Phytochrome A (phyA) is a versatile plant photoreceptor that mediates responses to brief light exposures (very low fluence responses, VLFR) as well as to prolonged irradiation (high irradiance responses, HIR). We identified the phyA-303 mutant allele of *Arabidopsis thaliana* bearing an R384K substitution in the GAF subdomain of the N-terminal half of phyA. *phyA-303* showed reduced phyA spectral activity, almost normal VLFR, and severely impaired HIR. Recombinant N-terminal half of PHYA bearing the *phyA-303* mutation showed poor incorporation of chromophore in *vitro*, despite the predicted relatively long distance (>13 Å) between the mutation and the closest ring of the chromophore. Fusion proteins bearing the N-terminal domain of oat phyA, β-glucuronidase, green fluorescent protein, and a nuclear localization signal showed physiological activity in darkness and mediated VLFR but not HIR. At equal protein levels, the *phyA-303* mutation caused slightly less activity than the fusions containing the wild-type sequence. Taken together, these studies highlight the role of the N-terminal domain of phyA in signaling and of distant residues of the GAF subdomain in the regulation of phytochrome bilin-lyase activity.

Plant photoreceptors monitor the cues provided by the light environment and trigger modifications that adjust growth and development to the prevailing conditions (1, 2). Phytochromes are sensors of red and far-red (FR) light that bind an open chain tetrapyrrrole chromophore (3, 4). *Arabidopsis thaliana* bears five phytochrome apoprotein genes (*PHYA* through *PHYE*) (5).

*phyA* is a versatile photoreceptor that induces seed germination in response to brief exposures to light (6, 7), which the seeds may experience during soil labor. *phyA* is also required to perceive the prolonged exposures to FR that the seedlings experience when they emerge from the soil under dense plant canopies (8). These two photoresponses mediated by *phyA* are of the types called very low fluence responses (VLFR) and high irradiance responses (HIR), respectively (9). Some physiological processes (e.g. inhibition of hypocotyl growth, unfolding of the cotyledons) exhibit both VLFR and HIR as two discrete phases of response, where VLFR saturates with infrequent FR pulses and HIR requires very frequent or continuous FR (10). HIR requires cis-acting elements at target gene promoters (11) and domains of the phyA molecule itself (12) that are dispensable for VLFR.

The N-terminal domain of phytochromes bears the chromophore attachment site (13) and provides differential spectral selectivity to phyA (more active under FR than red light) compared with phyB (more active under red light than FR) (14). The C-terminal domain contains a histidine kinase-related sequence motif that is able to mediate phosphorylation (15, 16), two PAS (domain named after Per, Arnt, and Sim) motifs important for downstream signaling in the context of the full molecule (17), and residues necessary for dimerization (18, 19) and targeting to the nucleus upon light activation (20, 21). Two recent findings have focused attention on the N-terminal domain of phytochromes. First, the N-terminal domain of phyB fused to green fluorescent protein (GFP), β-glucuronidase (GUS), and a nuclear localization signal (NLS) is physiologically active (21, 22). This result contradicts the previous extended consensus that the C-terminal domain of phytochromes was responsible for downstream signaling. Second, Wagner et al. (23) have determined the crystal structure of the conserved N terminus photosensory core of a bacteriophytochrome from *Deinococcus radiodurans* (*DrpBpH*).

The results presented here provide insight into the physio-

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†The on-line version of this article (available at http://www.jbc.org) contains supplemental Table S1.

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The abbreviations used are: FR, far-red light; *DrpBpH*, bacteriophytochrome from *D. radiodurans*; GAF, domain named after cyclic GMP, Adenylyl cyclase and PhIA; GFP, green fluorescent protein; GUS, β-glucuronidase; HIR, high irradiance response; lumi-R, red-shifted transient produced upon excitation of P₇₅; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; NLS, nuclear localization signal; PCB, phycocyanobilin; P₇₅, red and far-red light-absorbing forms of phytochrome; phyA, phyB, holo-protein of phytochromes A and B, respectively; PHYA, PHYB, apoprotein of phytochromes A and B, respectively; PHYA, phytochrome A encoding gene; phyA-65-WT, 65-kDa N terminus domain of wild-type oat phytochrome; phyA-65-R384K, 65-kDa N terminus domain of oat phytochrome with arginine 384 mutated for lysine; VLFR, very low fluence response; WT, wild type.
logical function and biochemical activity of the N-terminal domain of phyA. By using a forward genetics approach we have identified a mutation of the PHYA gene that impairs chromophore binding despite its relative distant position to the attachment site within the GAF domain. We also report on the biological activity of an N-terminal domain fragment of phyA and the impact of the aforementioned mutation on this activity.

EXPERIMENTAL PROCEDURES

Mutant Screening—Ethylmethane sulfonate mutagenized seeds of *A. thaliana* (accession Landsberg erecta; Lehle Seeds, Round Rock, TX) were sown in clear plastic boxes (175 × 225 mm² × 45 mm in height) containing 0.8% agar, incubated 3 days in darkness at 7 °C, and then transferred to continuous FR for 4 days (12).

Cloning and Sequencing of the phyA-303 Allele—Total mRNA was obtained with RNA Easy Plant kit (Qiagen) and used as a template for reverse transcription PCR to produce PHYA cDNA (primers are described in supplemental Table S1). The fragment was cloned into pUC19 vector and sequenced at Automatic DNA Isolation and Sequencing (Max-Planck-Institut für Züchtungsforschung).

Physiological Characterization—The wild-type (WT) strain and the phyA-201 (24), phyA-205 (25), hy1 and hy2 mutants (26–28) are all in the Landsberg erecta background. Fifteen seeds of each genotype were sown in clear plastic boxes and exposed to the different light treatments as described (12). Interference filters were used to provide light at 700, 710, 720 nm (CVI Laser Corp.), or 743 nm (Mainz Schott).

Hypocotyl length was measured with a ruler, and the angle between the cotyledons was measured with a protractor. Data corresponding to the 10 tallest seedlings from each box were averaged (one replicate) (12).

In the experiments involving chromophore feeding, 3 μl of biliverdin IXα solution of the indicated concentrations were added to each seed under green light immediately after the red light exposure used to induce germination. The boxes were kept in darkness at 23 °C for 20 h and then placed in darkness or under the various light treatments for 3 days before measurements.

Low Temperature in Vivo Phytochrome Fluorescence—Fluorescence emission spectra at low temperature (85 K) were recorded in the hypocotyl plus root of 4-day-old seedlings grown in darkness with a laboratory-designed spectrofluorometer based on two double grating monochromators as described (12).

PHYA Protein Blots—Aliquots of plant extracts (29) containing ~20 μg of crude protein were separated on SDS-polyacrylamide gels and blotted to polyvinylidene difluoride membranes (Millipore) for 1 h at ~200 mA. Immunodetection of *Arabidopsis* PHYA was performed using monoclonal antibody 073D (kindly provided by Richard Vierstra, University of Wisconsin). Detection of recombinant proteins and oat PHYA-65-GFP-GUS-NLS fusion proteins from transgenic plants was performed using monoclonal antibody Oat25 (kindly provided by Lee Pratt, University of Georgia). In all cases the blot was incubated overnight at 4 °C with the corresponding primary antibody. After washing, the membrane was incubated with 1:1000 dilution of horseradish peroxidase-conjugated antibody to mouse IgG (Sigma) for 1–2 h at room temperature. Bands were visualized by the chemoluminescent method. Band intensity was quantified with the Scion Image Software.

FIGURE 1. Identification of a novel phyA mutant allele. A, the mutant (Mut) shows partial induction of seed germination by FR. B, mutant seedlings show severely impaired hypocotyl growth and cotyledon unfolding responses of the seedling to FR and normal responses to red light (30 μeinstein m⁻² s⁻¹). C, sequencing the PHYA gene identified an R384K substitution. The mutant was named phyA-303. Physiological data are means and S.E. of three to six replicate boxes.
Heterologous Expression of PHYA Gene, Mutagenesis, and Protein Purification—For yeast expression the N-terminal domain of oat PHYA (nucleotides 1–1795, which corresponds to residues 1–595) was cloned into a derivative from the pHILD2 vector (Invitrogen), placing a His6 tail at the C-terminal end of the sequence. The R384K mutation was introduced by site-directed mutagenesis with the QuikChange Stratagene kit. The presence of the mutation was confirmed by sequencing. Plasmids were introduced into Pichia pastoris GS115, and expression was carried out according to the manufacturer’s instructions (Pichia expression kit; Invitrogen). For expression in bacterial host, the N-terminal cDNA from oat PHYA was cloned into pME8 vector. The mutation was produced as in the yeast system. Generation of the reverted gene was achieved by site-directed mutagenesis. Expression was accomplished in Escherichia coli Top10F’ strain (1-liter culture). For yeast and bacteria, cells were disrupted at liquid N2 temperature using an Ultra-Turrax, and the protein solution was clarified by ultracentrifugation at 14,000 rpm, 4 °C. The supernatant was used directly for chromophore assembly experiments. Detection of apoprotein was also checked in protein blots. His-tagged proteins were purified over the BD TALON™ metal affinity resin (Clontech Laboratories, Inc., Palo Alto, CA). Elution was carried out using imidazol, and buffer changes were performed by either dialysis with 10 K dialysis frames (Slide-A-Lyzer; Pierce) or by gel filtration using the PD-10 columns (Amersham Biosciences). All the primers are described in supplemental Table S1.

Chromophore Preparation—Phycocyanobilin (PCB) was extracted from Spirulina platensis as described (32). Biliverdin IXα was derived from bilirubin by oxidation (33) and was purified by high pressure liquid chromatography with the column Inertsil-3-C18, 7.6 × 250 mm and solvent EtOH:MeOH:Ace tone:H2O (20:20:20:40). Fractions were lyophilized and stored at −70 °C in darkness.

Plasmid Construction and Plant Transformation—Construction of the PHYA-65-WT-GFP-GUS-NLS chimeric gene was performed in several steps fusing all the four genes. The nuclear localization signal from SV40 was obtained by annealing of oligonucleotides (supplemental Table S1). The fragment encoding the N-terminal domain of PHYA from oat was amplified by PCR and fused to the sequences encoding the GFP-GUS-NLS fragment. The chimeric gene was generated in pBSKII and subcloned into pCHF3 vector under the 35S promoter. To generate the PHYA-65-R384K-GFP-GUS-NLS fusion, the fragment of the WT PHYA cDNA was replaced by the omt-mutated version. A. thaliana phyA-201 was transformed as described (34). Transformed plants were selected against 50 μg/ml kanamycin, and T2 seeds were tested for a 3:1 segregation of kanamycin resistance. T3 seeds of positive T2 plants were used for further experiments.

Confocal Microscopy—Transgenic seedlings were grown on agar (0.8% w/v) plates for 3 days in the dark. Etiolated seedlings were kept in the dark or exposed to either white light or FR. All subsequent manipulations were performed under green safelight. To visualize nuclei and cell walls, seedlings were stained with 0.1 μM aqueous solution of propidium iodide for 5–10 min (21). Fluorescence of GFP and propidium iodide was visualized by a confocal laser-scanning microscope (Zeiss LSM5, Pascal) with standard fluorescein isothiocyanate (GFP) and tetramethylrhodamine isothiocyanate filters (propidium iodide). Images
corresponding to dark were taken within the first minute of microscope observation. Similar results were obtained with hypocotyl and root samples. At least 10 seedlings were analyzed for each genotype, and representative images were processed using Adobe Photoshop 8.0 software.

RESULTS

Novel phyA Mutant Allele—To obtain mutants impaired in the HIR but retaining a significant VLFR we used mutagenized seeds of Arabidopsis thaliana and selected seedlings with long hypocotyl and poorly opened cotyledons under continuous FR (typical of poor HIR) produced by seeds that germinated under FR (typical of normal VLFR) (12). The mutant that we describe here was confirmed in the next generation by its ability to germinate under FR and its impaired hypocotyl growth and cotyledon angle responses under FR (Fig. 1A). No differences between the WT and the mutant were observed in darkness or under red light (Fig. 1B). The latter excluded mutations at loci involved in the synthesis of the chromophore, which are predicted to affect all phytochromes and therefore not only the response to FR but also to red light (see chromophore synthesis mutants hy1 and hy2 in Fig. 1B) (27, 28, 35). The null phyA-201 mutant failed to complement the mutant selected here (e.g. cotyledon angle under FR was <10° for the F1 generation of their cross), suggesting that the PHYA gene itself was affected. The PHYA gene was cloned and sequenced completely from two independent plants. A single base pair change (G-1337 to A) was observed, which resulted in the R384K substitution (Fig. 1C). The mutation falls in the GAF domain of PHYA, 61 amino acid residues away from the chromophore-bearing cysteine residue. The allele obtained here had not been reported earlier and was named phyA-303.

Physiological Characterization—For inhibition of hypocotyl growth and promotion of cotyledon unfolding, the VLFR saturates with infrequent FR pulses (e.g. one pulse every hour), whereas HIR requires very frequent or continuous excitation with FR (10). In the phyA-303 mutant, the VLFR (difference between hourly FR and darkness) of hypocotyl growth and cotyledon unfolding was close to the WT levels, but the HIR (difference between the effects of continuous and hourly FR) was severely impaired (Fig. 2, A and C). Another distinctive feature of the HIR is its strong fluence rate dependence (9). The phyA-303 mutant showed very weak fluence rate dependence (Fig. 2, B and D) but normal wavelength dependence of the phyA-mediated response (Fig. 2E).

Spectral Activity and Protein Abundance of phyA in phyA-303—Low temperature phytochrome fluorescence was severely reduced in dark-grown seedlings of phyA-303. Subtraction of the residual fluorescence signal observed in a null allele of phyA (i.e. the signal accounted for mainly by phyB) indicates that the phyA signal in the mutant is less than 0.2 of the WT signal (Fig. 3A). This result is consistent with the ΔΔ absorbance signal observed in seedling extracts (WT, 0.0048; phyA-303, 0.0019; null mutant phyA-201, 0.0010; i.e. the phyA-303 mutant retains 0.2 of the WT signal). The residual phytochrome photochemical activity in the mutant was qualitatively similar to that observed in the WT in terms of Pr-lumi-R photoequilibrium (Fig. 3B) and fluorescence spectrum (Fig. 3C). Immunochemically detected levels of PHYA were also reduced in the phyA-303, but this effect appears not as intense as the reduction in spectral activity (Fig. 3D).

Because the phenotype of the phyA-303 mutant is observed under FR and not in darkness, we investigated whether the differences in PHYA protein levels were larger under FR due to increased instability of the mutated protein. Seedlings were...
The residue phytochrome was a stronger reduction in low temperature used for FR (Fig. 4, A) and FR-irradiated samples of PHYA-65-WT, PHYA-65-R384K, and PHYA-65-R384K to R384K obtained in E. coli. D, absorption spectrum of PHYA-65-WT and PHYA-65-R384K with or without PCB and PCB alone. Insets, immunoblots with Oat25 antibody to demonstrate equal amounts of protein used in the assay.

grown for 3 days in darkness and then exposed to continuous FR for 7 h. Exposure of WT seedlings to FR caused a small but detectable decrease in protein levels (10). The response of the phyA-303 mutant was not proportionally larger than that observed in the WT (Fig. 3E).

**Mutation R384K Impairs Chromophore Incorporation**—The phyA-303 mutant showed a stronger reduction in low temperature fluorescence signal than in protein levels (Fig. 3). Because P_lumi-R photoequilibrium of the residual phytochrome was normal (Fig. 3B), one might propose that the mutation could reduce the efficiency of chromophore incorporation. To test this hypothesis in vitro we expressed WT and R384K mutated PHYA gene of Avena sativa, which for practical reasons is normally used for in vitro studies (16, 36–38), in two heterologous systems (E. coli or P. pastoris). We expressed the first 595 amino acids of oat PHYA (PHYA-65) because the spectroscopical features of this domain are almost identical to those of full-length phytochrome and the truncated protein is expressed in higher yields and shows an improved stability (39).

When incubated with PCB, PHYA-65-WT yielded typical spectrophotometric features with maximum absorbance differences at 654 and 713 nm between samples irradiated with red or FR (Fig. 4, A and C). After incubation of an equal amount of the mutated PHYA-65-R384K with PCB, the Δ absorbance signal was weak (Fig. 4, A and C) and did not increase even after overnight incubation (data not shown), thus precluding kinetics analyses (40). The presence and integrity of the WT and mutant PHYA were confirmed by MALDI-TOF mass spectrometry of the purified recombinant proteins (Fig. 4B). Although weak, the residual signal showed normal parameters (Fig. 4C). As a control experiment, WT PHYA was reconstituted from PHYA-65-R384K by site-directed mutagenesis to regenerate arginine. The regenerated WT PHYA was identical to the originally recombinant WT PHYA in terms of spectral behavior (Fig. 4C) and kinetics of chromophore incorporation (data not shown).

The primary defect of PHYA-65-R384K is in chromophore incorporation rather than in phototransformation. The absorption spectrum of PHYA-65-R384K plus chromophore was similar to that of the chromophore alone (Fig. 4D), indicating that PHYA-65-R384K fails to normally incorporate the chromophore and change its environment and spectral properties. PHYA-65-WT incorporated the chromophore and showed a maximum shift to 654 nm compared with free chromophore.

**Feeding with Biliverdin IXα Partially Rescues the phyA-303 Mutant**—To investigate whether chromophore incorporation in vivo was limiting for the physiological output, we cultivated WT and phyA-303 seedlings on agar supplemented with different doses of the chromophore precursor biliverdin IXα. The rationale behind this experiment was that if the inefficient incorporation of chromophore caused reduced responses to FR, increasing the amount of available chromophore might probably alleviate this defect. This expectation was met by the data (Fig. 5). In seedlings grown under continuous FR, addition of biliverdin IXα had no effects on the WT or the phyA-303 mutant. The hy1 (35) and partially rescued the phyA-303 mutant. The reduced protein levels observed in phyA-303 (Fig. 3D) could account for the failure of biliverdin IXα to fully restore a WT response to continuous FR. Addition of biliverdin IXα had no effect in phyA-303 seedlings grown under hourly pulses of FR (data not shown).

**Physiological Output of Oat PHYA Amino-terminal Domain**—The amino-terminal domain of PHYB is sufficient for signaling when fused to GFP, GUS, and NLS and expressed in a phyB null background (21, 22). Because our in vitro data had been
N-terminal Domain of Phytochrome A

A

| Darkness          | White light          |
|-------------------|----------------------|
| PHYA-65-WT        |                      |
| PHYA-65-R384K     |                      |

B

![Graph showing GUS activity relative to dark control for PHYA-65-WT and PHYA-65-R384K under Darkness and 3 h Red light conditions.]

C

![Graph showing hypocotyl length/mm against Log GUS Activity for PHYA-65-WT and PHYA-65-R384K.]

D

![Images showing PHYA-65-WT and phyA-201 growth under different light conditions.]

E

![Graph showing hypocotyl length relative to dark control for PHYA-65-WT and PHYA-65-R384K.]

F

![Graph showing hypocotyl length relative to dark control for different conditions.]

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obtained with the amino-terminal domain of oat WT and mutated PHYA (amino acids 1–595), we decided to evaluate the in vivo performance of the oat PHYA N-terminal domain fused to GFP, GUS, and NLS in a phyA-null mutant background in A. thaliana. Previous studies demonstrating activity of the N terminus had been done with phyB (21, 22); thus, an additional interest of this experiment was to test whether the N terminus of phyA is also sufficient for downstream signaling.

We obtained five independent transgenic lines homozygous for the PHYA transgene fusion (PHYA-65-WT lines) and four lines for the phyA-303 transgene fusion (PHYA-65-R384K lines). Taking advantage of the presence of GFP we investigated the cellular localization of the fusion proteins by confocal microscopy. Both PHYA-65-WT and PHYA-65-R384K showed nuclear expression in darkness and under illumination (Fig. 6A). The abundance of these fusion proteins measured by their GUS activity remained unaffected by the exposure of dark-grown seedlings to red light (Fig. 6B).

The physiological output of the different lines was plotted against their GUS activity driven by the fusion proteins. To our surprise, the length of the hypocotyl observed in independent transgenic lines grown in full darkness was inversely related to the GUS activity. This effect was somewhat larger for PHYA-65-WT than for PHYA-65-R384K (Fig. 6C). The two lines expressing the highest levels of GUS also showed unfolding of the cotyledons in darkness (Fig. 6D). The seedlings grown under continuous FR showed only a weak inhibition of hypocotyl growth by the light treatment, which reached a maximum of ~20% for the lines expressing the highest levels of GUS (Fig. 6E). Cotyledon unfolding retained dark control levels (e.g. mean ± S.E., cotyledon unfolding (in degrees) darkness, 70 ± 9; continuous FR, 65 ± 3). The PHYA-65-R384K lines had slightly less response than the PHYA-65-WT lines. Continuous and hourly pulses of FR were similarly effective to inhibit hypocotyl growth (Fig. 6F), indicating that the PHYA-65-WT or PHYA-65-R384K fusions were unable to produce a HIR.

The observed effect of PHYA-65-WT and PHYA-65-R384K on dark-grown seedlings could be accounted for by the action of the nuclear-localized protein fusions in their Pr form or by the Prn form of these molecules established by a previous light treatment, which reached a maximum of 80% for the lines expressing the highest levels of GUS (Fig. 6G). The phyA-303 mutant showed poor bilin incorporation in vitro (Fig. 4) even after prolonged incubation. The physiological phenotype could therefore be accounted for, at least in principle, by a combination of less apoprotein and poor chromophore attachment. In accordance with the latter concept, addition of chromophore to the growth medium (predicted to alleviate its inefficient incorporation rate) partially restored the phenotype under continuous FR (Fig. 5) and had no effects under pulsed FR. Therefore, the HIR appears to require a higher threshold of photochemically active phyA levels, but residual phyA has normal phototransformation parameters (Fig. 3) and physiological activity (Fig. 2D) (41). Furthermore, recombinant protein bearing the phyA-303 mutation showed poor bilin incorporation in vitro (42). The expression of PHYA-65-GFP-GUS-NLS fusions in a phyA null background restored a response to pulses of FR, which was not enhanced by continuous FR (Fig. 6, E and F).

The PHYA apoprotein catalyzes the formation of a thioether link with a bilin chromophore (42). The bilin lyase activity has been assigned to the GAF domain (43). Little is known, however, about the specific role played by different residues in the dual function of this domain as substrate and catalytic regulator. Slow or poor in vitro chromophore incorporation has been reported for proteins mutated close to the cysteine

FIGURE 6. Nuclear localization and physiological activity of PHYA-65-WT-GFP-GUS-NLS and PHYA-65-R384K-GFP-GUS-NLS fusion proteins expressed in A. thaliana seedlings of the phyA-207 background. A, fusion proteins are nuclear localized in darkness and upon exposure to light. Confocal images of hypocotyl cells from 4-day-old etiolated seedlings showing GFP or propidium iodide (red) fluorescence. B, fusion proteins are stable in the light. Immunoblots with Oat25 antibody against PHYA (left, signal only detectable for PHYA-65-WT and GUS activity (right, two independent transgenic lines per construct) from seedlings grown in darkness for 3 days and either left in darkness or exposed to red light for 3 h before harvest. C, hypocotyl length in dark-grown seedlings is inversely related to the abundance of fusion proteins measured by the GUS activity of independent transgenic lines. Data are means and S.E. (whenever larger than the symbols) of ten boxes for hypocotyl length and six boxes for GUS activity. D, the PHYA-65-WT lines with highest GUS activity show partial cotyledon unfolding in darkness. Representative seedling and average data (and S.E. from ten replicate boxes) from one of the two PHYA-65-WT lines with highest GUS activity. Seedlings of the phyA-207 mutant that do not express the fusion protein (photograph) or of the WT (not shown) exhibit no cotyledon unfolding in darkness. E, hourly pulses of FR (3 min, 200 μeinstein m⁻² s⁻¹) cause hypocotyl growth inhibition. Hypocotyl length under pulsed FR is expressed relative to dark controls (shown in panel C). Data as in panel C. F, continuous FR (10 μeinstein m⁻² s⁻¹) is not more effective than hourly FR. Data correspond to the three lines with highest GUS activity (see panels C and E).
N-terminal Domain of Phytochrome A

323, between amino acids 309 and 326 (43–45). Based on the recently published crystal structure of the photosensory core of DrBphP (23), the latter region involves residues in close contact with the chromophore.

The phyA-303 mutation affects a residue conserved among plant and bacterial phytochromes but poorly conserved among the generic sequence of the GAF domains (43). phyA-303 has a severe effect on chromophore incorporation despite the conservative nature of the Arg to Lys substitution in terms of amino acid charge and the lack of any impact on secondary structure. There are examples of substitutions that do not affect charge and yet have functional consequences. The R174K mutation of the PDE5-GAFa domain reduces its ability to bind cGMP in vitro because the substitution is located in a domain that interacts with cGMP (46) and is also predicted to alter hydrogen bonds. However, according to the DrBphP crystal structure, the R384K substitution (Arg-302 in DrBphP) is in an outer α-helix of the domain, more than 13 Å away from the chromophore (C ring of the open chain tetrapyrrole chromophore). A comparable conclusion (20 Å distance from the position equivalent to chromophore attachment site) can be reached by using homology modeling taking the crystal structure of the GAF domain of YKG9 (Protein Data Bank 1FSM) as template (46). Therefore, no direct interaction of the mutated site with the chromophore seems likely. The position equivalent to phyA Arg-384 in DrBphP (Arg-302) forms hydrogen bonds with both Lys-297 (phyA Val-379) and Glu-127 (phyA Glu-183). Modeling the introduction of the Arg to Lys mutation disrupts the interaction between the guanidinium group of Arg-302 and the oxygen carbonyl of Lys-297. Modeling to other rotamers of Lys without moving the backbone restores both hydrogen bonds through the single N atom present in the side chain of Lys. However, a weakening of these interactions by the Arg to Lys mutation disrupts the interaction rather than an effect of the Pr form. Finally, we had previously observed that in the WT irradiation protocols that cause seed exposure to light perceived by phyA and/or phyB in the hours immediately prior to germination (radicle protrusion) cause inhibition of hypocotyl extension in dark-grown seedlings (51). In the transgenics bearing PHYA-65-WT or PHYA-65-R384K fusions, the need for prolonged or delayed seed irradiation observed in the WT could be circumvented by the presence of a stable pool of N-terminal phyA.

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