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.

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 as 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 (Dehesh *et al.* 1993; Nagatani *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 noninductive conditions (Johnson *et al.* 1994; Monte 2003; Takano *et al.* 2005). The most striking *phyA* 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 (LDs) and short days (SDs), indicating that, in pea,

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doi: 10.1534/genetics.114.166785

Manuscript received June 2, 2014; accepted for publication July 3, 2014; published Early Online July 14, 2014.

Supporting information is available online at <http://www.genetics.org/lookup/suppl/doi:10.1534/genetics.114.166785/-DC1>.

Data availability: Illumina data in NCBI sequence read archive SRX557413.

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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 and Whitelam 2005; Franklin and Quail 2009; 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; Childs *et al.* 1997; Weller *et al.* 2001; Monte 2003; Takano *et al.* 2005).

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 LDs; however, under noninductive SDs, there is a slight acceleration of flowering (Monte 2003). In rice, loss of *PHYC* results in a slight acceleration of flowering under inductive SDs (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 are not due to redundancy because higher-order phytochrome mutants do not flower in a significantly different manner than single phytochrome mutants (Monte 2003; Takano *et al.* 2005; Osugi *et al.* 2011).

Exposure to LDs 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; Yan *et al.* 2006; Beales *et al.* 2007; Greenup *et al.* 2009). 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 (Yano *et al.* 2000; Song *et al.* 2010; Murphy *et al.* 2011; Campoli *et al.* 2012; Faure *et al.* 2012; Yang *et al.* 2014).

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 (Nemoto *et al.* 2003; Campoli *et al.* 2012).

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., Song *et al.* 2010; McClung 2011). 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 feed back 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* (Hazen *et al.* 2005; Onai and Ishiura 2005; McClung 2011). 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-Tryon *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* (Fowler *et al.* 1999; Park *et al.* 1999; Sawa *et al.* 2007). Thus, *GI* has separate roles in both the circadian clock and photoperiodic flowering pathways (Kim *et al.* 2007; Martin-Tryon *et al.* 2007; Sawa *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.* 2009).

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, Cloud-map (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

photoinduction 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.

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, 9.7 M). 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 12 ml 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 at <0.2% EMS, we did not observe many albinos in M2 plants. Based on these results we mutagenized ~4000 seeds at 0.6% EMS and ~12,000 seeds at 0.4% EMS. After EMS treatment, plants were vernalized at 5° for 4 weeks on moist paper towels. Vernalization for a minimum of 3 weeks is required for rapid flowering of Bd21-3 when grown under 16 hr of light (Ream *et al.* 2012). After vernalization, mutagenized seedlings were grown under 16 hr 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° for a minimum of 1 month to break dormancy (Barrero *et al.* 2012). To screen for delayed flowering mutants under 16 hr of light, we first vernalized imbibed M2 seeds at 5° in soil for 4 weeks before outgrowth at ~21° 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 University of Wisconsin 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) publicly available on Galaxy (Blankenberg *et al.*

2010; Giardine *et al.* 2010; Goecks *et al.* 2010). Mapping and variant calling (Supporting Information, Figure S2) were performed using the CloudMap pipeline with the Hawaiian Variant Mapping workflow as previously described (Minevich *et al.* 2012). As part of the pipeline, Burrows-Wheeler Aligner was used for mapping the 100-bp Illumina reads (Li and Durbin 2010), PICARD was used for removing duplicate reads (<http://picard.sourceforge.net>), Genome Analysis ToolKit 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° during the light period and 18° during the 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 Table S2.

Generation of transgenic *phyC-1* lines

A genomic *PHYC* fragment including 1 kb upstream from the start site was amplified from Bd21-3 DNA using the primer pairs listed in 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 (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).

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 >1 year in 20-hr day lengths (LDs), a highly inductive condition for *Brachypodium* flowering (Ream *et al.* 2014), and flowered only sporadically after a year of growth (Figure 1, A and 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-hr day lengths (SDs) to LDs, growth at higher temperatures, and growth in 24-hr 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 LDs, 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 SDs than in inductive LDs. In the mutants, however, there is little difference in leaf length between LDs and SDs, and the leaf length of the mutant is similar to that of WT in SDs. 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 (Figure S2A).

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 400-kb interval on the top arm of chromosome 1 (Figure S1). 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; Figure S2, A and B; also see *Materials and Methods*) optimized for *Brachypodium* using the recent genome sequence of Bd21-3 and Bd3-1 (Figure S2B; 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; Figure S2C). The delayed-flowering phenotype was rescued by introducing a genomic sequence of *PHYC*, including a 1-kb upstream sequence from the start site into the mutant, demonstrating that the *phyC* lesion is the causative mutation (Figure S2E).

The two other extremely delayed-flowering mutants also had lesions in *PHYC* (Figure 1D). We refer to these two

Figure 1 Phenotype of *phyC* mutants. (A) Wild-type Bd21-3 (WT) and *phyC-1* grown in a 20-hr photoperiod. Both plants germinated on the same day and a picture was taken of representative plants after 200 days. WT flowered on average after ~30 days and with 6–7 leaves, whereas *phyC-1* did not flower after a year of growth, producing >50 leaves on the parent shoot. Bar, 5 cm. (B) WT and the three mutant alleles of *phyC* grown in a 16-hr 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. Bar, 5 cm. (C) WT (+/+), heterozygous (+/-), and homozygous (-/-) plants grown in a 20-hr photoperiod for 90 days. WT flowered on average with 6–7 leaves, heterozygotes flowered between 6–15 leaves, and *phyC* mutants did not flower after a year of growth. Bar, 5 cm. (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 translocator protein) and Sim (single-minded protein) domain]; GAF, cGMP-stimulated 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.

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 nonsense mutation (W-520-stop) within the PHY domain (Figure 1D). Further confirmation that the causative lesions are in *PHYC* is that the *phyC* mutations cosegregate 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 (Figure S2D). The similar phenotypes of all three *phyC* alleles suggest that each lesion likely results in loss of *PHYC* function.

Figure 2 Leaf length of WT and *phyC-1* grown in long and short days. Leaf length (in centimeters) of Bd21-3 (WT) and *phyC-1* grown in long days (20 hr light/4 hr dark, A and C) and short days (8 hr light/16 hr dark, B and D). Picture taken at 4.5- to 5-leaf stage for both long-day and short-day grown plants. Values represent the average of six plants. The *phyC-1* used in this study had been backcrossed to Bd21-3 three times to reduce background mutations. Bar, 5 cm.

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 LDs 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 (zeitgeber, zt12–15), consistent with oscillation patterns in other cereals (Figure 3; Song *et al.* 2010). In the *phyC-1* background, however, expression of these genes is barely detectable throughout a 24-hr 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* (five 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.

Figure 3 Expression patterns of photoperiod-pathway genes. Gene expression patterns of (A) *FT*, (B) *FT2*, (C) *CO*, and (D) *PPD1* in Bd21-3 (solid lines) and *phyC-1* (shaded lines). Plants were grown in LDs (20-hr days) until the fourth-leaf stage was reached (Zadoks = 14) at which point the newly expanded fourth leaf was harvested at zt0, zt6, zt9, zt12, zt15, and zt20. The average of three biological replicates are shown $\pm$ 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).

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 hr light and 4 hr dark) and in free-run (LL) conditions (in plants that had been entrained to 12-hr light and 12-hr 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 4, A and B). In the LD conditions, the differences in *CCA1* and *TOC1* expression between *phyC* and WT are more pronounced (Figure 4, A and B).

For the evening-loop genes *GI* and *LUX*, loss of *PHYC* results in dampened expression (Figure 4, C and 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 LDs 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 *CHLOROPHYLL 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 LDs) (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 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 LDs, both genes are up-regulated during the cold, and *VRN1* levels are maintained postcold, whereas *VRN2L* levels drop back down to precold levels, 11 days postcold treatment (Figure 5). In the *phyC-1* background, *VRN1* and *VRN2L* are barely detectable prior to cold exposure (Figure 5, A and C). *VRN2L*

mRNA levels can fluctuate over a 24-hr 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-hr cycle (Figure S3). 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 5, A and C). Postcold, *VRN2L* mRNA levels return to precold levels in both WT and *phyC* (Figure 5, B and D). However, the postcold elevation of *VRN1* expression is not maintained in *phyC* (Figure 5, B and D), presumably because *FT* expression, which is lacking in *phyC* (Figure 5, E and F), is required for the postcold enhancement of *VRN1* expression (Distelfeld and Dubcovsky 2010; Ream *et al.* 2014; Woods *et al.* 2014).

Discussion

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

The grass family (Poaceae) originated ~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 LDs to flower. Our studies in *Brachypodium* demonstrate an essential role for *PHYC* in photoperiodic flowering (Figure 1 and Figure 3). While our article 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 2003).

In our screens to date all three extremely delayed flowering mutants result from lesions in *PHYC*. Phytochromes A–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

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 (WT, solid lines) and *phyC-1* (shaded lines). Plants were grown in LDs (20-hr days with average day temperature of 21° and night temperature of 18°, left column) until the fourth-leaf stage was reached at which point the newly expanded leaf was harvested at several time points throughout a 24-hr circadian cycle. Additionally plants were entrained under 12-hr days under constant temperatures of 21° until the third-leaf stage was reached at which point the plants were grown in continuous light (LL, right column) and the newly emerged third leaf was sampled every 4 hr for 48 hr. Shaded boxes represent subjective night. Values represent the average of three biological replicates $\pm$ 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 third-leaf stage in LDs (20-hr photoperiod) before transfer to 5° for 3 weeks also in LDs. After cold treatment, plants were grown in LDs. The third leaf was harvested at the end of vernalization (V) when the plants were at the fourth-leaf stage (A, C, and E). Nonvernalized (NV) controls were harvested at a similar developmental stage as the V samples (A, C, and E). After vernalization, plants were grown for 11 days (V+11) and the third leaf was harvested when the plants were at the sixth-leaf stage along with the third leaf from nonvernalized 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 four biological replicates $\pm$ standard deviation (three leaves per replicate). The experiment was repeated with similar results. qRT-PCR primers were optimized in Ream *et al.* (2014).

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 poads, 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; 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* (Figure 3 and Figure 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 *PPD1/PRR37*, *CO*, and *FT* in the *phyC* mutant is not surprising because it has been shown, for example, in rice and sorghum

that PRR37 regulates *CO* expression, and *CO* regulates *FT* expression (Koo *et al.* 2013; Yang *et al.* 2014). Our work shows that, unlike the case in rice and sorghum, in *Brachypodium*, *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*, *PHYC* is also at the top of this cascade (Chen *et al.* 2014 and Pankin *et al.* 2014).

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

As noted above, *PHYC*-mediated activation of *PPD1*/*PRR37* is the most “upstream” component so far identified in the cascade that leads to *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 LDs. In wheat and barley, *PPD1* promotes flowering in LDs 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 LDs as *prp37* plants are rapid flowering in LDs and exhibit higher *FT* expression (Murphy *et al.* 2011; Koo *et al.* 2013; Yang *et al.* 2014). Because loss of *PHYC* promotes flowering in noninductive 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 LDs 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* (Lv *et al.* 2014; Ream *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 noninductive SDs, 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.* 2014). 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* postcold is consistent with previous findings from *Brachypodium* as well as other temperate grasses indicating that *FT* enhances *VRN1* expression postcold (Sasani *et al.* 2009; Shimada *et al.* 2009; Ream *et al.* 2014; Woods *et al.* 2014). Additionally, the up-regulation of *VRN2L* during cold is photoperiod independent; however, the photoperiod pathway is required for *VRN2L* expression pre and postcold as *VRN2L* was barely detectable before and after cold (Figure 5, C and 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.

Acknowledgments

We thank Christopher Schwartz, Mark Doyle, and John Sedbrook for providing initial mutagenized seeds for screening *Brachypodium* mutants and isolating *phyC-1*. We thank undergraduate students Mary Kojima, Gerald Weiss, Jane Lee, Ka Yeun Jeong, Leah Varner, and Ryland Bednarek for assistance in screen. Additionally we thank Jill Mahoy and Heidi Kaeppler for providing transgenic material in *Brachypodium* and John Vogel and Sean Gordon for providing access to previously unpublished sequence data from Bd21-3 and Bd3-1 accelerating marker development. This work was funded in part by the National Science Foundation (grant no. IOS-1258126), the Great Lakes Bioenergy Research Center (Department of Energy Biological and Environmental Research Office of Science grant no. DE-FCO2-07ER64494),

a National Institutes of Health-sponsored predoctoral training fellowship to the University of Wisconsin Genetics Training Program to Daniel Woods, and the Gordon and Betty Moore Foundation and the Life Sciences Research Foundation for their postdoctoral fellowship support to Thomas Ream.

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Communicating editor: J. Borevitz

GENETICS

Supporting Information

<http://www.genetics.org/lookup/suppl/doi:10.1534/genetics.114.166785/-/DC1>

PHYTOCHROME C Is an Essential Light Receptor for Photoperiodic Flowering in the Temperate Grass, *Brachypodium distachyon*

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Figure S1 Diagram of the 5 chromosomes of *Brachypodium* and the corresponding indel and simple sequence repeat (SSR) markers that we have developed (unless otherwise specified in Table S1) using indel information from newly sequenced genomes of Bd21-3 and Bd3-1 (Gordon et al., 2014). Markers used for mapping mutants in Bd21-3 using Bd3-1 as a mapping partner. In bold are markers used for rough mapping. Additional markers on the diagram are shown to assist mapping other mutants. All markers are optimized to anneal at 51°C producing a product roughly 100bp in length. Markers resolve using a 3% sodium borate agarose gel as shown in the representative gel image which had been run for 30 minutes at 120 volts. The *phyC-1* allele mapped to the top arm of chromosome 1 indicated by the blue square. See Table S1 for primer sequences of markers.

Figure S2 (A) *phyC-1* F2 mapping population grown in 20-hour photoperiods, $n=512$. The majority of the population flowered between 20-39 days similar to the Bd21-3 and Bd3-1 controls. Heterozygous plants were identified via a tightly linked indel marker. The identified heterozygous plants were confirmed by progeny test. 23% of the population (116 plants) flowered like the *phyC-1* controls (did not flower for the duration of the experiment indicated by arrow, 149 days). DNA was extracted from the 116 delayed flowering plants and pooled DNA was sequenced using an Illumina Hiseq 2500. (B) Mapping frequency plots of the 5 chromosomes in *Brachypodium* showing a large peak between 5-6 Mb on the top arm of chromosome 1 overlapping previous rough mapping data. Frequency plots are binned into 1-Mb (gray) and 0.5-Mb (red) bins. (C) Following subtraction of variants present in the Bd3-1 crossing strain and our Bd21-3

reference strain, 1 coding variant remained in the 5-6 Mb mapping interval. Tabular list of the annotated variant provided by SnpEff. (D) dCAPs primer sequence for *phyC-2* and *phyC-3* designed to cut the Bd21-3 sequence but not the mutant variant using EcoRII. dCAPs primers were designed using the web-based software dCAPs Finder 2.0 (Neff et al., 2002). DNA was extracted from segregating M3 plants and the dCAPs primers were optimized for 55°C annealing temperature. PCR products were digested with EcoRII for 6 hours before resolving on a 3% agarose gel. (E) Picture of representative T0 plants grown in 20-hour photoperiod. Plant on left contains the rescue construct of a genomic *PHYC* fragment including 1 kb upstream of the translational start site of Bd21-3 *PHYC*; plant on right is T0 plant that does not contain the rescue construct. Nine independent transgenic lines exhibited rescue of the delayed flowering phenotype.

Figure S3 *VRN2L* mRNA expression throughout a 24 circadian cycle. Experimental samples identical as described in Figure 3.

Table S1 Indel Markers

Primer Name	Location	Forward Primer	Reverse Primer	Type	Reference
B1 2.8	2837148	CACCTCGCCCTTCTTACTCC	GTGGGTGTGTGTGGGTATGA	indel	This Paper
B1 5.2	5225657	CCTTCCAACTACTGTACCCAAAAC	GAAATGCAGAGATCCTGACTG	indel	This Paper
B1 5.5	5501626	ATACGCACTGGCCTCAAGC	CTCCCGGGGTTCTTCTG	indel	This Paper
B1 5.8	5809187	GTAATTCCTTGCTTAAGAG	ATAGGAGATCCATCGGAC	indel	This Paper
B1 6.1	6081471	TTGCATATATCATTATATCC	TGGATCTTACACAGATCCAC	indel	This Paper
B1 6.3	6280936	TATCTTTATTTGCCACTTG	TCTTTACTGGTATAAATATCTC	indel	This Paper
B1 6.5	6515371	AGGATACCTAATCGCGCTG	CGATCAGCTGAAATGCTCAA	indel	This Paper
B1 215	7060931	TCTTTGTTTCCCTTTTCCCC	AACGATGATTGGGACCAAAA	SSR	This Paper
B1 11.4	11374811	GCCACAGCAGCAATAATGT	CAGGGATTCATACACTTGATTAGTTC	indel	This Paper
B1 12.9	12894965	GCTGTGGCCATCAGTACACTT	AGGAAACATGTGTGTGTGTG	indel	This Paper
B1 14.7	14750599	TGGAACCATCTAGTAGCATCTGG	GGCTTCTTGGTAGCAAAATCAA	indel	This Paper
B1 19.7	19748276	GTCTCGTCACATATGGACCG	GATCATTCTTGCATCCACGTG	indel	This Paper
B1 23.67	23673189	GTGTTCTCGTCTGATGCCAC	CAACTAGGAAAGTGAGGGATC	indel	This Paper
B1 28.1	28114407	AGACAAGCGAAGGGTTACC	CAGGTATCGCCTCTTCACAGA	indel	This Paper
B1 29.8	29831845	TCACGCACGCATCGGTGAAG	AGCTAGTTTTCGGAATATAG	indel	This Paper
B1 38.6	38557197	AGAACACCCGTACAGTACAC	AGCGGTTTCGCATGTTAGCAC	indel	This Paper
B1 39.8	39817500	TTGGTGCCTCGGCTCGTGC	CGGATAAAAGTTCAAACCTGG	indel	This Paper
B1 43.5	43474416	GCGTCTACAGAAGAGCTTTC	AACTACTCGAGGAAACCTTCTC	indel	This Paper
B1 44.1	44141609	CAACGCAGCAATCAGTATTG	CATCGGACGACCTACCGT	indel	This Paper
B1 44.94	44943056	AGTACAATCTTACCACTGTGTC	GGTGGAAATGAGAAATTCGC	indel	This Paper
B1 45.3	45278913	AACAAACGCACATTAGTTAG	CTAGATATATGTGACATCGC	indel	This Paper
B1 46.5	46530000	TTGTAGGACTGCATCTCATG	AGTTTGTTGACTGAACACAG	indel	This Paper
B1 48.8	48865590	TAAACGAGTAGATTGCAACAAGC	TTTATGTCCTTGAATTGTTCCCT	indel	This Paper
B1 49.25		GTATGCCTCCGATACGAAT	GATGATGGTTATCACTAGGC	indel	This Paper
B1 50.53	50526328	TAATATGTGATGTTGCATCGA	ATTCTCAAGCAATGCTTGTC	indel	This Paper
B1 51.36	51355886	TAGCTGTAACAGGGTTGGAA	CCAATGGGTATTCAAGACTC	indel	This Paper
B1 51.9	51888868	AACGGTGCCTCTTCTGTTCT	GAGATGGTGGAGTGGAAC	indel	This Paper
B1 1567	57812395	AAGTCAAGGCGAGTCAGGAA	GCCACTGAAAGACTGCAACA	SSR	This Paper
B1 62.5	62484775	AGACAAAAAATGCCATTTCTAGC	TTTGTACGATAGAGTTGTGTTGATGA	indel	This Paper
B1 72.3	72323562	AAGCCACATGTGCAGAGGAT	GAACCTTCCCATTGTAAGAG	indel	This Paper
B1 74.4	74366750	AGGTGTTTGTGTCCTCCA	GGAAGCGTGCATTATCGATC	indel	This Paper
B2 3.3	3294880	ATCGATCGATGTGCCAC	CGCATGAATAACACATGATGG	indel	This Paper
B2 8.1	8124258	CTACATACTTCTTTTGTCTTAAG	CTCGATCACTCGCCTTATTACC	indel	This Paper
B2 8.6	8616254	CAGGATTTAATCAGATAGAAGCC	GTTTACGCAAGTGCCTGGTAG	indel	This Paper
B2 14.3	14355177	CACACGTGGAAGAGGACCTT	CCATGTGGTCAACCTCTCCT	indel	This Paper
B2 21.4	21469100	AATGGTCGACACACGTCCTT	CTAAACCATGCGCATTTTCC	indel	This Paper
B2 658	23000000	GAGCAAAAGGAAATCCCAACA	GCCCAGATCGAACTTGAATA	SSR	This Paper
B2 29.9	29965216	GAACCTGGACCGAGGGAGATAC	CGGCCGGGCTGGCTTCCGAG	indel	This Paper
B2 34.5	34469066	TCATTTTAACTTGGCACATGA	CTTATGCCACATGACGATCTGCA	indel	This Paper
B2 36.2	36231884	CCAAACAGTTCTGGGGCAAG	CTGTATTTTCCAGCCCATAGTG	indel	This Paper
B2 36.3	36323094	CAGTGGCTTGGCCTGTAAAC	GTGATCCATTGATGTGCTGC	indel	This Paper
B2 39.1	39123520	CAACCGACGGTTAGATATGTTGG	GTATTGTAATAATTATCTATAAGTCCAAC	indel	This Paper
ALB 179	40901313	TGATCAAGTGCAAGGAAACG	CACCGGAAGTGGAGAAGAAG	SSR	Vogel et al., 2009
B2 42.1	42175221	CAATGCTCAGCTCTAACCGAC	GGATTACAGAACTTCTCTTTCC	indel	This Paper
B2 46.6	46695113	CTAATCAAAGAAATAGCAATTCTC	GCTAGGGGATGAGTTATTGTCG	indel	This Paper
B2 47.5	47506114	CAAGGGACCTTGTCAAGCGAG	TCGATGTTGCCAACTGACCATC	indel	This Paper
B2 50.5	50530830	ACCATACCCTTGGGTTTTTCACGGAG	GGGGCATGTATATGCTTCTC	indel	This Paper
B2 1526	57	TCGATGTCCTCTTCTCCTC	TCGTTACCGTCAGCCAAAG	SSR	This Paper
B3 6.2	6205312	GAGTCGCCATTGTTGCTGTA	GCCAGAGTTTTCAGAACACATTA	indel	This Paper
B3 8.3	8277079	TAAGGTCACATCTTCGCAAC	CGGGCAATGAACCTCAAATA	indel	This Paper
B3 13.2	13229282	CGGTAATGGTGCCAGAATGT	TGTAATTCTGACCTGTGCCATC	indel	This Paper
B3 16.3	16330789	GCTGCGTATTGTCGGTGTTA	GTTGCCCCCAAGTTTCTACA	indel	This Paper
B3 565	19825154	TCGGTGGTAGGAGGTGGTAG	GCATTATTGAGCGCGTATGA	SSR	This Paper
ALB 022	20267283	GACCTCCCTGTCTCAGGT	ATTCTGCCAGCAAACT	SSR	Vogel et al., 2009
B3 22.8	22869632	GCTGATCATCGGGTGAGAAT	GAACATAATTGATCTGGCAATTGA	indel	This Paper
B3 29.5	29364425	AGAATCTGCACCGACAAAGG	AGGCTTAGCCACCGAAATTA	indel	This Paper
B3 36.7	36768127	GGTCTGGTCTACTCCCGTTAG	CTATGTTAAGGCCTAGTGGTAC	indel	This Paper
B3 36.8	36862036	CATTGGAATGTACTTCTCCG	CTCCGTACCATCTCAAAATGTC	indel	This Paper
B3 37	37078073	CCATATTAATTGTCTCAAATTTGTC	CCTGATATTTAATACTCCCGCCG	indel	This Paper
B3 45.2	45226850	TAACCTGCAAGCAATTCAGTG	CTGCAAAATCCACTGACGTGT	indel	This Paper
B3 46.95	46954407	GCTTAGGTTCCCTTTGGAAC	CCTGTCCTAAATCCAAACATAAGG	indel	This Paper
ALB348	52209350	CGACTCCAGGCCCTCACATATCTC	TGTGTGCCGAACATGTGAAAG	SSR	Vogel et al., 2009
B3 59.3	59320475	TCAGTCAGTCTTACCTGTGTTGC	CTTCAGTGGTGACGCAAGC	indel	This Paper
B4 6.4	6433520	AGACCGCACCTCCTAGGTGA	TCAACTAGTACGTTCTCCAGGAA	indel	This Paper
B4 12.9	12958087	TGAGCTACTGACGAATCTTGG	CAAAAGGGCGATCATGGTTA	indel	This Paper
B4 14.6	14616716	TCACTGGCTAACGGGAAGAT	ACGCTTACAAGGGGCTCAT	indel	This Paper
B4 31.9	31955520	TCCATCTGCTTTGTTCAAAG	TGTTTAGCAACCATGATGCC	indel	This Paper
B4 36.7	36779724	CGGTGCATGCATGATCGTCG	GCTTCTATGTGTAACAGGAGAAG	indel	This Paper
B4 43.1	43136667	CTGACTTATAGAAAACCAAGT	GCTAACCACTTTGCACCAG	indel	This Paper
B4 44.8	44836822	ACAGTAGATTGCAATAAGCAC	GAGGGCCGAGGTATATGTGA	indel	This Paper
B5 1	1015568	TTGTATAAGTATCTCCGGAC	GAGAGCTGGAGCCTGGAGT	indel	This Paper
B5 5.4	5396275	TCATCTTTCTTGTGTTGTGATG	TGACATGGCATGGTATTGAA	indel	This Paper
B5 11.1	11183240	ATGGAGGTGATGAGGCAGAG	GGGCTATCTGAAAACGACCA	indel	This Paper
ALB 363	13409608	CCGGGTATGTATGGCTCATC	ACCAAAGCAAAAATTTGCTCA	SSR	Vogel et al., 2009
B5 17	17054243	GTATAAGAGGTTTATGCTTTGTGC	TCCCGTGGATAATTGAGAG	indel	This Paper
B5 18	18035789	AAGAAATTAACAGCTGCACG	TCTCGAAAATACCCTAAGC	indel	This Paper
B5 20.4	20496533	TTACGCGTAACAAGAGTAGTGCA	AGGTTTGCAATTTGCCATCT	indel	This Paper
B5 595	26017525	ACATGCGCCCATAGACTGAT	CTCGCCCACTGTTAGTT	SSR	This Paper

Table S2 Real Time PCR Primers

Primer Name	Purpose	Forward Primer	Reverse Primer
FKF_Bradi4g16630	qRT-PCR	AGCTCCATGAGCTTTGCCTTGC	TGCACCCAGCATGGGTAGAATC
ZTL_Bradi1g33610	qRT-PCR	ACTGCCGGTTCTTGCAATGTAG	AACTACCGCGGCATCAACTAGG
CCA1_Bradi3g16515	qRT-PCR	AGCTTGGCAGCGCATAGAAGAG	AGCATGGCTTCTGATTTGCACAG
GI_Bradi2g05226	qRT-PCR	GCATCTCCCGAAATGCAGATGTC	CACAAAGCGCATCTACAACCTTTC
TOC1_Bradi3g48880	qRT-PCR	GCTGCGCATGAATGAGCTGTTG	TTTCTCGGCCAAGCCAAGCATC
CAB2_Bradi4g07380	qRT-PCR	TGAGAACCTCGCTGACCACAT	GGAACAAAGTTGGTGGCAAACGC
ELF3_Bradi2g14290	qRT-PCR	AGCTGCATAGGTTGATCAAAGTGC	AAGGCACGGATCTCCTTCAATAAG
PPD1_Bradi1g16490	qRT-PCR	CGATAATGGCAGTGGCACCCAAAG	TGTGGGCTGTCAATCTCCACAC
CO_Bradi1g43670	qRT-PCR	TGTGTGGTACCTTCGCAAGCTG	TCCAAGCCCATAACCACTCTGC
FT2_Bradi2g07070	qRT-PCR	AGTACTTGCACTGGCTGGTCAC	CCGAGCTGCTGGAATAGAAGGAAC
PHYC genomic	cloning genomic fragment	CACCCAAGAACACCACCTGTCTAC	TCAGAAGTTACTCTTACTTG