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ORIGINAL RESEARCH

Subfunctionalization of phytochrome B1/B2 leads to differential auxin and photosynthetic responses

Keisha D. Carlson | **Sneha Bhogale** | **Drew Anderson** | **Alondra Zaragoza-Mendoza** | **Andreas Madlung**

Department of Biology, University of Puget Sound, Tacoma, Washington

Correspondence
Andreas Madlung, Department of Biology, University of Puget Sound, 1500 N Warner St, CMB 1088, Tacoma, Washington 98416.
Email: amadlung@pugetsound.edu

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Abstract

Gene duplication and polyploidization are genetic mechanisms that instantly add genetic material to an organism's genome. Subsequent modification of the duplicated material leads to the evolution of neofunctionalization (new genetic functions), subfunctionalization (differential retention of genetic functions), redundancy, or a decay of duplicated genes to pseudogenes. Phytochromes are light receptors that play a large role in plant development. They are encoded by a small gene family that in tomato is comprised of five members: *PHYA*, *PHYB1*, *PHYB2*, *PHYE*, and *PHYF*. The most recent gene duplication within this family was in the ancestral *PHYB* gene. Using transcriptome profiling, co-expression network analysis, and physiological and molecular experimentation, we show that tomato *SlPHYB1* and *SlPHYB2* exhibit both common and non-redundant functions. Specifically, *PHYB1* appears to be the major integrator of light and auxin responses, such as gravitropism and phototropism, while *PHYB1* and *PHYB2* regulate aspects of photosynthesis antagonistically to each other, suggesting that the genes have subfunctionalized since their duplication.

KEYWORDS

auxin, gene duplication, photosynthesis, phototropism, phytochrome, subfunctionalization

1 | INTRODUCTION

Gene duplication is a powerful evolutionary mechanism that creates new genetic material on which natural selection can act (Moore & Purugganan, 2005). Duplication of genes can occur during polyploidization, also known as whole genome duplication (WGD), which may be the outcome of somatic genome doubling or the result of errors during meiosis leading to unreduced gametes (Bomblies & Madlung, 2014). While WGD is the most drastic form of gene duplication, other mechanisms can also lead to the duplication of individual genes. For example, unequal crossing over can lead to the formation of chromosomes with more or fewer copies of a given gene (known as tandem duplication), or transposons can copy and move genes within or between chromosomes (Panchy, Lehti-Shiu, & Shiu, 2016). Regardless by which mechanism they arise, duplicated genes can lead to the formation of multi-gene families and lay the foundation for evolutionary innovations (Van de Peer, Maere, & Meyer, 2009).

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One important family of plant genes are the phytochromes. Plants use both internal and external cues as signals to guide their growth and development, and to help them respond to their environment, such as to light quality and light quantity, temperature, moisture, or nutrient availability. Phytochromes (phys) are light-absorbing chromoproteins that consist of a chromophore and an apoprotein, which together transmit light signals and regulate gene expression in response to light (Chen & Chory, 2011; Franklin & Quail, 2010). The phy apoproteins are encoded by a multi-gene family that generally consists of a predominantly far-red (FR) responsive phy, phyA, and one or more predominantly red light (R) responsive phys. In Arabidopsis, the R responsive phys are encoded by four genes: *AtPHYB—AtPHYE*. Phylogenetically, gene duplication of an ancestral phytochrome gene first separated *PHYA/C* from the other PHYs. Subsequently, *PHYA* separated from *PHYC*, and *PHYB/D* from *PHYE* (Li et al., 2015; Mathews & Sharrock, 1997). Eventually, after the divergence of the *Brassicales*, *PHYB/D* separated into *PHYB* and *PHYD* genes in Arabidopsis (Mathews & Sharrock, 1997).

PHYs in tomato have not undergone the same phylogenetic evolution as in Arabidopsis. For instance, *SlPHYB1* and *SlPHYB2* (hereafter simply called *PHYB1* and *PHYB2*) are similar to *AtPHYB* and *AtPHYD* but these genes arose separately by a gene duplication event after the separation of the *Solanales* from the *Brassicales* about 110 Mya (Alba, Kelmenson, Cordonnier-Pratt, & Pratt, 2000; Pratt, Cordonnier-Pratt, Hauser, & Caboche, 1995), suggesting that any functional divergence of the duplicated genes would be unlikely to be the same in the two plant families. In contrast to Arabidopsis, mutation of *phyB1* in tomato results only in temporary red light insensitivity at a young seedling stage while *phyB1* adults look very similar in phenotype to WT tomato (Lazarova et al., 1998). In Arabidopsis and pea, *PHYB* plays a role during de-etiolation (Neff & Chory, 1998), chlorophyll production (Foo, Ross, Davies, Reid, & Weller, 2006), photo-reversible seed germination (Shinomura et al., 1996), timing of flowering (Khanna, Kikis, & Quail, 2003), the shade avoidance response (Keller et al., 2011), and the mediation of hormone responses (Borevitz et al., 2002), including lateral root initiation via auxin transport signaling (Salisbury, Hall, Grierson, & Halliday, 2007), polar auxin transport (Liu, Cohen, & Gardner, 2011), and seed germination via the regulation of abscisic acid (ABA) (Seo et al., 2006). Compared to Arabidopsis, much less is known about the functions of phys in the *Solanales*. In tomato, *PHYB1* is involved in hypocotyl inhibition, de-etiolation, and pigment production in R (Kendrick et al., 1994; Kendrick, Kerckhoffs, Tuinen, & Koornneef, 1997; van Tuinen, Kerckhoffs, Nagatani, Kendrick, & Koornneef, 1995). *PHYB2* plays a role in early seedling development (Hauser, Cordonnier-Pratt, & Pratt, 1998), and, in cooperation with *PHYA* and *PHYB1*, in the control of de-etiolation (Weller, Schreuder, Smith, Koornneef, & Kendrick, 2000). Analysis of *phyb1*;*phyb2* double mutants in tomato showed that a high level of redundancy exists between the two genes with respect to hypocotyl elongation during de-etiolation in both white light and R (Weller et al., 2000). Chlorophyll and anthocyanin production, on the other hand, was only reduced in the *phyB1* mutant and not in *phyB2*, but the *phyb1*;*phyb2* double mutant displayed a synergistic phenotype with less of both pigments than found in the *phyb1* mutant alone, suggesting that phyB2 contributes to pigment production in a significant manner (Weller et al., 2000). Subfunctionalization of the B-class phytochromes was also shown in maize, where *ZmPHYB1* was the predominant phy to regulate mesocotyl elongation in R, while *ZmPHYB2* was mainly responsible for the photoperiod-dependent transition from vegetative to floral development (Sheehan, Kennedy, Costich, & Brutnell, 2007).

To better understand to what degree subfunctionalization has occurred between tomato phyB1 and phyB2, we employed transcriptome profiling and co-expression network analysis. We found that tomato *PHYB1* and *PHYB2* exhibit both common and non-redundant functions. According to our analysis, two major areas of potential subfunctionalization are the regulation of genes involved in response to auxin and in photosynthesis. To verify the biological relevance of our genomic analyses, we tested *phyB1* and *phyB2* mutants for classical auxin responses, including phototropism and gravitropism, and for the rate of photosynthetic assimilation. We report here that phyB1 and phyB2 indeed differ in their involvement in some of these phenotypes, suggesting that the recent *PHYB* duplication in tomato has led to subfunctionalization that is different from those in maize or Arabidopsis.

2 | METHODS

2.1 | Plant materials and growth conditions

Solanum lycopersicum seeds of cultivar Moneymaker (Gourmet Seed, Hollister, CA, United States) and homozygous *phyB1* mutants (allele *tri*^{1}) and *phyB2* mutants (allele 2-1 (aka 70F), (Kerckhoffs et al., 1999; Weller et al., 2000) were used in all experiments. Both mutants used in this study were in the Moneymaker background (original source: Tomato Genome Resource Center, Davis, CA, USA). For RNAseq experiments, seeds were surface sterilized using 10% bleach for 15 min in ambient laboratory conditions and then sown on water-saturated, sterile filter paper in light-excluding plastic boxes. Plants were grown in a dark growth chamber at 25°C. Five-day-old seedlings of similar height were harvested under green safe light (522 nm LED), and flash-frozen in liquid nitrogen. Seedling handling and harvesting at room temperature under safelight conditions was limited to a few minutes of indirect exposure. The remaining seedlings were exposed to 60 min of red light (660 nm, using a custom-made LED display, 10 μmol m^{-2} s^{-1}) and then selected, harvested, and frozen as described for the dark-grown seedlings. Specimens were stored at −80°C until RNA was extracted. Tissue was grown in four biological replicates under the same conditions.

2.2 | RNA extraction and sequencing

Tissue was flash-frozen in liquid nitrogen and pulverized with a mortar and pestle. About 5 seedlings (~100 mg) were pooled per biological replicate for each genotype and condition. Total RNA was extracted using an RNeasy Plant Mini Kit (Qiagen) according

to the manufacturer's instructions. TruSeq stranded mRNA library construction was performed by the Research Technology Support Facility at Michigan State University. Paired end 125 bp reads were obtained using an Illumina Hi-Seq 2500 instrument. All data were uploaded for public use to NCBI's short read archive http://www.ncbi.nlm.nih.gov/sra/SRP108371.

2.3 | RNAseq differential expression analysis

RNAseq reads were mapped with HISAT2 to the SL3.0 version of the tomato genome with ITAG3.2 genome annotation from SolGenomics (www.solgenomics.org). First, phyB1 experiment reads and phyB2 experiment reads were mapped separately, and then they were mapped together. DESeq was used largely with default parameters to identify differentially expressed genes between wild type in the dark and wild type in R and between *phyB* mutants in the dark and in R, except that we used an alpha value of 0.05 for the multiple comparison adjustment. Genes identified in the phyB1 experiment alone as significantly differentially expressed (DE) by DESeq and with a abs(log2(fold change)) > 0.63, that is, changed by at least 1.5-fold between dark and R, were then looked at in the phyB1 comparison. If the gene had a log2(FC) that was significantly different from WT, we called the gene phyB1-regulated. To be characterized as significantly different, the difference between the log2(FC) in WT and *phyB1* had to be greater than the sum of the standard errors of the log2(FC) in WT and in *phyB1*. The process was repeated with the phyB2 experiment alone to identify phyB2-regulated genes.

2.4 | Co-expression analysis with WGCNA

From the data in which phyB1 and phyB2 experiment reads were mapped together, normalized read counts were obtained from DESeq. The variance of normalized expression was calculated across all samples (10 WT-D, 10 WT-R, 5 B1-D, 5 B1-R, 5 B2-D, 5 B2-R), and the top 8,000 most variable genes were identified. Their expression values were log transformed [log2(normalized read count + 1)] and used as input for WGCNA in R to identify co-expression modules. Beta was set to 10 for the adjacency function. Modules were obtained based on topological overlap and eigenvectors representing average expression of each module were correlated to condition (dark = 0, 60 min *R* = 1) and genotype (either *phyB1* = 1, *phyB2* and WT = 0 or *phyB2* = 1, *phyB1* and WT = 0).

2.5 | GO enrichment analysis

To determine which gene ontology (GO) categories were significantly enriched among the differentially regulated or co-expressed genes, we used the R package topGO (Alexa & Rahnenfuhrer, 2010; Alexa, Rahnenführer, & Lengauer, 2006). Only categories with p-values < 0.05 from Fisher's exact tests (weighted models) are reported. For topGO's "gene universe," GO annotations for *S. lycopersicum* were downloaded from the Panther Classification System (www.pantherdb.org, downloaded May 2017).

2.6 | Gravitropism

Wild type, *phyB1*, and *phyB2* seeds were sown at 12p.m., 5p.m., and 1p.m., respectively, to coordinate germination times (age-synchronized) and assure equal developmental stages at the time of experimentation. Seeds were sterilized by stirring for 15 min in 10% bleach in the dark and sown into light-excluding plastic boxes with saturated paper towels and filter paper under green light. Seeds were grown in the dark in a growth chamber at 25°C for 5 days. Age-synchronized seedlings were transferred under green light to 1% agar plates, placed either in dark, under R (135 μE) from the top, or in R from opposite sides (60 μE) and allowed to grow with the same gravity vector for 1 hr. Seedlings were then gravistimulated by rotating plates 90 degrees. Photographs were taken before gravistimulation (0 hr), after 4, 8, and 24 hr.

The angle of bending was measured with ImageJ. A three-way ANOVA (genotype, light condition, time) was performed in R followed by Tukey's post hoc test to determine statistically significant differences between groups.

2.7 | Phototropism

For phototropism experiments, age-synchronized seedlings (Moneymaker, *phyB1*, and *phyB2*) were grown in individual plastic scintillation vials filled with soil and incubated in the dark at 25°C for 5 days. Seedlings with similar hypocotyl length were then transferred to a black box illuminated with unilateral white light through a slit in the box. The plants were positioned such that their apical hook was facing away from the light source. Every hour over a time period of five hours, a set of plants was removed and scanned. The phototrophic bending angle of these plants was determined by ImageJ analysis, and data were plotted using R software. Data were analyzed by a two-way ANOVA (genotype, time) using the software R.

For qPCR analysis of *PHOT* genes, tomato seedlings were grown as for the phototropic experiments. Material was harvested and flash-frozen at the indicated times. Total RNA was extracted using an RNeasy kit (Qiagen) according to the manufacturer's instructions. Reverse transcription was performed using the iScript cDNA Synthesis kit (Bio-Rad) with the recommended incubation times and temperatures as follows: 25°C for 5 min, 46°C for 20 min, and 95°C for 1 min. QPCR was performed on a Bio-Rad Mastercycler C1000 using iTAQ Universal SYBR Green Supermix (Bio-Rad) with an incubation at 95°C for 3 min, followed by 40 cycles at 95°C for 10 s, and 60°C for 30 s. *SAND* (Solyc03g115810) and *RPL2* (Solyc10g006580) genes were used for normalization. Primer specificity was verified using the melt curves, and data were analyzed by the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001). Statistical analysis was performed using ANOVA (R version

3.4.1) on log10 normalized expression values. The primers are listed in Table S6. Three biological replicates were used with five seedlings per genotype and time point per biological replicate.

2.8 | Photosynthetic analysis and chlorophyll quantification

Six-week-old Moneymaker, *phyB1*, and *phyB2* plants grown in a growth chamber at 25°C under 16 hr of light were used for photosynthetic analysis and chlorophyll quantification. A LI-COR 6400XT portable photosynthesis system (LI-COR) with a standard leaf chamber and a LI-COR 6400 LED light source was used for photosynthetic efficiency measurement. To ensure best uniformity, we chose for analysis the terminal leaflet of the fourth youngest, fully developed leaf. Single leaflets still attached to the plant were clamped flat into the standard leaf chamber. The conditions in the leaf chamber were set at a reference CO_2 value of 400 mmol and a temperature of 21°C, and CO_2 uptake was measured at two different light intensities: 100 µmol photons m^{-2} s^{-1} and 1,500 µmol photons m^{-2} s^{-1}. Each leaf was placed in the standard leaf chamber before measurement and exposed to 2 min of light of the mentioned intensities in order to allow the plants to acclimate and CO_2 assimilation was measured. Matching was done after every plant to minimize errors. After measuring CO_2 assimilation, the leaf was photographed, and the leaf area was measured using ImageJ. Fresh weight of the respective leaf was also recorded, and chlorophyll was extracted in 5 ml of methanol for 72 hr in the dark at 4°C. Methanol extracts were analyzed by spectrophotometry and chlorophyll concentrations determined according to published procedures (Porra et al., 1989). Photosynthetic efficiency was calculated by normalizing the assimilation rate either for area or fresh weight. Three experimental replicates were performed with ~10 plants per genotype per replicate.

2.9 | Analysis of regulatory sequences

To compare the upstream regulatory region of *PHYB1* and *PHYB2*, we analyzed the 3-kb upstream of the transcription start site of each gene using the PLANTCARE database (Lescot et al., 2002). The sequences of PhyB1 (SlyPhyB1_SL2.50ch01_68870469.0.68867470) and PhyB2 (SlyPhyB2_SL2.50ch05_63510061.0.63507062) were obtained from Solgenomics (https://solgenomics.net/).

3 | RESULTS

3.1 | PhyB1 and PhyB2 differentially affect the transcriptome during photomorphogenesis in tomato seedlings

To determine if *PHYB1* and *PHYB2* have acquired different functions since the divergence from their common single-gene ancestor, we performed RNAseq analysis. We grew WT and *phyB1* and *phyB2* mutant seedlings for 5 days in the dark and compared them with individuals of the same genotypes and age that were also exposed to red light (R) for 60 min. We then identified genes that were differentially expressed in the mutants between dark and light (Table S1).

Using a threshold value of 1.5-fold upregulation or downregulation, we first filtered the data from the RNAseq analysis for genes that were statistically significantly upregulated or downregulated by light treatment in the WT. Of those genes, we considered a gene to be phyB1 or phyB2 regulated if it was either (a) upregulated or downregulated by light in the WT but not differentially regulated in the mutant, (b) oppositely regulated in the mutant compared to the WT, (c) significantly less strongly regulated in the mutant compared to the WT, or (d) more strongly regulated in the mutant compared to the WT. This data filtration yielded 121 phyB1-regulated genes, and 73 phyB2-regulated genes. In these gene sets, we identified functional enrichment gene ontology (GO) categories (Figure 1; Table S2). To identify traits possibly subfunctionalized between *PHYB1* and *PHYB2* mutants, we were particularly interested in GO categories that showed significant enrichment in one phyB-regulated gene set but not the other. GO categories significantly enriched in genes regulated by phyB1 included responses to auxin (GO: 0009733), responses to cytokinins (GO: 0009735), and protein phosphorylation (GO: 0006468). By contrast, phyB2-regulated genes did not fall into these three GO categories, but instead into GO categories such as defense response (GO: 0006952) and processes involving aromatic amino acid metabolism and biosynthesis (GO: 0009095 and GO: 0006558) (Table S2; Figure 1).

To gain additional insight into genes that were differentially affected by their mutations in either *PHYB1* or *PHYB2*, we employed transcriptional co-expression analysis of the top 8,000 most variably expressed genes across all conditions and found modules containing genes that due to their co-expression status were likely to have some degree of functional connectivity (Figure 2; Table S3). The yellow, blue, red, and light-cyan modules contained genes positively correlated to the *phyB1* mutation (i.e., they were more highly expressed in *phyB1* than in WT and *phyB2*) but negatively or not significantly correlated to the *phyB2* mutation. The opposite was true for the brown, salmon, turquoise, and green modules, which contained genes positively correlated to the *phyB2* mutation (i.e., they were more highly expressed in *phyB2* than in WT and *phyB1*) but not or negatively correlated to the *phyB1* mutation. These opposite expression patterns thus indicated diversified regulation between the two *PHYB* genes. Such diversified regulation was also seen, albeit not significantly, in the black, green-yellow, and cyan modules.

Modules containing genes that were regulated by light ("condition") included the tan module (negative correlation), and the green-yellow, magenta, green, midnight blue, and pink modules (positive correlation) (Figure 2). The green module was the only module containing genes that were significantly correlated with light (positively) and were also oppositely correlated with *phyB1* and *phyB2*. We looked for enriched GO functions in each co-expression module (Table S4). Among these

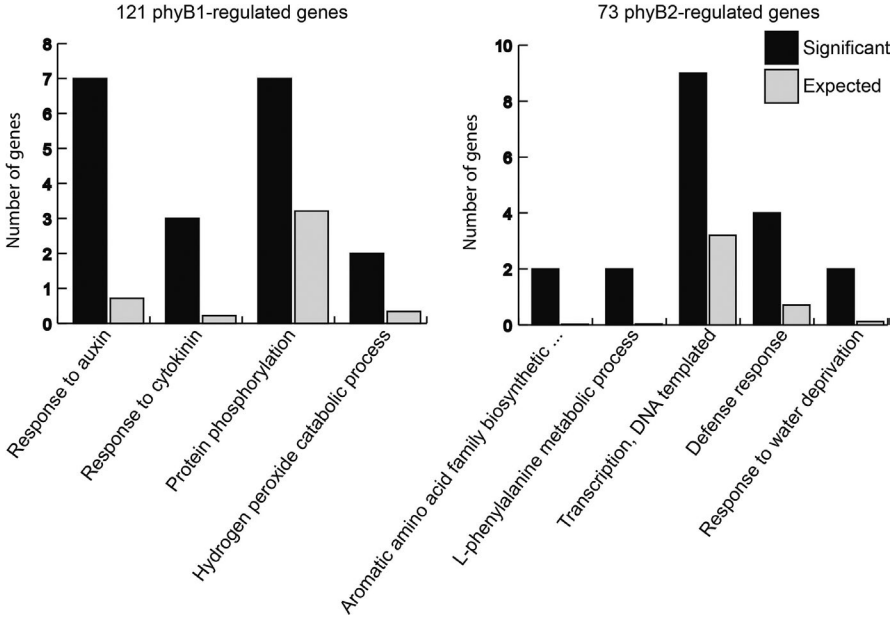

FIGURE 1 phyB1 and phyB2 regulate expression of genes involved in different biological processes. We identified 121 phyB1-regulated genes and 73 phyB2-regulated genes. Gene ontology functional enrichment analysis of these gene groups identified biological processes specifically regulated by phyB1 and phyB2. For all significant GO category enrichments, the black bars represent the number of genes with that annotation in that group (Significant) and the gray bars represent the expected number of genes with that annotation if representation was random (Expected)

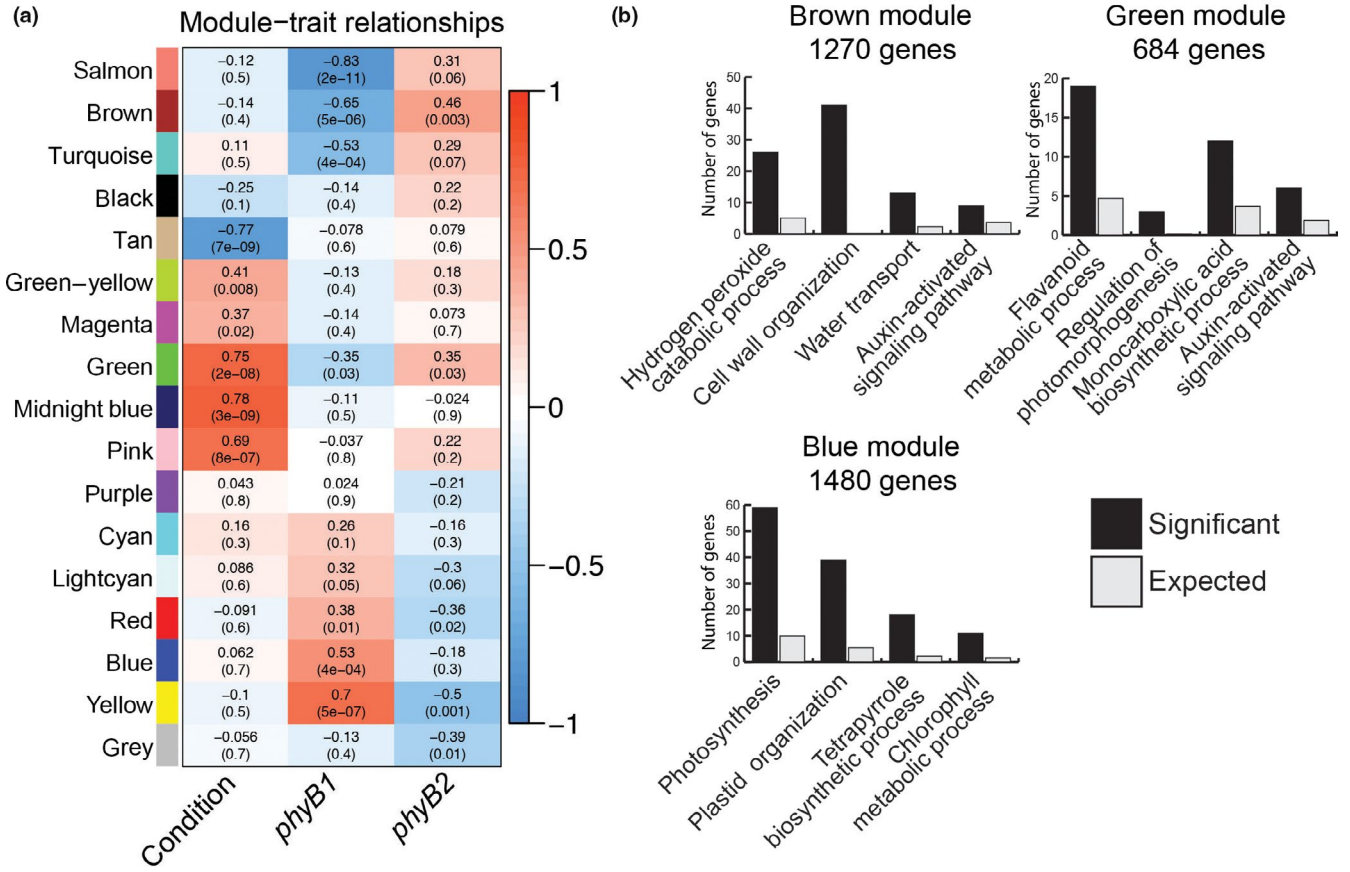

FIGURE 2 Co-expression modules show phyB1 and phyB2 differently regulate gene networks involved in auxin and photosynthesis related biological processes among others. (a) For each co-expression module (indicated by color) and the genes that did not fall into a co-expression module (gray), the average expression vector (eigenvector) across conditions and genotypes was correlated to condition (dark = 0, 60 min R exposure = 1) and genotype (*phyB1* column: WT and *phyB2* = 0, *phyB1* = 1; *phyB2* column: WT and *phyB1* = 0, *phyB2* = 1). R^2 values from the Pearson correlations are indicated in the heatmap by color according to scale on the right as well as by their printed value in the grid with p-values below in parentheses. (b) Gene ontology functional enrichment analysis identified biological processes central to each co-expression module. Displayed here are four enriched GO biological processes for the brown, green, and blue modules. The black bars represent the number of genes with that annotation in that group (Significant) and the gray bars represent the expected number of genes with that annotation if random (Expected)

functions were auxin-related processes, including auxin efflux (GO: 0010329), the auxin-regulated processes of gravitropism and phototropism (GO: 0009959, GO: 0009638), and auxin signaling (GO: 0009734), as well as photosynthesis-related processes (GO: 0009765, GO: 0009773, and GO: 0015979), in addition to a large number of other functional categories (Figure 2b and Table S4).

To determine areas of subfunctionalization between phyB1 and phyB2 in tomato, we combined information from our differential expression, co-expression and GO analyses to choose physiological functions for further testing and verification that transcriptomic differences had measurable effects on phenotypes. These functions were chosen based on (a) frequency of appearance in our data as being differentially regulated by phyB1 and phyB2, (b) statistical significance of our differential and co-expression analyses data, and (c) the number of genes on which individual enrichment analyses were based. Additionally, functions for further study were chosen if they were known from the literature to be regulated, at least in part, by phyB in Arabidopsis.

3.2 | PhyB1 and PhyB2 differentially modulate auxin responses in tomato seedlings

To determine if our gene expression analysis had predictive power on the plant's phenotype, we subjected wild type (WT) and *phyB1* and *phyB2* mutants to a variety of physiological experiments. Given that auxin-related processes had been implicated as differentially regulated by phyB1 and phyB2 in both differential expression and co-expression analyses, we tested if the auxin-related responses phototropism and gravitropism were differentially affected between *phyB1* and *phyB2* mutants when compared to the WT. Phototropism, the movement of plants toward a light source, is achieved by the perception of blue light via the photoreceptors PHOT1 and PHOT2, eventually leading to unequal distribution of auxin along the hypocotyl of a seedling exposed to unilateral light (Fankhauser & Christie, 2015). Differential auxin concentrations then result in unequal growth on the light versus dark side of the stem or hypocotyl leading to curvature toward the light source (Fankhauser & Christie, 2015). Indeed, when we exposed 5-day-old seedlings to unilateral white light (WL) over a period of three hours, *phyB1* hypocotyls displayed a significantly faster phototropic response (Figure 3) compared to the WT and *phyB2* plants, indicating a differential role of phyB1 and phyB2 in the phototropic response in tomato. This suggests that phyB1, but not phyB2, normally inhibits phototropic bending.

Our RNAseq differential gene expression analysis had found *PHOT1* to be differentially expressed in the WT dark versus WT red light comparison but the gene was not phyB1 or phyB2 regulated. *PHOT2* was not differentially expressed in either comparison. Since differences in the phototropic phenotype were recorded for seedlings grown under conditions different from those in our RNAseq experiment, we decided to check if gene expression differences of these receptors pivotal to the phototropic response might also be detectable between *phyB1* and *phyB2* mutants during phototropic stimulation. Testing *PHOT1* and *PHOT2* expression with qPCR at 0 and 3 hr of treatment with unilateral white light, we observed a decline in *PHOT1* and an increase in *PHOT2* expression over the 3-hr treatment (Fig. S1), but found no significant differences of gene expression between the two *phyB* mutants, suggesting that regulation of the *PHOT1* and *PHOT2* genes does not explain the measured phenotypic differences and instead indicates that the differences are likely due to differential gene regulation downstream of *PHOT1* and *PHOT2* (Fig. S1).

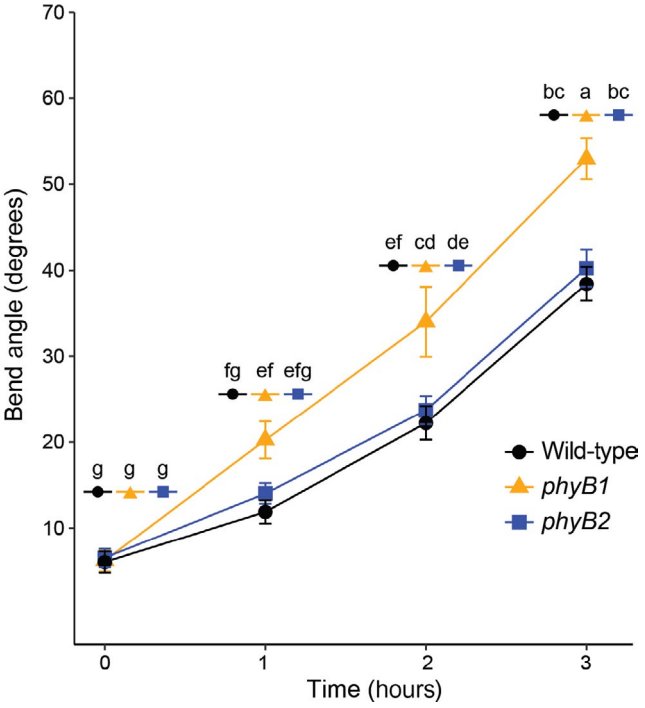

FIGURE 3 In white light, *phyB1* mutants show significantly faster phototropism than wild type or *phyB2* mutants. The average degree to which 5-day-old dark-grown seedlings bent toward unidirectional white light (bend angle) over 3 hr is shown. Error bars represent standard error. Combined data from three biological replicates are shown, n = 5 seedlings per genotype per time point per biological replicate. A two-way ANOVA with time and genotype was performed followed by Tukey's post hoc test using the software R. Shared letters represent no statistically significant difference

Since gravitropism, like phototropism, is a typical auxin-regulated response, we decided to test if gravitropism manifests itself differentially in the two *phyB* mutants in tomato. Five-day-old dark-grown seedlings were transferred to agar plates, either exposed to R or kept in the dark, and grown upright for 1 hr immediately after the transfer. Plates were then reoriented 90 degrees to induce a gravitropic response. We observed that in R the *phyB1* mutant responded statistically significantly faster to the altered gravity vector, which was especially obvious around 8 hr postgravistimulation, whereas in darkness the mutants responded to gravity at the same rate as WT (Figure 4). This experiment suggested that the differential auxin responsiveness between phyB1 and phyB2 also extends to differences in their gravitropic response. Interestingly, when we reduced the light levels from 135 to 60 $\mu mol*m^{-2}*s^{-1}$, the gravitropic response

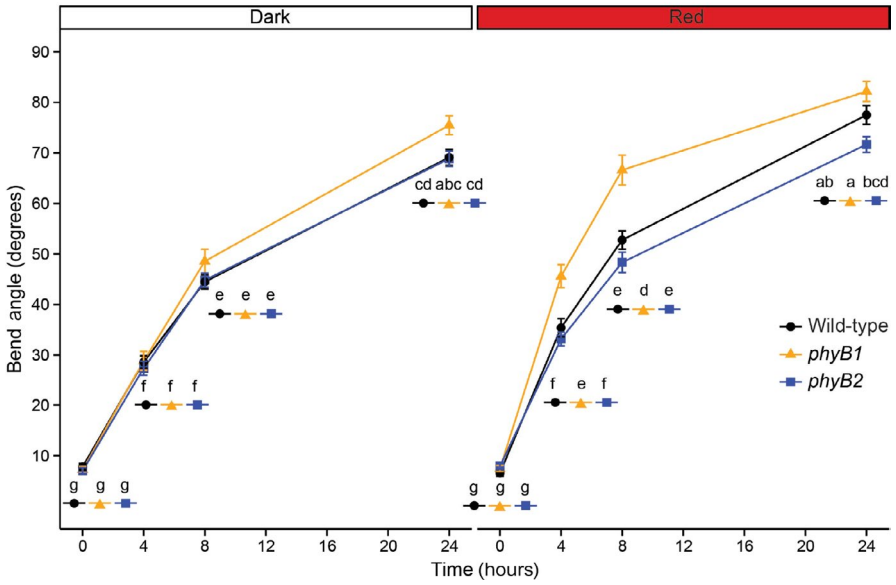

FIGURE 4 In R, *phyB1* mutants show significantly faster gravitropism than wild type or *phyB2* mutants. The average degree to which 5-day-old dark-grown seedlings bent toward the negative gravity vector (i.e., upwards) after gravistimulation over 24 hr is shown. Seedlings were either gravistimulated in the dark (left), or with 135 μmol photons m^{-2} s^{-1} of R. Error bars represent standard error. The dark and R plots each contain data from three biological replicates. *N* = 20 per genotype per time point per biological replicate. A three-way ANOVA with time, genotype, and light condition was performed followed by Tukey's post hoc test in R. Shared letters represent no statistically significant difference

differences between genotypes disappeared (Fig. S2), suggesting that the phyB1-mediated gravitropic response in tomato is also light intensity-dependent.

Since we only observed significantly greater gravitropic curvature in the *phyB1* mutants when the gravitropic experiment was done with high light intensity from the top but not with low light intensity from the side we wanted to exclude the remote possibility that in tomato phototropism can also be triggered by R alone, instead of requiring blue light. We therefore performed a series of control experiments in which we exposed seedlings to unilateral R light and measured their directional growth response over a period of three hours in a similar way to how we had performed the phototropic experiments shown in Figure 3. Not surprisingly (Fankhauser & Christie, 2015), our data showed that, like Arabidopsis, tomato does not have a red light phototropic response (data not shown), confirming that the enhancement in the gravitropic response of *phyB1* could not have been due to its enhanced phototropic response.

3.3 | PhyB1 and PhyB2 differentially modulate photosynthetic responses in tomato seedlings

Our transcriptional co-expression analysis had shown almost 60 photosynthesis-related genes to be enriched in the blue module, which contains genes with expression positively correlated with the *phyB1* mutation, but not significantly correlated with the *phyB2* mutation (Figure 2). We therefore decided to measure a variety of photosynthesis-related physiological parameters to test the hypothesis that gene duplication in PHYB had led to the subfunctionalization of regulation of genes involved in photosynthesis. We measured overall photosynthetic activity and related this activity to leaf size and fresh weight. Measuring overall leaf chlorophyll concentrations, we found differences between the WT and the two mutants but they were not statistically significantly different from each other (data not shown). Photosynthetic activity was not statistically significantly different between the three genotypes when the photosynthetic rate was normalized by leaf area regardless of light intensity (Figure 5a,c). However, when we normalized photosynthetic rate by fresh weight of the leaf portion used for the gas exchange analysis, we observed a statistically significant difference between the two phytochrome mutants. These differences between *phyB1* and *phyB2* were seen both in low and high light intensities (Figure 5b,d). Interestingly, the data suggest that phyB1 and phyB2 act antagonistically to each other and that *PHYB1* and *PHYB2* have subfunctionalized with respect to the role they play in regulating photosynthesis.

3.4 | Subfunctionalization of phyB1 and phyB2 is correlated with differences in the genes' regulatory region

Using the PlantCARE database (Lescot et al., 2002), we compared the 3-kb regulatory region immediately upstream from each gene's transcriptional start site (Table S5) and found a number of differences. Overall, *PHYB1* contained 17 recognized light-regulated cis-acting elements, while *PHYB2* only contained 7 such elements. The type of elements found in each gene's promoter region was also different. For example, the *PHYB1* promoter region contained 7 G-Box

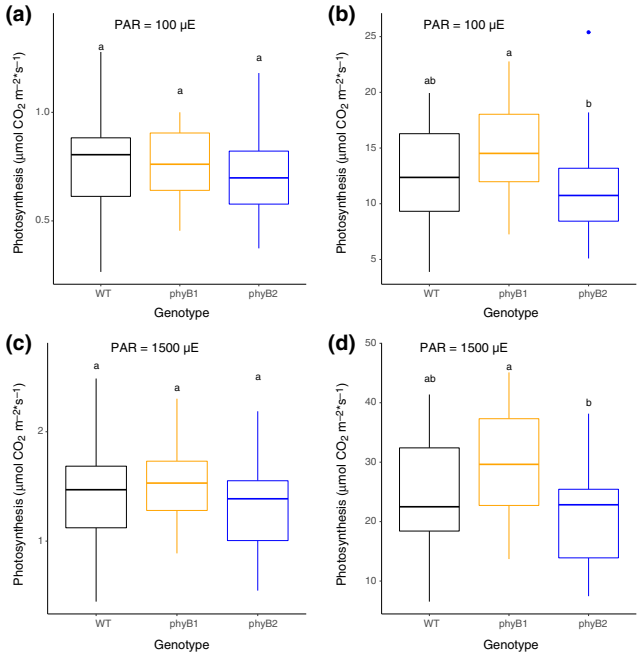

FIGURE 5 Photosynthetic activity is enhanced by phyB2 and repressed by phyB1 independent of light intensity. Photosynthetic activity was measured under varying light intensities in 6-week-old WT, *phyB1*, and *phyB2* mutants grown at 25°C (16 hr day/8 hr night) using a LiCOR 6400XT. Three biological replicates were performed with 10 plants per genotype per replicate. Data were normalized in two different ways either by leaf area (a and c) or by leaf area and fresh weight of the leaf tissue that was used for photosynthetic rate measurement. Data were statistically analyzed with a one-way ANOVA followed by a Tukey post hoc test using the software R. In each panel, data points not connected by a shared letter are statistically significantly different

elements, which bind *PHYTOCHROME INTERACTING FACOTRs* (*PIFs*) (Pham, Kathare, & Huq, 2018), while in *PHYB2*, there were only 2. Several other motifs were found only in one or the other phy gene (Table S5). Overall, the differential occurrence of light regulatory sequences suggests that transcription of these duplicated genes might be differentially regulated.

4 | DISCUSSION

Gene duplication is a major source of genetic material with the potential for the evolution of novel functions and the development of complexity of responses to the environment (Panchy et al., 2016). Retention of duplicated genes can either indicate that retained genes are positively selected to provide genetic redundancy (Zhang, 2012), that they are required to maintain proper dosage or genetic balance (Birchler & Veitia, 2014; Freeling & Thomas, 2006), or that duplication eventually led to the acquisition of novel or refined functions (Lynch & Conery, 2000; Ohno, 1970). PHY genes, in particular, have been estimated to be evolving at a faster rate (1.52–2.79 times) than the average plant nuclear gene, suggesting that diversification of the PHY gene family might respond either to selective pressure or to the absence of major evolutionary constraints (Alba et al., 2000).

We used differential mRNA expression and co-expression analysis to first evaluate the degree to which the PHY genes *PHYB1* and *PHYB2* have functionally diversified since their separation from a common ancestor gene and then to identify and verify physiological traits for which phyB has subfunctionalized since its gene duplication event. Our analysis indicated significant differences in the transcriptome of plants mutant in either *PHYB1* or *PHYB2*. On the other hand, after filtering, the overall number of genes that were regulated by phyB1 (121) and phyB2 (73) was relatively modest. Overall, our differential gene expression analysis showed that the group of genes regulated by phyB1 but not phyB2 was enriched in auxin response genes, and our co-expression analysis showed that those genes found in co-expression gene networks and that differentially correlated to phyB1 and phyB2 were enriched in auxin response and photosynthesis genes.

4.1 | Regulation of auxin responses by phytochrome B

In Arabidopsis, phototropic curvature is enhanced when plants are pre-treated with R for 2 hr before directional blue light (B) treatment (Janoudi, Konjevic, Apel, & Poff, 1992). This pre-treatment response is phyA-mediated, and not phyB-mediated (Parks, Quail, & Hangarter, 1996), although it has been shown that even without R pre-treatment, Arabidopsis phyA, phyB, and phyD promote phototropism (Whippo & Hangarter, 2004). Specifically for B intensities of greater than 1.0 $\mu mol^{*}m^{-2*}s^{-1}$ of light, phyB and phyD show functional redundancy with phyA, while at fluences of B around 0.01 $\mu mol^{*}m^{-2*}s^{-1}$, phyA was required for a normal phototropic response (Whippo & Hangarter, 2004). Additionally, Arabidopsis phyB has been shown to inhibit phototropism in shade-free environments (a high R/FR ratio), while mediating the phototropic response in the shade via PHYTOCHROME INTERACTING FACTORs (PIFs) and members of the *YUCCA* gene family (Goyal et al., 2016). Furthermore, it was shown that the quadruple mutant for phyB, phyC, phyD, and phyE has a normal phototropic response (Strasser, Sánchez-Lamas, Yanovsky, Casal, & Cerdán, 2010), confirming the notion that phyA is required in Arabidopsis for a normal low-fluence B-induced phototropic response. Direct connections between auxin signaling, phototropism and phytochrome involvement have been shown in Arabidopsis as well. Haga and colleagues (2014) used quadruple mutants in the *PINOID* (*PID*) and *WAVY ROOT GROWTH* (*WAG*) genes to show that phy upregulates the expression of *PIN-FORMED* (*PIN*) auxin transport proteins and suggested that PIN proteins were responsible for the R pre-treatment enhancement of phototropism.

Our data suggest that phototropism is differently regulated between tomato and Arabidopsis. Our genetic analysis shows that phyB1, but not phyB2, negatively regulates the phototropic

response in tomato (Figure 3). This in turn suggests that in tomato, phyB duplication led to a defined split between phyB1 and phyB2 with respect to phototropism, while in Arabidopsis phyB and phyD share redundancy, at least for its control of phototropism in response to R pre-treatment (Whippo & Hangarter, 2004). Additionally, while Arabidopsis work has shown phyB to be repressing phototropism in shade-free environments (Goyal et al., 2016), we saw that phyB2 in tomato is not involved in that response. Our RNAseq analysis supports the split in function also with respect to expression differences in the *PIN* genes that Haga and colleagues (2014) had proposed to play a role in phy-mediated phototropism: In tomato, our network analysis placed *SlPIN4* into the brown module, which is negatively correlated with the *phyB1* mutation but positively correlated with the *phyB2* mutation (Figure 2). Furthermore, *SlPIN4* was differentially regulated in response to R only in the *phyB2* mutant, but not in the *phyB1* mutant (Table S1). This differential sensitivity in auxin response signaling between the two subfunctionalized genes in tomato suggests one possible avenue for the two phy genes in tomato to differentially affect phototropic curvature.

Gravitropism, like phototropism, is an auxin-mediated differential growth response that results in directional elongation with respect to the gravity vector (Morita, 2010). Our data showed that phyB1, but not phyB2, represses gravitropism in R (Figure 4). This response is therefore similar to the phototropic response in that it is enhanced by the *phyB1* mutation. The role of phytochrome in the gravitropic response in less well understood than it is for phototropism. In Arabidopsis, but not in tomato, R perceived by both phyA and phyB results in strongly reduced shoot gravitropism (Liscum & Hangarter, 1993; Poppe, Hangarter, Sharrock, Nagy, & Schäfer, 1996) caused by *PIFs* that in R convert the gravity-sensing amyloplasts in the endodermis into other, non-gravity-sensing types of plastids (Kim et al., 2011). Interestingly, root gravitropism in white-light-grown Arabidopsis is diminished in *phyB* but not in *phyD* mutants (Correll & Kiss, 2005), suggesting subfunctionalization for this trait between the two genes in Arabidopsis. Interestingly, however, in Arabidopsis roots WT phyB promotes gravitropism, whereas in tomato shoots WT phyB1 inhibits it. Since R does not inhibit shoot gravitropism in 5-day-old dark-grown tomato seedlings, gravity sensing in the hypocotyl appears to follow a different signaling route than it does in Arabidopsis, but clearly phytochrome appears to play a role in both.

4.2 | Regulation of photosynthesis by phytochrome B

Our co-transcriptional analysis had suggested that photosynthesis genes were differentially affected by mutations in *PHYB1* versus *PHYB2* of tomato (Figure 2) and our physiological experiments had supported this finding (Figure 5). In Arabidopsis, phyB has previously been shown to increase photosynthetic rates, but only at light levels greater than 250 $\mu mol^{*}m^{-2*}s^{-1}$ (Boccalandro et al., 2009). Our data show that photosynthesis is enhanced in the *phyB1* mutant and reduced in the *phyB2* mutant compared to the WT response (Figure 5b,d), suggesting that in tomato phyB2, apparently antagonistically to phyB1, plays the role of increasing photosynthetic rates. Interestingly, it appears that this instance of subfunctionalization did not simply split the two phyB homologs into one serving the function of the parental gene while the other largely lost its participation in the process, but instead led to opposite regulation of the same process. Another difference between the Arabidopsis and tomato responses is that, unlike in Arabidopsis, the effects of phyB1 and phyB2 on photosynthesis are not light intensity-dependent in tomato, at least not at the two light intensities tested here. It is of note that differences in photosynthetic rates were only discernable in our analysis when we normalized carbon assimilation rates by fresh weight and leaf area as opposed to leaf area alone (Figure 5). Chlorophyll content in all genotypes was about the same but fresh weight per unit leaf area was highest in *phyB2* and lowest in *phyB1* among the three genotypes. This indicates that phyB1 promotes leaf thickness, water conservation or both, while phyB2 might promote transpiration (creating a net weight loss) or restrict leaf thickening. The conflict between gene functions of phyB1 and phyB2 could allow the plant to balance its photosynthetic and water needs depending on environmental conditions. More work is needed, however, to specifically assign those roles to the two phyB homologs in tomato.

4.3 | In tomato, subfunctionalization of phyB has led to equally important sister genes

The relatively recent duplication of phyB into separate homologs in different species provides a window into how gene duplication can result in different evolutionary trajectories. *PHYB* duplications in Arabidopsis and tomato both occurred after divergence of the Solanaceae and Brassicaceae (Li et al., 2015). In Arabidopsis, comparison of the coding sequence shows 48–56% amino acid identity between PHYA, PHYB, PHYC, and PHYE, but 80% identity between PHYB and PHYD (Clack, Mathews, & Sharrock, 1994). Amino acid identities between PHYB and PHYD in Arabidopsis, and between PHYB1 and PHB2 in tomato are similarly high in the two species (Hauser, Cordonnier-Pratt, Daniel-Vedele, & Pratt, 1995). Functional redundancy between PHYB and PHYD in Arabidopsis is high, but mutation in PHYD enhances the *phyB* mutant response with respect to leaf morphology, rosette leaf number (Franklin et al., 2003) and shade avoidance (Devlin et al., 1999; Franklin et al., 2003). While single mutation of *PHYD* in Arabidopsis leads to an increase in hypocotyl length in continuous R and white lights, the effect of *phyD* on the end-of-day (EOD) FR response was negligible until combined with a mutation in *PHYB* (Aukerman et al., 1997). With respect to leaf morphology and developmental traits, mutation in Arabidopsis PHYD resulted in none or only minor consequences on the phenotype while mutation in PHYB resulted in statistically significant phenotypic change (Aukerman et al., 1997). Analysis of the *phyB/D*

double mutant, however, showed that PHYD contributes residual function to phenotype in a manner redundant and subordinate to PHYB (Aukerman et al., 1997).

In tomato, divergence of the 5′ cis-regulatory regions in *PHYB1* and *PHYB2* has resulted in variability of the number and type of light response motifs, suggesting that this variation might be part of the reason for the genes' subfunctionalization. Duplication and gene divergence in tomato, in contrast to Arabidopsis, has resulted in two genes that have taken on specialized functions for a variety of developmentally important traits. This situation is not unlike that in maize. In maize, the two homologs of *ZmPHYB* showed complete redundancy for involvement in several morphological traits, such as plant height and stem diameter, while regulation of photoperiod-dependent flowering time was regulated only by *ZmPHYB2* (Sheehan et al., 2007). Early work on phyB1 and phyB2 in tomato describing the mutants had already noted that phyB1 and phyB2 played different roles in early seedling development, but described the genes as largely redundant in older plants (Weller et al., 2000). Our data suggest that in tomato, phyB1 inhibits auxin responses of phototropism and gravitropism (and phyB2 does not play a role) while phyB2 promotes and phyB1 inhibits photosynthesis.

We want to caution that in the absence of multiple alleles of *phyB1* and *phyB2* in our analysis, it is formally possible that unknown, secondary background mutations in the material could contribute to some of the observations we made in this study.

5 | CONCLUSIONS

Although phys are evolutionarily old genes and found in at least two copies, phyA and phyB, in all angiosperm species (Mathews, 2010), functional diversification is an ongoing process. PhyB is the phy homolog that has most recently duplicated again in some species (Mathews, 2010), including Arabidopsis (phyB/phyD), maize (phyB1/phyB2), and tomato (phyB1/phyB2). This latest round of duplication therefore lends itself well to analysis of variation in subfunctionalization of this important gene between species, and also provides a recent gene duplication event that plants have exploited for further specialization of their responses to light and the environment.

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CONFLICT OF INTEREST

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

AUTHOR CONTRIBUTIONS

A.M. conceived the original research experiment. K.C., S.B., D.A., A.Z-M., and A.M performed the experiments and analyzed the data. Specifically, K.C. and A.M. did the bioinformatic analysis and data interpretation; S.B. performed photosynthesis, phototropism, and promoter analysis experiments; K.C. performed the gravitropic experiments; A.Z-M. performed phototropism experiments; D.A. supervised plant growth and extracted RNA; K.C., S.B. and A.M. interpreted the data and wrote the article with contributions from all the authors. A.M. agrees to serve as corresponding author.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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