A point mutation in *Phytochromobilin Synthase* alters the circadian clock and photoperiodic flowering of *Medicago truncatula*

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Abstract

Plants use seasonal cues to initiate flowering at an appropriate time of year to ensure optimal reproductive success. The circadian clock integrates these daily and seasonal cues with internal cues to initiate flowering. The molecular pathways that control the sensitivity of flowering to photoperiod (daylength) are well described in the model plant Arabidopsis. However, much less is known in crop species, such as the legume family species. Here we performed a flowering time screen of a TILLING population of *Medicago truncatula* and found a line with late-flowering and altered light-sensing phenotypes. Using RNA-sequencing, we identified a nonsense mutation in the *Πηψτοςηρομοβιλιν Σψντηασε (ΜτΠΦΒΣ)* gene, which encodes an enzyme that carries out the final step in the biosynthesis of the chromophore required for phytochrome (*PHY*) activity. The analysis of the circadian clock in the MtpΦbs mutant revealed a shorter circadian period, which was shared with the *phyA* mutant. The *ΜτπΦβς* and *MtphyA* mutants showed downregulation of *FT* floral regulators *MtFTa1, MtFTb1/b2* and a shift in phase for morning and night core clock genes. Our findings show that PHYA is necessary to synchronize the circadian clock and integration of light signaling to promote expression of the *MtFT* genes to precisely time flowering.

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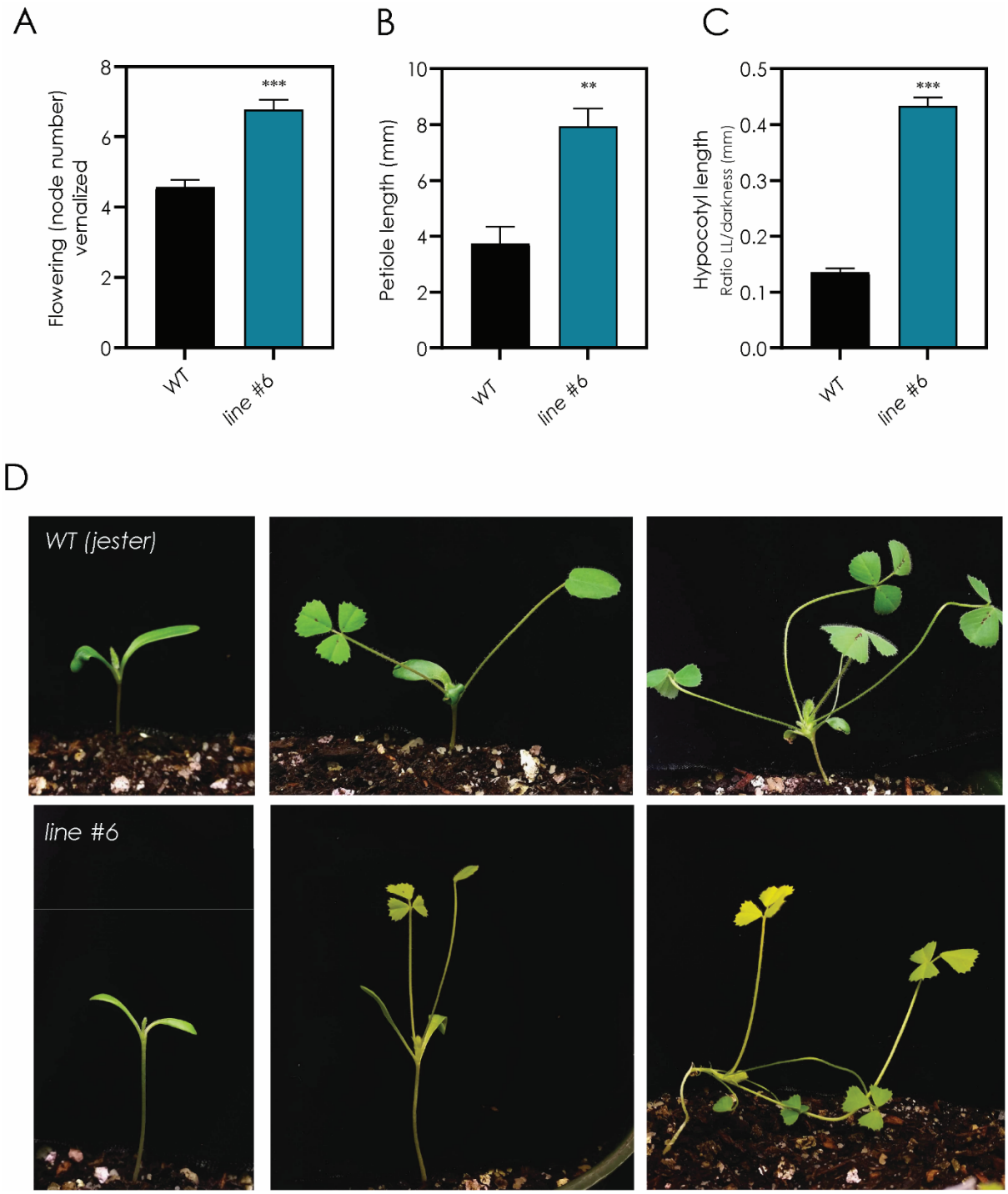


Figure 1. Characterisation of a late flowering mutant (*line#6*) with light-sensing deficiency. A) Flowering time of WT (Jester) and *line #6* of vernalised plants (14d at 4 °C) under LD conditions. **B)** petiole length (mm) and **C)** hypocotyl length (ratio mm LL/dark) under continuous light (LL). **D)** Photograph of WT (Jester) and *line #6* plant at the cotyledon stage, after the expansion of the first trifoliate and after the expansion of the fourth trifoliate leaf produced from the apical meristem. **p < 0.01, *** p< 0.001 (*t-student*). Error bars represent SEM.

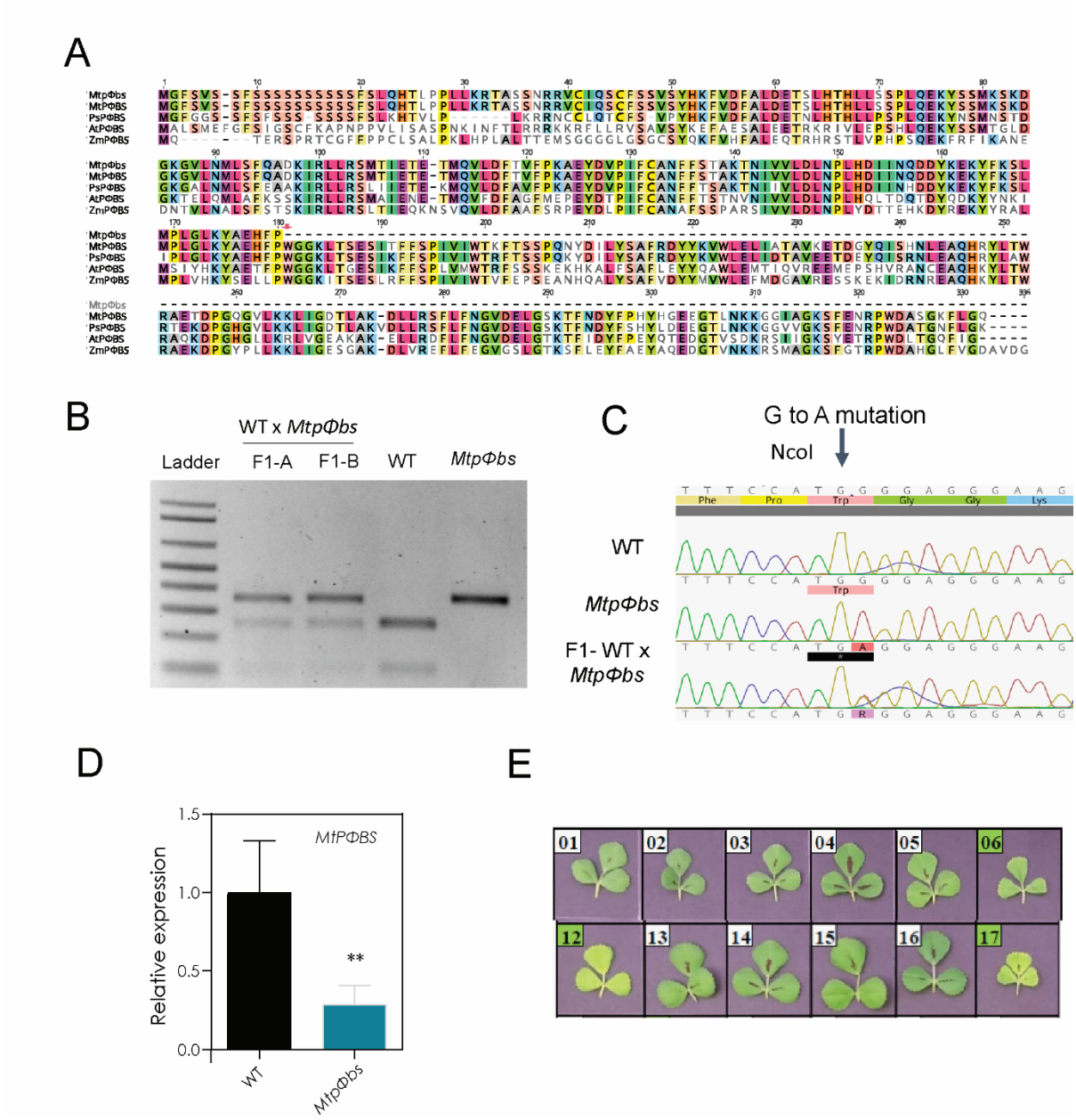
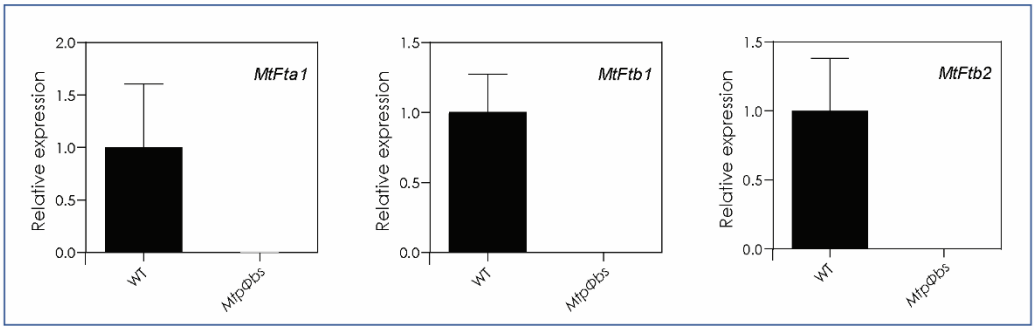


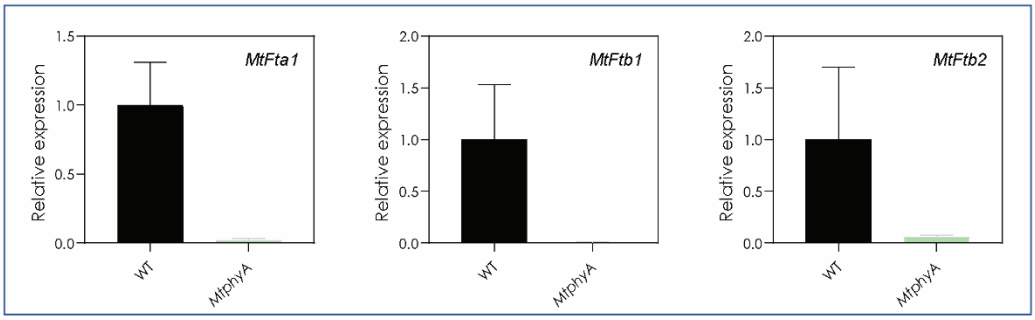
Figure 2. A point mutation in the gene *MtPφBS* is responsible for the photo-morphogenic and late-flowering phenotypes. A) Alignment of Medicago Medtr5g097080 (MtPφBS) protein sequence (region of exon 5) with the PΦBS sequence from other plant species (alignment tool in pairwise aligned using Geneious® (v.10.2.3). Arrow shows the location of the stop codon found in the *Mtpφbs* mutant. **B** *Mtpφbs* mutation G to A causes a disruption of a NcoI restriction site. NcoI restriction enzyme cuts PCR products from WT DNA (250bp + 100bp) but not *Mtpφbs* DNA (350bp). Successful cross/heterozygote F1 plants A and B PCR products (350pb +250bp + 100bp). **C.** Sequence conformation of the uncut products confirm the G to A change in the Mtpφbs mutant and a double (A/G) peak in F1 plants. **D** MtPΦBS expression levels were measured at ZT=2, primers were positioned upstream of the mutation site at the 5' end of the gene on either side of the exon1/exon2

boundary. Relative expression to WT normalized to housekeeper MtPDF2. **p < 0.01, *** p< 0.001 (*t-student*). Error bars represent SEM. **E.** Picture of representative trifoliate leaves of F2 segregating population. Green highlight displays genotypic *Mtpφbs* mutation confirmed using the NCO1 assay. White displays both heterozygotes and WT genotypes.

A


MtFta1
MtFtb1
MtFtb2
Relative expression
WT
MtphyB

B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MtFtb1
MtFtb2
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WT
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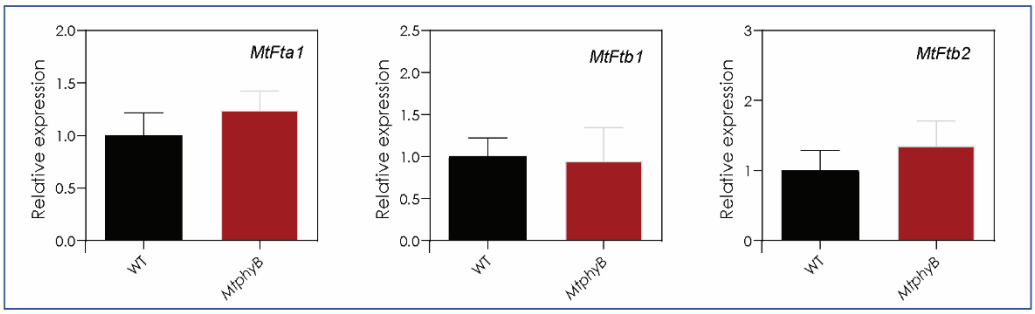
C


MtFta1
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Relative expression
WT
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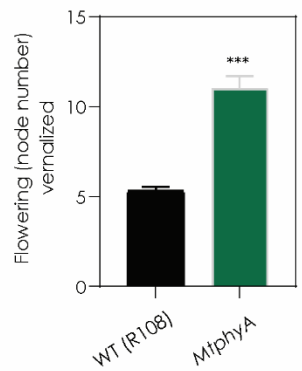
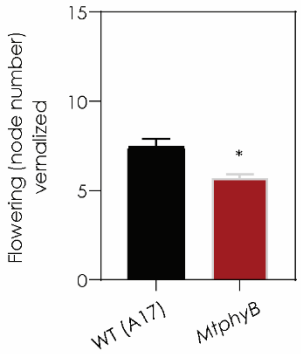

Flowering (node number)
vernalized
***
WT (R108)
MtphyA


WT (R108)
MtphyA

E


Flowering (node number)
vernalized
*
WT (A17)
MtphyB

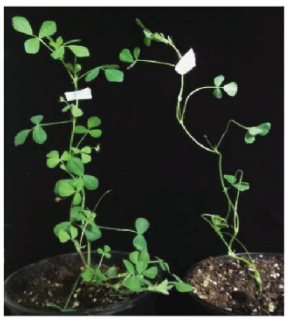

WT (A17)
MtphyB

Figure 3. Downregulation of FT genes are responsible for the late-flowering phenotype of *MtpΦbs*. Gene expression of the flowering FT genes, Fta1, FTb1 and FTb2 in **A)** *Mtpφbs,* ***B)*** *Mtphya* and ***C)*** *MtphyB* mutants, and respective WT in LD. Flowering time evaluated as number of nodes to first flower under Vernalised LD and photographs at time of first flower emerged for **D)** *MtphyA and* ***E)*** *MtphyB* and respective WT in VLD conditions. Relative transcript abundance was measured in the fully expanded trifoliate leaves of 14-day-old plants. Tissues were harvested 2h after dawn in LD conditions. Relative gene expression was measured by qRT-PCR with normalization to MtPDF2. Data are the mean ± SEM of three biological replicates and relative to the highest WT value. LD= long-day (16 h light/8 h dark). VLD = Vernalised lon-day. ***p < 0.001; **p<0.01, *p < 0.05 (Student t test).

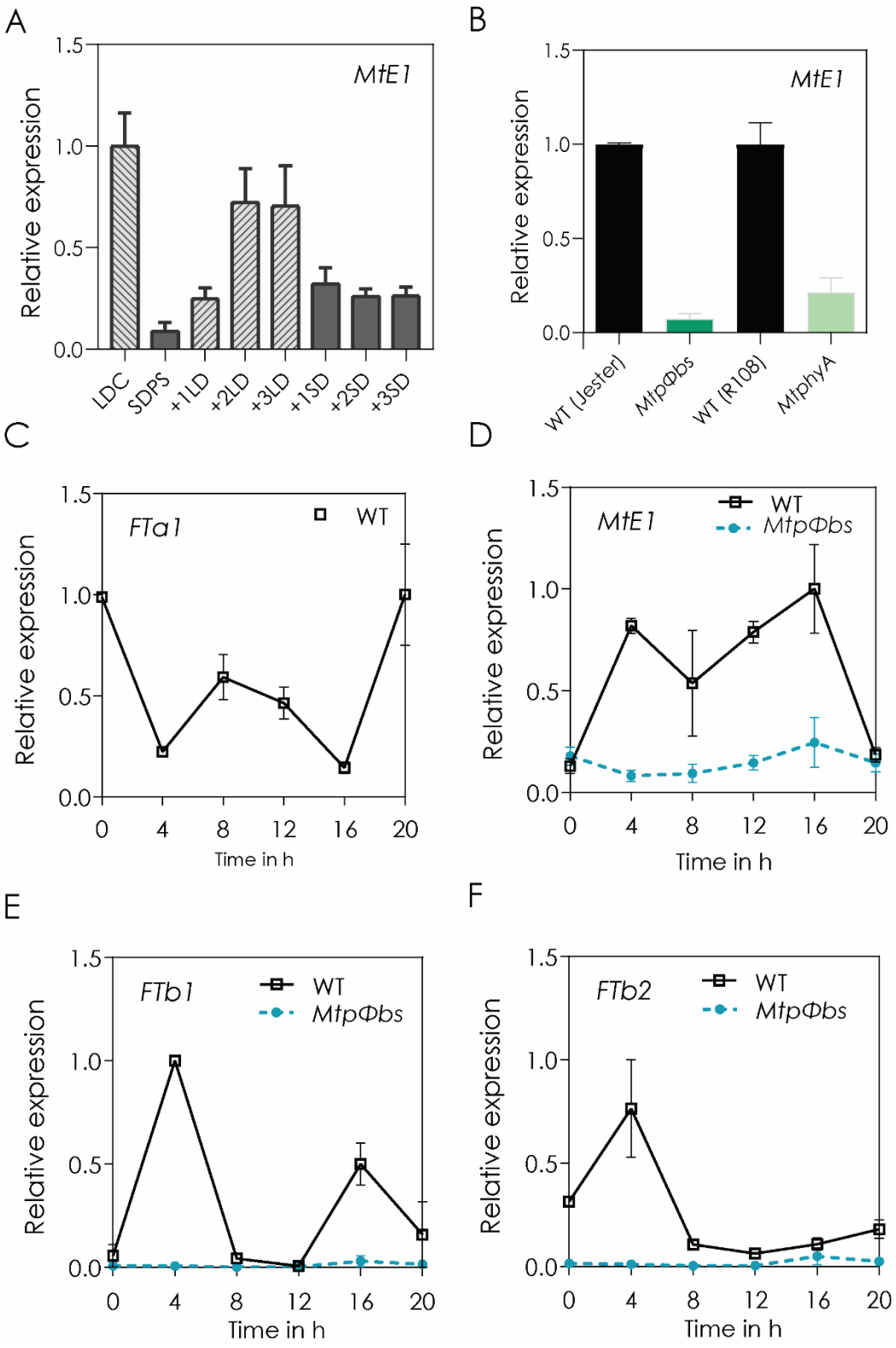


Figure 4. **The legume-specific flowering gene, MtE1, it is downregulated in the *Mtpφbs* mutant. A)** Response to changes in photoperiod. WT R108 leaves were collected at ZT4 of plants grown in LD and SD conditions. Plants grown in SD were then transferred to LD conditions for 1, 2, and 3 days before being transferred again to SD for 3 more days. E1 gene

expression of in **B)** *MtpΦbs* and *MtphyA mutan*t, and respective WT. Relative transcript abundance was measured 4h after dawn in LD conditions. Diurnal expression profiles of **C)** MtFta1, **D)** MtE1, **E)** MtFtb1 and **F)** MtFtb2. Plants were entrained for 21 days under LD conditions. Tissues were harvested every 4h for 1 day. Relative gene expression was measured by qRT-PCR with normalization to MtPDF2. Data are the mean ± SEM and relative to the highest WT value. SD = short day (8h light/16h dark), LD= long-day (16 h light/8 h dark).

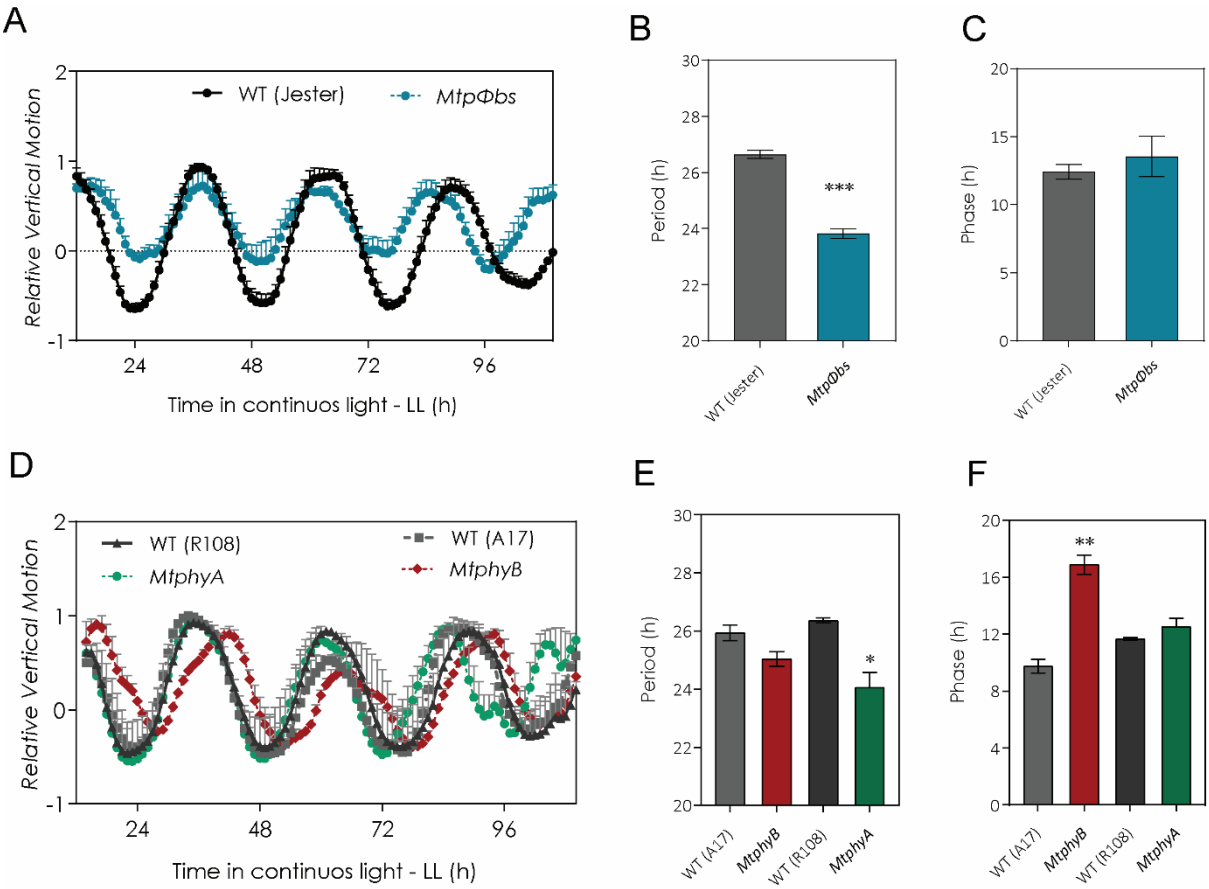


Figure 5. The *MtpΦbs* mutant has an altered circadian clock and it is shared with the *MtphyA* mutant. Circadian rhythm of cotyledon movement was recorded in plants entrained under long-day conditions (16 h light/8 h darkness) and then transferred to constant light (LL) for 5 days. Relative vertical motion was obtained for **A)** WT (Jester); black circles, *MtpΦbs* mutant blue circles; **D)** WT(R108); black triangle; *MtphyA green circles; WT(A17) grey squares and MtphyB, red circles. n=4 for all genotypes.* **B,E)** Period length of cotyledon movement estimated by fast Fourier transform– nonlinear least test (FFT-NLLS). **C,F)** Phase of cotyledon movement.. Error bars represent SEM. *p<0.05, **p<0.01, ***p < 0.001 (Student t-test). h=hours

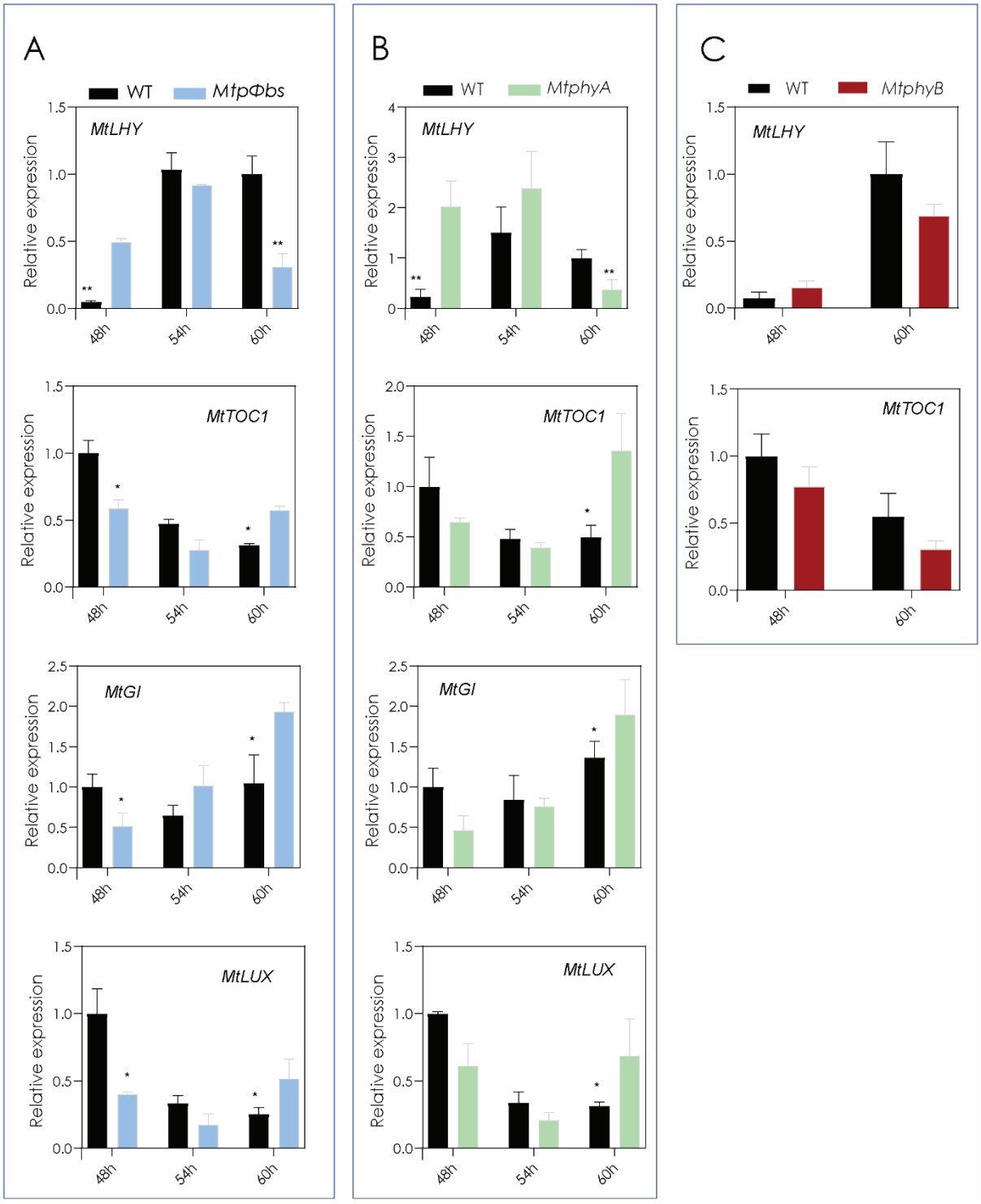


Figure 6. The *MtpΦbs* mutant has a shift in expression of the core clock genes. Gene expression of core clock genes in A) *Mtpφbs, B) MtphyA* and *C) MtphyB* mutants, and respective WT in LL3. Relative transcript abundance was measured in the fully expanded trifoliate leaves of 24-day-old plants. Tissues were harvested every 6h after dawn of the third day in LL after being entrained in LD for 21 days. Relative gene expression was measured by qRT-PCR with normalization to MtPDF2. Data are the mean ± SEM of three biological

replicates and relative to the highest WT value. **p<0.01, *p < 0.05 (Student t-test). LD= long-day (16 h light/8 h dark). LL3 = third day of free-running conditions (constant light).