**DIE NEUTRALS** and LATE BLOOMER 1 Contribute to Regulation of the Pea Circadian Clock

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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 HYOCOTYL 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*), barley (*Hordeum vulgare*; Hayama and Coupland, 2004; Trevisakis 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 HYOCOTYL (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 HYOCOTYLS 3 (FRY3), 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
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 (Fujiiwara 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 (Sericawawa 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-sensitive 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, 2005). We have recently identified late1 (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 apexal 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-Inensitive 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, (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
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
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 2. DNE Affects Rhythmic Expression of Clock Gene Homologs under Light/Dark Cycles.](image-url)
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 la 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 PsELF4 (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 PsELF4 (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),

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 earlier 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.

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.
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.
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, supporting 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 elf4-1 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; Matsuhashita 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.
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 (Wang and Tobin, 1998), and the residual low amplitude rhythm we observe reflect loss of HR function.
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 Proubona, 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 CO-like 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; Dalmals et al., 2008; Taddei 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 MtFTLe (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 *FT* 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 *ie*-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, phototron conditions, and grafting protocols (Hecht et al., 2007). Standard phototron 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* recombinant inbred line (Karimi et al., 2002) and confirmed by sequencing. The full-length *Ps* *ELF4(dne-1)* was detected as a cleaved amplified polymorphic sequence marker in the *JI281* recombinant inbred line (Karimi et al., 2002). The *Ps* *ELF4* was cloned in 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* (*AA471747*), and *Arabidopsis ELF4* (NM_128566, A12340080).

**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.** 3SS:Ps *ELF4* and 3SS: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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**DIE NEUTRALIS** and **LATE BLOOMER 1** Contribute to Regulation of the Pea Circadian Clock  
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