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Genetic and Molecular Analysis of Phytochromes from the Filamentous Fungus *Neurospora crassa*

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Phytochromes (Phys) comprise a superfamily of red-/far-red-light-sensing proteins. Whereas higher-plant Phys that control numerous growth and developmental processes have been well described, the biochemical characteristics and functions of the microbial forms are largely unknown. Here, we describe analyses of the expression, regulation, and activities of two Phys in the filamentous fungus *Neurospora crassa*. In addition to containing the signature N-terminal domain predicted to covalently associate with a bilin chromophore, PHY-1 and PHY-2 contain C-terminal histidine kinase and response regulator motifs, implying that they function as hybrid two-component sensor kinases activated by light. A bacterially expressed N-terminal fragment of PHY-2 covalently bound either biliverdin or phycocyanobilin in vitro, with the resulting holoprotein displaying red-/far-red-light photochromic absorption spectra and a photocycle in vitro. cDNA analysis of *phy-1* and *phy-2* revealed two splice isoforms for each gene. The levels of the *phy* transcripts are not regulated by light, but the abundance of the *phy-1* mRNAs is under the control of the circadian clock. Phosphorylated and unphosphorylated forms of PHY-1 were detected; both species were found exclusively in the cytoplasm, with their relative abundances unaffected by light. Strains containing deletions of *phy-1* and *phy-2*, either singly or in tandem, were not compromised in any known photoresponses in *Neurospora*, leaving their function(s) unclear.

Light is essential for life on earth, serving as a primary energy source for organisms ranging from single-celled bacteria to higher plants. Plants, in particular, contain a complex network of light perception and signal transduction systems that enables them to track and respond to fluctuations in multiple parameters within their light environment, including intensity, directionality, daily duration, and spectral quality. Plants employ at least three photoreceptor types for light perception, the cryptochromes and phototropins, which monitor the blue/UV region of the spectrum, and the phytochromes (Phys), which monitor the red-light (R)/far-red-light (FR) region (52). The generic Phy in higher plants is a soluble homodimer, consisting of two ~120-kDa polypeptides, each bearing a single bilin (or linear tetrapyrrole) chromophore. The bilin is bound covalently by an autocatalytic mechanism to an N-terminal pocket that, once assembled, serves as the sensory module (73). Through interactions between the bilin and the apoprotein, Phys reversibly photointerconvert between two stable conformers, an R-absorbing Pr form that is biologically inactive and an FR-absorbing Pfr form that is biologically active. Via interconversion between Pr and Pfr, Phys act as reversible switches in photoperception. The C-terminal half of Phys bears contacts for dimerization and sensory output activities, the nature of which is currently unclear (reviewed in references 58 and 71).

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The deluge of genomic sequence information has greatly expanded the Phy family, with new members added for plants, as well as cyanobacteria, eubacteria, actinobacteria, filamentous fungi, and possibly even slime molds (reviewed in references 50 and 72). Some Phys were even discovered that employ Pr as the active form or that work backwards, using Pfr and not Pr as the ground state. The breadth of this collection highlights the importance of light to both photosynthetic and nonphotosynthetic organisms, and it now provides new models to decipher the functions of these pigments. The filamentous fungus *Neurospora crassa* in particular offers the possibility of studying Phy function in a simple, genetically tractable eukaryote without the complications of photosynthesis.

A number of photoresponses are already known in *Neurospora* that are activated by blue light, including mycelial carotenoid biosynthesis, formation of vegetative spores (macroconidia), and phase shifting and photosuppression of circadian rhythms. Blue light also exerts its influence during the sexual cycle by inducing photodifferentiation and positive phototropism of perithecial beaks (reviewed in reference 20). Two important components of blue-light perception are the transcription factors WHITE COLLAR-1 (WC-1) and WHITE COLLAR-2 (WC-2) (4, 45, 46), which function as heterodimers in activating a battery of light-induced genes (22, 29). WC-1 binds a flavin chromophore and acts as the photoreceptor. Blue-light absorption triggers the multimerization of WC-1 and WC-2, presumably to allow multiple WC activation domains to act in tandem (22, 29). *wc-1*^{KO} and *wc-2*^{KO} strains are "blind" to all known blue-light-regulated processes (13, 16, 42, 44), which suggested initially that WC-1 is the only photoreceptor in this species. However, three more potential blue-light photoreceptors have been identified in the complete genomic sequence, including VIVID (VVD) and sequence relatives of

plant phototropin (PHOT) and cryptochrome (CRY) (20, 21). Upon binding a flavin to a light-oxygen-voltage domain similar to the LOV domain of WC-1, VVD photoregulates the gating of light input to the circadian clock, down regulates light-induced genes, and activates via a photoadaptation response a collection of light-induced genes in response to increased illumination (30, 65). VVD is expressed only in the light; this expression requires a functional WC-1/WC-2 complex, explaining why *wc-1*^{KO} and *wc-2*^{KO} strains are insensitive to blue-light responses regulated by VVD (13, 65). Similarly, the expression of *Neurospora* CRY is photoregulated by the WC-1/WC-2 complex (A. C. Froehlich, J. Loros, and J. C. Dunlap, unpublished data). Although the role(s) of this CRY is still unclear, it is possible that some of the actions attributed to WC-1/WC-2 are in fact directed by CRY. The PHOT-like gene does not appear to be light regulated, and a link to blue-light photobiology of *Neurospora* remains to be established (A. Mehra, C. Heintzen, and J. C. Dunlap, unpublished results). A possible green-light photoreceptor, NOP-1, whose sequence is related to that of opsin, has also been identified (6). Although no light-specific function has been ascertained for NOP-1, it assembles in vitro with all-*trans* retinal to form a green-light-absorbing pigment (7).

The discovery of two Phy sequences in the *Neurospora* genome (8, 24) adds additional complexity to its photobiology, especially since no R/FR-regulated biology has been found in this fungus. In the present study, we initiated a molecular genetic, biochemical, and physiological investigation of *phy-1* and *phy-2*. Analysis of the corresponding cDNAs provided the full-length amino acid sequences and revealed alternative splice isoforms that would synthesize severely truncated versions of each protein. Like other members of the Phy superfamily, recombinant PHY-2 assembles with bilins in vitro to generate R/FR photochromic pigments. We found that light does not regulate the abundance of either set of mRNAs, but the abundance of the *phy-1* transcripts is circadian-clock regulated. The PHY-1 protein exists in both unphosphorylated and phosphorylated forms; this phosphorylation state is not regulated by the circadian clock and does not appear to affect the abundance or subcellular localization of the holoprotein. A thorough study of the previously documented photobiology in *Neurospora* failed to identify functions for PHY-1 and -2, suggesting that these pigments control one or more novel photoresponses. To our knowledge, this report is the first in-depth analysis of Phys from the fungal kingdom.

MATERIALS AND METHODS

Strains and growth conditions. For most studies, the *N. crassa* strains used were as follows: 328-4 (*bd A*), 343-26 (*bd*; *phy-1*^{AF1}), 343-35 (*bd*; *phy-2*^{AF1} *a*), 343-49 (*bd*; *phy-1*^{AF1}; *phy-2*^{AF1} *a*), and 233-2 (*bd*; *wc-1*^{ER53} *a*) (42). The race tube assays used 343-44 (*bd*) and 343-42 (*bd*). For the analysis of light-induced transcription, 333-6 (*bd*) and 333-2 (*bd*; *phy-1*^{AF1} *A*) were used. The above-mentioned strains contain the *band* (*bd*) mutation, enabling the observation of the circadian control of conidiation. The R/FR growth rate assays employed 379-17, 379-8 (*phy-1*^{AF1}), 379-7 (*phy-2*^{AF1}), and 379-16 (*phy-1*^{AF1}; *phy-2*^{AF1}). Strain 379-4 was used for the microarray experiments.

The general conditions for growth and manipulation are described elsewhere (18). Liquid culture experiments were performed at 25°C as previously described (2, 17), using a growth medium containing 1× Vogel's, 2% glucose, 0.5% arginine, and 50 ng/ml biotin. For the expression analysis of *phy-1*, *phy-2*, *frq*, *al-2*, and *con-6* following light exposure, cultures were irradiated with ~40 μmol photons/m^2/s of white light (GE cool white fluorescent bulbs; F20T12.CW) of the indicated durations, following a 20-h dark incubation. R and FR experiments were performed using an E-30LED growth chamber equipped with R- and FR-emitting diodes (Percival Scientific, Inc., Perry, IA).

Race tube experiments using a medium containing 1× Vogel's, 0.1% glucose, 0.17% arginine, 50 ng/ml biotin, and 1.5% Bacto agar have been described elsewhere (2). Race tubes entrained with a constant light (LL) to constant dark (DD) transfer were grown in LL for 24 h (at 25°C) and then shifted to DD (at 25°C). Conidiation density was determined and calculations of period length were performed using CHRONO (59). For the growth rate studies, race tubes were inoculated and placed at 25°C in LL for 48 h; the ends were then sealed with parafilm, and the race tubes were transferred to the appropriate lighting conditions (25°C).

The perithecial phototropism assays were performed essentially as described by Harding and Melles, except that Westergaard's synthetic crossing medium was used (18, 28). In brief, strains were inoculated onto crossing plates and kept at 25°C in DD for 7 days. Fifty microliters of conidial suspension (strain 328-4) was pipetted as a thin line along the diameter of the plate, and the plates were returned to the dark. The plates were exposed to a 12-h light–12-h dark cycle (the light was provided by fluorescent lighting), with the plates positioned in a box with a 4-cm-wide opening so that the direction of the light was perpendicular to the line of perithecia. Perithecial beaks were scored ~14 days after inoculation, with the orientation of the beaks (toward, neutral, or away) scored relative to the direction of the light. For the dark-grown samples, an arbitrary "direction" was chosen for scoring purposes.

Targeted disruption of *phy-1* and *phy-2*. Constructions were generated in which the 5′ and 3′ sequence flanking each open reading frame (ORF) was appended to the hygromycin B phosphotransferase (*hph*) coding region, whose expression was driven by the *Aspergillus nidulans trpC* promoter, thus allowing selection by hygromycin B resistance. These deletion constructions were transformed into *Neurospora*, and hygromycin-resistant transformants containing single homologous integrations of the DNA were backcrossed to the wild type (wt) to obtain homokaryotic deletion strains, as judged by DNA gel blot analysis of genomic DNA (data not shown). A *phy-1*^{AF1}/*phy-2*^{AF1} double mutant was created by using the single mutants as parents in a sexual cross following standard methods.

The *phy-2* disruption was prepared with the pAF68 construct, which contains the *trpC*::*hph* selection marker flanked 5′ and 3′ by 0.9 kb and 1.7 kb, respectively, of the *phy-2* locus. pAF68 was created by three subsequent subcloning steps. The 5′ *phy-2* sequence was PCR amplified from genomic DNA using primers ACF93 (tacttagggcccTTCAAGTTACTAGCCCTATCACCAC) and ACF89 (ACTCGCATATGAACAGATCAGTGTC), digested with ApaI/XbaI, and ligated into ApaI/SpeI-digested pAF35 (22), resulting in pAF63. (Primer sequences in uppercase match the sequence of the target site, whereas lowercase sequences were added to the primer to aid in subsequent cloning of fragments.) The 3′ *phy-2* sequence was PCR amplified using primers ACF86 (ataagaatgcgg ccgcGTCTCGAATCTCAAGCCACCTTCAC) and ACF90 (CGCTTATGG-GAATAGTGTGCTGATG), digested with BamHI/NotI, and ligated into BamHI/NotI-digested pAF63, resulting in pAF64. The EcoRI fragment from pCSN44 (69), containing the *trpC* promoter, was ligated into EcoRI-digested pAF64, resulting in pAF68.

The pAF69 construct was used to generate the *phy-1* disruption and contains *trpC*::*hph* flanked 5′ and 3′ by 1.4 kb and 1.4 kb, respectively, of the *phy-1* locus. pAF69 was created by three subsequent subcloning steps. The 3′ *phy-1* sequence was PCR amplified from genomic DNA using primers ACF92 (GTACAAAGT TACCCACGCCTTGAAC) and ACF88 (ataagaatgcggccgcCCACTATACTA CAATCCCGCAGAAC), digested with MluI/NotI, and ligated into MluI/NotI-digested pAF35, resulting in pAF65. The 5′ *phy-1* sequence was PCR amplified using primers ACF87 (gctctagaGATATCACGCAGCTTCCTAAACAACAC) and ACF91 (ggactagtTATCTTCGGGAATGTCTGGCAAGTC), digested with SmaI/SpeI, and ligated into SmaI/SpeI-digested pAF65, resulting in pAF66. The SpeI/MluI fragment from pCSN44 containing the *trpC* promoter was ligated into SpeI/MluI-digested pAF66, resulting in pAF69.

The disruption constructs were digested (pAF68 with XhoI and pAF69 with KpnI), and the fragments containing the gene-*hph*-gene region were gel purified. The DNA fragments were transformed into *N. crassa* strain 87-74 (*his-3 bd a*) for pAF69 and strain 87-3 (*bd a*) for pAF68, following standard transformation protocols.

Quantitative real-time PCR analysis. Total RNA was prepared by the following procedure. Frozen mycelial samples were powdered using a mortar and pestle at liquid nitrogen temperatures, and 100 mg of this powder was homogenized in 1 ml of Trizol (Gibco BRL) by repeated pipetting. The extract was clarified by centrifugation at 4°C for 10 min at 12,000 × *g*, and 800 μl of the supernatant was mixed with 200 μl of chloroform for ~15 s. Samples were

centrifuged at 12,000 × *g* for 15 min at 4°C. The top aqueous phase (250 μl) was mixed with an equal volume of isopropyl alcohol and incubated for 10 min at room temperature. The RNA was collected by centrifugation for 10 min at 12,000 × *g* at 4°C. The pelleted RNA was washed with 1 ml of 75% ethanol, air dried, and resuspended in 100 μl of RNase-free water. The RNA concentration was determined by absorbance at 260 nm.

The RNA was treated with DNase I (Gibco-BRL) and used in a reverse-transcriptase reaction with random hexamers. Quantitative real-time PCR was performed with the generated cDNA, a pair of gene-specific primers, and SYBR Green reaction mixture (Applied Biosystems) using an ABI Prism 7700 Sequence Detection System (PE Biosystems) according to the manufacturer's instructions. The ribosomal L6 protein gene in *Neurospora*, which is not regulated by light or the circadian clock, was used for normalization (55). The gene-specific primer pairs were as follows: *frq*, 352F (TCGACATCGCAGAGGAGAAA)/417R (CAACGAAACCCCAGACGAGT); *L6*, L6R (GCGGATGGTCTTGCGG)/L6F (CAGAAATGGTACCCTGCTGAGG); *al-2*, al2-1 (CGACTCCGCATTGACCTGAT)/al2-2 (AGACCTCACGGCGAGATTTG); and *con-6*, con6-1 (TAATGTTTCCGAGGAAGCCA)/con6-2 (GGGTTCTTGTCGCCGTCGTC). Statistical differences between RNA transcript levels were tested by one-factor analysis of variance and a subsequent post hoc Dunnett's *t* test, where significance was set at a P value of <0.05.

Microarray analysis. Mycelial tissue was grown in liquid culture at 25°C for 10 h under fluorescent lights, transferred to DD, and then transferred to R or FR (~45 μmol photons/m^2/s) for 0, 0.5, 2, or 24 h. The duration of growth in DD was varied so that all cultures were grown for a total of 38 h. Two sets of biologically independent samples were harvested. RNA preparation, microarray preparation, hybridization, and scanning were done essentially as previously described (55). Each slide was hybridized with two targets, one "experimental," which was made from one of the R- or FR-treated samples, and one "reference RNA," which consisted of a DD sample. Experimental and reference RNAs were labeled with Cy3 and Cy5 dyes, respectively, and the dyes were switched in one of the biologically independent replicas of each sample. Data were analyzed using Gene Traffic Duo (Iobion Informatics LLC).

cDNA analysis. RNA was extracted from strain 328-4, which was exposed to a 30-min fluorescent-light pulse following 20 h growth in the dark. The RNA was DNase treated as described for the real-time PCR analysis and used to generate cDNA by reverse transcription with the Invitrogen Superscript Primary Strand kit and the Oligo dT primer. The cDNA, together with the following gene-specific primers, was subjected to PCR: *phy-1*, ACF87 (gctctagaGATATCACGCAGCTTCCTAAACAACAC)/ACF123 (CCGGTTTAGGTTGCTCGCCATTTAC), and *phy-2*, ACF85 (ggaattccTTCAAGTTACTAGCCCTATCACCAC)/ACF136 (ACTATTCCCATAAGCGAGCC). The products were separated by gel electrophoresis; the correct-size fragments were extracted from the gel and cloned into pCR4-TOPO (Invitrogen TOPO TA cloning kit). Multiple clones for each gene were then sequenced by standard methods and compared to the sequence data generated by the *Neurospora* genome project (http://www-genome.wi.mit.edu/annotation/fungi/neurospora/). Other GenBank entries for *phy-1* and *phy-2*, based on predictions of intron/exon structure, differ from our empirically generated cDNA analysis. Alignment of the putative PLD, GAF, and PHY regions from a collection of representative Phys were performed using CLUSTALX MAC v1.81 (http://www.embl.de/~chenna/clustal/darwin/) and displayed using MACBOX SHADE v2.15 (Institute of Animal Health, Pirbright, United Kingdom).

PHY-1 antibody production and protein analysis. The DNA sequence encoding the first 957 residues of PHY-1 was PCR amplified from the full-length cDNA clone (see above) using primers designed to add an NdeI site proximal to the start codon and a HindIII site immediately after codon 957. The product was digested and inserted into pET24a (Novagen), and the resulting C-terminally six-His-tagged expression construction was transformed into BL21-Codon Plus-RIL cells (Stratagene). Transformed cells were grown to logarithmic phase and induced with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 4 h at 37°C. Following sonication of the cells, a majority of the six-His-tagged PHY-1 polypeptide was in the inclusion body fraction, necessitating the addition of 6 M urea for solubilization. This soluble protein was purified by nickel chelate affinity chromatography using Ni-NTA Superflow agarose (QIAGEN) according to the manufacturer's directions. The eluate was dialyzed against phosphate-buffered saline buffer (137 mM NaCl, 10 mM KH_2PO_4, 100 mM Na_2HP0_4, 27 mM KCl, pH 7.4) and used directly as the antigen for injection into rabbits following standard procedures (Pocono Rabbit Farm and Laboratory, Canadensis, PA). *Neurospora* protein extractions, cellular fractionations, and immunoblot analyses were performed as previously described (22). Lambda protein phosphatase (λPPase) (New England Biolabs) treatments were performed at 30°C for 50 min on whole-protein extracts in which the EDTA concentration in the extraction buffer was reduced from 5 mM to 1 mM. Sodium vanadate was included to a final concentration of 20 mM.

Spectral analysis of PHY-2 (1-515). The region containing the chromophore-binding domain of PHY-2 (residues 1 to 515) was PCR amplified from reverse-transcribed mRNA from *Neurospora* mycelia using primers designed to add an NcoI site proximal to the start codon and a NotI site immediately after codon 515. The product was digested with NcoI and NotI and inserted into pET28b (Novagen), similarly digested, for expression in *Escherichia coli* with a C-terminal His6 tag. The PHY-2 (1-515) polypeptide was expressed in BL21 Codon Plus (DE3)-RIL cells as described previously (5) for 3 h following induction with IPTG. The crude soluble lysate was incubated for 50 min with either 13 μM biliverdin (BV) (Porphyrin Products, Logan, UT) or 10 μM phycocyanobilin (PCB) (provided by P. S. Song). The proteins were then purified from the soluble cell lysate by nickel chelate affinity chromatography (Novagen), and the buffer was exchanged for 70 mM Tris-HCl (pH 8.0)–1 mM Na_4-EDTA by ultrafiltration with a Centriprep YM-10 column. Absorbance spectra were determined after saturating R (690-nm) and FR (775-nm) irradiations. Binding of bilins was assayed by zinc-induced fluorescence of the holoprotein following sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (5).

Nucleotide sequence accession numbers. The DNA and protein sequence information has been deposited at the National Center for Biotechnology Information, and the GenBank accession numbers are DQ128077 for *phy-1* and DQ128076 for *phy-2*.

RESULTS

Organization of the *Neurospora* Phys. The genome of *Neurospora crassa* contains two genes that code for proteins with homology to previously identified Phys (5, 8, 10, 24). Following standard *Neurospora* nomenclature, these genes have been named *phytochrome-1* (*phy-1*) (NCU04834.1; linkage group VI) and *phytochrome-2* (*phy-2*) (NCU05790.1; linkage group VII) (8). To help map the intron/exon structures of *phy-1* and *phy-2* and thus deduce the full-length amino acid sequences of corresponding apoproteins, cDNAs spanning the entire ORFs of both genes were cloned and sequenced. The *phy-1* transcript is at least 4.9 kb and contains two introns of 57 and 87 bp (Fig. 1A). The *phy-2* transcript is at least 3.9 kb and contains five introns ranging in size from 57 to 76 bp (Fig. 1A). We did not map the exact 5′ and 3′ ends of the transcripts, so the transcription start and polyadenylation sites are not yet known.

The *phy-1* and *phy-2* loci are predicted to encode 1,536- and 1,169-amino-acid proteins, respectively. Like other members of the Phy superfamily, the PHY-1 and PHY-2 proteins both contain a signature cyclic GMP phosphodiesterase/adenyl cyclase/FhlA (GAF) domain, which clearly distinguishes them from other GAF-containing proteins (Fig. 1). Bracketing the GAF domain are a Per-Arndt-Sim-like domain (PLD) and a PHY domain. Together with the GAF domain, the PLD and the PHY domain act as the sensory input module by forming the pocket necessary for bilin binding and generating the appropriate contacts for stabilizing the Pr and Pfr forms. The C-terminal portions of PHY-1 and PHY-2 bear easily recognizable histidine kinase (HK) and receiver response (RR) domains, implicating these regions as the sensory output modules for two-component (TC) histidine kinase signaling cascades. The main difference between *Neurospora* PHY-1 and PHY-2 is an extension of ~160 amino acids at the N terminus and an additional ~200 amino acids separating the HK motif from the RR domain in PHY-1.

Alternative splicing of the *phy-1* and *phy-2* genes. Analysis of a population of *phy-1* and *phy-2* cDNAs detected alternatively spliced transcripts from both loci which could synthesize severely truncated polypeptides. The *phy-1* alternative transcript

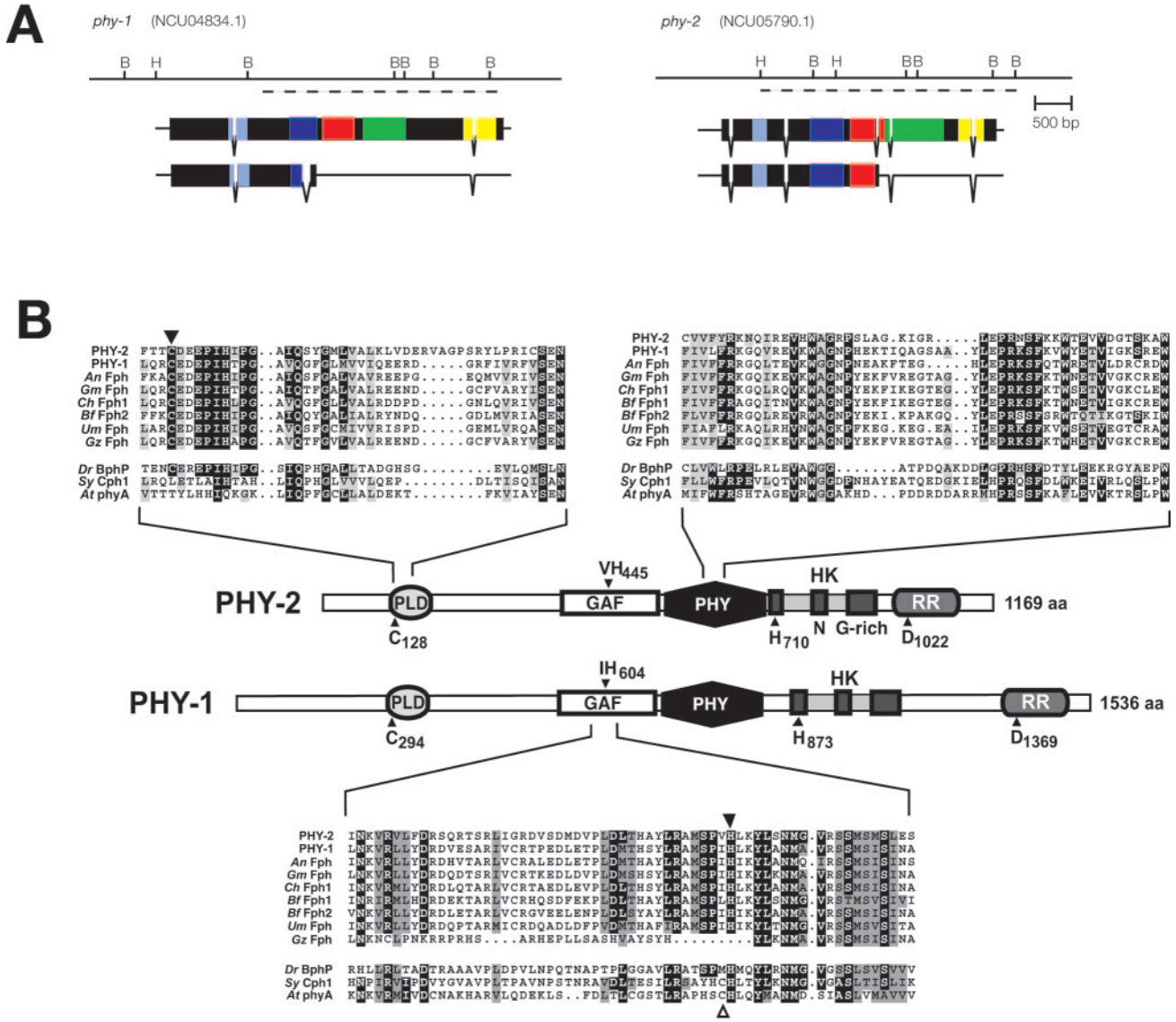

FIG. 1. Domain structure and amino acid sequence comparison of two *Neurospora* phytochromes. (A) Domains and intron/exon structures of PHY-1 (left) and PHY-2 (right). The two splice variants detected for each Phy are depicted directly below a schematic representation of the genomic DNA of each locus. The thin black line at the top represents the genomic locus, with letters indicating the relative positions of restriction sites: B, BamHI; H, HindIII. The thicker lines indicate the coding regions, with introns indicated by gaps. Conserved domains are highlighted: light blue, PLD; dark blue, GAF domain; red, PHY domain; green, HK domain; yellow, RR domain. The dashed lines indicate the genomic regions deleted in the deletion strains. (B) Amino acid sequence alignments of the PLD and the GAF and PHY domains of PHY-1, PHY-2, several predicted fungal phytochromes (Fphs), and single representatives of the BphPs, Cphs, and plant phytochromes. The position of each domain in Phys is shown using PHY-2 and PHY-1 to help locate the region in the Phy polypeptide. The top left alignment compares the sequences surrounding the N-terminal PLD used by *Agrobacterium tumefaciens* BphP1 to covalently bind bilins by a cysteine thioether linkage. The cysteine is identified by the arrowhead. The top right alignment shows the PHY domain. The bottom alignment includes the region of the GAF domain that binds bilins via a cysteine thioether linkage in Cphs and plant Phys or that may bind bilins by a histidine Schiff base linkage in some BphPs. The cysteine and histidine residues are identified by the open and closed arrowheads, respectively. Black and gray boxes denote identical and similar residues, respectively. Shown are *Arabidopsis thaliana* (*At*), *Aspergillus nidulans* (*An*), *Botryotinia fuckeliana* (*Bf*), *Cochliobolus heterostrophus* (*Ch*), *Deinococcus radiodurans* (*Dr*), *Giggerella moniliformis* (*Gm*), *Gibberella zeae* (*Gz*), *Synechocystis* PCC6803 (*Sy*), and *Ustilago maydis* (*Um*).

contains an additional intron in the GAF domain, resulting in a frame shift that upon translation would synthesize a 591-amino-acid polypeptide encompassing the N-terminal 564 residues plus an additional 27 residues. This truncation, if expressed, would lack the majority of the GAF domain and the distal PHY, HK, and RR domains, strongly suggesting that it would not be photochemically active (Fig. 1A). The alternative transcript from *phy-2* retains the third intron, located in the coding sequence for the PHY domain. If expressed, the resulting 684-amino-acid polypeptide, containing the N-terminal 670 residues plus an additional 14 residues encoded by an intron sequence, would have an intact GAF domain but would be

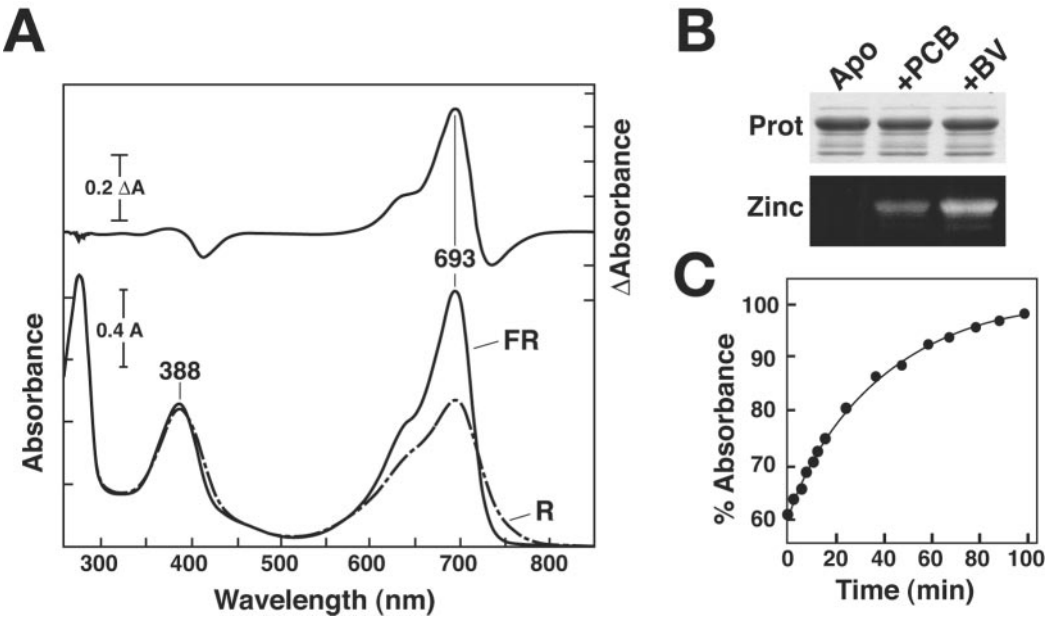

FIG. 2. Assembly and spectral properties of a recombinant N-terminal fragment of PHY-2 containing amino acids 1 to 515. (A) Absorbance and difference spectra of the PHY-2 (1-515) holoprotein assembled with BV following saturating irradiations with R and FR. The absorbance maxima of Pr are indicated. (B) Covalent binding of BV and PCB to PHY-2 (1-515). Apo–PHY-2 (1-515) was incubated with or without BV and PCB, purified by nickel-chelate affinity chromatography, and then subjected to SDS-PAGE and either stained for protein with Coomassie blue (top) or assayed for the bound bilin by zinc-induced fluorescence (bottom). (C) Dark reversion of PHY-2 (1-515) from Pfr to Pr. The holoprotein assembled with BV was photoconverted to an equilibrium mixture containing Pfr and Pr and then incubated at 24°C in the dark. Reversion of Pfr to Pr was monitored by the increase in absorbance at 693 nm.

missing part of the PHY domain and the entire HK and RR domains, suggesting that it could be photochemically active but incapable of signal transmission by itself (Fig. 1A). These alternative transcripts were detected from analysis of only a small collection of cDNAs (one out of eight *phy-1* cDNAs and two out of four *phy-2* cDNAs arising from the alternative spliced form), suggesting that they are relatively abundant and that more splice variants could be detected by more exhaustive cDNA analyses. While the function(s) of these alternative transcripts remains unknown, an intriguing possibility is that they express Phy variants with novel regulatory roles.

Assembly of PHY-2 apoprotein with bilins. To confirm that the PHY proteins indeed behave as typical Phys, we attempted to assemble the PHY-2 apoprotein with bilins and to examine the spectral behavior of the resulting chromoproteins. Unfortunately, the full-length version of PHY-2 expressed poorly in *Escherichia coli*, with nearly all of the protein found in the insoluble fraction. Truncations missing the C-terminal RR, HK, and PHY domains were equally insoluble. However, small amounts of soluble protein were obtained for a fragment encompassing just the N-terminal 515 residues. Because this region included the entire GAF domain necessary for autocatalytic attachment of the bilin and the PLD cysteine that likely provides the bilin attachment site, its use allowed us to test whether PHY-2 can bind bilins. As can be seen in Fig. 2, PHY-2 (1-515) readily bound both BV and PCB to form R/FR photochromic adducts. The characteristic Pfr absorbance was apparent upon BV binding, with a peak maximum of 693 nm (Fig. 2A). R irradiation converted this Pr to a "Pfr-like" state. While this Pfr form was substantially bleached relative to that obtained for full-length Phys, its absorption spectrum was reminiscent of those seen with similar truncations of bacterial and plant Phys missing the PHY domain (14, 31, 56). Consistent with the importance of the PHY domain in stabilizing the Pfr form (31), the BV adduct of PHY-2 (1-515) was unstable, as after photoconversion by R, Pfr rapidly cycled back to Pr by 100 min in the dark (Fig. 2C). The capacity to attach bilins covalently was confirmed by zinc-induced fluorescence of the chromophore following SDS-PAGE of the holoprotein. Incubation of Phy-2 (1-515) with either BV or PCB generated fluorescent adducts that were stable even following SDS denaturation (Fig. 2B).

Generation of gene replacements for *Neurospora phy-1* and *phy-2*. To help define the function(s) of PHY-1 and PHY-2 in *Neurospora*, portions of the ORF encoding each protein were individually deleted (Fig. 1A). Our designs of the deletion constructions were based on the initial predictions of the *phy-1* and *phy-2* genes in the partially annotated *Neurospora* genomic database. However, as additional genomic sequence information (http://www-genome.wi.mit.edu/annotation/fungi/neurospora/) and more careful annotations of each locus using cDNAs became available, we realized that these predictions had omitted coding sequence at the 5′ ends. The *phy-1* and *phy-2* deletions, therefore, still retain the potential to express the first 430 and 160 amino acids, respectively, so these alleles are designated *phy-1*$^{\Delta AF1}$ and *phy-2*$^{\Delta AF1}$. Even if expressed, these truncations would be expected to act as functional knockouts, given the absence of the GAF domain and other functional domains of the proteins.

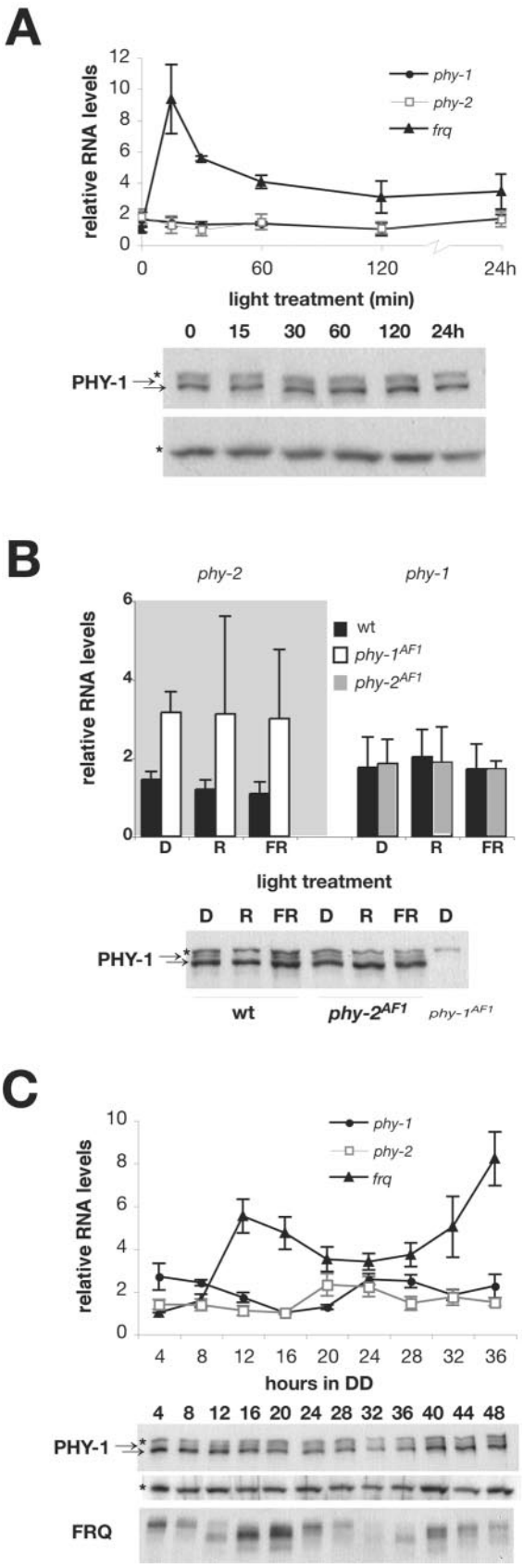

FIG. 3. Light and circadian regulation of *phy-1* and *phy-2* transcripts and PHY-1 protein. (A) (Top) Quantitative real-time PCR analysis of *phy-1*, *phy-2*, and *frq* transcript levels following white-light treatments varying from 0 to 24 h. The error bars are the standard errors of the mean (SEM); $n = 3$. (Bottom) Western blot analysis of PHY-1 following white-light treatments varying from 0 to 24 h. The arrows highlight the two forms of PHY-1. The asterisks indicate nonspecific bands which cross-react with the PHY-1 antisera. The lower blot is shown as a loading control. (B) (Top) Quantitative real-time PCR analysis of *phy-1* and *phy-2* transcript levels in the dark (D) or following 12 h of R or FR treatment. *phy-1* transcript levels were determined in wt and *phy-2*^{AF1} strains. *phy-2* transcript levels were determined in wt and *phy-1*^{Af1} strains. The data were normalized to the lowest values for each transcript, which were set to 1. The absolute levels of *phy-1* and *phy-2* transcripts, therefore, cannot be directly compared. The error bars are the SEM; $n = 3$. (Bottom) Western blot analysis of PHY-1 in the dark or following 12 h of red- or far-red-light treatment in wt and *phy-2*^{AF1} strains. The arrows and asterisks are the same as in panel A. (C) (Top) Quantitative real-time PCR analysis of *phy-1*, *phy-2*, and *frq* transcript levels in constant darkness throughout 1.5 circadian cycles. The error bars are the SEM; $n = 4$. (Bottom) Western blot analysis of PHY-1 and FRQ in constant darkness over a 48-h period. The arrows and asterisks are the same as in panel A.

Regulation of *Neurospora phy-1 and phy-2* gene expression. In higher plants, the levels of some *phy* transcripts are regulated by changes in both the quantity and quality of light (15). Transcription of the Phys, *cph1* and *cph2*, from the cyanobacterium *Synechocystis* PCC6803 is also down regulated by light, suggesting that similar regulation may occur in microorganisms (26, 57). To determine if the *Neurospora phy* genes are light regulated, quantitative real-time PCR analysis in which either the quantity or quality of light was varied was performed on RNA isolated from fungal mycelia. In one experiment, the wild-type strain was subjected to increasing doses of white light for 15 min to 24 h. (Similar treatments of *Arabidopsis thaliana* seedlings were previously shown to depress *PHYA* transcript levels [15]). Under these conditions, the levels of *frequency* (*frq*) mRNA, previously found to be rapidly induced by light (17), displayed a strong induction ($F_{5,12} = 6.5$; $P = 0.0039$) (Fig. 3A). However, for the *phy-1* and *phy-2* transcripts, there was no detectable change in mRNA abundance following any of the light treatments compared to an untreated control ($T = 0$ min) (*phy-1* [$F_{5,12} = 0.6$; not significant] and *phy-2* [$F_{5,12} = 0.5$; not significant]).

The light source used for the prior experiment was enriched in light between 400 and 550 nm with relatively low amounts in the R and FR regions of the spectrum (data not shown). If *Neurospora phy* genes are autoregulated or reciprocally regulated in a manner similar to that of plant *PHYA*, greater amounts of R and FR may be required to elicit changes in transcript levels. To test specifically for R/FR control, we continuously irradiated mycelia with high fluence rates (~30 μmol photons/m^2/s) of monochromatic R and FR provided by light-emitting diodes. (For *Arabidopsis*, as little as 0.3 μmol photons/m^2/s of FR can induce significant changes in *PHYA* mRNA levels after 24 h [9]). In addition to a wild-type strain, *phy-1* transcript levels were monitored in a *phy-2*^{AF1} strain and *phy-2* transcript levels were monitored in a *phy-1*^{AF1} strain. There was no difference in *phy-1* ($F_{2,6} = 1.6$; not significant) or *phy-2* ($F_{2,6} = 0.17$; not significant) transcript levels following the R or FR treatments in the wild type (Fig. 3B, top), suggesting that *phy-1* and *phy-2* transcripts are not regulated by R or FR.

Furthermore, we found no significant difference between the transcript levels in the wt and *phy* deletion strains, suggesting that the *Neurospora* PHYs are not required for reciprocal transcriptional regulation. Whether each of the PHYs autoregulates its own expression is not yet known.

Under a 12-h light–12-h dark photoperiod, all five *Arabidopsis PHY* genes (*PHYA* to *PHYE*) display diurnal patterns of expression, with transcript levels peaking during the light period. After entrainment, these oscillations persist during continuous light or dark, indicating that the expression of *Arabidopsis PHY* genes is regulated by the circadian clock (36, 70). To test for circadian regulation of the *phy-1* and *phy-2* transcripts, mycelia of approximately the same developmental age were harvested at 4-h intervals after transfer from light to DD (Fig. 3C). Through the action of the blue-light-sensing WC-1 photoreceptor, the light-to-dark transfer sets the clock to subjective dusk, after which the clock continues to run in constant darkness (reviewed in reference 23). Under these conditions, the circadian-clock-regulated *frq* transcript displays a strong circadian rhythm, with mRNA levels reaching a peak after ~12 to 16 h in constant darkness ($F_{8,27} = 7.3$; $P = 0.0001$) (2). With regard to *phy-2*, its transcript abundance fluctuated only slightly without any apparent circadian regulation after the dark transfer ($F_{8,27} = 2.0$; not significant). In contrast, the *phy-1* transcript appeared to be regulated in a circadian fashion ($F_{8,27} = 2.8$; $P < 0.05$), with transcript abundance gradually decreasing following the light-to-dark transfer, reaching a low after 16 to 20 h in the dark and then rising to a peak at 24 to 28 h, corresponding to circadian time 15 to 19, or subjective early evening (Fig. 3C). The *phy-1* transcript oscillated with an ~2-fold amplitude. *phy-1* and *phy-2* transcripts were not examined under constant-light conditions.

The circadian oscillation and the lack of light regulation of the *phy-1* transcript were confirmed by Northern blot analysis (data not shown). *phy-1* mRNA ran slightly higher than the largest *Neurospora* rRNA species, indicating that the *phy-1* transcript was at least 5 kb, in agreement with the cDNA analysis. We were unable to detect *phy-2* transcript by Northern blotting, suggesting that the transcript is expressed at very low levels under the growth conditions used here.

PHY-1 is modified by phosphorylation. To help detect the PHY-1 protein, we generated antiserum against the N-terminal 957 residues of PHY-1. (We also generated antiserum against the PHY-2 polypeptide but were unable to detect PHY-2 in *Neurospora* extracts.) The specificity of the antiserum was demonstrated by immunoblot analysis of extracts from wild-type and *phy-1*^{AF1} strains (Fig. 3B, bottom). At the approximate predicted molecular mass of PHY-1 (~168 kDa), two PHY-1 proteins were detected in wild-type extracts but were absent in extracts from the *phy-1*^{AF1} strain (Fig. 3B). The faster-migrating species was the more abundant form. The presence of two species suggested that PHY-1 is posttranslationally modified, with the slower-migrating species representing the modified form. To test whether phosphorylation might be involved, we treated crude extracts prior to SDS-PAGE with λPPase, a vanadate-sensitive phosphatase that can remove phosphates bound to serine, threonine, tyrosine, and histidine. The efficacy of this phosphatase treatment was confirmed using the highly phosphorylated FRQ protein as a control (25). As can be seen in Fig. 4A, FRQ migrates as a high-molecular-mass smear of species in untreated crude extracts (lane1). However, upon λPPase treatment, a lower-molecular-mass species (lane 2), whose formation can be effectively blocked by pretreatment with vanadate (lane 3), becomes predominant. When PHY-1 was examined following the same treatments, the slower-migrating PHY-1 species (present between the faster-migrating PHY-1 species and a slower-migrating nonspecific band detected with the anti-PHY-1 antibodies) was no longer present, presumably having been converted into the faster-migrating PHY-1 (lane 2). The conversion of the slower-migrating form to the faster-migrating form was fully inhibited by sodium vanadate, indicating that the change in mobility was not due to the activities of

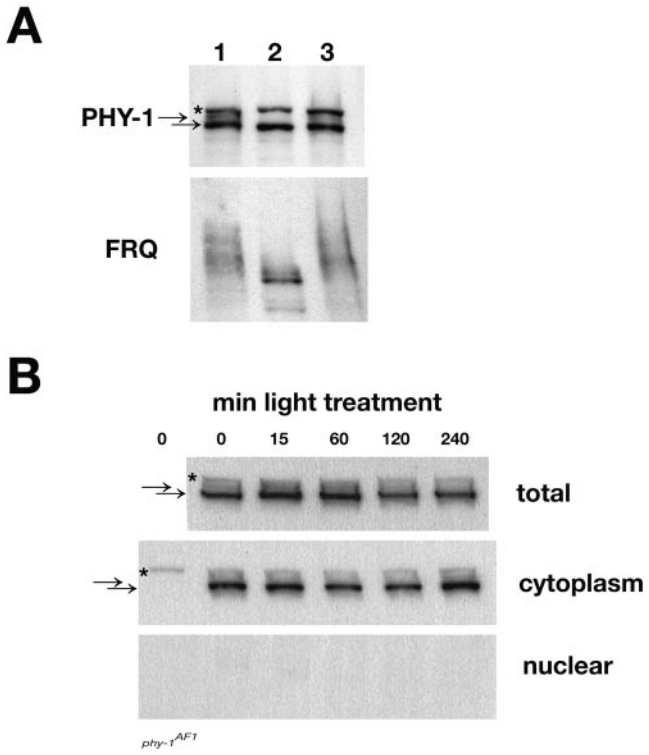

FIG. 4. PHY-1 is exclusively cytoplasmic and phosphorylated. (A) Western blot of PHY-1 and FRQ from a dark-grown culture harvested after 20 h and treated with λPPase. Lane 1, untreated sample revealing two PHY-1-specific bands (arrows) and a nonspecific band (asterisk). Lane 2, sample treated with λPPase, resulting in the disappearance of the slower-migrating PHY-1 band. Lane 3, sample treated with λPPase and sodium vanadate (a specific inhibitor of λPPase) containing both forms of PHY-1, indicating that the slower-migrating band is specifically due to phosphorylation. FRQ served as a control for the λPPase and sodium vanadate treatments. (B) Western blot analysis of PHY-1 in cellular fractions following light treatments indicates that both forms of PHY-1 are exclusively cytoplasmic, with light having no effect on localization. A wt strain was treated with white light for durations from 0 to 240 min; samples were harvested; and total, cytoplasmic, and nuclear fractions were run on an SDS-PAGE gel and blotted for PHY-1 (indicated by arrows). A single time-zero sample for a *phy-1*^{AF1} strain is shown on the left. Equal amounts of protein were loaded for each time point, as indicated by the similar intensities of the nonspecific band in each lane (indicated by an asterisk). Proper fractionation was assayed by immunoblotting with antibodies against WC-1, a predominately nuclear protein (data not shown).

contaminating proteases but was in fact due to phosphorylation of PHY-1 (lane 3). PHY-1 therefore exists in phosphorylated and unphosphorylated forms, with the latter being severalfold more predominant.

PHY-1 is a constitutively expressed cytoplasmic protein. Phytochromes have been historically classified as either of two types: type II, whose abundance is not dramatically affected by light, or type I phytochromes, which display a dramatic reduction in chromoprotein following light treatments. This drop is caused by a rapid transcriptional down regulation of the corresponding *PHY* gene combined with a more rapid degradation of the Pfr form of the chromoprotein (66, 68). To test for possible effects on PHY-1 abundance in *Neurospora*, we examined the abundances of the protein under various light regimes. White-light irradiations lasting from 15 min to 24 h had no effect on PHY-1 levels or on the relative amounts of phosphorylated versus unphosphorylated forms (Fig. 3A, bottom). Exposure to 12 h of R or FR also failed to alter PHY-1 abundance (Fig. 3B, bottom). Although fluctuations were seen in the levels of phosphorylated PHY-1 (e.g., a decrease in the wt under red light) (Fig. 3B, bottom), four independent replications failed to show a consistent pattern of regulation by phosphorylation. We therefore consider PHY-1 to be a light-stable type II phytochrome. The R and FR treatments were also performed in a *phy-2*^{AF1} strain (Fig. 3B, bottom), but no changes were seen in PHY-1 protein, mirroring the results seen for the *phy-1* transcript in a *phy-2*^{AF1} strain.

In *Arabidopsis*, transfer of plants from a 12-h light–12-h dark photoperiod to continuous white light generates a very low-amplitude cycling of PhyA and PhyC, but no significant circadian cycling of PhyB and PhyE (PhyD has not been examined) (66). To look for a similar circadian regulation of PHY-1, mycelia of approximately the same developmental age were harvested at 4-h intervals over 48 h after a light-to-dark transfer. Western blot analysis revealed that neither PHY-1 abundance nor its phosphorylation is circadian-clock regulated (Fig. 3C, bottom). Although some fluctuations in the levels of both forms of PHY-1 were observed over the time course, these changes were not reproducible in replicate experiments. As a positive control, FRQ abundance and phosphorylation can be seen to be circadian-clock regulated, as previously reported (25).

Arabidopsis light-induced transcription is mediated at least partly by a direct interaction of PhyA and PhyB with transcription factors (48, 53, 54), an action that is made possible by the light-regulated import of these phytochrome molecules into the nucleus. To study the subcellular localization of PHY-1, we utilized a biochemical method to isolate nuclei from *Neurospora* and detect PHY-1 by Western blot analysis. Figure 4B shows the results of Western analysis of PHY-1 in total, cytoplasmic, and nuclear fractions for samples harvested from 0 to 240 min following light treatment. Both the nonphosphorylated and phosphorylated forms of PHY-1 were found exclusively in the cytoplasmic fraction in the dark and following the various durations of light treatment. Time of day also did not effect this PHY-1 localization, with both forms of PHY-1 always being localized to the cytoplasm (data not shown).

Phenotypic analysis of *Neurospora phy-1*^{AF1} and *phy-2*^{AF1} strains. Although *Neurospora* is a nonphototrophic organism, light regulates many of its developmental processes. During the asexual phase, light induces mycelial carotenoid biosynthesis (27); increases and hastens vegetative-spore production (or conidiation) (35, 39); and sets the phase of the endogenous biological clock, which acts to regulate a variety of aspects of the life cycle of the organism (reference 62; reviewed in reference 47). During the sexual phase, light influences the formation of protoperithecia (19) and the phototropism of perithecial beaks (28). All of these responses have been shown to be blue-light regulated and therefore are not considered to involve phytochromes, historically viewed as R/FR sensors. However, given that most action spectra failed to extend into the FR region of the spectrum, that phytochromes also absorb blue light (Fig. 2A), and that some blue-light responses in plants are modified by phytochromes (e.g., resetting the circadian clock [67], inhibition of hypocotyl elongation [74], and flowering time [49]), we reexamined the photobiology of *Neurospora* using the *phy-1*^{AF1} and *phy-2*^{AF1} strains.

To determine if *phy-1* or *phy-2* plays a role in the *Neurospora* circadian system, the corresponding deletion strains were grown on race tubes using a high-light (~30 μmol photons/m^2/s)- or low-light (~1 μmol photons/m^2/s)-to-dark transfer to entrain the cultures. (Both high and low light were tested based on the observation in *Arabidopsis* that PhyA acts to transmit low-fluence blue and red light to the clock and PhyB transmits high-intensity red light to the clock [67]). The period and phase of the conidial banding were analyzed and compared in *phy-1*^{AF1}, *phy-2*^{AF1}, and two wt strains, all progeny from the same cross. The *phy-1*^{AF1} and *phy-2*^{AF1} strains had phases similar to those of the two wt strains (Fig. 5A and data not shown), suggesting that the *phy* genes do not play a role in light input to the *Neurospora* clock. A light-induced increase in *frequency* (*frq*) transcript levels is the central mechanism by which light input reaches the clock. The light induction of *frq* was also unaltered in a *phy-1*^{AF1} or a *phy-2*^{AF1} strain, further supporting the lack of involvement of the *phy* genes in light input to the *Neurospora* clock (Fig. 5D). The period lengths of the *phy-1*^{AF1} and *phy-2*^{AF1} strains following the light-to-dark transfers are also similar to those of the wt strains. Race tube experiments using a temperature step-up from 4°C to 25°C (in constant darkness) to entrain the cultures also resulted in wt periods and phases for the *phy-1*^{AF1} and *phy-2*^{AF1} strains (data not shown). Similarly, race tubes grown under R or FR showed no significant changes in period length for the wt or the *phy* deletion strains (data not shown). Taken together, it does not appear that PHY-1 or PHY-2 plays a central role in the *Neurospora* circadian clock.

The development of perithecia, the organs in which the sexual spores are formed, is light regulated in *Neurospora* (19). Blue light, but not red light, induces positive phototropism of the perithecial beaks (beak bending) to help eject spores in the direction of the light (28). To test for defects in phototropism of perithecial beaks, crossing plates were inoculated with one of three strains (wt, *phy-1*^{AF1}/*phy-2*^{AF1}, or *wc-1*^{ER53}), crossed with a wt strain, and then placed in directional lighting. The directions of the perithecial necks were then scored relative to the direction of the light; 82% of the wt perithecial necks pointed toward the light, whereas wt perithecia necks that developed in the dark displayed random growth directions (Fig. 5B). The *phy-1*^{AF1}/*phy-2*^{AF1} strain appeared wt for phototropism of perithecial beaks, with 88% of perithecial necks

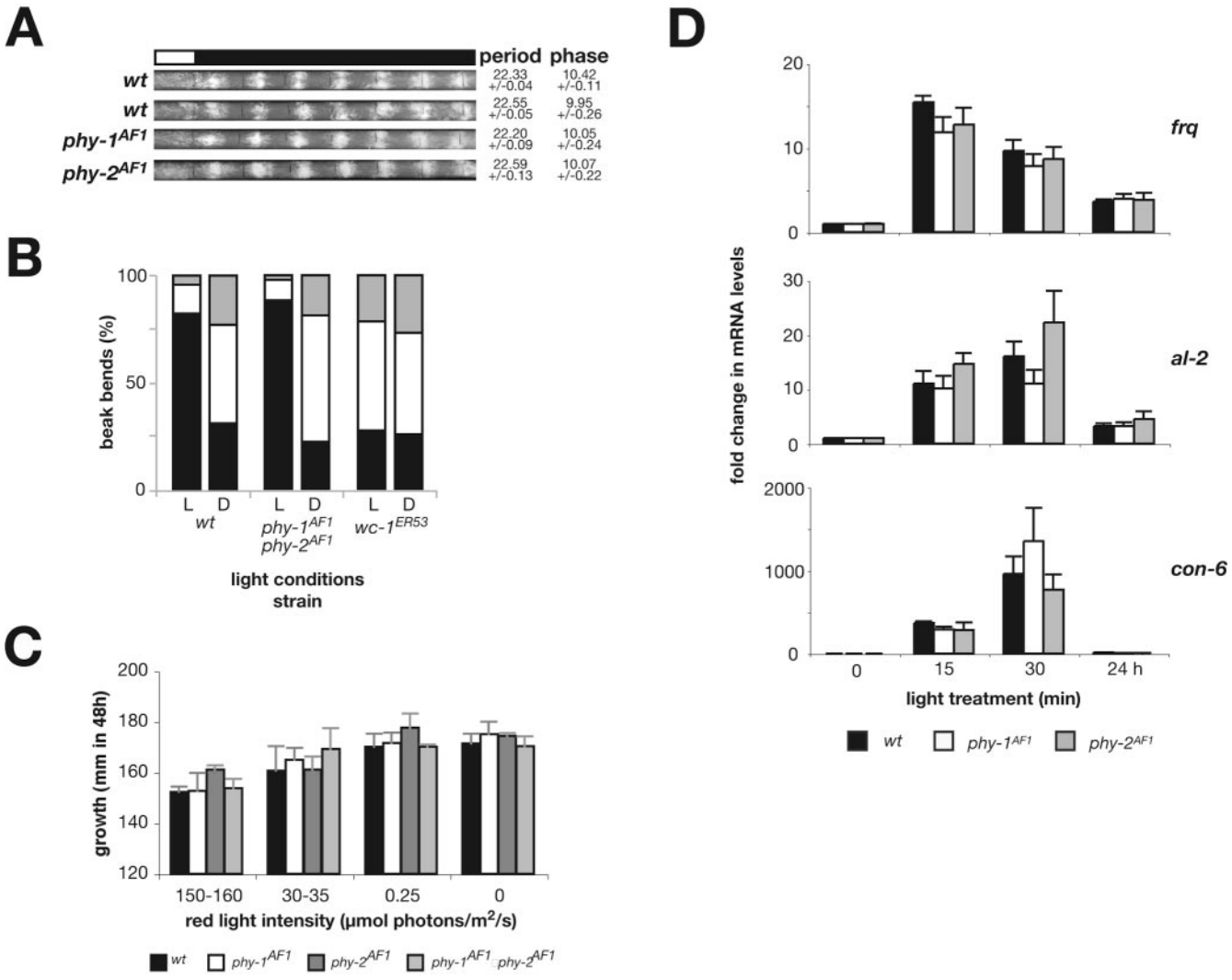

FIG. 5. PHY-1 and PHY-2 do not play a role in previously described *Neurospora* photobiology. *phy-1*^{AF1} and *phy-2*^{AF1} strains were assayed for defects in developmental processes known to be light regulated (A, B, and D) and for novel photobiology (C). (A) PHY-1 and PHY-2 do not play roles in light resetting of the *Neurospora* clock. Race tubes were inoculated with wt, *phy-1*^{AF1}, or *phy-2*^{AF1} strains, and the clock was reset with a light-to-dark transfer. The period and phase of conidial production in the *phy-1*^{AF1} and *phy-2*^{AF1} strains were similar to those in the wt. Average periods and phases with standard errors of the mean of six race tubes for each strain are shown, together with representative race tubes. (B) PHY-1 and PHY-2 are not required for phototropism of the perithecial beak. Protoperithecia were induced in wt, *phy-1*^{AF1}/*phy-2*^{AF1}, and *wc-1*^{ER53} mutant strains grown on petri plates. Duplicate plates for each strain were used in a cross and then placed in the dark or in directional lighting. The orientation of the resulting perithecial beaks (black, toward; white, neutral; gray, away) was scored relative to the direction of the light and plotted as a percentage of total perithecial beaks. L, light; D, dark. (C) The linear growth rates of wt and *phy* deletion strains are similar under various intensities of red light. Total linear growth in 48 h under conditions of constant red light was measured using race tubes. The error bars are the standard deviations (SD); $n = 3$. (D) Light induction of the *frq*, *con-6*, and *al-2* transcripts is normal in *phy-1*^{AF1} and *phy-2*^{AF1} strains. RNA was harvested from wt, *phy-1*^{AF1}, and *phy-2*^{AF1} strains grown in constant darkness or following 15 min, 30 min, or 24 h of light treatment. The transcript levels of *frq* (light induced to reset the clock), *al-2* (light-induced carotenogenesis gene), and *con-6* (light-induced conidiation gene) were determined using quantitative real-time PCR. The data are plotted with the zero-min values for each strain set to 1 so that the *y* axis indicates induction (*n*-fold) for each strain. The error bars are the SD; $n = 3$.

pointing toward the light. Under the same conditions, a random distribution was observed for the *wc-1* mutant, which is defective in white-light-induced perithecial phototropism (28).

Transfer of *Neurospora* cultures from the dark into the light triggers an increase in conidial production requiring the light induction of several conidiation, or *con*, genes (40, 41). We detected no gross defects in conidial production in *phy-1*^{AF1} and *phy-2*^{AF1} strains or in a strain containing both *phy* deletions (data not shown). On the molecular level, we also found no differences in the light induction of *con-6* and *con-10* genes in the *phy* deletion strains (Fig. 5D and data not shown). These results suggest that the *phy* genes do not play a role in the enhancement of conidiation by light. A dark-to-light transfer also stimulates carotenoid production in the mycelia, resulting in a very obvious increase in orange coloration. This carotenogenesis requires the light induction of the carotenoid bio-

synthesis genes *albino-1* (*al-1*), *albino-2* (*al-2*), and *albino-3* (*al-3*) (3, 43). Both light-induced carotenoid production and the abundance of the *al-1* and *al-2* transcripts in the *phy-1*^{AF1} and *phy-2*^{AF1} single and double mutants were indistinguishable from those of the wt (Fig. 5D and data not shown).

PHY-1 and PHY-2 are primarily predicted to function as sensors of R and FR, wavelengths for which no photobiological responses have been found in *Neurospora*. We therefore looked for novel photobiology, potentially R/FR regulated, which might require the activity of the *phy* genes. We first looked for gross effects of R/FR on *Neurospora* growth. Race tubes were inoculated and grown for 48 h under cool fluorescent lights; the growth fronts were marked and then transferred to continuous R or FR. After a subsequent 48 h of growth, the race tubes were removed and the linear growth of the cultures was measured. Although a slight decrease in growth with increasing light intensity is seen for all strains, we found no significant difference in the linear growth rate between wt, *phy-1*^{AF1}, *phy-2*^{AF1}, and *phy-1*^{AF1}/*phy-2*^{AF1} strains under conditions of constant R or FR of various intensities (0.25 to 160 μmol/photons/m^2/s) (Fig. 5C). There were also no gross morphological differences between the *phy* deletion strains and the wt under the conditions tested. Similar experiments were also performed using blue light with no difference found between the *phy* deletion strains and the wt (data not shown).

Although no appreciable difference in growth under constant R or FR illumination was detected in the *phy* knockout strains, it was possible that *Neurospora* could exhibit phototropism during the asexual stage of its life cycle. To assay for phototropism, *Neurospora* was cultured using numerous different physical setups combined with various directional-lighting configurations using standard fluorescent lighting, as well as custom-fabricated light-emitting diode arrays (blue, red, and white). Using standard race tubes, cultures were inoculated at one end and then placed in chambers with a light source at one or both ends of the chamber. Both the light source and the intensity were varied, enabling the testing of a variety of lighting configurations (e.g., low-intensity red light at one end of the chamber or high-intensity red at one end and low-intensity blue at the other). Modified race tubes that enabled inoculation in the middle of the tubes, as well as 150-mm petri plates, were also used in the chambers described above. We also developed a novel means of looking for effects of light on the fine branching structure of mycelia, using thin (1-mm) vertical gels similar to those used to run protein samples. Standard solid growth medium was poured between two glass plates, and *Neurospora* was inoculated at the top. The plates were positioned and masked so that light would reach the culture only from above or below. We could subsequently look for directional growth or effects on the mycelial branching pattern using a dissecting microscope. Stationary liquid cultures were also employed with a variety of lighting configurations. We failed to uncover any conditions or setup under which *Neurospora* displayed reproducible phototropism in wt strains or in the *phy* deletion strains (data not shown). We also considered the possibility that the PHYs might not be involved in light sensing per se but instead in heme and/or iron metabolism, so single and double *phy* mutant strains were cultured on race tubes with limited and different sources of iron; in no case was a significant difference in growth rate or gross morphology detected.

To complement the overt R/FR phenotypic analyses of wt and *phy* deletion strains, we used available *Neurospora* DNA microarrays to look at the molecular level for potential R/FR gene regulation that could be attributed to the *Neurospora* Phys. Mycelial tissue from a wt strain was collected from liquid cultures receiving R or FR treatment for 0, 0.5, 2, or 24 h. The arrays contained ~1,100 unique cDNA clones (described in reference 55) representing approximately 10% of the total predicted genes in the *Neurospora* genome. Using two biologically independent sets of samples, we did not see any reproducible and verifiable changes in gene expression due to any of the R or FR treatments (data not shown). The genes on the arrays were identified by expressed sequence tag sequencing of libraries made from nondifferentiating vegetative tissue grown in the dark, with the result that light-induced genes were predicted to be severely underrepresented on the arrays. It is therefore possible that the use of arrays containing a larger set of genes might uncover R/FR regulation in *Neurospora*.

DISCUSSION

The presence of two genes with homology to phytochromes in the *Neurospora* genome was unexpected given the absence of known red- or far-red-directed responses, despite many years of photobiological investigation. In this study, we undertook a biochemical, genetic, and physiological investigation of these two new members of the PHY superfamily in hope of learning more about the functioning of phytochromes in general, and in particular, their roles in *Neurospora*.

All five of the domains (the PLD and the GAF and PHY domains, followed by the HK and RR domains) identified in PHY-1 and PHY-2 are present in Phy sequences from the filamentuous fungi *Aspergillus nidulans*, *Botryotinia fuckeliana*, *Cochliobolus heterostrophus*, *Giggerella moniliformis*, *Gibberella zeae*, and *Ustilago maydis* (10, 72), suggesting that these domains are important to this Phy subfamily. Some of these other fungal Phys also contain regions with limited similarity to the additional sequences found in PHY-1. Interestingly, Phys have been conclusively found only in filamentous fungi and are not evident in the genomes of single-cell fungi, such as *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* (72), suggesting roles for the Phys in regulating the greater developmental complexity of the filamentous morphology.

The N-terminal halves of PHY-1 and PHY-2, consisting of the PLD and the GAF and PHY domains, are predicted to function as the light sensory input module. Like Phys from eubacteria, PHY-1 and PHY-2 do not contain the positionally conserved cysteine in the GAF domain used by plant and various cyanobacterial Phys to covalently bind the bilin via a thiol-ether linkage (reviewed in reference 72). Like other fungal Phys, a small hydrophobic residue (Val/Ile) is in this position (Fig. 1B). From a limited study of bacterial Phys, it appears that a cysteine within the PLD serves as the bilin attachment site in this subgroup (31, 37, 38). Similar to all other identified fungal Phy sequences, PHY-1 and PHY-2 contain this conserved cysteine (Fig. 1B). A 515-amino-acid recombinant fragment of PHY-2 containing just the PLD and the GAF domain was able to covalently attach both BV and PCB

to form R/FR photochromic adducts. These results confirm that PHY-2 and, most probably, PHY-1 are capable of autocatalytically attaching bilins and functioning as typical Phy photoreceptors. The BV–PHY-2 Pr absorption maximum (~693 nm) was slightly red shifted compared to those of plant Phys (~666 nm) and cyanobacterial phytochromes (Cphs) (~654 nm) but similar to BV-bacteriophytochromes (BphPs) (~698 nm). The PΦB/PCB precursor BV is used by BphPs as the chromophore, and it is likely that PHY-1 and PHY-2 also use BV as the natural chromophore, based on their similarity to bacteriophytochromes. However, BLAST searches of the *Neurospora* genome have so far failed to uncover a possible heme oxygenase that is required to generate BV from heme.

The C-terminal halves of PHY-1 and PHY-2, containing the HK and RR domains, appear to be hybrid TC-histidine kinase sensory output modules. Originally identified in bacteria, TC-HK phosphorelays are minimally composed of a sensor HK protein and a separate RR protein that helps many organisms sense and adapt to their environments. The appropriate environmental signal (e.g., nutrient levels, osmolarity, light, or oxygen levels) triggers the autophosphorylation of a conserved histidine residue by the HK domain in an ATP-dependent manner. The phosphoryl group is then transferred to a conserved aspartate residue in the RR, with this differential phosphorylation directing an appropriate output, such as a change in transcription, motility, or signaling, through a kinase cascade (reviewed in reference 61). The HK domains of PHY-1 and PHY-2 contain the conserved H box, including the positional conserved histidine that is the site of autophosphorylation, and the N and G boxes that participate in ATP binding (Fig. 1B). The phosphorylated form of PHY-1 could represent a more long-lived autophosphorylated species generated by this reaction sequence. The presence of the appended RR likely means that the histidine phosphate is transferred intramolecularly to the RR aspartate, rather than to a second separate RR polypeptide during signal transmission.

The absence of an output domain following the RR implies that additional phosphorelay steps are required before signal output by both PHY-1 and PHY-2. In similarly organized TC-HK systems, the RR aspartate-bound phosphate is transferred to the histidine of a separate histidine phosphotransferase (HPT) protein, and then ultimately, the phosphate is transferred to an aspartate in a second RR protein (reviewed in reference 61). The roles of these additional phosphorelay steps are unknown, but it has been speculated that this design enables integration of multiple input signals to a single output. *Neurospora* is predicted to contain nine additional hybrid HKs that could sense a variety of environmental signals (8, 10). One of these HKs, Nik-1/Os-1 (NCU02815.1), plays a role in hyphal development and may be involved in sensing osmolarity (1, 64). *Neurospora* is predicted to express only one HPT protein (NCU01489.1) and two RRs (NCU01895.1 and NCU02413.1). This small number suggests that the 11 hybrid HKs in *Neurospora* converge to activate only a few common pathways. Therefore, it is possible that the R/FR signaling by PHY-1 and PHY-2 are integrated with other HK signaling systems. The possibility that more than one signal is required before output could explain why we have so far failed to uncover any responses under PHY-1 or PHY-2 control using just R/FR as the signal.

The analysis of transcript regulation indicates that both *Neurospora phy* genes are expressed, with Northern analysis suggesting relatively low expression of *phy-2*. In *Arabidopsis*, transcription of *PHYA*, and to a lesser extent *PHYB*, is under negative control by light, with the accumulation of mRNA inversely related to the fluence rate (9, 15). R reduces *PHYA* transcript levels through the action of the PhyB protein, whereas FR reduces *PHYA* transcript levels through the action of the PhyA protein itself (9). We did not find changes in either light quality or quantity to have an effect on the levels of *Neurospora phy-1* or *phy-2* transcript. As such, the *Neurospora* PHYs are similar to *Arabidopsis PHYC*, *PHYD*, and *PHYE*, which display little or no photoregulation. At the protein level, light also causes a dramatic decrease in *Arabidopsis* PhyA protein and, to a much lesser extent, PhyB and PhyC (66, 68). However, similar to *Arabidopsis* PhyD and PhyE, we found no effect of light on PHY-1 protein levels. *phy-1*, but not *phy-2*, transcript exhibited a low-amplitude circadian rhythm under constant conditions. In contrast, PHY-1 protein levels lacked circadian regulation, consistent with the protein being quite stable compared to the 24-h time scale of the *phy-1* RNA rhythm. Interestingly, this type of regulation, circadian transcription but constitutive protein abundance, has also been seen for *Arabidopsis* PhyB and PhyC.

We found that neither light nor time of day alters PHY-1 cytoplasmic localization. In contrast, *Arabidopsis* PhyA and PhyB translocate to the nucleus following white-light treatment, with PhyA nuclear levels peaking within 10 min and PhyB requiring 6 h for maximal translocation (32–34, 60, 63, 76). The lack of light-regulated shuttling of PHY-1 into the nucleus, as well as the complete lack of PHY-1 in the nucleus, may be reasonable, considering the domain structure and potential signaling mechanism of PHY-1. As mentioned above, PHY-1 is a hybrid histidine kinase containing both a histidine kinase domain and a receiver domain within a single polypeptide. One of the most intensively studied hybrid histidine kinases, *S. cerevisiae*'s Sln1, is localized to the plasma membrane, where it detects osmotic stress to the cell. One of Sln1's downstream signaling partners is Skn7, which is a nucleus-localized transcription factor. Signaling between Sln1 in the plasma membrane and Skn7 in the nucleus is speculated to be mediated by the shuttling of the HPT protein Ypd1, which is small enough (~20 kDa) to freely diffuse into the nucleus (reviewed in reference 61). In an analogous fashion, PHY-1 and PHY-2, along with the 11 other hybrid histidine kinases, could be localized to the cytoplasm or plasma membrane, with a single HPT shuttling to nucleus-bound response regulator proteins.

Although the expression, regulation, and in vitro photochemistry of *Neurospora* PHY-1 and PHY-2 strongly suggest that they have a photobiological role in *Neurospora*, we have failed to identify specific processes under their control. Much of *Neurospora* photobiology is regulated by blue light in a WC-1/WC-2-dependent manner. However, it remains possible that some are also sensitive to R and/or FR, as is the case for many photoresponses in plants that involve multiple photoreceptors with overlapping functions (12). In the filamentous fungus *Aspergillus nidulans*, conidiation is elicited by exposure to red light in the range of 690 to 710 nm (51), with the same wavelengths also causing a delay in sexual sporulation (11). More recently, a role for red light, mediated by a phytochrome,

was seen in blocking sexual development in *Aspergillus* (7a). Interestingly, blue light (maximum, ~463 nm) has also been found to have the same effects on conidiation and sexual sporulation, but only in a strain with a mutation in the *bliA1* gene (11, 75). This suggests that blue-light regulation does exist in *Aspergillus* but that it is inhibited under standard laboratory conditions by a *bliA1*-dependent mechanism. A similar situation may exist in *Neurospora*, with blue-light regulation readily apparent but red/far-red signaling inhibited under laboratory conditions. This is supported by the fact that the known *Neurospora* photobiological processes are strongly regulated by many additional environmental factors, such as desiccation, gas concentrations, and available nutrients (e.g., conidiation is greatly increased by small increases in the amount of available carbon and inhibited by small increases in CO_2 levels.). R/FR regulation of *Neurospora*'s various developmental processes may occur only under specific conditions when inhibition of PHY signaling (potentially by some of the other nine HK proteins) is prevented. Systematically varying growth conditions may enable the empirical determination of conditions under which R/FR light perceived by the *Neurospora* phytochromes is translated into appropriate regulation of developmental processes, such as conidiation and carotenogenesis.

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