowering, whereas antisense transgenic plants flowered earlier than control plants in long days (Figure 6A). By contrast, in short days, antisense transgenic plants flowered slightly earlier than control plants, but sense transgenic plants were not noticeably different (Figure 6B). These results demonstrate that FyPP plays a major role in the photoperiodic control of flowering time in long days. The specific role of FyPP in flowering time control also is consistent with its predominant expression in floral organs (Figure 3). *FyPP* transgenic plants did not show any variations in plant morphology and flower architecture other than altered flowering

times, indicating that the primary role of FyPP is to control flowering initiation and that it is not involved directly in other growth and developmental processes.

Interestingly, the delayed flowering phenotype of sense transgenic plants was very similar to that of the phytochrome A null mutant (*phyA*), whereas the accelerated flowering phenotype of antisense transgenic plants was similar to that of the phytochrome B null mutant (*phyB*) (Bagnall et al., 1995) (Figure 6). These phenotypic similarities indicate that FyPP functions directly on the phytochromes by dephosphorylating certain Ser residues and acts as a negative regulator in the photoperiodic control of flowering. In agreement with this finding, the cryptochromes, which are phosphorylated by the phytochrome kinases, were not the dephosphorylation substrate for FyPP, and no interactions were detected between them in yeast coexpression assays (data not shown).

The high amino acid sequence homology (98%) with FyPP (Figure 1), the specific interactions with the phytochromes in a manner similar to that of FyPP (Figure 2), and the similar expression profiles (Figure 3) all strongly supported the idea that AtFyPPs are structurally and function-

ally homologous with FyPP. To further examine this possibility, the expression patterns of the *FyPP* transgene and intrinsic *AtFyPP* genes were analyzed in *FyPP* transgenic plants. RT-PCR, rather than RNA gel blot analysis, was used to distinguish their expression, because the two *AtFyPP* gene sequences share 92% identity. PCR primers were designed based on the sequence regions that are most divergent between the two gene sequences, and the expected sizes of the PCR products also were different from each other: 529 bp for *AtFyPP1* and 448 bp for *AtFyPP3*. In antisense transgenic plants that showed early flowering, the two Arabidopsis genes as well as the *FyPP* antisense transgene were suppressed drastically (Figure 7A). By contrast, expression of the intrinsic *AtFyPP* genes was unaffected in sense transgenic plants. Together with the observation that *AtFyPP* genes are expressed predominantly in floral organs, like *FyPP* (Figure 3D), this result indicates that AtFyPPs are functionally equivalent to FyPP.

To demonstrate unequivocally the functional equivalence, an AtFyPP3-deficient Arabidopsis mutant was isolated from a T-DNA insertion pool (ecotype Wassilewskija-2). DNA sequencing revealed that a T-DNA was inserted in the third intron of the *AtFyPP3* gene (Figure 7B, top). The absence of *AtFyPP3* gene expression in the knockout mutant was verified by RT-PCR (data not shown). The knockout mutant also exhibited an accelerated flowering phenotype (Figure 7B). However, it was not as prominent as that observed in antisense *FyPP* transgenic plants (Figure 6), possibly because the parental ecotype used to generate the T-DNA insertion pool was Wassilewskija-2, an early-flowering ecotype. Therefore, we conclude that FyPP and its functional orthologs, the AtFyPPs, play a common role in flowering time control.

## FyPP Functions Primarily through the Long-Day Flowering Pathway

Flowering in Arabidopsis, a facultative long-day plant, is regulated through a complex network of multiple genetic pathways that respond to a wide range of environmental and intrinsic developmental cues (Koornneef et al., 1998; Piñeiro and Coupland, 1998; Blázquez et al., 2001; Simpson and Dean, 2002). The long-day pathway promotes flowering initiation in long days, in which a group of clock genes are
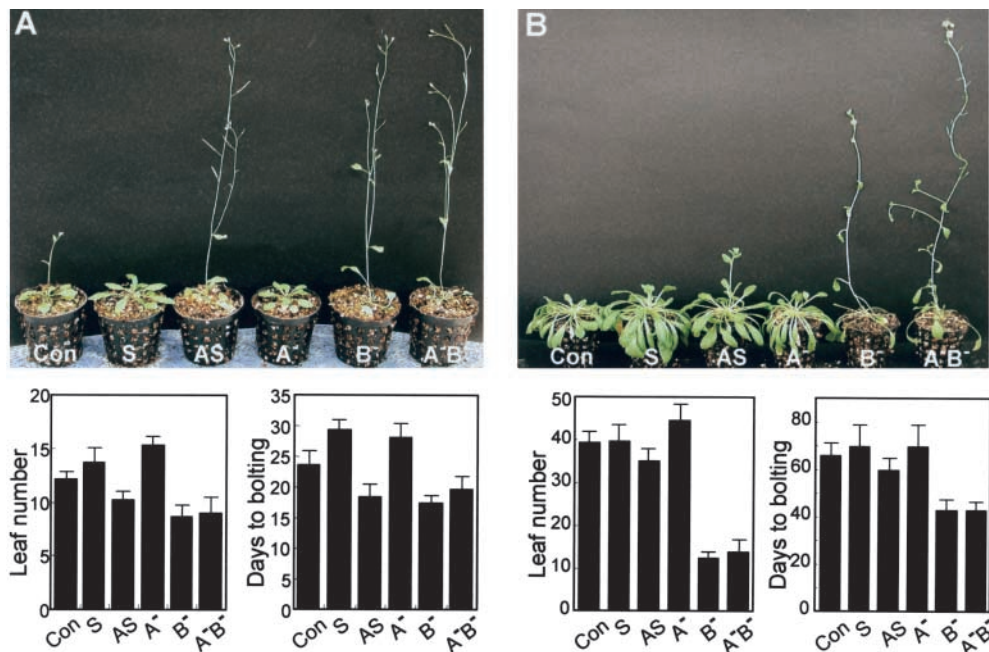


**Figure 6.** *FyPP* Transgenic Arabidopsis Plants.

Transgenic plants were grown in either long days (16 h of light/8 h of dark) or short days (8 h of light/16 h of dark).
**(A)** Transgenic plants grown in long days.
**(B)** Transgenic plants grown in short days.
The number of rosette leaves was counted at the day of bolting. Days to bolting refers to the days between the date when plants were put in the light and the date of bolting. Twenty plants were measured for each plant shown. Con, control; S and AS, sense and antisense *FyPP* transgenic plants, respectively; A⁻, phytochrome A null mutant; B⁻, phytochrome B null mutant; A⁻B⁻, phytochrome A and B double mutant.
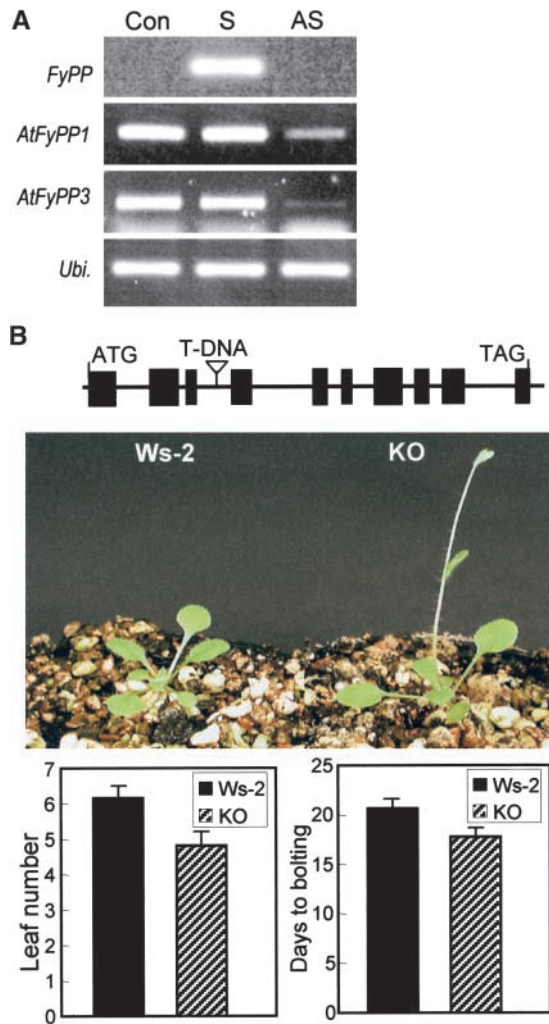
**Figure 7.** Functional Equivalence between FyPP and AtFyPPs.

**(A)** *AtFyPP* gene expression in *FyPP* transgenic Arabidopsis plants. Con, control; S and AS, sense and antisense *FyPP* transgenic plants, respectively; *Ubi.*, an Arabidopsis ubiquitin gene.

**(B)** Phenotype of an AtFyPP3-deficient Arabidopsis knockout plant compared with a wild-type plant. A T-DNA was inserted into the third intron of the *AtFyPP3* gene. Plants were grown in long days. Seventy plants of each line were used for each statistical measurement. Number of rosette leaves and days to bolting were as described for Figure 6. KO, knockout plant; Ws-2, wild-type plant (Wassilewskija-2).

involved. The gibberellic acid (GA) pathway is absolutely required for floral induction under noninductive short-day conditions, although the molecular nature of the input signal is unknown. Temperature and plant age and growth status also influence flowering.

CONSTANS (CO) gene expression is regulated by the circadian clock with a peak between 12 h after dawn and sub-

sequent dawn (Suárez-López et al., 2001). It also is regulated by daylength (Koornneef et al., 1998). It is relatively high when Arabidopsis plants are grown in long days but relatively low in short days. Our results indicated that FyPP and AtFyPPs regulate phytochrome-mediated light signals in flowering time control, mainly in long days (Figure 6A). Therefore, it was expected that *CO* expression, which accelerates flowering initiation exclusively in long days, would be altered in *FyPP* transgenic plants. To examine this possibility, wild-type and *FyPP* transgenic plants were grown for 3 weeks in long days, and plant materials were harvested 6 h after dawn. Quantitative RT-PCR/DNA gel blot analysis revealed that the *CO* transcript level decreased to half of the wild-type level in sense transgenic plants, whereas it increased ∼2.5-fold in antisense transgenic plants (Figure 8A). These observations support the notion that FyPP regulates phytochrome-mediated light signals in the long-day flowering pathway.

In addition to altered *CO* gene expression (Figure 8A), those of other flowering time genes also were influenced (Figure 8B). The transcript levels of the *GIGANTEA* and *FLOWERING LOCUS T* (*FT*) genes increased approximately threefold in antisense transgenic plants but decreased to a detectable level in sense transgenic plants. This finding also is consistent with their roles in flowering (Weigel et al., 1992; Bradley et al., 1997; Blázquez and Weigel, 2000).

Recent molecular genetic studies have shown that multiple input signals are integrated via the so-called floral pathway integrators, such as LEAFY (LFY), FT, and AGL20, that are regulated by GA as well as by light (Kobayashi et al., 1999; Lee et al., 2000; Samach et al., 2000). This entails possible cross-talk between light and GA signals in flowering time control, although direct molecular evidence is unavailable at present.

To determine whether GA or other factors are related to the light signals mediated by the phytochrome–FyPP interaction, the expression of the *GA1* and *SPY* genes involved in GA biosynthesis and signaling (Blázquez, 2000; Simpson and Dean, 2002) was analyzed in *FyPP* transgenic plants. The transcript levels were not significantly different from those in wild-type plants (Figure 8B), indicating that the GA pathway is not under the direct control of FyPP. The transcript levels of the *FLOWERING LOCUS C*, *TERMINAL FLOWER* (*TFL*), and *LFY* genes also were unaltered in *FyPP* transgenic plants (Figure 8B). Therefore, it is clear that FyPP plays its role through the long-day flowering pathway. To examine this notion further, the AtFyPP3-deficient plant (Figure 7B) was analyzed in a similar manner. The transcript levels of the *CO* and *FT* genes increased significantly, whereas that of the *SPY* gene did not (Figure 8C), confirming the primary role of FyPP in the long-day flowering pathway.

We then examined whether *AtFyPP* gene expression was regulated by daylength, as observed with *CO* gene expression. Interestingly, *AtFyPP3* was expressed at a higher level in long days than in short days, like *CO*, whereas *AtFyPP1*
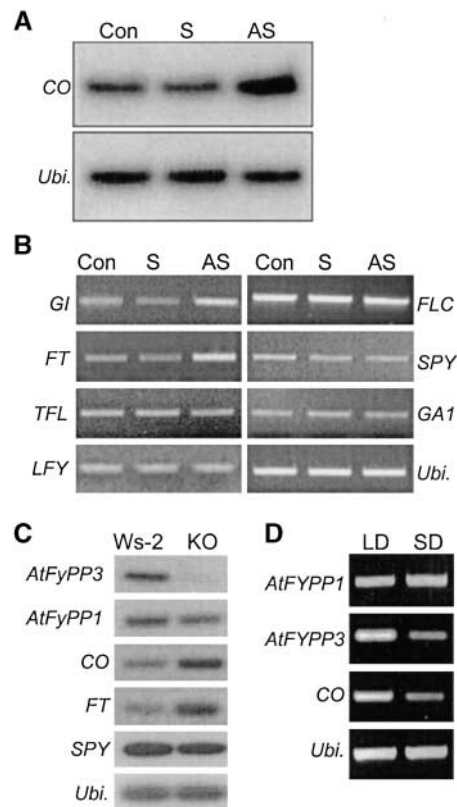
**Figure 8.** Flowering Time Gene Expression in *FyPP* Transgenic Arabidopsis Plants.

**(A)** *CO* gene expression. Plants were grown for 3 weeks in long days, and total RNAs were isolated from whole plants at 6 h after dawn. The *CO* transcript level was quantitated by RT-PCR/DNA gel blot analysis.
**(B)** Flowering time gene expression in *FyPP* transgenic plants.
**(C)** Flowering time gene expression in the AtFyPP3-deficient mutant.
**(D)** Daylength effects on *AtFyPP* gene expression. Arabidopsis plants were grown in either long days or short days.
Con, control plant; KO, knockout plant; LD and SD, long days and short days, respectively; S and AS, sense and antisense *FyPP* transgenic plants, respectively; *Ubi.*, an Arabidopsis ubiquitin gene; Ws-2, wild-type plant (Wassilewskija-2).

did not exhibit such differential expression (Figure 8D). These observations suggest that although AtFyPPs are biochemically equivalent, they play distinct roles in flowering time control. AtFyPP1 may be functional in both long-day and short-day conditions, but AtFyPP3 is required for phytochrome-mediated light signaling specifically in long days.

In conclusion, it is evident that a phytochrome kinase–associated PP2A modulates light signals in the photoperiodic control of flowering time in Arabidopsis. The phytochrome–FyPP interaction is specific to the long-day flowering pathway but is not related directly to other genetic pathways in flowering time control. It also is clear that protein phosphorylation is a

key molecular event in phytochrome kinase–mediated light signaling in plants, as demonstrated in various eukaryotic kinase signaling pathways.

## DISCUSSION

### Phytochrome Kinases Interact Functionally with a PP2A

Reversible protein phosphorylation is a feedback-control mechanism in various eukaryotic kinase signaling cascades (Stone et al., 1994; Westphal et al., 1998; Keyse, 2000). Recent molecular biological and biochemical evidence indicates that coordinated interactions between coupled protein kinases and phosphatases also play important roles in plants, especially in cell cycle control, in which a set of cyclin-dependent kinases and protein phosphatases are involved (Meszaros et al., 2000; Boniotti and Griffith, 2002). It also has been suggested that protein phosphorylation is an essential step in light signal transduction in plants and that protein phosphatase(s) possibly is involved in this process (Sheen, 1993; Harter et al., 1994; Chandok and Sopory, 1996; Fankhauser, 2000).

The eukaryotic phytochromes are unique red/far-red light receptors in that they perceive environmental light through the N-terminal chromophore binding domain and exert regulatory roles through the C-terminal domain. The C-terminal domain possesses structural elements required for interactions with downstream signaling mediators (Ni et al., 1998; Choi et al., 1999; Fankhauser et al., 1999). It also contains a motif that is similar to the prokaryotic His kinases. It is now generally accepted that the phytochromes are Ser/Thr-specific protein kinases that are regulated by light (Fankhauser and Chory, 1999). Furthermore, phytochrome autophosphorylation at certain Ser residues and phosphorylation of the phytochrome kinase substrates are essential for phytochrome function (Harter et al., 1994; Roux, 1994; Chandok and Sopory, 1996; reviewed by Nagy et al., 2000). Because the phytochromes are molecular light switches that regulate many photomorphogenic growth and developmental processes, biochemical and physiological activities should be controlled precisely. One potential molecular means to achieve this would be to modulate the phosphorylation status of the phytochromes, which requires that the intrinsic phytochrome kinase activity be coupled with a protein phosphatase(s). The recent identification of the phytochrome kinase substrates further supports this view.

Cryptochrome phosphorylation by the phytochrome kinases is regulated by light wavelength and required for the photoactivation of blue light responses (Ahmad et al., 1998). Notably, the photoactivated cryptochromes downregulate COP1 activity via direct protein–protein interactions in blue light–mediated photomorphogenic responses (Wang et al., 2001; Yang et al., 2001). Therefore, it is assumed that modulation of the phytochrome function by reversible

phosphorylation is an essential event that influences diverse plant growth and developmental processes.

Our experimental data demonstrate that FyPP and its functional orthologs in Arabidopsis, the AtFyPPs, are protein phosphatases that dephosphorylate the phytochrome kinases, providing molecular evidence for the presence of functional phytochrome kinase–phosphatase coupling in light signal transduction in plants. The phytochrome–FyPP interaction is influenced by the spectral conformation and phosphorylation status of the phytochromes. FyPP binds both phytochromes A and B, but with a relatively higher affinity for the latter (Figure 2). Transgenic plants with over-expressed or suppressed FyPP levels exhibit delayed- or early-flowering phenotypes (Figure 6) that mimic those observed in the *phyA* or *phyB* null mutant, respectively. All of these observations suggest that FyPP modulates both phytochrome A and B signals in flowering time control. However, it seems that FyPP has an opposite effect in each signaling pathway, a negative regulatory role in the former and a positive regulatory role in the latter. This finding may be related to the antagonistic roles played by phytochromes A and B in flowering time control (Mockler et al., 1999), although the exact molecular mechanisms remain to be examined. Genetic crosses between photoreceptor mutants and *FyPP* transgenic or knockout plants would clarify this point.

It is unlikely that FyPP is the only protein phosphatase that interacts directly with the phytochromes. Because the phytochromes regulate various aspects of plant photomorphogenesis, it is highly possible that more than one protein phosphatase would be involved, each for a specific photomorphogenic process. It will be interesting to determine whether multiple protein phosphatases interact with the same structural motif or with distinct motifs in the phytochrome molecule. This hypothesis reflects the fact that multiple phytochrome-interacting factors with apparently distinct roles interact with the phytochromes.

### Reversible Protein Phosphorylation Is a Primary Switch That Initiates Phytochrome Signaling

Phytochromes are autophosphorylated at distinct Ser residues, such as Ser-7, Ser-17, and Ser-598 (Lapko et al., 1999). Phosphorylation at these residues induces subtle conformational changes in the phytochrome molecule, which affect its interactions with downstream signaling mediators. Additionally, analysis of a S598A mutant phytochrome A revealed that phosphorylation at Ser-598 is critical for light regulation of phytochrome autophosphorylation and kinase activity (Fankhauser et al., 1999). Ser-598 is phosphorylated selectively in the Pfr phytochrome. Although it remains to be determined whether Ser-598 is the site dephosphorylated by FyPP or its orthologs, these observations strongly suggest that protein phosphorylation/dephosphorylation is an early biochemical event that initiates phytochrome signaling in conjunction with Pr↔Pfr phototransformation.

How does FyPP regulate the phytochrome signals? FyPP is localized in the cytoplasm, indicating that FyPP-mediated dephosphorylation of the phytochromes occurs in the cytoplasm. Phytochrome dephosphorylation by FyPP would mediate the light-induced nuclear localization of the phytochromes, as has been suggested (Ni et al., 1998, 1999). Alternatively, it may regulate the cytoplasmic function of the phytochromes. Our experimental data show that expression of the flowering time genes is affected greatly in *FyPP* transgenic plants with flowering time alterations (Figure 8B), supporting the idea that FyPP regulates the localization of phytochromes into the nucleus, where they function as the components of transcriptional regulator complexes (Ni et al., 1998). FyPP may either directly trigger the nuclear localization of the dephosphorylated phytochromes or indirectly facilitate the nuclear localization by releasing the phytochromes from certain cytoplasmic anchoring proteins, such as PKS1 (Fankhauser et al., 1999).

The physiologically active Pfr phytochrome is more readily dephosphorylated by FyPP (Figure 5). The Pfr phytochrome with an unphosphorylated Ser-598 residue has a reduced affinity for PKS1 (Fankhauser et al., 1999) but exhibits an increased affinity for PIF3 (Ni et al., 1998). It is more readily translocated into the nucleus, where it binds PIF3, Aux/IAA, or other unidentified transcription factors and regulates gene expression. It is possible that the light-activated phytochrome is desensitized by phosphorylation at Ser-598, the only residue that is phosphorylated specifically in the Pfr form, but sensitized by FyPP-mediated dephosphorylation, as has been suggested (Park et al., 2000a). A similar sensitization/desensitization mechanism has been well documented in the animal photoreceptor rhodopsin (Vishnivetskiy et al., 1999).

### FyPP Exerts a Flowering-Specific Role

Two major environmental factors that influence flowering time in Arabidopsis are light and temperature (vernalization). Long days and exposure to low temperature induce flowering (Blázquez et al., 2001; Simpson and Dean, 2002). Molecular mechanisms that underlie the photoperiodic control of flowering time have been studied extensively in recent years, and numerous flowering time genes have been identified (for reviews, see Levy and Dean, 1998; Simpson and Dean, 2002). The phytochromes and cryptochromes are involved in flowering time control but exhibit both synergistic and antagonistic coactions (Mockler et al., 1999; Mazzella et al., 2001), indicating that the light-signaling network involved is complicated.

Analyses of transgenic Arabidopsis plants with the *FyPP* gene demonstrate that the expression level of the *FyPP* or *AtFyPP* gene is in inverse proportion to the timing of flowering. Transgenic plants with reduced FyPP levels showed accelerated flowering, whereas those with increased FyPP levels showed delayed flowering, indicating that FyPP negatively

regulates flowering initiation. The altered flowering pheno-types observed in antisense and sense transgenic plants are very similar to those seen in the *phyA* and *phyB* mutants, re-spectively (Figure 6). Although the possibility that either phyA or phyB signal is regulated specifically by FyPP still exists, it is more likely that both signals are regulated by FyPP.

Long days represent a critical and sufficient environmen-tal factor that initiates flowering in Arabidopsis. *AtFyPP3* is expressed to a higher level in long days than in short days, as observed with *CO* (Suárez-López et al., 2001). CO has been suggested to control a signaling pathway that links daylength and the timing of flowering through genetic inter-actions with FT, TFL, and SOC1 (Onouchi et al., 2000; Suárez-López et al., 2001). FyPP and its orthologs may function in a similar manner. However, they do not seem to regulate all of the signaling pathways downstream of CO. FT gene expression was altered greatly in *FyPP* transgenic plants as well as in AtFyPP3-deficient mutant plants, but ex-pression of the *TFL* and *LFY* genes was not (Figures 8B and 8C). *AtFyPP1* is identical to *AtFyPP3* except for two resi-dues, and they share the same enzymatic activity and cation requirement. Both genes are expressed predominantly in floral organs. Unexpectedly, *AtFyPP1* expression was not influenced by daylength, unlike that of *AtFyPP3*, suggesting that it functions somewhat differently from *AtFyPP3*. It may play a constitutive role in phytochrome signaling. Therefore, it is assumed that phytochrome phosphorylation by FyPP or its orthologs is modulated precisely by more than one re-versible protein phosphorylation event for elaborate control of flowering time in plants, depending on daylength.

## METHODS

### Plant Materials and Growth Conditions

*Arabidopsis thaliana* plants (ecotypes Columbia and Wassilewskija-2) were grown in a controlled culture room at 22°C with a photoperiod of 16 h and RH of 70%. *Agrobacterium tumefaciens*–mediated trans-formation was performed by a modified floral-dip method (Clough and Bent, 1998). An Arabidopsis AtFyPP3-deficient mutant was iso-lated from a pool of T-DNA insertion lines (ecotype Wassilewskija-2; Wisconsin Arabidopsis Knockout Facility, Madison, WI). It had a T-DNA insertion in the third intron of the *AtFyPP3* gene, as deter-mined by DNA sequencing.

For light treatments, plants were grown in the dark or under vari-ous light wavelengths. Continuous white light was provided by fluo-rescent FLR40D/A tubes (Osram, Seoul, Korea) at 15 $\mu$mol·m$^{-2}$·s$^{-1}$. Red and far-red light (10 and 7 $\mu$mol·m$^{-2}$·s$^{-1}$, respectively) were pro-vided by red and far-red light-emitting diodes equipped in a red/far-red E-30LED1 growth chamber (Percival Scientific, Boone, IA).

### Yeast Two-Hybrid Screening

Yeast two-hybrid screening was performed using the MATCH-MAKER Two-Hybrid System (Clontech, Palo Alto, CA) according to

the manufacturer's instructions. The C-terminal half (residues 667 to 1122) of the Arabidopsis phytochrome A was fused in frame to the GAL4 DNA binding domain in the bait plasmid pGBT9. A cDNA library was constructed from 5-day-old dark-grown pea (*Pisum sativum*) seedlings into the phagemid vector pAD-GAL4-2.1 (Kang et al., 2001). Yeast strain HF7c was transformed with the pGBT9 construct and sub-sequently with the cDNA library phagemid constructs. Positive trans-formants (*his3*+ and *lacZ*+) were selected in the presence of 20 mM 3-aminotriazole to eliminate false-positive results. Phagemid vectors containing the cDNA clones were recovered by back transformation into *Escherichia coli* strain XL1-Blue and subjected to DNA sequencing.

### RNA Gel Blot Analysis and Comparative Reverse Transcription–PCR

Total RNA was extracted from plant materials using the RNeasy Plant Total RNA Isolation Kit (Qiagen, Valencia, CA). Routinely, 20 $\mu$g of each RNA sample was denatured in the denaturation buffer [20 mM 3-(*N*-morpholino)-propanesulfonic acid, 8 mM sodium acetate, and 1 mM EDTA] supplemented with 50% (v/v) formamide and 2.2 M formaldehyde at 65°C for 10 min, resolved on a 1.2% denaturing agarose gel, and transferred onto a Hybond-N$^+$ nylon membrane (Amersham Pharmacia, Buckinghamshire, UK). The membrane then was hybridized with $^{32}$P-labeled gene-specific probe.

Comparative reverse transcription (RT)–PCR was used for some quantitative purposes. Total RNA samples were pretreated exten-sively with RNase-free DNaseI to eliminate contaminating genomic DNA. Primary cDNA was prepared from 2 $\mu$g of total RNA using Pfu Turbo polymerase (Stratagene, La Jolla, CA) in a 20-$\mu$L reaction vol-ume, and 2 $\mu$L of the reaction mixture was used for subsequent RT-PCR in a 50-$\mu$L reaction volume. The RT-PCR runs were 15 to 28 cy-cles, depending on the linear range of PCR amplification for each gene, each cycle at 94°C for 1 min, 58°C for 30 s, and 72°C for 1 min. One additional cycle at 72°C for 7 min was performed after the last run to allow for trimming of incomplete polymerizations.

### Preparation of Phytochrome Photoreceptors

Oat (*Avena sativa*) phytochrome A was isolated from dark-grown oat seedlings for 5 days as described previously (Lapko and Song, 1995). Protein quality and spectral integrity were examined by SDS-PAGE, zinc blot, and spectral measurements. The protein purity was routinely >92%, and the specific absorbance ratio was 1.0 to 1.1.

The full-size Arabidopsis phytochrome B gene was cloned into the pGEM3Z(+) vector and subsequently subjected to in vitro translation using the TNT Quick Coupled Transcription/Translation System ac-cording to the procedure provided by the manufacturer (Promega, Madison, WI). One microgram of template DNA and 20 $\mu$Ci of $^{35}$S-Met (catalog No. AG1094; Amersham, Piscataway, NJ) were used in a 50-$\mu$L reaction volume. ATP was included at a final concentration of 1 $\mu$M. The reaction mixture was incubated at 30°C for 90 min. In vitro reconstitution with phycocyanobilin (PCB) was performed on ice under green safety light as described (Park et al., 2000b). Two mi-croliters of the PCB solution in DMSO at a concentration of 2 mM was used, and the reaction mixture was incubated for 1 h in the dark.

### In Vitro Pulldown Assays

Purified phytochrome A from dark-grown oat seedlings and in vitro translated Arabidopsis phytochrome B were used. The phytochromes

were either used directly or autophosphorylated in the presence of 0.5 mM cold ATP for 30 min at room temperature before use. They were then treated with red or far-red light to obtain the Pfr or Pr phytochrome, respectively. The FyPP–glutathione *S*-transferase (GST) fusion protein was expressed and purified from *E. coli* cells.

The in vitro pulldown assays were performed essentially as described previously (Kang et al., 2001) with some modifications. Briefly, 2 μg of the recombinant FyPP-GST fusion protein in PBS was first bound to glutathione–Sepharose 4B resin (Amersham Pharmacia) at 4°C for 20 min, and 2 μg of oat phytochrome A was added. The mixture was incubated at 30°C for 30 min. The resin then was washed thoroughly three times with PBS containing 5% Triton X-100, and the bound phytochrome A was eluted by boiling in double-distilled water for 10 min. The eluted samples were run on SDS-PAGE, transferred onto a Hybond-P$^+$ membrane (Amersham Pharmacia), and analyzed immunologically using a polyclonal anti-phytochrome A antibody.

In vitro pulldown assays with phytochrome B were performed in a manner similar to those with oat phytochrome A but using 5 μL of the in vitro translation mixture, which had been reconstituted in vitro with PCB.

### Protein Phosphatase Assays

Recombinant protein phosphatases were expressed as GST fusion proteins via the pGEX-4T-1 *E. coli* expression vector (Amersham Pharmacia). *E. coli* strain BL21 was used as the host for protein expression. Cell growth and induction conditions were as described previously (Park et al., 2000b). Protein phosphatase assays were performed essentially as described (Hériché et al., 1997) with a few modifications. Autophosphorylated oat phytochrome A was used as a substrate. One microgram of the substrate was incubated with 0.2 μg of protein phosphatase for up to 30 min at room temperature, analyzed by 12% SDS-PAGE, dried on 3MM paper, and subjected to autoradiography. An excess amount of cold ATP was included in the reaction mixtures at a final concentration of 1 mM to keep an equilibrating state of the autophosphorylated phytochrome substrate. Dephosphorylation efficiencies were quantified using ImageMaster VDS (Amersham Pharmacia).

Upon request, all novel materials described in this article will be made available in a timely manner for noncommercial research purposes.

### Accession Numbers

The GenBank accession numbers for the sequences described in this article are AF305635 (FyPP) and AF275664 (AtFyPP3). The accession numbers for the other sequences shown in Figure 1 are as follows: Q07098, Q07099, Q07100, P48578, and O04951 (Arabidopsis PP2A-1 to PP2A-5, respectively), S42558 and P48528 (Arabidopsis PPX-1 and PPX-2, respectively), S28173 (human PPX-1), AAA41930 (rat PPX-1), and AAD50050 (AtFyPP1).

### ACKNOWLEDGMENTS

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