PHYTOCHROME C regulation of photoperiodic flowering via *PHOTOPERIOD1* is mediated by *EARLY FLOWERING 3* in *Brachypodium distachyon*

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⚠ Expression of Concern

Following the publication of this article [1], concerns were raised regarding results presented in Fig 2. Specifically corresponding author informed PLOS that the underlying expression data reported in this study have been inappr manipulated.

The University of California, Davis confirmed that author DPW admitted to manipulation of the data underlying the presented in Fig 2.

The authors are working with PLOS to try and address these issues. Meanwhile, the *PLOS Genetics* Editors issu Expression of Concern to notify readers of the above issues.

19 Sep 2023: The PLOS Genetics Editors (2023) Expression of Concern: PHYTOCHROME C regulation of photo flowering via *PHOTOPERIOD1* is mediated by *EARLY FLOWERING 3* in *Brachypodium distachyon*. PLOS Gene e1010955. https://doi.org/10.1371/journal.pgen.1010955 | **View expression of concern**

Abstract

Daylength sensing in many plants is critical for coordinating the timing of flowering with the appropriate season. Tem adapted grasses such as *Brachypodium distachyon* flower during the spring when days are becoming longer. The p PHYTOCHROME C is essential for long-day (LD) flowering in *B. distachyon*. *PHYC* is required for the LD activation genes in the photoperiod pathway including *PHOTOPERIOD1* (*PPD1*) that, in turn, result in the activation of *FLOWE T* (*FT1*)/*FLORIGEN*, which causes flowering. Thus, *B. distachyon phyC* mutants are extremely delayed in flowering. that PHYC-mediated activation of PPD1 occurs via *EARLY FLOWERING 3* (*ELF3*), a component of the evening com circadian clock. The extreme delay of flowering of the *phyC* mutant disappears when combined with an *elf3* loss-of-f mutation. Moreover, the dampened *PPD1* expression in *phyC* mutant plants is elevated in *phyC/elf3* mutant plants c the rapid flowering of the double mutant. We show that loss of *PPD1* function also results in reduced *FT1* expressio delayed flowering consistent with results from wheat and barley. Additionally, *elf3* mutant plants have elevated expre *PPD1*, and we show that overexpression of *ELF3* results in delayed flowering associated with a reduction of *PPD1* a expression, indicating that ELF3 represses *PPD1* transcription consistent with previous studies showing that ELF3 b *PPD1* promoter. Indeed, *PPD1* is the main target of ELF3-mediated flowering as *elf3/ppd1* double mutant plants are flowering. Our results indicate that *ELF3* operates downstream from *PHYC* and acts as a repressor of *PPD1* in the p flowering pathway of *B. distachyon*.

Author summary

Daylength is an important environmental cue that plants and animals use to coordinate important life history events season. In plants, timing of flowering to a particular season is an essential adaptation to many ecological niches. Pe changes in daylength starts with the perception of light via specific photoreceptors such as phytochromes. In temper how daylength perception is integrated into downstream pathways to trigger flowering is not fully understood. Howev

components involved in the translation of daylength perception into the induction of flowering in temperate grasses h identified from studies of natural variation. For example, specific alleles of two genes called *EARLY FLOWERING 3* *PHOTOPERIOD1* (*PPD1*) have been selected during breeding of different wheat and barley varieties to modulate th response to maximize reproduction in different environments. Here, we show in the temperate grass model *Brachyp distachyon* that the translation of the light signal perceived by phytochromes into a flowering response is mediated b that *PPD1* is genetically downstream of *ELF3* in the photoperiodic flowering pathway. These results provide a genet understanding the photoperiodic response in temperate grasses that include agronomically important crops such as barley, and rye.

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Data Availability: All mutant lines are available upon request to the following email address: front_desk@bioche (Department of Biochemistry, University of Wisconsin) with no restrictions.

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Introduction

The transition from vegetative growth to flowering is an important developmental decision for which the timing is ofte influenced by the environment (e.g. [1–4]). This critical life history trait has been shaped over evolutionary time to en reproduction to coincide with the time of year that is most favorable for flower and seed development. Moreover, bre the timing of flowering in crops has been critical for adapting various crop varieties to changing environments and to (e.g. [5]).

In many plant species, changes in day-length and/or temperature provide seasonal cues that result in flowering duri time of year [1,6]. Many temperate grasses such as *Brachypodium distachyon* (*B. distachyon*), wheat, and barley th spring or early summer months in response to increasing day-lengths are referred to as long-day (LD) plants [7]. *B.* closely related to the core pooid clade comprising wheat, oats, barley, and rye and has a number of attributes that m attractive grass model organism suitable for developmental genetics research [8,9].

Variation in the LD promotion of flowering in temperate grasses such as wheat and barley can be due to allelic varia *PHOTOPERIOD1* (*PPD1*), a member of the pseudo-response regulator (PRR) gene family (*PPD1* is also known as *RESPONSE REGULATOR 37*;*PRR37*) [10,11]. Natural variation in *PPD1* resulting in either hypomorphic alleles as f or dominant *PPD1* alleles as found in tetraploid or hexaploid wheat impacts flowering [10–15]. Specifically, natural re mutations in the conserved CONSTANS, CONSTANS-LIKE and TIMING OF CAB EXPRESSION 1 (CCT) putative D domain in the barley PPD1 protein cause photoperiod insensitivity and delayed flowering under LD [11, 12], whereas

photoperiod insensitivity is linked to overlapping large deletions in the promoter region of *PPD1* in either the A [13] c
homeologs [10]. These deletions result in elevated expression of *PPD1*, particularly during dawn, causing rapid flow
under non-inductive SD conditions [13]. It is worth noting that although these wheat lines are referred to as photoper
(PI) varieties they still flower earlier under LD than under SD if the timing of flowering is measured as the emergence
spike (heading time) [16]. It has been hypothesized that the large deletion within the *PPD1* promoter might remove a
one or more transcriptional repressors [13]. To date, natural variation studies of flowering in *B. distachyon* have not
variation at *PPD1* and thus its role in LD flowering in *B. distachyon* is not known [17–21].

Variation in *EARLY FLOWERING 3* (*ELF3;* also known as *mat* and *eam*) impacts photoperiodic flowering in grasses
wheat [22,23], barley [24,25], and rice [26]. In these plants, natural variation in *ELF3* allows growth at latitudes that c
not be inductive for flowering, enabling these crops to be grown in regions with short growing seasons [5]. For exam
maturity (*eam*) loci have been used by breeders to allow barley to grow at higher latitudes in regions of northern Eur
growing seasons [24,27]. The *eam8* mutant in the barley ortholog of *ELF3*, is a loss-of-function mutation that accele
under SD or LDs [24,25] similar to *elf3* loss-of-function alleles described previously in the eudicot model *Arabidopsis*
thaliana) [28]. Moreover, loss of function of *ELF3* in *B. distachyon* also results in rapid flowering under SD and LD, a
of the *B. distachyon* ELF3 protein is able to rescue the *A. thaliana elf3* mutant, demonstrating a conserved role of *E*
across angiosperm diversification [29–31].

Work in *A. thaliana* has shown that *ELF3* is an important component of the circadian clock that acts as a bridge prot
trimeric protein complex that also contains *LUX ARRTHYHMO* (LUX), and *EARLY FLOWERING 4* (*ELF4*) and is ref
evening complex (EC) [32]. Loss-of-function mutations in any of the proteins that make up the EC results in disrupte
and rapid flowering [33–36]. The peak expression of the EC at dusk is involved in the direct transcriptional repressio
make up the morning loop of the circadian clock including *A. thaliana PRR7* and *PRR9*, which are paralogs of grass
PRR73 [37–39]. Recently, it has been shown that the EC also directly represses *PRR37*, *PRR95*, and *PRR73* in rice
rice ortholog of *PPD1)*, indicating conservation of the role of the EC across flowering plant diversification [40]. Furth
mutants in barley, wheat, and *B. distachyon* have elevated *PPD1* expression [23,24,30] indicating *ELF3* may impact
part via *PPD1*, but to what extent remains to be determined.

The photoperiod and circadian pathways converge in the transcriptional activation of florigen/*FLOWERING LOCUS*
leaves [6,41]. In temperate grasses, *PPD1* is required for the LD induction of *FT1*, whereas in *A. thaliana CONSTAN*
main photoperiodic gene required for *FT1* activation in LD [16,42,43]. There are two *CO*-like genes in temperate gra
Interestingly, in the presence of functional *PPD1*, *co1co2* wheat plants have a modest earlier heading phenotype su
are in fact mild floral repressors, but in the absence of *PPD1*, *CO1* acts as a flowering promoter under LD [16]. To d
co1co2 double mutants have been reported in *B. distachyon*. However, RNAi knock-down of *co1* results in a 30-day
flowering under 16h LD [44] and overexpression of *CO1* leads to earlier flowering in SD [44]. These results indicate
distachyon CO1 has a promoting role in flowering even in the presence of a functional *PPD1* gene, and suggest pot
differences in the role of *CO1* in the regulation of flowering between *B. distachyon* and wheat.

Once FT1 is activated by LD it interacts with the bZIP transcription factor FD which triggers the expression of the M
transcription factor *VERNALIZATION1* (*VRN1*) [45,46]. VRN1 in turn upregulates the expression of *FT1* forming a p
loop that overcomes the repression from the zinc finger and CCT domain-containing transcription factor *VERNALIZ*
[17,47–50]. The FT1 protein is then thought to migrate from the leaves to the shoot apical meristem, as shown in *A.*
rice [51,52], to induce the expression of floral homeotic genes including *VRN1*, thus converting the vegetative meris
meristem under favorable LD photoperiods.

Light signals are perceived initially by photoreceptors that initiate a signal transduction cascade impacting a variety
developmental responses to light [53]. The sensing of light is accomplished by complementary photoreceptors: phyt
perceive the ratio of red and far-red light, cryptochromes, phototropins, and Zeitlupe family proteins that detect blue
RESISTANCE LOCUS 8 that detects ultraviolet B light [54,55]. The phytochromes form homodimers that upon expo
to red light undergo a confirmation shift to an active form causing the activation of a suite of downstream genes [54]
plants to far-red or dark conditions causes reversion of the phytochromes to an inactive state [54].

There are three phytochromes in temperate grasses referred to as PHYTOCHROME A (PHYA), PHYTOCHROME B
PHYTOCHROME C (PHYC) [56]. Functional analyses of these phytochromes in temperate grasses revealed that P
play a major role in the LD induction of flowering because loss-of-function mutations in either of these genes results
delayed flowering [57–59] whereas loss-of-function mutations in *PHYA* in *B. distachyon* results in only a modest dela
under inductive LD, indicating PHYB and PHYC are the main light receptors required for photoperiodic flowering in t
grasses [29].The important role of PHYC in photoperiodic flowering is not universal as loss of *phyC* function in *A. th*

only has small effects on flowering [60,61].

In temperate grasses, PHYB and PHYC are required for the transcriptional activation of a suite of genes involved in pathway, including *PPD1*, *CO1*, and *FT1*, and ectopic expression of *FT1* in the *B. distachyon phyC* background resu flowering—a reversal of the *phyC* single-mutant, delayed-flowering phenotype [57,58,62]. Moreover, consistent with at the beginning of the photoperiodic flowering signal cascade, expression of genes encoding components of the cir are also severely dampened in the *phyB* and *phyC* mutant backgrounds [29,57–59]. An exception to this is that the *ELF3* is not altered in the temperate grass phytochrome mutants [29,58,59]. Recently, in *B. distachyon* it has been s PHYC can interact with ELF3, and this interaction destabilizes the ELF3 protein indicating that the regulation of ELF least in part at the protein level consistent with previous studies from *A. thaliana*, rice, and the companion study in w [23,29,40,63–66]. At present it is not clear to what extent the regulation of ELF3 by PHYs is critical for photoperiodic

Here, we show in *B. distachyon* by analyzing *phyC/efl3* double mutant plants that indeed the light signal perceived b phytochromes is mediated through ELF3 for photoperiodic flowering. The extreme delayed flowering of the *phyC* mu in the *phyC/elf3* double mutant which flower as rapidly as the *elf3* single mutant. Moreover, the expression profiles o photoperiod pathway are similar between *elf3* and *phyC/elf3* mutants compared to *phyC* mutants. Thus, *elf3* is comp to *phyC* at the phenotypic and molecular levels. Furthermore, we show strong, environment-dependent genetic inter between *ELF3* and *PPD1*, which indicates that *PPD1* is a main target of ELF3-mediated repression of flowering. The provide a genetic and molecular framework to understand photoperiodic flowering in the temperate grasses.

Results

Rapid flowering of *elf3* is epistatic to the delayed flowering of *phyC*

Previous studies in *B. distachyon* showed that PHYC can affect the stability of the ELF3 protein, and that the transcr *phyC* mutant resembles that of a plant with elevated ELF3 signaling [29]. Thus, it has been suggested that the extre flowering phenotype of the *phyC* mutant [58] could be mediated by ELF3 [29]. To test the extent to which the transla signal perceived by PHYC to control flowering is mediated by ELF3, we generated *elf3/phyC* double mutant plants a the flowering of the double mutant relative to that of *elf3* and *phyC* single mutants as well as Bd21-3 wild type under 8h-SD (Fig 1).

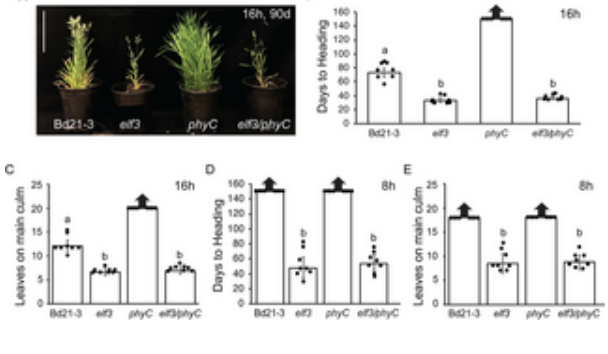

Fig 1.
The rapid flowering of the *elf3* mutant is epistatic to the delayed flowering of the *phyC* mutant (**A**) Representative Bd21-3 wild-type, *elf3*, *phyC* and *elf3/phyC* double mutant plants grown in a 16h photoperiod at 90d after germina 17cm. (**B, D**) Flowering times under 16h (**B**) or 8h daylengths (**D**) measured as days to heading of Bd21-3, *elf3*, *p elf3/phyC*. (**C**) Flowering phenotypes under 16h (**C**) or 8h daylengths (**E**) measured as the number of leaves on th culm at time of heading. Bars represent the average of 8 plants ± SD. Arrows above bars indicate that none of the flowered at the end of the experiment (150d). Letters (a, b) indicate statistical differences ($p < 0.05$) according to HSD test used to perform multiple comparisons.
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Under both 16h LD and 8 SD photoperiods, we found that *elf3* is epistatic to *phyC*. Specifically, in LD *elf3/phyC* doub flowered rapidly by 38 days with 6.9 leaves similar to *elf3* mutants that flowered by 34 days with 6.6 leaves (Fig 1A a contrast, *phyC* mutants had not flowered after 150 days with greater than 20 leaves when the experiment was termi Bd21-3 wild-type flowered by 72 days with 12 leaves consistent with previous studies [49,58]. In 8h SD, *elf3/phyC* d

also flowered rapidly by 54 days with 8.8 leaves similar to *elf3* mutants that flowered by 48 days with 8.5 leaves (Fig
contrast, both Bd21-3 wild-type and *phyC* mutants had not flowered by 150 days with >18 leaves when the experim
terminated (Fig 1D and 1E). These results indicate that the extreme delayed flowering mutant phenotype of *phyC* in
is mediated by ELF3.

To determine if *PHYC* affects the expression of *ELF3*, we analyzed *ELF3* mRNA levels across a diurnal light cycle (
dark). There were no significant differences in *ELF3* expression at any time point in the *phyC* mutant relative to wildt
indicating that *PHYC* does not affect the transcriptional profile of *ELF3* in *B. distachyon* consistent with results from
wheat (67, 69).

Effect of mutations in *PHYC* and *ELF3* on the transcriptional profiles of flowering time genes

To further understand how *PHYC* and *ELF3* affect flowering at a molecular level, we compared the mRNA levels of
orthologs of the photoperiod and vernalization pathway genes *FT1*, *VRN1*, *PPD1*, *VRN2*, *CO1*, and *CO2* across a d
16h LD in the *phyC* and *elf3* single mutants versus the *elf3/phyC* double mutant (Fig 2). We were particularly interes
determining how the expression profiles of "flowering-time genes" in the *elf3/phyC* double mutant compared to the *e*
single mutant. The newly expanded fourth leaf was harvested for gene expression analyses because at this develop
16h daylengths the meristems of all of the plant genotypes are at a vegetative stage and thus are developmentally e
Consistent with the rapid flowering of the *elf3* and *elf3/phyC* mutants, the mRNA expression levels of *FT1* and *VRN*
are significantly higher than the levels in wild-type and *phyC* mutants across all the time points tested (Fig 2A and 2
the overall expression profiles of *FT1* and *VRN1* in the *elf3* and *elf3/phyC* mutants were similar throughout the day.
contrast to the *phyC* mutant in which *FT1* and *VRN1* mRNA levels were lower than wild type throughout the day con
delayed flowering phenotype of *phyC*. Although the elevated levels of *FT1* and *VRN1* in both *elf3* and *elf3/phyC* are
their rapid flowering, the expression of the floral repressor, *VRN2*, exhibits a similar elevated expression profile throu
in both *elf3* and *elf3/phyC* relative to wild-type or *phyC* single-mutants (Fig 2E). The elevated *VRN2* expression leve
plants are consistent with previous results in *B. distachyon* and other grasses [23,29,30,40,64]. The transcriptional p
was similar in both the *elf3* and *elf3/phyC* mutants with elevated expression compared to wild-type between zt4-8 ar
than wild-type between zt12-20 (Fig 2C). A similar expression pattern was found for *Hd1* (the rice *CO* homolog) in th
double mutant in rice [40]. Consistent with previous reports, *CO1* expression levels remained low in *Brachypodium*
throughout a diurnal cycle [58]. By contrast *CO1* expression is increased in the *phyC* mutants in wheat [57] indicatin
difference in the regulation of *CO1* between these two species. Lastly, the *CO2* expression profiles were similar betw
elf3, and *elf3/phyC*, whereas *CO2* mRNA levels were lower in *phyC* throughout a diurnal cycle (Fig 2F). In summary
transcriptional profiles of *FT1*, *VRN1*, *VRN2*, *CO1*, and *CO2* are similar between *elf3* and *elf3/phyC* mutants consist
acting downstream from *PHYC* in the photoperiod flowering pathway.

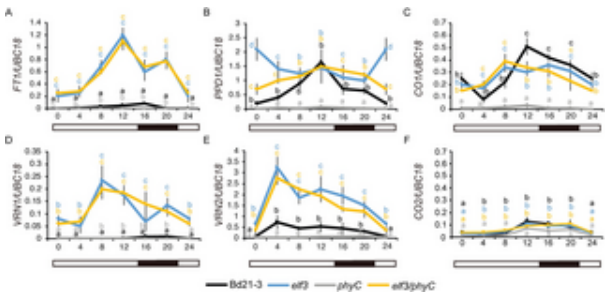

Fig 2. Effect of loss-of-function mutations in *ELF3* and *PHYC* on the transcriptional profiles of six flowering time genes in 16h LD.
Normalized expression of (**A**) *FT1*, (**B**) *PPD1*, (**C**) *CO1*, (**D**) *VRN1*, (**E**) *VRN2*, and (**F**) *CO2* during a 24h diurnal c
Bd21-3 (black line), *elf3* (blue line), *phyC* (gray line) and *elf3/phyC* double mutant (orange line). Plants were grow
until the fourth-leaf stage was reached (Zadoks = 14) at which point the newly expanded fourth leaf was harveste
zt8, zt12, zt16, and zt20. Note the zt0 value and zt24 value are the same. The average of four biological replicate
(three leaves per replicate). Error bars represent standard deviation of the mean. Data were normalized using *UB*
done in [49].
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The transcriptional profile of *PPD1* indicates a more complex interaction between *PHYC* and *ELF3*. In wild type, the
levels of *PPD1* peak at zt12 with the lowest expression level at dawn and during the evening consistent with previou

PPD1 expression patterns in *B. distachyon* [29,30] (Fig 2B). In both the *elf3* and *elf3/phyC* mutants, we observed inc expression relative to wild-type at dawn and during the evening with expression levels similar to wild-type at zt12. In increased expression of *PPD1* observed at dawn and during the night in the *elf3/phyC* background was significantly of the *elf3* single mutant suggesting *PHYC* may impact *PPD1* expression via additional genes beyond *ELF3*. In cont expression levels were reduced in the *phyC* mutant relative to wild type, *elf3*, and *elf3/phyC* mutants throughout a di consistent with the reduced *FT1* expression and delayed flowering phenotype of the *phyC* mutant.

Identification and mapping of a *ppd1* mutant in *B. distachyon*

To determine the role of *PPD1* in flowering in *B. distachyon*, the genome-sequenced, sodium-azide mutant line NaN predicted high-effect mutation impacting a splice acceptor donor site in *PPD1* (BdiBd21-3.1G0218200) was obtained Genome Institute (JGI) ([65]; **https://phytozome-next.jgi.doe.gov/jbrowse/**). A quarter of the NaN610 M3 seeds re segregating for an extremely delayed flowering phenotype (Fig 3B–3D).

Due to the high mutant load of these NaN mutant lines, we validated through mapping that the delayed flowering ph associated with *PPD1* (Fig 3E and 3F). We backcrossed NaN610 with Bd21-3 and confirmed a quarter of the plants population (n = 380) were delayed flowering, demonstrating the recessive nature of the mutant. Three Derived Clea Polymorphic Sequences (dCAPs) markers closely linked with *PPD1* were developed based on the variant's informat NAN610 line, with one of the dCAPs primers located within the *PPD1* locus itself (Fig 3E and S1 Table). This approa to map the causative lesion to within a 1Mb interval (13.1Mb-14.2Mb) on the top arm of chromosome 1, demonstrati flowering phenotype is tightly linked with *PPD1* (Fig 3E).

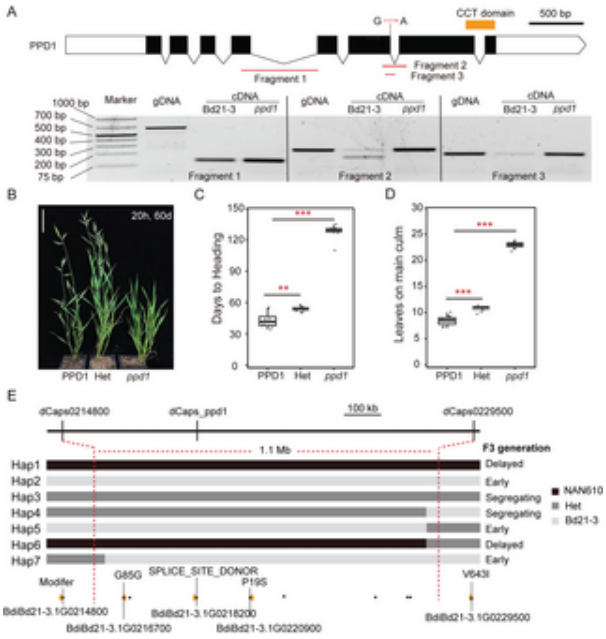

Fig 3. Identification of a *ppd1* mutant.
(**A**) Gene structure of *PPD1* showing the location of the nucleotide change of the sodium azide-induced mutation indicates the region that encodes the CCT domain. Below the gene structure diagram is a gel image of the revers transcription polymerase chain reaction (PCR) (30 cycles of amplification) showing PCR products of *PPD1* cDNA and *ppd1* mutant plants. The location of primers used in each reaction are shown in the diagram above the gel im Representative photo of Bd21-3, heterozygous, and homozygous *ppd1* plants grown in a 20h LD. Picture was tak germination in 20h LD, bar = 5cm. (**C** and **D**) Flowering time was measured as days to heading (**C**) and the numb on the parent culm at time of heading (**D**), ** indicates statistical differences ($p < 0.01$), *** indicates statistical diff 0.001) by Student's t-test. (**E**) Fine mapping of *ppd1* in a population of 380 BC1F2 individuals. Individuals with se haplotypes were identified by three dCAPS markers and flowering times of each haplotype were determined in th generation. Black, grey, and light grey rectangles represent NAN610, heterozygous, and Bd21-3 genotypes, resp Variants around the *PPD1* locus from the NAN610 line are shown with black dots, and yellow arrows indicate the genes within the mapped interval with the specific effect on the coding region indicated.
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To confirm that the predicted splice site mutation does in fact impact the splicing of *PPD1*, we sequenced the mRNA *ppd1* NaN610 mutant line and Bd21-3 (Fig 3A). We found that the splice site mutation resulted in the mis-splicing of generating a reading frame shift resulting in a truncated protein lacking the conserved CCT domain (Fig 3A). The ex delayed flowering of the *B. distachyon ppd1* mutant is consistent with the *ppd1* null mutants described in wheat, whi days to head under inductive LD conditions [16,43], demonstrating *PPD1* is required for LD flowering broadly within grasses.

Genetic interactions between *ELF3* and *PPD1* under long and short days

We and others have shown that *PPD1/PRR37* expression is increased in an *elf3* mutant background in *B. distachyo* wheat (Fig 2B; [29,30,40,66]). Moreover, a CHIPseq analysis of ELF3 demonstrated that *PPD1* is directly bound by of-day-responsive manner [29,40]. Thus, ELF3 acts as a direct transcriptional repressor of *PPD1* but the extent to w explains the rapid flowering in the *elf3* mutant has not been tested. Therefore, we generated an *elf3/ppd1* double mu the genetic interactions of these two genes under a highly inductive 20h LD, inductive 16h LD, and non-inductive 8h

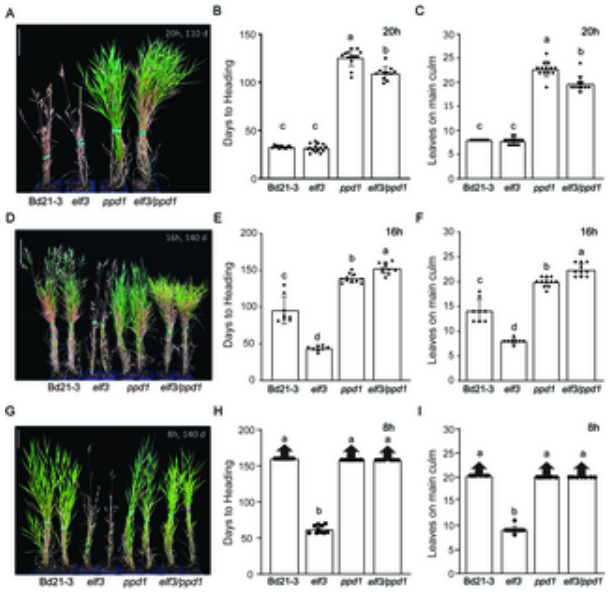

Fig 4. Genetic interactions between the delayed flowering *ppd1* mutant and the rapid flowering *elf3* mutant.
Representative image of Bd21-3 wild-type, rapid flowering *elf3* mutant, delayed flowering *ppd1* mutant, and delay *elf3/ppd1* double mutant grown in a 20h photoperiod (**A**), 16h photoperiod (**D**), and 8h photoperiod (**G**). Picture w after 110d, for the 20h LD (**A**) and 140d after germination for the 16h LD and (**D**) 8h SD. Scale bar = 5cm. (**B**, **E**, times under 20h (**B**), 16h (**E**), 8h (**G**) measured as days to heading of Bd21-3, *elf3*, *ppd1*, and *elf3/ppd1*. Floweri under 20h (**C**), 16h (**F**), and 8h (**I**) measured as the number of leaves on the parent culm at time of heading. The experiment was repeated three times. The first experiment resulted in *ppd1* plants that stopped producing new le wild type. One possibility for the cessation of new leaf production in *ppd1* plants in this experiment is that the mer transitioned to flowering, but then did not proceed to heading. However, in two subsequent experiments *ppd1* pla continually produced new leaves for the duration of the experiment similar to wild type and this data is shown in (all three experiments are shown in S1 Data for Fig 4. When grown under non-inductive conditions for 120 days o *B. distachyon* plants flower; we consider this a stochastic flowering response because the majority of plants do n Bars represent the average of 8 plants ± SD. Arrows above bars indicate that none of the plants flowered at the e experiment (150d, >20 leaves). Letters (a, b, c, d) indicate statistical differences ($p < 0.05$) according to a Tukey's used to perform multiple comparisons.
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Under all photoperiods, the *elf3/ppd1* double mutant flowered significantly later than the *elf3* single mutant (Fig 4). I under 20h LD, the *elf3/ppd1* double mutant flowered earlier than *ppd1* by 16.2 days forming 3.0 fewer leaves where days *elf3/ppd1* mutant flowered significantly later than *ppd1* by 13.7 days with 2.5 more leaves. In 8h SD, only *elf3* were able to flower; Bd21-3, *ppd1*, and *elf3/ppd1* all failed to flower by the end of the experiment. It is also worth not *elf3/ppd1* double mutants are still able to respond to different photoperiods, with longer days resulting in significantly flowering plants than under shorter days (Fig 4B and 4E and 4H). These results indicate that there are strong genet

between *ELF3* and *PPD1* under different photoperiods, that *PPD1* is a key flowering regulator downstream of *ELF3*, is a residual photoperiodic response that is independent of these two genes.

Effect of mutations in *ELF3* and *PPD1* on the transcriptional profiles of flowering time genes

To understand how *ELF3* and *PPD1* affect flowering at a molecular level, we measured the mRNA levels of *FT1*, *VR* *VRN2*, *CO1*, and *CO2* in the *elf3* and *ppd1* single mutants and the *elf3/ppd1* double mutant across a diurnal cycle in As noted before, *FT1* and *VRN1* expression levels were elevated in the *elf3* mutant background; however, in the *elf* mutant, expression of these genes remained low and resembled the expression profile of *ppd1* single mutants (Fig 5 low expression levels of *FT1* and *VRN1* in *ppd1* and *ppd1/elf3* mutants is consistent with the delayed flowering pher of these mutants in 16h LD. The *VRN2* expression profile was similar between wild type and *ppd1* mutant plants wit expression levels at dawn and increased expression throughout the light cycle before expression levels dropped in t 5E). Interestingly, *VRN2* expression levels are similarly elevated throughout the day in *elf3* and *elf3/ppd1* mutants co type (Fig 5E).

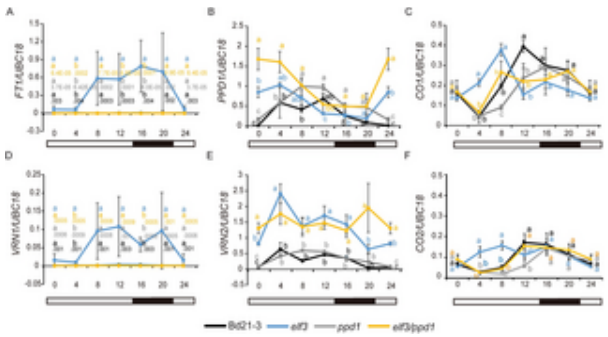

Fig 5. Effect of loss-of-function mutations in *ELF3* and *PPD1* on the transcriptional profiles of six flowering-time genes in 16h LD. The fourth newly expanded leaves were harvested every 4h over a 24-hour period; three biological replicates (tw replicate) were harvested at each time point for each genotype. Diurnal expression of *FT1* (**A**), *PPD1* (**B**), *CO1* (**C** *VRN2* (**E**), and *CO2* (**F**) were detected in Bd21-3 (black line), *elf3* (blue line), *ppd1* (grey line) and *elf3/ppd1* doub line). Bars represent the average of three biological replicates ± SD. Letters (a, b, c, d) indicate statistical differen 0.05) according to a Tukey's HSD test used to perform multiple comparisons, letter color corresponds to the four genotypes. The black, gray, and orange lines overlap given the scale used to show *ELF3* expression in the same orange line is arbitrarily shown on top. Specific expression values are shown below the lettered statistical test. Ra included in S1 Data file.
https://doi.org/10.1371/journal.pgen.1010706.g005

Consistent with the expression patterns of *PPD1* in wild type and *elf3* shown in Fig 2, the expression levels of *PPD1* wild-type and the *elf3* mutant has increased *PPD1* expression relative to wild type at dawn and during the evening (expression levels in the *ppd1* mutant should be interpreted with caution because we do not know the effect of the sp mutation on the mRNA stability. Significantly higher levels of *PPD1* expression were observed in *ppd1* relative to wil and ZT16, and in *elf3/ppd1* relative to *elf3* at dawn. However, the expression patterns of *PPD1* were most similar be and wild type and between *elf3/ppd1* and *elf3* (Fig 5B).

CO1 and *CO2* expression both exhibit peak expression in wild type at zt12 with expression dampening in the evenin with previous reports [29,58]. Interestingly, expression levels of *CO1* and *CO2* were elevated between zt4-8 in *elf3* c wild-type. However, at zt16 and zt20, expression levels were similar in wild type, *elf3*, and *elf3/ppd1* mutants, where expression of *CO1* was reduced in *elf3* compared to wild type. In contrast, the expression levels of *CO1* and *CO2* w *ppd1* compared to the other lines at zt8. In the *elf3/ppd1* mutants, *CO1* and *CO2* expression was most similar to *pp* morning and most similar to *elf3* in the evening (Fig 5C and 5F). These results indicate complex interactions betwee ELF3 in the regulation of *CO1* and *CO2*.

Constitutive expression of *ELF3* results in delayed flowering and lower *PPD1*, *FT1*, and *VRN1* expression levels

In our previous study, we showed that overexpression of *ELF3* in the *elf3* mutant background results in strongly dela ([30], Fig 6A). However, this was done in the T0 generation, so we evaluated the flowering time and expression of d

flowering-time genes in the T1 generation. We grew four *UBI*::*ELF3/elf3* transgenic lines alongside Bd21-3 and *elf3* photoperiod, and harvested the newly expanded fourth leaf at zt4. This time point was chosen because expression critical genes such as *CCA1*, *TOC1*, *LUX*, *PPD1*, *VRN2*, *CO1*, and *CO2* were significantly different in the morning in mutant compared with wild-type [29,30]; Figs 2 and 5). We first confirmed that all of the *UBI*::*ELF3/elf3* transgenic lin elevated *ELF3* mRNA levels, and found indeed there is a significant increase of *ELF3* expression in the transgenic l To understand how *UBI*::*ELF3/elf3* affects flowering, we evaluated expression levels of *FT1*, *VRN1*, *PPD1*, *VRN2*, *C* wild type, *elf3*, and *UBI*::*ELF3/elf3*. Consistent with the delayed flowering, *FT1* and *VRN1* expression levels of *UBI*:: reduced relative to wildtype compared to elevated levels *elf3* relative to wildtype (Fig 6D and 6G). Also, the expressi *VRN2*, *CO1*, and *CO2* were decreased in *UBI*::*ELF3/elf3*, indicating ELF3 is playing a broad repressive role in regul domain containing genes responding to photoperiodic flowering.

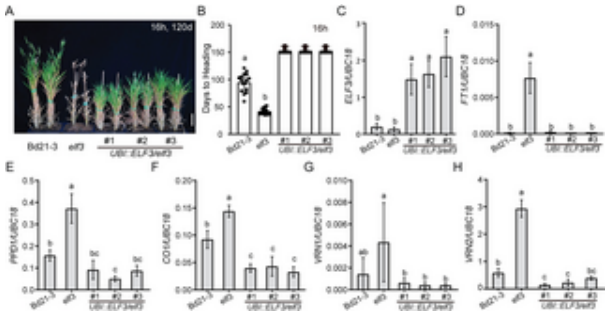

Fig 6. Overexpression of *ELF3* in the *elf3* mutant delays flowering.
(**A**) Representative image of Bd21-3 wild type, *elf3*, and three independent transgenic lines of *UBI*::*ELF3* in the *e* background grown in a 16h photoperiod. Images were taken 120d after germination. Bar = 5 cm. The fourth newl leaves were harvested at zt4 in 16h. (**B**-**I**), Normalized expression of *ELF3* (**C**), *FT1* (**D**),*PPD1* (**E**), *CO1* (**F**), *VRN* (**H**) in Bd21-3 wild type, *elf3*, and three *UBI*::*ELF3/elf3* transgenic lines. Expression of *CO2* is shown in S2 Fig. Ba the average of four biological replicates ± SD.
https://doi.org/10.1371/journal.pgen.1010706.g006

Discussion

The phytochromes *PHYC/PHYB* and *ELF3* connection

B. distachyon has an obligate requirement for LD to flower [8,49,67]. Previous studies have shown the important rol PHYC and ELF3 play in photoperiodic flowering in *B. distachyon* [29,30,58]. Specifically, mutations in *phyC* result in delayed flowering whereas loss-of-function mutations in *elf3* result in rapid flowering in either LD or SD [30,58]. Furt mutants resemble plants grown in SD both morphologically and at the transcriptomic level regardless of day-length mutants resemble plants grown in LD both morphologically and at the transcriptomic level regardless of day-length [Thus, we were interested in exploring the genetic relationships between *PHYC* and *ELF3*. The extreme delayed flow phenotype observed in *phyC* mutant plants is mediated by *ELF3* because *phyC/elf3* double mutants flower rapidly in similar to *elf3* mutants. Similar genetic interactions between *phyB* and *elf3* were also found in wheat in the compani suggesting these interactions are likely to be conserved broadly in temperate grasses. Loss-of-function mutations in also result in delayed flowering similar to *phyC* [59]. At present, no null *phyB* alleles have been reported in *B. distac* PHYB is able to heterodimerize with PHYC in *B. distachyon* and wheat [29, 57], and both *phyB* [59] and *phyC* [57] m extremely late flowering in wheat suggesting that both PHYs are likely required for photoperiodic flowering in the ten grasses, perhaps because PHYB/PHYC heterodimers are required for flowering regulation.

Phytochrome regulation of ELF3 at the post-translational level rather than at the transcriptional level is likely to be th interaction impacting flowering. In *A. thaliana*, *B. distachyon*, and wheat, *phyB/phyC* mutants do not impact the circa of *ELF3* mRNA levels [29,59,68]. However, in all three species PHYB and PHYC have been shown to interact with t protein, but the stability of the ELF3 protein upon exposure to light differs between *A. thaliana* and temperate grasse Specifically, in *A. thaliana*, PHYB contributes to the stability of the ELF3 protein during light exposure leading to ELF at the end of the day [63,68], whereas in rice ELF3 is degraded and or modified during light exposure in a PHY-med [40]. In temperate grasses, ELF3 protein accumulates during the night and is rapidly degraded or modified upon ligh [29,67], and this is likely to be a PHY mediated response as well.

The differences in how phytochromes impact the stability of the ELF3 protein might explain the contrasting flowering the *phyB/phyC* mutants between *A*. *thaliana* and temperate grasses. In *A*. *thaliana*, *phyB* mutants flower more rapid in either LD or SD and *phyC* mutants flower earlier under SD [60], whereas in temperate grasses *phyB* or *phyC* mut extremely delayed in flowering [57–59]. However, ELF3 acts as a flowering repressor in both *A*. *thaliana* and grasse *thaliana* PHYB stabilizes the ELF3 protein; therefore, in *phyB* mutants, ELF3 is no longer stable leading to rapid flow contrast, in temperate grasses and rice, in the absence of *phyB* or *phyC* the ELF3 protein is more stable leading to flowering.

Interestingly, overexpression of ELF3 results in extremely delayed flowering in *B*. *distachyon* [29,30] (Fig 6A and 6B regulation of ELF3 appears to occur at the protein level, one might not expect that overexpression would cause such flowering delay. However, if the ELF3 protein is expressed at a high level such that the degradation machinery is un much of the ELF3 protein during LD, then a strong flowering delay might occur. In support of this idea, the delayed f overexpression of ELF3 is mitigated when plants are grown under constant light versus 16h LD [29]. It is worth notin overexpression of ELF3 generally leads to delayed flowering in different plant species, there is considerable variatio magnitude of this delayed flowering [29,63,66] (Fig 6A and 6B).

Similar genetic interactions between *ELF3* and *PHYB* have also been observed in rice which is a SD-flowering plant rice-specific *ELF3* paralogs [70]. Mutations in either paralog results in delayed flowering in SD or LD in contrast to th flowering observed in temperate grasses containing *elf3* mutations [40,71,72]. Also in contrast to the situation in tem *phyB* mutants flower more rapidly than wild type in rice [73]. Despite the flowering differences of the *elf3* and *phyB* m between rice and temperate grasses, the flowering phenotype of *phy* mutants is ELF3 mediated because in both rice temperate grasses *elf3* is epistatic to *phyB or phyC* [Fig 1; 40, 67]. Moreover, PHYB and ELF3 proteins interact impa modification of ELF3 by light [40]. The opposite roles that phytochromes and *elf3* have on flowering in rice and temp is likely due, at least in part, to the reverse role that the downstream *PPD1/PRR37* gene has on flowering. *PPD1* is a flowering in LD temperate grasses but is a repressor of flowering in SD grasses such as rice [11,16,74,75] (Fig 3).

The *ELF3* and *PPD1* connection

The extremely delayed flowering of *B*. *distachyon ppd1* mutant plants under LD is similar to the extremely delayed h mutants in wheat [16]. However, a previous study in *B*. *distachyon* using a CRISPR induced *ppd1* mutant allele whic deletion in the sixth exon of *PPD1* has a milder delayed flowering phenotype with plants taking around 40 days to flo LD, whereas the mutant *ppd1* plants presented here flower around 120 days in 20h LD [29]; Figs 3 and 4). In both s type Bd21-3 plants flower on average between 25–30 days in 20h LD consistent with previous reports in *B*. *distachy* The differences in flowering time between the two *B*. *distachyon ppd1* mutant alleles suggests that the CRISPR indu is a weaker hypomorphic allele than the *ppd1* mutant allele characterized in this study. This is further supported by t *ppd1* allele described here has an extremely delayed flowering phenotype similar to the null *ppd1* wheat allele [16].

The *ppd1/elf3* double mutant is delayed in flowering relative to *elf3* mutant plants indicating that *PPD1* is downstrea photoperiodic flowering. This is also consistent with the elevated *PPD1* expression levels observed at dawn and dus mutant relative to wild-type in temperate grasses [30,70] (Fig 5). Indeed, ELF3 binds to the *PPD1/PRR37* promoter *distachyon*, wheat, and rice indicating ELF3 is a direct transcriptional repressor of *PPD1/PRR37* in grasses [29,40,6 not have any known DNA binding activity and thus, the direct repression is likely to be due to ELF3's interaction with transcription factor which, from studies in *A*. *thaliana*, recognizes GATWCG motifs that are also found in the *PPD1* p grasses [37,38,67]. Interestingly, photoperiod insensitivity in wheat is associated with deletions in the *PPD1* promote remove the LUX binding site and results in elevated *PPD1* expression at dawn similar to the *PPD1* expression dyna in *elf3* and *lux* mutant plants [10–13,39,77,78]. In the companion wheat paper, ChIP-PCR experiments show ELF3 e the DNA region around the LUX binding site in the *PPD1* promoter, which is present within the region deleted in pho insensitive wheats. These results demonstrate that removal of the evening complex binding site leads to elevated ex *PPD1* and accelerated heading under SD in many photoperiod-insensitive wheats [66].

The characterization of *elf3/ppd1* mutant plants under different photoperiods reveal complex interactions between th and their downstream targets depending on the environment. For example, *elf3/ppd1* mutant plants are earlier flowe mutant plants under 20h day-lengths, but are later flowering under 16h and 8h day-lengths. This is in contrast to *elf3* in wheat, which head earlier than *ppd1* under a 16h day-lengths indicating that *ELF3* can delay heading independen this condition [66]. Thus, there are differences between *B*. *distachyon* and wheat in the effects of *ELF3* on heading i of *PPD1*. We speculate that these differences may be related to the different interactions observed between *CO1* an flowering genes (e.g. PHY) in these two species. For example, in wheat *phyC* and *ppd1* mutants, *CO1* expression le elevated compared to wild type, whereas in *B*. *distachyon CO1* expression is reduced in both mutants [57,58,66].

Materials and methods

Plant materials and growth conditions

The rapid flowering mutant *elf3* and four *UBI*::*ELF3*/*elf3* transgenic lines in *B*. *distachyon* were previously characteri
the delayed flowering *phyC* mutant [58]. All the mutants used for phenotyping and expression in this study were bac
least twice with the wild-type Bd21-3 accession. Seeds were imbibed in the dark at 5°C for three days before plantin
photoperiods 8h-SD (8h light/16h dark), 16h-LD (16h light/ 8h dark), and 20h-LD (20h light/ 4h dark) were used. For
plants were grown at the University of California-Davis in growth chambers with metal halide and sodium bulbs as th
and a temperature of 22°C during light periods and 17°C during dark periods. Light intensity was approximately 300
plant height. For Figs 4 and 5, plants were grown at the University of Wisconsin-Madison in growth chambers with T
bulbs (5000 K), and light intensity was approximately 200umol m-2s-1 at plant height. Temperatures averaged 22°C
periods and 18°C during dark periods. Flowering time was estimated by measuring days to heading and leaves on t
time of heading. Recording of days to heading was done as the days from seed germination to the first emergence o

Generation of *elf3/phyC* and *elf3/ppd1* double-mutant lines

Epistasis analysis between *phyC*, *ppd1*, and *elf3* was studied by generating *elf3/phyC* and *elf3/ppd1* double mutants
were crossed with *elf3* and *elf3/phyC* homozygous double mutant plants were selected in a segregating F2 populati
genotyping using primers in S1 Table. Similarly, *ppd1* was crossed with *elf3*, and *elf3/ppd1* homozygous double mut
were selected by genotyping using primers in S1 Table in the segregating F2 population. Flowering time of *elf3/ppd*
were estimated by growing with Bd21-3, *elf3*, *ppd1* side by side in 8h SD, 16h LD, and 20h LD, and *elf3/phyC* doubl
were grown in 8h SD and 16h LD.

RNA extraction and qPCR

The method for RNA extraction, cDNA synthesis and quantitative PCR (qPCR) is described in [49]. Primers used fo
expression analyses are listed in S1 Table.

Statistical analyses

Comparison of more than two genotypes were performed by using *agricolae* package in R [79]. Statistically significa
among different genotypes were calculated by using one-way analysis of variance (ANOVA) followed by a Tukey's H
Student's t-test was used for analyzing the difference between two genotypes, significant if $P < 0.05$.

Supporting information

S1 Fig. Effect of loss of function mutations in *PHYC* on the transcriptional profile of *ELF3*.
https://doi.org/10.1371/journal.pgen.1010706.s001
(TIF)

S2 Fig. Normalized expression of *CO2* detected in Bd21-3, *elf3*, and three *UBI*::*ELF3/elf3* transgenic lines.
https://doi.org/10.1371/journal.pgen.1010706.s002
(TIF)

S1 Table. Primers used in this study.
https://doi.org/10.1371/journal.pgen.1010706.s003
(PDF)

S1 Data. S1_Data.xlsx file contains all raw data used in this study.
https://doi.org/10.1371/journal.pgen.1010706.s004
(XLSX)

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