PHYTOCHROME C is an essential light receptor for photoperiodic flowering in the temperate grass, *Brachypodium distachyon*

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ABSTRACT

We show that in the temperate grass, *Brachypodium distachyon*, *PHYTOCHROME C* (*PHYC*), is necessary for photoperiodic flowering. In loss-of-function *phyC* mutants, flowering is extremely delayed in inductive photoperiods. *PHYC* was identified as the causative locus by utilizing a mapping by sequencing pipeline (Cloudmap) optimized for identification of induced mutations in Brachypodium. In *phyC* mutants the expression of Brachypodium homologs of key flowering time genes in the photoperiod pathway such as *GIGANTEA* (GI), *PHOTOPERIOD 1* (*PPD1/PRR37*), *CONSTANS* (CO), and florigen/FT are greatly attenuated. *PHYC* also controls the day-length dependence of leaf size as the effect of day-length on leaf size is abolished in *phyC* mutants. The control of genes upstream of florigen production by *PHYC* was likely to have been a key feature of the evolution of a long-day flowering response in temperate pooid grasses.

INTRODUCTION

Flowering during a particular season is often critical for reproductive success, and thus when to initiate flowering is a critical developmental decision in the life cycle of many plant species (*e.g.*, Amasino, 2010). In many plants, initiation of flowering is driven by perception of changes in day length, a phenomenon known as photoperiodism (Garner and Allard, 1920). Plants adapted to temperate climates often perceive the lengthening days of spring as a
signal to initiate reproduction (e.g., Andres and Coupland, 2012); such plants are known as long-day plants.

Light signals such as light quality and length of day are perceived by several types of photoreceptors including phytochromes and cryptochromes (e.g., Lin, 2000). Phytochromes detect the levels and ratio of red (R) and far-red (FR) light in the environment (e.g., Quail, 2002). Upon absorption of red light, phytochromes photoconvert to an active form that initiates a signal transduction cascade leading to a range of developmental responses such photomorphogenesis (e.g., Quail, 2002).

Flowering plants typically contain three types of phytochromes referred to as PHYTOCHROME A (PHYA), PHYTOCHROME B (PHYB), and PHYTOCHROME C (PHYC) (Mathews, 2010). Certain groups of plants, such as diploid grasses, have only one copy of PHYA, PHYB, and PHYC, although lineage-specific duplications and losses of phytochrome genes have occurred in many groups of flowering plants (Mathews, 2010). Phytochrome mutants in the model plant systems Arabidopsis (Arabidopsis thaliana, Brassicaceae) and rice (Oryza sativa, Poaceae) have contributed to the elucidation of the functional roles of the various phytochromes. PHYA is required for seedling establishment as phyA mutants do not display de-etiolation responses and have elongated hypocotyls (Nagatani et al., 1993; Parks and Quail, 1993; Dehesh et al., 1993). However, loss of PHYA in both Arabidopsis and rice has no effect on flowering in inductive photoperiods, and only a modest effect in non-inductive conditions (Johnson et al., 1994; Takano et al., 2005; Monte et al., 2003). The most striking
A flowering phenotype is in garden pea (*Pisum sativum*, Fabaceae); loss-of-function *phyA* mutants exhibit a 20-day delay in flowering and a similar growth habit in long days (LD) and short days (SD), indicating that, in pea, *PHYA* is the major photoreceptor for the photoperiodic flowering response (Weller et al., 1997).

*PHYB* is involved in the shade-avoidance response as *phyB* mutants exhibit many of the hallmarks of the shade-avoidance response even when not grown in shade. Thus, the role of *PHYB* in the red-light activated form is to suppress the shade-avoidance response (Franklin et al., 2009; Franklin et al., 2005; Quail et al., 1998; Li et al., 2012). Accordingly, *phyB* mutants flower more rapidly than wild-type (WT) plants in a range of species in which the shade-avoidance response triggers flowering such as Arabidopsis, pea, rice and sorghum (Halliday et al., 1994; Monte et al., 2003; Takano et al., 2005; Weller et al., 2001; Childs et al., 1997).

Studies of *PHYC* have revealed minor roles in early seedling development and shade-avoidance responses in both Arabidopsis and rice (Franklin et al., 2003; Takano et al., 2005). In Arabidopsis, loss of *PHYC* function does not have any effect on flowering under inductive LD; however, under non-inductive SD, there is a slight acceleration of flowering (Monte et al., 2003). In rice, loss of *PHYC* results in a slight acceleration of flowering under inductive SD (Takano et al., 2005). Thus, in both rice (a short-day plant) and Arabidopsis (a long-day plant) genetic and physiological studies indicate that *PHYC* is a weak floral repressor in SDs. However, in Arabidopsis *PHYC* is not functional in the absence
of other phytochromes and thus any roles for PHYC require other phytochromes as well (Hu et al., 2013).

The modest effects of single phytochrome mutants on flowering in inductive photoperiods in Arabidopsis and rice is not due to redundancy because higher-order phytochrome mutants do not flower in a significantly different manner than single phytochrome mutants (Monte et al., 2003; Takano et al., 2005; Osugi et al., 2011).

Exposure to LD promotes flowering in Arabidopsis and most temperate grasses including Brachypodium (Ream et al., 2012). Genes encoding components of the photoperiod pathway, defined primarily from studies in Arabidopsis and rice, are typically present in all plant genomes examined including Brachypodium (Higgins et al, 2010). Variation in the LD promotion of flowering of temperate grasses such as wheat and barley appears to be due to allelic variation at *Photoperiod-H1 (PPD1)* and *FT* (Turner et al., 2005; Greenup et al., 2009; Beales et al., 2007; Yan et al., 2006). Light and the circadian clock regulates the timing of *PPD1* expression; *PPD1* in turn is required for the expression of the gene encoding the mobile floral signal FT in leaves, and FT travels from leaves to the meristem to activate floral homeotic genes (e.g., Song et al., 2010). Recently, differences in *FT* expression levels have been shown to correlate with flowering time in Brachypodium accessions (Ream et al., 2014).

In Arabidopsis, rice, and sorghum, the role of *PRR7/PRR37 (PPD1* homologs) in *FT* expression is mediated through the circadian timing of expression of *CONSTANS (CO*, named *Hd1* in rice), and also potentially by
directly affecting $FT$ activity (Song et al., 2010, Yang et al., 2014; Campoli et al., 2012; Faure et al., 2012; Yano et al., 2000; Murphy et al., 2011). Mutations or natural allelic variation in CO homologs that affect flowering have not been reported in temperate grasses; however, CO may have a role in grass flowering control because overexpression of CO in barley leads to up-regulation of $FT$ and more rapid flowering (Campoli et al., 2012), and CO from wheat is able to rescue an $hd1$ mutant in rice suggesting wheat CO is functionally conserved (Campoli et al., 2012; Nemoto et al., 2003).

Photoperiod-pathway gene expression is controlled, in part, by light signals that entrain the circadian clock. In Arabidopsis, the circadian clock is composed of several interconnected feedback loops including a core oscillator, a morning loop, and an evening loop; these clock components appear to be broadly conserved across flowering plants (e.g., McClung, 2011; Song et al., 2010). The core oscillator of the plant circadian clock involves an antagonistic relationship between the MYB-related transcription factors $CIRCADIAN CLOCK ASSOCIATED1$ (CCA1) and $LATE ELONGATED HYPOCOTYL$ (LHY) and the pseudo-response regulator (PRR) $TIMING OF CAB1$ (TOC1); CCA1 and LHY have expression peaks in the morning and repress the early evening-expressed $TOC1$ (Alabadi et al., 2002). The genes $LHY/CCA1$ in turn promote the expression of two $TOC1$ relatives $PRR7$ and $PRR9$, which feedback and repress $LHY/CCA1$, forming what is referred to as the “morning-feedback loop” (McClung, 2011). In addition to $LHY/CCA1$, the “morning loop” also involves evening-expressed genes $EARLY FLOWERING3$ (ELF3), $EARLY FLOWERING4$
(ELF4) and LUX ARRHYTHMO also known as PHYTOCLOCK1 (LUX/PCL1), which, in contrast with LHY/CCA1, are involved in the repression of PRR7 and PRR9 (McClung, 2011; Hazen et al., 2005; Onai and Ishiura, 2005). An additional input pathway involves GI, which is involved in the regulation of TOC1; TOC1 in turn represses GI expression during the evening (Locke et al., 2006; Martin-tyron et al., 2007; Ito et al., 2008). Through interaction with the Flavin-binding, Kelch repeat, F-box protein (FKF1), GI positively regulates the expression of flowering-time genes CO and FT (Park et al., 1999; Fowler et al., 1999; Sawa et al., 2007). Thus, GI has separate roles in both the circadian clock and photoperiodic flowering pathways (Martin-Tyron et al., 2007; Sawa et al., 2007; Kim et al., 2007). Lastly, GI from Brachypodium has a similar expression profile as Arabidopsis GI, interacts with the same proteins via a yeast two-hybrid assay, and is able to rescue the Arabidopsis gi mutant, indicating that it likely plays a similar functional role promoting flowering in Brachypodium (Hong et al., 2010).

Here we show the light receptor PHYC plays a major role in floral initiation in the temperate grass model Brachypodium distachyon as phyC mutants are extremely delayed in flowering. To streamline the identification of mutations in Brachypodium, we have adapted the bioinformatics-mapping pipeline, Cloudmap (Minevich et al., 2012; available on GALAXY), for use in identifying Brachypodium mutants. We show that PHYC is required for the transcriptional activation of several photoperiod-pathway genes including PPD1, CO, and FT, and that loss of PHYC alters the expression of certain circadian input and output
genes, revealing a difference in how light is perceived for the photo-induction of flowering between the well-studied plants Arabidopsis and rice compared to the temperate grass Brachypodium. Lastly we demonstrate that the photoperiod pathway is not required for the up-regulation of vernalization genes in the cold, and thus the phyC mutant provides a useful genetic background to explore the vernalization pathway without the compounding effects of the photoperiod pathway.

RESULTS

Isolation of extremely delayed flowering mutants in Brachypodium

To explore the molecular mechanisms controlling the initiation of flowering in temperate grasses, we screened for Brachypodium mutants that were delayed in flowering. We identified three independent mutants with an extreme delayed-flowering phenotype. These mutants remained vegetative for over a year in 20 hour day-lengths (LD), a highly inductive condition for Brachypodium flowering (Ream et al., 2014), and flowered only sporadically after a year of growth (Figure 1A-B). Upon flowering, the spikelets had a normal morphology and produced viable seed (data not shown).

We explored a range of different environmental treatments in an attempt to cause more rapid flowering in the mutants; treatments included long cold exposure (vernalization), shifts from short 8 hour day-lengths (SD) to LD, growth at higher temperatures, and growth in 24 hour day-lengths. However, none of
the treatments promoted flowering in the mutants (data not shown). Thus, it appeared the mutant lines had lesions in an essential flowering pathway.

In addition to the extreme delay of flowering, another, albeit more subtle, phenotype of this group of mutants was that in LD the mutants have shorter leaves and thus the plants are more compact than WT (Figure 2). In WT, leaf length is day-length dependent; leaves are shorter in non-inductive SD than in inductive LD. In the mutants, however, there is little difference in leaf length between LD and SD, and the leaf length of the mutant is similar to that of WT in SD. This is consistent with the mutants possessing a defect in day-length sensing. There is no difference in the rate of leaf initiation between mutant and WT in SD or LD photoperiods (data not shown).

**Extremely delayed flowering mutants map to **PHYTOCHROME C**

In all three mutants, plants heterozygous for the mutation were measurably delayed in flowering (Figure 1C). The clear heterozygous phenotype permitted use of heterozygotes for genetic studies. To create mapping populations, the heterozygous plants (which are in the Bd21-3 accession) were crossed to the polymorphic accession Bd3-1. Bd3-1 was chosen because it has flowering behavior identical to Bd21-3, and, most importantly, no flowering-time variation occurs in an F2 population derived from these two accessions indicating that no modifier loci of flowering behavior are present (Supplemental Figure 2A).

Using indel markers designed from the newly sequenced genomes of Bd21-3 and Bd3-1 (Gordon et al., 2014), the mutation in one line was initially
mapped to a 400kb interval on the top arm of chromosome 1 (Supplemental Figure 1). Further fine mapping using whole-genome sequence data from bulked DNA from 116 mutant plants from a segregating population was done using the Cloudmap pipeline (Minevich et al., 2012; Supplemental Figure 2A-B; also see methods) optimized for Brachypodium using the recent genome sequence of Bd21-3 and Bd3-1 (Supplemental Figure 2B; Gordon et al., 2014). The Cloudmap results overlap with our initial indel mapping data, and the only lesion present in all 116 mutants is a missense mutation (N-782-K) in the second PAS-fold domain of the light receptor PHYTOCHROME C; we refer to this allele as phyC-1 (Figure 1D; Supplemental Figure 2C). The delayed-flowering phenotype was rescued by introducing a genomic sequence of PHYC, including 1kb upstream sequence from the start site, into the mutant demonstrating that the phyC lesion is the causative mutation (Supplemental Figure 2E).

The two other extremely delayed-flowering mutants also had lesions in PHYC (Figure 1D). We refer to these two additional alleles as phyC-2, which contains a missense mutation (T-789-I) also within the second PAS-fold domain, and phyC-3, which contains a non-sense mutation (W-520-stop) within the PHY domain (Figure 1D). Further confirmation that the causative lesions are in PHYC is that the phyC mutations co-segregate perfectly with the delayed-flowering phenotype in segregating M3 populations. Specifically, all 35 delayed-flowering mutants in segregating M3 phyC-2 families and all 20 delayed-flowering mutants in segregating M3 phyC-3 families were homozygous for their respective EMS lesion, whereas all phenotypically wild-type plants either did not carry the EMS
lesion or were heterozygous for the wild-type and mutant alleles (Supplemental Figure 2D). The similar phenotypes of all three phyC alleles suggest that each lesion likely results in loss of PHYC function.

**PHYTOCHROME C is essential for the expression of photoperiod-pathway genes.**

To understand how PHYC affects flowering at a molecular level, we measured the mRNA levels of Brachypodium orthologs of the photoperiod-pathway genes FT, CO, and PPD1/PRR37 (as identified by Higgins et al., 2010) and FT2 (which is the closest FT paralog). Brachypodium accessions have an obligate requirement for LD (Ream et al., 2014), and LD conditions were chosen for this analysis because growth in LD provides the greatest difference in flowering time between phyC and WT. In WT, the expression levels of FT, FT2, CO and PPD1/PRR37 all peak around the middle of the photoperiod (zt12-zt15, zt=zeitgeber), consistent with oscillation patterns in other cereals (Figure 3; Song et al., 2010). In the phyC-1 background, however, expression of these genes are barely detectable throughout a 24-hour circadian cycle (Figure 3). These results demonstrate that PHYC is required for the expression of CO, PPD1/PRR37, FT and the FT paralog FT2.

To determine if increased FT expression alone can compensate for the flowering delay in phyC-1, we overexpressed FT in the phyC-1 background using a UBI:FT construct (described in Ream et al., 2014). Transformation of plants heterozygous for the phyC-1 allele enabled us to control for the effects of
particular transgene positions because self-pollination of the transformed phyC-1 heterozygote produced both phyC-1 mutants and PHYC WT plants with the same transgene. All plants carrying the UBI:FT (5 independently transformed lines) flowered rapidly within 20 days with no difference observed in flowering between homozygous phyC-1 plants and WT (data not shown). This demonstrates that FT, as expected, is downstream of PHYC in the flowering pathway, and that FT expression alone is sufficient to rescue the delayed flowering phenotype of a phyC lesion.

**PHYC affects the expression of circadian clock components**

To determine whether or not PHYC affects expression of circadian clock components, we analyzed the expression of Brachypodium orthologs, identified in Higgins et al. (2010), of core oscillators CCA1 and TOC1, the evening-loop genes GI, LUX, ELF3 and ZTL, and clock-output genes FKF and **CHLOROPHYLL A-B BINDING PROTEIN (CAB2)** in a diurnal light cycle (20 h light and 4 h dark) and in free-run (LL) conditions (in plants that had been entrained to 12 h light and 12 h dark cycles) (Figure 4). In our conditions, all of the genes in WT behaved as expected based on a previous report in Brachypodium (Filichkin et al., 2011).

In the phyC mutant, the mRNA levels of core oscillators CCA1 and TOC1 oscillate relatively “normally” in LL (i.e., similar to WT) although peak expression is somewhat lower than in WT (Figure 4A-B). In the LD conditions, the
differences in CCA1 and TOC1 expression between phyC and WT are more pronounced (Figure 4A-B).

For the evening-loop genes GI and LUX, loss of PHYC results in dampened expression (Figure 4C-D). In phyC, LUX oscillates; however, LUX amplitude is dampened in both the LL and LD conditions. The effect of loss of PHYC is even more severe for GI; in phyC, GI mRNA is barely detectable, and in LD does not oscillate like WT. Like GI, the GI-interacting protein FKF had peak expression levels around the middle of the light period in WT; however, in phyC peak expression of FKF was lower than WT and the peak of expression was shifted toward dusk (Figure 4E). In contrast with LUX and GI, we observed no expression differences in the circadian clock components ELF3 or ZTL between WT and phyC in both the LL and LD conditions (data not shown).

We also examined the expression of CHOROPHYLL A/B BINDING PROTEIN (CAB2) a clock-output gene involved in photosynthesis. In the phyC background in both LL and LD, CAB2 mRNA levels were higher than WT (for instance note the nearly 50-fold higher level at zt20 in LD) (Figure 4F). Thus, PHYC is a negative regulator of CAB2.

The circadian clock clearly plays a critical role in the regulation of flowering in plants (e.g., Imaizumi et al., 2010). However, to what extent the observed alterations of the mRNA levels of clock components contribute to the delayed flowering phyC phenotype remains to be determined.
**PHYC is not required for up-regulation of VRN1 and VRN2L in the cold**

To determine whether or not the lesion in *PHYC* and the resulting disruption of the photoperiod pathway affects expression of the vernalization genes *VERNALIZATION 1* (*VRN1*) and *VERNALIZATION 2-like* (*VRN2L*) (*VRN2L* is the closest homolog of wheat *VRN2* (Yan et al., 2004) in Brachypodium (Ream et al., 2012)), expression levels of *VRN1* and *VRN2L* and, as a control, *FT* were measured before, during and 11 days after cold treatment. Consistent with previous findings (Ream et al., 2014), in WT the expression of *VRN1* and *VRN2L* levels is detectable without cold in LD, both genes are up-regulated during the cold, and *VRN1* levels are maintained post cold whereas *VRN2L* levels drop back down to pre-cold levels, 11 days post cold treatment (Figure 5). In the *phyC-1* background, *VRN1* and *VRN2L* are barely detectable prior to cold exposure (Figure 5A,C). *VRN2L* mRNA levels can fluctuate over a 24-hour cycle in barley (Trevaskis et al., 2006), and thus the lower *VRN2L* levels in *phyC* could result from a shift of peak expression. However, the lower *VRN2L* expression in *phyC* is maintained throughout a 24-hour cycle (Supplemental Figure 3). Despite lower expression in *phyC* prior to cold exposure, during cold treatment both genes are up-regulated in *phyC* similar to that in WT (Figure 5A,C). Post cold, *VRN2L* mRNA levels return to pre-cold levels in both WT and *phyC* (Figure 5B,D). However, the post-cold elevation of *VRN1* expression is not maintained in *phyC* (Figure 5B,D), presumably because *FT* expression, which is lacking in *phyC* (Figure 5E,F), is required for the post-cold enhancement of
VRN1 expression (Distelfeld and Dubcovsky, 2010; Woods et al., 2014; Ream et al., 2014).

**DISCUSSION**

**PHYC plays a major role in photoperiodic flowering in Pooids**

The grass family (Poaceae) originated approximately 70 million years ago as forest understory in tropical conditions, and most tropical grasses require SD conditions or are day neutral with respect to flowering (Kellogg, 2001). However, Pooideae, one of the most species-rich grass subfamilies, radiated and diversified into temperate ecosystems at higher latitudes, and part of the adaptation to higher latitudes was the evolution of a LD requirement for flowering (Stromberg 2005; Sandve et al., 2008). Brachypodium is a temperate grass within the subfamily Pooideae, sister to the crown pooids, which comprise economically important cereals such as wheat (*Triticum aestivum*), barley (*Hordeum vulgare*), oats (*Avena sativa*) and rye (*Secale cereale*). Brachypodium and all crown pooids studied to date require LD to flower. Our studies in Brachypodium demonstrate an essential role for PHYC in photoperiodic flowering (Figure 1,3). While our paper on the role of PHYC in Brachypodium was undergoing revision, it was reported that *phyC* mutations also result in delayed flowering in wheat supporting PHYC’s role regulating photoperiodic flowering broadly within Pooideae (Chen et al., 2014). Thus, the acquisition by PHYC of a major role in flowering is likely to have been part of the evolutionary pathway to a LD requirement for flowering in the Pooideae. The importance of PHYC to LD flowering may be grass or monocot specific as *phyC* mutations do not have a
pronounced effect on flowering in the LD plant model Arabidopsis (Monte et al., 2003).

In our screens to date all three extremely delayed flowering mutants result from lesions in PHYC. Phytochromes A, B, and C are present as single copy genes in Brachypodium. Thus, if another phytochrome was an essential partner with PHYC in photoperiod sensing, one would expect to find extremely delayed flowering mutants in the partner as well.

The phyC mutant alleles characterized in this study result in a severely delayed flowering phenotype similar to the maintained vegetative phase (mvp) mutant in diploid wheat (Triticum monococcum) (Shitsukawa et al., 2007). The mvp mutation is an ion-beam induced deletion of several linked genes including the wheat orthologs of the floral homeotic gene APETALA/FRUITFUL (called VERNALIZATION1 (VRN1) in grasses) and PHYC (Distelfeld and Dubcovsky, 2010). In tetraploid wheat (cultivar Kronos), loss of PHYC activity results in a lack of FT expression and a substantial delay of flowering, but the delay of flowering is not as strong as that exhibited by the mvp mutant (Chen et al., 2014). As noted by Chen et al. (2014), the difference between the mvp phenotype in diploid wheat and the loss of PHYC phenotype in tetraploid wheat may be due to the additional genes deleted in the mvp mutant. That three independent Brachypodium phyC mutants all have a severe delayed flowering phenotype indicates that in a close relative of the crown pooids loss of PHYC alone can create an mvp-like phenotype.
PHYC allelic variation has been associated with flowering time variation in Arabidopsis, barley, and pearl millet (*Pennisetum glaucum*) (Balasubramanian et al., 2006; Nishida et al., 2013; Pankin et al., 2014 in press; Saidou et al., 2014). For example, pearl millet grown in Niger over the course of three decades has been selected for a shorter life cycle and more rapid flowering to maintain yield in regions where the growing season is becoming shorter due to changes in precipitation patterns (Vigouroux et al., 2011). This shift to earlier flowering is associated with a specific allele at the PHYC locus, which has increased in frequency over the past three decades (Vigouroux et al., 2011). It will be interesting to determine whether or not PHYC contributes to the wide range of variation for flowering time that has been documented in accessions of Brachypodium (Ream et al., 2014). Whole-genome sequence data from many accessions is currently being generated and will enable a future study of association between possible allelic variation at PHYC with flowering (http://genome.jgi.doe.gov/genome-projects/).

**Flowering “circuitry” in grasses**

Our work shows that in Brachypodium PHYC is required for proper expression of *PPD1/PRR37, CO*, two *FT* homologs, and *GI* (Figures 3 and 4) which are all genes for which the modulation of expression is critical for the initiation of flowering in a range of plants (e.g., Song et al., 2010). We also find that loss of *PHYC* results in perturbations in the expression patterns of other circadian clock components and clock output genes (Figure 4).
That there is the same effect on expression of \textit{PPD1/PRR37}, \textit{CO}, and \textit{FT} in the \textit{phyC} mutant is not surprising because it has been shown, for example, in rice and sorghum that PRR37 regulates \textit{CO} expression, and \textit{CO} regulates \textit{FT} expression (Koo et al., 2013; Yang et al., 2014). Our work shows that, unlike the case in rice and sorghum, in Brachypodium, \textit{PHYC} is a major photoreceptor at the top of this cascade. It has recently been shown that in wheat and barley, which are closely related to Brachypodium, \textit{PHYC} is also at the top of this cascade (Chen et al., 2014 and Pankin et al., 2014 in press).

Lack of \textit{FT} expression in \textit{phyC} mutants (Figure 3) is likely to account for the severe delay in flowering. Although there are several \textit{FT} homologs in Brachypodium, a recent study indicates that one of them, which is phylogenetically most closely related to Arabidopsis \textit{FT}, is the primary promoter of flowering as knockdown of its expression results in a severe delay of flowering (Lv et al., 2014). We have shown that this \textit{FT} homolog is not expressed in \textit{phyC} mutants and that overexpression of this \textit{FT} is able to rescue the delayed flowering phenotype of \textit{phyC}. The closely related \textit{FT} gene, \textit{FT2}, can also promote flowering in Brachypodium (Wu et al., 2013) and is also undetectable in the \textit{phyc} background; thus, loss of \textit{FT2} expression might also contribute to the delayed flowering phenotype of the \textit{phyC} mutant.

As noted above, \textit{PHYC}-mediated activation of \textit{PPD1/PRR37} is the most “upstream” component so far identified in the cascade that leads to \textit{FT} induction and flowering in Brachypodium. The different photoperiodic flowering behavior of SD-flowering grasses such as rice and sorghum and LD-flowering temperate
grasses such as wheat, barley, and Brachypodium is likely due to opposite roles of *PPD1/PRR37* in the regulation of *FT/Hd3a* in LD. In wheat and barley, *PPD1* promotes flowering in LD as *ppd1* mutants are delayed in flowering and exhibit nearly undetectable *FT* expression (Turner et al., 2005; Shaw et al., 2013). However, in rice and sorghum the *PPD1* ortholog (referred to as *PRR37*) represses flowering in LD as *prr37* plants are rapid flowering in LD and exhibit higher *FT* expression (Koo et al., 2013; Yang et al., 2014; Murphy et al., 2011). Because loss of *PHYC* promotes flowering in non-inductive photoperiods in rice (Takano et al., 2005), *PHYC* may also be required for the expression of *PRR37* in rice. Some of the difference in flowering between Brachypodium and rice *phyC* mutants may also be due to the presence of *Early heading date 1* (*Ehd1*) which promotes flowering in rice by activating *FT* via a separate pathway from *CO* (Doi et al., 2004); however, this gene has been lost in Brachypodium (Higgins et al., 2010).

**phyC mutants enable other flowering pathways to be studied in Brachypodium independent of photoperiod effects**

Many temperate grasses require prolonged exposure to cold prior to exposure to inductive LD in order to flower; the process by which cold exposure enables flowering is known as vernalization. The vernalization response in barley and wheat is mediated by a feedback loop formed by *VRN1*, *VRN2*, and *FT* (Distelfeld and Dubcovsky, 2010). *VRN2* expression inhibits flowering because *VRN2* inhibits *FT* induction (Yan et al., 2004). During the cold *VRN1* is
up-regulated, and VRN1 represses VRN2 expression to allow LD induction of FT (Yan et al., 2003). FT enhances VRN1 expression, “locking in” VRN2 repression and the promotion of flowering (Yan et al., 2006; Shimada et al., 2009). The proposed feedback model resulting from studies in wheat and barley appears to be largely conserved in Brachypodium (Ream et al., 2014; Lv et al., 2014) except that the role of VRN2L is less clear as it is up-regulated in the cold (Ream et al., 2014).

In Brachypodium, there is an obligate requirement for inductive photoperiods in order to flower, and this obligate requirement is not affected by vernalization. Thus, Brachypodium plants will not flower in non-inductive SD even if first extensively vernalized (Ream et al., 2014). Day-length can however, affect the expression of VRN2L and FT; thus, in some environmental conditions and/or genetic backgrounds day-length can affect the vernalization requirement (Ream et al., 2012, 2014; Colton-Gagnon et al., 2013). That the photoperiodic flowering pathway is not operative in phyC mutants enabled us to study the role of the photoperiod pathway in the regulation of VRN1 and VRN2L during and after cold exposure by comparing WT and the phyC mutant in identical conditions. VRN1 and VRN2L are up-regulated during the cold to the same level as WT in phyC indicating that transcriptional activation of VRN1 and VRN2L is photoperiod pathway independent (Figure 5). The inability of phyC mutants to maintain elevated VRN1 expression and induce FT post-cold is consistent with previous findings from Brachypodium as well as other temperate grasses indicating that FT enhances VRN1 expression post-cold (Woods et al., 2014; Ream et al., 2014;
Shimada et al., 2009; Sasani et al., 2009). Additionally, the up-regulation of VRN2L during cold is photoperiod independent; however, the photoperiod pathway is required for VRN2L expression pre- and post-cold as VRN2L was barely detectable before and after cold (Figure 5C-D). In general, because loss of PHYC abolishes photoperiod sensing, the phyC mutant background could be useful to explore other developmental or biochemical pathways independent of any complicating effect of growing plants in different photoperiods.

MATERIALS AND METHODS

Mutant Screen
The inbred strain Bd21-3 was mutagenized using ethyl methanesulfonate (EMS) as adapted from Caldwell et al., 2004 (Sigma M0880-5G, 9.7M). A range of EMS concentrations were initially evaluated ranging from 0.2%, 0.3%, 0.4%, 0.5%, 0.6%, 0.7%, and 0.8% EMS on ~100 seeds in 12mL water to achieve an acceptable balance of mutant load with germination and fecundity rates. We observed equal germination rates across the EMS concentrations and water only control tested. However, at >0.6% EMS the seedling vigor and fecundity plummeted whereas <0.2% EMS we did not observe many albinos in M2 plants. Based on these results we chose, we mutagenized approximately 4000 seed at 0.6% EMS and approximately 12,000 seed at 0.4% EMS. After EMS treatment, plants were vernalized at 5°C for four weeks on moist paper towels. Vernalization for a minimum of three weeks is required for rapid flowering of Bd21-3 when
grown under 16 h of light (Ream et al., 2012). After vernalization, mutagenized seedlings were grown under 16 h of light in a greenhouse with supplemented high pressure sodium lighting. Plants flowered uniformly and M2 seeds were harvested after plants had senesced. M2 seeds were kept at ~20°C for a minimum of 1 month to break dormancy (Barrero et al., 2012). To screen for delayed flowering mutants under 16 h of light we first vernalized imbibed M2 seed at 5°C in soil for four weeks before outgrowth at ~21°C in the greenhouse condition as described above. *phyC-1* was backcrossed three times with Bd21-3 to reduce background mutations (was able to backcross by using heterozygous plants) and when possible this seed was used in all subsequent experiments.

**Identification of the *phyC-1* candidate gene using mapping by sequencing**

**Cloudmap software and segregation analysis**

Segregating mutants in an F2 mapping population were pooled and DNA extracted using a modified CTAB protocol (Lodhi et al., 1994) with the addition of RNAse and Proteinase K steps. DNA was sequenced at the UW-Madison Biotechnology sequencing facility using an illumina HiSeq 2500. The sequencing facility generated the sequencing library following established illumina protocols. Reads were mapped against the Bd21-3 sequence available at brachypodium.org (Gordon et al., 2014). We optimized the Cloudmap bioinformatics pipeline (Minevich et al., 2012) for mapping EMS induced mutations in Brachypodium (Bd21-3) publically available on Galaxy (Goecks et al., 2010; Blankenberg et al., 2010; Giardine et al., 2005). Mapping and variant
calling (Supplemental Figure 2) was performed using the CloudMap pipeline with the Hawaiian Variant Mapping workflow as previously described (Minevich, et al. 2012). As part of the pipeline, BWA was used for mapping the 100bp Illumina reads (Li et al., 2010), PICARD was used for removing duplicate reads (http://picard.sourceforge.net), GATK was used for variant calling and variant subtraction (DePristo et al., 2011), and SnpEff was used for variant annotation (Cingolani et al., 2012).

**Plant Growth and Flowering Time Measurements**

Growth and scoring of plants were done as described in Ream et al. 2014. Growth chamber temperatures averaged 21°C during the light period and 18°C during dark period.

**qRT-PCR**

RNA extraction and expression analysis was done as detailed in Ream et al., 2014. Primer pairs used to amplify each gene are listed in Supplemental Table S2.

**Generation of transgenic phyC-1 lines**

A genomic PHYC fragment including 1kb upstream from the start site was amplified from Bd21-3 DNA using the primer pairs listed in Supplemental Table S2. DNA of the correct size was gel extracted (Qiagen) and cloned into pENTR-D-TOPO (Life Technologies) using the manufacturer’s protocol. Clones were
verified by sequencing. pENTR clones containing the PHYC genomic fragment were recombined into pIPKB001 (Himmelbach et al., 2007) using Life Technologies LR Clonase II following the manufacturer’s protocol. Clones were verified by sequencing in pIPKB001 and then transformed into chemically competent Agrobacterium tumefaciens strain Agl-1. Plant callus transformation of heterozygous phyc-1 was performed as described (Vogel and Hill, 2008) by the Great Lakes Bioenergy Research Center Brachypodium Transformation Facility. Independent transgenic lines were genotyped for the transgene using gene-specific forward and pIPKB001 reverse primer (Supplemental Table S2). The UBI:FT construct described in Ream et al., 2014 was transformed into heterozygous phyC-1 plants following the above protocols and the putative transgenic plants were genotyped as described in Ream et al., 2014.

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25
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Figure Legends:

Figure 1. Phenotype of phyC mutants

(A) Wild-type Bd21-3 (WT) and phyC-1 grown in a 20-hour photoperiod. Both plants germinated on the same day and a picture was taken of representative plants after 200 days. WT flowered on average around 30 days and with 6-7 leaves whereas phyC-1 did not flower after a year of growth producing more than 50 leaves on the parent shoot. Scale bar= 5cm. (B) WT and the three mutant alleles of phyC grown in a 16-hour photoperiod after 6 weeks of vernalization. WT flowered on average after 20 days of growth and at 4-5 leaves whereas none of the phyC mutants flowered after a year of growth (12 plants per line, repeated with similar results). Picture taken after 50 days. Scale bar= 5cm. (C) WT (+/+), heterozygous (+/-) and homozygous (-/-) plants grown in a 20 hour photoperiod for 90 days. WT flowered on average with 6-7 leaves, heterozygotes flowered between 6-15 leaves, and phyC mutants did not flower for a year of growth. Scale bar= 5cm. (D) Gene structure of PHYC showing the location, nucleotide changes, and corresponding amino-acid changes of the EMS-induced mutations of the three mutant alleles, phyC-1, phyC-2, and phyC-3. Domain structure of phytochrome includes, PAS2 and PAS (Per (period circadian protein) Arn (Ah receptor nuclear translocater protein) and Sim (single-minded protein) domain); GAF, cGMP-stimulaed phosphodiesterase, Anabena adenylate cyclases, and Escherichia coli FhlA domain; PHY, phytochrome domain; HA, His kinase A (phosphoacceptor) domain; HD, Histidine kinase, DNA gyrase B and HSP90-like ATPase domain.
Figure 2 Leaf length of WT and phyC-1 grown in long and short days

Leaf length (cm) of Bd21-3 (WT) and phyC-1 grown in long days (20 hour light/4 hours dark, A & C) and short days (8 hour light/16 hour dark, B & D). Picture taken at 4.5-5 leaf stage for both long-day and short-day grown plants. Values represent the average of 6 plants. The phyC-1 used in this study had been backcrossed to Bd21-3 three times to reduce background mutations. Scale bar=5cm

Figure 3. Expression patterns of photoperiod-pathway genes

Gene expression patterns of (A) FT, (B) FT2, (C) CO, and (D) PPD1 in Bd21-3 (black lines) and phyC-1 (grey lines). Plants were grown in LD (20 hour days) until the 4th leaf stage was reached (Zadoks=14) at which point the newly expanded 4th leaf was harvested at zt0, zt6, zt9, zt12, zt15 and zt20. The average of three biological replicates are shown +/- standard deviation (three leaves per replicate). This experiment has been repeated with similar results. Expression is normalized to UBC18 as done in Ream et al., (2014).

Figure 4. Expression patterns of circadian clock and clock-output genes.

Gene expression patterns of circadian clock genes (A) CCA1, (B) TOC1, (C) LUX, (D) GI, and clock-output genes (E) FKF, (F) CAB2 in Bd21-3 (wild-type=WT, black lines) and phyC-1 (grey lines). Plants were grown in LD (20 hour days with average day temperature of 21°C and night temperature of 18°C, left column) until the 4th leaf stage was reached at which point the newly expanded leaf was harvested at several time points throughout a 24-hour circadian cycle. Additionally plants were entrained under 12 hour days under constant
temperatures of 21°C until the 3rd leaf stage was reached at which point the plants were grown in continuous light (LL, right column) and the newly emerged 3rd leaf was sampled every 4 hours for 48 hours. Shaded boxes represent subjective night. Values represent the average of three biological replicates +/- standard deviation (three leaves per replicate). The experiment was repeated with technical replicates and similar results were obtained. Expression is normalized to UBC18 as done in Ream et al., (2014).

Figure 5. VRN1, VRN2L, and FT gene expression in leaves of vernalized plants during and after cold treatment.

Seedlings were grown to the 3rd leaf stage in LD (20-hour photoperiod) before transfer to 5°C for 3 weeks also in LD. After cold treatment, plants were grown in LD. The third leaf was harvested at the end of vernalization (V) when the plants were at the 4th leaf stage (A,C,E). Non-vernalyzed (NV) controls were harvested at a similar developmental stage as the V samples (A,C,E). After vernalization, plants were grown for 11 days (V+11) and the 3rd leaf was harvested when the plants were at the 6th leaf stage along with the 3rd leaf from non-vernalyzed controls at the same developmental stage (NV+11). Gene expression is shown relative to 21-3 NV samples (A-F). The phyC-1 used in this study had been backcrossed three times. Bars represent the average of 4 biological replicates +/- standard deviation (three leaves per replicate). The experiment was repeated with similar results. qRT-PCR primers were optimized in Ream et al., (2014).
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5