Sorghum Phytochrome B Inhibits Flowering in Long Days by Activating Expression of SbPRR37 and SbGHD7, Repressors of SbEHD1, SbCN8 and SbCN12

Shanshan Yang1, Rebecca L. Murphy1, Daryl T. Morishige1, Patricia E. Klein2, William L. Rooney3, John E. Mullet1*

1 Department of Biochemistry and Biophysics, Texas A&M University, College Station, Texas, United States of America, 2 Department of Horticultural Sciences and Institute for Plant Genomics and Biotechnology, Texas A&M University, College Station, Texas, United States of America, 3 Department of Soil and Crop Sciences, Texas A&M University, College Station, Texas, United States of America

Abstract

Light signaling by phytochrome B in long days inhibits flowering in sorghum by increasing expression of the long day floral repressors PSEUDORESPONSE REGULATOR PROTEIN (SbPRR37, Ma1) and GRAIN NUMBER, PLANT HEIGHT AND HEADING DATE 7 (SbGHD7, Ma6). SbPRR37 and SbGHD7 RNA abundance peaks in the morning and in the evening of long days through coordinate regulation by light and output from the circadian clock. 58 M, a phytochrome B deficient (phyB-1, ma3*) genotype, flowered ~60 days earlier than 100 M (PHYB, Ma3) in long days and ~11 days earlier in short days. Populations derived from 58 M (Ma1, ma3, Ma5, ma6) and R.07007 (Ma1, Ma3, ma5, Ma6) varied in flowering time due to QTL aligned to PHYB/phyB-1 (Ma3, Ma5, and GHD7/ghd7-1 (Ma6). PHYC was proposed as a candidate gene for Ma5 based on alignment and allelic variation. PHYB and Ma5 (PHYC) were epistatic to Ma1 and Ma6 and progeny recessive for either gene flowered early in long days. Light signaling mediated by PhyB was required for high expression of the floral repressors SbPRR37 and SbGHD7 during the evening of long days. In 58 M (phyB-1) these genes were highly expressed in long and short days. Furthermore, SbCN15, the ortholog of rice Hd3a (FT), is expressed at low levels in 100 M but at high levels in 58 M (phyB-1) regardless of day length, indicating that PhyB regulation of SbCN15 expression may modify flowering time in a photoperiod-insensitive manner.

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* Email: jmullet@neo.tamu.edu

Introduction

Flowering time has a significant impact on plant adaptation to agro-ecological environments, biomass accumulation and grain yield [1]. Floral initiation is regulated by plant development, photoperiod, shading, temperature, nutrient status, and many other factors [2–5]. Signals from many input pathways are integrated in the shoot apical meristem (SAM) through regulation of the meristem identity genes LEAFY (LFY) and APETALA1 (AP1), which are activated during transition of the SAM from a vegetative meristem to a floral meristem. Long day (LD) plants, such as Arabidopsis, flower earlier in LD compared to short days (SD). In contrast, SD plants, such as rice and sorghum, show delayed floral initiation under LD conditions. Photoperiod regulated flowering is mediated by light signaling from photoreceptors and output from the endogenous circadian clock consistent with external coincidence models of flowering time regulation [6]. Photoperiod sensitive Sorghum bicolor genotypes delay floral initiation when grown under LD conditions. Sorghum genotypes with reduced photoperiod sensitivity have been identified and used by breeders because they flower early and at similar times in both long and short days, enhancing grain production [7]. In contrast, bioenergy sorghum is highly photoperiod sensitive, flowering in long day environments only after an extended phase of vegetative growth, thereby increasing biomass accumulation and nitrogen use efficiency [1,6].

Photoperiod regulated flowering requires perception of light and signaling by plant photoreceptors such as the red/far-red light sensing phytochromes (Phy), blue light/ultraviolet wavelength sensing cryptochromes (Cry), phototropins, and Zeatupes [9,10]. Phytochromes play an important role in flowering time regulation in most plants including rice [11], barley [12], and sorghum [13]. The sorghum genome encodes three phytochrome genes, PHYA, PHYB and PHYC. Quail et al. (1994) established a standard
nomenclature for phytochrome where PHY corresponds to phytochrome apoproteins, while phytochrome or phy indicates presence of the holoprotein, the fully assembled chromoprotein with chromophore covalently attached to the apoprotein [14]. Since all phytochrome proteins referred in this study are presumed to be holoproteins, Phy is used to represent wild type holoprotein, while phy is used to represent mutant versions of the holoprotein.

Inactivation of PhyB results in early flowering in long days [13]. Phytochromes are soluble chromoproteins that contain an N-terminal photosensory domain and a C-terminal dimerization moiety. There are three sub-domains in the N-terminal moiety: PAS (PER, ARNT and SIM), GAF (cGMP phosphodiesterase, adenylate cyclase, Fh1A) and PHY (phytochrome-specific GAF-related), which form a unique structure, the “light-sensing knot” [15]. The PAS/GAF domains transduce light signals and the C-terminal domain, consisting of two PAS and HKRD (histidine-kinase-related domain), is responsible for dimerization and nuclear localization.

The central oscillators of the plant circadian clock are encoded by TIMING OF CAB EXPRESSION 1 (TOC1), CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY) [16]. Rhythmic expression of these central oscillators modulates the expression of GIGANTEA (GI), an output gene of the circadian clock, GI, in concert with other factors, activates expression of CONSTANS (CO), a zinc-finger transcription factor that plays an essential role in photoperiod regulation of flowering time in Arabidopsis [17], rice [18] and sorghum [19]. In Arabidopsis, CO is stabilized and accumulates during the evening of long days through the action of Cry1, Cry2 and PhyA, where it activates expression of FT and flowering. In SD, CO is not stabilized during the evening because CO expression occurs in darkness [20]. FT is produced in leaves and translocated to the SAM where it binds to FD. In Arabidopsis, FT together with SUPPRESSOR OF OVEREXPRESSION OF CONSTANS (SOC1), promotes expression of meristem identity gene LFY and AP1, leading to floral transition [20].

The core of photoperiod regulatory pathway GI-CO-FT is present in Arabidopsis, a LD plant, and the SD plants rice and sorghum. In rice, OsGI, HEADING DATE 1 (Hd1), and HEADING DATE 3a (Hd3a) are orthologs of GI, CO, and FT, respectively [21]. Hd1 (OsoGI) delays flowering time in LD in rice and activates flowering in SD. In addition, Itoh et al. [22] identified a pair of genes in rice, EARLY HEADING DATE 1 (EHD1) and GRAIN NUMBER, PLANT HEIGHT AND HEADING DATE 7 (GHD7) that regulate flowering in response to day length by modifying expression of Hd3a (florigen). EHD1 activates Hd3a expression and induces floral transition. In contrast, GHD7, a homolog of wheat VRN2 [23], represses flowering in LD by down-regulating EHD1 and Hd3a. In maize, 25 FT-like homologs were identified and designated as Zea mays CENTRORADIALIS (ZCN) genes. ZCN8 was identified as a source of florigen [24]. SbCN8 (ortholog of ZCN8) and SbCN12 (ortholog of ZCN2) have been proposed to encode florigen in sorghum [19,25,26]. In sorghum, CO activates flowering in SD by inducing expression of ShEHD1, SbCN8 and SbCN12, whereas in LD, CO activity is inhibited by ShPBR37 [19].

More than 40 flowering time QTL have been identified in sorghum [27] and maturity loci Ma1–Ma6, modify photoperiod sensitivity [7,28,29]. Dominance at Ma1–Ma6 delays floral initiation in long days. Ma3 encodes phytochrome B, indicating that light signaling through this photoreceptor is required for photoperiod sensitive variation in flowering time [13]. Ma6 was identified as ShGHD7, a repressor of flowering in long days [26]. In LD, SbGhd7 increases photoperiod sensitivity by inhibiting expression of the floral activators ShEHD1, ShCN12 and ShCN8. Ma1 was identified as ShPBR37, a floral repressor that acts in LD [25]. The orthologs of ShPBR37 in wheat and barley, PhDPERIOD 1 (Ppd1, Ppd-H1, Ppd-D1a) [30,31] and rice OsPBR37 [32], also modulate flowering time in response to photoperiod. In LD, ShPBR37 inhibits expression of ShEHD1, ShCN12, and ShCN8, resulting in repression of flowering [23]. Moreover, ShPBR37 modulates photoperiod sensitivity and floral repression in an additive fashion together with ShGHD7 [26]. Expression of ShPBR37 and ShGHD7 is regulated by the circadian clock and light, suggesting common upstream regulation [26].

The current study focused on elucidating how phytochrome B regulates flowering time in response to day-length in sorghum. We report that PHYB is required for light activation of ShPBR37 and ShGHD7 expression in the evening of long days, resulting in repression of ShEHD1, ShCN12, ShCN8 and floral initiation.

Materials and Methods

Phenotypic analysis of sorghum flowering time

The maturity loci and flowering dates of all sorghum lines used in this study are listed in Table S1. To characterize the difference in flowering time between different genotypes and day-length, 100 M and 58 M were planted in Metro-Mix 200 (Sunshine MYP; Sun Gro Horticulture) and grown in a greenhouse in LD (14 h light/10 h dark) and SD (10 h light/14 h dark) conditions. Days to mid-anthesis were recorded and plants were photographed. 100 M plants (n = 5) and 58 M plants (n = 9) were grown in LD and phenotyped for days to anthesis (Figure 1A). The mean days to flowering for 100 M was 126 days (± 4 days) and 62 days (± 5 days) for 58 M, a significant difference in flowering times for these genotypes (p-value < 0.001, Welch two sample t-test). Under SD, 100 M plants (n = 7) and 58 M plants (n = 5) were used for analysis of flowering time (Figure 1B). The mean days to flowering for 100 M was 59 days (± 4 days) and for 58 M, 48 days (± 1 days), a significant differences in days to flowering (p-value < 0.001). To establish the interaction between PhyB and photoperiod, factorial ANOVA was run with photoperiod and PhyB alleles as factors. The significance of the effects of PhyB alleles, day-length and PhyB:day-length interaction were detected (p-value < 0.001). All statistics were run in R 3.1.0. The two-way interaction graphs were plotted using the “HH” package in R.

Sequencing of PHYB alleles

To identify coding alleles in the PHYB gene, the full-length genomic sorghum PHYB genes from historical sorghum cultivars were amplified as three overlapping segments by PCR (Phusion High-Fidelity DNA polymerase, New England BioLabs, Inc). The amplified PCR products were cleaned and concentrated (QIAquick PCR Purification kit, QIAGEN). PCR products were separated by electrophoresis on 1% agarose gels. Specific PCR products were excised and purified (QIAquick Gel Extraction Kit, QIAGEN). The purified PCR products were sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and the Applied Biosystems 3130xl Genetic Analyzer. All primers used for sequencing were designed using PrimerQuest™ software (Integrated DNA Technologies, Inc) and are shown in Table S2. Sequences v4.0 (Gene Codes) was used for sequence assembly and alignment with the B73x23 whole genome sequence of Sorghum bicolor (version 1.4) downloaded from Phytozome v8.0 (http://www.phytozome.net/). The SIFT (sorting intolerant from tolerant) program (http://sift.jcvi.org/) was utilized to predict whether an amino acid substitution affects protein function, based
on the degree of conservation of amino acid residues in sequence alignments derived from closely related sequences.

QTL analysis of PHYB action

The sorghum cultivar 58 M (Ma1Ma2ma3Ma4Ma5ma6) was crossed to R.07007 (Ma1ma2Ma3Ma4ma5Ma6) to generate a population for QTL analysis. F1 generation plants were self-pollinated to produce F2 populations from which F3 populations were derived by self pollination. F2 and F3 populations were planted in the greenhouse and grown under long day conditions (14 h light/10 h dark). Days to mid-anthesis of panicles of plants from the F2 and F3 populations were recorded. The median, standard error, and range of Days to Flowering and the number of plants of each genotype analyzed from the F2 and F3 populations are shown in Table 3. For analysis of epistatic interaction, three-way ANOVA was run to detect the effect of allelic variation in three maturity genes (Ma3, Ma5 and Ma6) and three two-way interactions (Ma3:Ma5, Ma3:Ma6, Ma5:Ma6). The significance of the effects of single genes and genetic interactions were detected (p-value < 0.001). All statistics were run in R 3.1.0. The two-way interaction graph was plotted using the “HH” package in R.

For genotyping, genomic DNA of 86 F2 individuals and 132 F3 individuals was extracted from leaf tissue using the FastDNA Spin Kit (MP Biomedicals). Template for sequencing on an Illumina GAIIx sequencer was generated following the standard Digital Genotyping (DG) protocol [33]. Genotypes of all individuals from both populations were identified. The genetic map was constructed using the Kosambi mapping function in MAPMAKER v3.0 with 285 markers from the F2 population and 653 markers from the F3 population. QTL were mapped using the genetic map and the Composite Interval Mapping (CIM) function in WinQTL Cartographer v2.5 [34]. Significant LOD thresholds for QTL

![Figure 1. Photographs of the sorghum lines 100 M and 58 M for flowering time phenotype.](A) Photograph of 100 M (left) and 58 M (right) grown for 109 days in LD (14 h light/10 h dark). 100 M and 58 M flowered after 126 days and 62 days respectively. (B) Photograph of 100 M (left) and 58 M (right) grown in a greenhouse in SD for 53 days (10 h light/14 h dark). 100 M flowered after 59 days and 58 M flowered after 48 days. LD: long days. SD: short days. DTF = number of days to flowering time. Scale bar is 8.6 cm.
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Table 1. Sequence analysis of PHYB coding alleles in different sorghum lines.

| Exon 1 | Exon 1 | Exon 3 | Exon 4 | Sorghum Genotypes |
|--------|--------|--------|--------|-------------------|
| Nucleotide Variation | CAC… | A… | G | A… | C–G |
| Protein Modification | His… | Asp… | Gly | Premature stop codon | Leu… Val |
| Mutation Position (AA #) | 31 | 308 | 1023 | 1113 |
| Alignment with PHYB in Arabidopsis (AA #) | 32 | 293 | 1007 | 1096 |
| Phytochrome Domain | GAF(N) |
| PHYB (Ma3 or ma3) | – | – | – | – | BTx623, 100 M, 90 M, R.07007, Hegari, Tx7000, BTx642, SC56, Shallu, BTx3197 |
| phyB-1 (ma3) | – | – | + | – | 58 M |
| phyB-2 | + | + | – | + | IS3620C |

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The specificity of each gene was run on the 7900HT Fast Real-Time PCR System with SDS (VIC Probe) and rRNA Forward/Reverse Primer. All reactions were carried out using Power SYBR Green PCR Master Mix (Applied Biosystems). 18S rRNA was selected as the internal control reference and the reactions were performed using the TaqMan Universal PCR Master Mix Protocol with rRNA Probe (VIC Probe) and rRNA Forward/Reverse Primer. All reactions were run on the 7900HT Fast Real-Time PCR System with SDS v2.3 software (Applied Biosystems). The specificity of each gene expression assay was determined by the serial dilution method [36] (Table S3). Relative expression was determined by the comparative cycle threshold (ΔΔCt) method [36] with calibration from most highly expressed samples. The calculated primer efficiencies were used to adjust data for relative quantification by the efficiency correction method [37]. Each relative expression value was derived from an average of three technical replicates and three biological replicates. The individual expression data points presented as 2−ΔΔCt [38]. The significance (p-values) of the difference in expression between genotypes were detected using Welch two sample t-test in R 3.1.0 based on three technical replicates and three biological replicates. P-values were calculated either for certain time points of the day or all time points of the day.

**Results**

**PHYB alleles in diverse sorghum lines**

Sorghum genotype 58 M, a photoperiod insensitive early flowering line, has the genotype *ma3*/*ma3* corresponding to the *phyB-1* allele [13]. This allele contains a frame shift mutation that results in a prematurely terminated PhyB lacking regions of the protein necessary for dimerization and biological activity. To confirm and extend prior analysis of PHYB diversity in sorghum, alleles from several sorghum lines that vary in photoperiod sensitivity were sequenced and compared. The coding sequence of PHYB from BTx623 and 100 M (both *Ma3*) was 7285 bp in length consisting of four exons encoding a protein with 1178 amino acid residues. PHYB sequences from R.07007, Hegari, Tx7000, BTx642, SC56, Shaliu and BTx3197 were identical to BTx623 and 100 M (*Ma3*). The PHYB sequence from 58 M (*ma3*), referred to as *phyB-1* (Table 1), contains a mutation that
Table 2. Information on flowering time QTL identified in the 58MxR.07007 F2 population.

| QTL | Maturity Locus | Chromosome Number | Position (cM) | LOD score | Physical Interval | Additive Effect | Dominant Effect | R² |
|-----|----------------|-------------------|---------------|-----------|-------------------|----------------|----------------|----|
| 1   | Ma5            | Ch_1              | 1.8           | 8.66      | 6139583–9077991   | -17.09         | 18.19          | 0.1964 |
| 2   | Ma3            | Ch_1              | 99.4          | 24.21     | 60402909–61604749 | 12.55          | 16.09          | 0.1408 |
| 3   | Ma6            | Ch_6              | 7.2           | 12.09     | 203707–1716581    | 12.83          | 5.81           | 0.1549 |
| Total|                |                   |               |           |                   | 49.21%         |                |     |

*aPosition of likelihood peak (highest LOD score).
*bPhysical Interval: physical coordinate interval spanning 1 LOD interval across the likelihood peak.
*cAdditive Effect: A positive value means the delay of flowering time due to R.07007 allele. A negative value means the delay of flowering time due to 58 M allele.
*dDominant Effect: A positive value means dominance for the delay of flowering time.
*eR² (coefficient of determination): percentage of phenotypic variance explained by the QTL.

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In sorghum, SbPRR37 (Ma1), and SbGHD7 (Ma6) are primary determinants of photoperiod sensitivity in Ma3, and SbGHD7 (Ma6) is the primary determinant of flowering time in LD. The SbPRR37 gene in Ma1 is expressed in an additive fashion to inhibit flowering, and the SbGHD7 gene in Ma6 is expressed in a dominant fashion to delay flowering time. The SbGHD7 gene in Ma6 is located on chromosome 6, spanning a physical interval from 203707–1716581 bp.

**PhyB** is epistatic to Ma1 (SbPRR37) and Ma6 (SbGHD7) in sorghum. When grown in a greenhouse in LD during December–February at 85 days, 100 M flowered in 69 days (±2 days) (Figure 1B). Therefore, the factorial ANOVA with photoperiod and **PhyB** alleles as factors indicated the effects of **PhyB** on flowering time in LD (p-value < 0.001) (Figure S1-A).

**PhyB** affects flowering time in LD and SD. The sorghum maturity standards, 100 M and 58 M, were constructed from Milo genotypes that contain alleles of Ma1 (SbPRR37) and Ma6 (SbGHD7) to modify flowering time [28]. The genotype 58 M is photoperiod insensitive, flowers early in LD and SD, and has the ghd7-1 null allele from 58 M was associated with early flowering in LD. The third flowering time QTL near the proximal end of chromosome 1 (chromosome 1:6,139,583–9,077,991) had a LOD score of 8.66 and a physical interval from 6139583–9077991 bp.

**SbPRR37** and **PhyB** interact epistatically. When grown in a greenhouse in LD during December–February at 85 days, 100 M flowered in ~180 days (±2 days), whereas 58 M flowered in ~136 days (±2 days) (Figure 1A). This result refutes the suggestion that *SbPRR37* and **PhyB** activity in 58 M reduces the ability of 58 M to inhibit flowering in LD [27]. The factorial ANOVA with **PhyB** day-length and **PhyB**-allele interactions as factors indicated the effects of **PhyB** on flowering time in SD (p-value < 0.001) (Figure S1-A).

PhyB affects flowering time in LD and SD. The sorghum maturity standards, 100 M and 58 M, were constructed from Milo genotypes that contain alleles of Ma1 (SbPRR37) and Ma6 (SbGHD7) to modify flowering time [28]. The genotype 58 M is photoperiod insensitive, flowers early in LD and SD, and has the ghd7-1 null allele from 58 M. The third flowering time QTL near the proximal end of chromosome 1 (chromosome 1:6,139,583–9,077,991) had a LOD score of 8.66 and a physical interval from 6139583–9077991 bp.
LOD score of 8.7 and explained 19.6% percent of phenotype variance. This QTL was tentatively identified as Ma5 because R. 07007 was reported to be recessive for Ma5, a rare allele in sorghum [29]. No QTL aligned with Ma1 as expected because both 58 M and R. 07007 contain dominant alleles of Ma1 (ShPrrr37). The three flowering time QTL were also identified in the corresponding F3 population (data not shown).

Plants from the F2/3 population are homozygous for Ma1, a repressor of flowering in LD, but varied in alleles of Ma3, Ma5, and Ma6. Three-way ANOVA was used to analyze the effect of allelic variation in three maturity genes (Ma3, Ma5, and Ma6) on flowering time, and three two-way interactions (Ma3:Ma5, Ma3:Ma6, Ma5:Ma6) showed that allelic variation of the three Ma genes and three two-way interactions were significant (p-values <0.001). The three two-way interaction graphs between Ma3:Ma5, Ma3:Ma6, and Ma5:Ma6 are shown in Figure S1-B–D. Progeny with the genotypes Ma3_Ma5.Ma6_ and Ma3_.Ma5_Ma6ma6 showed that allelic variation of the three Ma genes and three two-way interactions were significant (p-values <0.001). The three two-way interactions between Ma3:Ma5, Ma3:Ma6, and Ma5:Ma6 are shown in Figure S1-B–D. Progeny with the genotypes Ma3_Ma5_Ma6_ and Ma3_.Ma5_Ma6ma6 flowered later than genotypes that were homozygous recessive for ma3R, showing that PHYB is epistatic to the floral repressors encoded by Ma1 and/or Ma6 (Figure 2B; Figure S1-B,C). Progeny with the genotype Ma3_.Ma5_Ma6_ (101–129 days) flowered later than plants with the genotype Ma3_Ma5_.Ma6ma6 (60–91 days), consistent with increased floral repression due to Ma6 in Ma1 dominant backgrounds. The effect of Ma6 was delayed flowering with varying extents in different genetic backgrounds ranging from 14 days in ma3R.ma3R.Ma5_.ma6ma6 (60–91 days) to 29 days in Ma3_.ma5ma5, and ~9 days in ma3R.ma3R.ma5ma5. Furthermore, it was noted that progeny lacking PhyB with a dominant Ma6 allele showed a significant range of flowering times (42–75 days), suggesting that additional genes and/or environmental factors affect Ma6 action in this genetic background (Figure 2B; Table 3). A similar wide range of flowering time (59 days) was observed among plants with the genotype Ma3_.ma5ma5ma6_ (Figure 2C; Table 3). In addition, plants with the genotype Ma3.Ma5. Ma6_ flowered later in LD than plants with the genotypes Ma3ma5ma5ma6_ or Ma3ma5ma5ma6ma6 (Figure 2C; Figure S1-D). This shows that Ma5 is also required for late flowering in LD in Ma1Ma3 backgrounds and that Ma5 is epistatic to Ma1 and Ma6. Plants with the genotype ma3Rma3R.Ma5_.Ma6ma6 and Ma3_.ma5ma5ma5ma6ma6 flowering early and in a similar number of days as genotypes that are homozygous recessive for both ma3R and ma5 (ma3Rma3R.Ma5_.ma6ma6) indicating that the products of both Ma3 and Ma5 are required in LD for delayed flowering mediated by Ma1 (ShPrrr37).

The requirement for both PhyB and the product of Ma5 to observe delayed flowering in LD led us to examine the Ma5 locus for candidate genes that might explain this interaction. The Ma5 locus is located on SBI-01 and spans a large number of genes including several genes known to affect flowering time in other plants, including AP1, CK2, and PHYC. PHYC appeared to be the best candidate gene for Ma5 because PhyC modifies flowering time in rice specifically in LD, similar to Ma5 in sorghum [39]. PhyB stabilizes PhyC, and PhyB:PhyC act as heterodimers in both Arabidopsis [40,41] and rice [39], consistent with the co-dependence observed between PHYB and Ma5 in this study. Comparison of PHYC sequences from BTx623 (Ma5), 100 M (Ma5), and R.07007 (ma5) revealed four differences in PhyC amino acid sequence between BTx623 and R.07007, and two

### Table 4. Sequence analysis of PHYC coding alleles in different sorghum lines.

| Exon 1 | Exon 2 | Sorghum Genotypes |
|--------|--------|-------------------|
| Nucleotide Variation | G>T | G>A | T>C | G>T | PHYC-1 (Ma5) |
| Protein Modification | Gly>Val | Gly>Arg | Val>Ala | Glu>Asp | PHYC-2 |
| Mutation Position (AA #) | 124 | 160 | 198 | 226 | 954 |
| Alignment with PHYB in Arabidopsis (AA #) | 160 | 198 | 190 | 922 | 954 |
| Phytochrome Domain | PAS(N) | PAS(N) | PAS-GAF Loop | HKRD(C) | PHYC-1 (ma5) |
| PHYC-1 (Ma5) | - | - | - | - | BTx623 |
| PHYC-2 | - | + | + | - | 100 M, 90 M |
| PHYC-1 (ma5) | + | + | + | + | R.07007 |

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differences between 58 M/100 M and R.07007 (Table 4). The latter amino acid variants occur in the PAS domain (Gly:Val) and HKRD domain (Glu:Asp) and SIFT analysis [42] indicated these changes could affect the function of PhyC. These results are

Figure 3. Relative expression of \textit{SbPRR37} and \textit{SbGHD7} in 100 M (Ma3/PHYB) and 58 M (ma3/phyB-1) in LD and SD. 100 M (solid black line) and 58 M (dashed red line) plants were entrained LD (14 h light/10 h dark) or SD (10 h light/14 h dark) and sampled for one 24 h cycle, followed by 48 h in LL (continuous light and temperature). The grey background corresponds to time when plants are in darkness. Relative gene expression was determined every 3 hours by qRT-PCR. Arrows represent morning peaks of expression and arrowheads represent evening peaks of expression. (A) In LD, the second peak (arrowhead) of \textit{SbPRR37} expression in the evening (~15 h) is missing in the \textit{phyB} deficient line, 58 M. (B) In SD, the second peak (arrowhead) of \textit{SbPRR37} is absent in both 100 M and 58 M. (C) In LD, the second peak (arrowhead) of \textit{SbGHD7} expression in the evening (~15 h) is attenuated in 58 M. (D) In SD, the second peak of \textit{SbGHD7} is attenuated in both 100 M and 58 M. Each data point of relative expression was based on data from three technical replicates and three biological replicates. Error bars indicate SEM. doi:10.1371/journal.pone.0105352.g003

differences between 58 M/100 M and R.07007 (Table 4). The latter amino acid variants occur in the PAS domain (Gly:Val) and HKRD domain (Glu:Asp) and SIFT analysis [42] indicated these changes could affect the function of PhyC. These results are
consistent with PHYC as the candidate gene for Ma5. Further analysis is underway to test this assignment.

**PhyB modulates expression of SbPRR37 and SbGHD7 in long days**

Expression of SbPRR37 and SbGHD7 in leaves is regulated by light and gating by the circadian clock [25,26]. The influence of PhyB on SbPRR37 and SbGHD7 expression was analyzed using 100 M (PHYB) and 58 M (phyB-1) plants grown for 32 days in LD then entrained for 7 days in LD or SD (Figure 3). Following entrainment, leaf samples were collected from plants for one 24 h LD or SD light-dark cycle, then from plants exposed to continuous light and temperature consistent with regulation by the circadian clock (Figure 3, 24–72 h). In leaves of 100 M in LD (Figure 3A/C, dashed red lines), SbPRR37 and SbGHD7 showed an increase in RNA abundance in the morning (arrow) but only a small increase in expression in the evening (arrowhead) compared to 100 M (Figure 3A, p-value < 0.1; Figure 3C, p-value < 0.05). These results indicate that PhyB is required for elevated evening expression of SbPRR37 and SbGHD7 in LD in 100 M.

When 100 M and 58 M plants were entrained and assayed in SD, the morning peak of SbPRR37 expression was of similar amplitude in both genotypes and expression of SbPRR37 was low.

**Figure 4.** Expression of SbCO, SbEhd1, SbCN8/12/15 in 100 M (Ma3/PHYB) and 58 M (ma3R/phyB-1) in LD and SD. Relative RNA levels in leaves of 100 M (solid black lines) and 58 M (dashed red lines) entrained and sampled in LD (14 h light/10 h dark) or SD (10 h light/14 h dark) for 24 h followed by 24 h in LL (continuous light and temperature). Relative expression levels were determined every 3 hours by qRT-PCR analysis. The gray shaded areas represent the dark periods. (A) SbCO, (B) SbEHD1, (C) SbCN8, (D) SbCN12, (E) SbCN15. Each data point of relative expression is based on three technical replicates and three biological replicates. Error bars indicate SEM. doi:10.1371/journal.pone.0105352.g004
and expression levels were also elevated in the second subjective day (Figure 3B/D). In 58 M, the evening peak of SbPRR37 and SbGHD7 reappeared during the first subjective day, however overall expression was attenuated relative to 100 M during the second subjective day.

**PhyB modulates expression of CO, Ehd1, SbCN8, SbCN12 and SbCN15**

In 100 M entrained to LD, the sorghum ortholog of CON- STANS (SbCO) shows peaks of expression at dawn (24 h) and in the evening (15 h) that are regulated by SbPRR37, the circadian clock, and day length [25]. In 58 M entrained and sampled in LD, the amplitude of the peak of SbCO expression at dawn (24 h) was reduced compared to 100 M (Figure 4A, p-value<0.05). The peak of SbCO expression at dawn was also reduced and of similar amplitude in plants entrained and sampled in SD (Figure 4A, lower). These results show that the peak of SbCO expression at dawn is dependent on PhyB, most likely because expression of SbPRR37 in the evening phase of LD is dependent on PhyB (Figure 3A). In contrast, the evening peak (15 h) of SbCO expression was similar in both LD and SD in 100 M and 58 M indicating that PhyB does not significantly modulate SbCO expression at this time (15 h) of day.

**EHD1** is an activator of Hd3a, one of the florigens in rice [43]. The sorghum ortholog of Hd3a is SbCN15. Expression of SbEHD1 increases when 100 M is transferred from LD to SD in parallel with increased expression of SbCN8 (ortholog of ZCN8 [24]) and SbCN12 (ortholog of ZCN12) that have been proposed to encode florigens in sorghum [19,25,26]. SbPRR37 and SbGHD7 repress expression of SbEHD1 in 100 M entrained in LD [25,26]. Therefore SbEHD1 expression in 58 M and 100 M was quantified and compared to determine if PhyB modulates SbEHD1 expression. In LD, SbEHD1 RNA abundance peaked in the evening and was up to ~100-fold higher in 58 M relative to 100 M throughout the time course (Figure 4B, upper; Figure S2-A, p-value<0.001). In LD, expression of SbEHD1 was high in both genotypes and peaked during the night (Figure 4B, lower; Figure S2-A).

In 58 M entrained and analyzed in LD, expression of SbCN8 (Figure 4C, upper) and SbCN12 (Figure 4D, upper) peaked early in the morning and the relative abundance of RNA derived from these genes was elevated more than ~100-fold relative to their levels in 100 M (Figure S2-B/C, p-values<0.001). In SD, SbCN8 (Figure 4C, lower) and SbCN12 (Figure 4D, lower) expression was similar in both genotypes. Similarly, SbCN15 (Hd3a) expression was increased up to ~60-fold in 58 M compared to 100 M in LD and SD (Figure 4E; Figure S2-D, p-values<0.001) at all time points assayed, indicating that PhyB mediated repression of SbCN15 expression occurs regardless of photoperiod.

PhyB could be inducing SbPRR37 and SbGHD7 expression directly, and/or indirectly by altering output from the circadian clock. To determine if allelic variation in PHYB affected clock gene expression, TOC1 and LHY/CCA1, the central oscillators, and GI, a mediator of clock output were examined (Figure S3). In LD and SD, TOC1, LHY and GI expression in 38 M and 100 M peaked at similar times and most of these genes showed similar amplitude of expression, although expression of GI was approximately 2-fold lower in 58 M. Although three biological replicates at the indicated time points may not be sufficient to detect all biologically significant variation present, the small fold differences of circadian clock genes do not appear sufficient to explain the large variation in SbPRR37 and SbGHD7 expression observed in
Ma3 vs. ma3<sup>5</sup> backgrounds. PHYB and PHYC RNA levels were similar in 100 M and 58 M plants in LD and SD (data not shown).

**Discussion**

Sorghum genotypes used for grain production are typically photoperiod insensitive and flower in 55–75 days when planted in April in locations such as College Station, Texas where day lengths increase during the early portion of the growing season. Early flowering in grain sorghum helps avoid adverse weather and insect pressure during the reproductive phase, thereby enhancing yield. In contrast, highly photoperiod sensitive energy sorghum genotypes planted in the same location will not initiate flowering for 175 days until mid-September when day lengths decrease to less than 12.2 h [1,29]. Delayed flowering results in long duration of vegetative growth of energy sorghum, increasing biomass yield [8] and nitrogen use efficiency [8]. The importance of optimal flowering time for sorghum productivity led us to investigate the genetic and molecular basis of variation in this trait in sorghum.

Variation of flowering time of sorghum germplasm grown in LD environments is caused principally by differences in photoperiod sensitivity, although shading, GA, temperature, length of the juvenile phase among other factors also affect this trait [7]. A model summarizing information about photoperiod regulation of flowering time in sorghum is shown in Figure 5. In LD, flowering is delayed in photoperiod sensitive sorghum by the additive action of the floral repressors, ShbPRR37 (Ma1) and ShbGhd7 (Ma6) [25,26,28,29]. ShbPRR37 and ShbGhd7 repress expression of the grass specific floral activator, ShbEHD1. In addition, ShbPRR37 inhibits the activity of CO, another activator of flowering in sorghum [19]. The floral activators, ShbEhd1 and ShbCO, induce expression of ShbCN8 and ShbCN12, the proposed sources of FT in sorghum. ShbCN15, the ortholog of Hd3a and a source of florigen in rice [21], may also be a source of florigen in sorghum. The circadian clock is shown regulating expression of ShbG1, ShbCO, ShbPRR37 and ShbGhd7, and light regulating expression of ShbGhd7 and ShbPRR37 as shown in previous studies [25,26].

Photoperiod has minimal impact on flowering time in sorghum genotypes such as SM100 that encode null versions of ShbPRR37 and ShbGhd7 [25,26]. Presence of functional alleles of either gene increases photoperiod sensitivity and a further delay in flowering is observed when both genes are present in dominant Ma3Ma5 backgrounds. Expression of ShbPRR37 and ShbGhd7 is regulated by light and the circadian clock. Both genes show peaks of RNA abundance in the morning and again in the evening in LD and both peaks of RNA are attenuated in darkness. Importantly, the evening peak of expression is attenuated in SD when this phase occurs in darkness, indicating a requirement for light signaling during the evening to maintain sufficiently high levels expression of ShbPRR37 and ShbGhd7 to inhibit flowering. The morning and evening peaks of ShbPRR37 and ShbGhd7 expression observed in sorghum in LD is a pattern of expression first observed in photoperiod versions of this C4 grass. In Arabidopsis, PRR7, the ortholog of ShbPRR37, shows a single peak of clock-regulated expression during the morning [44]. In rice, ShbGhd7 shows a single peak of clock-gated expression in the morning of LD [22]. It will be interesting to determine if the dual peak pattern of PRR37 and GHD7 expression observed in sorghum is found in other related C4 grasses such as pearl millet, Miscanthus and sugarcane.

The current study focused on characterizing the light-signaling pathway that regulates ShbPRR37 and ShbGhd7 expression in response to day length. Previous studies showed that sorghum genotypes lacking PHYB (58 M, phyb-1) flower earlier in LD compared to near isogenic genotypes (100 M) expressing PHYB, demonstrating that light signaling through this photoreceptor is required for photoperiod sensitive variation in flowering time [13]. The current study showed that PhyB (Ma3) is epistatic to genes encoding the floral repressors ShbPRR37 and ShbGhd7 and that PhyB is required for photoperiod-regulated expression of these genes. Moreover, 58 M, a genotype lacking functional PhyB, showed attenuated expression of ShbPRR37 and ShbGhd7 during the evening of LD compared to 100 M (PhyB). In SD, expression of the floral repressors was similar in 58 M and 100 M. Taken together, these results indicate that in sorghum PhyB is required for light signaling in LD that results in elevated expression of ShbPRR37 and ShbGhd7 during the evening.

The molecular basis of PhyB induced expression of ShbPRR37 and ShbGhd7 during the evening of long days is unknown but could involve other photoreceptors and intermediary transcription factors such as PIFs [45]. Detailed studies in rice showed that PhyA, PhyB and PhyC modulate flowering time [39]. PhyC in particular plays a role in natural variation of flowering time in pearl millet [46], Arabidopsis [47], and wheat [48]. In Arabidopsis, a long day plant, PhyB destabilizes CO, an action countered by Cmy, PhyA and SPA in LD, leading to floral induction [20]. In rice, phyb mutants flower early in LD and SD similar to sorghum. Interestingly, rice phyC mutants flower early only in LD [39]. In addition, in rice, both PhyB and PhyC are required to induce GHD7 expression, where PhyB alone causes some repression of GHD7 mRNA levels [49]. This indicates that in rice PhyB regulates floral induction in both LD and SD, while PhyC modifies flowering time selectively in LD. The stability of PhyC is reduced in the absence of PhyB in rice and Arabidopsis [40]. PhyB increases PhyC stability, and chromophore-containing PhyB:PhyC heterodimers are required for PhyC activity [41]. Therefore, in sorghum the requirement for PhyB in photoperiod sensitive flowering time may be because PhyB increases PhyC stability and through formation of PhyB:PhyC heterodimers.

Genetic analysis of the role of PHYB in sorghum was examined using a population dominant for Ma1 (ShbPRR37) and segregating for alleles of PHYB (Ma3), Ma5, and ShbGhd7 (Ma6). The presence of Ma1 in all progeny of the population caused delayed flowering in LD unless the expression or activity of Ma1 (and in some genotypes Ma1 and Ma6) was altered by recessive alleles of Ma3 or Ma5. The analysis showed that plants homozygous for null alleles of PHYB (phyb-1) in Ma5<sup>b</sup> backgrounds had reduced photoperiod sensitivity and flowered earlier in LD compared to plants encoding PhyB. Similarly, progeny homozygous for recessive alleles of Ma5, in Ma3<sup>5</sup> backgrounds, showed reduced photoperiod sensitivity and flowered earlier in LD. The results indicated that both PHYB and Ma5 are epistatic to Ma1 and Ma6. Progeny recessive for either gene flowered earlier in LD, but showed a range of flowering times, indicating that other genes and/or environmental factors affected flowering time in these backgrounds, although with reduced response to photoperiod.

Interestingly, PHYB and Ma5 appear to be co-dependent or acting at a similar point in the regulatory pathway because allelic differences at Ma5 did not affect flowering time significantly in phyb-1 backgrounds and vice versa. R.07007 (Ma3ma5) and 58 M (ma3<sup>5</sup>Ma5) showed attenuated expression of ShbPRR37 and ShbGhd7 in the evening of LD [25] and this study) indicating that both Ma3 (PhyB) and Ma5 are required for elevated expression of the sorghum floral repressors during the evening of LD. In searching for an explanation for this co-dependence, we found the Ma5 locus spans several genes known to affect flowering time including PHYC and that the sequence of PhyC in R.07007 (ma5) contained amino acid changes that could potentially modify the function of this protein. The hypothesis that Ma5 corresponds to
**PHYC** is consistent with studies showing that PhyC modifies flowering in an LD specific manner in rice, similar to *Ma5* [39]. In addition, PhyC stability is dependent in part on PhyB and PhyC activity requires the formation of functional heterodimers with PhyB (and other phytochromes) [41]. If sorghum PhyC is regulated by PhyB in a manner similar to their counterparts in rice, this would explain why *Ma5* (presumptive **PHYC**) activity is not observed in *phyB-1* backgrounds. Experiments designed to test this hypothesis are currently underway.

In Arabidopsis, **CO** expression peaks once per day in the evening and the amplitude of **CO** expression is regulated by blue light/GI-FKF1-ZTL mediated turnover of CDF1, a repressor of **CO** expression [50]. PRR7 also modifies **CO** expression through repression of CDF1 expression [51]. In sorghum, **ShCO** expression peaks twice each day, at dawn and again in the evening in LD. The peak of **ShCO** expression at dawn is attenuated in SD ([25] and this study) and in genetic backgrounds lacking ShBPR37 [19]. It is possible that ShBPR37 modulates **ShCO** expression by repressing sorghum orthologs of CDF1 as occurs in Arabidopsis [51]. The peak of **ShCO** expression at dawn in LD was not observed in the sorghum genotype lacking PhyB (58 M). Since PhyB is required for elevated ShBPR37 expression in the evening of LD, and ShBPR37 has been shown to induce elevated expression of **ShCO** at dawn, it is likely that lack of PhyB induced expression of ShBPR37 during the evenings of LD explains the observed expression of **ShCO** in 58 M.

In rice, *Hd3a*, a member of the PEBP gene family, encodes an FT protein that acts as a florigen [52]. In maize, **ZCN8** and possibly **ZCN12** are sources of florigen [24, 53]. Sorghum encodes orthologs of *Hd3a* (**SbCN15**), **ZCN8** (**SbCN8**) and **ZCN12** (**SbCN12**). **SbCN8** and **SbCN12** expression is regulated by day length and by alleles of ShBPR37, ShGH7D7, and **PHYB** in a manner consistent with these genes being sources of florigen in sorghum. In prior studies, **SbCN15** expression was modulated to only a small extent in photocperiod and in mutants of ShBPR37 and ShGH7D7 that affect flowering time, suggesting that this gene was not an important target of photoperiod regulation [25, 26]. In the current study, expression of **SbCN15** was found to be ~60-fold higher in leaves of 58 M (**phyB-1**) compared to 100 M (**PHYB**) in both LD and SD. If **SbCN15** functions as a source of florigen as in rice, photoperiod independent repression of **SbCN15** expression by PhyB suggests that this gene may be responsible for early flowering induced by shading [7]. 58 M plants exhibit shade avoidance responses including longer leaf blades and sheaths, fewer tillers, narrower leaf blades, less leaf area, and more rapid stem elongation [7]. In Arabidopsis, light signaling through PhyB represses shade avoidance responses, and PhyB deficient mutants have elongated stems and an early flowering phenotype associated with “constitutive shade avoidance” [54]. Information on photocperiod regulated flowering time in sorghum described in this paper will hopefully facilitate analysis of flowering time variation caused by shading and other environmental factors.

### Supporting Information

**Figure S1** ANOVA interaction graphs showing (A) Day-length:PhyB (Day:Genotype) interaction. (B-D) Three two-way interactions (Ma3:Ma5, Ma3:Ma6, Ma5:Ma6) in the 58Ma5,07007 F2/F3 population. (TIF)

**Figure S2** Fold differences of **SbEHD1**, **SbCN8**, **SbCN12** and **SbCN15** RNA abundance at peaks of expression in 100 M and 58 M grown in LD (14 h light/10 h dark) or SD (10 h light/14 h dark). Positive fold difference values indicate higher mRNA levels detected in 58 M. (A) **SbEHD1**, (B) **SbCN8**, (C) **SbCN12**, (D) **SbCN15**. The time point corresponding to peak expression is shown below each graph. (TIF)

**Figure S3** Relative expression levels of circadian clock genes and **GI** in 100 M (black solid line) and 58 M (red dashed line) under either LD (14 h light/10 h dark) or SD (10 h light/14 h dark) conditions. The gray shaded area represents the dark period. The first 24 h covers one light-dark cycle, followed by 24 h of continuous light. (A) **GI**. (B) **TOC1**. (C) **LHY**. Each data point of relative expression corresponds to three technical replicates and three biological replicates. Error bars indicates SEM. (TIF)

**Table S1** Genotypes and flowering dates of sorghum lines. (DOCX)

**Table S2** Primer sequences used for **PHYB** alleles amplification and sequencing. (DOCX)

**Table S3** Primer sequences and amplification efficiency for qRT-PCR. (DOCX)

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### Author Contributions

Conceived and designed the experiments: SY RLM DTM. Performed the experiments: SY RLM DTM. Analyzed the data: SY RLM. Contributed reagents/materials/analysis tools: PEK WLR. Contributed to the writing of the manuscript: SY DTM JEM.

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