The Plant Cell, Vol. 14, 1591–1603, July 2002, www.plantcell.org © 2002 American Society of Plant Biologists

Missense Mutation in the PAS2 Domain of Phytochrome A Impairs Subnuclear Localization and a Subset of Responses

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Phytochrome A signaling shows two photobiologically discrete outputs: so-called very-low-fluence responses (VLFR) and high-irradiance responses (HIR). By modifying previous screening protocols, we isolated two Arabidopsis mutants retaining VLFR and lacking HIR. Phytochrome A negatively or positively regulates phytochrome B signaling, depending on light conditions. These mutants retained the negative but lacked the positive regulation. Both mutants carry the novel *phyA-302* allele, in which Glu-777 (a residue conserved in angiosperm phytochromes) changed to Lys in the PAS2 motif of the C-terminal domain. The *phyA-302* mutants showed a 50% reduction in phytochrome A levels in darkness, but this difference was compensated for by greater stability under continuous far-red light. phyA-302:green fluorescent protein fusion proteins showed normal translocation from the cytosol to the nucleus under continuous far-red light but failed to produce nuclear spots, suggesting that nuclear speckles could be involved in HIR signaling and phytochrome A degradation. We propose that the PAS2 domain of phytochrome A is necessary to initiate signaling in HIR but not in VLFR, likely via interaction with a specific partner.

INTRODUCTION

Phytochrome is a small family of red light (R) and far-red light (FR) photoreceptors comprising five isoforms, phytochrome A through phytochrome E (phyA through phyE), in Arabidopsis (Neff et al., 2000). phyA and phyB are dimeric cytoplasmic proteins in dark-grown seedlings that migrate to the nucleus upon light activation (Kircher et al., 1999; Yamaguchi et al., 1999). The N-terminal domain bears an open chain tetrapyrrol chromophore. The C-terminal domain contains motifs that are important for interaction with the nuclear transcription factor PIF3 (Ni et al., 1998, 1999; Martinez-García et al., 2000), the phytochrome kinase substrate PKS1 (Fankhauser et al., 1999), nucleoside diphosphate kinase 2 (Choi et al., 1999), and cryptochrome (Ahmad et al., 1998).

The pattern of a response—graded, multiphasic, with a threshold, with biorhythmic oscillations, et cetera—depends on the architecture of the signaling network (Becskei et al., 2001). Seedling deetiolation under continuous FR is mediated by phyA (Nagatani et al., 1993; Parks and Quail, 1993; Whitelam et al., 1993). Even if a single end point process is considered (e.g. inhibition of hypocotyl growth), two components of phyA activity can be distinguished, demonstrating the bistable or binary nature of the response (Casal et al., 2000): the very-low-fluence response (VLFR) component, which saturates with infrequent (e.g., single or hourly pulses) activation of phyA by very low fluences of R or FR, and the high-irradiance response (HIR) component, which requires sustained activation of phyA with higher fluences of FR.

The architectural features of the phyA signaling network that give rise to the biphasic response have not been elucidated. Nevertheless, VLFR and HIR can be dissected genetically by the *vlf1*, *vlf2*, *fhy3*, and *dim/dwarf1/eve1* loci that operate downstream of phyA (Yanovsky et al., 1997, 2000; Luccioni et al., 2001). These two components also can be

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Article, publication date, and citation information can be found at www.plantcell.org/cgi/doi/10.1105/tpc.000521.

separated molecularly because the HIR requires a region of target gene promoters that is not necessary for VLFR (Cerdán et al., 2000). Furthermore, activation of phyA in the VLFR mode (by a pulse of R) negatively regulates phyB signaling, whereas activation of phyA in the HIR mode (by several hours of FR) positively regulates phyB signaling initiated by a subsequent R pulse (Cerdán et al., 1999).

Together, these observations are consistent with a model in which signaling downstream of phyA branches out into two cascades, depending on the mode in which the photoreceptor is activated. The point at which divergence is initiated has not been established. The aim of this work was to identify mutants selectively impaired in the HIR and not in the VLFR component to gain insight into the molecular differences between these two response modes.

RESULTS

Novel Alleles of *phyA* That Show Deficient Hypocotyl Growth Inhibition but Normal Seed Germination

To obtain Arabidopsis mutants with impaired HIR but retaining VLFR, both germination of mutagenized seed and subsequent seedling growth were conducted under continuous FR (Figure 1). phyA-mediated promotion of seed germination is a VLFR (Botto et al., 1996; Shinomura et al., 1996) because the response is saturated with hourly pulses of FR and continuous FR has no additional effect (Figure 2A). In contrast, phyA-mediated inhibition of hypocotyl growth is predominantly a HIR because continuous FR is significantly more effective than hourly pulses of FR.

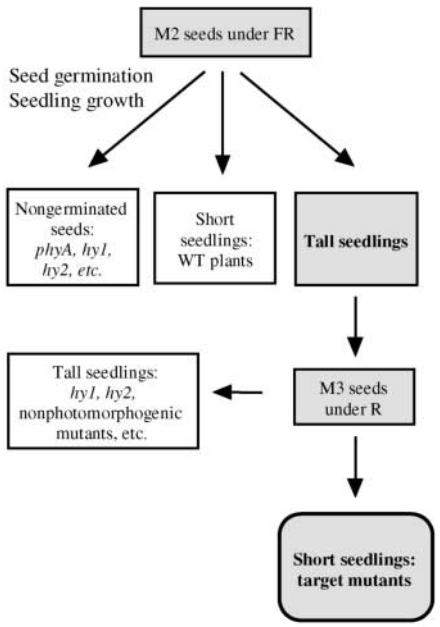


Figure 1. Screening Protocol Used to Identify Mutants Affected More Strongly in HIR Than in VLFR of phyA.

WT, wild type.

Tall seedlings under continuous FR were selected from M2 seeds (Figure 1). Normal seedlings under pulsed R were selected from M3 seeds of these mutants to avoid phytochrome chromophore and nonphotomorphogenic hypocotyl growth mutants (Figure 1). Two independent lines (designated *phyA-302-1* and *phyA-302-2* based on the evidence described below), showing seed germination under FR (Figure 2A), normal inhibition of hypocotyl growth under R, and long hypocotyls under continuous FR (Figure 2B), were chosen for further studies. The two mutants failed to complement the null *phyA-201* mutant (Figure 3).

In etiolated seedlings, the levels of phyA detectable immunologically and by fluorescence signals were reduced by ~50% in both mutant lines (Figures 4A and 4B). The fluorescence spectra (Figure 4C), the extent of Pr-to-lumi–R (the first photoproduct stable at low temperatures) conversion at 85K upon illumination with 639 nm (0.24 to 0.3), and the extent of Pr-to-Pfr conversion at 273K upon illumination with 680 nm (0.55 to 0.65) were not affected significantly by the *phyA-302* mutations. Because the abundance of phyA normally is reduced by continuous FR compared with darkness or hourly FR pulses (Casal et al., 2000), immunoblot analysis also was conducted with dark-grown 3-day-old seedlings transferred to continuous FR for 8 h. Although *phyA-302* mutants had less phyA in dark-grown control seedlings, the residual phyA was more stable than in the wild type (Figure 4D).

The Two Missense Mutations Occur in Amino Acid 777 of phyA

The *phyA* genes of the two mutants and of the wild type were cloned and sequenced completely. The two independent mutants contain the same Guanidine-to-Adenine mutation that results in the change of amino acid 777 from Glu to Lys (Figure 5; amino acid numbering based on the chromophore attachment site at Cys-323).

phyA-302:Green Fluorescent Protein Fusion Proteins Fail to Produce Nuclear Speckles

Null *phyA* mutant seedlings of Arabidopsis were transformed with the Arabidopsis *PHYA*:green fluorescent protein (*GFP*) or *phyA-302*:*GFP* genes under the control of a 35S promoter. The *PHYA*:*GFP* transgene fully rescues the *phyA* null mutant phenotype (Kim et al., 2000). The *phyA-302*:*GFP* transgene rescued the *phyA* null mutant phenotype under hourly pulses of FR (hypocotyl length [mm] in the wild type was 11.8 ± 0.2 in darkness and 10.8 ± 0.2 in

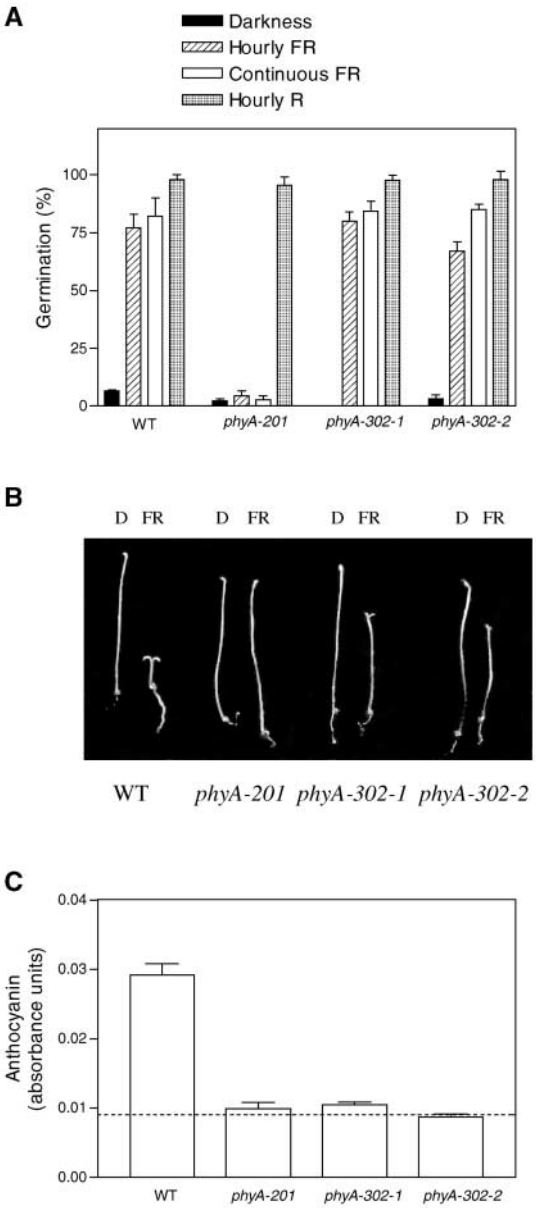


Figure 2. *phyA-302* Mutants Show Normal Induction of Seed Germination **(A)**, Weak Hypocotyl Growth Inhibition and Cotyledon Unfolding **(B)**, and No Promotion of Anthocyanin Synthesis **(C)** under Continuous FR.

Data are means and SE of six **(A)** or five **(C)** replicate boxes. In **(C)**, the dashed line shows absorbance levels in dark-grown seedlings. D, darkness; WT, wild type.

pulsed FR [$P < 0.05$]; hypocotyl length in the null *phyA* mutant was 11.3 ± 0.4 in darkness and 11.2 ± 0.2 in pulsed FR [$P > 0.1$]; hypocotyl length in the null *phyA* mutant expressing the phyA-302:GFP transgene was 13.3 ± 0.3 in darkness and 12.2 ± 0.3 in pulsed FR [$P < 0.05$]).

Continuous FR reduced hypocotyl length in the wild type dramatically (2.0 ± 0.1 mm), but it had no more effect than pulsed FR in the phyA-302:GFP transgenic plants (12.1 ± 0.3 mm). This pattern of response agrees with the behavior of the *phyA-302* mutants (see below). The localization of phyA:GFP and phyA-302:GFP fusion proteins was investigated in transgenic seedlings grown in darkness and subsequently transferred to continuous FR.

In the seedlings that remained as dark controls, the nuclei showed no fluorescence associated with the presence of phyA (Figure 6). phyA:GFP or phyA-302:GFP was present in the nuclei of plants exposed to 1, 3, 9, or 24 h of continuous FR, indicating that the *phyA-302* mutation did not affect nuclear translocation. However, although phyA:GFP was able to form speckles in >90% of the nuclei (Kim et al., 1999; Kircher et al., 1999; Hisada et al., 2000), plants bearing the phyA-302:GFP fusion protein failed to form these spots (Figure 6), which were observed in only 10% of the nuclei after 24 h of FR and never in nuclei corresponding to the hook region of the hypocotyl.

Sustained and Transient Activation with FR Are Equally Effective in *phyA-302*

In the *phyA-302* mutants, hypocotyl growth inhibition, which is dominated by the HIR component, was more affected than seed germination, which is dominated by the VLFR component. To compare VLFR and HIR of the same end point responses (hypocotyl growth and cotyledon unfolding), etiolated seedlings were allowed to germinate in darkness for 24 h after a saturating R pulse and transferred to either hourly pulses of FR, which saturate the VLFR but are insufficient to cause a HIR, or continuous FR at a fluence rate that is sufficient to initiate a HIR (Casal et al., 2000).

The wild type showed hypocotyl growth inhibition and cotyledon unfolding under hourly FR (i.e., VLFR), but continuous FR was significantly more efficient, suggesting a strong contribution of a HIR (Figure 7). In the *phyA-302-1* and *phyA-302-2* mutants, pulsed and continuous FR produced similar effects, indicating the absence of a HIR. The weak *phyA-205* allele (Nagatani et al., 1993; Reed et al., 1994), included for comparative purposes, showed reduced responses to both pulsed and continuous FR, but the latter was significantly more effective than hourly FR. Thus, in contrast to the *phyA-302* mutants, the *phyA-205* allele showed a reduced VLFR but retained some HIR (Figure 7).

We also investigated the effects of pulsed and continuous FR in the *hy1* and *hy2* mutants, which have severely reduced levels of phytochrome (Koornneef et al., 1980). These mutations affect chromophore synthesis genes and not apoprotein genes (Davis et al., 1999; Muramoto et al., 1999; Kohchi et al., 2001). Although photomorphogenesis under FR is very weak in these mutants, both showed stronger effects under continuous FR than under pulsed FR (angle between the cotyledons [°] in the wild type was 4 ± 1 in

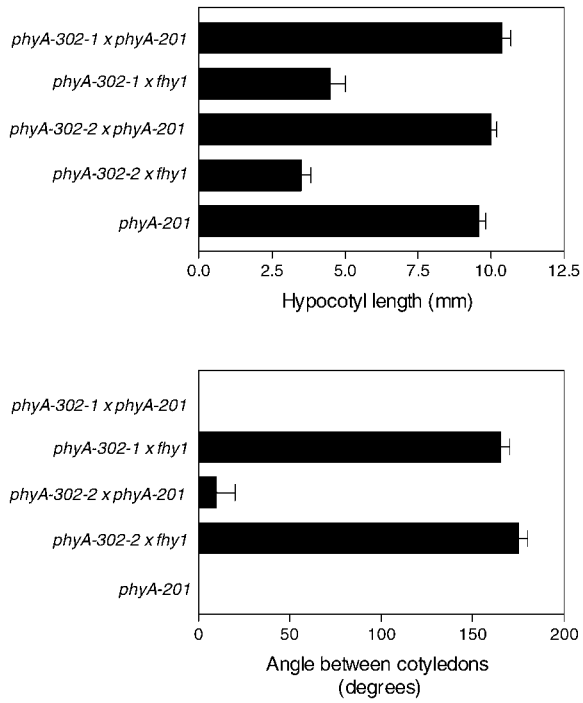


Figure 3. Complementation Tests of the Mutants That Germinate but Fail to Deetiolate Normally under FR.

The novel mutants were used as mother plants, and *phyA-201* and *fhy1* were used as pollen donors. Data are means and SE of 8 to 12 replicate plants.

darkness, 108 ± 3 in pulsed FR, and 180 ± 0 in continuous FR; angle between the cotyledons in *hy1* was 1 ± 1 in darkness, 3 ± 1 in pulsed FR, and 11 ± 2 in continuous FR; angle between the cotyledons in *hy2* was 1 ± 1 in darkness, 5 ± 1 in pulsed FR, and 17 ± 5 in continuous FR). These results indicate that a reduction of photoactive phyA levels does not necessarily eliminate the HIR in Arabidopsis.

Weak Fluence Rate Dependence of the Effects of Continuous FR in *phyA-302*

One of the distinctive features of phyA activity under continuous FR is its strong fluence rate dependence. In the wild type, both hypocotyl growth and cotyledon unfolding increased in response to the fluence rate of continuous FR between 0 and 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (Figure 8). In the *phyA-302* mutants, hypocotyl growth inhibition saturated at the lowest fluence rate of continuous FR tested (0.1 $\mu mol \cdot m^{-2} \cdot s^{-1}$), and cotyledon unfolding showed weak fluence rate dependence at >0.1 $\mu mol \cdot m^{-2} \cdot s^{-1}$. At this fluence rate, hypocotyl growth inhibition and cotyledon unfolding showed wild-type levels (Figure 8).

Continuous FR Fails to Promote Anthocyanin Synthesis in *phyA-302*

In Arabidopsis, continuous FR promotes anthocyanin synthesis, but hourly pulses of FR have negligible effects on this process compared with dark controls (Yanovsky et al., 2000). This finding indicates that anthocyanin synthesis is dominated by the HIR component, whereas the VLFR component makes at most a weak contribution. If the *phyA-302* mutations cause a general failure of HIR, the promotion of anthocyanin synthesis by continuous FR should be impaired severely in *phyA-302-1* and *phyA-302-2*. This expectation was met by the data (Figure 2C). Thus, the *phyA-302* mutants showed wild-type promotion of seed germination, intermediate inhibition of hypocotyl growth and cotyledon unfolding, and no promotion of anthocyanin by continuous FR (Figure 2). Across different processes, the effect of the mutations was inversely related to the effect of pulsed FR compared with continuous FR.

Inductive Responses in *phyA-302*

To investigate inductive responses (i.e., the effects of light pulses) in more detail, the seedlings were exposed to hourly pulses predicted to establish a series of calculated proportions of phytochromes in the Pfr form (Pfr/P). The wild type showed biphasic responses of hypocotyl growth and cotyledon unfolding (Figure 9). The first phase (which saturates at Pfr/P of 10%) is the VLFR mediated by phyA, and the second phase (observed between Pfr/P of 33 and 87%) is the R/FR reversible response mediated by phyB (Yanovsky et al., 1997). In the *phyA-302* mutants, the VLFR was affected only partially (Figure 9). Protein levels are similar under hourly FR pulses compared with those in darkness (Casal et al., 2000), suggesting that on a protein basis (Figure 4), the mutated phyA photoreceptor retained normal efficiency for VLFR.

Analysis of the Interaction between phyA-302 and phyB Signaling

The interaction between phyA and phyB signaling is synergistic when phyA operates in the HIR mode and antagonistic when phyA operates in the VLFR mode (Cerdán et al., 1999). To investigate whether the *phyA-302* mutants retained these interactions, the seedlings were exposed daily to a brief pulse of R or FR in factorial combination with or without 3 h of FR (Figure 10A). The effect of R pulses compared with FR pulses is mediated by phyB (Cerdán et al., 1999). In seedlings exposed only to brief pulses, phyA oper-

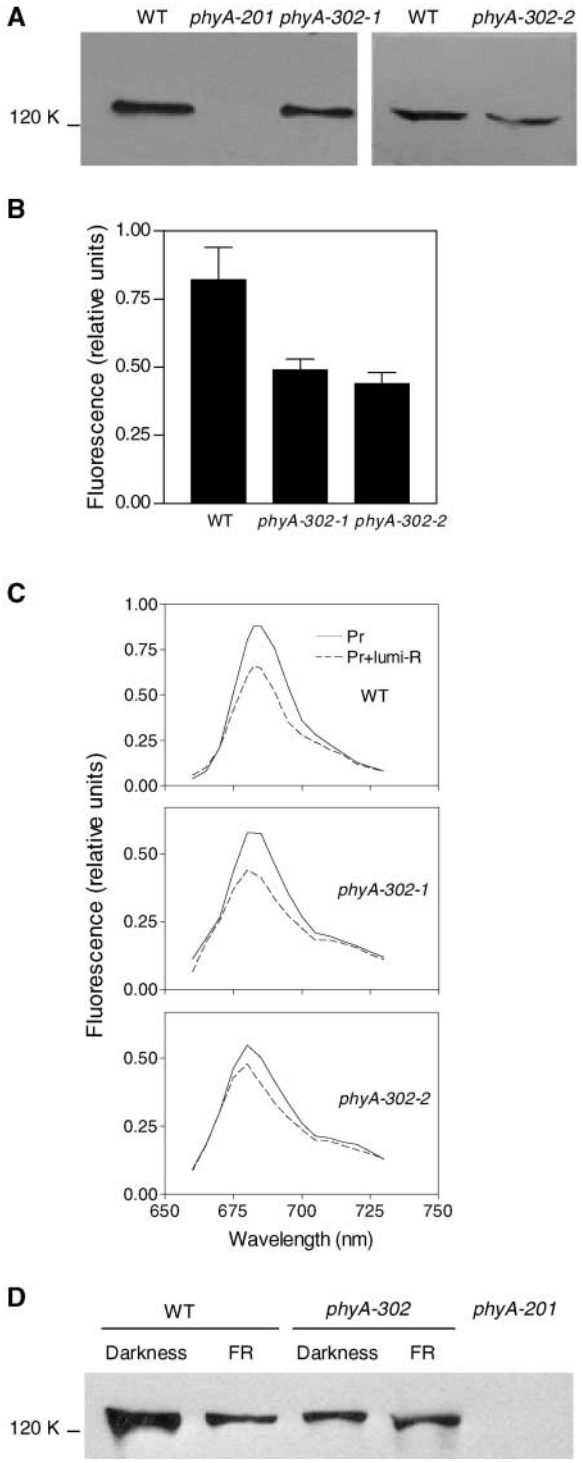


Figure 4. Etiolated Seedlings of the *phyA-302* Mutants Contain Less phyA, but This phyA Is More Stable under Continuous FR.

(A) Immunoblots of 4-day-old etiolated seedlings.

(B) Low-temperature fluorescence of phytochrome in etiolated hypocotyls.

ated in the VLFR mode, whereas in those seedlings exposed to 3 h of FR, phyA operated predominantly in the HIR mode. In the absence of 3-h FR exposures, wild-type seedlings failed to unfold the cotyledons in response to a daily pulse of R compared with FR (Figure 10B; the difference is shown in Figure 10C).

Because daily exposure to a pulse of R induces cotyledon unfolding in *phyA* null mutants but not in the *phyA phyB* double mutant, phyA signaling in the VLFR mode is predicted to antagonize phyB signaling (Cerdán et al., 1999). In wild-type seedlings exposed daily to 3 h of FR, a subsequent R pulse caused cotyledon unfolding. Both phyA and phyB were required for this amplification effect, indicating a synergistic action of both photoreceptors when phyA operates in the HIR mode (Cerdán et al., 1999). In the *phyA-302* mutants, a pulse of R given alone was not effective (Figure 10). This finding indicates that in terms of the antagonistic effect, the mutated phyA molecule behaved like wild-type phyA.

However, in contrast to what was seen in the wild type, daily exposure to 3 h of FR completely failed to amplify the response to a R versus FR test pulse (Figure 10). This finding indicates that in terms of the synergistic effect, the mutated phyA molecule lacks functional activity. In other words, in seedlings exposed to 3 h of FR followed by a R pulse, cotyledon unfolding was observed in the wild type (as a result of the amplification of the response by 3 h of FR) and in the *phyA* null mutant (in which the response to R is constitutive) but was very poor in the *phyA-302* mutants (in which the constitutive response was minimal and amplification was null).

DISCUSSION

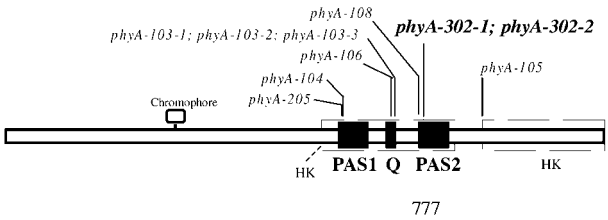
Novel *phyA* Alleles Affected in the PAS2 Domain

Screening for mutants able to germinate but not to deetiolate normally under continuous FR resulted in the isolation of two independent *phyA-302* plants carrying a missense mutation (Glu to Lys) at amino acid 777 (Figure 5). The traditional protocol for the screening of *phyA* mutants, which involves the induction of germination of chilled seeds by white light before transfer to FR (Nagatani et al., 1993; Parks and Quail, 1993; Whitelam et al., 1993), has yielded a number of

(C) Low-temperature fluorescence spectra of phytochrome in etiolated hypocotyls.

(D) Immunoblot of 3-day-old seedlings transferred to FR for 8 h.

Protein gel blots are representative of three independent determinations. Fluorescence data are means and SE of seven independent sets of measurements. WT, wild type.



phyA-302	P I FG TD E FG W C T**K** W NP A M S K L
PHYA Arab.	P I FG TD E FG W C T**E** W NP A M S K L
PHYA Sola.	P I FG TD Q FG W C S**E** W NS A M T M L
PHYA Pisu.	P I FG TD E FG W C T**E** W NP A M S K L
PHYA Nico.	P I FG TD Q FG W C S**E** W NS A M T K L
PHYA Oryz.	P I FG AD E FG W C S**E** W NA A M T K L
PHYA Aven.	P I FG AD E FG W C S**E** W NA A M T K L
PHYB Arab.	P I FA AD E NT C C L**E** W NM A M E K L
PHYC Arab.	P I F I TN E NG V C S**E** W NN A M Q K L
PHYD Arab.	P I FA AD E NT C C L**E** W NT A M E K L
PHYE Arab.	P I FA SD E NA C C S**E** W NA A M E K L

Figure 5. Location of the Missense Mutation in *phyA-302-1* and *phyA-302-2*.

The positions of other C-terminal missense *phyA* mutations are indicated for comparative purposes. The amino acid sequence in the region of the *phyA-302* mutation is compared with sequences of wild-type *PHYA* from Arabidopsis and other angiosperms as well as other Arabidopsis phytochromes. *Arab*., Arabidopsis; *Aven*., *Avena sativa*; HS, His kinase–related domains; *Nico*., *Nicotiana tabacum*; *Oryz*., *Oryza sativa*; *Pisu*., *Pisum sativum*; Q, Quail box; *Sola*., *Solanum tuberosum*.

null and leaky *phyA* alleles that do not coincide with the mutation reported here (Dehesh et al., 1993; Whitelam et al., 1993; Reed et al., 1994; Xu et al., 1995).

Several *phyA* and *phyB* mutations that do not alter phyA or phyB protein levels, spectral activity, or dimer formation are located between amino acids 632 and 768 (amino acid numbering based on the chromophore attachment site at Cys-323), particularly amino acids 715, 716, and 727 (Figure 5) (Quail et al., 1995; Wagner and Quail, 1995; Xu et al., 1995). This region, often designated the Quail box (amino acids 714 to 731) (Song, 1999), is important for phytochrome interaction with partners such as PIF3 (Ni et al., 1998, 1999) and nucleoside diphosphate kinase 2 (Choi et al., 1999). The *phyA-302* mutations are not in the Quail box (Figure 5). They do not affect the His kinase–related domain present between amino acids 889 and 1117 (Schneider-Poetsch and Braun, 1991; Yeh and Lagarias, 1998) that is involved in phytochrome interaction with PKS1 (Fankhauser et al., 1999) and that participates in phyB function (Krall and Reed, 2000).

There is a second region within the C terminus, between amino acids 593 and 825, related to the His kinase domain of the cyanobacterial phytochrome Cph1 (Yeh and Lagarias, 1998), that contains the Quail box, two individual PAS domains (amino acids 621 to 684 and 767 to 817) (Taylor and Zhulin, 1999), and the *phyA-302* mutations. Actually, these mutations fall within the PAS2 domain (Figure 5) in an amino acid conserved in PAS2 of angiosperm phytochromes but not in the PAS1 domain of phyA or the PAS domain of photoreactive yellow protein (Taylor and Zhulin, 1999).

phyA-302 Mutants Retain VLFR but Lack HIR

phyA-mediated responses have two components whose relative contributions to the output depends on the developmental context: the VLFR, which saturates with infrequent pulses of very low fluences of R or FR, and the HIR, which requires sustained excitation with FR and shows strong fluence rate dependence. The *phyA-302* mutants retained VLFR but lacked HIR (Figures 3, 7, and 8).

The failure of HIR in *phyA-302* is not a general (nonspecific) consequence of reductions in phyA signaling. The *phyA-205* allele, which carries a missense mutation (Val to Met) at amino acid 631 (Reed et al., 1994) (Figure 5) and retains normal protein levels and photochemical activity (Quail et al., 1995), shows weak phyA-mediated effects but retains a significant HIR (the difference between hourly and continuous FR in Figure 7). Similarly, *phyA-103*, *phyA-104*, *phyA-106*, and *phyA-108*, which carry missense mutations within the Quail box, and *phyA-105*, which carries a Ala-to-Val mutation at position 893 (Figure 5), all affect VLFR (Parks et al., 1996), but they retain the strong fluence rate dependence typical of HIR (Xu et al., 1995).

The loss of HIR in *phyA-302* is not the consequence of reduced phyA levels. Although the *phyA-302* mutation caused a 50% reduction in protein levels in dark-grown seedlings, this difference was compensated for by the greater stability of phyA under FR in *phyA-302* than in the wild type (Figure 4D). Furthermore, reduced phyA levels do not eliminate HIR per se. The *phyA-109* mutant has low protein levels but retains fluence rate–dependent inhibition of hypocotyl growth (Xu et al., 1995), and *hy1* and *hy2* chromophore mutants have very little active phyA (Koornneef et al., 1980), yet they show a stronger residual response to continuous FR than pulsed FR, which is typical of HIR.

The *phyB-101* mutation involves a Glu-to-Lys change at the amino acid position equivalent to *phyA-302*, and the recombinant mutant protein produced by the *phyB-101* allele shows enhanced Pfr-to-Pr dark reversion (Elich and Chory, 1997). Increased dark reversion accounts for the *phyB-101* phenotype, which is weak under continuous R (which counteracts dark reversion) and resembles *phyB*-null alleles in its failure to respond to the Pfr level established before darkness (Elich and Chory, 1997). We have not analyzed dark reversion in *phyA-302*, but it is obvious that if this mutation causes Pfr dark reversion of phyA (which is absent in Landsberg *erecta*; Eichenberg et al., 2000), this should have a

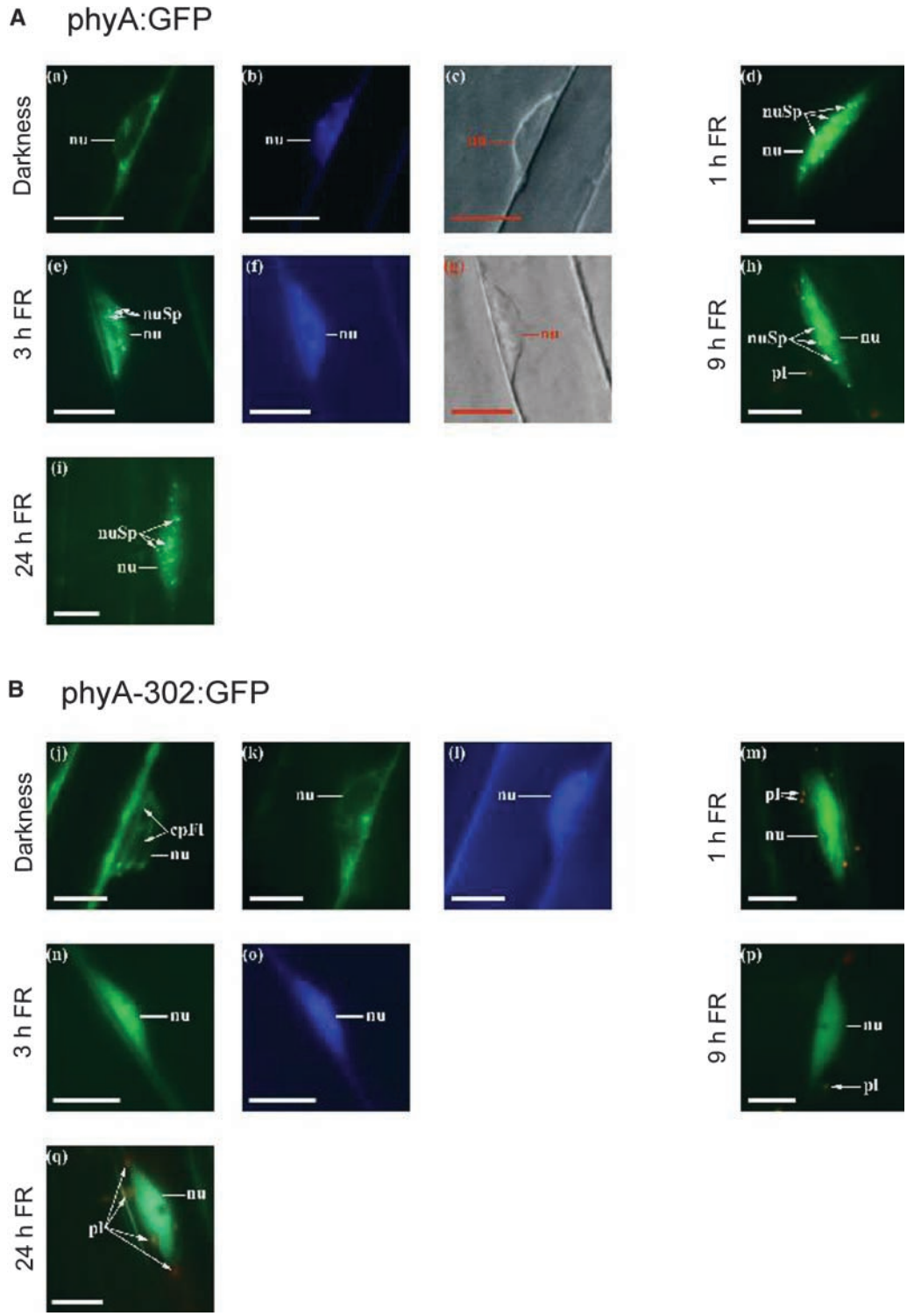


Figure 6. Arabidopsis phyA:GFP and phyA-302:GFP Fusion Proteins Translocate to the Nucleus, but phyA-302:GFP Fails to Produce Nuclear Speckles under FR.

(A) Representative nuclei of transgenic seedlings expressing phyA:GFP.

(B) Representative nuclei of transgenic seedlings expressing phyA-302:GFP.

The seedlings were kept in darkness (**[a]**, **[b]**, **[c]**, **[j]**, **[k]**, and **[l]**) or transferred to continuous FR for 1 h (**[d]** and **[m]**), 3 h (**[e]**, **[f]**, **[g]**, **[n]**, and **[o]**), 9 h (**[h]** and **[p]**), or 24 h (**[i]** and **[q]**). The subcellular localization of the GFP fusion proteins was investigated by fluorescence microscopy (**[a]**, **[d]**, **[e]**, **[h]**, **[i]**, **[j]**, **[k]**, **[m]**, **[n]**, **[p]**, and **[q]**). 4′,6-Diamidino-2-phenylindole staining (**[b]**, **[f]**, **[l]**, and **[o]**) and differential interference contrast images (**[c]** and **[g]**) are included (**[a]** to **[c]**, **[e]** to **[g]**, **[k]** to **[l]**, and **[n]** to **[o]**; each show identical nuclei). Nuclei (nu), nuclear spots (nuSp), plastids (pl), and cytoplasmic fluorescence (cpFl) are indicated. Bars = 10 μm.

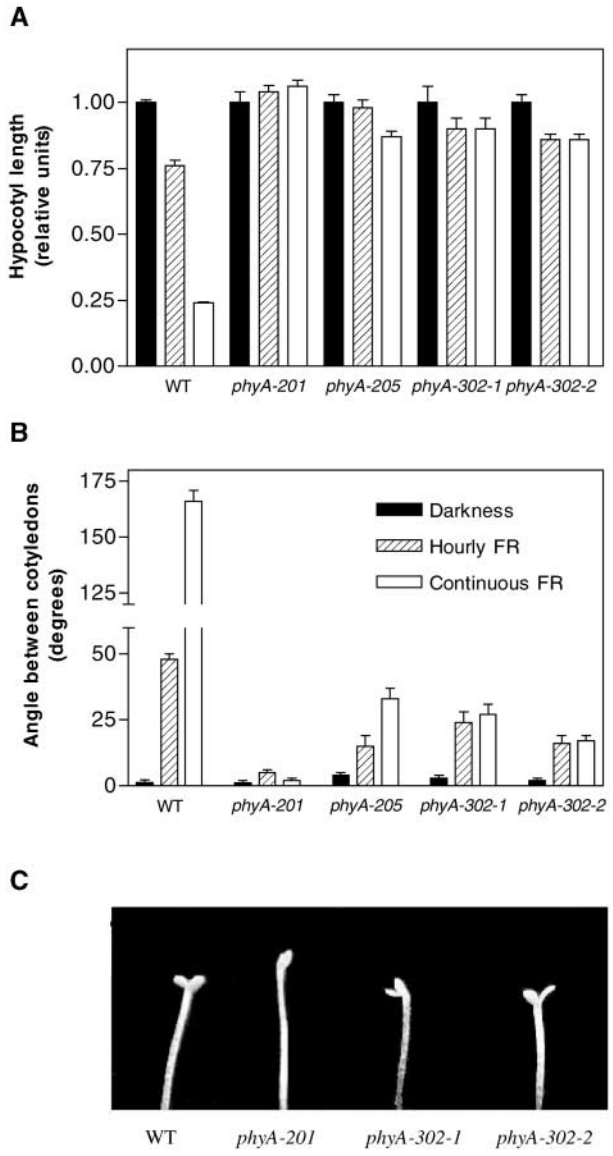


Figure 7. At Equal Total Fluence, Hourly and Continuous FR Are Equally Effective at Inhibiting Hypocotyl Growth and Promoting Cotyledon Unfolding in the *phyA-302* Mutants, whereas Continuous FR Is More Effective Than Pulsed FR in Wild-Type and *phyA-205* Seedlings.

The seedlings were grown under continuous FR of 10 $\mu mol \cdot m^{-2} \cdot s^{-1}$, hourly FR (3 min/h) of 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$, or in darkness. Data are means and SE of 12 replicate boxes. WT, wild type.

(A) Hypocotyl length relative to dark controls (symbols as in **[B]**).

(B) Angle between the cotyledons.

(C) Detail of the cotyledons under hourly pulses of FR.

greater effect on the response to pulsed FR than to continuous FR (i.e., the phenotype should be opposite to the one observed here).

The region between amino acids 730 and 821 serves as a homodimerization interface bearing two surface anti-parallel β-strands (amino acids 735 to 750 and 780 to 800) (Song, 1999). However, the *phyA-302* mutation did not affect dimerization (data not shown), which is consistent with observations in mutated phyB (Wagner and Quail, 1995). A response to continuous FR typical of phyA is mediated by chimeric phytochrome molecules bearing the N-terminal domain of phyA and the C-terminal domain of phyB (Wagner et al., 1996). This finding implies that any determinant of HIR present in the C terminus also should be found in phyB. This is the case for the Glu residue present at amino acid 777 in Arabidopsis phyA and conserved in phyB (Figure 5).

The Glu-to-Lys mutation observed in *phyA-302* mutants severely affected the fluence rate dependence of phyA action under FR (Figure 8), but the same mutation in the equivalent position of phyB did not impair fluence rate dependence under continuous R (Wagner and Quail, 1995). Thus, different processes would underlie the fluence rate dependence of phyA- and phyB-mediated responses. In accordance with this interpretation, the fluence rate dependence of phyB-mediated responses to continuous R appears to be attributable to Pfr-to-Pr dark reversion (Kretsch et al., 2000), a kinetic component that is absent for phyA in Landsberg *erecta* (Eichenberg et al., 2000).

The wild type of Arabidopsis shows little morphological response to a daily pulse of R. This treatment, however, is effective in both *phyA* null mutants and wild-type seedlings exposed daily to a few hours of continuous FR before the R pulse. These observations have led to a model in which phyA in the VLFR mode (i.e., under R) negatively regulates phyB signaling, whereas phyA in the HIR mode (i.e., under continuous FR) positively regulates phyB signaling (Cerdán et al., 1999). The *phyA-302* mutants behaved neither like the wild type nor like a *phyA* null mutant because they failed to respond to daily R pulses given alone or after a daily FR pretreatment (Figure 10). This observation is consistent with the ability of *phyA-302* to retain VLFR and its failure to exhibit HIR.

phyA-302:GFP Fusion Proteins Fail to Produce Nuclear Speckles

Both phyA and phyB are present in the cytosol in etiolated seedlings and migrate to the nucleus upon illumination, where they localize to discrete subnuclear sites, forming spots that are smaller and more numerous in the case of phyA (Kim et al., 1999; Kircher et al., 1999; Yamaguchi et al., 1999; Hisada et al., 2000). The phyA-302:GFP fusion protein showed no obvious abnormalities with regard to translocation to the nucleus but failed to produce nuclear speckles (Figure 6). This observation suggests that Glu-777 in the

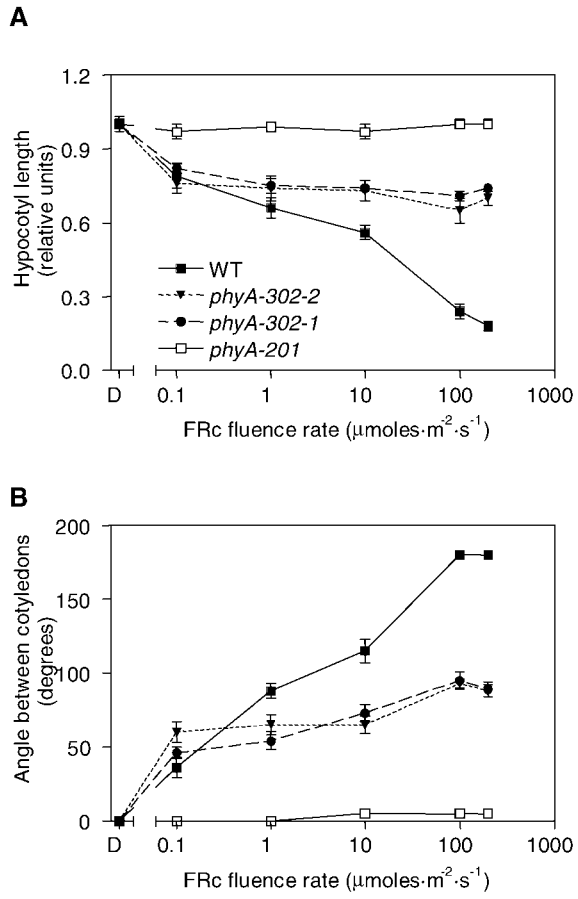


Figure 8. Weak Fluence Rate Dependence of Hypocotyl Growth Inhibition **(A)** and Cotyledon Unfolding **(B)** by Continuous FR in the *phyA-302* Mutants.

Symbols in **(B)** are as indicated in **(A)**. Data are means and SE of six replicate boxes. WT, wild type.

PAS2 domain of phyA could be part of a speckle retention signal (Kim et al., 1999).

In animal cells, many nuclear factors localize either partially or completely in distinct "bodies" or subnuclear compartments, some of which contain factors involved in the processing and transcription of RNA (Lamond and Earnshaw, 1998). In addition to phytochromes, few plant proteins are known to form nuclear spots: COP1, a nuclear repressor of photomorphogenesis (Stacey and von Arnim, 1999); cryptochromes, blue light photoreceptors (Mas et al., 2000); LAF1, a transcription factor involved in photomorphogenesis (Ballesteros et al., 2001); and Rpn6, a proteasome non-ATPase regulatory subunit that is part of complexes apparently involved in responses to environmental stresses (Peng et al., 2001).

Mutations that occur in the WD-40 repeat domain of COP1 (Stacey and von Arnim, 1999) or in Glu-777 of the PAS2 domain of phyA affect both subnuclear localization and biological activity, suggesting a functional role for spot formation. The phenotype of *phyA-302* mutants could result from either a failure of the mutated protein to colocalize with partners or impaired protein–protein interactions. Nuclear spots that contain phyA might represent foci for transcriptional complexes, a role that would be consistent with the action of phytochrome as a direct regulator of transcription factor activity (Ni et al., 1999). Failure to form such foci could impair HIR-specific transcriptional changes. Nuclear foci also might be involved in protein degradation, as speculated by Mas et al. (2000). This effect could account for the enhanced stability of the phyA-302 protein under continuous FR.

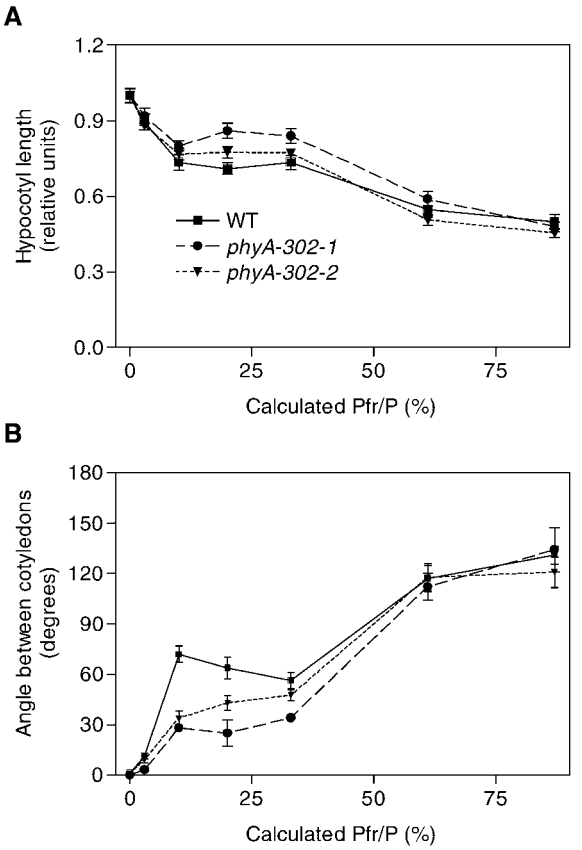


Figure 9. VLFR of phyA- and phyB-Mediated Responses of Hypocotyl Growth **(A)** and Cotyledon Unfolding **(B)** in Wild-Type and *phyA-302* Seedlings.

Seedlings were grown under hourly R/FR pulses providing the indicated calculated proportion of phytochrome in the Pfr form. The first phase is the VLFR and the second phase (Pfr/P $>$ 30%) is the phyB-mediated effect. Symbols in **(B)** are as indicated in **(A)**. Data are means and SE of 16 replicate boxes. WT, wild type.

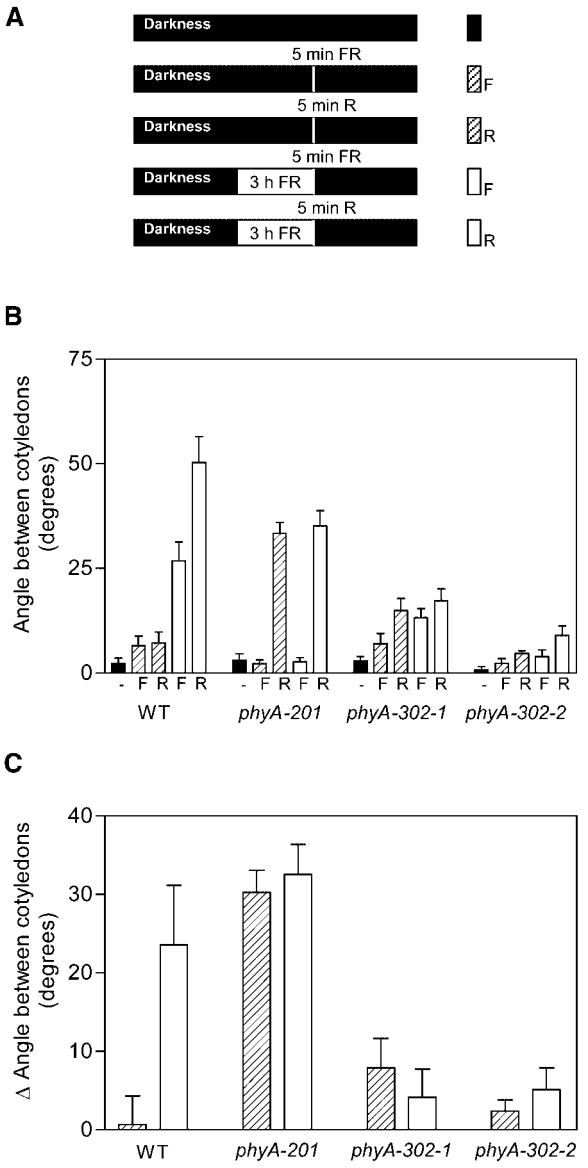


Figure 10. The *phyA-302* Mutants Retain the phyA-Mediated Negative Modulation of phyB Signaling but Fail to Amplify phyB Signaling in Response to Prolonged Exposures to FR.

(A) Protocol and symbols. Seedlings were grown in complete darkness or exposed daily to a 5-min pulse of FR (F) or R, or to 3 h of FR followed immediately by a 5-min pulse of either FR or R.

(B) Angle between the cotyledons. Data are means and SE of seven or eight replicate boxes. WT, wild type.

(C) The difference in angle between cotyledons caused by a R compared with a FR 5-min daily pulse was calculated for the seedlings exposed or not exposed to 3 h of FR.

Implications for phyA Signaling

The change from Glu (acidic side chain) to Lys (basic side chain) caused by the *phyA-302* mutations is predicted to alter the electrostatic environment of the peptide region, which could impair phyA interaction with other proteins. Actually, PAS domains often are involved in protein–protein interactions in signaling (Taylor and Zhulin, 1999). Furthermore, the mutation affects a peptide region containing Trp residues 774 and 778 (773 and 777 in oat phyA) that undergoes detectable conformational changes during phototransformation and becomes exposed preferentially in the Pfr form (Park et al., 2000). At least in the context of the full-length C-terminal domain of phyB, the Glu-to-Lys mutation at position 838 (which is equivalent to the *phyA-302* mutation) impairs the binding of PIF3 (Ni et al., 1998).

Binding of nucleoside diphosphate kinase 2 to the C-terminal domain of phyA is disrupted by the *phyA-103* mutation (Choi et al., 1999), but to the best of our knowledge, more C-terminal mutations have not been tested. The region close to the junction between the Quail box and the kinase motif (Figure 5) also could affect phyA kinase activity, as proposed recently by Park et al. (2000). However, the putatively impaired binding or activity of PIF3, nucleoside diphosphate kinase 2, or PKS1 does not offer a direct explanation for the lack of HIR in *phyA-302*. Antisense or knockout seedlings with reduced or no PIF3 (Ni et al., 1998), nucleoside diphosphate kinase 2 (Choi et al., 1999), or PKS1 (Fankhauser et al., 1999) retain full or weakly affected HIR.

Taking into account the fact that VLFR and HIR can be dissected genetically (Yanovsky et al., 1997, 2000; Luccioni et al., 2001) and at the level of target genes (Cerdán et al., 2000), it is tempting to speculate that the Glu-777 residue in the PAS2 domain is involved in HIR signaling via interaction with a partner not necessary for VLFR. Nuclear speckles could be a site for such interaction, and eventually a site for phyA destruction after signaling. There are several examples in nonplant systems in which signaling bifurcates at the receptor protein itself via specific docking sites for different signaling proteins (Pawson, 1995), creating a network of interconnected pathways that help coordinate cellular responses to the environment.

METHODS

Plant Material

Mutagenized seeds of *Arabidopsis thaliana* (ecotype Landsberg *erecta*) were purchased from Lehle Seeds (Round Rock, TX). For the mutant screening, the seeds were incubated in boxes (175 × 225 mm^2 × 45 mm in height) containing 0.8% agar for 3 days at 7°C before transfer to the specific protocol conditions (Figure 1). The wild type was Landsberg *erecta*. *phyA-201* (Nagatani et al., 1993), *phyA-205* (Reed et al., 1994), and *hy1* and *hy2* (Koornneef et al., 1980;

Davis et al., 1999; Muramoto et al., 1999; Kohchi et al., 2001) were included in some experiments for comparative purposes.

The construction of the *35S*:*PHYA*:green fluorescent protein (*GFP*) chimeric gene containing the full-length *PHYA* cDNA from Arabidopsis in the binary vector pPCV 812 and its introduction in the *phyA* null background of Arabidopsis have been described (Kim et al., 2000). To produce the *35S*:*PHYA-302*:*GFP* chimeric gene, the *phyA-302* point mutation was introduced in the full-length *PHYA* cDNA by PCR and transferred to the 35S-GFP/nopaline synthase binary expression plasmid described previously (Kircher et al., 1999; Kim et al., 2000). Transgenic plants harboring GFP constructions were produced in the *phyA-211* mutant Columbia background according to the method of Koncz et al. (1994). Several positive lines with varying levels of PHYA-302:GFP were analyzed in preliminary experiments, and the wild-type and mutant lines chosen exhibited similar intensities of GFP fluorescence.

Experimental Setting

In germination experiments, 25 seeds were sown in clear plastic boxes (40 × 33 mm^2 × 15 mm in height) on two layers of filter paper saturated with distilled water, incubated in darkness at 6°C for 3 days, and further incubated at 25°C either in darkness or exposed to different light treatments. Germinated seeds (radicle protrusion) were counted 4 days later. In hypocotyl growth and cotyledon unfolding experiments, 15 seeds of each genotype were sown as described for germination experiments. After chilling, the boxes were exposed to a red light (R) pulse to induce homogeneous germination, incubated in darkness at 25°C for 24 h, and transferred to the light or dark treatments for 3 days before measurements.

Hypocotyl length was measured to the nearest 0.5 mm with a ruler, and the largest 10 seedlings from each box (i.e., one replicate) were averaged. The angle between the cotyledons was measured with a protractor in the same seedlings used for length measurements, and the 10 values obtained per box also were averaged before statistical analysis. For anthocyanin experiments, 50 seeds were sown per box according to the procedure used for hypocotyl growth experiments. After 3 days of light treatment, the seedlings were harvested for extraction with 1 mL of 1% (w/v) HCl methanol. Absorbance at 530 nm was measured and corrected for chlorophyll absorption by subtracting 0.25 absorbance at 657 nm.

Hourly pulses of far-red light (FR; 3 min/h at 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$) and continuous FR (10 $\mu mol \cdot m^{-2} \cdot s^{-1}$) were provided by incandescent lamps in combination with a water filter, a red acetate filter, and six 2-mm-thick blue acrylic filters (Paolini 2031; La Casa del Acetato, Buenos Aires, Argentina). The calculated Pfr/P was 10%. In the experiments in which responses were plotted against calculated Pfr/P, the seedlings were exposed to 3-min hourly light pulses (10 to 50 $\mu mol \cdot m^{-2} \cdot s^{-1}$). Incandescent lamps were combined with a water filter and an RG9 filter (calculated Pfr/P of 3%; Schott, Mainz, Germany), one red plus one green acetate filter (calculated Pfr/P of 33%), or one red acetate filter (calculated Pfr/P of 61%). R (calculated Pfr/P of 87%) was provided by light-emitting diodes.

Protein Extraction and Immunoblot Analysis

Extracts were prepared from samples harvested on ice according to Martinez-García et al. (1999) and subjected to SDS-PAGE in 1.5-mm-thick, 4.5/7.5% stacking/resolving gels (Mini Protean II; Bio-Rad, Richmond, CA). Proteins were electroblotted to nitrocellulose (0.45-μm pore size; Sigma, St. Louis, MO) for 45 min in a Milliblot-SDE system (Millipore, Bedford, MA) according to the manufacturer's instructions. The remaining protein binding capacity was blocked with 1% skim milk, 50 mM Tris-Cl, and 200 mM NaCl, pH 7.4, for 30 min at 37°C. The monoclonal antibody 073D raised in mouse against *Avena* phyA (Somers et al., 1991) was kindly provided by Peter Quail (University of California, Berkeley, and U.S. Department of Agriculture Plant Gene Expression Center, Albany, CA).

The blot was incubated overnight at 7°C with 10 mL of this primary antibody at a dilution of 1:1000. After washing, the membrane was incubated with a 1:500 dilution of affinity-isolated alkaline phosphatase–conjugated antibody to mouse IgG developed in goat (Sigma). The bands were visualized by incubating the blots in 0.1 M Tris, pH 9.5, 100 mM NaCl, and 5 mM $MgCl_2$ containing 0.165 mg/mL 5-bromo-4-chloro-3-indolyl phosphate (*p*-toluidine salt) and 0.33 mg/mL nitroblue tetrazolium (both Sigma).

Phytochrome Fluorescence

Fluorescence data were obtained as described in Sineshchekov et al. (1998) from 3- to 4-day-old seedlings grown in complete darkness at 26°C on filter paper moistened with tap water. The cotyledons were cut off to eliminate protochlorophyllide interference, and 10 hypocotyls plus roots were glued with a 50:50 water:glycerol mixture to a Plexiglas plate of the sample holder. Fluorescence emission spectra at low temperature (85K) were recorded with the use of a laboratory-designed spectrofluorimeter based on two double-grating monochromators. The source of exciting light (reduced by ~100-fold with the use of neutral filters to avoid photochemical effects) and actinic light at 639 nm was a 5-mW laser diode (RLD-635; Taiwan).

For each experimental sample, three emission spectra usually were measured: (1) in the etiolated state (state 0); (2) after phototransformation induced by the saturating actinic light of Pr into the first photoproduct stable at low temperatures (lumi–R) (state 1); and (3) after Pr photoconversion into Pfr at 273K by monochromatic light at 680 nm (state 2). To obtain emission spectra of phytochrome, the spectrum of the *phyA-201 phyB-1* Arabidopsis double mutant was subtracted from the experimental spectra of the samples after normalization at 660 nm (at which phytochrome emission is negligible), and the phytochrome difference spectrum was divided by the intensity of background emission at 660 nm to correct for sample mass. The noise level during the registration of the emission spectra was 5 to 7%, and the smoothing of the spectra was performed by eye.

DNA Sequencing

Genomic DNA was isolated from plants of the wild type and of the *phyA* alleles identified here according to the procedure described by Rogers and Bendich (1988). The complete *PHYA* gene (including exons and introns) was amplified by PCR using a pair of primers designed on the basis of a previously published sequence of the gene (Dehesh et al., 1993). The PCR mixture was 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 2 mM $MgCl_2$, 200 μM each deoxynucleotide triphosphate, 25 pmol of each primer, 2 μL of the genomic DNA preparation, and 2.5 units of Taq polymerase (Gibco BRL) in a final volume of 25 μL.

The mixture was heated to 94°C for 4 min and subjected to 35 cycles of 30 s at 94°C, 30 s at 55°C, and 210 s at 72°C. The reaction products were analyzed on 0.8% agarose gels. The size of the products was ~4.8 kb. The corresponding fragments were extracted from the gels and subcloned in the pUC19 plasmid (Sambrook et al., 1989) according to standard protocols. Both strands of the cloned

phyA genes were sequenced using the recombinant plasmids as templates with an automated DNA sequencer (Alf Express II; Amersham Pharmacia) and 10 pairs of primers internal to the PCR product designed on the basis of a published sequence (Dehesh et al., 1993).

Epifluorescence and Light Microscopy

Dark-grown seedlings (3 days old) were either kept in the dark or exposed to 1, 3, 9, or 24 h of continuous FR (21 $\mu mol \cdot m^{-2} \cdot s^{-1}$). All subsequent manipulations were performed under dim green light. The upper halves of the hypocotyls were collected, transferred to glass slides, and analyzed with an Axiovert microscope (Zeiss, Oberkochem, Germany). Excitation of GFP was performed with standard fluorescein isothiocyanate filters. Representative nuclei were photographed with an Axiocam camera and Axiovision software (Zeiss). Only epidermal cells of the upper quarter of the hypocotyl were analyzed.

To minimize the nuclear import of fusion proteins induced by microscopic light, photographs of the GFP fluorescence were taken during the first minute of microscopic analysis. 4′,6-Diamidino-2-phenylindole staining of nuclei was performed by incubating the seedlings in an aqueous solution containing 10% DMSO (v/v) and 10 μg/mL 4′,6-diamidino-2-phenylindole. For optimal presentation, the images were processed using Adobe Photoshop 5.0 (Adobe Systems Europe, Edinburgh, UK).

ACKNOWLEDGMENTS

We thank Peter Quail (University of California, Berkeley) for his kind provision of the phyA monoclonal antibody and the ABRC (Ohio State University, Columbus) for seed batches. This work was supported by grants from Fondo Nacional de Ciencia y Técnica (BID 1201/OC-AR-PICT 06739), the University of Buenos Aires (TG59), and the Fundación Antorchas (A-13622/1-40) to J.J.C., Consejo Nacional de Investigaciones Científicas y Técnicas Grant 4527/96 to R.J.S., a Howard Hughes Medical Institute International Scholar Fellowship, a Hungarian Foundation for Basic Science Grant (T-02584), and a Wolfgang Paul Award to F.N., Deutsche Forschungsgemeinschaft Grant SFB 592 to E.S., and Russian Foundation for Fundamental Research Grant 99-04-48730 to V.A.S.

Received November 14, 2001; accepted March 19, 2002.

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