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The Fast-Evolving *phy-2* Gene Modulates Sexual Development in Response to Light in the Model Fungus *Neurospora crassa*

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ABSTRACT Rapid responses to changes in incident light are critical to the guidance of behavior and development in most species. Phytochrome light receptors in particular play key roles in bacterial physiology and plant development, but their functions and regulation are less well understood in fungi. Nevertheless, genome-wide expression measurements provide key information that can guide experiments that reveal how genes respond to environmental signals and clarify their role in development. We performed functional genomic and phenotypic analyses of the two phytochromes in *Neurospora crassa*, a fungal model adapted to a postfire environment that experiences dramatically variable light conditions. Expression of *phy-1* and *phy-2* was low in early sexual development and in the case of *phy-2* increased in late sexual development. Under light stimulation, strains with the phytochromes deleted exhibited increased expression of sexual development-related genes. Moreover, under red light, the *phy-2* knockout strain commenced sexual development early. In the evolution of phytochromes within ascomycetes, at least two duplications have occurred, and the faster-evolving *phy-2* gene has frequently been lost. Additionally, the three key cysteine sites that are critical for bacterial and plant phytochrome function are not conserved within fungal *phy-2* homologs. Through the action of phytochromes, transitions between asexual and sexual reproduction are modulated by light level and light quality, presumably as an adaptation for fast asexual growth and initiation of sexual reproduction of *N. crassa* in exposed postfire ecosystems.

IMPORTANCE Environmental signals, including light, play critical roles in regulating fungal growth and pathogenicity, and balance of asexual and sexual reproduction is critical in fungal pathogens' incidence, virulence, and distribution. Red light sensing by phytochromes is well known to play critical roles in bacterial physiology and plant development. Homologs of phytochromes were first discovered in the fungal model *Neurospora crassa* and then subsequently in diverse other fungi, including many plant pathogens. Our study investigated the evolution of red light sensors in ascomycetes and confirmed—using the model fungus *Neurospora crassa*—their roles in modulating the asexual-sexual reproduction balance in fungi. Our findings also provide a key insight into one of the most poorly understood aspects of fungal biology, suggesting that further study of the function of phytochromes in fungi is critical to reveal the genetic basis of the asexual-sexual switch responsible for fungal growth and distribution, including diverse and destructive plant pathogens.

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Beyond its role supplying the energy for photosynthesis, and thus its direct or indirect energetic support of most forms of life on earth, light also serves as a nearly ubiquitous source of temporal and spatial information concerning environmental changes. Most fungi, like many microbes and most plants, cannot rapidly navigate their environment to move toward desirable conditions. Unlike many microbes, a fungus can comprise a large biomass extending across two or three spatial dimensions as well as time, thereby experiencing diverse environmental conditions. Intense light—a key indicator of environmental changes—is detrimental to most fungi and other microbes over large periods of their life history (1–4). Nevertheless, it serves as one of the direct environmental signals by which they can regulate life cycle deci-

sions (5) and is a facet of fungal biology that has been targeted for the prevention of plant pathogenesis (6). Accordingly, fungal gene expression responds rapidly and dynamically to light stimulation (7–11), presumably and in some cases demonstrably through the action of sensory molecules. Although effects of light on the pathogenicity, reproduction, and life cycle of ascomycetous fungi have been studied for a long time, little genetic analysis of light-responsive pathways has been conducted in these fungi. There is a lack of knowledge about fungal responses to the light spectrum in non-model species, which include many plant-associated fungi. Moreover, even in model species, such as *Neurospora crassa* and *Aspergillus nidulans*, the systems biology behind fungal photobiology has been found to be highly complicated (5, 8, 12, 13).

Four kinds of sensory molecules, which bind chromophores that respond to blue, green, and red light, respectively, have been discovered in fungal genomes. The blue light sensors, which are proteins with a flavin chromophore, have been most intensively investigated in plants, animals, microbes, including fungi (5, 8, 14, 15). In *Neurospora crassa*, blue light regulates circadian rhythms and fungal response to stress agents (16–19). Blue light also plays a role in carotenoid production, conidiation, protoperithecium formation, and phototropism of perithecial beaks (20–23). For example, under nutrient starvation, exposure of *N. crassa* to brief pulses of blue light can induce formation of protoperithecia or conidiation (24, 25), and blue light affects the polarity of the perithecial beak (21, 22, 26). Rhodopsins (opsins) sense blue and green light and have been found in various fungal genomes (27). Homologs of red-light-sensing proteins, known as phytochromes (PHY) in plants, have also been identified in *Neurospora*, *Aspergillus*, *Sclerotinia*, and some other fungal species (6). Plant and fungal phytochromes share an origin with bacterial phytochromes that bind their chromophore through a thioether bond to conserved cysteine residues, but they followed two distinct evolutionary trajectories subsequent to their divergence (5, 28, 29). In plants, the phytochromes exist as two distinct but photoreversible forms: the red-light-absorbing form (Pr) and the far-red-light-absorbing form (Pfr). The Pfr form is generally considered the active form, interacting with various transcription factors to regulate plant development (30, 31).

Fungal phytochromes, in contrast, are light-regulated “hybrid” histidine kinases that carry a histidine kinase (HK) domain in addition to the N-terminal response regulator (RR) domain in the output module (32–34). Within the photosensory module at the N terminus, there are three domains—PAS, GAF, and PHY (phytochrome). Light-driven HK activity has been reported in phytochrome FphA of *Aspergillus* species, in which full asexual reproduction (conidiation) requires stimulation by both blue light and red light to effectively inhibit sexual development, and sexual reproduction of these fungi usually requires culture under complete darkness (35–44). After binding to the biliverdin chromophore, both FphA and its *Neurospora* homolog, PHY-2, exhibit spectral properties similar to phytochrome in plants (33, 34, 37). FphA also interacts with *A. nidulans* proteins Light response A (LreA) and LreB, which together form a complex homologous to the *Neurospora* White Collar Complex (WCC) (40). In *A. nidulans* exposed to white light, the LreA/LreB complex represses asexual development and promotes sexual development, whereas FphA represses sexual development and promotes asexual development (2, 38–40, 45); in contrast, asexual development in *A. fumigatus* is neither influenced by blue light nor red light nor by mutation of *lreA* or *fphA* orthologs (46). Light-dependent gene activation in *A. nidulans* was reported as strictly dependent on function of the phytochrome: FphA regulates expression of *clock-controlled gene A* (*ccgA*), probably via White Collar-regulated histone H3 acetylation (13). Upregulation of *ccgA* in response to red light in *A. nidulans* was almost entirely abrogated by deletion of *fphA*. In contrast, the phytochrome in the fungal entomopathogen *Beauveria bassiana*—which lacks a sexual cycle—was reported to control conidiation in response to red/far-red light and daylight length (47). In *N. crassa*, there are two phytochromes, PHY-1 and PHY-2 (48). Genetic and molecular analysis of *N. crassa* phytochromes revealed a chromophore binding capability of PHY-2 *in vitro*, and phosphorylated and unphosphorylated forms of PHY-1 exclu-

sively in the cytoplasm (37). Transcript levels of *phy-1* and *phy-2* were not demonstrably regulated by light, and no photoresponse phenotypes have been identified in common photobiological assays, including vegetative growth, phototropism, conidiation assays, and assays of perithecial beak development (37). In asexual development under darkness, expression of *phy-1* was not detectable in *N. crassa* and *Neurospora tetrasperma*, and *phy-2* was expressed significantly higher in *N. tetrasperma* than in *N. crassa*. Under the same dark culture conditions, the two genes were similarly upregulated across protoperithecial development within both of the *Neurospora* species, as well as between the orthologs (49). This correlation implies that expression of *phy-1* and *phy-2* is not induced by light but rather is constrained by the initiation of sexual development in both *Neurospora* species. Recently, a complex model of the regulation of expression of conidiation genes *con-10* and *con-6* by photoreceptors was proposed for *N. crassa*, in which the activity of WCC was suggested to be negatively regulated by several photoreceptors, including cryptochrome (*cry-1*), opsin (*nop-1*), and PHY-2 proteins (23).

Sexual and asexual reproduction in ascomycetous fungi can be viewed as two modes of reproduction competing for resources, with one process inhibiting the other and vice versa. The balance between the two modes is generally regulated by combined environmental and developmental signals (43). The fungus *N. crassa* presents a potential model for investigating this balance, especially for non-strict plant pathogens and endophytic fungi (50–54). Its asexual ecology is accessible and well known, it is often found in postfire environments with both endophytic and saprotrophic lifestyles, colonizing exposed dead plant substrates during fast growth and distribution (55). In these environments, *N. crassa* produces a large amount of small conidiospores via repeated asexual reproduction (56). In contrast, sexual reproductive structures of *Neurospora* are not commonly observed in natural settings, but initial colonization after fire very likely occurs via thermally cued germination of robustly persistent ascospores in the soil (57, 58). *N. crassa* produces impact-, desiccation-, and heat-resistant ascospores under environmental conditions that are unfavorable to fast growth. Initiation of sexual reproduction starts with the accumulation of a few thin-walled cells. Then, in 12 to 18 h, these cell masses darken and expand to form mature protoperithecia (59). There is little known regarding how light affects sexual development in *N. crassa*. Inhibition of sexual development by a phytochrome responding to red light has been reported in *Aspergillus nidulans*, which preferentially undergoes sexual reproduction in the dark (5, 38). In contrast, *Neurospora* is known for fast growth and colonization via asexual reproduction in the postfire environment, and sexual reproduction in *N. crassa* is often triggered by a low nutrient level and requires specific light signals during late perithecial development: i.e., precise regulation of sexual reproduction negatively mediated by light is critical, especially during the initiation stage. Both asexual development and sexual development of its congener *N. intermedia* have been observed to occur above ground, perithecia have been found under the surface of plant epidermal tissue, and well-developed beaks have been found protruding through cracked, burned plant tissue (58), indicating a developmental program in which light likely plays a key and important role.

Genome-wide expression measurements provide detailed information about gene activity during the processes and facilitate the design of focused experiments to reveal how genes respond to

TABLE 1 Transcriptomic data analyzed for this study

Expt	Species	Conditions	Accession no.	Reference
Asexual development	<i>Neurospora crassa</i>	Bird medium, constant light, wild type	GSE26209	60
Sexual development	<i>N. crassa</i>	Carrot medium, constant light, wild type	GSE41484	62
	<i>N. tetrasperma</i>	Carrot medium, constant light, wild type	GSE60255	63
	<i>N. discreta</i>	Carrot medium, constant light, wild type	GSE60266	63
	<i>Fusarium graminearum</i>	Carrot medium, constant light, wild type	GSE61865	64
	<i>F. verticillioides</i>	Carrot medium, constant light, wild type	GSE61865	64
Light response	<i>N. crassa</i>	Bird medium, dark and light conditions, wild-type and KO strains	GSE8932	7

environmental signals. In return, new understanding of the roles that genes play across development expands our ability to interpret transcriptomic observations. Here, based on published transcriptomic data previously unexamined for these questions, we reveal expression patterns of phytochromes during sexual development in *N. crassa*, *N. tetrasperma*, and *N. discreta* as well as two species of *Fusarium*. We designed and executed assays to directly compare developmental behaviors between wild-type strains and phytochrome knockout (KO) mutants under different light conditions at developmental stages where phytochromes were especially abundantly or meagerly expressed. To investigate genome-wide impacts on gene expression of phytochromes, we used a Bayesian approach to reanalyze genome-wide expression data collected at multiple time points for wild-type and KO strains of phytochromes under different light stimulation conditions. To assess the potential ecological roles of these light-dependent genes, we reconstructed the gene phylogenies of phytochromes from diverse annotated genomes in ascomycetous fungi.

RESULTS

Expression of phytochromes was upregulated during sexual development in *Neurospora crassa* and related fungi. The genome-wide gene expression was profiled during the course of asexual development (60) and over sexual development in *N. crassa* (61, 62), *N. tetrasperma* and *N. discreta* (63), and two *Fusarium* species (64) (Table 1). Across asexual development in *N. crassa*, expression of *phy-1* and *phy-2* exhibited sequential—but not statistically significant—downregulation. Across sexual development, expression of *phy-1* and *phy-2* exhibited sequential upregulation; expression of *phy-2* increased over 6-fold ($P < 0.01$) starting at 48 h after crossing (Fig. 1). Phytochromes in *N. tetrasperma*, *N. discreta*, and two *Fusarium* spp. also exhibited increased expression during sexual development (see Fig. S1 in the supplemental material), measured as reads per kilobase of exon model per million mapped reads (RPKM) (65). Five genes, including *wc-1*, *wc-2*, *vvd*, *cry*, and *velvet*, are known as blue-light-responsive

genes. They exhibited a peak expression at 48 h after crossing, a stage characterized by meiosis-specific expression and ascus/ascospore development. Unlike upregulated phytochromes, these genes were generally downregulated during late perithecial development, except *cry*, in *N. crassa* (see Fig. S2 in the supplemental material).

Knockouts of phytochromes affected light-induced expression of conidiation-related genes and early sexual developmental genes. A hierarchical regulatory program governing responses to white light has been described in *N. crassa* based on analysis of transcriptional profiles of cultures held in white light up to 6 h (7). These extensive data were reanalyzed with a new focus on *phy-1* and *phy-2* knockout strains and a *phy-1 phy-2* double-knockout strain (see Tables S1 and S2 in the supplemental material). The $\Delta phy-1$, $\Delta phy-2$, and $\Delta phy-1 \Delta phy-2$ gene deletion strains all exhibited genome-wide impacts on gene expression patterns and levels (Fig. 2; see Fig. S3 in the supplemental material). Among eight light-responsive transcription factors investigated, WC-1, VVD (VIVID, a PAS protein), and SUB-1 (submerged protoperithecia-1) strongly influenced early or late light-responsive genes (affecting 3% of the *N. crassa* genome). A weaker but similar influence was observed for CSP-1 (conidial separation-1), SAH-1 (short aerial hyphae-1), VAD-3 (vegetative asexual development-3), NOP-1 (new eukaryotic ops-in-1), and a hypothetical protein, NCU03643 (4). Overall one-third or more of the genome showed significantly higher expression ($P < 0.05$) in $\Delta phy-2$, and $\Delta phy-1 \Delta phy-2$ strains compared with wild-type and $\Delta phy-1$ strains, especially under light stimulation longer than 60 min (see Fig. S3 in the supplemental material). Similar results were obtained imposing an exclusive effect-size threshold of 2-fold for biological significance. Cell-type differentiation genes were significantly enriched for upregulation in single- and double-knockout mutants shortly after exposure to light (see Table S2A in the supplemental material). Some differentially expressed cell-type differentiation genes had roles in conidiation, including *con-8* (NCU10997), sporulation protein gene *sps19*

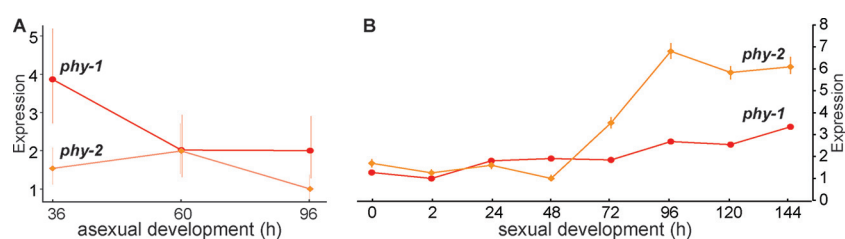


FIG 1 Expression of *N. crassa* phytochrome genes *phy-1* and *phy-2*. (A) Expression level of phytochromes across asexual development, assessed using gene expression microarrays (60). (B) Expression of phytochromes during sexual development using transcriptome sequencing (62). Expression levels are presented in fold change relative to the lowest time point, which was normalized to 1. Error bars represent 95% confidence intervals.

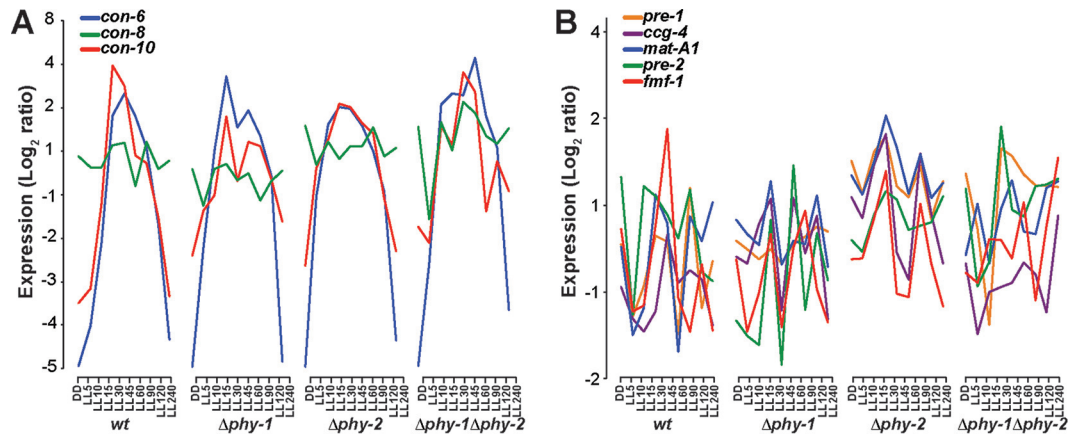


FIG 2 Wild-type and phytochrome knockout strains all respond dynamically to light, but respond differently, especially for genes involved in sexual reproduction. Disruption of phytochromes affected expression of early sexual development genes in response to light but did not markedly affect expression of conidiation genes. (A) The conidiation genes *con-6*, *con-8*, and *con-10*, showed similar expression profiles among wild-type and mutant strains. (B) Highly correlated upregulation of expression of sexual development-related genes occurred immediately following light stimulation in the $\Delta phy-2$ KO strain. The log₂ ratio of expression level was estimated based on analysis of microarray data (7). Expression is depicted under conditions of constant darkness (D) and following light exposure (5 to 240 min [LL5 to LL240]).

(NCU07958), high conidial production gene *hcp-1* (NCU07221), G-protein gene *gna-1* (NCU06493), and conidiophore development gene *hym-1* (NCU03576 [51, 66]). We observed a typical photoadaptation response (acute light induction followed by a decrease in expression for carotenoid biosynthesis genes [7, 8, 67]) for the three conidiation genes *con-6*, *con-10*, and *con-8* (Fig. 2A). The $\Delta phy-2$ knockout strain exhibited highly correlated upregulation for genes related to sexual development right after exposure to light (Fig. 2B), including mating type locus *mat-A1* (NCU01958), pheromone precursor coding gene *ccg-4* (NCU02500), and pheromone receptor coding genes *pre-1* (NCU00138) and *pre-2* (NCU05758).

Knockouts of phytochromes exhibit phenotypes of altered initiation of sexual development in *Neurospora crassa*. Light influenced the production of protoperithecia in both wild-type and knockout strains (Fig. 3; see Fig. S4 and S5 in the supplemental material). The greatest abundance of perithecia formed in cultures grown in darkness and, in the case of the $\Delta phy-2$ strain, also under red light (Fig. 3; see Fig. S5). We examined the role of the *phy* genes in sexual development on synthetic complete medium (SCM) and

carrot media under darkness and exposed to consistent red, far-red, blue, and white light conditions, by culturing crosses between wild-type strains and *phy-1* and *phy-2* knockout strains (see Fig. S4 and S5A and B in the supplemental material). Under diverse light conditions, sexual development manifests itself similarly in *N. crassa* cultured on SCM and carrot medium. However, perithecial morphology is markedly easier to assess in SCM cultures: orange-colored conidia, profuse along the crossing zone, seriously obscure the orange protoperithecia and young perithecia, making the discernment of sexual phenotypes extremely challenging to perform against the similarly orange background of the carrot medium. Cultured under constant blue light, both $\Delta phy-1$ and $\Delta phy-2$ strains produced protoperithecia and then perithecia slightly earlier than the wild-type strains; however, this phenotype was irregular, exhibiting levels of variation among replicates and differences between mating types that rendered any strong conclusions in this culture condition unwarranted in our judgment (see Fig. S5C). Perhaps extensive experimentation could enlighten the reasons for this variability and provide a means to yield a consistent phenotype under blue light. Exposed

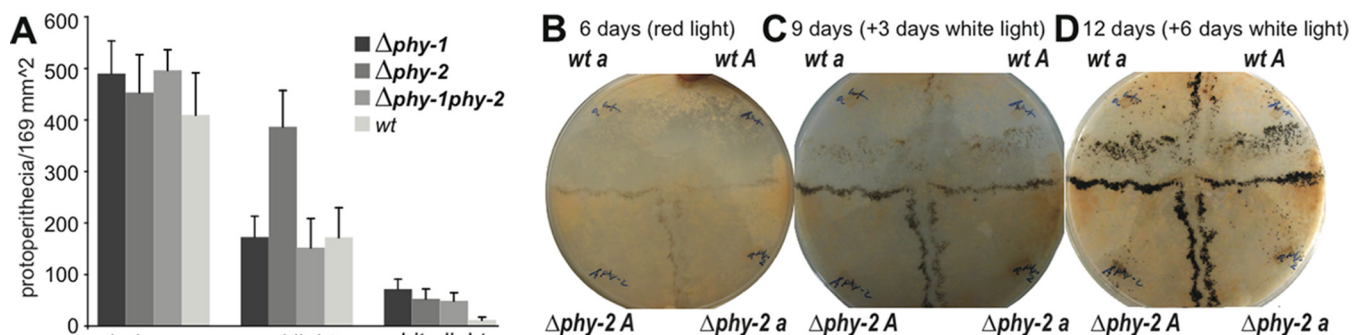


FIG 3 Effects of light on sexual development in *N. crassa*. (A) Abundance of protoperithecia in constant darkness or exposed to red light, and white light for the $\Delta phy-1$, $\Delta phy-2$, $\Delta phy-1\Delta phy-2$, and wild-type strains after 6 days of incubation. Error bars represent standard deviations. (B to D) The $\Delta phy-2$ strain was cultured for 6 days under constant red light and exhibited early sexual development of protoperithecia and perithecia. (B) Protoperithecia and early perithecia formed on the $\Delta phy-2$ strain side. (C) The asymmetry was even more evident after the cultures were placed under 3 days of constant white light for an additional 3 days. (D) The wild-type side of the crossing plate begins to mature at 6 days after shifting to continuous white light.

to constant red light, mating pairs of $\Delta phy-2$ strains produced protoperithecia much earlier than did mating pairs of wild-type and $\Delta phy-1$ strains (Fig. 3B to D; see Fig. S4). Pairs of $\Delta phy-1$ strains exhibited almost the same phenotypes as mating pairs of wild-type strains under red-light conditions. A double-knockout *mat* a $\Delta phy-1 \Delta phy-2$ strain was generated, and under all light conditions, phenotypes of this mutant exhibited the same phenotypes as the $\Delta phy-1$ strain. Under weak white light, $\Delta phy-2$ strains produced protoperithecia slightly earlier than did mating pairs of the wild type. Under intense white light, the earlier development of perithecia in mutants was too slight to be readily apparent, implying the possibility of light intensity or other light-dependent regulations. In cultures that were tightly sealed, in cultures grown on conidiation medium under any light conditions, in all cultures grown in the dark, and in late perithecial development stages, no developmental difference observed between knockout and wild-type strains. Cosegregation experiments were performed to confirm that the intended deletion is responsible for the mutant phenotype. Cosegregation of the hygromycin resistance marker and earlier protoperithecia phenotypes was observed for $\Delta phy-1$ and $\Delta phy-2$ knockout mutants. All 30 individual ascospore progenies that were resistant to hygromycin from a cross of wild-type and $\Delta phy-2$ strains exhibited dramatically earlier production of protoperithecia cultured under red light (see Fig. S5D for three representative images). Phenotypes under the blue light condition in the cosegregation experiment were ambiguous. For the $\Delta phy-1$ strain, only 12 out of 30 ascospore progeny from a cross with the wild type exhibited both hygromycin resistance and weaker protoperithecial production.

Evolutionary history of phytochromes is revealed with available fungal genomes. Phytochrome genes experienced several duplication and loss events during the evolution of higher fungi (subkingdom Dikarya) that are likely related to their function in divergent taxa. Accordingly, across ascomycetes, homologs of *N. crassa phy-2* are fast evolving. ModelTest (68) suggested HKY85 (Hasegawa-Kishino-Yano 85) as an optimal model for assessing phytochrome evolutionary history. The relative ratio test in HyPhy (69) exhibited elevated numbers of nucleotide substitutions in *phy-2* compared to *phy-1* ($P < 0.01$). Furthermore, *phy-2* exhibited a higher nonsynonymous mutation rate than *phy-1* (0.63 versus 0.33, $P < 0.01$, calculated with CODEML of PAML [70, 71]). The index of substitution saturation (*Iss*, 0.71) and the critical index of substitution saturation (*Iss.c*, 0.76), are not significantly different (72), indicating that synonymous mutations are substantially saturated for phytochromes over the time scale of the evolution of ascomycetes (minimum most recent common ancestor [MRCA] of 400 million years ago [mya] [73]).

The molecular phylogeny of fungal phytochromes and their functional domains suggested a single origin of ascomycete phytochromes (Fig. 4; see Fig. S6 and Table S2B and C in the supplemental material); no significant conflicts in topology were observed among phylogenies obtained using likelihood, Bayesian parsimony, or distance criteria. The polytomy of the phylogeny based only on the sensory module, including the P2, GAF, and PHY domains, implies quick diversification and rapid settling into conserved function (Fig. 4A; see Fig. S7 in the supplemental material). *N. crassa phy-2* and its homologs were part of a strongly supported clade present in 96% of bootstrap replicates (bootstrap proportions [BP]) and 1.0 Bayesian posterior probability (BPP), referred to here as the *phy-2* clade. *N. crassa phy-1* and its ho-

mologs were part of a second strongly supported clade (BP = 99%, BPP = 1.0) referred to here as the *phy-1* clade. Phytochromes in *Aspergillus* and related species formed the *fphA/phy-1* clade, which included two phytochrome genes in two closely related genomes, *A. fumigatus* and *A. fischeri*. The phylogeny indicated that *phy-2* homologs and other phytochromes in ascomycetes were the results of gene duplications. There were subsequent losses of *phy-2* in many ascomycetes, especially in the *Dothideomycetes* and *Sordariomycetes*, and many losses of *phy-1* in the *Leotiomycetes* (see Fig. S6 and S7 in the supplemental material). With the sole exception of *Saitoella*, no phytochrome was identified in annotated yeast genomes in *Saccharomycotina* and *Taphriomycotina*, the deepest-diverging lineages in *Ascomycota*.

Amino acid sequences of PHY-1 associated with functional domains were highly conserved in fungi (Fig. 4B). Three to four cysteines located in the PAS and GAF domains serve as chromophore-binding sites: two of these cysteine residues are conserved in bacteria (the 294th and 633rd), and two (the 580th and 651st) are conserved in plants (74). *N. crassa* PHY-1 has cysteines in all four of these positions (Fig. 4B). All ascomycete phytochromes examined featured at least one of the cysteines conserved in plants, except in the fast-evolving phytochrome lineages and in the two basidiomycete outgroups.

DISCUSSION

We have demonstrated that in the model filamentous fungus *Neurospora crassa*, light affects the initiation of sexual development via phytochromes, mainly the fast-evolving *phy-2* gene. Expression of these two genes was at its nadir at early sexual development. Consistent with previous studies (7, 8, 37), our data demonstrate that expression of *phy-1* and *phy-2* is not regulated by light, yet show for the first time that their functions are light dependent. Deletions of *phy-2* and/or *phy-1* positively impacted genome-wide expression levels and patterns, especially for genes regulating mating and early sexual development, implying a negative regulatory role for *phy-2* in sexual reproduction initiation in the response to light. Strains with knockouts of phytochromes also exhibited phenotypes of altered initiation of sexual development in *N. crassa*; protoperithecial development commenced earlier in the $\Delta phy-2$ strain than in the wild type when cultured under red light, suggesting that phytochromes are regulating the balance between asexual and sexual reproduction in *N. crassa* as in *A. nidulans* (40). The repressive regulation imposed mainly by the phytochrome *phy-2* gene impacts both the timing of the transition to sexual development and the abundance with which sexual development is initiated in *N. crassa*. Our observations provide evidence that phytochromes may play a role regulating the asexual-sexual balance in numerous fungi. Considering its unique postfire ecology, the use of light signals and nutrient sensing to balance asexual and sexual development could ensure that *N. crassa* can maximize its spatial expansion and biomass via quick asexual growth on rich, easily acquired nutrients, before turning to sexual development for the production of genetically diversified, resistant sexual structures and spores that can persistently await the next fire event.

Despite the individual function required to retain them both within single lineages, there may be a degree of functional redundancy between the phytochrome paralogs. In *N. crassa*, the $\Delta phy-1$ and $\Delta phy-2$ strains exhibited no malfunctions in growth and development in a previous study (37). Furthermore, our analysis of amino acid conservation demonstrates that all of the recognized

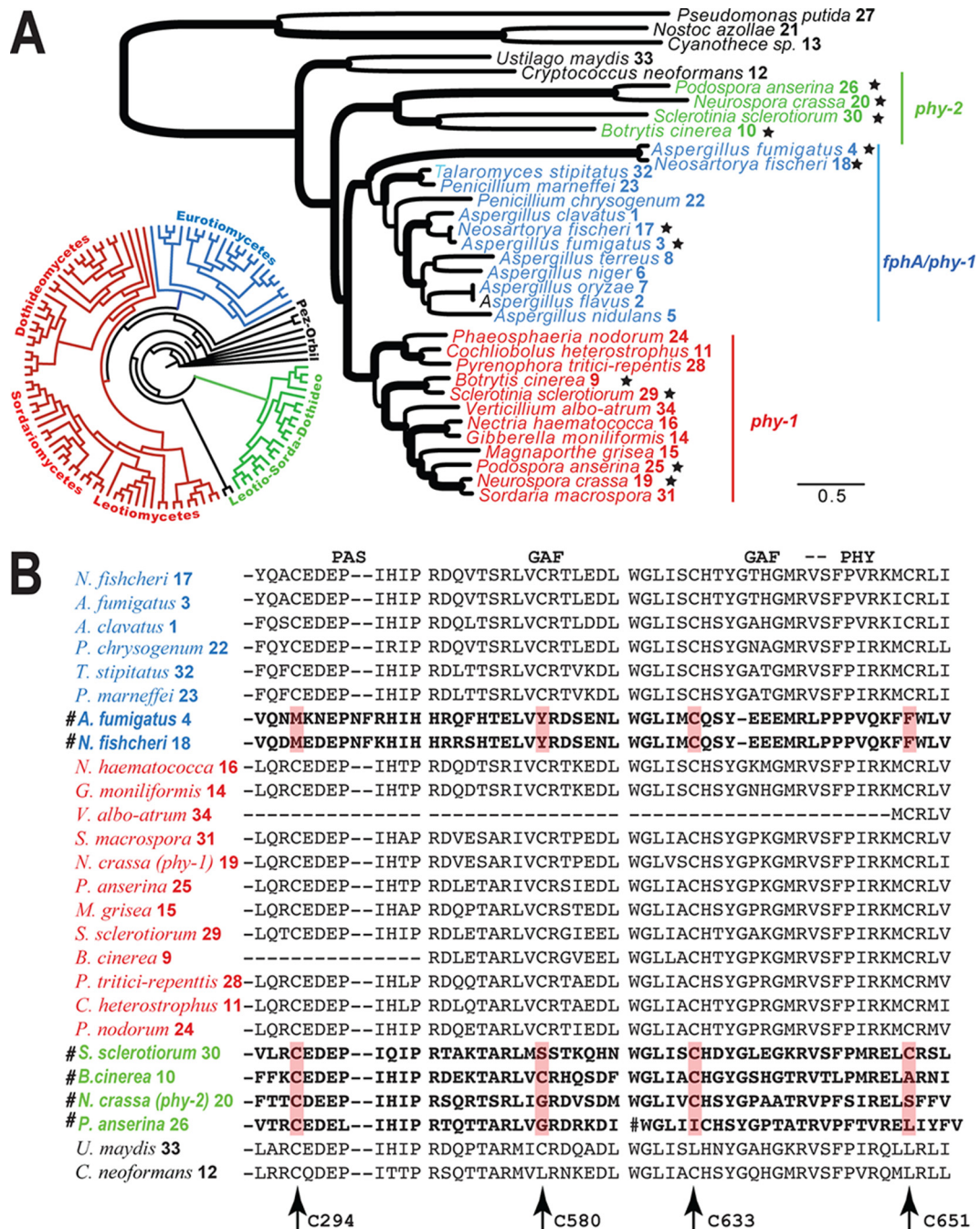


FIG 4 Evolution of phytochromes in fungi. Homologs were color coded for *phy-1* (red), *phy-2* (green), and *fphA* (blue), and sequences were numbered as in Table S2B in the supplemental material. (A) Maximum likelihood phylogeny of fungal phytochromes. Branches with strong support (BP of >85% or BPP of >0.98) are in boldface. Species with multiple copies of phytochromes are marked with stars. A circular tree shows a distribution of phytochromes in major ascomycetous groups. (Phylogenetic details are provided in Fig. S6 in the supplemental material.) (B) Excerpts of amino acid alignments of the PAS, GAF, and PHY domains. The cysteine sites putatively functioning in chromophore binding are indicated by arrows. PHY-1 of *N. crassa* was used as a reference for positions of the cysteine sites. Fast-evolving phytochromes are marked with the symbol #.

domains of the sensory and output modules of PHY-1 and PHY-2 are conserved between paralogs. Generally, strong conservation is equated to essentiality and importance to wild-type fitness (75). However, tests of this assumption using genome-wide data have led to contradictory conclusions (76). The functions of genes that duplicate and then are retained are often split between paralogs

(*de novo* functions are—arguably—rarely gained via gene duplication [77, 78]). The results from our sexual development assay under the blue light condition were indicative but noisy, implying that additional pathways, including some role for *phy-1*, might be actively regulating sexual development in a light-dependent manner.

Inference of the detailed role of *phy-1* in sexual development is made challenging by the complex regulatory effects of light on both asexual and sexual development. In a previous study (37), expression of *phy-1* was not observed to be affected by light conditions, yet was under the control of the circadian clock, which is regulated mainly by blue light. Full cosegregation was not observed here for the Δ *phy-1* strain, which suggested that observed variable phenotypes of earlier protoperithecial production in the Δ *phy-1* strain could be evidence of a less robust regulatory apparatus, greater sensitivity to microenvironmental variation, or possibly other segregating mutations. The complexity of interactions among light reception genes is clear in *A. nidulans*, where function of *fphA* is likely realized through histone H3 acetylation by blue light sensors LreA and velvet protein VeA on clock-controlled gene *cgc-4* and where both asexual reproduction and sexual reproduction are regulated by light responses of many genes (13). Upon light exposure, in *N. crassa* the White Collar Complex (WCC) binds to hundreds of genomic regions as a transcription factor—including the promoter region of *Phy-1* (19). Knowledge of the multiple effects of blue light on *N. crassa* fruiting body maturation would assist efforts to develop an assay that yielded consistent results. The level of detail of knowledge of the circadian response to light during asexual development is striking in comparison to how little is understood regarding how light is perceived and used to modulate sexual reproduction—a challenging but potentially highly informative component of the photobiology of *Neurospora* and of fungi in general.

Considering the overall conservation of these phytochrome genes, the functional divergence that we do observe between PHY-2 and PHY-1 is slightly puzzling. Except for the highly divergent copy of phytochrome in *Aspergillus fumigatus* and *Neosartorya fischeri*, all examined fungal phytochromes are conserved at the chromophore-binding cysteine site (Cys-294 in *N. crassa* PHY-1) in the PAS domain. However, additional cysteine sites in the GAF and PHY domains are conserved among *phy-1* and *phy-1/fphA* homologs, whereas the PHY-2 amino acid sequence features a glycine or serine instead of cysteine at two of these sites (Cys-580 and Cys-651). The conserved cysteine sites at key locations within phytochromes are critical for chromophore binding and function (28, 29). Cyanobacterial photosensors that have evolved insertions of additional cysteines within the GAF domain all share a ground-state absorbance of near-UV to blue light and a common mechanism of light perception (79). Absorption spectra for *phy-1* and *phy-2* are needed to test if additional cysteines in the GAF domain might also restrict the photosensory range of *phy-1* to shorter wavelengths of visible light in *N. crassa*. Since the blue light sensor WCC is known to play multiple roles in *Neurospora* circadian biology, asexual development and sexual development (8, 16, 22), including binding to the *phy-1* promoter region (19), an additional response mediated by *phy-1* and by blue light would be complex to characterize. Accordingly, we observed inconsistent precociousness of sexual development among Δ *phy-1* knockout strains during development under blue light. The phenotype of the Δ *phy-2* strain was more perceptible under red light than under blue or white light; both complexities of phenotype imply the existence of diverse regulatory interactions affecting the asexual-sexual switch.

Repression of sexual development in *N. crassa* in response to reception of light by phytochromes is probably a secondary or fine-tuning regulatory mechanism: production of perithecia is not

fully suppressed in wild-type strains even under intense white light under laboratory conditions. Accordingly, while phytochromes are functioning to sense changes in light and to ensure that protoperithecial development begins under appropriate conditions, other environmental factors and interactions can significantly influence sexual development. Intriguingly, *phy-1* and *phy-2* knockout mutants exhibited a wild-type phenotype when cultured under intense white light, implying that under natural conditions, initiation of sexual development is regulated by an interaction of light sensors that are sensitive to different components of the spectrum. As evidence of even further complexity, the *phy-1 phy-2* double knockout exhibited a wild-type phenotype cultured under blue, red, and white light, so that the consonant effects of Δ *phy-1* and Δ *phy-2* are antagonistic rather than synergistic with regard to the asexual-sexual life history decision. Since both the asexual development and sexual development of *N. crassa* are stimulated by different light conditions, further investigation of the absorbance spectrum and gene interactions of *phy-1* and *phy-2* would facilitate investigation of the regulatory interactions mediated by different light sensors. Detailed sensing of environmental factors likely helps fungal species to precisely program developmental regulation of life history decisions.

The examination of the function and evolution of phytochromes in ascomycetes provides additional insight into how fungi adapt and respond to environmental signals. In *Aspergillus nidulans*, *fphA* mutants exhibited an increase in the number of sexual fruiting bodies compared to the wild type under white light but not in the dark (38). Unlike *N. crassa*, which requires light during late sexual development, *Aspergillus* reproduces sexually underground in darkness. The function of *fphA* in repressing sexual reproduction is considered an adaptation to *Aspergillus* ecology (5). Catlett et al. (32) reported no phytochromes in early lineages in *Ascomycota*. We did not find phytochromes in any available yeast genomes in the *Saccharomycotina*, but we identified a phytochrome in *Taphrina* and in the yeast *Saitoella*, deeply diverging ascomycetous lineages in *Taphrinomycotina*. A very short phytochrome was found in the largest sequenced ascomycete genome, that of *Tuber melanosporum* (80), an underground mycorrhizal species in *Pezizomycetes*. Interestingly, aquatic fungus *Loramyces juncicola*, which grows on submerged substrates, has eight phytochrome-like proteins—the largest number observed in genomes examined here.

Plant pathogens *Sclerotinia sclerotiorum* and *Botrytis cinerea* are very closely related, yet show divergent regulation of life history regarding sporulation type: while *S. sclerotiorum* produces only ascospores and not conidia, *B. cinerea* is dispersed mainly via conidia (81). Both pathogens can survive during winter in the form of sclerotia, from which sexual apothecia or conidiophores arise, extend beyond covering duff or soil, and then release sexual or asexual spores to attack budding and blooming plants. In contrast to *Aspergillus* and *Neurospora*, asexual sporulation in *B. cinerea* is inhibited by blue light but can be promoted by far-red light, and sexual development of *S. sclerotiorum* is regulated by light in a narrow range of UV to blue light (36, 82). Furthermore, the stipe bearing the apothecia of *S. sclerotiorum* exhibits phototropism in red light that is not essential for apothecial development (82), and stipe morphology appears to be regulated in part by a CRY-DASH type photolyase/cryptochrome (83). Two to three phytochromes are found in *B. cinerea* and *S. sclerotiorum*, which exhibit evidence of domain losses and substitutions at key func-

tional sites, including a histidine-to-alanine change at the histidine kinase-related domain in the *S. sclerotiorum* homolog of PHY-2. Disruption of *Bcphy3* in *B. cinerea* (one of the two orthologs of *N. crassa phy-2*) led to a significant reduction in growth, production of sclerotia, and pathogenicity (84). Although only a single phytochrome (a Phy-1 homolog) is identified in pathogenic *Fusarium*, expression of this phytochrome gene in two *Fusarium* species exhibited upregulation during the late sexual reproduction, as seen in *phy-2* in *N. crassa*. There has been no report of the effects of red light on *Fusarium* development and pathogenicity. Measurement of the absorption spectrum and better identification of the roles of conserved *phy-1* in *N. crassa* would be helpful for investigation of the function of the single *phy-1* homolog in *Fusarium*, and further study of the function and evolution of phytochromes in fungi such as *Aspergillus*, *Sclerotinia*, *Botrytis*, and *Neurospora* has great potential to reveal the genetic basis of sexual development in response to light perception by rapidly and slowly evolving copies of red light sensors in ascomycetous fungi, including diverse and destructive plant pathogens.

MATERIALS AND METHODS

Strains and growth conditions. To examine the strains and growth conditions for genome-wide profiles of gene expression during asexual and sexual development, the *N. crassa* strains FGSC4200 (*mat a*) and FGSC2489 (*mat A*) were provided by the Fungal Genetic Stock Center. The conditions for growth were as described in detail elsewhere (60, 61). For assays of gene expression across asexual development, the strains were grown on Bird medium (85). Assays of gene expression across perithecial development were conducted on both synthetic crossing medium (SCM) and carrot agar (86).

Expression of phytochromes in asexual and sexual development. Expression of phytochromes in asexual and sexual development and genome-wide gene expression in *Neurospora* and *Fusarium* species were assessed using microarrays and RNA sequencing (Table 1). LOX v1.6 (87) and BAGEL (88, 89) were used to estimate gene expression levels across the time courses of this study.

Assessment of phenotypes of the knockout mutants. To assess the phenotypes of the knockout mutants, *N. crassa* gene knockouts were acquired from the Fungal Genetic Stock Center, including knockout strains FGSC11235 (*mat a*) and FGSC11236 (*mat A*) for $\Delta phy-1$ and FGSC11240 (*mat a*) and FGSC11241 (*mat A*) for $\Delta phy-2$. A double-knockout *mat a* strain, 560-8 (*phy-1::hph; phy-2::hph*), was generated for a previous study (7) and was investigated in this study as well. All KO strains used in this study were produced and verified by Southern blotting (90). Verification of the genotype of the $\Delta phy-1$ and $\Delta phy-2$ gene deletion mutants was performed by PCR-based genotyping (see Fig. S8 in the supplemental material), following a highly accurate methodology (91, 92).

Gene expression phenotypes of the KO strains. To determine the gene expression phenotypes of the KO strains, data from 40 microarrays originally reported by Chen et al. (7) and available from the GEO database (GSE8932) were reanalyzed. These data included measurements for the wild-type, $\Delta phy-1$, $\Delta phy-2$, and $\Delta phy-1 \Delta phy-2$ double-knockout strains at all sample points. The signal intensities of MRAT (the median of the set of background-corrected single-pixel ratios for all pixels with the spot) were compared between the reference and samples, and the ratios were used as input for a Bayesian analysis of gene expression levels (BAGEL) (88, 89). Data from serial data points were combined and treated as replicates for four different light conditions, including constant dark ("DD" [as in reference 7]), short light exposure (1,200 lx provided with cool white fluorescent bulbs) exposure up to 30 min (LL5, -10, -15, and 30), medium light exposure from 45 to 90 min (LL45, -60, and -90), and long light exposure from 120 to 240 min (LL120 and -240). BAGEL output was then further analyzed to identify genes with significant changes in expression among different samples, as well as to identify gene enrichment pat-

terns for different treatments on different genotypes, using the Functional Catalogue annotation scheme (93).

Developmental phenotypes of the KO strains. Using developmental phenotypes of the KO strains to screen for developmental phenotypes of phytochromes, crosses were prepared between opposite mating types on synthetic complete medium (SCM [94]). All permutations of crosses were performed in triplicate, and fungi were cultured at room temperature (25°C) under five light conditions, including complete darkness, constant white light provided by Ecolux bulbs with two intensity settings of 14 $\mu\text{mol}/\text{m}^2/\text{s}$ and about 700 $\mu\text{mol}/\text{m}^2/\text{s}$, respectively, and constant red light (670 nm), far-red light (735 nm), and constant blue light (470 nm), which was provided by an E-30LED incubator (Percival Scientific) as the sole light source with a high intensity of 450 $\mu\text{mol}/\text{m}^2/\text{s}$. Abundance of protoperithecia was assayed using a 100 by 100 by 15-mm gridded petri dish (Fisher Scientific). Plates with 40 ml SCM seeded with 3,000 conidia (hemocytometer counts) were incubated for 6 days. Numbers of protoperithecia were recorded for four blocks in the center of each plate, each 13 by 13 mm², with two biological replicates. All comparisons were based on *mat A* strains. Under the blue light condition for all tested strains, protoperithecia were produced in unevenly distributed patches and were concealed under dense aggregations of conidia: thus, we elected not to attempt to count protoperithecia under this condition. Development of protoperithecia and perithecia was examined to reveal the impact of light on sexual development between wild-type and KO strains. Opposite mating types were inoculated on opposite sides of small plates (9 cm in diameter) or in alternate quadrants of larger plates (14 cm in diameter).

Complementation of the knockout mutants. For complementation of the knockout mutants, cosegregation experiments were performed to ensure that the intended deletion is responsible for the mutant phenotype (95, 96). A hygromycin resistance cassette at the location of the deletion mutation provides a selectable marker (97). To assess cosegregation, the *mat A* $\Delta phy-1$ and $\Delta phy-2$ KO strains were crossed with a wild-type strain (FGSC4200 *mat a*). Thirty individual ascospore progenies were isolated and tested for resistance to hygromycin and genotyped by PCR to confirm the existence of *phy-1* and *phy-2* in hygromycin-resistant strains. Their phenotypes were then examined when cultured on SCM under red and blue light. Cosegregation of hygromycin resistance and the observed phenotype constitutes evidence that the observed phenotype was a result of the deletion of the specified gene.

Molecular evolution analyses. For molecular evolution analyses, multiple BLAST searches were performed against the NCBI and JGI databases (98) for homologs of *N. crassa* PHY-1 (NCU04834) and PHY-2 (NCU05790) in other fungal genomes. Bacterial phytochromes were downloaded to root the phylogeny (5). A set of 116 unique amino acid sequences from major groups of *Ascomycetes* was assembled from 302 phytochrome-like proteins acquired from protein BLAST searches (minimum length of 700 amino acids and sequence similarity above 40%). To confirm our finding of no phytochrome-like proteins in the *Saccharomyces* yeasts, a more relaxed search (minimum length of 300 amino acids and sequence similarity above 20%) was performed against all available yeast genomes in *Saccharomyces*. Amino acid sequences were aligned using SATÉ-II (99) with MAFFT as the aligner, MUSCLE as the merger, and RAXML as the tree estimator under the WAG model. The robustness of branching topologies was estimated with 1,000 maximum likelihood searches of bootstrapped sequence data using PhyML (100) under the WAG model. Robustness of the topology was further confirmed by comparison to phylogenetic analyses applying parsimony and distance criteria, performed with PAUP* 4.0 (101), as well as Bayesian analyses using MrBayes 3.2 (102). Branches with bootstrap proportions (BP) higher than 85% or posterior probability (PP) higher than 0.98 were considered significantly supported. A smaller data set that included homologs of *Phy-1* and *Phy-2* in species closely related to *Neurospora* and homologs of *FphA* in species closely related to *Aspergillus* was prepared for further analyses. ModelTest (68) was used to select an appropriate model of nucleotide substitution for the fungal phytochromes, and the relative ratio test in

HyPhy (69) was applied to evaluate differences in substitution rates between lineages of interest. Synonymous and nonsynonymous mutation rates of fungal phytochromes were calculated with CODEML of PAML (70, 71), and substitution saturation was estimated with index of substitution saturation analysis (72). Evolutionary conservation in the sequence and structure of each domain in phytochromes was calculated by Bayesian inference, specifying a WAG substitution model with ConSeq at the ConSurf (103) online server (104, 105).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.02148-15/-DCSupplemental>.

- Figure S1, TIF file, 0.7 MB.
- Figure S2, TIF file, 0.6 MB.
- Figure S3, TIF file, 1.3 MB.
- Figure S4, TIF file, 1.7 MB.
- Figure S5, PDF file, 2 MB.
- Figure S6, TIF file, 1.2 MB.
- Figure S7, TIF file, 0.8 MB.
- Figure S8, TIF file, 0.4 MB.
- Table S1, XLSX file, 1.5 MB.
- Table S2, PDF file, 0.3 MB.

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