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ORIGINAL RESEARCH

Regulation of monocot and dicot plant development with constitutively active alleles of phytochrome B

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Abstract

The constitutively active missense allele of Arabidopsis phytochrome B, *AtPHYB*^{Y276H} or *AtYHB*, encodes a polypeptide that adopts a light-insensitive, physiologically active conformation capable of sustaining photomorphogenesis in darkness. Here, we show that the orthologous *OsYHB* allele of rice phytochrome B (*OsPHYB*^{Y283H}) also encodes a dominant "constitutively active" photoreceptor through comparative phenotypic analyses of *AtYHB* and *OsYHB* transgenic lines of four eudicot species, *Arabidopsis thaliana*, *Nicotiana tabacum* (tobacco), *Nicotiana sylvestris* and *Solanum lycopersicum* cv. MicroTom (tomato), and of two monocot species, *Oryza sativa* ssp. japonica and *Brachypodium distachyon*. Reciprocal transformation experiments show that the gain-of-function *c*onstitutive *p*hotomorphogenic (*cop*) phenotypes by *YHB* expression are stronger in host plants within the same class than across classes. Our studies also reveal additional *YHB*-dependent traits in adult plants, which include extreme shade tolerance, both early and late flowering behaviors, delayed leaf senescence, reduced tillering, and even viviparous seed germination. However, the strength of these gain-of-function phenotypes depends on the specific combination of *YHB* allele and species/cultivar transformed. Flowering and tillering of *OsYHB*- and *OsPHYB*-expressing lines of rice Nipponbare and Kitaake cultivars were compared, also revealing differences in *YHB/PHYB* allele versus genotype interaction on the phenotypic behavior of the two rice cultivars. In view of recent evidence that the regulatory activity of *At*YHB is not only light insensitive but also temperature insensitive, selective *YHB* expression is expected to yield improved agronomic performance of both dicot and monocot crop plant species not possible with wild-type *PHYB* alleles.

KEYWORDS
flowering regulation, light signal transduction, photobiology, photoperiodism, phytochrome, shade avoidance

1 | INTRODUCTION

Plants possess an array of photoreceptors that mediate real-time light acclimation responses to optimize light capture and energy resource allocation. Among the best studied of these sensors are the phytochromes, which mainly monitor red (R) and far-red (FR) light fluences informing growth and developmental decisions that primarily impact competition with neighboring plants (Ballare & Pierik, 2017; Casal, 2013; de Wit, Galvao, & Fankhauser, 2016). Phytochromes sense visible light using a linear tetrapyrrole (bilin) chromophore that is covalently linked to a conserved cysteine residue in a multidomain apoprotein (Anders & Essen, 2015; Burgie & Vierstra, 2014; Rockwell, Su, & Lagarias, 2006). Plant PHY apoproteins comprise an N-terminal photosensory "light input" module consisting of highly conserved PAS, GAF, and PHY domains, and a more diverged C-terminal regulatory "signal output" module with two PAS domains and a histidine kinase-related domain (HKRD) (Nagatani, 2010). Photoisomerization of the C15 double bond of the bilin chromophore initiates the reversible interconversion between the inactive R-absorbing Pr form and the active FR-absorbing Pfr form of holophytochromes (phys), triggering structural changes throughout the polypeptide that are recognized by downstream signaling effectors (Bae & Choi, 2008; von Horsten et al., 2016; Pham, Kathare, & Huq, 2018).

As master regulators of plant growth and development, phys influence seed germination, vegetative growth, photoperiodic flowering, storage organ development, photosynthesis, senescence, shade avoidance responses (SARs), and phase/amplitude of the circadian clock (Demotes-Mainard et al., 2016; Franklin & Quail, 2010). For this reason, phys have been a target of crop improvement efforts for over three decades (Keller, Shanklin, Vierstra, & Hershey, 1989; Smith, 1992; Smith, Casal, & Jackson, 1990). Three major *PHY* lineages are present in flowering plant genomes, *PHYA*, *PHYB*, and *PHYC*, with gene expansion and/or loss having occurred in some eudicot species (Alba, Kelmenson, Cordonnier-Pratt, & Pratt, 2000; Clack, Mathews, & Sharrock, 1994; Karve et al., 2012; Mathews, 2010; Sheehan, Farmer, & Brutnell, 2004; Takano et al., 2005). Transgenic phytochrome-based crop improvement efforts have predominantly exploited *PHYA* overexpression because, unlike phyB, phyA remains active in FR-enriched shade light (Ganesan, Lee, Kim, & Song, 2017). SARs that include rapid internode elongation, enhanced apical dominance, reduced photosynthesis efficiency, premature flowering and increased susceptibility to pathogen infection, reduce crop yields as plant density is increased (Carriedo, Maloof, & Brady, 2016). Triggered when phyB is inactivated by FR light, SARs are suppressed by Pfr-phyB, whereas *phyB* mutants exhibit constitutive SARs even under direct sunlight (Casal, 2013). Despite its inactivation by FR, *PHYB* overexpression can suppress shade-induced internode elongation and increase photosynthetic activity (Boccalandro et al., 2003; Hennig, Poppe, Unger, & Schafer, 1999; Husaineid et al., 2007; Karve et al., 2012; McCormac, Smith, & Whitelam, 1993; Sharkey, Vassey, Vanderveer, & Vierstra, 1991; Wagner, Tepperman, & Quail, 1991). However, *PHYB* overexpressors can flower early in non-inductive photoperiods (Bagnall et al., 1995; Hajdu et al., 2015; Krall & Reed, 2000; Oka et al., 2004; Song et al., 2015; Wallerstein, Wallerstein, Libman, Machnic, & Whitelam, 2002; Wu, Zhang, Li, & Fu, 2011; Zhang, Stankey, & Vierstra, 2013), late in inductive and non-inductive photoperiods (Bagnall & King, 2001; Halliday, Thomas, & Whitelam, 1997; Hwang et al., 2014; Song et al., 2015), at the same time as WT (Endo, Nakamura, Araki, Mochizuki, & Nagatani, 2005; Palagyi et al., 2010; Schittenhelm, Menge-Hartmann, & Oldenburg, 2004; Thiele, Herold, Lenk, Quail, & Gatz, 1999; Usami, Matsushita, Oka, Mochizuki, & Nagatani, 2007; Zheng, Yang, Jang, & Metzger, 2001), and even exhibit novel phenotypes inconsistent with SAR suppression (Viczian, Klose, Adam, & Nagy, 2017).

These observations underscore our incomplete understanding of phyB's regulatory roles in plants and also challenge the tacit assumption from model systems that these roles will be conserved in all plant species. It is well established that phyB signaling requires formation of stable and transient complexes with transcription regulators, components of the proteasome, and factors that affect the circadian clock (Bae & Choi, 2008; Viczian et al., 2017; Wang & Wang, 2014). PhyB also can form heterodimers with other phys, that is, with phyC-E in Arabidopsis (Clack et al., 2009; Sharrock & Clack, 2004). Hence, the relative abundances of phyB homodimers and these heterodimeric species change when phyB levels are altered. The regulatory behavior is even more complicated when phyB from one plant species is expressed in another, since the affinity of the introduced phyB with endogenous phys and/or with other downstream signaling components cannot be assumed to be the same as that occurring in the host. While this complexity accounts for the difficulty to predict the phenotypic consequences of phyB expression, it also implicates the potential of tailored phyB expression to modify selective aspects of light-mediated development of crop plants without affecting others.

Here, we exploited *YHB* alleles of rice (*Oryza sativum*) and Arabidopsis *PHYB*, the latter of which has been proven to be constitutively active regardless of the light conditions (Hu, Su, & Lagarias, 2009; Su & Lagarias, 2007). We reasoned that, as dominant alleles, *YHBs* could be used to suppress SARs in any plant cultivar to mitigate deleterious consequences on crop performance at high planting densities. Our studies examined the phenotypic consequences of heterologous expression of *AtYHB* in three eudicot species, tomato (*Solanum lycopersicum*) and two *Nicotiana* species (*N. tabacum* and *N. sylvestris*), and in the monocot species rice. Homologous *OsPHYB*- and *OsYHB*-expressing rice lines were constructed for comprehensive comparative analyses, which also enable assessment of the relative potency of homologous and heterologous *YHB* alleles on rice development. It is noteworthy that homologous overexpression of *OsPHYs* in rice has not yet been reported until now. We also examined the effects of heterologous *OsYHB* expression in the temperate model grass *Brachypodium distachyon*. Our studies illustrate the potential of *YHB*-based tools to alter photomorphogenic development in both dicot and monocot crop plants.

2 | EXPERIMENTAL PROCEDURES

2.1 | Construction of *OsPHYB*-, *OsYHB*-, and *AtYHB*-expressing transgenic rice lines

The rice transformation binary vector pSK63 containing the maize Ubiquitin promoter (Christensen & Quail, 1996), and a NOS terminator was modified from pSK61 kindly provided by Dr. Venkatesan Sundaresan at UC Davis (Kumar, Wing, & Sundaresan, 2005). pSK61 was digested with *Kpn*I and *Sac*I to remove the DsRed sequence and replace it with the MCS region from the pBS KS+ vector containing a unique *Spe*I cloning site. The full-length rice *OsPHYB* cDNA of *indica* subspecies in the RPB6 vector was kindly provided by Dr. Peter Quail (Plant Gene Expression Center) (Note: three polymorphisms exist between *indica* and *japonica OsPHYB* sequences). *OsPHYB* was subcloned into the pGEM®-T vector (Promega) with primers oWH7 (5′-atcGGTACCATGGCCTCGGGTAGCCGCGCCACG-3′) and oWH8 (5′-gatACTAGTTGGTTGACCGAATAGTTATGCG-3′). *pGEM-OsPHYB*^{Y283H} (*OsYHB*) was created by PCR-mediated site-directed mutagenesis with primers oWH9 (5′-GACCGCGTTATGGTG**C**ACAGGTTCCATGAGGATG-3′) and oWH10 (5′-CATCCTCATGGAACCTGT**G**CACCATAACGCGGTC-3′). *Kpn*I and *Spe*I digested *OsPHYB* and *OsYHB* from the aforementioned clones were ligated into pSK63 to obtain the corresponding plant transformation constructs *pSK63-pUbi::OsPHYB* and *pSK63-pUbi::OsYHB*. These two constructs were transformed into the Agrobacterium strain EHA105, and further transformed into *Oryza sativa* ssp. *japonica* cv Nipponbare and Kitaake, respectively, by the UC Davis Plant Transformation Facility (http://ptf.ucdavis.edu). Primers oWH16 (5′-TTGAAGACATTCGGGCCAGAAC-3′) and oWH20 (5′-GCTGGAGCAAACCTCACCATGC-3′) were used for PCR genotyping of the transgene (amplicons are 2,105 and 988 bp from the genomic DNA and cDNA transgene templates, respectively). To overexpress *AtYHB* in rice, the $_{P}$*35S::AtYHB::*$_{T}$*RBCS* cDNA expression cassette from the *pJM61-35S::AtYHB* plasmid (Su & Lagarias, 2007) was excised with *Pme*I and *Sfo*I and subcloned into the pCAMBIA1300 vector at the *Ecl*136II restriction site to create pPIPRA321 that confers hygromycin resistance *in planta*. The pPIPRA321 was transformed into the rice cv. Kitaake by the aforementioned facility to create *35S::AtYHB*/Kitaake lines.

2.2 | Heterologous *AtYHB*-overexpressing tobacco and tomato transgenic lines, and *OsYHB*-overexpressing Arabidopsis and *Brachypodium* transgenic lines

pJM61-35S::AtYHB (Su & Lagarias, 2007) was transformed into two tobacco species *Nicotiana sylvestris* and *N. tabacum* cv Maryland Mammoth and into tomato *Solanum lycopersicum* cv MicroTom by the aforementioned facility. To overexpress *OsYHB* in Arabidopsis, *pSK63-pUbi::OsYHB* was double digested with *Kpn*I and *Xba*I; the cDNA sequence was ligated into the *pJM61-35S::AtPHYB* vector (Su & Lagarias, 2007) that was similarly digested to delete the *AtPHYB* sequence. The resultant construct was transformed into Agrobacterium GV3101 and further transformed into Arabidopsis accession Col-0 using the floral dip method (Clough & Bent, 1998). To overexpress *OsYHB* in *Brachypodium*, *pSK63-pUbi::OsYHB* was transformed into the *Brachypodium distachyon* inbred line Bd21-3 by Dr. John Vogel's group at USDA-ARS, Western Regional Research Center (Bragg et al., 2012; Vogel & Hill, 2008). Standard genetic practice was employed in obtaining at least two independent genetically single-insertion homozygous transgenic lines of each species for phenotypic analysis.

2.2.1 | Rice *phyB-6* mutant

Five rice *phyB* mutant alleles were previously reported (Takano et al., 2005). We obtained an independent rice *phyB* null mutant in the Nipponbare cultivar, named accordingly as *phyB-6* (line #AMWA08; NCBI Accession # CL523988) from the CIRAD Centre of France (http://orygenesdb.cirad.fr) (Perin et al., 2006; Sallaud et al., 2004). Primers oWH15 (5′-CGCTCATGTGTTGAGCATAT-3′) and oWH16 were used to detect the mutant allele (~0.8 kb), and oWH16 and oWH17 (5′-CCCACATGCACAGAATACAGGC-3′) to detect the WT allele (~1.1 kb).

2.2.2 | Rice growth conditions and phenotypic measurements

Rice seeds were dehusked, surface sterilized with 2% w/v sodium hypochlorite for 30 min with shaking, and rinsed 4 times thoroughly with sterile water. Seeds were then submerged in water at 37°C for 2 days for germination promotion prior to transfer to 1× MS growth medium (for seedling measurements) or on soil (for adult plant measurements). A SANYO LED light was used for red light source (peak at 654 nm). Hypoxic germination condition was achieved by submerging rice seeds 4 cm under water level in test tubes. For observing rice root morphology, 0.2% (w/v) gellan gum (www.PhytoTechLab.com) rather than phytagar was used as the solidification agent to create transparent MS medium. Gellan gum needed at least 40 min of autoclave to completely dissolve. Greenhouse supplemented with light source was used for flowering test in natural day-length conditions (30°C during day and 25°C at night) in Davis, California, USA (Latitude 38°32′42″N). Conviron® growth chambers equipped with Philips MH400/U ED37 400 W Mogul Clear Metal Halide lamps were also used for additional short-day flowering test (10 hr L/14 hr D, constant 28°C).

2.2.3 | Physiological and phenotypic characterization of non-rice plants

For dark flowering studies, *AtYHB*^{g} (genomic *YHB*) transgenic Arabidopsis plants (Su & Lagarias, 2007) were grown on horizontal

petri dishes of 1× MS medium supplied with 2% w/v sucrose at 20˚C in darkness for up to 14 weeks. For shade response measurements, Arabidopsis seedlings were grown on MS medium under continuous white light (Wc, 75 μmol m^{-2} s^{-1}, R/FR ratio = 2.5) for 7 days at 20˚C, then transferred to an FR light-rich chamber under the same light fluence rate with a reduced R/FR ratio of 0.5 for additional 24 hr. Seedlings were harvested at 0 hr (no shade), 2, 4, 8 and 24 hr after shade exposure. Owing to poor germination on MS media, seeds of both tobacco species were directly germinated on potting soil for seedling measurement. Tobacco plants were grown in an extended-day greenhouse at 25°C under LD photoperiod (16 hr L/8 hr D) or in a Conviron® growth chamber at 20°C under short-day condition (SD) photoperiod (8 hr L/16 hr D, ~250 μmol m^{-2} s^{-1} light intensity) for flowering and adult plant phenotypic measurements. No-flowering *N. Sylvestris* under SD were kept for two years and *N. tabacum* cv Maryland Mammoth under LD for one year before being discarded. Tomato and *Brachypodium* both were grown in Conviron® growth chambers (~100 μmol m^{-2} s^{-1}, under LD 16 hr L/8 hr D or SD 8 hr L/16 hr D (only for tomato) photoperiods) at 20°C for phenotypic analysis. *Brachypodium* plants were vernalized (4˚C) for 10 days when they were one month old. Days to flowering were determined when primary stems with visible floral buds were 20 cm (or 25 cm) long for *N. sylvestris* (or for *N. tabacum* cv Maryland Mammoth), when the first flower opened for tomato, or when the spike emerged for *Brachypodium*.

2.2.4 | Transmission electron microscopy

The second leaves of 5-day-old, dark- or continuous red light (Rc)-grown seedlings of Kitaake rice cultivar were cut into small pieces under dim green light and immediately fixed in the Karnovsky's fixative solution. The sample processing and TEM image acquiring were essentially same as before (Hu et al., 2009). The samples were sectioned longitudinally.

2.2.5 | Quantitative RT-PCR

Arabidopsis seedlings from the shade response experiment, and 5-day-old rice seedlings grown under light or in darkness as specified in the results were snap-frozen in liquid nitrogen. Then, total RNAs were extracted using RNeasy Plant Mini Kit (Qiagen). cDNA was synthesized from DNase I-treated total RNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche) for Arabidopsis samples and using the SuperScript™ III kit (Invitrogen) for rice samples following manufacturer's instructions. Quantitative PCR was performed using SYBR® Green PCR Master Mix in the ABI 7,300 real-time PCR system (Applied Biosystems) for Arabidopsis genes, and later using the SsoAdvanced Universal SYBR Green Supermix kit in the CFX96 real-time PCR detection system (Bio-Rad) for rice genes. Primers used for qRT-PCR are listed in Table S4. Specificity of qPCR amplicons was confirmed by melt curve analysis and agarose gel visualization. Reference genes *UBQ10* (At4g05320) and *EF1a* (Os03g08020) were used to normalize gene expression levels for Arabidopsis and rice, respectively. Expression values are presented as mean ± *SD* from at least three technical replicates of pooled RNA samples.

2.2.6 | Microarray analysis

Two biological replicates of 5-day-old seedlings of Nipponbare rice cultivar grown in darkness or under Rc (50 μmol m^{-2} s^{-1}) at 28°C were harvested in the subjective morning, immediately frozen in liquid N_2 and stored at −80°C until RNA extraction. Two independent *Ubi::OsYHB*/Nip lines #1 and #9 were used for microarray work. RNeasy Plant Mini Kit was used for total RNA extraction and cleanup. Five-hundred nanogram total RNA was used for synthesis and fragmentation of linearly amplified RNA (aRNA) using the 3'IVT Express Kit (Affymetrix). Fifteen microgram fragmented aRNA was hybridized with the GeneChip Rice Genome Array (Affymetrix) that contains probes representing 51,279 rice transcripts from two rice cultivars. Data were processed using the analysis pipelines described previously (Hu et al., 2009; Smyth, 2004). All microarray data were deposited in NCBI Gene Expression Omnibus with an accession number GSE36320.

2.2.7 | Immunoblot analysis

Protein extractions were performed as previously described (Su & Lagarias, 2007). Equal amount of proteins (100 μg/lane; determined by BCA protein assay with BSA as protein standard) were separated on 7.5% Mini-PROTEAN® TGX™ precast gel (Bio-Rad) or 4%–20% ExpressPlus™ PAGE precast gel (GenScript) and then electroblotted onto an Immobilon®-FL PVDF membrane (Millipore). Membranes were blocked with Odyssey® blocking buffer (LI-COR) for at least 1 hr at RT. Mouse monoclonal anti-AtphyB B1 (1:300), rabbit polyclonal anti-OsphyB (1:1,000) (Takano et al., 2005), and anti-alpha tubulin (Thermo Scientific, 1:1,000) antibodies were used for immunodetection of AtphyB/AtYHB, OsphyB/OsYHB, and tubulin, respectively. After washing 5× with TBST (10 min each time), membranes were incubated with IRDye 800CW conjugated goat-anti-Mouse or goat-anti-rabbit IgG (H + L) (LI-COR, 1:5,000), washed, and then scanned using the Odyssey infrared imaging system (LI-COR) for visualizing immuno-reactive bands.

3 | RESULTS

3.1 | Arabidopsis *AtYHB* confers light-independent photomorphogenesis and shade insensitivity

We previously demonstrated that expression of the dominant gain-of-function $AtPHYB^{Y276H}$ (*AtYHB*) allele confers *c*onstitutive *p*hotomorphogenic (*cop*) phenotypes upon Arabidopsis seedlings

regardless of the light conditions (Hu et al., 2009; Su & Lagarias, 2007). When supplied with sucrose, dark-grown *AtYHB*-expressing Arabidopsis plants could proceed into adult stage, producing 8.1 ± 1.1 leaves (n = 18) before transitioning to flowering (Figure 1a). Such *AtYHB* plants lacked the typical apical dominance behavior, as multiple short inflorescence shoots emerged concomitantly from the rosette during a 14-week growth period. These observations reveal that *AtYHB* sustains prolonged plant photomorphogenic development in a light-independent manner. Since *At*YHB is poorly photoactive (Su & Lagarias, 2007), we further tested whether *AtYHB* plants retained sensitivity to changes in the R/FR ratio. To do so, we measured transcript levels of four shade-inducible genes, *ATHB2*, *PIL1*, *IAA29*, and *HFR1*, in the wild type and two transgenic lines expressing a *35S::AtPHYB* (WT) construct or an *AtYHB* genomic fragment driven by its native promoter (*YHB*^{g}). Consistent with previous studies (Roig-Villanova, Bou, Sorin, Devlin, & Martinez-Garcia, 2006; Salter, Franklin, & Whitelam, 2003; Sessa et al., 2005), all four genes were acutely induced in WT plants after 2 hr exposure to simulated shade, *that is*, low R/FR white light, and then underwent a rapid decay in transcript abundance (Figure 1b). Elevated expression of two of these genes, that is, *PIL1* and *HFR1*, re-occurred 24 hr later. By contrast, *35S::AtPHYB* and *AtYHB*^{g} transgenes suppressed shade-induced expression of these four genes (Figure 1b). These results established that native promoter-driven *AtYHB*, which led to accumulation of near wild-type level of *At*YHB protein (Figure 1b, bottom panel), was as effective as overexpressed *AtPHYB* in blocking the rapid transcriptional response to shade.

3.2 | Heterologous expression of *AtYHB* alters photomorphogenesis of eudicot species

To validate *YHB's* potential in regulating photomorphogenesis of other plant species, we examined the phenotypic consequences of heterologous expression of *35S::AtYHB* in two tobacco cultivars, *Nicotiana sylvestris* (abbreviated as Syl) and *N. tabacum* cv. Maryland Mammoth (abbreviated as MM), and one tomato species, *Solanum lycopersicum* cv. MicroTom (abbreviated as MT). Two independent transgenic lines for each species were secured, for which *At*YHB accumulation was confirmed by immunoblot assay (Figure S1a,b,c). *AtYHB* expression conferred *cop* phenotypes in darkness and enhanced light sensitivity for seedlings of all three species (Figure 2a,b,i). Notably, dark-grown *AtYHB*-expressing tomato seedlings accumulated high levels of anthocyanin in their hypocotyls; the emerging purple hypocotyls empirically became a phenotypic hallmark of homozygous *AtYHB* transgenic tomato (Figure 2i, Figure S2d). For both tobacco species, *AtYHB* expression rendered plants with compact rosettes and dark green foliage (Figure S2a,b); adult plants later exhibited severe dwarfism (Figure 2g, Figure S2c). The phenotypic consequences of *AtYHB* expression in adult tomato were mild although statistically significant (Figure 2j, Figure S2e,f), probably because MicroTom already is a dwarf cultivar (Carvalho et al., 2011; Marti, Gisbert, Bishop, Dixon, & Garcia-Martinez, 2006).

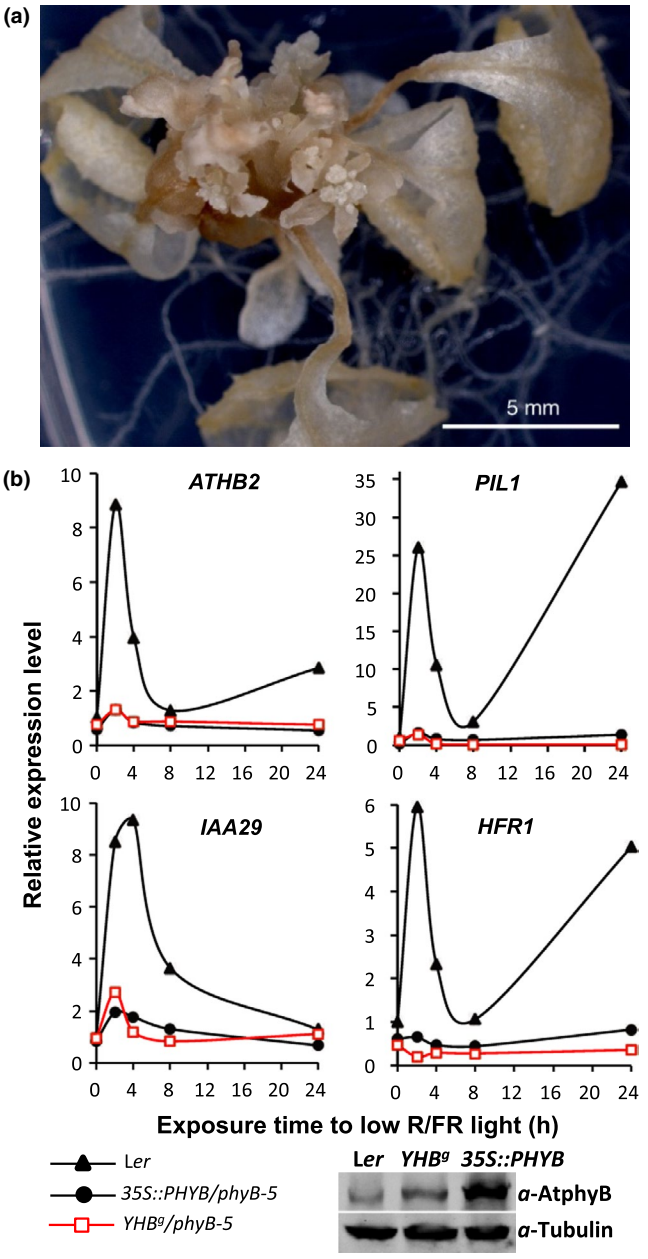

FIGURE 1 *AtYHB*-expressing Arabidopsis plants can flower in the dark and also are shade insensitive. (a) A representative *AtYHB*^{g}*/phyA-201phyB-5* plant grown in darkness for ten weeks on 2% w/v sucrose-containing MS medium. (b) Time-course qRT-PCR measurements of transcript levels of four shade-inducible genes after transferring plants to simulated shade. *UBQ10* serves as the reference gene for normalization. Immunoblot comparison of AtphyB/AtYHB protein levels is shown at the bottom right

The tobacco Syl cultivar is a long-day (LD) plant; both WT and transgenic plants neither flowered, nor bolted, within 2-year growth period under SD. By contrast, *AtYHB* expression delayed flowering of Syl plants by about one month under LD (Figure 2c,e). The tobacco MM cultivar is a qualitative short-day plant harboring a null *ft* (*FLOWERING LOCUS T*) mutation that prevents flowering under LD (Garner & Allard, 1920, 1923; Lifschitz et al., 2006). Both *AtYHB* transgenic and MM WT plants remained vegetative under LD as

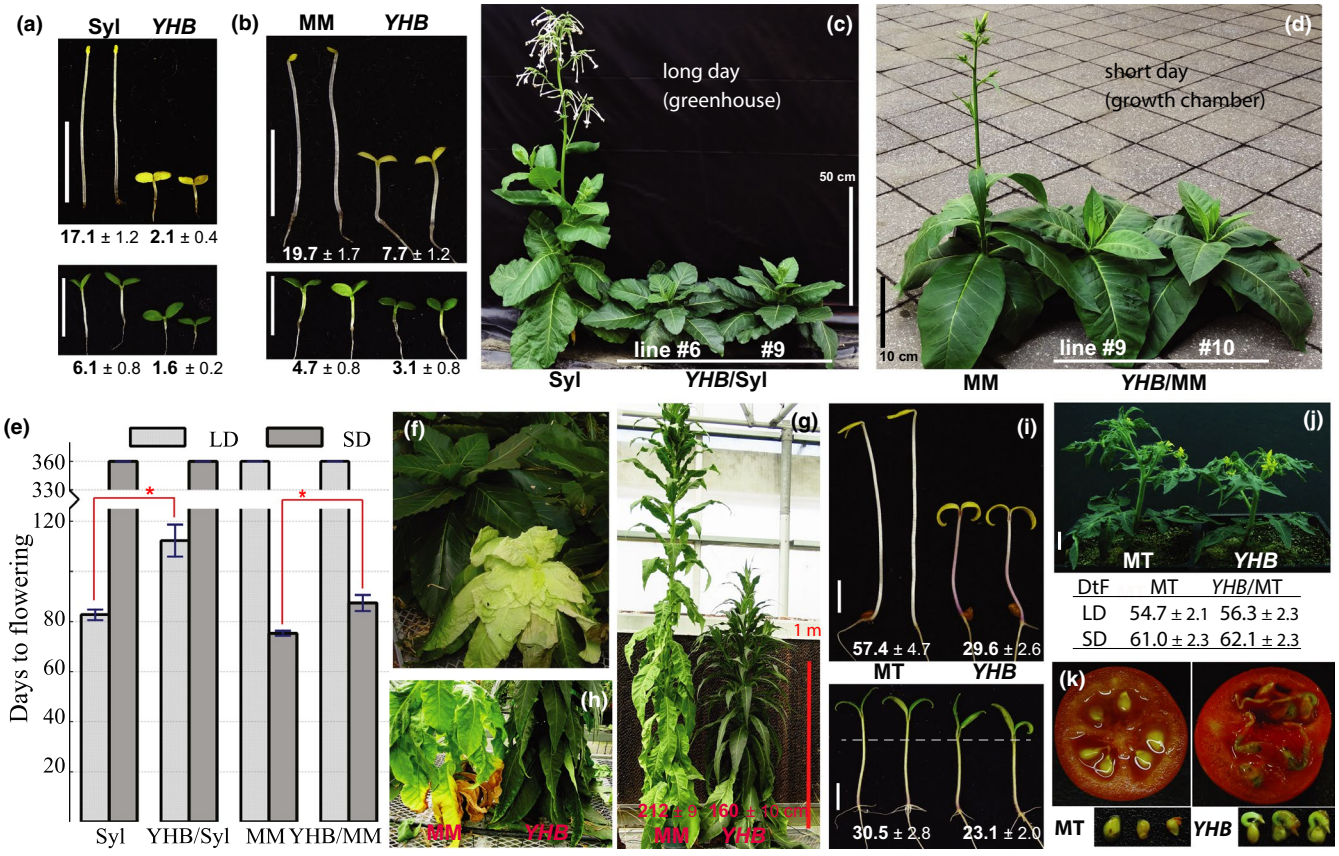

FIGURE 2 Heterologous expression of *AtYHB* in eudicot crop plant species confers constitutive photomorphogenesis, shade insensitivity, and altered day-length sensitivity. (a,b) Seven-day-old, dark- (top) and Rc-grown (bottom) WT and *35S::AtYHB* seedlings of *Nicotiana sylvestris* (Syl) and *N. tabacum* cv. Maryland Mammoth (MM); values represent mean hypocotyl length (mm) ± *SD* ($n \geq 30$). (c) LD greenhouse-grown 105-day-old Syl and *AtYHB*/Syl plants. (d) SD growth chamber-grown 79-day-old MM and *AtYHB*/MM plants. (e) Days to flowering (DtF) of the two WT tobacco species and corresponding *35S::AtYHB* transgenics under different photoperiods. Mean values are from two independent transgenic lines (*statistical significance $p < .0001$, $n \geq 10$). (f) The yellowish *AtYHB*/Syl transgenic plant had been completely shaded by a neighboring plant for more than 40 days, but did not show shade avoidance syndrome. (g) LD greenhouse-grown 6-month-old MM and *AtYHB*/MM plants. Values represent mean plant height ± *SD* ($n = 6$). (h) Comparative leaf senescence of 8-month-old greenhouse-grown WT MM and transgenic *AtYHB*/MM plants under LD photoperiods. (i) Seven-day-old, dark- (top) and Rc-grown (bottom) WT and *35S::AtYHB* seedlings of tomato cultivar, *Solanum lycopersicum* cv. Microtom (MT); values represent mean hypocotyl length (mm) ± *SD* ($n \geq 40$). (j) Comparative stature and flowering phenotypes of SD - and LD-grown WT and *AtYHB* transgenic MT plants, shown are mean values of DtF ± *SD* ($n \geq 9$). (k) Comparative vivipary phenotype of WT and *AtYHB* transgenic MT seeds inside ripened fruits. Scale bar = 1 cm if not otherwise labeled

expected (Figure 2e,g). By contrast, *AtYHB* expression delayed flowering of MM plants by about ten days under SD (Figure 2d,e). For MT tomato—a day-neutral plant, *AtYHB* expression did not alter flowering phenotype significantly (Figure 2j).

AtYHB expression also affected other traits of these dicot plant species. Under LD greenhouse growth conditions, *35S::AtYHB*/Syl plants produced such large and dark green leaves that shaded neighboring plant leaves became albino (Figure 2f). Despite exposure to such extreme shade, the transgenic plants lacked shade-induced responses, that is, stem elongation and accelerated flowering, confirming their shade tolerance. Delayed senescence of *35S::AtYHB*/MM plant leaves was evident (Figure 2h). *AtYHB* expression in MT also enhanced seed vivipary (Figure 2k, Figure S2g). However, no significant effect of *AtYHB* on fruit weight and seed number per fruit of MT plants was observed (Figure S2h).

3.3 | Expression of rice *YHB* (*OsYHB*) induces constitutive photomorphogenesis in two japonica rice cultivars

Molecular and physiological functions of monocot phys have been best characterized for the model crop species rice (*Oryza sativa*) based upon loss-of-function mutant analyses (Jumtee et al., 2009; Liu et al., 2012; Osugi, Itoh, Ikeda-Kawakatsu, Takano, & Izawa, 2011; Takano et al., 2001, 2005, 2009). To examine the phenotypic consequences of *OsYHB* expression in rice, we introduced the *Ubi::OsPHYB*^{Y283H} (*OsYHB*) overexpression construct into two cultivars of *Oryza sativa* ssp. japonica, Nipponbare and Kitaake. For comparative studies, *Ubi::OsPHYB* (WT) transgenic lines were also generated. At least two genetically single-insertion, homozygous lines were obtained for each combination of cultivars and constructs,

and expression of *OsPHYB* or *OsYHB* transgenes was confirmed transcriptionally and immunochemically (Figure 3a, Figure S1d; Table S1). WT Nipponbare, the *phyB-6* mutant (see Figure S3 for characterization of this new mutant line) and *Ubi*::*OsPHYB*/Nip seedlings all exhibited elongated coleoptiles in darkness, whereas coleoptiles of dark-grown *Ubi*::*OsYHB*/Nip lines were ~3-fold shorter, similar to those of Rc-grown control lines (Figure 3b,c). In addition, the first and second leaves of dark-grown *Ubi*::*OsYHB*/Nip seedlings were significantly shorter than those of other three dark-grown genotypes. Not surprisingly, leaf lengths of Rc-grown *Ubi*::*OsYHB*/Nip and *Ubi*::*OsPHYB*/Nip seedlings were both shorter than those of WT, indicating that overexpressed *OsPHYB*/*OsYHB* enhanced seedling red light sensitivity. Leaves of the *phyB-6* mutant were significantly longer than those of WT under Rc (Figure 3c), consistent with the reported phenotype of other rice *phyB* mutants (Takano et al., 2005). In the Kitaake cultivar, *OsYHB* also conferred *cop* phenotypes similar to those of the *OsYHB*/Nip lines (Figure S4a). Taken together, these measurements show that *OsYHB* functions as a dominant gain-of-function allele by inhibiting elongation of above-ground tissues in a light-independent manner.

OsYHB-promoted *cop* phenotypes were also manifest in rice mesocotyls and roots. In the dark, mesocotyls of WT seedlings typically elongated 1 ~ 5 mm (seedlings examined $n > 30$). By contrast, dark-grown *Ubi*::*OsYHB* did not have recognizable elongation of mesocotyls (Figure 3d, Figure S4b), same as light-grown seedlings. The growth of the seminal root, the primary root emerging from germinated rice

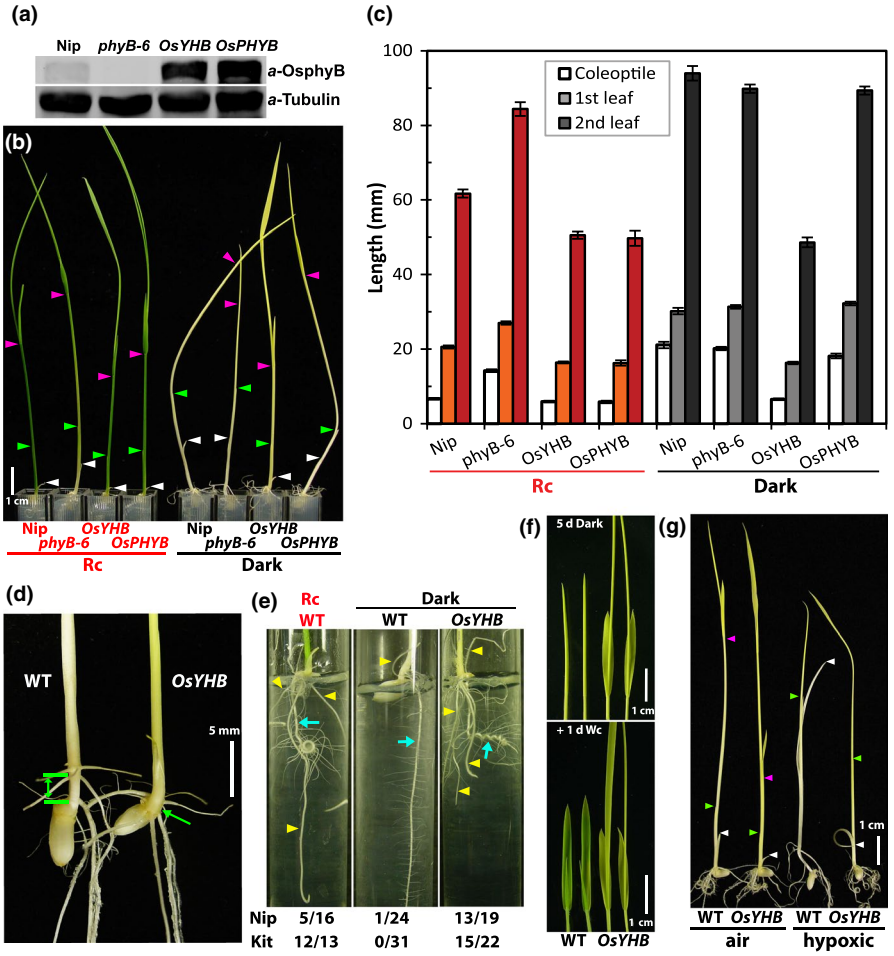

FIGURE 3 Expression of *OsYHB* confers constitutive photomorphogenesis to rice seedlings. (a) Comparative immunoblot analysis of OsPHYB/OsYHB protein levels in dark-grown Nipponbare WT, *phyB-6* mutant, and *pUbi*::*OsYHB* and *pUbi*::*OsPHYB* transgenic lines. (b) Representative 7-day-old seedlings grown under continuous red light (Rc, 50 µmol m^{-2} s^{-1}) or in darkness. (c) Comparative lengths of the coleoptile, first leaf and second-leaf sheath of 7-day-old Nipponbare seedlings, mean ± *SEM* ($n \geq 12$). (d) Comparative mesocotyl elongation of 7-day-old dark-grown WT and *OsYHB* transgenic Nipponbare seedlings. (e) Comparative crown and seminal root development of 7-day-old Rc-grown WT, dark-grown WT and *OsYHB* Kitaake seedlings. Cyan arrows and yellow arrowheads indicate seminal roots and crown roots, respectively; numbers of seedlings with coiled seminal root tips out of numbers of tested seedlings from Nipponbare and Kitaake cultivars are listed below the images. (f) Comparative blade expansion of 5-day-old dark-grown seedlings (top) and second-leaf greening after additional 1 day Wc exposure (bottom) in the Kitaake cultivar. (g) Suppression of *OsYHB*-dependent *cop* phenotypes of Kitaake seedlings under hypoxic germination conditions, that is, submergence under 4 cm deep water. White, green and magenta arrowheads in (b) and (g) indicate the apexes of coleoptiles, first leaves and second-leaf sheaths, respectively. Phenotypes from panels (d) to (g) were found in both Nipponbare and Kitaake cultivars, but are only shown from one cultivar

seeds, is known to be regulated by both phyA and phyB (Shimizu et al., 2009). In darkness, WT rice roots typically grow straight and possess few lateral crown roots. Dark-grown *Ubi::OsYHB* seedlings instead developed shortened seminar roots with multiple crown roots—a phenotype similar to Rc-grown WT seedlings (Figure 3e, Figure S4c). Approximately 70% of the dark-grown *Ubi::OsYHB* seedlings exhibited coiled roots, consistent with previous analyses of Rc-grown rice (Shimizu et al., 2009).

A closer examination of the second-leaf blades of dark-grown *Ubi::OsYHB* and WT seedlings indicated that the former had expanded as if they were grown in the light, in contrast to the latter. Upon light exposure, the second-leaf blades of WT promptly expanded and greened, while those of *Ubi::OsYHB* mostly remained yellowish with possible greening a few days later (Figure 3f, Figure S4d). It was known that dark-grown *AtYHB* Arabidopsis seedlings (>3-day-old) are photobleached and die upon light exposure, due to *At*YHB-mediated suppression of protochlorophyllide reductase A (*PORA*) expression and activation of tetrapyrrole biosynthetic pathway that collectively results in phototoxicity of the dark-accumulated protochlorophyllide (Hu & Lagarias, 2017). For *OsYHB* rice plants, however, the third leaves green normally after light exposure. Such de-etiolation differences likely arise from the mild twofold downregulation of *OsPORA* by *OsYHB* or red light in rice (Table S2, see below) compared with the ~30-fold downregulation of *AtPORA* by *AtYHB* in dark-grown Arabidopsis (Hu & Lagarias, 2017; Hu et al., 2009).

Upon submergence in deep water, germinating rice seedlings respond to the lack of oxygen (hypoxia) by inhibiting root and shoot growth while exaggerating coleoptile elongation (Magneschi & Perata, 2009). To test whether *OsYHB* seedlings retain responsiveness to submergence, we compared the growth of WT and *OsYHB* seedlings germinated in the dark 4 cm below the water level. The experiment revealed that submergence strongly, but not completely, suppressed the *cop* phenotypes of *OsYHB* seedlings (Figure 3g, Figure S4e). The lengths of coleoptiles and primary leaves of submerged *OsYHB* seedlings were much longer than those of aerially grown seedlings (WT and *OsYHB*), yet still shorter than those of the submerged WT.

3.4 | *OsYHB* promotes light-independent chloroplast differentiation

Transmission electron microscopy (TEM) next was used to examine the effect of *OsYHB* expression on rice chloroplast development. Mesophyll cells of dark-grown WT were relatively round or ellipsoidal, which contained small, undifferentiated etioplasts displaying prominent prolamellar bodies (Figure 4a,b). By contrast, mesophyll cells of Rc-grown WT were larger and more irregular in shape due to the increased size and number of differentiated chloroplasts (Figure 4c). Plastids of Rc-grown WT contained well-organized thylakoids (Figure 4d). Mesophyll cell ultrastructure of dark-grown *Ubi::OsYHB* was more similar to Rc-grown WT than dark-grown WT (Figure 4e). Moreover, plastids of dark-grown *Ubi::OsYHB* frequently contained parallel thylakoid membranes, indicative of light-independent plastid differentiation (Figure 4f). Such differentiation was incomplete, however, presumably due to the lack of chlorophyll synthesis in darkness (compare Figure 4d,f). Notably *AtYHB* also only triggers partial plastid differentiation in dark-grown Arabidopsis plants (Hu et al., 2009).

3.5 | The transcriptome of dark-grown *OsYHB* rice seedlings resembles that of Rc-grown WT

Affymetrix rice genome arrays were used to compare transcriptomes of 5-day-old *OsYHB*-expressing Nipponbare lines with those of the WT. Employing previous protocols and pipelines for *AtYHB* Arabidopsis plants (Hu et al., 2009), transcriptomes were determined for two biological replicates of dark-grown WT (Nip-D) and *Ubi::OsYHB* (*Os*YHB-D) lines and of Rc-grown WT (Nip-Rc) and *Ubi::OsYHB* (*Os*YHB-Rc) lines. The transcriptome data were highly reproducible, with correlation coefficient values from each pair of

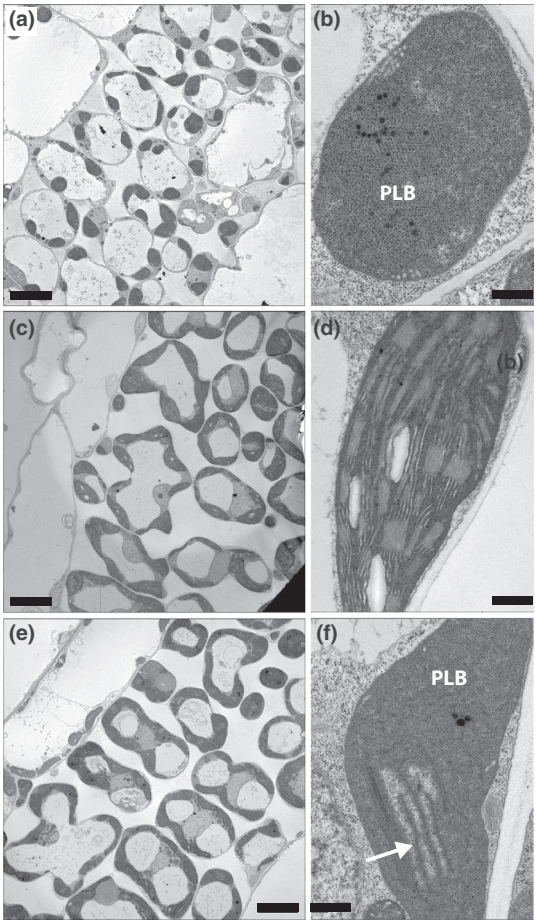

FIGURE 4 Transmission electron microscopy reveals light-independent chloroplast development in leaves of dark-grown *OsYHB* rice. (a,b) Kitaake (WT) in darkness, (c,d) Kitaake in Rc (50 μmol m^{-2} s^{-1}), (e,f) *Ubi::OsYHB*/Kit in darkness. (a,c,e) illustrate multiple cells and (b,d,f) focus on one plastid. Bars = 10 μm in (a,c,e) and 0.5 μm in (b,d,f)

replicates varying from .984 to .996. Of the 57,381 probe sets corresponding to 51,279 transcripts on the array, 28,184 (49.1% of the total) were expressed in the seedlings. To simplify the analysis, each probe set was assigned to a single transcript corresponding to one gene model.

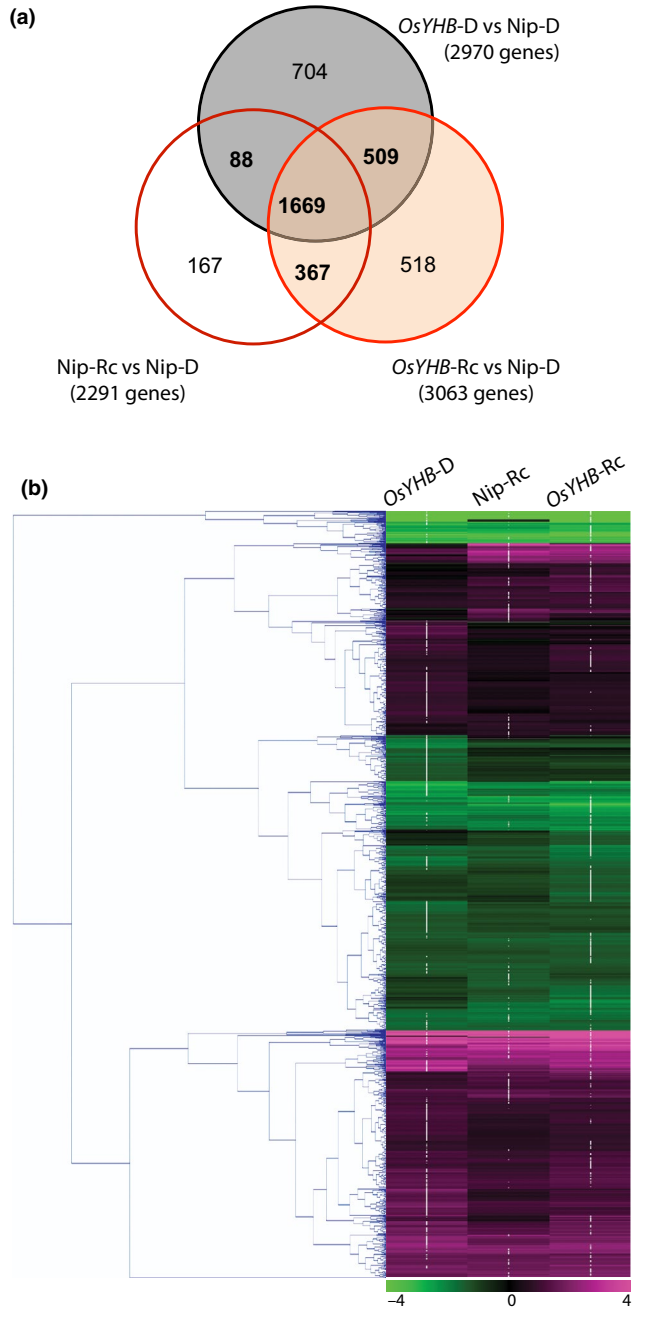

FIGURE 5 Transcriptome of dark-grown *OsYHB*-expressing Nipponbare rice mimics that of the Rc-grown wild type. (a) Venn diagram of differentially expressed transcripts (statistically significant and at least twofold difference) of dark-grown *Ubi::OsYHB*/Nip, and Rc50-grown WT (Nipponbare) and *Ubi::OsYHB*/Nip. (b) Expression pattern clustering heatmap of 4,022 transcripts differentially regulated by red light and/or *OsYHB*. The numerical values for the green-to-magenta gradient bar represent $\log_2$-fold change of gene expression relative to the Nip-D group, with magenta denoting expression induction, green repression and dark no change. White dots denote absolute maximum of expression change for each gene among the three groups

In comparison with the Nip-D transcriptome, 2,291, 2,970, and 3,063 genes with statistically significant and twofold (SSTF) expression difference were identified in Nip-Rc, *Os*YHB-D, and *Os*YHB-Rc transcriptomes, respectively (Figure 5a, Dataset S1). The gene overlap was largest between Nip-Rc and *Os*YHB-Rc at 89%, while the overlap between Nip-Rc and *Os*YHB-D was also significant (Figure 5a). Among the 4,022 genes differentially regulated by *Os*YHB and/or Rc, 1669 (41%) were shared by all experimental groups. As expected, genes functioning in light harvesting, photosynthetic electron transfer, carbon fixation, and biosynthetic metabolic processes were enriched in the shared core gene set. Hierarchical clustering showed that expression patterns of these 4,022 genes were qualitatively similar across experimental groups (Figure 5b). In short, transcriptomic profiling supported that *OsYHB*, as a gain-of-function allele, phenocopies the Rc-dependent gene regulatory networks in a light-independent manner.

Quantitative real-time PCR (qRT-PCR) was employed to additionally validate microarray-measured gene expression. The two methods gave consistent results for 10 out of 12 selected genes representing various patterns of expression changes (Table S2). The transcript levels of these genes in the dark-grown *Ubi::OsPHYB* line (*Os*PHYB-D) were also measured by qRT-PCR, which showed similarity with those in Nip-D seedlings, reinforcing that light was required to activate the *Os*phyB-dependent transcriptional network despite *Os*phyB being overexpressed. Notably, three of the 12 tested genes, that is, Os03g54000, Os01g72370, and Os03g51530, displayed red light-dependent and *OsYHB*-independent expression pattern (Table S2). Expression changes in four genes, that is, *RBCS*, *LHCB*, *OsPORA*, and *OsPIF4*, were also quantified in the Kitaake WT and *OsYHB*/Kit lines by qRT-PCR. The results confirmed that their expression patterns were in agreement with those in the Nipponbare background (Table S3).

The nature of microarray probe sets and signal detection accounts for discrepancies in expression levels of a small number of genes. First, the probe set for *OsPHYB* was designed to recognize the 3'UTR region; it therefore could not detect the overexpression level of the *Ubi::OsYHB* transgene lacking the native 3'UTR (Table S1). Second, saturation of hybridization signals on arrays limits the precise detection of induction of highly abundant transcripts. It was known that Rc illumination dramatically induces the transcript levels of *RBCS* and *LHCB* in WT rice, but not in the *phyABC* null mutant (Takano et al., 2009). qRT-PCR validated the strong induction of both genes in *Os*YHB-D (Tables S2,S3). By contrast, microarray showed no significant change in *RBCS* expression across experimental groups. This was because the *RBCS* transcript levels in Nip-D were already close to the maximum of recordable hybridization signal; the additional large increase in *RBCS* expression therefore was not reliably estimated by microarray. Similar inconsistencies had been seen for highly expressed Arabidopsis *CAB* genes that differed by more than 20-fold in expression comparing microarray and qRT-PCR estimates (Hu et al., 2009). Lastly, the rice genome array did not cover all gene

models, for example, *LHCB*. The *LHCB* expression levels acquired by qRT-PCR clearly showed significant transcript up-regulation by *Os*YHB or Rc exposure (Tables S2,S3).

3.6 | The effect of *OsYHB* and *OsPHYB* overexpression on rice flowering is cultivar-dependent

Flowering is a crucial developmental trait regulated by photoperiod, temperature, and endogenous signals. Based on the early flowering phenotype of *phyB* mutants in model eudicots and monocots, it is widely accepted that phyB delays flowering (Andres & Coupland, 2012; Franklin & Quail, 2010; Lee & An, 2015). Paradoxically, *PHYB* overexpression also leads to early flowering in many plant species (Hajdu et al., 2015). In the rice Nipponbare cultivar, both *Ubi::OsPHYB* and *Ubi::OsYHB* transgenic plants headed as early as the *phyB-6* mutant regardless of the photoperiod in a greenhouse environment (Figure 6a and Figure S5a). The early flowering behavior of *Ubi::OsYHB*/Nip plants was less evident under 10 hr L/14 hr D photoperiod in Conviron® growth chambers, due to greater growth variation of *OsYHB* plants than those grown in the greenhouse (Figure 6a). In the Kitaake cultivar, *OsYHB* delayed flowering by a few days compared to the WT under all photoperiodic conditions, while *OsPHYB* promoted flowering under non-inductive LD and had no significant effect under SD photoperiods (Figure 6b). In non-inductive LD, OsphyB induces the *Ghd7* expression, whose function is to repress the flowering-promoting genes *Ehd1* and *Hd3a*, thereby delaying flowering (Itoh, Nonoue, Yano, & Izawa, 2010). Harboring a non-functional *Ghd7* allele, the Kitaake cultivar is relatively insensitive to LD-dependent repression of flowering, and heads early in both SD and LD photoperiods (Itoh et al., 2010; Kim, Choi, Jung, & An, 2013; Xue et al., 2008). The paradoxical early flowering of *Ubi::OsPHYB*/Nip and *Ubi::OsYHB*/Nip plants is reminiscent of the very early flowering phenotype of *35S::AtPHYB* Arabidopsis plants grown in non-inductive SD conditions (Bagnall et al., 1995; Krall & Reed, 2000). While the mechanism whereby Os*PHYB/OsYHB* promotes flowering is unclear, the flowering-suppressive effect of *OsYHB* in the Kitaake cultivar suggests that *Ghd7* may play both positive and negative roles in photoperiod-dependent floral regulation by OsphyB in rice.

3.7 | *OsYHB* and *OsPHYB* reduce tiller number and stature of adult rice plants

Coincident with their early flowering phenotypes, both the *phyB-6* mutant and *Ubi::OsYHB/OsPHYB* transgenic Nipponbare plants had significantly reduced tiller numbers regardless of photoperiods (Figure 6c,d, and Figure S5a). Time-course measurements showed that *OsYHB/OsPHYB* overexpression repressed tiller outgrowth long before heading date, whereas WT Nipponbare kept developing more tillers over a longer period. This observation suggests that early heading of rice plants conferred either by the lack of endogenous phyB or by *Os*phyB/*Os*YHB over-accumulation is not the direct cause for the inhibition of tiller development. For the Kitaake cultivar, the tillering inhibition by *OsYHB/PHYB* overexpression was significant in LD (consistently two tillers fewer than WT) but marginal in SD conditions (Figure 6e,f). Together, overexpression of *OsPHYB/OsYHB* in rice neither increased tiller numbers nor promoted branching, contrasting with the branching-promotion effect of *AtPHYB/AtYHB* overexpression in eudicots. Because WT and transgenic Kitaake plants did not dramatically differ in flowering and tillering behaviors, we further compared other growth traits for this cultivar. Both *OsPHYB* and *OsYHB* overexpressors exhibited similarly reduced plant height by about 15% compared to WT (Figure S5b). The WT plants exhibited spreading architecture after seed maturation, owing to panicle weight-conferred stem bending. By contrast, both *Ubi::OsYHB* and *Ubi::OsPHYB* plants possessed relatively upright stems and compact architectures (Figure S5c)—a potentially beneficial trait agronomically.

3.8 | *OsYHB* overexpression induces constitutive photomorphogenesis and modestly promotes flowering in the model temperate grass *Brachypodium distachyon*

The effect of heterologous *OsYHB* expression was next examined in another monocot *Brachypodium distachyon* (inbred line Bd21-3). Immunoblot assays confirmed that four obtained independent transgenic lines express high levels of *Os*YHB protein (Figure 7a). Seedlings of these lines exhibited typical *cop* phenotypes, that is, coleoptiles of dark-grown transgenics were much shorter than those of dark-grown WT, but similar to those of red light-grown WT and transgenics (Figure 7b). *OsYHB* conferred a shorter adult plant stature in comparison with WT (Figure 7c,e). Lastly, *OsYHB* promoted early flowering by about four days; the effect is mild yet statistically significant by comparison with the WT control (Figure 7d). Thus, overexpression of *OsYHB* promoted flowering in both rice and *Brachypodium* in a dominant, gain-of-function manner.

3.9 | Reciprocal heterologous expression of *YHBs* in Arabidopsis and rice elicits weaker phenotypes than homologous expression

Heterologous expressions of *AtYHB* in other eudicots (tobacco and tomato) and of *OsYHB* in another monocot (*Brachypodium*) conferred strong *cop* seedling phenotypes. To further test whether YHB function is conserved across eudicots and monocots, we performed reciprocal expression experiments. Firstly, *OsYHB* was expressed in Arabidopsis (Col-0 accession) under the control of the constitutive 35S promoter. Dark-grown *35S::OsYHB*/Col seedlings exhibited *cop* phenotypes weaker than *35S::AtYHB*/Col seedlings, as evident by their longer hypocotyls (>3-fold longer) and

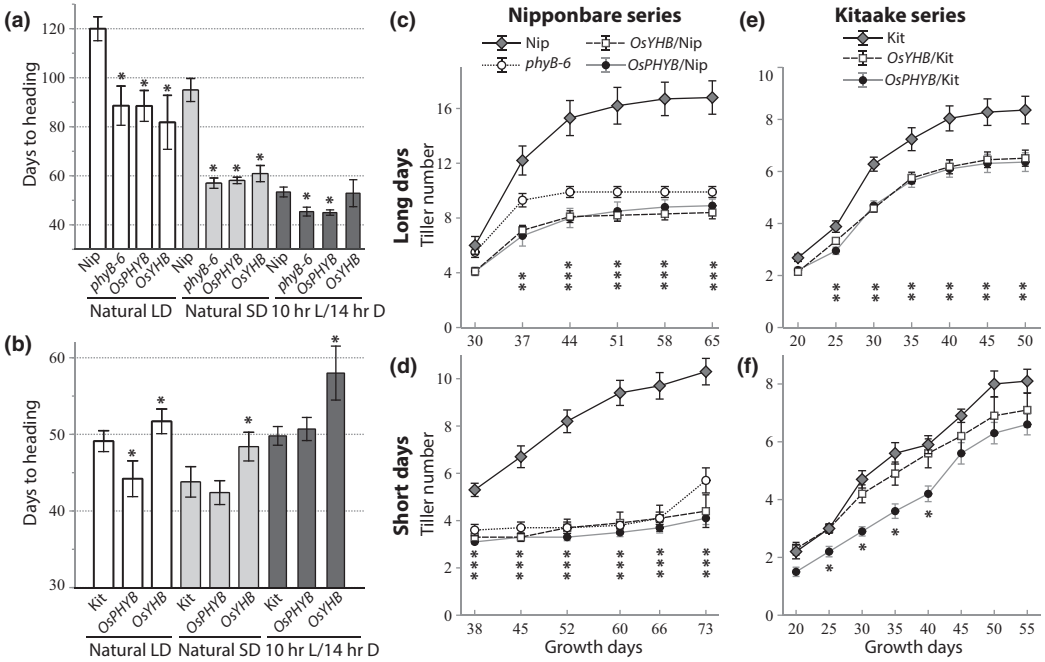

FIGURE 6 The influence of *OsPHYB* and *OsYHB* overexpression on photoperiod-regulated flowering and tillering in the rice Nipponbare and Kitaake cultivars. (a) *OsPHYB* and *OsYHB* transgenic lines and *phyB-6* mutants in the Nipponbare cultivar similarly exhibit early heading. (b) *OsYHB* delays, while *OsPHYB* may or may not promote, heading in the Kitaake cultivar. For (a-b), mean ± *SD*, *n* = 9~29 from two independent lines except for *OsPHYB*/Nip (only one line). Plants grown under natural long-day (LD) and short-day (SD) photoperiod were raised in the greenhouse, whereas plants grown under the 10 hr L/14 hr D were raised in growth chambers. (c-d) *OsPHYB* and *OsYHB* transgenic lines and *phyB-6* mutants in the Nipponbare cultivar develop significantly less tillers than WT under both LD (c) and SD (d) photoperiods. (e-f) Overexpression of *OsPHYB* and *OsYHB* in the Kitaake cultivar significantly reduces tiller numbers under LD (e) or has marginal effects under SD (f) photoperiods. For (c-f), mean ± *SEM*, *n* = 9~40 from two or three independent lines. **p* < .005 based on Dunnett's test comparing each transgenics or mutant to the wild-type control in one-way ANOVA

smaller cotyledons (Figure 8a). Under low fluence rate white light, *35S::OsYHB*/Col seedlings were significantly shorter than Col and just slightly longer than *35S::AtYHB*/Col seedlings, indicating that *OsYHB* function in Arabidopsis is enhanced by illumination (probably due to the synergistic activity of endogenous Arabidopsis phys). Consistent with that *AtPHYB*-overexpressing Arabidopsis plants flower very early in non-inductive SD photoperiod (Bagnall et al., 1995; Hajdu et al., 2015; Krall & Reed, 2000), *35S::AtYHB*/Col plants flowered much earlier than Col (Figure 8b). Flowering of *35S::OsYHB*/Col plants was also promoted, which was statistically significant although phenotypically lesser impressive (Figure 8b). For the reciprocal experiment, in which *AtYHB* was heterologously overexpressed in rice (Kitaake cultivar), weaker *cop* seedling phenotypes were also observed by comparison to the effect of homologous *OsYHB* overexpression (Figure 8c-e). Coleoptiles and first leaves, but not the second leaves, of dark-grown *35S::AtYHB*/Kit seedlings were significantly shorter than those of Kit WT (Figure 8d,e). The stature of adult *35S::AtYHB*/Kit plants was in between those of WT Kit and *Ubi::OsYHB*/Kit plants, demonstrating that AtYHB suppressed rice growth more weakly than OsYHB (Figure 8f; Table 1). Reduced tiller number under LD, reduced seed number, and reduced seed weight found among *Ubi*:*OsYHB*/Kit plants, were not, or only marginally, found among *35S::AtYHB*/Kit plants (Table 1). Taken together, *YHB* genes expressed heterologously across eudicot and monocot plants do exhibit conserved constitutive gain-of-function activity, but their regulatory potency is diminished, presumably due to reduced compatibility with the endogenous downstream signaling components in the heterologous host.

4 | DISCUSSION

Our work demonstrates that the tyrosine-to-histidine missense alleles of representative eudicot and monocot *PHYBs*, a.k.a. *YHBs*, encode light-insensitive "signaling-active" proteins, providing compelling support for the conclusion that *YHB* alleles of all angiosperm phyBs similarly will confer light-independent signaling activity. In this regard, the *YHB* allele of the liverwort phytochrome, $MpPHY^{Y241H}$ from *Marchantia polymorpha*, also yields a constitutively active protein that promotes nuclear photobody formation, MpPIF protein degradation, gemma germination, lateral growth of regenerated thalli, and up-regulation of light-responsive genes—all in the absence of light (Inoue et al., 2016; Nishihama et al., 2015). Despite its unique photobiological mode of action, the *YHA* allele of Arabidopsis *PHYA* is also constitutively active (Rausenberger et al., 2011; Su & Lagarias, 2007). These results indicate that this conserved Tyr residue in the GAF domain performs an important role in light-mediated signal

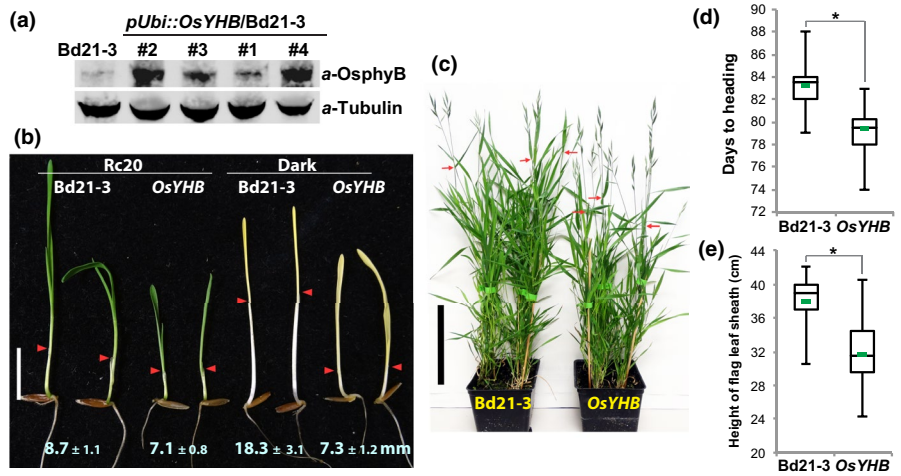

FIGURE 7 Heterologous expression of *pUbi::OsYHB* in *Brachypodium distachyon* (inbred line Bd21-3) supports constitutive seedling photomorphogenesis and promotes early flowering of light-grown adult plants. (a) Immunoblot detection of *Os*YHB protein in dark-grown, 10-day-old seedlings of four independent transgenic lines using the cross-reacting polyclonal anti-*Os*phyB antibody. (b) Six-day-old WT Bd21-3 and *Ubi::OsYHB*/Bd21-3 transgenic seedlings (from two independent lines) grown under continuous red light (20 µmol m^{-2} s^{-1}) or in darkness on MS media. Red arrowheads indicate coleoptile tips; values are coleoptile lengths represented as mean ± *SD* (*n* = 10); scale bar = 10 mm. (c) Photograph of 88-day-old WT and transgenic plants grown under 16 hr L/8 hr D photoperiods; red arrows indicate the flag leaf sheaths of primary tillers; scale bar = 10 cm. (d,e) Box plots of days to heading (d) and heights of the flag leaf sheath of primary tillers (e) for plants grown under 16 hr L/8 hr D photoperiods; short green lines indicate means, *indicates statistical significance (Student's *t* test, $p < .0001$); $n = 18$

transduction by all streptophyte phys, possibly even in the early diverging streptophyte algal species.

4.1 | YHB-mediated gain-of-function flowering phenotypes are plant cultivar-dependent

AtPHYB-overexpressing Arabidopsis plants are early flowering in non-inductive SD conditions—a paradoxical result in view of the phyB role in delaying flowering (Bagnall et al., 1995; Krall & Reed, 2000). Heterologous overexpression of *AtPHYB* in field-grown potato also leads to early flowering (Boccalandro et al., 2003). The present studies corroborate these observations for monocot species by demonstrating that (a) *OsPHYB*/*OsYHB*-overexpressing Nipponbare rice plants flower very early in both inductive SD and non-inductive LD conditions, and (b) *OsYHB*-overexpressing *Brachypodium* plants flower significantly earlier than WT. In Arabidopsis, the direct interaction between the E3-ubiquitin ligase SPA1 and over-accumulated phyB in its active Pfr form at night appears to be responsible for this early flowering phenotype via prolonged stabilization of CONSTANS (Hajdu et al., 2015). Consistent with this model, *cop1* and *spa1* mutants both flower early in non-inductive SD conditions due to enhanced expression of *FT* at night (Hajdu et al., 2015; Laubinger et al., 2006; Yu et al., 2008). In rice under non-inductive LD conditions, phyB has been shown to delay flowering by up-regulating *Ghd7*, thereby inhibiting expression of the flowering signal integrator *Ehd1* that promotes expression of florigen genes *Hd3a* and *RFT1* (Itoh et al., 2010; Osugi et al., 2011; Xue et al., 2008). Another major photoperiodic regulator of *Hd3a* expression is the rice CONSTANS orthologue Hd1, which appears to promote or repress flowering in SD or LD, respectively (Du et al., 2017; Izawa et al., 2002). We hypothesize that *OsPHYB*/*OsYHB* overexpression triggers early flowering in rice by transcriptionally/translationally regulating the Ghd7-Ehd1-Hd3a/RFT1 pathway and/or Hd1 activity. In the *Ghd7*-deficient, day-neutral Kitaake cultivar, *OsPHYB* overexpression only marginally promotes flowering in LD while, by contrast, *OsYHB* delays flowering (Figure 6). This result indicates that Ghd7 is critical for "interpreting" the enhanced phyB signal. Loss-of-function mutations in the rice *COP1* orthologue *PPS* (*PETER PAN SYNDROME*) also lead to early flowering in both SD and LD photoperiods independent of a functional Hd1 (Tanaka et al., 2011). This suggests that the Ghd7-Ehd1-Hd3a/RFT1 pathway and the PPS E3 ubiquitin ligase complex, both regulated by phyB in rice, contribute to the early flowering behavior of *OsPHYB*/*OsYHB* overexpressors. On the other hand, our studies show that overexpression of *PHYB*/*YHB* does not always confer early flowering. *AtYHB* overexpression significantly delays flowering of two tobacco species with opposite photoperiod sensitivity (Figure 2c-e). Similar results were reported in *AtPHYB*-overexpressing tobacco plants previously (Halliday et al., 1997). Overexpression of a cabbage *BrPHYB* in Arabidopsis was shown to slightly delay flowering in SD (Song et al., 2015). *AtYHB* overexpression did not affect flowering of the day-neutral tomato plants (Figure 2j). Therefore, the actual phenotypic consequence of *PHYB*/*YHB* overexpression on flowering depends on the genetic background of a particular plant species and likely depends on the origin of the transgene introduced as well as its expression pattern and level. Flowering is regulated by multiple positive and negative signaling pathways that are under clock control (Andres & Coupland, 2012; Boss, Bastow, Mylne, & Dean, 2004; Shim, Kubota, & Imaizumi, 2017). Hence, the effectiveness of *PHYB*/*YHB* alleles for regulation of flowering time cannot be

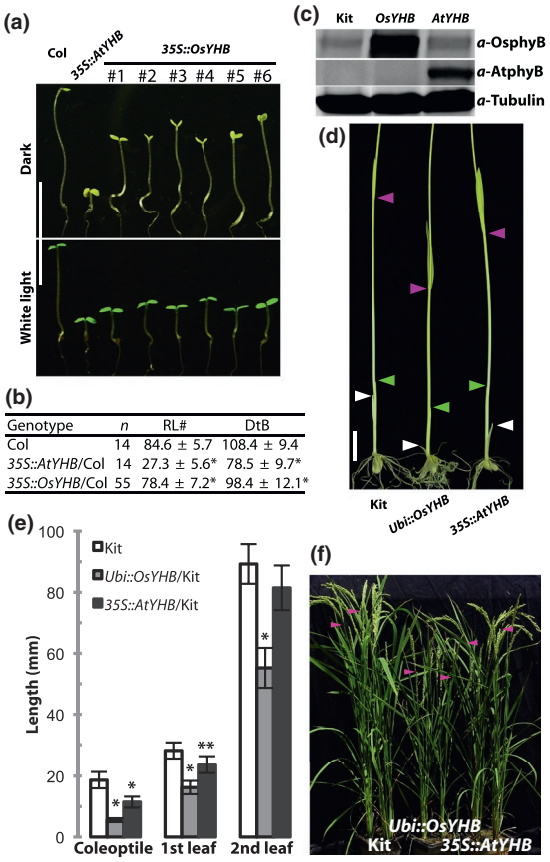

FIGURE 8 Heterologous *YHB* transgenes confer weaker gain-of-function phenotypes than homologous *YHB* transgenes. (a) Heterologous expression of rice *OsYHB* in Arabidopsis (Col ecotype); six independent *35S::OsYHB*/Col lines are shown; white light is 10 µmol m^{-2} s^{-1}, bar = 1 cm. (b) OsYHB moderately promotes early flowering in Arabidopsis under short-day conditions, **t* test *p*-values < .005 in comparison with Col; *n*, number of plants (for WT) or independent lines analyzed; RL#, rosette leaf number; DtB, days to bolting. (c) Immunoblot detection of rice and Arabidopsis YHB proteins in transgenic rice (Kitaake cultivar). (d) Heterologous expression of Arabidopsis *AtYHB* in rice; shown are representative dark-grown 7-day-old seedlings of WT Kit, *Ubi::OsYHB*/Kit #7 and *35S::AtYHB*/Kit #4 lines; white, green, and magenta arrowheads indicate the apexes of coleoptiles, 1st leaves, and 2nd leaf sheaths, respectively; bar = 1 cm. (e) Mean lengths ± *SD* (*n* = 8) of above-ground tissues of three genotypes. *t* test *p*-values of comparison to Kitaake: *<.001, **<.005. (f) Adult rice plants grown under LD for 65 days; magenta arrowheads indicate the last stem nodes of primary tiller (two plants per genotype)

easily predicted and will need to be examined empirically with each elite line of target crop plant species.

4.2 | Shoot branching and seed yield are negatively impacted by YHB overexpression

Mutant analyses establish that phyB promotes shoot branching in eudicots and monocots (Figure 6) (Finlayson, Krishnareddy, Kebrom, & Casal, 2010; Kebrom, Burson, & Finlayson, 2006; Kebrom & Mullet, 2016; Reddy & Finlayson, 2014). Overexpression of *AtPHYB* in eudicots consistently leads to more branches (Thiele et al., 1999). By contrast, *OsPHYB*/*OsYHB*-overexpressing Nipponbare rice plants develop fewer tillers than WT—a result also seen in the *phyB-6* mutant (Figure 6). This unexpected phenotype is concomitant with the early heading phenotypes of *OsPHYB/OsYHB* transgenic rice and may be mediated by the Ghd7 pathway. This interpretation is supported by the observations that (a) tiller number reduction was less dramatic in the Ghd7-deficient Kitaake cultivar, and (b) Ghd7 acts downstream of phyB in promoting more tillers (Weng et al., 2014). The underlying molecular mechanism of this effect awaits further investigation.

Since *OsPHYB*/*OsYHB* overexpression reduces tiller number in both rice cultivars regardless of photoperiod, with the effect being more dramatic in Nipponbare, the panicle number and total grain yield of transgenics will inevitably be lessened (Figure 6). For LD-grown Kitaake plants, the difference in heading dates between transgenics and WT was minor, which was thus ideal for comparative analysis of grain number per panicle and grain weight (Table 1). The measurements indicated that enhanced levels of *Os*YHB negatively impact grain yield index in the *japonica* rice background. Two early studies reported that heterologous overexpression of *AtPHYA* in rice reduced plant stature (Garg et al., 2006; Kong et al., 2004). In the sp. *indica* (*O. sativa* L. Pusa Basmati-1) background, transgenic *AtPHYA* rice plants developed more panicles and had 6 ~ 21% yield improvement in greenhouse experiments (Garg et al., 2006). In the sp. *japonica* (*O. sativa* L. Nakdong) by contrast, transgenic *AtPHYA* rice grown in paddy fields developed fewer tillers resulting in reduced yield despite their larger grain size (Kong et al., 2004). The latter study is consistent with our findings, suggesting that the difference may be subspecies/cultivar-dependent. It is noted that heterologously overexpressed *AtYHB* in Kitaake rice only modestly reduced plant stature, without affecting tiller number or grain yield index (Table 1). Additional studies are needed to dissect the roles of cultivar, promoter and allele choice on tillering and grain yield of phy-expressing transgenic rice.

Regarding the negative impact of YHB on seed yield, reduced seed yield was also noted for *35S::AtYHB/N. sylvestris* plants despite their extended vegetative growth compared with WT controls (Figure 2, Figure S2c, and data not shown). Moreover, numerous previous studies did not report seed yield enhancement in *35S::AtPHYB* Arabidopsis plants despite the apparent increase in photosynthetic capacity (Kreslavski et al., 2018). We attribute this to increased photo-assimilation products being preferably retained for vegetative biomass production and/or inefficiently mobilized upwards to developing seeds during seed set. By contrast, heterologous overexpression of *AtPHYB* in potato did improve tuber number in greenhouse and high-density field growth experiments, albeit with reduction in tuber weight (Boccalandro et al., 2003; Thiele et al., 1999). These studies indicate that the source-to-sink relationships are altered by *PHYB* overexpression and that much remains to be understood regarding the molecular basis of phyB's regulation of biomass

TABLE 1 Phenotypic comparison of *Ubi::OsYHB*/Kit and *35S::AtYHB*/Kit transgenic rice

		Ubi::OsYHB/Kit		*35S::AtYHB*/Kit	
Genotype	**Kitaake (WT)**	**#4**	**#7**	**#1**	**#4**
Days to heading (days)	49.1 (0.3)	52.2 (0.3)**	50.5 (0.3)*	48.7 (0.4)	50.8 (0.3)**
Tiller number	8.4 (0.5)	5.6 (0.5)**	6.7 (0.6)**	8.1 (0.5)	8.4 (0.9)
Height of last node (cm)	75.4 (1.1)	56.5 (0.9)**	54.7 (1.6)**	60.8 (0.9)**	71.1 (1.0)*
Seed # of primary panicle	80.7 (3.0)	65.2 (2.4)**	64.0 (2.5)**	68.1 (3.6)*	78.6 (3.6)
Seed weight (g/1,000)	24.7 (0.1)	22.3 (0.3)**	22.7 (0.5)**	25.0 (0.3)	23.8 (0.2)*

Note: Data presented as mean (*SEM*); $n \geq 15$. Statistical significance in comparison with wild type by *t* test, $^{**}p$-value < .001, $^{*}p$-value < .01.

redistribution into seed, stem, and root storage organs. It may be feasible to exploit *YHB* alleles to selectively alter biomass partitioning to maximize yield and quality of leaf, seed, stem, and root tuber crop plant species by appropriate spatiotemporal regulation of *YHB* transgene expression under dynamic light environment.

4.3 | *PHYA* and *PHYB/YHB* overexpression phenotypes are species-dependent

Typical phenotypes of *AtYHB*-expressing tobacco and tomato plants, that is, dark green foliage, delayed leaf senescence, compact rosette, dwarfism,and enhanced anthocyanin accumulation, are all in line with earlier transgenic studies in which monocot *PHYA*s were expressed in tobacco or tomato (Boylan & Quail, 1989; Cherry, Hershey, & Vierstra, 1991; Keller et al., 1989; Nagatani, Kay, Deak, Chua, & Furuya, 1991; Stockhaus et al., 1992). Similarly, "cross-class" expressed monocot *PHYA*s in Arabidopsis regulated plant growth in a way similar to endogenous phyB in white and red light (Boylan & Quail, 1991; Halliday, Bolle, Chua, & Whitelam, 1999; Kneissl, Shinomura, Furuya, & Bolle, 2008). Rice plants expressing Arabidopsis *PHYA* also exhibited significantly reduced stature (Garg et al., 2006; Kong et al., 2004), consistent with phenotypes of *OsYHB*/*OsPHYB*-overexpressing rice plants reported here. By contrast, eudicot *PHYA* expression in another eudicot or monocot *PHYA* expression in another monocot, that is, same-class expression, both fail to significantly alter the phenotype of host plants in white or red light (Clough, Casal, Jordan, Christou, & Vierstra, 1995; Heyer, Mozley, Landschutze, Thomas, & Gatz, 1995; Shlumukov, Barro, Barcelo, Lazzeri, & Smith, 2001). Such phenomenon may be attributed to enhanced stability of cross-class heterologously expressed phyAs that can function similarly to phyBs as low fluence rate sensors. Indeed, cross-class heterologously expressed phyAs were significantly less labile than endogenous phyAs of host plants, indicating that heterologous Pfr-phyAs were more resistant to the host protein degradation machinery (Boylan & Quail, 1989, 1991; Cherry et al., 1991; Garg et al., 2006; Kong et al., 2004; Stockhaus et al., 1992). This unique characteristic of phyA overexpression has been exploited for engineering crops with better agronomic traits (Ganesan et al., 2017; Gururani, Ganesan, & Song, 2015). It remains interesting to compare the functional interface of heterologous phyAs with the nuclear trafficking machinery in the same-class and cross-class transgenic lines. Nevertheless, as illustrated in Figure 8, our studies clearly show that PHYB/YHB overexpression confers stronger phenotypes in host plants within the same class than across classes. Although we cannot fully discount differences in YHB expression levels between species in part to explain these species-specific effects, an early study indicated that PHYB-dependent phenotypes saturate when its expression level exceeds that of endogenous PHYB by as little as threefold (Wagner, Koloszvari, & Quail, 1996). In view of the strong promoters used and the consistency of the phenotypes observed for multiple transgenic lines, it is likely that heterologous YHB expression well exceeds that of the endogenous PHYB. We therefore interpret the distinct phenotypes of heterologous and homologous YHB plants to mirror intrinsic differences in the biochemical activities of the heterologous and homologous YHB proteins.

4.4 | Prospects for biotechnological applications of *YHB* alleles

In view of the importance of phyB to plant growth, biomass and crop yield in major crop plants such as maize for example (Wies, Mantese, Casal, & Maddonni, 2019), we envisage a number of applications for crop plant improvement with novel *PHYB* alleles, *for example*, suppression of SARs, alteration of shoot and root dormancy, tillering, tuberization, among other phyB-dependent processes. As dominant missense alleles, *YHBs* should be feasible to generate in the native chromosomal context by CRISPR-Cas technology to yield new varieties of "unconventionally bred" elite crop lines without alteration of other loci. Moreover, YHB proteins are both light- and temperature-insensitive (Huang et al., 2019; Jung et al., 2016). Hence, their regulatory properties are not subject to the natural variation in these abiotic factors in the field unlike wild-type phyBs, and selective *YHB*

expression is expected to yield phenotypic outcomes distinct from wild-type *PHYB*-expressing lines, for example, in tissues such as meristems and roots in which the light environment restricts phyB activity. Our studies indicate that the success of biotechnological applications of both heterologous and homologous *YHB* alleles will depend on the strength of their coupling with the endogenous phyB regulatory pathways of each crop plant species. These include interactions with other phys and with downstream effectors, for example, PIFs, COP1, among many others, which are difficult to predict in new plant species. For these reasons, the effectiveness of *YHB* alleles therefore must be determined empirically for each target crop plant species, many of which are highly inbred and already have had extensive genetic modifications of natural light- and temperature-sensing pathways.

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CONFLICT OF INTEREST

The authors declare no conflict of interest associated with the work described in this article.

AUTHOR CONTRIBUTIONS

W.H. and J.C.L. conceived the project; W.H. performed experiments and analyzed data; R. F-B. and C. C-H. contributed to new germplasm sources (*AtYHB*-expressing tomato and Kitaake rice lines). The manuscript was written by W.H. and J.C.L. and approved by all authors.

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SUPPORTING INFORMATION

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