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DIE NEUTRALIS and *LATE BLOOMER 1* Contribute to Regulation of the Pea Circadian Clock [W]

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The *DIE NEUTRALIS* (*DNE*) locus in garden pea (*Pisum sativum*) was previously shown to inhibit flowering under noninductive short-day conditions and to affect a graft-transmissible flowering signal. In this study, we establish that *DNE* has a role in diurnal and/or circadian regulation of several clock genes, including the pea *GIGANTEA* (*GI*) ortholog *LATE BLOOMER 1* (*LATE1*) and orthologs of the *Arabidopsis thaliana* genes *LATE ELONGATED HYPOCOTYL* and *TIMING OF CHLOROPHYLL A/B BINDING PROTEIN EXPRESSION 1*. We also confirm that *LATE1* participates in the clock and provide evidence that *DNE* is the ortholog of *Arabidopsis EARLY FLOWERING4* (*ELF4*). Circadian rhythms of clock gene expression in wild-type plants under constant light were weaker in pea than in *Arabidopsis*, and a number of differences were also seen in the effects of both *DNE*/*ELF4* and *LATE1*/*GI* on clock gene expression. Grafting studies suggest that *DNE* controls flowering at least in part through a *LATE1*-dependent mobile stimulus, and *dne* mutants show elevated expression of a *FLOWERING LOCUS T* homolog under short-day conditions. However, the early flowering of the *dne* mutant is not associated with altered expression of a previously described *CONSTANS*-like gene. Collectively, our results characterize the clock system and reveal its importance for photoperiod responsiveness in a model legume.

INTRODUCTION

In many species, photoperiod is an important environmental signal influencing the onset of flowering, and rapid advances have recently been made in understanding how plants sense and respond to photoperiod. Most of this progress has come from studies in *Arabidopsis thaliana*, but more recent work has expanded to several other species, including rice (*Oryza sativa*) and the temperate cereals wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*; Hayama and Coupland, 2004; Trevaskis et al., 2007). At the most general level, photoperiodic flowering results from photoperiod-specific expression of genes in the *FLOWERING LOCUS T* (*FT*) family. The biochemical function of FT proteins is unclear, but they have been shown to move from leaf to apex and interact with bZIP transcription factors to regulate inflorescence identity genes (Kobayashi and Weigel, 2007; Turck et al., 2008). While several mechanisms contribute to the photoperiod-specific expression of *FT* genes in the leaf, all appear to involve interactions between light and the circadian clock (Doi et al., 2004; Imaizumi and Kay, 2006; Hayama et al., 2007; Jung et al., 2007; Fujiwara et al., 2008).

Circadian clocks are molecular oscillators that generate output rhythms of ~24 h under constant conditions, which can be entrained to a cycle of exactly 24 h by diurnal variations in light or temperature. The molecular nature of the plant circadian clock is best understood in *Arabidopsis* and is thought to consist of three interlocking negative feedback loops in which myb transcription factors COLD CLOCK ASSOCIATED 1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY) reciprocally regulate the expression of the pseudo-response regulator TIMING OF CHLOROPHYLL *A/B* BINDING PROTEIN EXPRESSION 1 (TOC1) and several other related proteins (Gardner et al., 2006; McClung, 2008). A number of other genes whose biochemical function is less well understood also have an important influence on the clock, including *EARLY FLOWERING 4* (*ELF4*), *GIGANTEA* (*GI*), and *LUX ARRHYTHMO* (*LUX*), which are proposed to be core clock components (Hazen et al., 2005; Locke et al., 2005; McWatters et al., 2007), and *EARLY FLOWERING 3* (*ELF3*), *TIME FOR COFFEE* (*TIC*), and *FAR-RED ELONGATED HYPOCOTYLS 3* (*FHY3*), which are thought to function in gating of light signals to the clock (McWatters et al., 2000; Allen et al., 2006; Ding et al., 2007).

In *Arabidopsis*, up to 15% of genes show rhythmic cycling of transcript abundance under constant conditions, including genes acting in a wide variety of different metabolic processes, emphasizing the importance of circadian regulation for adaptation to the daily light/dark cycle (Gardner et al., 2006; McClung, 2008). The specific importance of the clock for photoperiodic flowering is demonstrated by the circadian regulation of many *Arabidopsis* flowering genes and the fact that many *Arabidopsis* mutants with a primary effect on clock also show altered photoperiod responses. In *Arabidopsis*, the main output mechanism by which the clock controls flowering is through the rhythmic

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regulation of the B-box transcription factor CONSTANS (CO), such that *CO* expression occurs during the light period under long days (LDs) but not short days (SDs) (Suárez-López et al., 2001; Yanovsky and Kay, 2002). More recently, other mechanisms for clock regulation of *FT* have been proposed to act independently of CO, through ELF3 (Kim et al., 2005), *miRNA172* (Jung et al., 2007), and the MADS protein SHORT VEGETATIVE PHASE (Fujiwara et al., 2008).

The nature of the circadian clock in other species is much less well understood than in *Arabidopsis*. One recent study in *Lemna gibba* used RNA interference to address the conservation of the core clock mechanism (Serikawa et al., 2008) and demonstrated important roles for *LHY*, *GI*, and *ELF3* homologs in regulation of *Arabidopsis* CCA1 and *TOC1* reporters in a transient expression system. Although expression studies have been conducted in various other species, functional analyses have otherwise been limited to overexpression studies in rice (Murakami et al., 2003, 2007). The identification of flowering time genes *Heading date 6* in rice and *Photoperiod -H1* in barley as homologs of *Arabidopsis* genes implicated in clock function (Takahashi et al., 2001; Turner et al., 2005) does suggest that photoperiod response in these species also depends on normal function of the circadian system. Comparative studies in rice and potato (*Solanum tuberosum*) have identified *CO*-like genes as clock outputs important for regulation of *FT* expression and photoperiod response (Kojima et al., 2002; Rodríguez-Falcón et al., 2006), and involvement of *CO* in photoperiod-dependent *FT* expression has been inferred from expression studies in poplar (*Populus* spp; Böhlenius et al., 2006). However, CO-independent clock regulation of *FT* genes has also been demonstrated in both rice and *Pharbitis* (Doi et al., 2004; Hayama et al., 2007). suggesting that as in *Arabidopsis*, *CO*-like genes may not be the only clock output necessary for photoperiod-responsive growth and flowering.

Another model system prominent in early genetic studies of flowering time control is garden pea (*Pisum sativum*). Pea is the best-studied legume model for control of flowering, and more than a dozen major flowering loci have been identified, including several that affect photoperiod responsiveness and graft-transmissible signals (Weller et al., 1997; Weller, 2007). We recently identified *LATE BLOOMER 1* (*LATE1*) as the pea ortholog of *Arabidopsis GI* and showed it to be necessary for promotion of flowering, the production of a mobile flowering stimulus, and induction of a *FT* homolog under LD conditions (Hecht et al., 2007). We also described the isolation of pea orthologs of *Arabidopsis* clock genes *LHY* (previously called *MYB1*), *TOC1*, and *ELF4* (Hecht et al., 2005) and showed that *LATE1* influences diurnal expression rhythms of several of these genes (Hecht et al., 2007).

In contrast with *LATE1*, other pea photoperiod response loci inhibit flowering under SD conditions. Mutants at the *STERILE NODES* (*SN*), *DIE NEUTRALIS* (*DNE*), and *PHOTOPERIOD* (*PPD*) loci all flower earlier than the wild type in SDs and show characteristics of LD-grown wild-type plants, such as reduced branching and rapid termination of apical growth (Murfet, 1971; King and Murfet, 1985; Taylor and Murfet, 1996). Little is known about the molecular nature of these loci, but the fact that many early-flowering photoperiod response mutants in *Arabidopsis* affect the circadian clock has suggested that these pea genes may also affect the circadian system (Weller, 2005). We have been characterizing the effect of these mutants on the circadian system and pursuing a candidate gene approach to identify the *SN*, *DNE*, and *PPD* genes (Weller, 2007). In this study, we show that the *DNE* and *LATE1* genes function in the pea circadian clock. We also provide evidence that *DNE* is an ortholog of *Arabidopsis ELF4* and examine its interactions with *LATE1* in the control of flowering and photoperiod responsiveness. Our results make a significant contribution to comparative genetics of the plant circadian clock and identify both similarities and differences with the *Arabidopsis* model. They also argue against the long-standing hypothesis that the photoperiod response in pea is primarily determined through the action of a mobile flowering inhibitor.

RESULTS

The *dne* Mutant Shows Early, Photoperiod-Insensitive Flowering

The *DNE* locus is known from a single mutant allele, *dne-1*, which was isolated in the cv Torsdag genetic background (King and Murfet, 1985). The *dne* mutant flowers early in SDs and shows other traits characteristic of a wild-type plant grown in LDs, including reduced branching, rapid termination of apical growth after flowering, and rapid senescence (Figure 1). The *dne* mutant thus appears to show constitutive activation of a LD developmental program and is essentially unresponsive to photoperiod differences.

This is true regardless of the genotype at the *LE* locus, which governs the synthesis of the active gibberellin, GA_1 (Lester et al., 1997; see Supplemental Figure 1 online). Under LDs, the *dne* mutant does typically flower slightly earlier than the wild type, but the wild type and *dne* are otherwise similar in phenotype (King and Murfet, 1985).

The *dne* Mutant Shows Altered Rhythms of Gene Expression under Light/Dark Cycles

The early-flowering phenotype of the *dne* mutant is similar to that of *Arabidopsis* circadian clock mutants *elf3*, *elf4*, *lux*, and the *cca1 lhy* double mutant (Hicks et al., 1996; Doyle et al., 2002; Mizoguchi et al., 2002; Hazen et al., 2005), and we therefore considered that *dne* might have a defect in rhythmic expression of clock gene homologs. We previously found that the pea genes *LHY*, *TOC1*, *ELF4*, and *LATE1* show LD expression rhythms that are similar to *Arabidopsis* and that these are altered in a *late1* mutant (Hecht et al., 2007).

Figure 2 shows that *dne* had no clear effect on expression of *LHY* under either SDs or LDs, nor on *TOC1* under LDs (Figures 2A and 2B). However, under SDs, the expression rhythm of *TOC1* in the wild type showed a relatively sharp peak at dusk (ZT8) and dropped significantly by ZT12, whereas in the *dne* mutant, *TOC1* expression continued into the night, remaining high at ZT12 (Figure 2A), suggesting a small phase delay. *ELF4* expression in the wild type under SDs showed a sharp peak

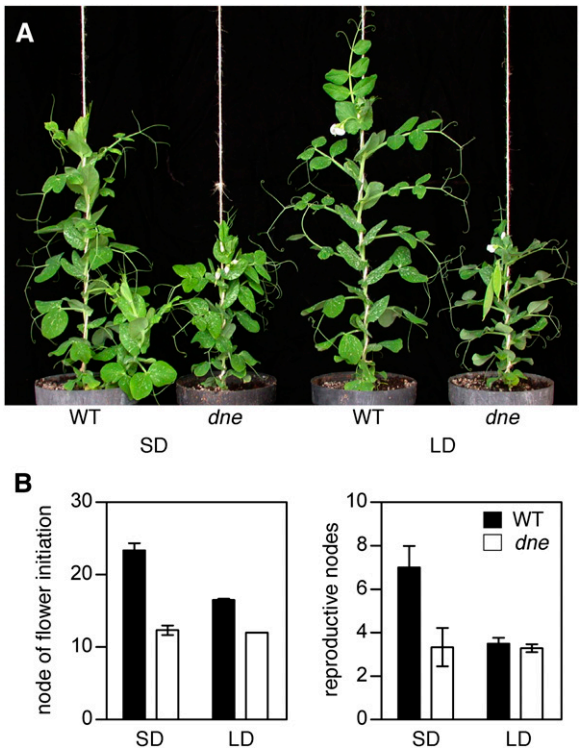

Figure 1. The *dne* Mutant Is Early Flowering and Insensitive to Photoperiod.

(A) Representative 6-week-old wild-type line NGB5839 and isogenic *dne-1* mutant plants.

(B) Node of flower initiation (left) and final number of reproductive nodes. Data are mean ± SE for $n = 6$ to 8 plants.

All plants were grown in the phytotron under standard SD or LD conditions.

early in the night (ZT12), whereas in the *dne* mutant, the peak occurred at dusk (ZT8) (Figure 2A). The earlier rise of *ELF4* expression during the day and the earlier drop during the night are consistent with a phase advance in *dne*. Under LDs, the shape of the wild-type *ELF4* rhythm differed from SD, with a broader peak from ZT12 to ZT16 (Figure 2B). In LDs, the *ELF4* rhythm in *dne* peaked late in the day (ZT12) and also showed a small phase advance relative to the wild type. The *dne* mutation also affected *LATE1* expression under both SDs and LDs (Figures 2A and 2B). There was no clear indication of a phase shift under either photoperiod, but *LATE1* transcript levels were higher in *dne* than in the wild type throughout the night, similar to the effect of the *sn* mutant on *LATE1* (Hecht et al., 2007). Expression of the *TOC1*-related genes *PSEUDO-RESPONSE REGULATOR 37* (*PRR37*) and *PRR59* in SDs was apparently unaffected by *dne* (Figures 2A and 2B), whereas in LDs, expression of *PRR59* in *dne* was elevated relative to the wild type during the night (Figure 2B). In summary, the *dne* mutant affects the diurnal expression of *TOC1*, *ELF4*, *LATE1*, and *PRR59* under SD and/or LD photoperiods but had no apparent effect on *LHY* or *PRR37* expression.

DNE and *LATE1* Affect Rhythms of Clock Gene Expression in Constant Light

We were next interested to examine whether *DNE* might affect circadian rhythms. The circadian clock has not been directly examined in pea, but in *Arabidopsis*, most circadian analyses have been performed under constant white light (LL), and robust rhythms for leaf movement and gene expression generally persist over several circadian cycles. We initially found that the strong expression rhythms seen for *LHY*, *TOC1*, *LATE1*, and *ELF4* in wild-type pea seedlings under diurnal cycles were significantly damped during the first subjective night after transfer to LL of moderate irradiance, resulting in lower peak levels for *LHY* and *ELF4* and higher trough levels for *TOC1* and *LATE1* (see Supplemental Figure 2 online). However, under LL of lower irradiance, clear rhythmic expression was maintained for all four genes through at least one circadian cycle (Figure 3). For *ELF4*, the rhythm was maintained for at least 48 h with a strong amplitude similar to the LD rhythm, whereas rhythms for *LHY*, *LATE1*, and *TOC1* showed some damping by the second circadian cycle, toward trough levels for *LHY* and intermediate levels for *TOC1* and *LATE1* (Figure 3). Although rhythms were only followed for two full circadian cycles, all four genes gave some indication of a shorter period, with peaks 18 h apart for *ELF4* and 21 h apart for *LHY* and *TOC1*.

We also examined the effect of the *dne* and *late1* mutations in the same experiment (Figures 3A and 3B). The clearest effects were seen for *late1*, which showed substantial reduction in the peak expression level for all four genes and little evidence of any residual rhythm (Figure 3B). By contrast, *dne* had more subtle effects, including an apparent small phase advance of *TOC1* and *LATE1* expression in the second circadian cycle, suggestive of a shorter period. Interestingly, there is also a suggestion that the phase difference for *ELF4* expression between wild type and *dne* under light/dark cycles may diminish after transfer to LL.

DNE and *LATE1* Also Affect Rhythms of Clock Gene Expression in Constant Darkness

Rhythmic expression of clock genes also persisted after transfer of wild-type plants from entraining conditions to constant darkness (DD) but differed from LL in several respects (Figure 4). The strongest rhythm in DD was seen for *LHY* expression, which, as in LL, showed only moderate damping over the two circadian cycles (Figure 4A). In contrast with LL, the *ELF4* rhythm was strongly damped in DD, although still clearly rhythmic. Both *ELF4* and *LHY* rhythms showed periods of close to 24 h. *TOC1* expression under DD was not clearly rhythmic and, in contrast with LL, damped to a low rather than intermediate level, appearing to lose the induction during the subjective day, instead of the repression phase during the subjective night in LL (Figure 3). Finally, the rhythm of *LATE1* under DD was very similar to LL both in amplitude and apparent period shortening.

As in LL, *late1* had clear effects on expression rhythms of *LHY* and *ELF4* under DD, essentially eliminating the expression of both genes. The *late1* mutation also appeared to eliminate rhythmic expression of *LATE1* itself, which although expressed, showed no discernable rhythm in *late1* (Figure

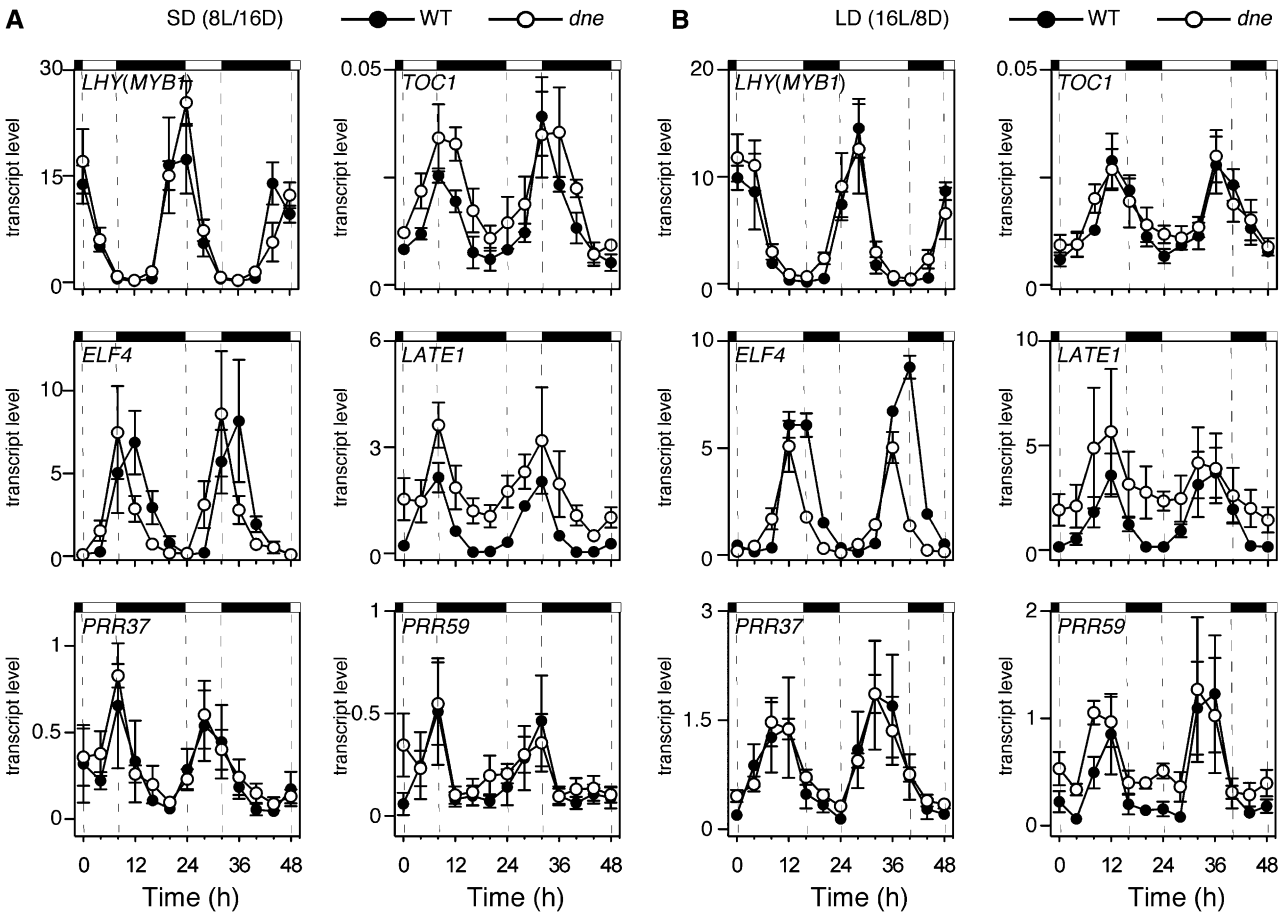

Figure 2. *DNE* Affects Rhythmic Expression of Clock Gene Homologs under Light/Dark Cycles.

(A) SD conditions (8-h photoperiod).

(B) LD conditions (16-h photoperiod).

All plants were grown for 21 d from sowing at 20°C before harvesting commenced. Data are mean ± SE for $n = 3$ biological replicates, each consisting of pooled material from two plants. Day and night periods are indicated by open and closed bars, respectively, above the graph.

4B). *TOC1* expression was already low and essentially arrhythmic in DD in wild-type seedlings and was not significantly affected by *late1*. In the *dne* mutant, *LHY* expression continued to cycle in DD, but with an apparently shorter period, with peaks at ZT45 and ZT60 compared with ZT48 and ZT69 in the wild type. No clear effect of *dne* on *TOC1* or *LATE1* expression was detected, but the residual rhythm of *ELF4* itself was also altered in the *dne* mutant under DD, with a lower peak expression level and a phase advance of ~8 h compared with the wild type (Figure 4A).

Genetic and Physiological Interaction of *DNE* and *LATE1* in the Control of Flowering

As both *DNE* and *LATE1* appear to have a primary influence on the circadian clock, it seemed possible that both genes might affect flowering through the same pathway, and to test this, we constructed a *dne late1* double mutant. Figure 5A shows that, under LD, the *dne late1* mutant is similar in overall appearance to the *late1* single mutant, with delayed senescence, increased branching, and an increased number of reproductive nodes compared with the wild type. Despite these similarities, the double mutant initiated the formation of its first flower at a much lower node than in the single *late1* mutant (Figure 5B). Interestingly, however, the growth of flowers at the first few reproductive nodes of *dne late1* plants was arrested at an early stage (Figure 5A, inset), and fully developed, open flowers were not produced until approximately the node at which flowering commenced in the *late1* single mutant (Figure 5A). The *dne* mutation was thus clearly able to promote the initiation of flowering in the absence of *LATE1*, but *LATE1* clearly influenced the subsequent development of these early-initiated flower primordia and was epistatic to *DNE* in other respects.

Previous studies suggested that *LATE1* is necessary for the production of a mobile stimulus of flowering (Hecht et al., 2007), and the *LATE1–DNE* interaction raised the possibility that *DNE* might act, in part, through the same mobile signal. Figure 5C shows that under SDs, *dne* graft stocks possessing three or four

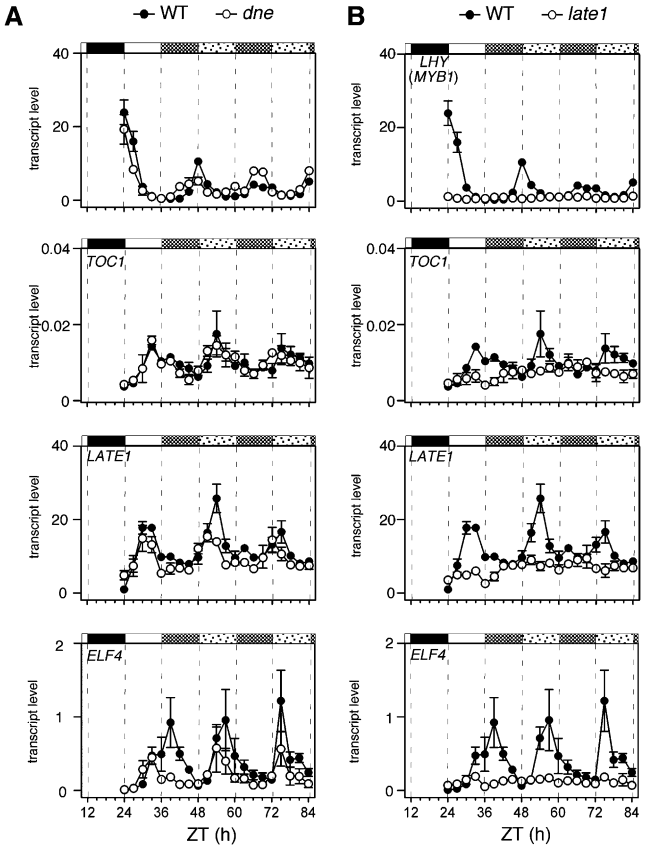

Figure 3. *DNE* and *LATE1* Affect Circadian Rhythms of Clock Gene Homologs in LL.

Plants were grown in growth cabinets under a light/dark cycle (12L:12D) at 20°C for 21 d before transfer to continuous white light at 25 μmol m^{-2} s^{-1} at ZT36. Data are mean ± SE for $n = 2$ biological replicates, each consisting of pooled material from two plants. Zeitgeber time (ZT) refers to the time since lights-on of the last full entraining cycle. Bars above the graph refer to periods of light (open or stippled bars) or darkness (closed bars). The heavy and light stippled bars refer to periods of subjective night and subjective day, respectively, during the period of continuous light.

(A) Expression of clock genes in the wild type and *dne*.

(B) Expression of clock genes in the wild type and *late1*.

true foliage leaves strongly promoted flowering of wild-type scions relative to wild type–on–wild type self-grafts. By contrast, flowering of *dne* scions grafted to wild-type stocks was not significantly delayed compared with *dne* self-grafts (P = 0.74). This implies that the early flowering of the *dne* mutant in SDs is associated with increased production of a mobile stimulus, rather than reduced production of an inhibitor as previously suggested (King and Murfet, 1985). Moreover, in *dne late1* double mutant stocks, the ability of *dne* to promote flowering of wild-type scions was completely blocked by the *late1* mutation (Figure 5C). This suggests that *LATE1* not only controls a mobile flowering signal in LD, but also acts downstream of *DNE* in the regulation of a similar signal in SDs.

Effects of *DNE* on Expression of *CO* and *FT*-Like Genes

In *Arabidopsis*, one of the main ways the circadian clock influences flowering is through control of the expression rhythm of the *CO* gene. Pea and *Medicago* both possess a single group Ia *CO*-like gene (*COLa*) that is orthologous to the *CO*/*COL1*/*COL2* clade in *Arabidopsis* and shares the diurnal expression pattern of *COL1* and *COL2* but not *CO* (Hecht et al., 2007; P. Diwadkar, R.E. Laurie, and R.C. Macknight, unpublished data). In a previous study, we showed that although *late1* affected the diurnal regulation of several clock-related genes and impaired the induction of an *FT* homolog, *FTL*, there was no clear effect of *late1* on the expression rhythm of *COLa* (Hecht et al., 2007). Figure 6A shows that there was also no significant difference in the expression rhythm of *COLa* under SDs between the wild type and *dne*.

We also examined whether the *dne* mutation might also affect expression of *FTL* and how *dne* and *late1* might interact in this respect. Figure 6B shows that in SDs, where the early-flowering phenotype of the *dne* mutant is most evident, *FTL* expression in leaf tissue was significantly higher in *dne* than in the wild type. In LDs, *FTL* expression was significantly lower in *late1* than in the wild type, consistent with our previous report (Hecht et al., 2007), whereas the expression level in *dne* was not significantly different from wild type (P = 0.19). However, *FTL* expression in the *dne late1* double mutant was low like the *late1* single mutant (Figure 6), despite the fact that it initiated flowering much earlier (Figure 5). Taken together, these results suggest (1) that *DNE* and *LATE1* interact to control flowering and other photoperiodic traits through a mobile signal; (2) that regulation of *FTL* expression correlates with the response to photoperiod; and (3) the promotion of flower initiation by *DNE* in LDs can occur independently of *LATE1* and transcriptional regulation of the *FTL* gene.

DNE Is the Likely Pea Ortholog of *Arabidopsis ELF4*

The results from expression analyses demonstrate that the *dne* mutant affects the rhythmic expression of clock genes, suggesting that *DNE* might itself be a homolog of a known clock-related gene. We therefore investigated whether any homologs of known clock-related genes were located in the *DNE* genomic region, making use of the genomic resources of the related legume *Medicago truncatula*. Database searches identified a *Medicago ELF4* homolog that mapped to a region of chromosome 3 syntenic to the region of pea linkage group III containing the *DNE* locus (see Supplemental Figure 3 online), suggesting that Ps *ELF4* (monitored in the expression experiments in Figures 2 to 4 above) could be a candidate for *DNE*. We therefore extended the previously reported partial sequence of Ps *ELF4* (Hecht et al., 2005) to obtain a full-length cDNA. The Mt *ELF4* and Ps *ELF4* genes cluster with other legume *ELF4*-like genes and At *ELF4* in a well-supported clade of apparent *ELF4* orthologs (see Supplemental Figure 4 online). Three other *ELF4*-like (*ELF4-L*) sequences from *Medicago* cluster in another well-supported clade with previously described *Arabidopsis ELF4*-like genes *ELF4-L2*, *ELF4-L3*, and *ELF4-L4* (Khanna et al., 2003; see Supplemental Figure 4 online). We mapped Ps *ELF4* close to marker R12_320, previously shown to be closely linked to *DNE* (Rameau et al., 1998) (see Supplemental Figure 3 online),

confirming the conserved genomic location of these genes in pea and *Medicago*. Sequencing of Ps *ELF4* from *dne-1* revealed a mutation predicted to replace Gln-64 (CAG) with a stop codon (TAG) (Figure 7A), which cosegregated perfectly with the early-flowering phenotype in >500 progeny from segregating families. This shows that *DNE* is tightly linked to the *ELF4* gene at a distance of <0.2 centimorgans.

Alignment of ELF4 sequences revealed a highly conserved central domain, but little sequence similarity in the short C- and N-terminal extensions (Figure 7A). As the truncated ELF4 protein in the *dne* mutant would lack most of the conserved central domain, it is likely to be largely functionally inactive, and we tested this by complementation in *Arabidopsis*. *Arabidopsis elf4* mutants are unable to sense daylength and flower early under both LDs and SDs, with elongated hypocotyls and petioles (Doyle et al., 2002; McWatters et al., 2007). Figures 7B to 7E

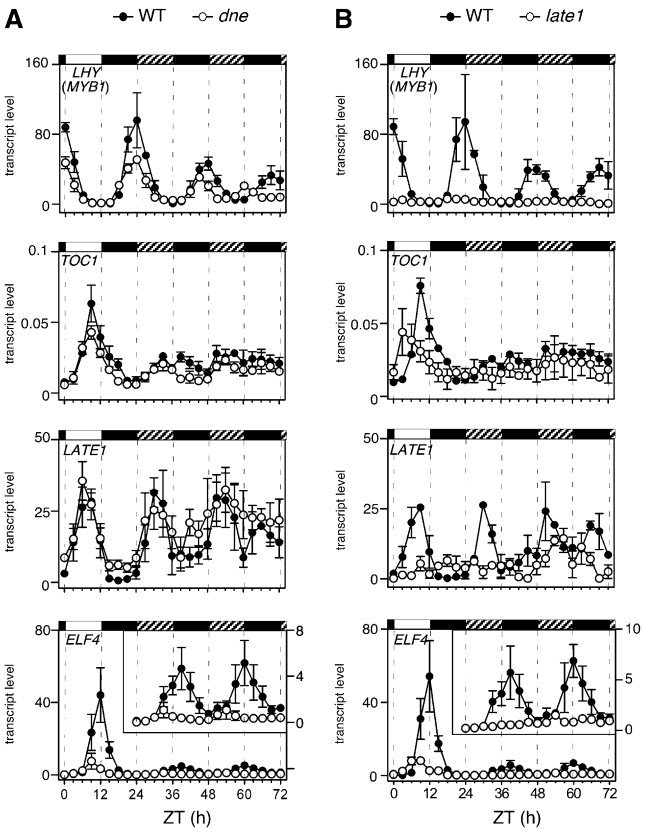

Figure 4. *DNE* and *LATE1* Affect Circadian Rhythms of Clock Gene Homologs in DD.

Plants were grown in growth cabinets under a light/dark cycle (12L:12D) at 20°C for 3 weeks before transfer to continuous darkness. Data are mean ± SE for $n = 2$ to 3 biological replicates, each consisting of pooled material from two plants. Zeitgeber time (ZT) refers to the time since lights-on of the last full entraining cycle. Bars above the graph refer to periods of light (open bars) or darkness (closed or hatched bars). The hatched bars indicate the periods of subjective day during the period of continuous darkness.

(A) Expression of clock genes in the wild type and *dne*.

(B) Expression of clock genes in the wild type and *late1*.

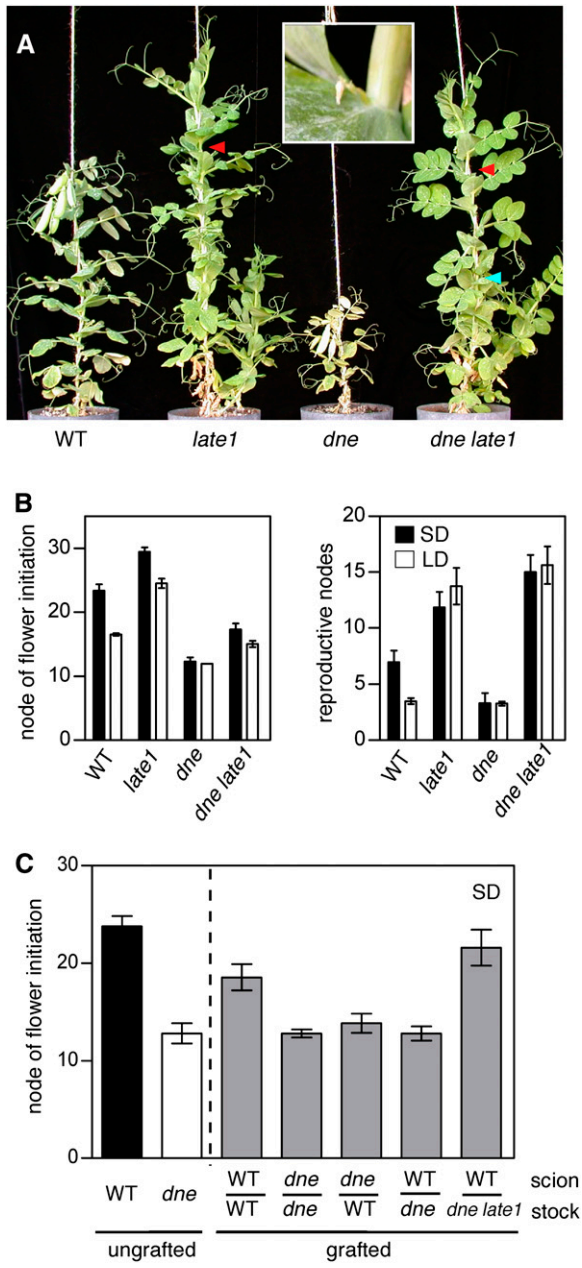

Figure 5. Interaction of *LATE1* and *DNE* in the Control of Flowering.

(A) Representative 8-week-old plants grown under LDs. Inset shows the first initiated flower primordium in the *dne late1* mutant, which is also indicated by the blue arrowhead in the main panel. The red arrowheads indicate the node of first open flower in *late1* and *dne late1*.

(B) Node of flower initiation (left) and final number of reproductive nodes (right). Data are mean ± SE for $n = 6$ to 8 plants.

(C) Node of flower initiation in ungrafted controls, self-grafts, and reciprocal grafts between the wild type, *dne*, and *dne late1*. Data are mean ± SE for $n = 12$ plants.

All plants were grown in the phytotron under standard SD or LD conditions.

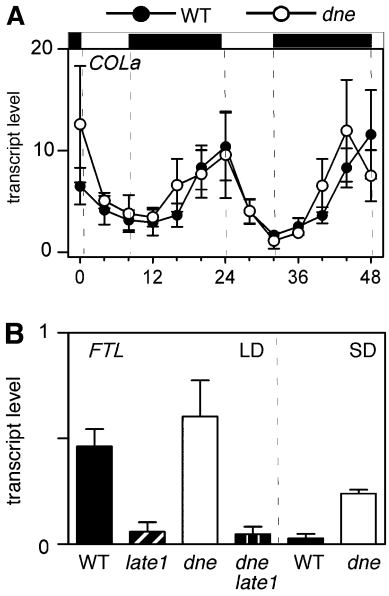

Figure 6. Effect of *dne* on Expression of *CO* and *FT* Homologs.

(A) Diurnal rhythms of *COLa* transcript accumulation in 21-d-old plants grown in SDs.

(B) Relative transcript levels of *FTL* in LDs (20-d-old plants) and SDs (35-d-old plants).

All plants were grown in growth cabinets at 20°C under either SD (8 h) or LD (16 h) conditions. Data are mean ± SE for $n = 3$ biological replicates, each consisting of pooled material from two plants.

show that Ps *ELF4* expressed under the control of the 35S promoter complemented the *Arabidopsis elf4-1* mutation under both LDs and SDs, strongly delaying flowering in a manner similar to *35S:*At *ELF4*. By contrast, overexpression of Ps *ELF4* carrying the *dne-1* mutation had much less of an effect than wild-type Ps *ELF4* under both photoperiods despite comparable expression levels (see Supplemental Figure 5 online), and plants continued to produce elongated petioles, suggesting the Ps *ELF4* activity had been mostly eliminated by the *dne-1* mutation. Under SDs, however, flowering time was significantly later than *elf4-1*, suggesting some residual function of the truncated *dne-1* protein (Figure 7). Nevertheless, the strong impairment of Ps ELF4 function caused by this mutation and the tight cosegregation of the *dne* mutant phenotype with the mutation strongly support a conclusion that *DNE* is Ps *ELF4*.

DNE Also Regulates Stem Elongation

In addition to effects on flowering, the circadian clock regulates other traits, including rhythmic regulation of hypocotyl elongation and leaf expansion (Dowson-Day and Millar, 1999). As *Arabidopsis ELF4* is also proposed to function in phyB-mediated deetiolation (Khanna et al., 2003), we also examined deetiolation in the *dne* mutant. Mutant *dne* seedlings were indistinguishable from the wild type in both darkness and white light but showed shorter internodes under red, blue, and far-red light, with the proportionately strongest effect seen under blue (Figure 8A). This is in clear contrast with the elongated hypocotyl phenotype seen in the *elf4* mutant (Khanna et al., 2003). By contrast, the *dne* mutant and the wild type did not differ in leaf expansion in darkness or under any light condition (Figure 8A).

We also examined whether Ps *ELF4* could complement the hypocotyl elongation phenotype of the *Arabidopsis elf4* mutant. Figure 8B shows that overexpression of Ps *ELF4* in the *Arabidopsis elf4-1* mutant resulted in shortened hypocotyls that were comparable in length to the wild type (Wassilewskija [Ws]). No change in hypocotyl length was observed in seedlings overexpressing the *dne-1* mutant ELF4 protein, supporting the conclusion from the flowering-time experiment (Figure 7) that the *dne-1* mutation severely impairs Ps ELF4 protein function. This result also suggests that the difference in elongation phenotype of the *dne* and *elf4* mutants is due to a species-specific context for DNE/ELF4 protein function, rather than being inherent to the two proteins.

DISCUSSION

Early studies of photoperiod-responsive flowering in pea centered on the physiological and genetic analysis of three loci necessary for inhibition of flowering under noninductive SD photoperiods: *SN*, *DNE*, and *PPD* (Murfet, 1971; King and Murfet, 1985; Taylor and Murfet, 1996). More recent studies have identified genes necessary for promotion of flowering under inductive LD photoperiods, including *PHYA* (Weller et al., 2004) and *LATE1*, the ortholog of *Arabidopsis GI* (Hecht et al., 2007), but the primary physiological role and molecular nature of the *SN*, *DNE*, and *PPD* loci have remained unclear. Here, we show that *DNE* is necessary for the normal rhythmic regulation of circadian clock genes and identify *DNE* as the pea ortholog of *Arabidopsis ELF4*.

ELF4 is thought to be a core component *Arabidopsis* circadian clock and functions in the *CCA1/LHY-TOC1* feedback loop of the central oscillator. *ELF4* also plays a role in the entrainment of the clock, functioning as part of the light input pathway. Despite these important roles, little is known about the function of *ELF4*-like genes outside of *Arabidopsis*, and true orthologs of *ELF4* may not exist in monocots (Khanna et al., 2003; Murakami et al., 2007). The identification of *DNE* thus provides the first opportunity to examine the function of this gene in another species.

Rhythmic Expression of Pea Clock Genes

Diurnal expression rhythms described here for *LHY*, *TOC1*, *ELF4*, and *LATE1* are consistent with a previous report (Hecht et al., 2007) and are similar to those of the corresponding *Arabidopsis* genes (Fowler et al., 1999; Matsushika et al., 2000; Doyle et al., 2002; Mizoguchi et al., 2002) with peak expression of *LHY* in the morning and peak expression of *TOC1*, *ELF4*, and *LATE1* in the evening. Clearly rhythmic expression is also seen for pea genes under low irradiance LL, but these rhythms are strongly damped at higher irradiances (Figure 3; see Supplemental Figure 2 online). A direct comparison with *Arabidopsis* data is difficult due to incomplete reporting of growth conditions in many published studies, but it is clear that strong rhythms do persist

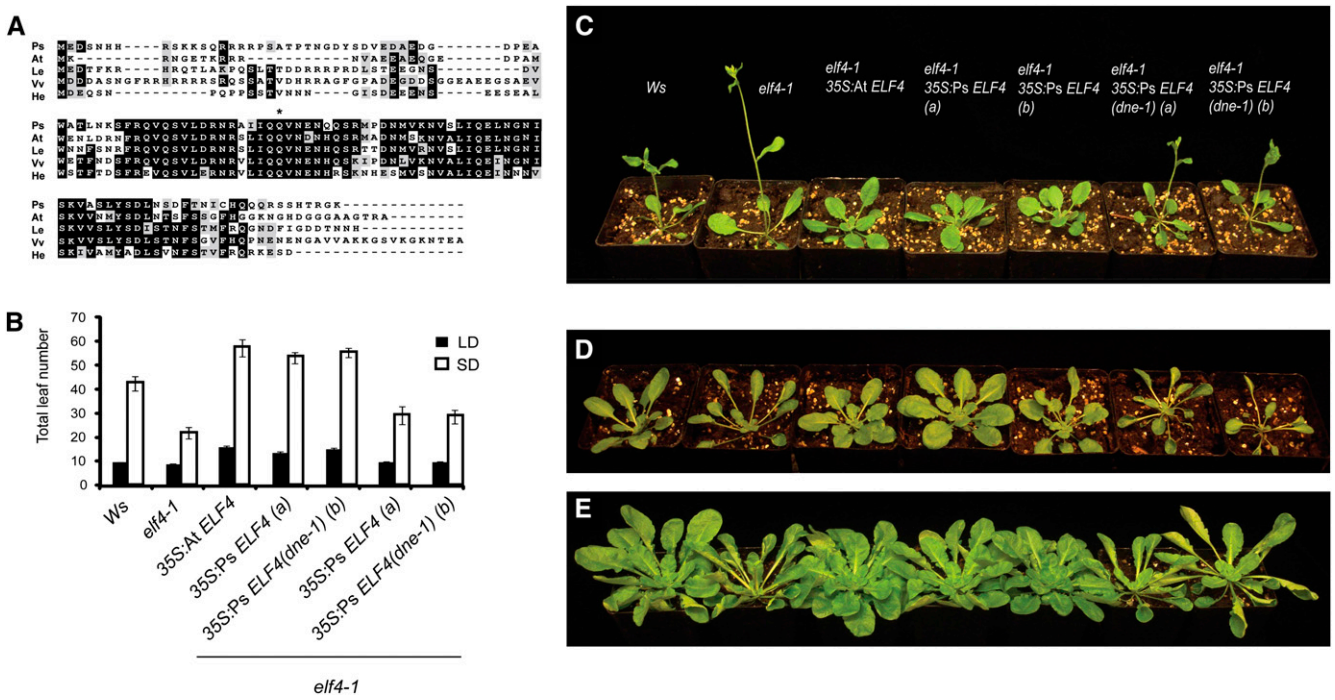

Figure 7. *DNE* Is the Likely Pea Ortholog of *Arabidopsis ELF4*.

(A) Alignment of ELF4 protein sequences. Conserved amino acids are shaded in black, and the location of the Gln-64 residue altered by the *dne-1* mutation is indicated by an asterisk. Ps, *Pisum sativum*; At, *Arabidopsis thaliana*; Le, *Lycopersicon esculentum*; Vv, *Vitis vinifera*; He, *Helianthus exilis*.
(B) to **(E)** Complementation of the *Arabidopsis elf4* mutant. All plants were grown in growth cabinets at 20°C in either LD (16 h) or SD (8 h) conditions.
(B) Total leaf number at flowering of *Arabidopsis* plants in LDs and SDs. Data are mean ± SE for $n = 3$.
(C) Representative plants grown in LDs. Flower induction has occurred in *elf4-1* and *elf4-1* expressing mutated Ps *ELF4* (*dne-1*), whereas other plants have not yet flowered.
(D) Representative plants grown in SDs, showing elongated petioles in *elf4-1* and *elf4-1* expressing mutated Ps *ELF4* (*dne-1*), whereas other plants have normal petiole length.
(E) Representative plants grown in SD, showing flower induction in *elf4-1* and *elf4-1* complemented with mutated Ps *ELF4* (*dne-1*), whereas other plants have not yet flowered.

for all four genes under LL irradiances above 60 μmol m^{-2} s^{-1} (e.g., Park et al., 1999; Alabadi et al., 2001; Doyle et al., 2002; Mizoguchi et al., 2002; Hazen et al., 2005). In addition, even under low-irradiance LL, pea genes show signs of damping by the second circadian cycle. These apparent differences deserve further study but in general do suggest that pea and *Arabidopsis* differ in their regulation by light.

In *Arabidopsis*, selective impairment of circadian rhythms under LL is reported for mutants in *ELF3*, *TIC*, *LUX*, and *FHY3*, genes that are all thought to have a role in input of light signals to the clock (Hicks et al., 1996; McWatters et al., 2000; Covington et al., 2001; Hall et al., 2003; Hazen et al., 2005; Allen et al., 2006). One explanation for the suppressed rhythmicity seen for several pea genes under higher irradiances of LL could be that our standard wild-type line NGB5839 is a natural mutant with reduced function of one of these genes or in another gene needed for light input to the clock. In the future, this could presumably be evaluated using standard release-from-light and phase-response assays. It will also be important to determine whether this unusual circadian phenotype is common to other cultivars or, indeed, to the entire species. In this respect, it is notable that most garden pea cultivars (including NGB5839) and many spring-sown field pea cultivars carry recessive alleles at the *HIGH RESPONSE* (*HR*) locus (Murfet, 1973; Lejeune-Hénaut et al., 2008), which confer earlier flowering under SDs and a reduction in the flowering response to photoperiod. The light input mutants *elf3*, *lux*, and *tic* are also early flowering in short days (Zagotta et al., 1996; Hall et al., 2003; Hazen et al., 2005), and it will therefore be of interest to test if the weaker LL rhythms we observe reflect loss of *HR* function.

In addition to the unexpected damping or loss of rhythms under LL, we also observed differences in the expression rhythms of pea clock genes in DD in comparison to their *Arabidopsis* counterparts. The patterns of *ELF4* and *TOC1* expression in DD are similar in pea and *Arabidopsis*, with both rhythms damping rapidly to trough and median levels, respectively (Strayer et al., 2000; Doyle et al., 2002), and rhythmic *GI*/*LATE1* expression persists in both species (Park et al., 1999; Figure 4). However, the persistence of rhythmic Ps *LHY* expression in DD (Figure 4) differs from expression patterns of *CCA1* and At *LHY*, which both damp rapidly to near trough levels (Wang and Tobin, 1998), and the residual low amplitude rhythm we

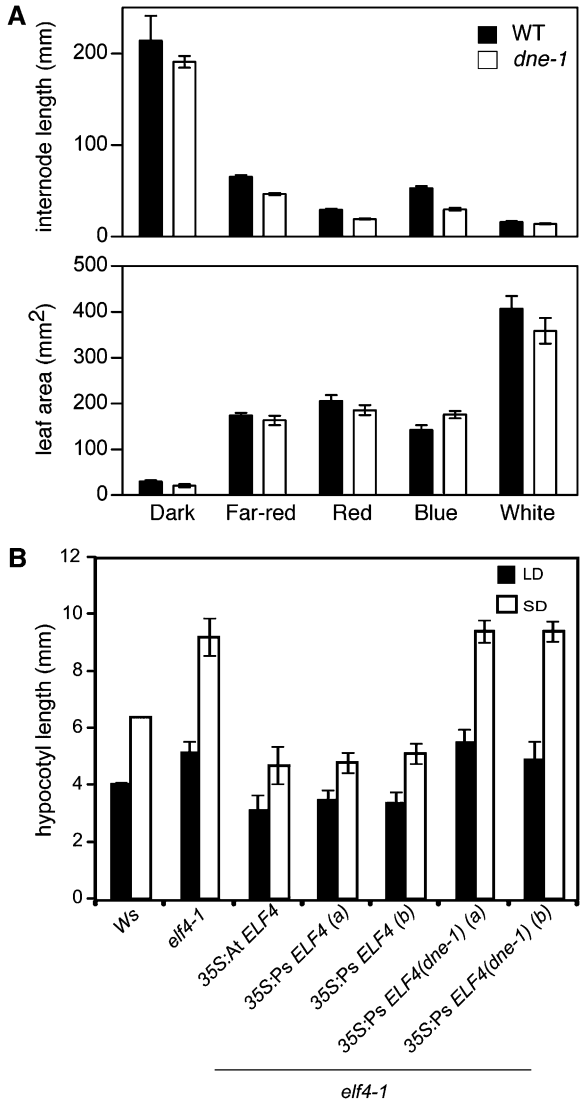

Figure 8. Effects of *DNE* on Stem Elongation.

(A) Effect of the *dne* mutation on spectral sensitivity for deetiolation responses. Seedlings were grown for 14 d from sowing under continuous light or darkness. Internode elongation was quantified as the length between nodes 1 and 3, and leaf area was estimated as the product of the length and width of a single leaflet from leaf 3.

(B) Hypocotyl length of *Arabidopsis* plants showing elongated hypocotyl in *elf4-1* and *elf4-1* complemented with mutated Ps *ELF4* (*dne-1*) compared with normal hypocotyl lengths in wild-type plants and in *elf4-1* mutant plants complemented with wild-type Ps *ELF4* (*DNE*).

All plants were grown in growth cabinets under continuous far-red, red, or blue light at 15 μmol m^{-2} s^{-1} **(A)** or white light at 100 μmol m^{-2} s^{-1} (**[A]** and **[B]**). Data are mean ± SE for n = 8 to 12 **(A)** or n = 12 to 20 **(B)**.

observed for pea *ELF4* is not apparent in the *Arabidopsis* data (Doyle et al., 2002). In addition, whereas in *Arabidopsis* the period of expression for several clock genes in DD is generally longer than 24 h (Hicks et al., 1996; Wang and Tobin, 1998), the rhythms of Ps *LHY* and *LATE1* expression rhythms under DD appeared significantly shorter than 24 h.

In summary, despite the fundamental importance of the circadian clock, significant differences in the expression of clock genes are evident between *Arabidopsis* and pea. This suggests that the function of clock components and mechanisms for clock entrainment may differ between plant species. Interestingly, a similar conclusion has been drawn from work in another legume, *Phaseolus* (Kaldis and Prombona, 2006).

Roles of *DNE* and *LATE1* in Diurnal and Circadian Rhythms

To assess the effect of the *dne* mutation on the pea circadian clock, we analyzed the expression of pea clock genes under both LL and DD. We found that in contrast with *Arabidopsis elf4* mutations, which severely impair rhythmic expression of *LHY*, *CCA1*, *TOC1*, and *ELF4* under LL (Doyle et al., 2002; McWatters et al., 2007), *dne* has only relatively minor effects on phase of *TOC1*, *LATE1*, and *ELF4* in LL, and all four genes cycle with amplitude similar to the wild type. In DD, the *dne* mutation also causes a reduction in amplitude and a phase advance in the *DNE* rhythm and an apparent period shortening of the *LHY* rhythm in DD. Less is known about the effects of *Arabidopsis elf4* in DD except that, as in LL, it severely impairs the *CCA1* expression rhythm (Doyle et al., 2002). Overall, these results suggest that *DNE* may have a more subtle role in clock regulation than *Arabidopsis ELF4*, that there may be a greater degree of redundancy within the family of *ELF4*-like genes in pea, or that there may be some residual DNE activity in the *dne-1* mutant, as suggested from the *Arabidopsis* complementation experiments. However, regardless of which of these explanations may be true, it is evident that a strong effect of the *dne-1* mutation on photoperiodic flowering is associated with only relatively minor effects on circadian rhythms of clock gene expression.

Under LD cycles, *Arabidopsis elf4* had no effect on *CCA1* expression, while under SDs the *CCA1* rhythm in *elf4* showed a reduced ability to anticipate dawn and an increased sensitivity to light immediately after dawn (McWatters et al., 2007). By contrast, the pea *LHY* rhythm was not significantly affected by *dne* under SDs or LDs, and we found no clear evidence for impaired anticipation of dawn in *dne*, although this could in part reflect the lower resolution of our measurements and in future should be examined in more detail. It will also be interesting to examine whether *DNE*, like *Arabidopsis ELF4*, has a role in the light induction of *LHY* and in the gating of light signals to the clock. It is notable that in SDs the *dne* mutation causes a phase delay in *TOC1* expression but a phase advance in expression of *DNE* itself, despite both genes being expressed in the evening. This is difficult to reconcile with a primary effect of *DNE* on the core clock mechanism and may instead reflect a role in light input. Such a role may also be suggested by the fact that the timing of peak *TOC1* expression is less sensitive to photoperiod in *dne* than in the wild type.

Other comparisons also suggest that *DNE* does not have a simple interaction with the putative core clock components *LHY* and *TOC1*. For example, in DD, where *dne* clearly affected the *LHY* rhythm, it had little effect on expression of the evening genes *TOC1* and *LATE1* (Figure 4A), but under SD cycles, where the rhythm of *LHY* was not affected, *dne* did affect both *TOC1* and

LATE1 (Figure 2A). This could imply either that *dne* mutation may independently affect light signaling to *LHY* and *TOC1* and/or that it may affect the coupling of *LHY* and *TOC1* expression. In *Arabidopsis*, most analyses of the core clock mechanism have been conducted in constant light, where coupling of antiphased *CCA1*/*LHY* and *TOC1* expression rhythms are normally observed. However, it has been shown in deetiolating *Arabidopsis* seedlings that *ELF4* can act independently of *TOC1* to regulate *CCA1*/*LHY* expression and that rhythmic *TOC1* expression does not completely depend on the regulation of *CCA1*/*LHY* (Kikis et al., 2005). The presence of an additional factor X necessary for the coupling of the *TOC1*-*GI* loop to *CCA1*/*LHY* has been predicted from computational modeling, and *ELF4* has been proposed as one candidate for X (Locke et al., 2005; Zeilinger et al., 2006). Given the complexity of the circadian clock, more detailed comparisons between pea and *Arabidopsis* will require the application of similar modeling approaches in both species.

We previously showed that *LATE1* has a role in regulating diurnal rhythms in expression of several clock genes under LD cycles (Hecht et al., 2007), implying that *LATE1* might function in the circadian clock, and this has now been confirmed by the finding that *late1* eliminates rhythmic expression of *LHY* and *DNE* after transfer to LL or DD (Figures 3B and 4B). Thus, without *LATE1* function, several putative core components of the pea circadian clock are significantly misregulated under both constant and photoperiodic conditions. The effects of *late1* on *LHY* and *TOC1* expression in LL are similar to those reported for *gi* mutants in *Arabidopsis* (Fowler et al., 1999; Park et al., 1999; Mizoguchi et al., 2005; Gould et al., 2006), whereas effects of *gi* on *ELF4* expression have not been reported. A major role of *GI* in the *Arabidopsis* clock is suggested to be the light-dependent regulation of TOC1 protein stability (Kim et al., 2007), but computational modeling also suggests that *GI* may contribute to the function of a hypothetical clock component Y necessary for regulation of *TOC1* expression (Locke et al., 2006). Under LD cycles, *gi* mutations (in contrast with *late1*) reduce the amplitude but do not affect the phase of *LHY* expression. This suggests that either the mechanisms by which *GI* and *LATE1* affect *LHY* expression may be fundamentally different or that the additional effect of *late1* on *LHY* phase may be a combined effect of the *late1* and *hr* mutations.

Coupling of *DNE* and *LATE1* to Flowering Output Pathways

Previous models for flowering in pea proposed that the *dne* mutation blocks production of a mobile inhibitor of flowering in SDs (King and Murfet, 1985), a suggestion difficult to reconcile with current understanding of photoperiodic flowering in *Arabidopsis*, where the primary target of clock regulation (FT) acts as a mobile stimulus (Turck et al., 2008). However, we show here that in grafts with leafy donor tissue, the major effect of *DNE* in SDs is to inhibit a graft-transmissible flowering stimulus (Figure 5C) and that increased ability of the *dne* mutant to promote flowering across a graft union is associated with elevated expression of the *FT*-like gene *FTL*. The correlation between flowering time, effect on a mobile stimulus, and *FTL* expression is also seen for the late-flowering photoperiod response mutant *late1* under LDs (Hecht et al., 2007).

Together, these results are superficially similar to those from *Arabidopsis* showing that the induction of *FT* expression is necessary for the LD response and that *ELF4* and *GI* act in opposite ways to regulate expression of *FT* (Suárez-López et al., 2001; Doyle et al., 2002). In *Arabidopsis*, this regulation occurs at least in part through *CO* (Doyle et al., 2002; Mizoguchi et al., 2005). However, as reported previously for the *late1* mutant (Hecht et al., 2007), *dne* also had no effect on the *COLa* expression rhythm under conditions where its flowering phenotype is strongest (Figure 6A). It is possible that other *COL* genes may have assumed the function of *Arabidopsis CO*, and this can now be addressed using reverse genetics (Hecht et al., 2005; Dalmais et al., 2008; Tadege et al., 2008).

We also used a *dne late1* double mutant to examine the interaction between *DNE* and *LATE1* in control of flowering. In most respects *late1* and *dne* show a straightforward interaction in which *late1* is epistatic to *dne*, with respect to overall phenotype in both SDs and LDs (Figures 5A and 5B) and graft-transmissible effects on flowering in SDs (Figure 5C), suggesting that *LATE1* is necessary for *DNE* effects on both a mobile flowering stimulus and on general photoperiod responsiveness. However, a more complex interaction between *dne* and *late1* in the control of flower initiation is suggested by the early flower initiation of flower primordia in the *dne late1* double mutant. This distinct phenotype of the *dne late1* double suggests that *dne* can affect flowering independently of *late1*, a conclusion that is superficially contradictory to the observation that *late1* completely blocked the effect of *dne* on flowering in graft stocks (Figure 5C). However, these two experiments differ in that the intact *dne late1* plants carried the *dne* and *late1* mutations in all tissues, whereas the grafted plants carried the mutations in the graft stock only, which might mean that the overall effect of the *dne* mutation was less in these plants. Alternatively, the difference could reflect the existence of a heterogenous mobile flowering signal and differential effects of *dne* and *late1* on components of such a signal. In this respect, an interesting feature of the early initiation in *dne late1* is that it occurs in the apparent absence of any increase in *FTL* expression level in leaf tissue. This indicates that *DNE* can act independently of both *LATE1* and *FTL* specifically to regulate the induction of flowering. One possible interpretation for these results is that the role of *FT* in pea may not be performed by *FTL* alone but by one or more additional *FT*-like genes.

In *Arabidopsis*, the *FT* family has two members, *FT* and *TSF*, which have similar regulatory characteristics and functions (Yamaguchi et al., 2005), but several recent studies have shown that the *FT* family in other species is expanded relative to *Arabidopsis*, with individual members showing distinct patterns of regulation with respect to daylength, season, and tissue specificity (Izawa et al., 2002; Faure et al., 2007; Danilevskaya et al., 2008; Igasaki et al., 2008) and interactions with different downstream partners (Li and Dubcovsky, 2008). In *M. truncatula*, the *FT* gene family consists of five genes, and the pea *FTL* gene described here is the apparent ortholog of Mt *FTLe* (Hecht et al., 2005; see Supplemental Figure 6 online). If the early initiation of flowering in the *dne late1* double mutant is due to expression of another *FTL* gene, this would imply the existence of at least two pea *FTL* genes induced under LD: one associated with general

photoperiod responsiveness and one with a narrower role in initiation of flowering. We have recently found that in *Medicago* both *FTLe* and *FTLa* are upregulated under LD and that *FTLa* has a significant role in regulation of flower induction under LD (R.E. Laurie, M. Tadege, K. Mysore, J.L. Weller, and R.C. Macknight, unpublished data). It will be of interest to determine whether this is also the case in pea, whether these genes are differentially regulated by *DNE* and *LATE1*, and whether they have distinct functions. Unraveling the roles of the different legume *FTL* genes and understanding how they are regulated will be the focus of future work.

METHODS

Plant Material, Growth Conditions, and Grafting

The origins of the *le-3*, *dne-1*, and *late1-2* mutants have been described previously (King and Murfet, 1985; Hecht et al., 2007). Seedling deetiolation experiments (Figure 8) gene expression studies (Figures 2 to 4 and 6) and *Arabidopsis thaliana* flowering experiments (Figure 7) were conducted in growth cabinets at 20°C, whereas photoperiod and grafting experiments (Figures 1 and 5) 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 for 8 h with white light from fluorescent tubes at an irradiance of 10 μmol m^{-2} s^{-1} to give a 16-h LD. Spectral scans for all artificial light sources used can be viewed at http://www.utas.edu.au/glasshouse/gh_facilities.html.

Gene Isolation, Mapping, and Molecular Genotyping

The full-length Ps *ELF4*/*DNE* cDNA was obtained by rapid amplification of cDNA ends-PCR using the BD-SMART RACE cDNA amplification kit (Clontech) and gene-specific primers (ELF4-GSP2 and ELF4-2R for the 5′ region). All PCR fragments were cloned in pGEM-T easy (Promega) and sequenced at the Australian Genome Research Facility. The *dne-1* mutation was detected as a cleaved amplified polymorphic sequence (marker, and cosegregation with the *dne* phenotype was confirmed in segregating progenies from several different crosses. For mapping of Ps *ELF4*/*DNE*, a polymorphism was identified and scored as a derived cleaved amplified polymorphic sequence marker in the JI281 × JI399 recombinant inbred line population (Hall et al., 1997). All primer details are given in Supplemental Table 1 online. For phylogenetic trees shown in Supplemental Figures 4 and 6 online, amino acid sequences of proteins related to ELF4 and FT were aligned using ClustalX (Thompson et al., 1997) Distance and parsimony-based methods were used for phylogenetic analyses in PAUP*4.0b10 (http://paup.csit.fsu.edu/) using the alignments shown. The tree in Supplemental Figure 4 online is rooted with a putative ELF4 ortholog from the chlorophyte *Chlamydomonas reinhardtii* as the outgroup, whereas the tree of FT-related proteins in Supplemental Figure 6 online is rooted at the midpoint between the FT clade and the other clades.

Complementation Studies

The *Arabidopsis elf4-1* mutation in the Ws background has been previously described (Doyle et al., 2002). Full-length cDNA fragments for *ELF4* were generated by PCR from pea (*Pisum sativum*) wild-type line NGB5839 and the isogenic *dne-1* mutant, and from *Arabidopsis* accession Ws, using primers listed in Supplemental Table 1 online. The cDNA fragments were recombined into the binary vector pB2GW7 (Invitrogen) using Gateway cloning (Karimi et al., 2002) and confirmed by sequencing. To measure hypocotyl length, seeds were surface sterilized and plated on 4 g/L Murashige and Skoog without sucrose and 8 g/L agar. Plates were stored at 4°C in the dark for 48 h and transferred into growth chambers with the appropriate light regimes.

Gene Expression Studies

Harvested tissue consisted of both leaflets from the uppermost fully expanded leaf. Samples were frozen in liquid nitrogen and total RNA extracted using the Promega SV Total RNA isolation system (Promega). RNA concentrations were determined using Ribogreen RNA quantification reagent (Molecular Probes) in a Picofluor fluorometer (Turner Biosystem). Reverse transcription was conducted in 20 μL with 1 μg of total RNA using the ImProm-II reverse transcription system (Promega) according to the manufacturer's instructions. RT-negative (no enzyme) controls were routinely performed to monitor for contamination with genomic DNA. First-strand cDNA was diluted five times, and 2 μL was used in each real-time PCR reaction. Real-time PCR reactions using SYBR green chemistry (Quantace Sensimix) were set up with a CAS-1200N robotic liquid handling system (Corbett Research) and run for 50 cycles in a Rotor-Gene RG3000 (Corbett Research). Two technical replicates and two to three biological replicates were performed for each sample. Transcript levels for experimental genes were evaluated against the constitutive gene *ACTIN* (*ACT*) as previously described (Weller et al., 2009). Primer sequences are given in Supplemental Table 1 online.

Accession Numbers

Genomic and cDNA sequences are deposited in GenBank/EMBL under the following accession numbers: AY830926 (Ps *ELF4* genomic/cDNA), FJ609177 (*PRR37* cDNA), FJ609178 (*PRR37* genomic), FJ609179 (*PRR59* cDNA), and FJ609180 (*PRR59* genomic). Accession numbers for other sequences used are as follows: *P. sativum ACTIN* (X68649), *LHY* (AY826730), *TOC1* (AY830927), *LATE1* (EF185297), *COLa* (AY830921), and *FTL* (AAX47174), and *Arabidopsis ELF4* (NM_129566, At2G40080).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Effect of *dne* Mutation on Flower Initiation in the Tall (*LE*) Genetic Background.

Supplemental Figure 2. Circadian Regulation of Pea Clock Gene Expression in LL at 150 μmol m^{-2} s^{-1}.

Supplemental Figure 3. Comparative Map of Pea Linkage Group III and *Medicago* Chromosome 3.

Supplemental Figure 4. Phylogram for ELF4-Like Protein Sequences Aligned with ClustalX and Rooted to Cs ELF4.

Supplemental Figure 5. *35S:*Ps *ELF4* and *35S:*Ps *ELF4(dne-1)* Are Expressed at Similar Levels in *Arabidopsis elf4-1* Plants.

Supplemental Figure 6. Phylogram for FTL Protein Sequences Aligned with ClustalX.

Supplemental Table 1. Primers Sequences Used in Gene Isolation, Mapping, and Mutation Detection and Real-Time PCR.

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