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Identification of *LATE BLOOMER2* as a *CYCLING DOF FACTOR* Homolog Reveals Conserved and Divergent Features of the Flowering Response to Photoperiod in Pea

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The molecular pathways responsible for the flowering response to photoperiod have been extensively studied in *Arabidopsis thaliana* and cereals but remain poorly understood in other major plant groups. Here, we describe a dominant mutant at the *LATE BLOOMER2* (*LATE2*) locus in pea (*Pisum sativum*) that is late-flowering with a reduced response to photoperiod. *LATE2* acts downstream of light signaling and the circadian clock to control expression of the main photoperiod-regulated *FT* gene, *FTb2*, implying that it plays a primary role in photoperiod measurement. Mapping identified the *CYCLING DOF FACTOR* gene *CDFc1* as a strong candidate for *LATE2*, and the *late2-1D* mutant was found to carry a missense mutation in *CDFc1* that impairs its capacity to bind to the blue-light photoreceptor FKF1 in yeast two-hybrid assays and delays flowering in Arabidopsis when overexpressed. Arabidopsis *CDF* genes are important negative regulators of *CONSTANS* (*CO*) transcription, but we found no effect of *LATE2* on the transcription of pea *CO-LIKE* genes, nor on genes in any other families previously implicated in the activation of *FT* in Arabidopsis. Our results reveal an important component of the pea photoperiod response pathway and support the view that regulation of *FTb2* expression by photoperiod occurs via a *CO*-independent mechanism.

INTRODUCTION

In plants, a key factor in the transition from vegetative to reproductive development is the ability to detect and respond to changes in photoperiod. This capacity enables plants to synchronize flowering time with seasonal conditions that are conducive to maximum reproductive fitness. For instance, wild progenitors and primitive cultivars of rice (*Oryza sativa*) originating from tropical regions are typically short-day (SD) plants with a long basic vegetative growth phase that maximizes accumulation of resources before flowering in response to lengthening nights at the end of summer (Wu et al., 2013). By contrast, the progenitors of many important crop species known to have originated in the Fertile Crescent of southwest Asia, such as wheat (*Triticum* spp), barley (*Hordeum vulgare*), pea (*Pisum sativum*), lentil (*Lens culinaris*), and flax (*Linum usitatissimum*), are long-day (LD) plants, flowering in response to the lengthening days of spring and enabling seed maturation and harvest before the arrival of late summer droughts (Nakamichi, 2015).

The relaxation of such photoperiod requirements for flowering is considered to have been essential to the spread and cultivation of numerous crop species (Nakamichi, 2015). For example, it is thought that reduced function at LD repressor loci enabled cultivation of rice at higher latitudes, where the growing season is restricted to spring and summer, and long photoperiods would otherwise be noninductive (Takahashi and Shimamoto, 2011; Gómez-Ariza et al., 2015). Similarly, molecular evidence suggests that it was a loss of function in genes conferring a strong LD requirement that first permitted ancestral forms of wheat, barley, pea, and lentil to flower early and be cultivated as spring crops at higher latitudes in Europe where summers are short (Turner et al., 2005; Comadran et al., 2012; Weller et al., 2012).

Because of the relevance of photoperiod responsiveness to plant adaptation in new cropping environments, the underlying molecular processes have received considerable attention, particularly in the model species *Arabidopsis thaliana* and rice (Shrestha et al., 2014). In both species, studies of photoperiod response variants have identified genes in the *FT* family as the ultimate targets of photoperiod regulation and key factors in communication between photoperiod sensing in leaves and meristem identity gene networks at the shoot apex (Suárez-López et al., 2001; Ishikawa et al., 2005).

In Arabidopsis, the B-box transcription factor CONSTANS (CO) plays an important role in the LD-specific induction of *FT* expression through direct interaction with the *FT* promoter (Tiwari et al., 2010). *CO* expression is diurnally regulated by the circadian clock, with peak expression occurring at night during SD, but in the afternoon under LD (Suárez-López et al., 2001). During peak *CO* expression in LD, the blue light photoreceptor FLAVIN BINDING KELCH REPEAT

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F-BOX1 (FKF1) is activated by light and forms a complex with GIGANTEA (GI; Sawa et al., 2007; Song et al., 2012). This complex binds to and directs the degradation of CYCLING DOF FACTOR (CDF) proteins, which are transcriptional repressors of *CO* and *FT*, allowing *CO* expression and flowering to occur (Imaizumi et al., 2005). Light-activated FKF1 also binds directly to CO and contributes to its stabilization (Song et al., 2012). In contrast to LD conditions, *CO* expression under SD occurs in the dark period, when FKF1 is not active. As a result, FKF1-dependent release of *CO* expression and stabilization of CO protein do not occur, resulting in persistent repression of *FT* (Sawa et al., 2007; Song et al., 2012).

Evidence from several other photoperiodic species also indicates an important role for *CO* homologs in photoperiod responsiveness. In rice, the *CO*-like gene *Heading date 1* (*Hd1*) is involved in photoperiod measurement and photoperiod-specific regulation of genes in the *FT* family (Brambilla and Fornara, 2013), but unlike Arabidopsis *CO*, it serves a dual function as both a promoter of *FT* under SD and an inhibitor under LD (Izawa et al., 2002; Kojima et al., 2002). Functional analyses in potato (*Solanum tuberosum*) and sugar beet (*Beta vulgaris*) have also demonstrated a role for *CO*-like genes in photoperiod responses (Chia et al., 2008; González-Schain et al., 2012). Evidence for conserved *CO* function is less clear in other species such as barley and poplar (Campoli et al., 2012; Hsu et al., 2012).

In legumes, the molecular basis of photoperiod measurement has also attracted much interest. However, most research has focused specifically on light perception, circadian clock function, or genes from the *FT* family (e.g., Hecht et al., 2007, 2011; Liew et al., 2009; Kong et al., 2010; Watanabe et al., 2012), and comparatively little is known about the mechanism by which clock and photoreceptor inputs are integrated to provide photoperiod-specific regulation of *FT* genes. Recent analysis of *CONSTANS-LIKE* (*COL*) genes in the temperate LD legume *Medicago truncatula* suggests that the central role of *CO* in photoperiod measurement is not conserved in temperate legumes (Wong et al., 2014), and it seems likely that *CO*-independent pathways may play a greater role in regulating photoperiod sensing in legumes generally.

We previously conducted a mutant screen to isolate late-flowering photoperiod-insensitive mutants that might define genes encoding components of the photoperiod sensing mechanism in pea and identified one such gene, *LATE BLOOMER1* (*LATE1*), as the pea ortholog of Arabidopsis *GI* (Hecht et al., 2007). In this study, we describe a second locus that contributes to photoperiod responsiveness, *LATE2*. We present genetic and molecular evidence that *LATE2* is a *CDF* homolog that can regulate *FT* expression and flowering time without affecting the expression of *CO*-like genes. Together with results from previous studies, this supports the idea that *CO*-like genes do not have a significant role in the photoperiod response of pea and other temperate legumes.

RESULTS

A New Photoperiod Response Locus, *LATE2*, Defined by a Dominant Late-Flowering Mutant

Screening of an EMS-mutagenized population of the dwarf pea line NGB5839 under 18-h LD conditions identified a line with several characteristics of a photoperiod response mutant, including delayed flowering, an extended flowering phase, and an increased tendency for basal branching (Hecht et al., 2007; Figures 1A and 1B). These phenotypes cosegregated and showed dominant inheritance in the F2 population of a cross to the NGB5839 progenitor (Figure 1C). Crosses to other late-flowering photoperiod response mutants *phyA-1* and *late1-2* returned wild-type progeny in the F2, indicating that the mutant defined a new

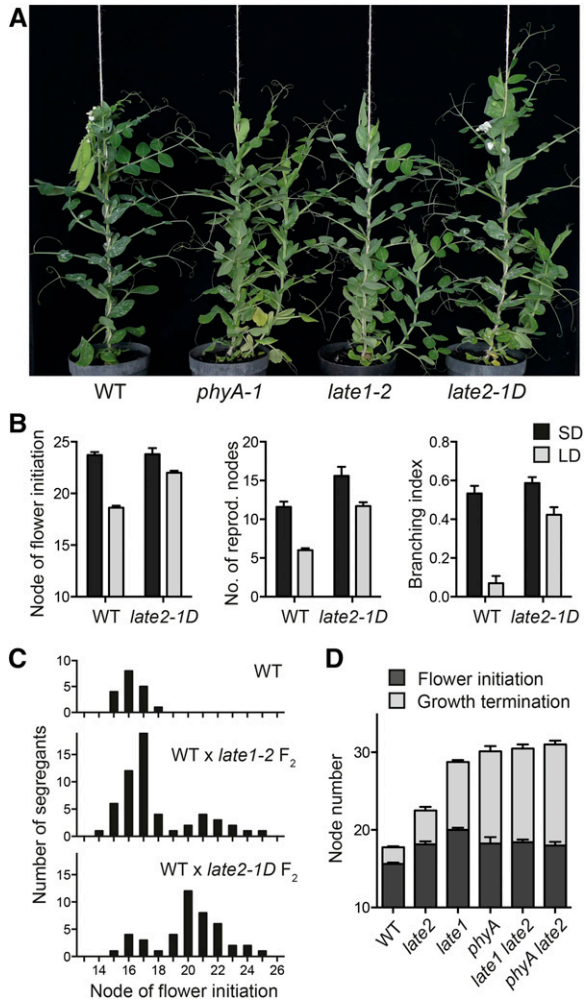

Figure 1. The Dominant *late2-1D* Mutant Shows Reduced Responsiveness to Photoperiod.

(A) Comparison of *late2-1D* with photoperiod response mutants *phyA-1* and *late1-2* and their wild-type progenitor line NGB5839 (WT) grown under LD conditions.

(B) Effect of photoperiod on flowering and other traits in wild-type (NGB5839) and *late2-1D* plants. Values represent mean ± SE for $n = 6$ to 8 replicates.

(C) Distribution of flowering time in F2 progeny of the cross NGB5839 (WT) × *late2-1D*. The distribution in an F2 progeny of the cross wild type × *late1-2* is shown for comparison.

(D) Genetic interaction of *late2-1D* with *phyA-1* and *late1-2* in control of flower initiation and growth termination under LD conditions. Values represent mean ± SE for $n = 6$ to 8 replicates.

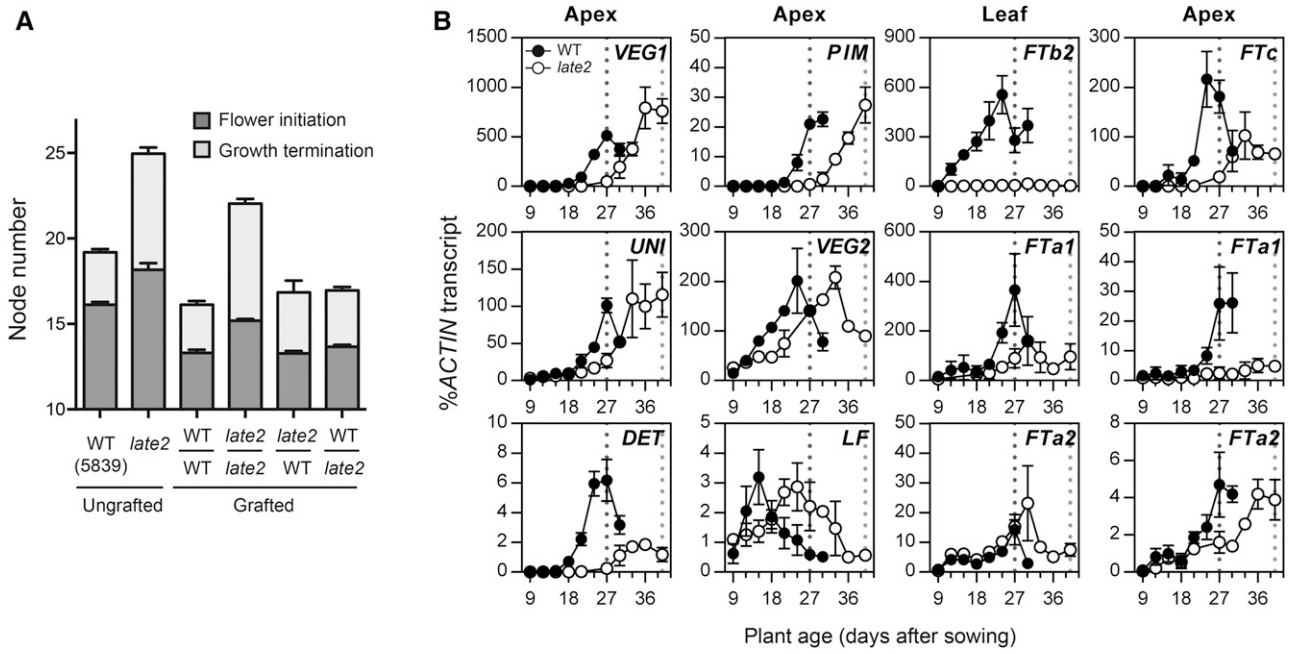

Figure 2. The *late2-1D* Mutation Affects Graft-Transmissible Flowering Signals and Induction of Flowering Genes.

(A) Node of flower initiation and growth termination for graft combinations of wild-type NGB5839 (WT) and the *late2-1D* mutant, in comparison to intact and self-grafted controls. For each graft combination, the genotypes of scion (top) and stock (bottom) are shown, separated by a horizontal line. Six-day-old shoots (scions) were excised above the cotyledons and the epicotyl grafted onto the uppermost internode of 3-week-old stocks. Values represent mean ± SE for $n = 10$ to 20 plants grown in LD.

(B) Gene expression in the wild type (NGB5839) and the *late2-1D* mutant during development under continuous light. Relative transcript levels were determined in dissected shoot apices or the second-uppermost fully expanded leaf. Mean values ± SE are shown for $n = 2$ to 3 biological replicates, each consisting of pooled material from two plants. Developing floral buds were first visible in the wild type 27 d after sowing and in *late2-1D* 40 d after sowing (broken lines).

locus, which we termed *LATE2*, with the single mutant allele designated *late2-1D*. A comparison of phenotypes under LD and SD conditions showed that, like the *phyA-1* and *late1-2* mutants, the *late2-1D* mutant flowered later than the wild type under LD conditions and showed a reduced response to photoperiod (Figure 1B). However, the *late2-1D* phenotype under LD was clearly weaker than that of *late1* and *phyA* null mutants (Weller et al., 1997; Hecht et al., 2007; Figures 1A and 1D). To test the genetic relationship of *LATE2* to *LATE1* and *PHYA*, we generated double mutants for *late2-1D* with the null *late1-2* and *phyA-1* alleles (Weller et al., 2004; Hecht et al., 2007). Figure 1D shows that neither the *late2 late1* nor the *late2 phyA* double mutants initiated flowering later or produced more reproductive nodes than the respective single mutants, consistent with all three mutations affecting the same pathway. The marginally earlier flowering in the *late1 late2* double mutant relative to *late1* mutant controls ($P = 0.03$) shown in Figure 1D was not consistently observed in segregating populations.

The *late2-1D* Mutation Impairs Graft-Transmissible Promotion of Flowering and Disrupts Expression of *FT* Genes

We previously showed that the late-flowering LD phenotypes of the photoperiod response mutants *phyA* and *late1* can be rescued by grafting to wild-type stocks (Weller et al., 1997; Hecht et al., 2007). A similar grafting experiment was performed to examine whether the same might be true for *late2-1D*. Figure 2A shows that self-grafted *late2* scions flowered approximately two nodes later than self-grafted wild-type scions and produced more than twice the number of reproductive nodes at maturity ($P < 0.0001$ in both cases). However, grafting *late2* scions to wild-type stocks fully restored the node of flower initiation (13.28 ± 0.14 versus 13.31 ± 0.18 in wild-type self-grafts; $P = 0.87$) and significantly reduced the number of reproductive nodes (3.56 ± 0.20 versus 6.85 ± 0.27 in *late2* self-grafts; $P < 0.0001$). These results show that like *phyA* and *late1*, the *late2-1D* mutation impairs the production of a graft-transmissible signal.

Previous work distinguished two different graft-transmissible signals in pea that are correlated to the expression of different *FT* genes (Hecht et al., 2011). The graft-transmissible promotion of flowering by LD is closely associated with the induction of the *FTb2* gene, which does not occur in wild-type plants grown under SD or in *late1* mutants (Hecht et al., 2011). We therefore examined whether *late2-1D* might also be defective in *FTb2* regulation. Under constant light, the *late2-1D* mutant flowered four nodes later than the wild type (20.4 ± 0.24 nodes versus 16.2 ± 0.13), and Figure 2B shows this was reflected in a delay in induction of *VEGETATIVE1* (*VEG1*), a developmental marker for legume inflorescence development (Berbel et al., 2012), which was first

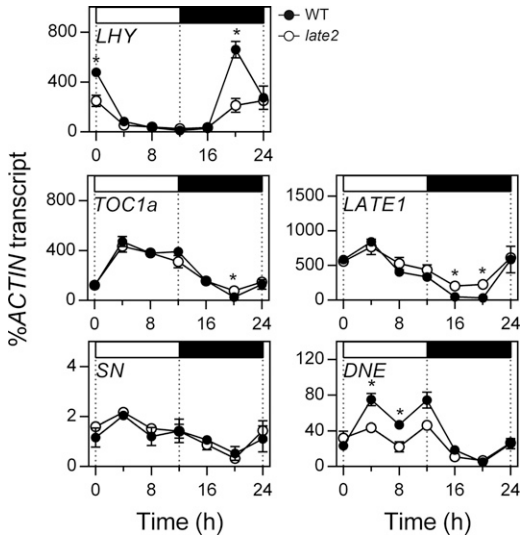

Figure 3. The *late2-1D* Mutation Does Not Affect the Rhythmic Expression Patterns of Circadian Clock Genes under SD Cycles.

Transcript levels were determined in the uppermost fully expanded leaf of 3-week-old wild-type (NGB5839) and *late2-1D* mutant plants grown under a 12-h photoperiod at 20°C. Mean values ± SE are shown for $n = 2$ to 6 biological replicates, each consisting of pooled material from two plants. Asterisks indicate significantly different values ($P \leq 0.05$). Day and night periods are respectively indicated by white and black bars above the graph. Note that both genotypes carry the *hr* mutation (Weller et al., 2012).

detectable above background at 18 d after sowing in the wild type and 27 d in *late2-1D*. Other flowering genes expressed at the shoot apex also showed a delay in induction or in the timing of peak expression levels in *late2-1D*, including *APETALA1* homolog *PROLIFERATING INFLORESCENCE MERISTEM* (*PIM*), *LEAFY* ortholog *UNIFOLIATA* (*UNI*), *FD* homolog *VEGETATIVE2* (*VEG2*), and *TERMINAL FLOWER1* homologs *DETERMINATE* (*DET*) and *LATE FLOWERING* (*LF*), which is generally consistent with the delay in flowering. The *late2-1D* mutant also completely failed to induce expression of *FTb2* in leaves throughout the entire time course examined, even though flower initiation occurred within the time frame of the experiment, ~40 d after sowing. Expression of other *FT* genes was also altered in *late2-1D*, but to a lesser extent, with weaker induction of *FTa1* in leaves and delayed induction of both *FTa1* and *FTc* in shoot apical tissue. Overall, this pattern of *FT* gene misregulation is very similar to that described for the *late1* mutant (Hecht et al., 2011).

The *late2-1D* Mutant Does Not Affect Photomorphogenesis or the Rhythmic Expression of Circadian Clock Genes

As in Arabidopsis and other species, the photoperiod response in pea depends both on photoreceptor signaling and the circadian clock (Weller et al., 2004, 2012; Hecht et al., 2007; Liew et al., 2009, 2014), and we considered that the *late2-1D* phenotype could therefore result from a defect in either of these systems. In order to test whether the mutation influenced clock function, we examined the rhythmic expression of several central circadian clock genes under light/dark cycles. Figure 3 shows that under a 12-h-light/12-h-dark diurnal cycle, the expression rhythms of clock genes *LATE ELONGATED HYPOCOTYL* (*LHY*), *TIMING OF CAB EXPRESSION 1a* (*TOC1a*), and *STERILE NODES* (*SN*) were unaffected by the *late2-1D* mutation. The rhythmic phase of other clock-related genes *LATE1* and *DIE NEUTRALIS* (*DNE*) was also unaffected by *late2-1D*, although there were significant differences in expression level of both genes at several points in the diurnal cycle, suggesting that *LATE2* could potentially influence the expression of these genes in a manner unrelated to their rhythmic regulation. Similar patterns and effects of *late2-1D* were also seen under LD conditions (Supplemental Figure 1).

We also examined whether the *late2-1D* mutation affected seedling responses to light. Figure 4 shows that both *phyA* and *late1* mutations disrupt normal photomorphogenesis, consistent with previous reports (Weller et al., 1997; Hecht et al., 2007). In contrast, *late2-1D* mutants did not differ from the wild type for either stem elongation or leaf expansion under monochromatic red, blue, or far-red light conditions ($P > 0.05$ for each comparison). This indicates that the *late2-1D* flowering phenotype is unlikely to be the result of a primary defect in light perception or signaling and suggests that *LATE2* may participate specifically in the photoperiod response mechanism.

The *late2-1D* Mutant Carries a Substitution of a Highly Conserved C-Terminal Residue in a *CYCLING DOF FACTOR* Homolog

In order to narrow the range of potential candidate genes, we mapped *LATE2* in the F2 progeny of a cross between *late2-1D* and cv Térèse ($n = 219$). Initial results placed *LATE2* in linkage group VII, in a region syntenic with the middle of *M. truncatula* chromosome 4 (Supplemental Figure 2). Several genes potentially related to flowering were identified within this region, including homologs of the MADS domain gene *SHORT VEGETATIVE PHASE* (*SVP*), *HUA2*, and a *CYCLING DOF FACTOR* (*CDF*) gene. Fine-mapping of *LATE2* in relation to these genes identified recombination with *SVP* and *HUA2* but not with the *CDF* gene, indicating a distance of <0.3 cM between *LATE2* and this *CDF*, and defining an interval comprising 226 annotated genes in the corresponding region of the *M. truncatula* genome. Apart from the *CDF* gene, no other genes with reported effects on flowering were present in this interval.

In Arabidopsis, the *CDF* genes comprise a small subgroup of *DOF* transcription factor genes distinguished by their rhythmic expression and ability to delay flowering when overexpressed (Imaizumi et al., 2005; Fornara et al., 2009). Phylogenetic analysis of legume *DOF* genes identified three distinct subclades, designated here as *CDFa*-*CDFc*, within the group II DOF clade (Yanagisawa, 2002). This clade includes Arabidopsis *CDF1*-*CDF3* and *CDF5*, which share redundant roles and a similar expression pattern (Fornara et al., 2009), and potato *CDF1*-*CDF5* (Figure 5A; Supplemental Figure 3). The *CDF* gene identified as a positional candidate for *LATE2* in our mapping population corresponded to *CDFc1* in this analysis. Other legume DOF proteins fall outside this core clade and are more closely related to Arabidopsis CDF4 and related proteins (Supplemental Figure 3). Overall, the phylogenetic relationships of the legume clades to specific *CDF* genes in Arabidopsis

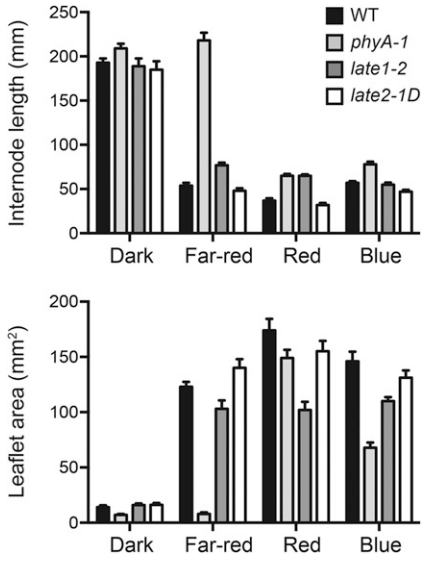

Figure 4. The *late2-1D* Mutation Does Not Affect Photomorphogenesis.

Internode elongation (upper panel) and leaflet area (lower panel) of wild-type, *phyA-1*, *late1-2*, and *late2-1D* seedlings grown from sowing in darkness or under monochromatic red, blue, or far-red light (15 μmol m^{-2} s^{-1}). Internode length was measured as the length between nodes 1 and 3, and leaflet area was estimated as the product of the length and width of the larger of the leaflets from leaf 3. Values represent mean ± SE for n = 4 to 14 plants.

and potato are not well resolved, but the clades appear to predate legumes, as a more extensive analysis shows that they include genes from non-legume taxa (Supplemental Figure 3). A duplication is apparent within each of the legume *CDFa*, *CDFb*, and *CDFc* clades, such that the basic complement of genes appears to be six, as seen in chickpea (*Cicer arietinum*), or 12 in tetraploid soybean (*Glycine max*; Figure 5A). Other legume taxa may have suffered the loss of one or more genes, as bean (*Phaseolus vulgaris*) *CDFb2*, *M. truncatula CDFa1*, and pea *CDFa1* and *CDFa2* do not appear in any sequence databases. In addition, the *CDFc2* gene has undergone multiple duplications in both pea and *M. truncatula*, with the multiple copies in *M. truncatula* arranged in tandem (Supplemental Table 1).

Sequencing of *CDFc1* cDNA in the wild type and *late2* revealed a C1348T transition resulting in a substitution of arginine by tryptophan at residue 450 (Figure 5B). As expected, a marker for this mutation also cosegregated perfectly with the *late2-1D* phenotype (Supplemental Figure 2). The affected amino acid is located within the FKF1 binding domain defined in Arabidopsis and potato (Imaizumi et al., 2005; Kloosterman et al., 2013) and is one of several highly conserved basic residues within this C-terminal region suggested to potentially influence the CDF-FKF1 interaction (Figure 5C), strongly implicating this mutation as the likely cause of the *late2-1D* phenotype.

In Arabidopsis, the three genes most closely related to *PsCDFc1* (*AtCDF1-3*) all show rhythmic expression patterns, with a morning peak under LD (Imaizumi et al., 2005; Fornara et al., 2009), and we found this also to be true for *PsCDFc1* (Figure 5D). This expression pattern contrasted with those of *LATE1* and *FKF1*, which both showed peaks in the middle of the day (Figure 5D). We also consulted the pea gene expression atlas (Alves-Carvalho et al., 2015) for information about tissue specificity of *PsCDFc1* expression and found it to be expressed most strongly in leaf tissue, similar to *AtCDF1* (Imaizumi et al., 2005). In addition, among the six other *CDF* genes shown in Figure 5A, only two (*PsCDFb1* and *PsCDFc2-1*) showed significant expression in the gene atlas. These genes also showed a similar pattern of expression to *PsCDFc1* but were expressed at levels ~4-fold and 12-fold lower than *PsCDFc1*, respectively.

The Function of the CDFc1 Protein Is Altered by the R450W Mutation

The nature of the R450W mutation suggested that the *late2-1D* flowering phenotype might be due to an inability of PsCDFc1 to bind to PsFKF1, resulting in impaired light-dependent degradation of PsCDFc1 and persistent transcriptional repression of *FTb2*. To test this idea, we used a yeast two-hybrid assay system to examine the ability of both wild-type and R450W mutant forms of PsCDFc1 to interact with the C-terminal half of PsFKF1 (Supplemental Figure 4; Alves-Carvalho et al., 2015), which contains several KELCH repeats involved in protein-protein interactions (Nelson et al., 2000; Imaizumi et al., 2005). We first confirmed that none of the bait or prey constructs showed autoactivation (Supplemental Figure 5A). Yeast two-hybrid assay results in Figure 6A show that the wild-type form of CDFc1 interacts strongly with FKF1, whereas the *late2-1D* R450W variant does not. This provides a clear demonstration that the regulation of CDFc1 is likely to be impaired by the R450W mutation and supports the idea that this mutation is the cause of the late flowering phenotype in the *late2-1D* mutant.

To further test this idea, we confirmed that the R450W variant also impaired the ability of PsCDFc1 to interact physically with Arabidopsis FKF1 (Supplemental Figure 5B), and we next compared the ability of wild-type and mutant forms of *PsCDFc1* to influence flowering when expressed in Arabidopsis. Figures 6B and 6C show that expression of *PsCDFc1* from the 35S promoter had no effect on the flowering time of wild-type Arabidopsis plants grown under LD, whereas expression of the *late2-1D* R450W variant significantly delayed flowering in multiple independent transformed lines. This delayed flowering was also reflected in reduced levels of endogenous *CO* and *FT* transcript (Figure 6C), showing that the mutant form of PsCDFc1 is more effective than the wild-type form in repressing flowering through the *CO*/*FT* pathway. Together, these results strengthen the evidence that the R450W mutation is indeed the cause of the *late2-1D* late-flowering phenotype and show that when expressed in transgenic Arabidopsis, the mutant PsCDFc1 is capable of repressing flowering via *CO* in a manner similar to the endogenous Arabidopsis CDF1. However, the lack of effect of Pro*35S:PsCDFc1* was in contrast to previous reports that overexpression of *AtCDF1* from the 35S promoter causes late flowering (Imaizumi et al., 2005). This could reflect differences in the relative expression levels or location of the two proteins, or in the efficiency with which they are processed by the Arabidopsis FKF1/GI degradation mechanism. Consistent

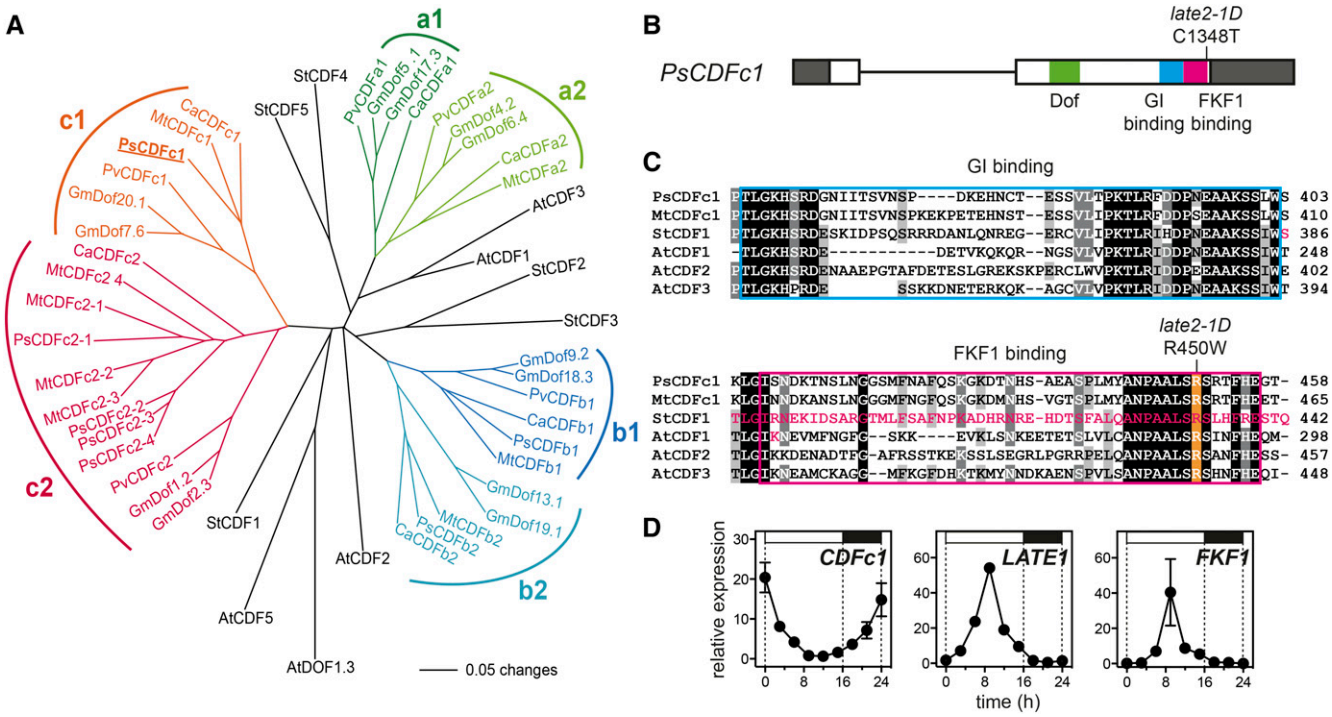

Figure 5. The *late2-1D* Mutant Carries a Mutation in a CDF Homolog.

(A) Phylogram of the legume *CDF* family with reference to CDF genes from Arabidopsis and potato. Branches with bootstrap values <55% obtained from 1000 trees have been collapsed. The analysis is based on the sequence alignment shown in Supplemental File 1. Sequence details are given in Supplemental Table 1.

(B) Diagram of the pea *CDFc1* gene showing the nature and location of the mutation in *late2-1D* in the FKF1 binding domain. Exons are shown as boxes, with coding sequence in white and untranslated regions in dark gray. Functional domains are indicated.

(C) Alignment of pea CDFc1 and other CDF homologs showing conserved residues in the GI binding (blue box) and FKF1 binding (pink box) domains. The arginine affected by the *late2-1D* mutation is highlighted in orange. The residues deleted in the naturally occurring potato StCDF1.2 and 1.3 variants, and altered in the Arabidopsis AtCDF1 K253A variant, all of which affect FKF1 binding (Imaizumi et al., 2005; Kloosterman et al., 2013), are indicated in pink. Shading indicates degree of conservation (black = 100%, dark gray = 80%, and light gray = 60%).

(D) Comparison of rhythmic expression patterns for *CDFc1*, *LATE1*, and *PsFKF1* under LD conditions. Transcript levels were determined in the uppermost fully expanded leaf of 3-week-old wild-type (NGB5839) plants grown under a 16-h photoperiod at 20°C. Mean values ± SE are shown for $n = 2$ biological replicates, each consisting of pooled material from two plants. Day and night periods are indicated by white and black bars, respectively, above the graph. At, *Arabidopsis thaliana*; Ca, *Cicer arietinum*; Gm, *Glycine max*; Mt, *Medicago truncatula*; Ps, *Pisum sativum*; Pv, *Phaseolus vulgaris*; St, *Solanum tuberosum*.

with this interpretation, we found that when *PsCDFc1* was instead expressed from the Arabidopsis *SUC2* promoter, which drives expression specifically in phloem companion cells, a small delay in flowering was observed (Supplemental Figure 6).

LATE2 Does Not Affect the Expression of *COL* Genes

In Arabidopsis, overexpression of *CDF1* results in strong repression of *CO* expression in the afternoon and during the night under LD conditions (Imaizumi et al., 2005). A similar effect was seen in lines expressing a mutant form of the CDF1 protein that can no longer bind to FKF1 (Imaizumi et al., 2005). This regulatory relationship is conserved in potato, where lines carrying a truncated, degradation-resistant form of the potato CDF1 protein show increased repression of *CO* expression in comparison to the wild type (Kloosterman et al., 2013). Previously, we described 11 members of the *CO*-like gene family in legumes and showed that the temperate LD legume *M. truncatula* has only a single *CO* co-ortholog (*COLa*), which does not affect flowering (Wong et al., 2014). In addition, both *M. truncatula COLa* and its pea ortholog exhibit a morning phased diurnal expression rhythm that lacks the afternoon peak characteristic of *CO* and is more similar to that of Arabidopsis *COL1* and *COL2* (Hecht et al., 2007; Wong et al., 2014).

In view of the apparent conservation in CDF function between Arabidopsis and potato, we first examined whether the *late2-1D* mutation might also affect expression of the pea *COLa* ortholog. Figure 7A shows that the diurnal rhythm of *COLa* expression is very similar in the wild type and *late2-1D* and thus provides no evidence that *late2-1D* may affect flowering through regulation of *COLa* transcript level. This is consistent with the previous finding that a null *late1* mutation also does not appear to influence the *COLa* expression rhythm (Hecht et al., 2007).

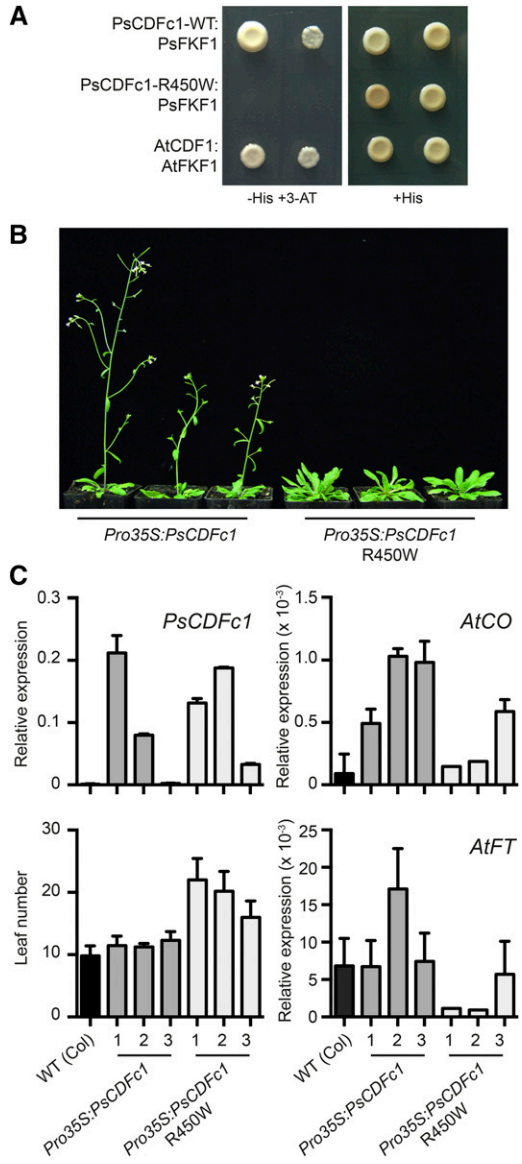

Figure 6. The R450W Mutation in *late2-1D* Affects the Function of the CDFc1 Protein.

(A) Yeast two-hybrid analysis of the interaction between pea FKF1 and the wild-type and R450W mutant form of CDFc1. The previously characterized interaction between Arabidopsis FKF1 and CDF1 proteins (Imaizumi et al., 2005) was included as a positive control. Each panel shows bait/prey interactions in both orientations, either FKF1/CDF (left) or CDF/FKF1 (right). Each clone is shown after 4 d of growth at 30°C on selective medium (with 3-amino-1,2,4-triazole added; -His+3-AT) and nonselective medium (+His). Negative controls are shown in Supplemental Figure 5.

(B) Representative plants from three independent Arabidopsis lines (Columbia accession) transformed either with wild-type or R450W mutant forms of pea CDFc1, grown under LD conditions.

(C) Relative expression levels at ZT 15.45 of *PsCDFc1*, *AtCO*, and *AtFT* and leaf number at flowering in transgenic Arabidopsis lines. Labels 1 to 3 denote independent transformants. Values represent mean ± SE for $n = 2$ biological replicates (expression data) and $n = 6$ to 10 plants (leaf number).

Using a combination of database searches and PCR-based approaches, we isolated 10 further pea *COL* genes orthologous to the previously described *M. truncatula COLb-k* genes (Wong et al., 2014; Supplemental Figures 7 and 8). We then examined whether the expression of any of these genes was altered in the *late2-1D* mutant by performing RNA-sequencing with RNA from leaf tissue of 3-week-old seedlings. We also included the *late1* mutant in this experiment, with the reasoning that as the Arabidopsis ortholog *GI* regulates *CO* through an effect on CDF proteins, any gene necessary for *LATE2* regulation of *FTb2* should show similar misregulation in both *late1-2* and *late2-1D* mutants in pea. In an attempt to minimize any light-driven rhythmic component to the differential regulation, plants were grown under conditions of continuous light from sowing. Under these conditions, both *late1-2* and *late2-1D* mutants showed a characteristic late-flowering phenotype similar to that seen under a standard 16-h photoperiod (Figure 7B). In order to verify the absence of any expression rhythms under these continuous light conditions, we used RT-qPCR to examine expression of clock and *COL* genes in leaf tissue of 3-week-old wild-type plants over a 24-h time course. Supplemental Figure 9 confirms that rhythmic regulation was effectively absent for key clock genes and the 11 members of the pea *COL* family. RNA-seq analysis of a single time point showed that, as expected, the *late2-1D* mutant had a negligible level of *FTb2* transcript, similar to *late1-2*, and much lower than the wild type (Figure 7D). Figure 7C confirmed that *COLa* transcript levels did not differ significantly between wild-type and *late2-1D* mutants. In the *late1-2* mutant, *COLb* showed a small but statistically significant increase in expression relative to the wild type, while *COLe* and *COLk* transcript levels were slightly reduced; however, there was no corresponding misregulation of any of the *COL* genes in *late2-1D* mutants (Figure 7C).

LATE2 Does Not Affect the Expression of Other Genes Implicated in the Activation of *FT* in Arabidopsis

While *CO* homologs have been implicated in photoperiod-specific *FT* expression across a range of diverse species (Kojima et al., 2002; Navarro et al., 2011; Yang et al., 2014), recent evidence from species within the Brassicaceae suggests this may reflect convergent evolution (Simon et al., 2015) and raises the possibility of *CO*-independent mechanisms for photoperiod regulation of flowering in other species. One possible mechanism is suggested by recent results from sugar beet, where the CCT-domain pseudo-response regulator gene *BTC1* and the B-box (BBX) gene *B2* have similar roles in the regulation of *FT* genes and are proposed to act together to confer a *CO*-like activity (Pin et al., 2012; Dally et al., 2014). In addition, in Arabidopsis, a number of other transcription factors have been shown to directly activate *FT*, including members of the CIB (CRYPTOCHROME-INTERACTING BASIC-helix-loop-helix) and PIF (PHYTOCHROME INTERACTING FACTOR) families of bHLH (basic helix-loop-helix) proteins (Liu et al., 2008; Kumar et al., 2012; Liu et al., 2013). Members of the *AP2* family of transcription factors have also been implicated as regulators of photoperiodic flowering and *FT* expression (Jung et al., 2007).

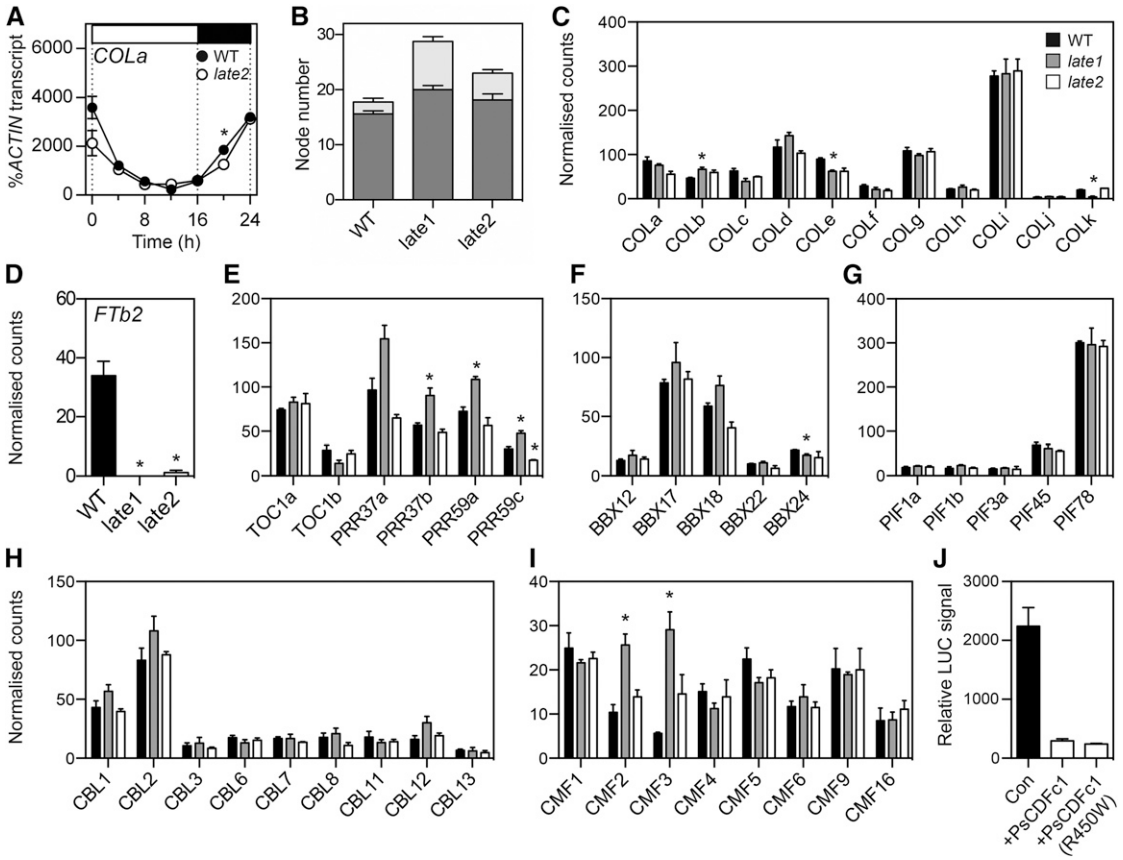

Figure 7. Effects of *LATE2* on *FTb2* Expression Are Not Mediated by Expression Changes in Key Transcription Factor Families and May Result from Direct Transcriptional Repression.

(A) Expression of *COLa* in wild-type NGB5839 (WT) and *late2-1D* mutant plants under a 16-h LD photoperiod. Transcript levels were determined in the second uppermost fully expanded leaf of 3-week-old plants grown at 20°C. Mean values ± SE are shown for $n = 2$ biological replicates, each consisting of pooled material from two plants. Day and night periods are indicated by white and black bars, respectively, above the graph.

(B) Flowering phenotypes of *late1-2* and *late2-1D* mutants grown from sowing under continuous white light. Values represent mean ± SE for $n = 8$ replicates. Dark gray, node of flower initiation; light gray, node of growth termination.

(C) to **(I)** Transcript levels of *FTb2* **(D)** and genes in the *COL* **(C)**, *PRR* **(E)**, *BBX* **(F)**, *PIF* **(G)**, *CBL* **(H)**, and CMF **(I)** families in expanded leaf tissue from wild-type, *late1-2*, and *late2-1D* mutants grown from sowing under continuous white light. Values represent mean ± SE for $n = 3$ biological replicates, each consisting of pooled material from two plants. Asterisks indicate values significantly different from the wild type: $P \leq 0.05$ for **(A)** and $P \leq 0.025$ for **(C)** to **(I)**.

(J) Luciferase activity driven from the *M. truncatula FTb1* promoter:LUC construct coinfiltrated with *Pro35S:GUS* (control, denoted as "Con"), *Pro35S:PsCDFc1* (wild type), or *Pro 35S:PsCDFc1* (mutant R450W) and transiently expressed in *N. benthamiana* leaves. Data represents mean ± SE for $n = 8$, 3, and 10 independently infiltrated plants, for control, *PsCDFc1* wild-type, and *PsCDFc1* mutant respectively.

To look for evidence that *LATE2* might act through one or more similar genes in pea, we next annotated all *CCT*, *BBX*, *CIB*/*BEE*-like (*CBL*), *PIF*, and *AP2* family genes in the recently released pea transcriptome and other publicly available pea transcript resources (Franssen et al., 2011; Kaur et al., 2012; Alves-Carvalho et al., 2015). We identified a total of 7 *PRR* genes, 14 *CBL* genes, 12 additional *BBX* genes, 9 *PIF* genes, 5 *AP2* family genes, and 16 additional *CCT MOTIF FAMILY* (*CMF*) genes in pea (Supplemental Figures 7, 8, and 10 to 12). Transcript levels for expressed genes in these families did not differ significantly between the wild type and *late2-1D*, with the exception of *PRR59c*, which showed a small reduction (Figures 7E to 7I; Supplemental Figure 13). In the *late1* mutant, expression of *PRR59c* was significantly elevated relative to the wild type, along with *PRR37b*, *PRR59a*, *CMF2*, and *CMF3*, while *BBX24* showed a small but significant reduction in expression. However, overall, none of these genes in any of the families examined showed comparable expression changes in both *late2-1D* and *late1-2*, suggesting that the absence of *FTb2* induction in these mutants is unlikely to result from misregulation of these genes at the transcriptional level. In addition, consistent with the data in Supplemental Figure 1, we found no significant misregulation of *LATE1*/*GI*, *FKF1*, or other clock-related genes in the *late2-1D* mutant (Supplemental Figure 13), with the possible exception of *LHY*, which showed a reduction in expression at the margin of significance ($P = 0.025$).

In the absence of any candidate transcription factors showing clear coregulation with *FTb2*, we considered the possibility that CDFc1 might be able to repress the transcription of *FTb2* through direct interaction with its promoter. In addition to transcriptional repression of *CO*, Arabidopsis *CDF1* has been reported to associate with the *FT* promoter (Sawa and Kay, 2011; Song et al., 2012), and it has been suggested that *FKF1*-dependent removal of CDF1 may contribute to *FT* regulation in a *CO*-independent manner (Song et al., 2012). As the available pea genome sequence is incomplete in the region of *PsFTb2*, we were unable to isolate the *PsFTb2* promoter, and as an alternative we used the promoter of the *M. truncatula FTb1* gene, which is known to show qualitative induction under LD, similar to *PsFTb2* (Laurie et al., 2011). Figure 7J shows that in the *Nicotiana benthamiana* transient assay system, constructs expressing either the wild-type or the *late2-1D* variant of *PsCDFc1* from the 35S promoter were able to significantly repress expression of the *LUCIFERASE* reporter driven from the *MtFTb1* promoter, relative to control. This result provides evidence that the PsCDFc1 protein is capable of directly repressing the expression of *MtFTb1* and suggests that the same is likely to be true for *PsFTb2*.

DISCUSSION

In comparison to Arabidopsis and cereal systems, our understanding of the genetic mechanisms conferring photoperiod responsiveness in legumes is less advanced. Although several previous studies in pea and soybean have characterized components of the photoperiod response pathway and confirmed the conserved importance of light signaling and the circadian clock (Weller et al., 2001; Hecht et al., 2007; Liew et al., 2009; Xia et al. 2012; Watanabe et al., 2012), the mechanism by which light and clock signals are integrated to confer photoperiod-specific induction of *FT* genes is still not clear. In this study, we characterized a component of the pea photoperiod response pathway, *LATE2*, and identified it as a *CDF* gene that appears to act downstream of light signaling and the circadian clock to repress the expression of *FTb2*, the main photoperiod-regulated *FT* gene in pea.

A Conserved Role for the *LATE2*/*CDFc1* Gene in the Pea Photoperiod Response

In the Arabidopsis photoperiod response pathway, CDF proteins appear to have a primary role as indirect repressors of *FT* expression, acting via transcriptional repression of the direct *FT* activator, *CO*. Light-dependent binding of the FKF1-GI complex to a region near the C terminus of CDF proteins targets them for degradation in the afternoon, which releases CO from repression and allows the induction of *FT* and flowering (Imaizumi et al., 2005; Sawa et al., 2007; Fornara et al., 2009). A similar interaction is seen between potato homologs of these proteins (Kloosterman et al., 2013). In both species, CDF variants with disrupted FKF1 binding domains are no longer subject to posttranslational regulation by the FKF1-GI complex, and this leads to accumulation of CDF protein and persistent repression of *CO* and *FT* transcription (Imaizumi et al., 2005; Kloosterman et al., 2013). The *late2-1D* mutant is a late flowering, dominantly inherited photoperiod response mutant that carries a mutation in a *CDF* gene, *CDFc1*, which is tightly linked to the *LATE2* locus (Figures 1 and 5; Supplemental Figure 2). The mutation directs the substitution of a highly conserved residue (R450W) within the FKF1 binding domain of the CDFc1 protein and impairs its physical interaction with PsFKF1 (Figures 5 and 6), suggesting that LD-specific CDFc1 protein degradation may also be impaired. Consistent with this interpretation, overexpression of this mutant form in transgenic Arabidopsis delays flowering (Figure 6). Together, these findings support the R450W substitution as the cause of the *late2-1D* late-flowering phenotype and indicate an endogenous role for CDFc1 in repression of flowering under noninductive conditions.

Like *LATE2*, the pea *GI* ortholog *LATE1* also participates in photoperiod-specific induction of *FTb2* (Hecht et al., 2007, 2011), and the similarity of late-flowering phenotypes in *late2-1D*, *late1*, and the double mutant is consistent with these two genes acting in the same pathway (Figure 1). In addition, the patterns of rhythmic expression for all three genes (*LATE2*, *FKF1*, and *LATE1*) are similar to their Arabidopsis equivalents (Figure 5D; Supplemental Figure 1; Imaizumi et al., 2005; Fornara et al., 2009), suggesting a similar temporal pattern of interaction within the daily cycle. These data are consistent with the conservation in pea of a mechanism in which orthologs of GI and FKF1 interact to regulate CDF protein stability. The conservation of this mechanism within legumes is also supported by a study showing interaction of FKF1, GI, and CDF homologs in soybean (Li et al., 2013). In the future, it will be of interest to test other facets of this mechanism directly, including FKF1 function and regulation of CDF protein abundance.

Other aspects of the *late2-1D* phenotype raise questions about whether *LATE2* might influence flowering through other mechanisms. We also observed small effects of the *late2-1D* mutation on the expression levels of several genes related to circadian clock function. These differences seem unlikely to represent a significant change to clock function, as they occur at different points in the daily cycle and do not appear to constitute a change in rhythmic phase, nor are they reflected in changes to expression levels or rhythmic phases of other clock-related or clock-regulated genes such as *SN*/*LUX* or *TOC1a*. In this respect, the effects of *LATE2* are different from those previously reported for *LATE1* (Hecht et al., 2007; Liew et al., 2009). In addition, the observed differences are difficult to reconcile with the known functions of these genes in flowering time control (as, for example, *DNE* is known to inhibit flowering in SD, but its expression is lower in the late-flowering *late2-1D* mutant; Figure 3; Supplemental Figure 1). We conclude that these differences are unlikely to be important for the observed effects of *LATE2* on flowering, but further work may be needed to establish this definitively.

The Regulatory Relationship between *CDF* and *CO* Homologs Is Not Conserved in Pea

In contrast to the apparent conservation of the FKF1/GI/CDF mechanism in pea and its role in the regulation of photoperiod-specific *FT* expression, there is no evidence for a conserved role of *CO*-like genes as flowering time regulators. Diurnal expression dynamics of the pea *CO* co-ortholog *COLa* do not conform to those of Arabidopsis *CO* and are not altered in photoperiod response mutants such as *late1* and *dne*, which influence the

circadian clock (Hecht et al., 2007; Liew et al., 2009). The observation that expression of *COLa* under LD conditions was also unaffected by the *late2-1D* mutation (Figure 7A) further indicates that the regulatory relationship between *CDF* and *CO* seen in Arabidopsis and potato is not present in pea and that *COLa* is unlikely to mediate the effects of *LATE2* on *FTb2*. Further support for this conclusion is provided by recent observations that a null mutant for *M. truncatula COLa* does not have a flowering phenotype under LD and that *MtCOLa* does not promote flowering when overexpressed in Arabidopsis (Wong et al., 2014). Interestingly, recent evidence suggests that Group Ia *COL* genes in soybean may contribute to delay of flowering under noninductive conditions (Cao et al., 2015), although the significance of this for the overall response to photoperiod has yet to be explored in detail.

Demonstration that a close homolog of *CO* contributes to flowering time control and the photoperiod response in rice (Hayama et al., 2003) has generated speculation that a "CO/FT" module could be widely conserved as a central feature of the photoperiod response mechanism in angiosperms (Hayama and Coupland, 2004; Izawa, 2007; Valverde, 2011). However, this assumption has increasingly been challenged by observations inconsistent with such a model (Ballerini and Kramer, 2011). The recent study of Simon et al. (2015) is particularly definitive, showing that the characteristic regulation and function of Arabidopsis *CO* arose from a tandem duplication within the Brassicaceae, which placed the *CO* promoter adjacent to multiple DOF binding sites and thereby under the control of CDF proteins. This implies that *CO*-like genes are unlikely to be important for flowering time control in all species, but that where they are, this role has evolved independently from the one well known in Arabidopsis and may involve a different regulatory mechanism or represent convergent evolution. Consistent with these conclusions, *COLa* genes in both pea and *M. truncatula* show a morning-phased expression pattern similar to that of the Arabidopsis *COL1* and *COL2* genes (Figure 7; Ledger et al., 2001; Hecht et al., 2007), which is likely to represent an ancestral, CDF-independent pattern of regulation for this class of *COL* genes (Simon et al., 2015).

LATE2 Does Not Regulate Genes Homologous to Direct Transcriptional Activators of Arabidopsis *FT*

If the pea *CO* ortholog does not mediate the effect of *LATE2* on *FTb2* expression and flowering, then how else might this be achieved? One possibility is that CO function could be assumed by another protein in the wider COL family, or by association of two different proteins, each containing one of the two domains (BBX and CCT) characteristic of COL proteins, as has recently been proposed for *FT*-dependent bolting control in sugar beet (Pin et al., 2012; Dally et al., 2014). Another possibility is that this role may be performed by an unrelated protein that acts in a manner analogous to CO: as an activator of *FTb2* transcription that is itself transcriptionally repressed by *LATE2* under SD. In Arabidopsis, several proteins other than CO have been demonstrated to directly bind to the *FT* promoter and activate transcription, including members of the PIF and CIB families of bHLH transcription factors (Liu et al., 2008, 2013; Kumar et al., 2012). However, in a comprehensive analysis of genes in the *COL*, *CCT*, *BBX*, *PIF*, and *CIB* families, we did not identify any with expression that was dependent on *LATE1* and *LATE2* and correlated with expression of *FTb2* or flowering time (Figure 7). This implies that none of these genes serves as an intermediate in the regulation of *FTb2* expression by *LATE2*.

Other Possible Mechanisms for the *CO*-Independent Response to Photoperiod in Pea

Another explanation for our findings is that a function analogous to that of CO may not exist in pea, and the mechanism for regulation of *FTb2* by FKF1/GI/CDF may be more direct or occur via alternative mechanism(s). Evidence in Arabidopsis suggests that CDF1 protein, in addition to binding to the *CO* promoter (Imaizumi et al., 2005), can also bind directly to the *FT* promoter to repress transcription under noninductive conditions (Song et al., 2012), and the FKF1/GI complex might therefore relieve this direct repression by promoting CDF degradation. Other observations in Arabidopsis indicate that GI can induce *FT* expression independently of *CO*, and this is suggested to operate through interaction with direct repressors of *FT* including *SVP* and *TEM* (Sawa and Kay, 2011) and regulation of miR172 abundance (Jung et al., 2007). Whether or not CDF proteins participate in either of these mechanisms has not been thoroughly examined, but one recent study reports a regulatory link between *AtCDF2* and miR172 levels (Sun et al., 2015). However, as none of the *AP2* family of miRNA172 target genes showed differential expression in our RNA-seq experiment (Supplemental Figure 13), it seems the latter mechanism is unlikely to operate in pea. In Arabidopsis, both FKF1 and GI have also been reported to associate with the *FT* promoter (Imaizumi et al., 2005; Sawa and Kay, 2011), suggesting that they could have a more direct role in activation of *FT* and that interaction between GI, FKF1, and CDF proteins at the *FT* promoter may be significant. The ability of *PsCDFc1*/*LATE2* to repress transcription from the photoperiod-regulated *MtFTb1* promoter (Figure 7J) provides an indication that direct regulation of *FT* genes may indeed be an important mechanism through which CDF proteins regulate flowering in temperate LD legumes and might even form a central feature of the photoperiod response mechanism in this group. Whether PsGI/LATE1 and PsFKF1 are able to activate target *FT* genes directly and how levels of LATE1, LATE2, and FKF1 proteins may be regulated by photoperiod are intriguing questions for future research.

CO-independent mechanisms for *FT* upregulation have been reported in several other species. In rice, the main pathway for photoperiod-specific flowering acts in parallel to *Hd1*/*CO* and involves the action of *EARLY HEADING DATE1*, which encodes a B-type response regulator, with no clear ortholog in Arabidopsis. This gene is in turn suppressed by Ghd7, a CCT domain protein with similarity to wheat VERNALIZATION2 (Trevaskis et al., 2006; Xue et al., 2008). There is also evidence in rice that the Dof transcription factor OsDOF12 can regulate the expression of the *FT* homolog *Hd3a* without effect on *Hd1*/*CO* expression (Li et al., 2009). In soybean, the B3 transcription factor-like gene *E1* is rhythmically regulated and acts downstream of light signaling to repress expression of the *FT* genes *FT2a* and *FT5a* under noninductive LD (Xia et al., 2012). Interestingly, E1 also acts by promoting expression of the *FTb*-class gene *FT4*, an inhibitor of

flowering (Zhai et al., 2014), suggesting that it may have the capacity to act as a transcriptional activator. *E1*-like genes exist in temperate legumes, including pea, but a transcript from the *E1* ortholog was not detected in our RNA-seq data set. Whether these genes may participate in the long-day photoperiod response mechanism is not yet known.

Any explanation for the *CO*-independent integration of light and clock signals for photoperiod responsiveness in pea must also take into account the role of *PHYA*, another important component, which has a similar mutant phenotype to those of *late1* and *late2-1D* (Figure 1). In Arabidopsis, PHYA promotes *FT* expression by regulating CO activity, opposing the COP1-dependent degradation of CO protein (Yanovsky et al., 2001; Valverde et al., 2004; Laubinger et al., 2006; Jang et al., 2008). However, it also mediates light input to the circadian clock (Somers et al., 1998), suggesting the potential to influence the rhythmic expression of *CO* transcript. In addition to its effects on CO, COP1 also acts together with ELF3 to promote degradation of GI (Yu et al., 2008), representing a third potential mechanism through which light could affect *FT* expression. Pea orthologs of all of these genes have been functionally characterized, and it will be interesting in the future to explore the effects of *PHYA*, the *COP1* ortholog *LIP1* and the *ELF3* ortholog *HR* on the expression and/or protein stability of *LATE1* and *LATE2.*

Increasing evidence that the role of *CO* in photoperiodic regulation of flowering time is not widely conserved raises fundamental questions about the evolution of photoperiod sensing mechanisms in plants. At present, our understanding of these mechanisms in species where there is no clear role of *CO*-like genes is in its infancy. In this study, we established an important role for a *CDF* gene in a major crop group where there is mounting evidence against a conserved regulatory role for *CO*-like genes in flowering time. Our results provide evidence that a FKF1/GI/CDF module in pea controls *FT* expression and contributes to photoperiod measurement. However, they also suggest that *CO* homologs are not targets of this module and point to the existence of an alternative mechanism, possibly one in which it is more directly involved in *FT* regulation. In the future, clarification of the precise means by which *LATE2* regulates flowering time will be a key step in understanding photoperiod regulation in legumes and the basis for diversification of photoperiod response mechanisms. One plausible hypothesis is that direct regulation of *FT* genes by a GI/FKF1/CDF module could be an ancestral mechanism for flowering time control that has been reinforced in various taxa by other, diverse mechanisms. It will thus be of interest to determine whether this is a widespread phenomenon.

METHODS

Plant Material, Growth Conditions, and Grafting

As with the previously described *phyA-1* and *late1-2* mutants (Weller et al., 1997; Hecht et al., 2007), the *late2-1D* mutant was derived from EMS mutagenesis of pea (*Pisum sativum*) line NGB5839, a dwarf derivative of 'Torsdag' carrying the *le-3* and *hr* mutations (Lester et al., 1999; Weller et al., 2012) using the mutagenesis protocol described by Weller et al. (1997). Plants used for both the photomorphogenesis experiment (Figure 4) and expression experiments (Figures 2B, 3, and 7; Supplemental Figures 1, 9, and 13) were conducted in growth cabinets at 20°C. All other experiments were conducted in the Hobart phytotron using previously described growth media, light sources, phytotron conditions, and grafting protocols (Hecht et al., 2007). Standard phytotron SD conditions consisted of an 8-h photoperiod of natural light, which was extended with white light from fluorescent tubes and incandescent globes at an irradiance of 10 μmol m^{-2} s^{-1} to provide a LD photoperiod. Branching index (Figure 1B) represents the ratio of the total length of lateral branches to the total height of the main shoot axis.

Mapping

The *late2-1D* × Térèse F2 population consisted of 219 plants. F3 seed from plants displaying the mutant phenotype were screened for segregation of the *late2-1D* phenotype in order to determine the genotype of F2 plants. Markers used in the linkage analysis were designed to target the introns of genes identified in the relevant interval of the *Medicago truncatula* genome (v4.0; www.jcvi.org/medicago) that were also present in pea sequence databases in GenBank (www.ncbi.nlm.nih.gov). Details of these markers and their method of detection are provided in Supplemental Table 2.

Phylogenetic Analysis

For phylogenetic analyses, genes were identified by performing BLAST searches at NCBI (www.ncbi.nlm.nih.gov), Phytozome (phytozome.jgi.doe.gov), and the pea RNA-seq gene atlas (bios.dijon.inra.fr/FATAL/cgi/pscam.cgi), with reciprocal BLAST searches performed against Arabidopsis at TAIR (www.arabidopsis.org) to confirm gene identity. For each alignment (Supplemental Files 1 to 8), full-length amino acid sequences were aligned using ClustalX (Thompson et al., 1997) or the MAFFT algorithm in Geneious (v8.1.7; Biomatters) and adjusted manually, where necessary, using GeneDoc (version 2.7.000; Nicholas and Nicholas, 1997; http://www.psc.edu/biomed/genedoc). Using these alignments, distance-based methods were used for phylogenetic analyses in PAUP* 4.0b10 (http://paup.csit.fsu.edu/), using either 1000 or 10,000 bootstrap replications, as indicated in the figure legends.

Gene Expression Studies

Harvested tissue used in RT-qPCR experiments (Figures 2B, 3, and 7A) consisted of both leaflets from a fully expanded leaf or dissected apical buds (~2 mm) from two plants. Samples were frozen in liquid nitrogen and total RNA extracted using the SV Total RNA Isolation System (Promega). RNA concentrations were determined using a NanoDrop 8000 (Thermo Scientific). Reverse transcription was performed in 20 μL with 1 μg of total RNA using the Tetro cDNA synthesis kit (Bioline). RT-negative (no enzyme) controls were included to monitor for genomic DNA contamination. First-strand cDNA was diluted five times and 2 μL was used in each RT-qPCR. RT-qPCR reactions using SYBR green chemistry (SensiFast; Bioline) were set up with a CAS-1200N robotic liquid handling system (Corbett Research) and run for 50 cycles in a Rotor-Gene Q (Qiagen). Two technical replicates and at least two biological replicates were performed for each sample. Primer details are included in Supplemental Table 3.

Yeast Two-Hybrid Assay

For the yeast two-hybrid assay (Figure 6A), full-length coding sequences of *PsCDFc1* and *AtCDF1* and the KELCH repeats region of *PsFKF1* and *AtFKF1* (Imaizumi et al., 2005; amino acids 296 to 636 and 284 to 619, respectively) were amplified from wild-type pea cv NGB5839 and *Arabidopsis thaliana* (Col-0) cDNA. In addition, the R450W allele of *PsCDFc1*, comprising the full-length coding sequence, was isolated from the *late2-1D* mutant. These sequences were then cloned in-frame into the pCR8/GW/TOPO entry vector (Invitrogen) and transferred by Gateway LR reaction (Invitrogen) into both pDEST32 (bait; BD) and pDEST22 (prey; AD) vectors

(Invitrogen). Primers used to amplify these fragments are provided in Supplemental Table 3. Using the method described by de Folter and Immink (2011), the haploid yeast strains PJ69-4α and PJ69-4A were transformed with bait and prey constructs, respectively, and mated to create diploid colonies containing both interaction partners. Clones containing empty pDEST32 and pDEST22 vectors were also created as controls to test autoactivation. Diploid colonies were subcultured onto Synthetic Complete (SC) medium lacking leucine (Leu) and tryptophan (Trp). Individual colonies were suspended in 200 μL and were directly spotted onto nonselective medium (SC -Leu -Trp) and selective medium also lacking histidine (SC -Leu -Trp -His) with 35 mM 3-amino-1,2,4-triazole added and grown at 30°C for 4 d to test interactions. Negative controls and controls confirming interaction between AtFKF1 and PsCDFc1 are shown in Supplemental Figure 5.

Construct Preparation and Plant Transformation

For the Arabidopsis expression studies (Figure 6; Supplemental Figure 6), pCR8/GW/TOPO entry clones were generated for the wild-type (NGB5839) and R450W (*late2-1D*) alleles of PsCDF1. Entry clones were then linearized with either *Xba*I or *Xho*I (New England Biolabs) and transferred by LR reaction (Invitrogen) into the pB2GW7 vector, which contains the CaMV 35S promoter. The *PsCDF1* entry clones were also transferred by LR reaction into a version of the pB2GW7 vector where the CaMV 35S promoter was replaced with the *SUC2* promoter (Lee et al., 2013). For the transient assays (Figure 7J), the *M. truncatula FTb1* (Medtr7g006630.1) promoter:LUC construct was generated by PCR amplifying from genomic DNA a 453-bp promoter fragment (from an upstream *Hind*III site to the *FTb1* ATG translation initiation site, which was converted to a *Nco*I site; GenBank ID NC_016413.2, chr7:777018-776566) and cloning it into the pGreen 0800-5-LUC binary vector (Hellens et al., 2005). The GUS (*Escherichia coli* uidA) gene was also cloned into the pB2GW7 vector and used as a control in Figure 7J. The binary vectors were transformed into *Agrobacterium tumefaciens* GV3101 by electroporation. Arabidopsis (Col-0) transformation was conducted using the method described by Martinez-Trujillo et al. (2004). For transient expression in *Nicotiana benthamiana* leaves (Figure 7J), infiltrations were performed as described by Hellens et al. (2005), and expression was assayed 5 d after infiltration.

RNA-Seq Experiment and Data Analysis

Plants were grown in constant light for 3 weeks, and three leaflets (one each from leaves 6, 7, and 8) were harvested and pooled from two plants per replicate. As for gene expression studies, samples were frozen in liquid nitrogen and total RNA extracted using the SV Total RNA Isolation System (Promega). RNA concentration and RNA quality were determined using a Fragment Analyzer (Advanced Analytical). Sequencing library preparations were conducted with 1 μg of total RNA using the Truseq Stranded Total RNA library preparation kit with Ribozero Plant (Illumina). Three replicates per genotype were prepared separately. Each replicate was sequenced on a Miseq sequencer using a Miseq Reagent v3 150 cycles kit (Illumina). Quality checking of sequenced reads was performed using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Mapping of sequenced reads was performed using STAR (Spliced Transcript Alignment to a Reference; Dobin et al., 2013) on the pea transcriptome (PsUniLowCopy; Alves-Carvalho et al., 2015), and mapping results are presented in Supplemental Table 4. Raw read counts were obtained using the R package Rsubread (Liao et al., 2013) and normalized using the DESeq method (Love et al., 2014).

Statistical Analysis

For statistical analysis of the data presented in Figures 2, 3, and 7A, a two-tailed unpaired *t* test (with Welch's correction for Figure 3) was performed, while data in Figure 4 were analyzed using a two-way ANOVA with Dunnett's multiple comparison test. Differences between wild-type and mutant normalized count data in Figures 7C to 7I were also subjected to a two-tailed unpaired *t* test, with an adjusted threshold P value of 0.025 to account for the two comparisons performed for each gene. All tests were performed using GraphPad Prism (v6.05; GraphPad Software).

Accession Numbers

The accession numbers for genes newly reported in this study can be found in Supplemental Table 1 and in the supplemental figures.

Supplemental Data

Supplemental Figure 1. Rhythmic expression patterns of circadian clock genes under LD cycles.

Supplemental Figure 2. Position of *LATE2* in pea linkage group VII.

Supplemental Figure 3. Phylogram of the legume CDF family and related DOF transcription factors.

Supplemental Figure 4. Phylogram of the legume FKF1 protein family.

Supplemental Figure 5. Yeast two-hybrid controls.

Supplemental Figure 6. Leaf number at flowering in both wild-type and transgenic Arabidopsis lines expressing wild-type and R450W forms of *PsCDFc1* from the *SUC2* promoter.

Supplemental Figure 7. Phylogram of the legume BBX protein family.

Supplemental Figure 8. Phylogram of the legume CCT protein family.

Supplemental Figure 9. Expression patterns of clock-related and *COL* family genes under constant light.

Supplemental Figure 10. Phylogram of legume CIB/BEE-like (CBL) proteins within the bHLH subfamily XII.

Supplemental Figure 11. Phylogram of legume PIF-like proteins within the bHLH subfamily VII(a+b).

Supplemental Figure 12. Phylogram of the legume AP2 protein family.

Supplemental Figure 13. Transcript levels in expanded leaf tissue from wild-type, *late1-2*, and *late2-1D* mutants grown from sowing under continuous white light.

Supplemental Table 1. Details of sequences for DOF transcription factors used in phylogenetic analyses and alignments.

Supplemental Table 2. Mapping marker details.

Supplemental Table 3. Primer details.

Supplemental Table 4. RNA-seq mapping results.

Supplemental References.

Supplemental File 1. Alignment for Figure 5A.

Supplemental File 2. Alignment for Supplemental Figure 3.

Supplemental File 3. Alignment for Supplemental Figure 4.

Supplemental File 4. Alignment for Supplemental Figure 7.

Supplemental File 5. Alignment for Supplemental Figure 8.

Supplemental File 6. Alignment for Supplemental Figure 10.

Supplemental File 7. Alignment for Supplemental Figure 11.

Supplemental File 8. Alignment for Supplemental Figure 12.

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AUTHOR CONTRIBUTIONS

J.L.W., S.R., R.C.M., V.H., and F.C.S conceived and designed the experiments. S.R., F.C.S., V.H., J.K.V.S., R.L., and J.L.W. performed the experiments. J.B. and G.A. contributed new analytical tools. S.R., F.C.S., V.H., J.K.V.S., J.L.W., R.L., and R.C.M. analyzed the data. S.R., J.L.W., and F.C.S. wrote the article.

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