Light-Independent Phytochrome Signaling Mediated by Dominant GAF Domain Tyrosine Mutants of Arabidopsis Phytochromes in Transgenic Plants

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The photoreversibility of plant phytochromes enables continuous surveillance of the ambient light environment. Through expression of profluorescent, photoinsensitive Tyr-to-His mutant alleles of Arabidopsis thaliana phytochrome B (PHYBY276H) and Arabidopsis phytochrome A (PHYAY242H) in transgenic Arabidopsis plants, we demonstrate that photoconversion is not a prerequisite for phytochrome signaling. PHYBY276H-expressing plants exhibit chromophore-dependent constitutive photomorphogenesis, light-independent phBY276H nuclear localization, constitutive activation of genes normally repressed in darkness, and light-insensitive seed germination. Fluence rate analyses of transgenic plants expressing PHYBY276H, PHYAY242H, and other YGAF mutant alleles of PHYB demonstrate that a range of altered light-signaling activities are associated with mutation of this residue. We conclude that the universally conserved GAF domain Tyr residue, with which the bilin chromophore is intimately associated, performs a critical role in coupling light perception to signal transduction by plant phytochromes.

INTRODUCTION

Life forms living at or near the earth’s surface possess a diverse array of photosensory receptors that trigger cellular signaling cascades to regulate adaptive biological responses to light at the organismal level (Chen et al., 2004; Franklin and Whitelam, 2004; Schäfer and Nagy, 2005). The cohort of photoreceptors is most complex in photosynthetic organisms that must cope with the dual threat of too much or too little light (Falciatore and Bowler, 2005; Lariguet and Dunand, 2005). Land plants possess red/far-red light (R/FR)–absorbing phytochromes (Rockwell et al., 2006), UV-A/blue light (B)–sensing crytochromes and phototropins (Briggs and Christie, 2002; Lin and Todo, 2005), and UV-B photoreceptors (Ballare´, 2003) for perception of the full range of the solar light spectrum (Franklin et al., 2005; Wang, 2005). In flowering plants, phytochromes are encoded by a small family of genes that have arisen by repeated gene duplication of a eukaryotic phytochrome progenitor during the course of evolution (Mathews, 2006). The phytochrome family in the model dicot species Arabidopsis thaliana consists of five genes denoted PHYA-E (Sharrock and Quail, 1989; Clack et al., 1994), while monocot species appear only to possess representatives of the PHYA-C families (Sawers et al., 2005). Genetic analyses have facilitated characterization of the functions of individual phytochrome family members in plants, and null mutants of all five Arabidopsis phytochromes have been isolated. Light-labile phyAs appear primarily responsible for sensing very low light fluences and for adaptation to FR-enriched shade environments (reviewed in Casal et al., 1997; Sineshchekov, 2004), while the light-stable phytochromes phyB-E mediate the classic R/FR photoreversible responses and adaptation to elevated fluence rates of R (Reed et al., 1993; Franklin et al., 2003a, 2003b). The overlapping and distinct functional roles of phytochromes are further underscored by the recent observation that phyA can also mediate irradiance-dependent responses to R (Franklin et al., 2007).

Our current understanding of the structure and molecular function of phytochromes has greatly benefited from the isolation of photoreceptor mutants (reviewed in Quail, 1997). Although the majority of phytochrome mutants so far identified have been loss-of-function alleles (for a recent list of phy mutant alleles, see Supplemental Table 2 in Rockwell et al. (2006)), alleles of phytochromes with increased sensitivity to light and weak constitutive photomorphogenesis (COP) phenotypes have also been reported (Kretsch et al., 2000; Weller et al., 2004; Dieterle et al., 2005; Mateos et al., 2006). The latter are still dependent on light for function, so their functions cannot be analyzed without activating other photobiological processes. Despite robust screens for their identification, constitutively active mutants of phytochromes have not yet been isolated. Constitutively active alleles would thus be invaluable tools for characterizing the unique molecular functions of individual phytochromes.

This investigation reports the unexpected discovery of a new class of plant phytochrome mutants that exhibit light-independent signaling activity. Here, we document that dominant gain-of-function activity is caused by mutation of a conserved GAF domain Tyr residue (YGAF) previously shown to be critical for photoactivation of the cyanobacterial phytochrome Cph1 (Fischer and Lagarias, 2004; Fischer et al., 2005). Mutation of this Tyr also strongly inhibits photoactivation of plant phytochromes (Fischer et al., 2005), so we expected that YGAF mutants of Arabidopsis...
phyA and phyB would exhibit reduced light-signaling activity. To the contrary, the poorly photoactive, profluorescent Tyr276His allele of PHYB (PHYB<sup>Y276H</sup>) not only complements phyB mutants as effectively as the wild-type PHYB allele but leads to COP development, light-independent activation of gene expression, and general R/FR insensitivity. Transgenic plants expressing the Tyr276His allele of PHYA (PHYA<sup>Y276H</sup>) also display weak COP development and dominant-negative, phyA-deficient phenotypes under continuous FR. Comparative analyses of transgenic plants expressing other Y<sup>GAF</sup> mutant alleles of PHYB demonstrate a critical role of this Tyr in coupling light perception with downstream signaling.

RESULTS

**PHYB<sup>Y276H</sup> Is a Dominant, Biologically Active Phytochrome B Allele That Can Complement PhyB-Deficient Arabidopsis Mutants**

To investigate the biological activity of Y<sup>GAF</sup> mutants of *Arabidopsis* phytochromes, we prepared transgenic lines expressing cDNA and genomic constructs of wild-type and Y<sup>GAF</sup> mutant alleles of PHYA and PHYB under control of the cauliflower mosaic virus 35S promoter or the native PHY promoters (see Supplemental Figure 1 online for T-DNA maps and Supplemental Table 1 online for transgenic lines constructed). Multiple transformants were selected in various genetic backgrounds (typically more than five), from which multiple homozygous T3 lines were generated. Phenotypic data for representative plant lines for each construct are discussed below, and detailed phenotypic data for all lines are compiled in the supplemental data online.

Our initial studies focused on PHYB<sup>Y276H</sup>-expressing transgenic plant lines expressing the cauliflower mosaic virus 35S promoter–driven cDNA construct. Since overexpression of wild-type PHYB confers enhanced white (W) and R light sensitivity to transgenic plants (Wagner et al., 1991), we compared light-grown wild-type PHYB- and PHYB<sup>Y276H</sup>-expressing transgenic plants in both Landsberg erecta (Ler) wild-type and phyB-5 null mutant backgrounds (Figures 1A and 1B). Despite the poor photoconvertibility of phyB<sup>Y276H</sup> (Fischer et al., 2005; see Supplemental Figure 2 online), the apparent light-exaggerated phenotypes of transgenic plants overexpressing PHYB<sup>Y276H</sup> were indistinguishable from those of plants overexpressing the wild-type PHYB allele. Hypocotyl and internode lengths of PHYB<sup>Y276H</sup>-expressing transgenic plants were considerably shorter than those of untransformed Ler wild-type plants (see Supplemental Figure 3 online), and PHYB<sup>Y276H</sup> plants possessed smaller rosettes under continuous white light (data not shown). PHYB<sup>Y276H</sup> overexpression also rescued the characteristic elongated phenotype of phyB null mutants as effectively as both the wild-type PHYB allele and a previously described wild-type PHYB-green fluorescent protein (GFP) chimera (Yamaguchi et al., 1999; Figure 1). These results indicate that phyB<sup>Y276H</sup> is biologically active in light-grown plants.

We next compared the phenotypes of PHYB<sup>Y276H</sup>- and PHYB-expressing transgenic lines grown under continuous R light. R is the optimal light quality for conversion of the red-absorbing Pr form of phytochrome to the FR-absorbing, signaling-active Pfr form (Furuya and Schäfer, 1996). Both cDNA and genomic constructs were introduced into Ler wild-type, phyB single mutant and phyA phyB double mutant backgrounds. As shown in Figure 1C and Supplemental Figure 3A online, overexpression of PHYB<sup>Y276H</sup>, wild-type PHYB, or PHYB-GFP alleles all strongly inhibited hypocotyl growth under R regardless of the genetic background. The elongated hypocotyl phenotypes of R-grown phyB and phyA phyB seedlings were strongly suppressed by expression of the genomic PHYB<sup>Y276H</sup> allele despite the near wild-type level of PHYB protein production (Figure 1D). Indeed, phyB<sup>Y276H</sup> appeared more active than wild-type phyB as evidenced by the greater suppression of hypocotyl growth by PHYB<sup>Y276H</sup> compared with the wild-type PHYB allele in the phyA phyB double mutant background. Taken together, these results indicate that PHYB<sup>Y276H</sup> encodes a functional phytochrome.

**Dark-Grown PHYB<sup>Y276H</sup>-Expressing Transgenic Plants Exhibit COP**

Flowering plants grown in darkness etiolate, undertaking an adaptive developmental program characterized by rapid hypocotyl mesocotyl epicotyl elongation growth, repression of hook opening, cotyledon/leaf expansion, and altered plastid development, known as skotomorphogenesis (von Arnim and Deng, 1996). Etiolation facilitates emergence from soil until sufficient light is available for photoautotrophic growth. Although phytochromes are critical photosensors that regulate deetiolation of developing seedlings following skotomorphogenesis, phyA, phyB, and phyA phyB null mutants etiolate normally, demonstrating that phyA and phyB do not actively repress skotomorphogenesis in darkness (Smith et al., 1997), nor does phytochrome overexpression affect etiolation, because dark-grown seedlings overexpressing phytochromes are indistinguishable from wild-type and null mutants (Smith, 1994). It was thus surprising to observe that PHYB<sup>Y276H</sup>-expressing plants were fully deetiolated in complete darkness (Figure 2; see Supplemental Figure 3C online). This COP phenotype included inhibition of hypocotyl elongation, pronounced hook opening, and cotyledon expansion. Because chlorophyll synthesis requires light, neither control lines nor PHYB<sup>Y276H</sup> transgenic plants greened in darkness. The PHYB<sup>Y276H</sup> COP phenotype starkly contrasts with skotomorphogenetic development of untransformed Ler wild-type, phyA, phyB, and phyA phyB null mutants as well as all transgenic lines expressing the wild-type PHYB or PHYB-GFP alleles (Figure 2A).

In T2 generations, dark-grown seedlings of multiple independent transformants of both cDNA and genomic alleles of PHYB<sup>Y276H</sup> displayed COP phenotypes regardless of their genetic backgrounds. For PHYB<sup>Y276H</sup> cDNA transformations, we observed six of nine COP kanamycin-resistant segregants in phyA phyB backgrounds, and we observed six of nine COP kanamycin-resistant segregants in phyA phyB backgrounds. For transformation of the phyA phyB background with the PHYB<sup>Y276H</sup> genomic allele, COP phenotypes were observed in 8 of 8 transformants. Segregation analyses of heterozygous lines established that PHYB<sup>Y276H</sup> is a dominant allele; one copy proved sufficient to confer COP phenotypes in all genetic backgrounds analyzed (data not shown). These results indicate that PHYB<sup>Y276H</sup> is a completely penetrant, dominant gain-of-function allele.
Immunoblot analysis indicated that the COP phenotype mediated by the genomic PHYB<sup>Y276H</sup> allele was not due to elevated expression of the PHYB<sup>Y276H</sup> mutant protein (Figure 1D). PHYB<sup>Y276H</sup>-dependent COP phenotypes were also unaltered by FR irradiation immediately following imbibition (data not shown), a treatment that would photorevert a hypothetical stable pool of Pfr present in seeds prior to imbibition if such a pool was present. Moreover, PHYB<sup>Y276H</sup>-expressing seedlings continued to develop true leaves when sucrose was present as a carbon source, while seedlings expressing wild-type PHYB alleles showed arrested development with very long hypocotyls, unexpanded cotyledons, and no true leaves (Figures 2B and 2C). Taken together, these results indicate that PHYB<sup>Y276H</sup> alleles encode constitutively active phytochrome mutants that do not require light for activation.

**Bilin Chromophore Is Required for the Gain-of-Function Activity of the PHYB<sup>Y276H</sup> Mutant Protein**

It is well established that the bilin chromophore of phytochromes is essential for light signaling (Koornneef and Kendrick, 1994; Terry, 1997; Montgomery et al., 2001; Sawers et al., 2002). To
test the hypothesis that bilin chromophore is required for the gain-of-function activity of the \( \text{PHYB}^{Y276H} \) mutant, we compared the phenotypic consequence of introducing \( \text{PHYB}^{Y276H} \) and wild-type \( \text{PHYB} \) genomic alleles into both bilin-deficient \( \text{hy1} \) \( \text{phyA} \) \( \text{phyB} \) triple and bilin-producing \( \text{phyA} \) \( \text{phyB} \) double mutant backgrounds. These analyses show that \( \text{hy1-1} \), a null allele that is deficient in the bilin chromophore biosynthetic enzyme heme oxygenase (Muramoto et al., 1999), strongly suppressed the gain-of-function activity of the \( \text{PHYB}^{Y276H} \) allele as measured by seedling hypocotyl growth (Figure 3; see Supplemental Figure 4 online). Although dark-grown \( \text{PHYB}^{Y276H} \) transgenics in the \( \text{hy1} \) \( \text{phyA} \) \( \text{phyB} \) background possessed shorter hypocotyls than the untransformed \( \text{hy1} \) \( \text{phyA} \) \( \text{phyB} \) parent, \( \text{PHYB}^{Y276H} \) transgenics were significantly more etiolated in bilin-deficient \( \text{hy1} \) \( \text{phyA} \) \( \text{phyB} \) backgrounds compared with \( \text{PHYB}^{Y276H} \) transgenics in bilin-producing \( \text{phyA} \) \( \text{phyB} \) backgrounds that exhibited a strong COP phenotype (Figures 3A and 3B). By contrast, this phenomenon was not observed in \( \text{hy1} \) \( \text{phyA} \) \( \text{phyB} \) and \( \text{phyA} \) \( \text{phyB} \) backgrounds transformed with the wild-type \( \text{PHYB} \) genomic allele, both of which showed normal skotomorphogenesis.

To test whether the incomplete suppression of the \( \text{PHYB}^{Y276H} \) dependent COP phenotype by \( \text{hy1} \) reflected partial synthesis of bilin chromophore via one of the three other heme oxygenases in \textit{Arabidopsis} (Davis et al., 2001; Muramoto et al., 2002; Emborg et al., 2006), we performed an additional set of phenotypic comparisons under R (Figure 3C). These experiments showed that the \( \text{hy1} \) mutation does not fully eliminate the light response as long as some \( \text{PHYB} \) protein is present. This indicates that residual chromophorylation of \( \text{PHYB} \) protein occurs in the \( \text{hy1} \) mutant. This conclusion is supported by the R-dependent hypocotyl growth inhibition of \( \text{hy1} \) \( \text{phyA} \) seedlings, whose hypocotyl lengths were \( \sim 66\% \) of dark-grown wild-type \textit{Ler} (Figure 3C). Strikingly, the hypocotyl lengths of \( \text{hy1} \) \( \text{phyA} \) plants under R are indistinguishable from those of \( \text{hy1} \) \( \text{phyA} \) \( \text{phyB} \) mutants that had been transformed with either \( \text{PHYB} \) or \( \text{PHYB}^{Y276H} \) genomic alleles (Figure 3C). The latter suggests that the residual activity...
of wild-type phyB in the hy1 phyA phyB background is nearly identical with the light-independent activity of the phyBY276H mutant in the same genetic background under R. Taken together, these results indicate that bilin chromophore is required for the light-independent signaling activity of the PHYB\textsuperscript{Y276H} protein.

**PhyB\textsuperscript{Y276H} Holoprotein Constitutively Localizes to Nuclear Bodies and Activates the Expression of Light-Regulated Genes in Both Light and Darkness**

The subcellular localization of phytochrome is tightly correlated with its photoregulatory activity (Yamaguchi et al., 1999; Kircher et al., 2002). Cytoplasmically tethered phytochrome is functionally inactive even in the presence of light (Huq et al., 2003), while nuclear-localized phytochrome requires light activation for regulatory function (Matsushita et al., 2003). Therefore, nuclear-localized Pfr is needed for full signaling activity. Once in the nucleus, phytochrome accumulates in nuclear bodies or speckles, and the size of phyB nuclear bodies depends on continuous Pfr generation by light (Chen et al., 2003). Nuclear body formation also correlates with hypocotyl growth inhibition, and phyB mutants deficient in speckle formation are poorly active, supporting the conclusion that nuclear body formation plays a direct role in signal transduction (Chen et al., 2005). Because of the constitutive signaling activity of the phyB\textsuperscript{Y276H} mutant, we tested whether light is required for its nuclear localization. The phyB\textsuperscript{Y276H} holoprotein is strongly fluorescent (Fischer et al., 2005), so its localization could readily be monitored by fluorescence microscopy. In contrast with the light-dependent nuclear localization of a wild-type phyB-GFP chimera used as a control (Yamaguchi et al., 1999), phyB\textsuperscript{Y276H} was found in the nucleus in both dark- and in light-grown plants (Figure 4A). The presence of red fluorescent nuclear bodies in dark-grown PHYB\textsuperscript{Y276H} transgenics clearly indicates that both nuclear translocation and nuclear body formation of phyB\textsuperscript{Y276H} are light independent, a result fully consistent with the constitutive signaling activity of this mutant. The facile detection of phyB\textsuperscript{Y276H} fluorescence in planta also implies that the full-length mutant has similar photochemical properties to those previously reported for recombinant truncations in vitro (Fischer et al., 2005; see Supplemental Figure 2 online).

To ascertain whether the COP phenotype of dark-grown PHYB\textsuperscript{Y276H} transgenics reflects misexpression of light-regulated genes, RT-PCR analysis was performed for chalcone synthase (CHS) and chlorophyll a/b binding protein (CAB)—two well-studied phrase-replete phyA phyB and chlorophyll-deficient hy1 phyA phyB backgrounds. Values are mean ± SD (n = 50).

**Figure 3.** Phytochrome Chromophore Biosynthesis Is Required for the Gain-of-Function Activity of PHYB\textsuperscript{Y276H}.

(A) Transgenic plants with or without the hy1 mutation were grown in darkness for 6 d on sucrose-free media.

(B) Relative hypocotyl length of dark-grown seedlings in (A) normalized to that of Ler shows the differential activity of PHYB\textsuperscript{Y276H} in chromatophore-replete phyA phyB and chromatophore-deficient hy1 phyA phyB backgrounds. Values are mean ± SD (n = 50).

(C) Relative hypocotyl length of seedlings grown on sucrose-free media under 20 \( \mu\text{mol m}^{-2} \text{s}^{-1} \) continuous R. Mean hypocotyl lengths (± SD; n = 50) are normalized to those of dark-grown Ler seedlings. The shorter hypocotyl lengths of hy1/phyA, PHYB/phyB/phyA/phyB, and PHYB\textsuperscript{Y276H} hy1/phyA/phyB plants compared with the hy1/phyA/phyB parent line indicate that sufficient bilin chromophore is present in the hy1/mutant background to maintain reduced but significant signaling activity of phyB and phyB\textsuperscript{Y276H}.

(D) Immunoblot analysis of PHYB protein level was performed as in Figure 1.
light-responsive genes (Quail, 1991). While the untransformed Ler wild type requires light for measurable expression of either gene, phyB<sup>Y276H</sup> strongly activated expression of both genes in darkness regardless of the presence of wild-type PHYB alleles (Figure 4B). For light-grown plants, little difference was detected in the amount of CAB or CHS gene expression between Ler wild-type and PHYB<sup>Y276H</sup> transformants. Taken together, the observed light-independent nuclear localization of phyB<sup>Y276H</sup> and misexpression of light-regulated genes indicate that the Tyr<sup>276</sup>His mutation bypasses both light-dependent signaling processes.

Other Y<sub>GAF</sub> Alleles of PHYB Complement the PhyB-Deficient Phenotype of the phyA phyB Double Mutant

Saturation mutagenesis of the Y<sub>GAF</sub> residue in the cyanobacterial phytochrome Cph1 showed that, although this Tyr is not essential for bilin attachment, it is critical for normal photochemistry (Fischer et al., 2005). Besides the intensely fluorescent Y<sub>GAF</sub>H mutant of Cph1, three other amino acid substitutions (Glu, Gln, and Trp) similarly enhanced Cph1 fluorescence. By contrast, most other amino acid substitutions yielded poorly photoactive and nonfluorescent Cph1 holoproteins with more cyclic, deprotonated bilin chromophores, while the Cph1Y<sup>176</sup>R mutant bound a porphyrin (Fischer et al., 2005). To compare the phenotypic consequences of the different classes of Y<sub>GAF</sub> mutant of phyB in planta, we transformed the phyA phyB null background with genomic PHYB<sup>Y276Q</sup>, PHYB<sup>Y276I</sup>, and PHYB<sup>Y276R</sup> alleles to produce photoreceptors predicted to have fluorescent/extended, nonfluorescent/cyclic, and porphyrin chromophores, respectively. We also performed comparative spectroscopic analysis of recombinant versions of these PHYB mutant alleles expressed in Escherichia coli (see Supplemental Figures 2C and 2D online). With the exception of PHYB<sup>Y276R</sup>, which failed to form a tetrapyrrole adduct, the spectroscopic properties of the Y<sub>GAF</sub> mutants of phyB were in good agreement with those of the equivalent Y<sub>GAF</sub> mutants of Cph1 (Fischer et al., 2005). Like phyB<sup>Y276H</sup>, all of the Y<sub>GAF</sub> mutants of phyB proved to be essentially photoinactive, with <10% photoactivity of wild-type phyB. 

Homozygous PHYB<sup>Y276Q</sup>, PHYB<sup>Y276I</sup>, and PHYB<sup>Y276R</sup> lines were selected and grown under R for phenotypic comparison with untransformed Ler wild-type, phyA, phyB, and phyA phyB controls as well as with phyA phyB transformants harboring PHYB wild-type and PHYB<sup>Y276Q</sup>, PHYB<sup>Y276I</sup>, and PHYB<sup>Y276R</sup> alleles (Figure 5A; see Supplemental Figure 5 online). Both PHYB<sup>Y276Q</sup> and PHYB<sup>Y276I</sup> alleles restored R-mediated hypocotyl growth inhibition and cotyledon expansion seen in the Ler wild-type. By contrast, the PHYB<sup>Y276R</sup> allele failed to complement the phyA phyB mutant under the same experimental conditions. Since Y<sub>GAF</sub> mutant protein was present in all transgenic lines, albeit at slightly different levels (Figure 5B), the observed phenotypic complementation under R demonstrates signaling activity for all Y<sub>GAF</sub> mutants except phyB<sup>Y276R</sup>. Phenotypic comparisons of 5-week-old adult plants grown under continuous W were fully consistent with the R-grown seedling experiments (Figure 5C). The PHYB<sup>Y276H</sup> and PHYB<sup>Y276Q</sup> alleles proved more effective than PHYB<sup>Y276I</sup> in complementing the elongated internode phenotype of the phyA phyB mutant in white light, and the PHYB<sup>Y276R</sup> allele similarly...

Figure 4. PhyB<sup>Y276H</sup> Localizes to the Nucleus, Forms Nuclear Bodies (Specckles), and Activates the Expression of Light-Regulated Genes in Darkness.

(A) Comparative subcellular localization of phyB-GFP and PHYB<sup>Y276H</sup>-derived phyB proteins in 5-d-old dark- and continuous W-grown (80 µmol m<sup>−2</sup> s<sup>−1</sup>) seedlings was performed by fluorescence confocal microscopy. PhyB-GFP (B-GFP) was visualized using GFP optics (green), phyB<sup>Y276H</sup> (BY<sup>276</sup>H) was visualized using Texas Red optics (red), and nuclei were identified by 4',6-diamidino-2-phenylindole (DAPI) staining (blue). Merged images represent overlapping DAPI and GFP images (for phyB-GFP) or overlap of the DAPI with Texas Red images (for phyB<sup>Y276H</sup>). Bars = 10 µm.

(B) Light-independent expression of CAB and CHS transcripts was observed in dark-grown PHYB<sup>Y276H</sup> plants by RT-PCR. Ethidium bromide-stained agarose gels contain 10 µL of PCR product per lane. ACT transcript levels are shown as a control.
proved inactive. These data demonstrate that, although the YGAF residue is critical for phytochrome’s photochemical activity, the YGAF residue of phyB can be replaced with other amino acids that considerably inhibit the efficiency of its photoactivation yet still retain significant signaling activity.

We also examined the dark-grown phenotypes of all transgenic lines. PHYB_Y276Q-expressing plants exhibited a COP phenotype when grown in darkness, although this phenotype was not as pronounced as in plants expressing the PHYB_Y276H allele (Figure 6A). While PHYB_Y276Q-expressing plants had shorter hypocotyls and slightly expanded cotyledons relative to wild-type plants, plants expressing PHYB_Y276R and PHYB_Y276R alleles showed normal skotomorphogenesis, demonstrating that these alleles do not encode constitutively active phyBs (Figure 6A; see Supplemental Figure 5D online). Given the comparable levels of PHYB protein accumulation in PHYB_Y276Q- and PHYB_Y276H-expressing plants (Figure 5B), these results suggest that phyB_Y276Q is not as active as phyB_Y276H.

To more fully characterize the light responsiveness of the YGAF mutants, fluence rate response measurements of hypocotyl growth were undertaken for seedlings grown under continuous R. Since these experiments employed YGAF transgenic lines in a phyA phyB genetic background, the endogenous phyA and phyB photoreceptors should not interfere with the measurement of YGAF mutant activity. As shown in Figure 6B, phyB_Y276H proved fully activated under all fluences of R, while no activity of PHYB_Y276R-expressing plants could be detected at any fluence rate. Hypocotyl lengths of dark-grown PHYB_Y276H seedlings were indistinguishable from those of seedlings exposed to any fluence rate of R, confirming that the constitutive gain-of-function activity of phyB_Y276H is independent of red light.

By contrast with PHYB_Y276H transgensics, the phenotypes of PHYB_Y276Q- and PHYB_Y276I-expressing plants were both strongly regulated by the fluence rate of R, similar to those of plants harboring wild-type PHYB alleles (Figure 6B), demonstrating that both PHYB_Y276Q and PHYB_Y276I alleles encode functional photoreceptors that require R illumination for full activity. PHYB_Y276Q-expressing seedlings were shorter than both the Ler wild type and the phyA phyB mutant transformed with a wild-type PHYB allele at all fluence rates of R, indicating that phyB_Y276Q possesses both light-independent and light-dependent regulatory activity. By contrast, PHYB_Y276I appears to encode a less active phytochrome that requires continuous irradiation for sustained activation. To test this hypothesis, hypocotyl lengths of YGAF seedlings grown under 8-h-R/16-h-dark cycles were compared with those grown under continuous R, demonstrating that PHYB_Y276I seedlings were particularly sensitive to the two light regimes (Figure 6C). For PHYB_Y276I seedlings grown under 8-h-R/16-h-dark cycles, hypocotyls were ~50% longer than seedlings grown under continuous R, which contrasts with a 20% difference for plants expressing a wild-type PHYB allele (i.e., Ler and PHYB-expressing plants). These results indicate that phyB_Y276I may require sustained activation for full activity, suggesting enhanced dark reversion of its light-activated Pfr form. PhyB mutants with enhanced dark reversion have been previously described, and many show reduced signaling activity under low fluence rates of light (Elich and Chory, 1997; Sweere et al., 2001).
To further characterize the light responsiveness of the Y\textsuperscript{GAF} mutants, fluence response measurements of hypocotyl growth were also undertaken for seedlings grown under continuous FR light, conditions that specifically activate phyA signaling (Furuya and Schaefter, 1996). Since the Y\textsuperscript{GAF} mutants were introduced into the phyA phyB background, such experiments might reveal whether the Y\textsuperscript{GAF} mutations alter the light specificity of phyB signaling. In this regard, both PHYB\textsuperscript{Y276H} and PHYB\textsuperscript{Y276Q} alleles apparently restore the strong FR-dependent hypocotyl growth inhibition and cotyledon expansion responses that are deficient in the phyA phyB double mutant (Figures 7A and 7B; see Supplemental Figure 5E online). Since both alleles encode constitutively active photoreceptors in darkness (Figure 2), the apparent phyA regulatory activity is likely to be independent of FR irradiation per se. In contrast with the PHYB\textsuperscript{Y276H} and PHYB\textsuperscript{Y276Q} alleles, the PHYB\textsuperscript{Y276I} and PHYB\textsuperscript{Y276R} alleles proved inactive under FR (Figures 7A and 7B). Taken together, these experiments show that FR does not reverse the constitutive gain-of-function activities of the phyB\textsuperscript{Y276H} and phyB\textsuperscript{Y276Q} mutants—a result that strongly contrasts with the striking inhibition of wild-type phyB by FR.

**PHYB\textsuperscript{Y276H} - and PHYB\textsuperscript{Y276Q}-Expressing Transformants Exhibit Light-Independent Seed Germination**

*Arabidopsis* seed germination is primarily regulated by phyA and phyB (Shinomura, 1997). Since seeds immediately following imbibition possess little phyA owing to its light lability, seed germination of many plant species is primarily promoted by R and inhibited by FR. Indeed, *Arabidopsis* seed germination is strongly inhibited by a short pulse of far-red light (FRp) that photoconverts the Pfr form of phyB already present in the seed to its inactive Pr form (Shinomura, 1997). The promotive effect of a pulse of red light (Rp) can be strongly reversed by a subsequent FRp via the so-called low fluence response—a response that is mostly mediated by phyB (Shinomura et al., 1996; Shinomura et al., 1998). To test the phenotypic consequence of Y\textsuperscript{GAF} alleles on seed germination and to avoid the contribution of endogenous phyA and phyB to this response, we determined the light dependence of seed germination of PHYB\textsuperscript{Y276H}- and PHYB\textsuperscript{Y276Q}-expressing lines in the phytochrome-deficient phyA phyB background (Figure 7C). Seed germination efficiency of both Y\textsuperscript{GAF} mutant lines was \textasciitilde 100% both in complete darkness and under all light regimes tested (i.e., Rp, FRp, Rp/FRp, and Rp/FRp/Rp). By comparison, the frequencies of seed germination of Ler, phyA, and phyA phyB control lines were strongly stimulated by red light (Rp) and reversed by far-red light (FRp) (Figure 7C; data not shown). These studies not only confirm that the phyB\textsuperscript{Y276H} and phyB\textsuperscript{Y276Q} mutants are constitutively active but also demonstrate that light-independent promotion of seed germination by both Y\textsuperscript{GAF} mutants is not FR inhibited.

**The Y\textsuperscript{GAF} Allele of PHYA Exhibits Gain-of-Function Activity in Darkness and Acts as a Dominant-Negative Mutant under Continuous FR Light**

To evaluate the biological activity of the Y\textsuperscript{GAF} mutant of PHYA (PHYAY\textsuperscript{Y242H}), transgenic plants expressing both wild-type PHYA
PHYAY242H mutant alleles under the control of the native PHYA promoter were constructed in Ler wild-type and phyA null mutant backgrounds (see Supplemental Figure 1 and Supplemental Table 1 online). Hypocotyl lengths of dark- and continuous FR–grown seedlings of homozygous lines were then measured (Figure 8; see Supplemental Figure 6 online). As expected (Smith, 1994), expression of the wild-type PHYA allele did not significantly affect skotomorphogenesis of Ler wild-type or PHYA-complemented phyA seedlings (Figure 8A), and the PHYA transformant in the Ler background was hypersensitive to continuous FR compared with Ler (Figures 8B and 8C; see Supplemental Figure 6 online). By contrast, PHYAY242H expression in Ler wild-type and phyA backgrounds yielded dark-grown seedlings with open cotyledons and slightly shorter hypocotyls—a phenotype similar to but not as striking as PHYBY276H-expressing transformants (Figure 8A). However, under FR, PHYAY242H-expressing seedlings in the Ler background developed significantly longer hypocotyls compared with seedlings possessing only wild-type PHYA alleles (Figure 8B). These results indicate that, at this fluence rate of FR, the PHYAY242H allele encodes a dominant-negative protein that attenuates the signaling output activity of wild-type phyA.

To more fully examine the photoregulatory activity of the PHYAY242H-derived photoreceptor, fluence rate response measurements for hypocotyl growth inhibition were performed (Figure 8D). These studies showed that the activity of phyAY242H was independent of the fluence rate of FR, in striking contrast with the strong fluence rate dependence of seedlings possessing wild-type PHYA alleles (Figure 8D). Indeed, the wild-type PHYA allele fully complemented the FR-dependent inhibition of hypocotyl elongation of the phyA mutant, restoring the fluence rate response to that of the Ler wild type at fluence rates of FR above 2 μmol m⁻² s⁻¹ (Figure 8D). Interestingly, the light-independent gain-of-function activity of phyAY242H in the wild-type Ler background seen in darkness was balanced by a light-dependent dominant-negative activity at higher fluence rates. At low fluence rates of FR, PHYAY242H-expressing seedlings in the Ler background had shorter hypocotyls than the Ler wild type (as observed in darkness), while at high fluence rates, the former had longer hypocotyls (Figure 8D). This result shows that PHYAY242H functions as a constitutive gain-of-function allele in darkness and a dominant-negative allele under elevated fluence rates of FR. Immunoblot analysis further showed that PHYAY242H accumulated to lower levels than wild-type PHYA in the wild-type Ler background seen in darkness was balanced by a light-dependent dominant-negative activity at higher fluence rates. At low fluence rates of FR, PHYAY242H-expressing seedlings in the Ler background had shorter hypocotyls than the Ler wild type (as observed in darkness), while at high fluence rates, the former had longer hypocotyls (Figure 8D). This result shows that PHYAY242H functions as a constitutive gain-of-function allele in darkness and a dominant-negative allele under elevated fluence rates of FR. Immunoblot analysis further showed that PHYAY242H accumulated to lower levels than wild-type PHYA in all dark-grown genotypes examined (Figure 8C; see Supplemental Figure 6 online). This indicated that the constitutive photomorphogenetic activity of PHYAY242H was not due to elevated accumulation of the PHYAY242H mutant protein. Taken together, these results suggest that phyAY242H adopts a light-independent, signaling active state that is similar to that of phyBY276H but less active than fully activated wild-type phyA.

DISCUSSION

YGAFH Mutants of phyB and phyA Are Constitutively Active

The observation that plants expressing YGAFH mutants of phyA and phyB exhibit constitutive photomorphogenetic development in darkness establishes that the functional consequence of this mutation is the light-independent activation of both photoreceptors. While the extent of the gain-of-function activities is difficult to quantify from our data, the signal output appears greater for phyBY276H than phyAY242H. In this regard, hypocotyl growth...
inhibition and cotyledon expansion of dark-grown seedlings appeared fully activated in PHYB<sup>Y276H</sup> transformants, while PHYA<sup>Y242H</sup> seedlings only showed partial activation of these photomorphogenetic responses. We interpret the observed gain-of-function activities of both photoreceptors as reflecting the same structural consequence (i.e., that the Y<sup>GAF</sup>H mutation confers a conformation that mimics the photoactivated Pfr form for both phytochromes).

Fluence rate response curves indicate that the regulatory output activity of phyB<sup>Y276H</sup> is fluence rate independent, exceeding that of fully light-activated wild-type phyB (Figure 6). We attribute the light-independent hyperactivity of phyB<sup>Y276H</sup> to two factors: one, the inability to fully photoconvert wild-type phyB to 100% Pfr; and two, the lack of phyB<sup>Y276H</sup> dark reversion. In this regard, red light can only produce a maximum of 85% Pfr for phyA owing to the spectral overlap of Pr and Pfr forms (Lagarias et al., 1987). The percentage of photoconversion is even lower for phyB due to dark reversion, especially under lower fluence rates of light (Hennig and Schäfer, 2001). Since the light-independent activation of the phyB<sup>Y276H</sup> mutant is not constrained by the photoequilibrium limitations of the wild type and its lack of dark reversion would sustain its activation in darkness (or under low fluence rates of light), the hyperactivated phenotype of the PHYB<sup>Y276H</sup> plants is consistent with a photoinsensitive, non-dark-reverting allele of activated phyB.

Our studies also show that the activity of phyA<sup>Y242H</sup> is not affected by illumination with FR light in the phyA genetic background (Figure 8D). We therefore conclude that phyA<sup>Y242H</sup> also adopts a light-independent activated conformation, although one that is apparently less active than fully FR-activated wild-type phyA. The partial activity of phyA<sup>Y242H</sup> can be attributed to the different modes of action of phyA and phyB photoreceptors (Reed et al., 1994; Furuya and Schäfer, 1996). Owing to its light lability and to the negative feedback of its own transcription, phyA accumulation in plants is strongly light regulated (Quail, 1991). This light lability is responsible for the low abundance of phyA in light-grown plants and for its elevated level in dark-grown plants. Indeed, preliminary immunoblot analysis indicates that PHYA levels are extremely low in light-grown PHYA<sup>Y242H</sup> plants, very similar to those in wild-type Ler (data not shown). The COP phenotype of dark-grown PHYA<sup>Y242H</sup>-expressing seedlings is therefore unlikely to represent previously light-activated phyA in seed embryos, as was recently reported for plants constitutively expressing a PHYA-GFP-β-glucuronidase chimera (Mateos et al., 2006).

The dominant-negative phenotype of PHYA<sup>Y242H</sup>-expressing transgenic plants in wild-type Ler backgrounds also is consistent with the reduced specific activity of phyA<sup>Y242H</sup> compared with fully photoactivated wild-type phyA. Since phyA is an obligate
homodimer (Jones and Quail, 1986; Henning and Schäfer, 2001), heterodimers between phyAY242H and wild-type phyA subunits could explain the dominant-negative phenotype. Indeed, heterodimerization could also explain the dominant-negative phenotypes observed by expression of variously truncated (and potentially weakly functional alleles) of both PHYA and PHYB in wild-type genetic backgrounds (Boylan et al., 1994; Wagner et al., 1996). Based on an elegant two-pulse experimental setup, Shinomura et al. (1998) proposed that the active form of phyA that mediates the FR high irradiance response is not Pfr but a Pr species that has been cycled through Pfr. We therefore conclude that the dominant-negative regulatory activity of phyAY242H under high fluence rates of FR reflects a photostable Pfr-like state that is not as active as cycled Pr. Hence, in the Ler genetic background, heterodimers between wild-type phyA and phyAY242H mutant subunits can never be fully activated by FR, leading to the dominant-negative phenotype at elevated fluence rates.

The Biological Activities of Other YGAF Mutants of PhyB Implicate a Critical Role of the GAF Domain Tyr Residue in Coupling Light Perception to Signaling Output

Our studies show that the phenotypic consequence of the mutation of the YGAF residue of phytochrome B is strongly dependent on the particular amino acid substitution chosen. His and Gln substitutions both confer gain-of-function COP activity in darkness, suggesting that the two amino acid substitutions have similar effects on structural and biological properties of phyB. In contrast with the light-independent constitutive activity of phyBY276H, phyBY276Q exhibits some light R sensitivity (Figure 6B). It is interesting that both YGAFH and YGAFQ substitutions have similar effects on the spectroscopic properties of Cph1 (i.e., the two mutants are strongly fluorescent and both possess extended, protonated bilin chromophores) (Fischer et al., 2005). This suggests that the structural perturbations in the chromophore binding pocket that give rise to the fluorescence gain-of-function of these YGAF mutants mimic the light activation of phytochrome.

The correlation of biological activity with the spectral properties of specific YGAF mutations is further underscored by the photoproducive consequences of the expression of PHYBY276H and PHYBY276R alleles—neither of which confers a COP-like phenotype to transgenic plants. The observation that PHYBY276H complements the phyB deficiency of the phyA phyB double mutant under both W and R is of particular interest. Indeed, full complementation by PHYBY276L was unexpected in view of its poor photoconvertibility and the cyclic, deprotonated chromophore of the phyBY276L mutant observed in vitro (see Supplemental Figure 2 online). PHYBY276L-derived mutant plants are considerably taller than those possessing wild-type PHYB alleles, particularly under light/dark cycles. This supports the interpretation that the light-activated Pfr form of phyBY276L is unstable, possibly exhibiting enhanced reversion to the inactive Pr form in darkness. However, phyB mutant alleles with increased dark reversion are typically hypomorph (Elich and Chory, 1997). These results suggest that either the specific activity of the light-activated Pfr form of phyBY276L exceeds that of wild-type phyB or that continuous prolonged exposure to light enables the phyBY276L chro-
transcription of light-regulated genes require both phytochromes, nuclear migration, nuclear body (speckle) formation, and (B) A cellular model for phytochrome signaling. For wild-type phytochrome-NLS, triggering nuclear translocation of phytochrome. For the wild type, PSD. We envisage that this conversion exposes the C-terminal regulatory domain (CTRD) that potentially are transduced via the PHY subdomain of the YH*. This results in release (or uncoupling) of the CTRD domain from the YGAFH mutant of phytochrome B, since YH* activates these processes in refer to cytoplasmic and nuclear localization, respectively. shown. In YGAFH PHYB (or PHYA) apoproteins and the Pr form of the wild type (left), the photosensory domains (PSDs) are tightly associated with the C-terminal regulatory domains (CTRDs). This association masks a cryptic nuclear localization signal (NLS) located in the PAS repeat region within the CTRD that is specific to plant phytochromes (Chen et al., 2005). Activation occurs by Pr-to-Pfr photoconversion for the wild-type (green arrow) or by assembly of the YGAFH mutant apoprotein (YH) with phytochromobilin (PφB) to produce the activated holoprotein species YH*. This results in release (or uncoupling) of the CTRD domain from the PSD by chromophore-mediated allosteric changes within the GAF domain that potentially are transduced via the PHY subdomain of the PSD. We envisage that this conversion exposes the CTRD-localized NLS, triggering nuclear translocation of phytochrome. For the wild type, this conversion is metastable and can be reversed both by FR irradiation (red arrow) or by dark reversion. (B) A proposed model of phytochrome protein conformational changes is shown. In YGAFH PHYB (or PHYA) apoproteins and the Pr form of the wild type (left), the photosensory domains (PSDs) are tightly associated with the C-terminal regulatory domains (CTRDs). This association masks a cryptic nuclear localization signal (NLS) located in the PAS repeat region within the CTRD that is specific to plant phytochromes (Chen et al., 2005). Activation occurs by Pr-to-Pfr photoconversion for the wild-type (green arrow) or by assembly of the YGAFH mutant apoprotein (YH) with phytochromobilin (PφB) to produce the activated holoprotein species YH*. This results in release (or uncoupling) of the CTRD domain from the PSD by chromophore-mediated allosteric changes within the GAF domain that potentially are transduced via the PHY subdomain of the PSD. We envisage that this conversion exposes the CTRD-localized NLS, triggering nuclear translocation of phytochrome. For the wild type, this conversion is metastable and can be reversed both by FR irradiation (red arrow) or by dark reversion. (B) A proposed model of phytochrome protein conformational changes is shown. In YGAFH PHYB (or PHYA) apoproteins and the Pr form of the wild type (left), the photosensory domains (PSDs) are tightly associated with the C-terminal regulatory domains (CTRDs). This association masks a cryptic nuclear localization signal (NLS) located in the PAS repeat region within the CTRD that is specific to plant phytochromes (Chen et al., 2005). Activation occurs by Pr-to-Pfr photoconversion for the wild-type (green arrow) or by assembly of the YGAFH mutant apoprotein (YH) with phytochromobilin (PφB) to produce the activated holoprotein species YH*. This results in release (or uncoupling) of the CTRD domain from the PSD by chromophore-mediated allosteric changes within the GAF domain that potentially are transduced via the PHY subdomain of the PSD. We envisage that this conversion exposes the CTRD-localized NLS, triggering nuclear translocation of phytochrome. For the wild type, this conversion is metastable and can be reversed both by FR irradiation (red arrow) or by dark reversion. (B) A proposed model of phytochrome protein conformational changes is shown. In YGAFH PHYB (or PHYA) apoproteins and the Pr form of the wild type (left), the photosensory domains (PSDs) are tightly associated with the C-terminal regulatory domains (CTRDs). This association masks a cryptic nuclear localization signal (NLS) located in the PAS repeat region within the CTRD that is specific to plant phytochromes (Chen et al., 2005). Activation occurs by Pr-to-Pfr photoconversion for the wild-type (green arrow) or by assembly of the YGAFH mutant apoprotein (YH) with phytochromobilin (PφB) to produce the activated holoprotein species YH*. This results in release (or uncoupling) of the CTRD domain from the PSD by chromophore-mediated allosteric changes within the GAF domain that potentially are transduced via the PHY subdomain of the PSD. We envisage that this conversion exposes the CTRD-localized NLS, triggering nuclear translocation of phytochrome. For the wild type, this conversion is metastable and can be reversed both by FR irradiation (red arrow) or by dark reversion.

Montgomery and Lagarias [2002] for discussion of the various domains of phytochromes). Indeed, recent work has shown that the PHY domain can be cross-linked to a residue lying near this surface in the bacteriophytochrome Agp1 (Noack et al., 2007). The YGAF mutations may therefore disrupt photochemistry due to the proximity of this residue to, and its interaction with, the chromophore that can derepress signaling by distorting a domain-domain interaction surface. The distinct biological activities of the various YGAF mutants examined here strongly imply that this Tyr performs a critical role in coupling light perception to signal transduction.

With regard to transcriptional regulation, these studies show that two representative light-regulated genes, CHS and CAB, are activated in PHYB<sup>Y276H</sup> seedlings grown in darkness. Misregulation of CHS expression is notable since its transcription is mainly B/UV-light dependent in Arabidopsis (Batschauer et al., 1996), while CAB transcription is known to be strongly phytochrome dependent (Chory et al., 1993). The observed light-independent expression of these two light-regulated genes implies that phyB<sup>Y276H</sup> constitutively regulates the nuclear factors that mediate the transcription of these genes. Notable among these are members of the PIF3 family of basic helix-loop-helix transcription factors (Bailey et al., 2003), components of COP1 and DET1-containing complexes that target the degradation of the bZIP transcription factor HY5 (Yi and Deng, 2005), and factors that participate in nuclear body formation (Chen et al., 2003). Recent work has shown that photoactivated phyB can interact with various members of the PIF3 family within the nucleus, presaging their phosphorylation and protein turnover (Oh et al., 2004; Al-Sady et al., 2006). The previously reported interaction between phyB’s C terminus and COP1 (Yang et al., 2001) suggests that photoactivated phyB may directly interact with COP1 to regulate HY5 accumulation. In this regard, a direct interaction between the Pr form of phyA and COP1 has been shown to be responsible for the rapid turnover of phyA (Seo et al., 2004). This type of light-dependent interaction could also influence the E3-ligase activity of COP1 toward other regulators of photomorphogenesis—an interaction that we propose to be light independent for phyB<sup>Y276H</sup>. Figure 9B compares the light-independent signaling processes that appear constitutively activated by the phyB<sup>Y276H</sup> mutant (in black) and the light-dependent processes that are regulated by wild-type phytochromes (in green and red) when bilirn chromophore is present.

**Agronomic Applications of YGAF Mutants to Regulate Light Responses of Crop Plant Species**

Identification of a constitutively active allele of plant phytochromes not only represents a new tool to elucidate the molecular mechanism of phytochrome signaling but also holds great potential for crop improvement. We have shown that seed germination can be dramatically altered by expression of PHYB<sup>Y276H</sup> (and PHYB<sup>Y276D</sup>); hence, the potential for enhancing germination of certain crop plant species in shade environments is quite real. PHYB<sup>YGAFH</sup> alleles also should be particularly effective for inhibiting shade avoidance responses. Based on the data presented here, we expect that the constitutive activity of phyB<sup>Y276H</sup> will counteract the enhanced elongation growth response of

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**Figure 9. Proposed Mechanism of Light-Independent Light Signaling by YGAFH Mutant Phytochromes.**

(A) A proposed model of phytochrome protein conformational changes is shown. In YGAFH PHYB (or PHYA) apoproteins and the Pr form of the wild type (left), the photosensory domains (PSDs) are tightly associated with the C-terminal regulatory domains (CTRDs). This association masks a cryptic nuclear localization signal (NLS) located in the PAS repeat region within the CTRD that is specific to plant phytochromes (Chen et al., 2005). Activation occurs by Pr-to-Pfr photoconversion for the wild-type (green arrow) or by assembly of the YGAFH mutant apoprotein (YH) with phytochromobilin (PφB) to produce the activated holoprotein species YH*. This results in release (or uncoupling) of the CTRD domain from the PSD by chromophore-mediated allosteric changes within the GAF domain that potentially are transduced via the PHY subdomain of the PSD. We envisage that this conversion exposes the CTRD-localized NLS, triggering nuclear translocation of phytochrome. For the wild type, this conversion is metastable and can be reversed both by FR irradiation (red arrow) or by dark reversion.

(B) A cellular model for phytochrome signaling. For wild-type phytochromes, nuclear migration, nuclear body (specle) formation, and transcription of light-regulated genes require both PφB chromophore binding (black arrows on left) and red light activation (green arrows). While the relationship of nuclear body (nb) formation to PIF-dependent transcription, proteosome-mediated protein turnover of these factors, and COP1- and DET1-dependent repression pathways remains unresolved, all of these phyB-mediated signaling processes are reversed by FR light (red arrows) and/or by dark reversion (black dashed arrows). By contrast, Pφ-B binding is sufficient for light-independent activation of the YGAFH mutant of phytochrome B, since YH* activates these processes in the absence of light (solid black arrows). The superscript “c” and “n” refer to cytoplasmic and nuclear localization, respectively.

3.6 Å of the chromophore D-ring in the recent high-resolution crystal structure of the bacteriophytochrome DrBphP (Wagner et al., 2007). YGAF also lies within 3.5 Å of the conserved aromatic residue at position 203 in DrBphP. Residue 203 is part of a conserved, solvent-accessible surface in the available crystal structures of truncated PSDs (Wagner et al., 2005, 2007; Rockwell and Lagarias, 2006) that may constitute a docking site for domains absent in these structures, such as the PHY domain.
plants to FR-enriched shade- and reflected-light environments (Schmitt et al., 1995; Smith and Whitelam, 1997; Franklin and Whitelam, 2005; Izaguirre et al., 2006). Phytochrome-mediated shade avoidance responses are often counterproductive in high-density agricultural venues, where they contribute to significant losses in crop yield (Kasperbauer, 1987; Smith et al., 1990). With the appropriate choice of promoter, expression of PHYB[Y276H] allelism ultimately may also prove useful for regulation of many aspects of plant development that are affected by light, including seed germination, elongation growth, flowering, seed yield, and senescence.

METHODS

Plant Materials, Growth Conditions, and Phenotypic Analyses

Arabidopsis thaliana ecotype Ler wild-type and phyA-201, phyB-5, phyA-201 phyB-5, hy1-1 phyA-201, and hy1-1 phyA-201 phyB-5 mutants (all in Ler ecotype) were obtained from colleagues or The Arabidopsis Information Resource (http://www.arabidopsis.org/). The ProSS5:PHYB-GFP-expressing line PBG-5 in phyB-5 (Yamaguchi et al., 1999) was used as a control for fluorescence microscopy. Seedlings were grown at 20°C on 0.8% (w/v) agar media (Phytobond; Caisson Laboratories) containing half-strength MS salt, half-strength vitamin solution, and 1% (w/v) agar for 6 d, and mean hypocotyl lengths (± SD) of 50 seedlings were determined using ImageJ software (http://rsb.info.nih.gov/ij). SNAP-LITE light sources (Quantum Devices) were used for red (662 ± 15 nm) and FR light (730 ± 15 nm). Philips F48T12 cool white VHO 1LP fluorescent lights were used as the continuous white light source with a fluence rate of 50 to 100 μmol m⁻² s⁻¹. For germination experiments, seeds were surface sterilized and sowed on top of four layers of moist filter papers, followed by treatment with saturated FR light (18 mmol m⁻² s⁻¹) and kept in the dark at 4°C for 3 d prior to the induction of germination. Imibed seeds were kept at 20°C in darkness or treated with <1.5-min saturating pulses of R (1.5 mmol m⁻² s⁻¹), FR (5 mmol m⁻² s⁻¹), R (1.5 mmol m⁻² s⁻¹) followed by FR (5 mmol m⁻² s⁻¹), or R (1.5 mmol m⁻² s⁻¹) followed by FR (5 mmol m⁻² s⁻¹) and then by R (1.5 mmol m⁻² s⁻¹), and incubated in darkness at 23°C for 6 d before germination efficiencies were scored. The mean of germination efficiency was calculated from at least three independent experiments (n = 100 seeds per experiment).

Plant Transformation Constructs

The Arabidopsis PHYB cDNA plant transformation vector pJM61 was described previously (Maloof et al., 2001). Construction of the PHYB[Y276H] cDNA plant transformation vector encoding the PHYB[Y276H] mutant entailed two cloning steps. The PBS-PHYB[Y276H]_ST plasmid was initially constructed by mutagenizing the plasmid PBS-PHYB-ST (Fischer et al., 2005) using the QuikChange site-directed mutagenesis kit (Stratagene) with forward and reverse primers 5'-GGTTATGATCGTGTTATGGTT-3' and 5'-GGTTATGATCGTGTTATGGTT-3'. The mutagenized regions were excised with SacII and EcoRI and cloned into the similarly restricted PHYB[Y276H] vector construction (encoding the PHYB[Y276H] mutant), the PHYA coding region was amplified with Phylolyase polymerase (Stratagene) using forward and reverse primers 5'-AGAAGCTTACGT-CAGGCTCTAGGCCGACT-3' and 5'-CTAATGCATCTTATTAGGATCCTAGCTGTCACCACTAC-3'. To express PHYA and PHYB[Y276H] cDNAs under the control of the PHYA promoter (ProPHYA), the PHYA promoter was amplified with Phylolyase polymerase using Columbia genomic DNA as template and the primers 5'-GAATTCTGTTGAATGTTAATACGGA-CTCGAGATTGTCGCGGATGAG-3' and 5'-GAAGCTTGAG-GATACCCCATTGTTGTACACACAGAC-3'. The PCR product was blunt-ended cloned into a Bluescript II KS⁺ restricted with EcoRV to yield PBS-PHYA. Plasmid PBS-PHYA[Y276H] was generated using the QuikChange site-directed mutagenesis kit with plasmid PBS-PHYA as template and the following primers: 5'-GGTTATGATCGTGTTATGGTT-3' and 5'-CTAATGCATCTTATTAGGATCCTAGCTGTCACCACTAC-3'. To express PHYA and PHYB[Y276H] cDNAs under the control of the PHYA promoter (ProPHYA), the PHYA promoter was amplified with Phylolyase polymerase using Columbia genomic DNA as template and the primers 5'-GGTTATGATCGTGTTATGGTT-3' and 5'-CTAATGCATCTTATTAGGATCCTAGCTGTCACCACTAC-3'. The PCR product was blunt-ended cloned into a Bluescript II KS⁺ restricted with EcoRV to yield PBS-35S:PHYA, PBS-35S:PHYB[Y276H], PBS-35S:PHYA[Y276H], PBS-35S:PHYB[Y276H], PBS-35S:PHYA[Y276H], PBS-35S:PHYB[Y276H], and PBS-35S:PHYA[Y276H] cDNA constructs. Transgenic lines segregating ~3:1 for antibiotic resistance in the T2 generation were selected, and the T3 or T4 homozygous generation was used for photographs, protein extraction, and phenotypic analyses.

Total Protein Extraction and Immunoblot Analysis

For total protein extraction, 6-d-old dark-grown seedlings were frozen in liquid nitrogen, ground into powder, and extracted with hot SDS buffer (165 mM Tris-HCl, pH 6.8, 5.1% [w/v] SDS, 5 mM EDTA, 5 mM EGTA, 5% [w/v] β-mercaptoethanol, and 1 mM PMSF) and boiling for 1 min. The soluble fraction was clarified by centrifugation, and proteins were precipitated with methanol-chloroform extraction (Wessell and Flugge, 1984). Protein pellets were dissolved in 50 mM Tris-HCl, pH 6.8, containing 2% (w/v) SDS, and total protein concentration was determined by BCA protein assay reagent using BSA as standard (Pierce). Equal amounts of proteins were separated on SDS-PAGE (Laemmli, 1970) and electroblotted to a polyvinylidene difluoride membrane. Mouse monoclonal anti-PHYA 073D, anti-PHYB B6-B3, and anti-α-tubulin antibodies (Sigma-Aldrich) were used for immunodetection of PHYA, PHYB, and tubulin, respectively. After washing, blots were incubated with alkaline

TAAACGAGCTCATAACCC-3'; for PHYB[Y276H] (5'-GGTTATGATCGTGTTATG- GGTCAAAGTTGCAGTTAAGGAGGAC-3' and 5'-GGTTATGATCGTGTTATG- GGTCAAAGTTGCAGTTAAGGAGGAC-3'); and for PHYB[Y276H] (5'-ATAACCA- TGAAGTTTCATGAAGATGATCAC-3' and 5'-ATAACCA-TGAAGTTTCATGAAGATGATCAC-3' and 5'-ATAACCA-TGAAGTTTCATGAAGATGATCAC-3').
phosphatase-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology). Immunoreactive bands were visualized by incubating blots with NBT/BCIP reagent (Pierce).

RT-PCR Analysis

Total RNA was isolated from 7-d-old dark- and light-grown seedlings using TRIzol reagent (Invitrogen). First-strand cDNA was synthesized using the StrataScript first-strand synthesis system (Stratagene), and 1 μL of first-strand cDNA was used for 25 μL of PCR reaction. CAB, CHS, and actin (ACT) transcripts were amplified using the following primer sets: CAB-F, 5′-TAAAGGCGTCAAGGTTCGCC-3′, and CAB-R, 5′-TACCATGATGCATGATTC-3′ (Usami et al., 2004); CHS-F, 5′-GGATCACC-AACAGTGAACAC-3′, and CHS-R, 5′-TTCCTCGGCATGATGATTC-3′ (Mehrtens et al., 2005); Actin-F, 5′-ATGAAGATTAAGGTGTGGA-3′, and Actin-R, 5′-TCCGAGTTGAAAGGGTAC-3′ (Abe et al., 2004). PCR reactions were performed using the following cycle: 94°C for 2 min and 94°C for 30 s, 56°C for 30 s, and 72°C for 40 s for 35 cycles, followed by 72°C for 10 min. Ten microliters of PCR reactions were separated on 2% TAE gels and visualized with ethidium bromide staining.

Fluorescence Microscopy for Phytochrome Localization

Five-day-old dark- and light-grown (continuous white light, 80 μmol m−2 s−1) seedlings were stained with 50 ng/mL of DAPI in PBS buffer for 30 min followed by destaining in PBS buffer for 10 min. DAPI-stained seedlings were transferred to a microscope slide immersed in PBS buffer under a cover slip and examination by fluorescence microscopy at the MCB Microscopy Imaging Facility. An Olympus FV1000 confocal laser scanning microscope equipped with an LD violet diode laser (405 nm, 25 mW), a multiline Ar laser (457 nm, 488 nm, 515 nm, Total 30 mW), a HeNe-G laser (633 nm, 10 mW) using DAPI (EX 405, EM 425/75), GFP (EX 488, EM500/55), and CY-5 (EX 633, EM 650LP) filter sets were used to visualize DNA, phyB-GFP, and phyBY276H, respectively.

Supplemental Data

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

Supplemental Figure 1. Schematic Diagram of Binary Vector Constructs.

Supplemental Figure 2. Spectra of PCB Adducts of Recombinant Wild-Type and YGAF Mutants of At PHYA and At PHYB.

Supplemental Figure 3. Hypocotyl Lengths of Multiple PHYB276H Transgenic Lines Grown in Continuous Red Light and Darkness.

Supplemental Figure 4. Relative Hypocotyl Lengths of Multiple PHYB276H Transgenic Lines in Phytochromes and the Chromophore-Deficient Background Grown in Darkness and Continuous Red Light.

Supplemental Figure 5. Hypocotyl Lengths of Multiple Transgenic Lines Expressing PHYB YGAF Mutant Alleles Grown in Continuous Red Light, Darkness, and Continuous Far-Red Light.

Supplemental Figure 6. Hypocotyl Lengths of Multiple PHYA1242H Transgenic Lines Grown in Continuous Far-Red Light and Darkness.

Supplemental Table 1. Transgenic Plant Lines Expressing Wild-Type and YGAF Mutant Phytochromes.

Supplemental Methods.

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Correction

Yi-shin Su and J. Clark Lagarias (2007). Light-Independent Phytochrome Signaling Mediated by Dominant GAF Domain Tyrosine Mutants of Arabidopsis Phytochromes in Transgenic Plants. Plant Cell 19: 2124–2139.

Figure 3A should be labeled D instead of R.

In Figure 7A, the labels have been corrected for the control lines. The control lines labeled phyB, phyA, phyA/phyB, and B have been changed to phyA, phyA/phyB, B#2, and B#14, respectively. The corrected figures appear below.

![Corrected Figure 3A](image)

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**Figure 3.** Phytochrome Chromophore Biosynthesis Is Required for the Gain-of-Function Activity of PHYB<sup>Y276H</sup>.

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Figure 3. (continued).
(A) Transgenic plants with or without the hy1 mutation were grown in darkness for 6 d on sucrose-free media.
(B) Relative hypocotyl length of dark-grown seedlings in (A) normalized to that of Ler shows the differential activity of PHYB^{Y276H} in chromophore-replete phyA phyB and chromophore-deficient hy1 phyA phyB backgrounds. Values are mean ± SD (n = 50).
(C) Relative hypocotyl length of seedlings grown on sucrose-free media under 20 μmol m^{-2} s^{-1} continuous R. Mean hypocotyl lengths (± SD; n = 50) are normalized to those of dark-grown Ler seedlings. The shorter hypocotyl lengths of hy1/phyA, PHYB/hy1/phyA/phyB, and PHYB^{Y276H}/hy1/phyA/phyB plants compared with the hy1/phyA/phyB parent line indicate that sufficient bilin chromophore is present in the hy1 mutant background to maintain reduced but significant signaling activity of phyB and phyB^{Y276H}.
(D) Immunoblot analysis of PHYB protein level was performed as in Figure 1.

Figure 7. Signaling Activities of phyB^{Y276H} and phyB^{Y276Q} Are Not Inhibited by Continuous FR Illumination.
(A) Comparative analysis of seedling development of 6-d-old plants grown under 20 μmol m^{-2} s^{-1} continuous FR. One representative line from transformation of phyA/phyB is shown.
(B) Comparative fluence response curves for hypocotyl growth indicate that phyB^{Y276H}-mediated growth suppression is FR light independent. Each data point represents the mean of 50 seedlings ± SD.
(C) Comparative analysis of seed germination phenotypes of the wild type and phyA phyB mutant transformed with genomic PHYB^{Y276H} or PHYB^{Y276Q} alleles.
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