# *EARLY FLOWERING3* Redundancy Fine-Tunes Photoperiod Sensitivity[1][OPEN]

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Three pea (*Pisum sativum*) loci controlling photoperiod sensitivity, *HIGH RESPONSE* (*HR*), *DIE NEUTRALIS* (*DNE*), and *STERILE NODES* (*SN*), have recently been shown to correspond to orthologs of Arabidopsis (*Arabidopsis thaliana*) circadian clock genes *EARLY FLOWERING3* (*ELF3*), *ELF4*, and *LUX ARRHYTHMO*, respectively. A fourth pea locus, *PHOTOPERIOD* (*PPD*), also contributes to the photoperiod response in a similar manner to *SN* and *DNE*, and recessive *ppd* mutants on a spring-flowering *hr* mutant background show early, photoperiod-insensitive flowering. However, the molecular identity of *PPD* has so far remained elusive. Here, we show that the *PPD* locus also has a role in maintenance of diurnal and circadian gene expression rhythms and identify *PPD* as an *ELF3* co-ortholog, termed *ELF3b*. Genetic interactions between pea *ELF3* genes suggest that loss of *PPD* function does not affect flowering time in the presence of functional *HR*, whereas *PPD* can compensate only partially for the lack of *HR*. These results provide an illustration of how gene duplication and divergence can generate potential for the emergence of more subtle variations in phenotype that may be adaptively significant.

Plant responses to photoperiod are known to depend on interaction between light perception and the circadian clock. This interaction has been extensively explored in Arabidopsis (*Arabidopsis thaliana*), where the circadian clock consists of a network of genes that form several interlocking feedback loops and influences flowering time through control of several direct and indirect regulators of the florigen gene *FLOWERING LOCUS T* (*FT*; Andrés and Coupland, 2012; Song et al., 2012; Millar, 2016). While the overall architecture of the clock is complex and the details are still a matter of debate (Nagel and Kay, 2012; McClung, 2014), one group of genes has emerged as being particularly significant for clock entrainment and photoperiodism. The myb transcription factor gene *LUX ARRHYTHMO* (*LUX*; also known as *PHYTOCLOCK1*) and two other plant-specific genes *EARLY FLOWERING3* (*ELF3*) and *ELF4* have similar mutant phenotypes, exemplified by early, photoperiod-insensitive flowering, elongated hypocotyls, and loss of circadian rhythmicity under constant conditions (Hicks et al., 1996; Doyle et al., 2002; Hazen et al., 2005; Onai and Ishiura, 2005; Anwer et al., 2014). The proteins encoded by these genes form a complex termed the evening complex (EC), in which the ELF3 protein is suggested to act as a molecular scaffold and signaling hub, connecting ELF4 with LUX (Nusinow et al., 2011; Huang et al., 2016). ELF4 appears to have a role in determining the subcellular location of the complex (Herrero et al., 2012) and, thus, possibly in directing the activity of the LUX transcription factor. Loss of any one of these proteins compromises the EC function (Nusinow et al., 2011), an interaction that is likely to explain the generally similar phenotypes of *elf3*, *elf4*, and *lux* mutants.

The EC is integral to circadian clock function and gating of light input to the clock (Huang and Nusinow, 2016). EC proteins are involved in regulation of a number of clock-associated genes, including *TIMING OF CAB EXPRESSION1* (*TOC1*), *GIGANTEA* (*GI*), *LUX*, *PSEUDO RESPONSE REGULATOR7* (*PRR7*), and *PRR9*, and act to repress expression of these genes during the night (Huang and Nusinow, 2016). In some cases, this appears to reflect direct binding of LUX to LUX binding site elements in the promoters of these genes (Helfer et al., 2011; Nusinow et al., 2011; Chow et al., 2012; Herrero et al., 2012; Mizuno et al., 2014). In turn, the expression of EC genes and formation of the EC itself are also tightly regulated by the circadian clock through multiple regulatory mechanisms (Herrero et al., 2012; Choudhary et al., 2015). The *ELF3*, *ELF4*, and *LUX* promoters are bound by the key clock protein CCA1, a morning phased myb transcription factor that

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acts together with its paralog LATE ELONGATED HYPOCOTYL (LHY) to repress evening-phased genes during the day (Lu et al., 2012; Adams et al., 2015; Nagel et al., 2015; Kamioka et al., 2016). Autoregulation of the EC may also be achieved through binding of the LUX protein to its own promoter (Helfer et al., 2011). In addition to its regulation of and by other clock components, the EC is also regulated at multiple levels by light and temperature pathways, and this may be one important mechanism through which the clock can be entrained by environmental variation (Huang and Nusinow, 2016).

The EC proteins not only participate directly in clock function but also have a major role in several important clock-regulated outputs, although the molecular links between the EC genes and output pathways connecting EC activity to specific developmental processes have only been investigated in detail in a few cases. The most prominent example is the regulation of hypocotyl elongation in which the EC represses the expression of the growth-promoting PHYTOCHROME-INTERACTING FACTOR (PIF) transcription factors PIF4 and PIF5 during the night (Nusinow et al., 2011; Seaton et al., 2015). Although it is clear that the EC also plays a major role in control of photoperiodic flowering, the mechanism for this has not been fully elucidated. ELF3 has been shown to facilitate the control of GI protein stability by the CONSTITUTIVE PHOTOMORPHOGENIC1 ubiquitin ligase (Yu et al., 2008), and ELF4 controls GI localization within the nucleus and may prevent its access to target promoters (Kim et al., 2013). As GI is well known as an activator of *FT* (Suárez-López et al., 2001; Jung et al., 2007; Sawa and Kay, 2011), both of these could constitute mechanisms by which the EC could regulate *FT* and flowering time. In addition, although it is clear that participation in the EC is a key role for ELF3, ELF4, and LUX proteins, it also seems likely that the individual functions of ELF3 and ELF4 are not limited to this, as they have been reported to interact independently with other circadian clock, photoperiod pathway, and light signaling components (Liu et al., 2001; Kim et al., 2013; Kaiserli et al., 2015; Nieto et al., 2015).

The importance of EC genes for circadian rhythms and photoperiod responsiveness has been confirmed more recently in other plant groups, including legumes and cereals. Interestingly, in both crop groups, mutations in *ELF3* genes have been shown to contribute to the expansion in range of species through alteration of photoperiod responsiveness and the associated impact on yield (Faure et al., 2012; Matsubara et al., 2012; Weller et al., 2012; Zakhrabekova et al., 2012; Alvarez et al., 2016; Lu et al., 2017). In crop species, a role for *ELF4* function has been clearly established only in the legume species pea (*Pisum sativum*; Liew et al., 2009), but mutations in *LUX* orthologs have also been shown to cause early flowering and impaired photoperiod response in pea and in the cereals einkorn wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*; Mizuno et al., 2012; Campoli et al., 2013; Liew et al., 2014), suggesting that the EC genes are likely to be intimately linked to the photoperiod response mechanism in both crop groups.

In the long-day legume species pea, the *ELF3* ortholog *HR* has a central role in photoperiod adaptation, with a null mutation conferring partial loss of photoperiod responsiveness and earlier flowering under short photoperiods, and this appears to have been central to adaptation of the crop to spring sowing and expansion to higher latitudes (Weller et al., 2012). Naturally occurring and induced mutations in the *LUX* ortholog *SN* completely eliminate responsiveness to photoperiod (Liew et al., 2014), whereas a mutant for the *ELF4* ortholog *DNE* enhances the effect of the *hr* mutation but has little effect when functional *HR* is present (Liew et al., 2009, 2014). In addition to these three EC homologs, mutations at two other loci confer early, photoperiod-insensitive flowering in pea: a dominant mutation in the *PHYTOCHROME A* (*PHYA*) gene (Weller et al., 2004) and recessive mutations at the *PHOTOPERIOD* (*PPD*) locus (Arumingtyas and Murfet, 1994; Taylor and Murfet, 1996; Murfet and Taylor, 1999), whose molecular identity is not yet known. In this study, we report that the *ELF3* gene has undergone duplication in legumes and show that the second *ELF3* gene, *ELF3b*, corresponds to the *PPD* locus. We also show that *PPD* is largely redundant with *HR* in control of flowering and confirm that unlike in Arabidopsis, none of the pea EC genes have a significant role in stem elongation.

## RESULTS

### *PPD* Contributes to Photoperiodic Flowering

Two previously described recessive alleles, *ppd-1* and *ppd-2*, were induced by gamma irradiation in pea cv Borek (Arumingtyas and Murfet, 1994; Taylor and Murfet, 1996). We subsequently identified a third recessive mutant allele, *ppd-3*, from EMS mutagenesis of the pea line NGB5839 (Hecht et al., 2007), which has been widely used as a reference wild-type line. Both of these parental lines carry the same *hr* mutation (Weller et al., 2012). Figure 1 and Supplemental Figure S1 confirm that in contrast to the photoperiod-responsive wild type ($P < 0.001$), the *ppd-3* allele confers early flowering ($P < 0.001$) regardless of photoperiod, equivalent to *ppd-1* and *ppd-2* (Taylor and Murfet, 1996) and to *sn* and *dne* mutants (Murfet, 1971; King and Murfet, 1985; Liew et al., 2009, 2014). As all three *ppd* mutations appeared similar in their phenotypic effects, we used the *ppd-3* mutant to explore the roles of *PPD*, as it was generated in the same genetic background as other relevant mutants, including *sn* and *dne*.

### *PPD* Affects the Expression of *FT* Genes

Previous studies have shown that the early flowering phenotypes of *sn* and *dne* mutants under short-day (SD) conditions are associated with elevated expression levels of several *FT* homologs, similar to the inductive effect of long days (LDs) observed for the wild type (Hecht et al., 2011; Liew et al., 2014). We used

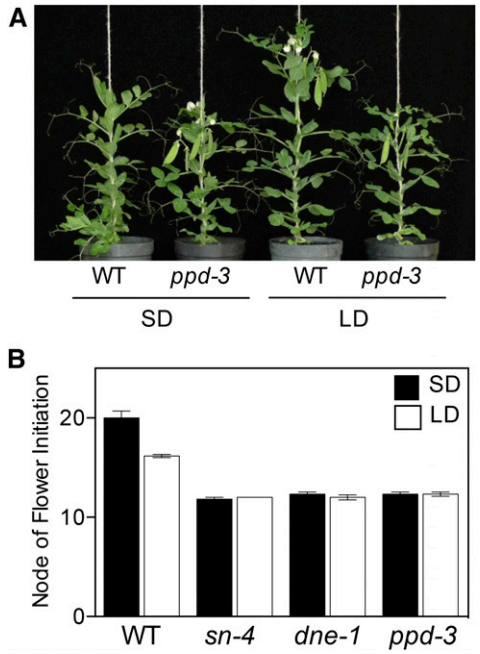


**Figure 1.** Phenotypic comparison of photoperiod-insensitive early flowering pea mutants. A, Representative 7-week-old wild-type (WT) line NGB5839 and isogenic *ppd-3* mutant plants. B, Comparison of the response to photoperiod in *ppd-3* and similar mutants *sn-4* and *dne-1* in the NGB5839 genetic background. Data represent mean ± SE for $n = 5$ to 6 plants. All plants were grown in the phytotron under SD (8L:16D) or LD (16L:8D) conditions using extended natural daylight. The progenitor line NGB5839 carries the *hr* mutation.

qRT-PCR to compare the expression of pea *FT* genes in *ppd-3* and these other mutants over the course of development under SDs. Figure 2A shows that under SD conditions, the inflorescence identity marker *PROLIFERATING INFLORESCENCE MERISTEM* (*PIM*) is expressed 2 to 3 weeks earlier in apical tissue of *sn-4*, *dne-1*, and *ppd-3* mutants than in the wild type, as expected in view of their early flowering phenotypes. The *FTb2* gene appears to be the main target for photoperiod regulation of flowering in pea as it is strongly induced in leaf tissue during commitment to flowering under LDs but is not significantly expressed in SDs (Hecht et al., 2011). Figure 2B shows that in contrast to the wild type, all three mutants showed significant *FTb2* expression in expanded leaf tissue under SDs, whereas *FTb2* transcript was not detectable in equivalent wild-type tissue. Importantly, *FTb2* transcript levels were detectable above background in all three mutants from the first time point at day 11, prior to the first detectable induction of *PIM* (at day 18 in *sn-4* and *dne-1* or day 25 in *ppd-3*).

A second pea *FT* gene, *FTa1*, has an important role in promotion of flowering, is expressed in expanded leaf tissue, and may contribute to a second mobile signal (Hecht et al., 2011). However, unlike *FTb2*, *FTa1* does not appear to be directly involved in the response to photoperiod, as it shows basal expression under SDs, is relatively weakly induced by LD after floral commitment, and *fta1* mutants, although late-flowering, are still capable of responding to photoperiod (Hecht et al., 2011). Nevertheless, expression of *FTa1* was also elevated in leaf tissue of *sn-4*, *dne-1*, and *ppd-3* mutants (Fig. 2B), and its induction in shoot apical tissue

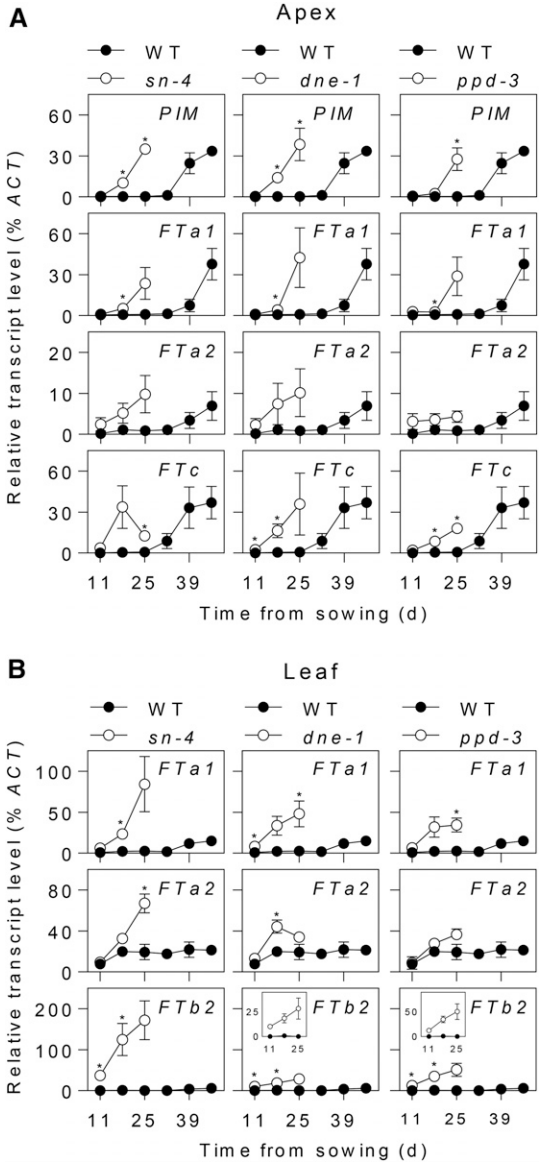


**Figure 2.** *FT* genes are misregulated in photoperiod-insensitive early flowering pea mutants. Developmental regulation of *FT* genes and the floral marker *PIM* in NGB5839 (WT) and the *ppd-3* mutant are shown in comparison to *sn-4* and *dne-1* mutants. Plants were grown under an 8-h photoperiod in growth cabinets and samples harvested weekly until the appearance of visible flower buds. Transcript levels were determined in dissected shoot apex or uppermost fully expanded leaflet tissue and are shown relative to the *ACTIN* reference gene. Each sample consisted of pooled material from two plants, and each data point represents the mean ± SE for $n = 2$ to 3. Asterisks indicate differences between wild-type and mutant values where $P < 0.05$.

occurred 2 to 3 weeks earlier than the wild type, in parallel with *PIM* (Fig. 2A). As previously described, a third *FT* gene, *FTc*, is only expressed in shoot apex tissue and in wild-type plants is also induced in parallel with *PIM* under both SDs and LDs (Hecht et al., 2011; Fig. 2A). *FTc* induction also occurred 2 to 3 weeks earlier in all three mutants relative to the wild type. Although minor differences in the expression profiles and timing of *PIM* and *FT* induction were observed, these results overall show that early flowering in all three mutants is associated with a similar pattern of derepression of several *FT* genes.

### *PPD* Affects the Maintenance of Circadian Rhythms

The similarity of the *ppd* mutant phenotype to those of the *dne* and *sn* mutants suggested that *PPD*, like *SN* and *DNE*, might also affect the circadian clock. To test this possibility, we compared diurnal and circadian expression patterns of key clock-related genes in the wild type and *ppd-3.* Figure 3 shows that the *ppd-3* mutation influences the diurnal expression rhythms of several clock-associated genes under both conditions. Interestingly, the effects of *ppd-3* were more pronounced under LD conditions where *DNE*, *TOC1a*, *LATE1*, and *PRR59a* expression rhythms showed a small but significant phase advance relative to the wild type (Supplemental Fig. S2). Trough expression levels for the evening genes *LATE1* and *PRR59a* were also significantly higher in *ppd-3* than in the wild type, a difference also apparent under SDs. In contrast, there was no clear evidence of any effect of *ppd-3* on expression of *PRR37a*, consistent with earlier reports on *sn* and *dne* mutants (Liew et al., 2009, 2014).

We also examined whether the *ppd-3* mutation also affected expression rhythms after transfer of plants from entraining photoperiod cycles to constant darkness. Figure 4 shows that after transfer to constant darkness, rhythms of *LHY*, *ELF4*, and *LATE1* were maintained for two cycles in both the wild type and *ppd-3*, but peaks occurred increasingly earlier in *ppd-3*, suggesting the mutant may affect the rhythmic period, and this was again confirmed through statistical analysis (Supplemental Fig. S2). Although clear rhythms of *TOC1a* expression in constant darkness were not apparent, the overall level of expression in *ppd-3* remained lower than the wild type. Collectively these results are similar to those previously reported for the *sn-4* (Liew et al., 2014) and *dne-1* mutations (Liew et al., 2009), indicating a small but clear effect of the *ppd-3* mutation on rhythmic expression of several key circadian clock components, which suggests that the primary role of *PPD* may also be related to clock function.

### *PPD* Is the Second of Two Duplicate *ELF3* Genes in Pea

Initial studies reported that *PPD* was located toward the top of linkage group II near loci *Aatp* and *Rms3* (Murfet and Taylor, 1999). We refined this

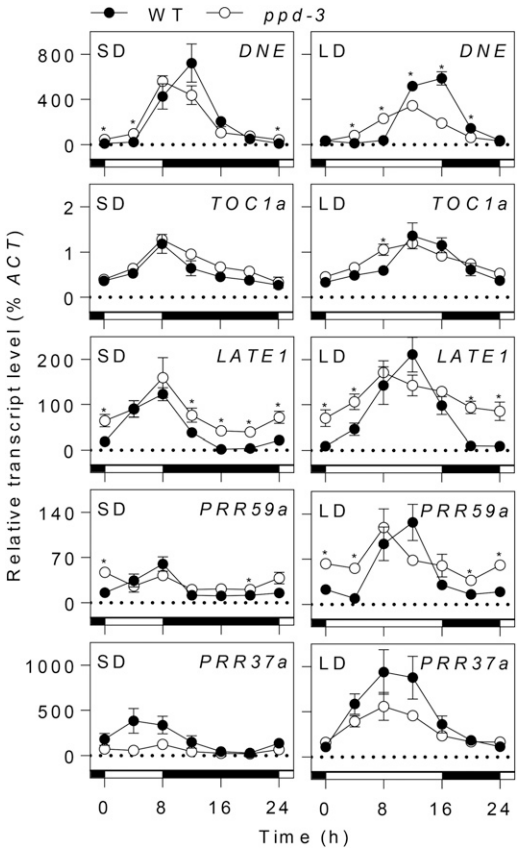


**Figure 3.** *PPD* affects diurnal expression rhythms of clock-related genes. Transcript levels were determined in the uppermost fully expanded leaf of 3-week-old NGB5839 (WT) and *ppd-3* plants grown under SD (8L:16D) or LD (16L:8D) conditions at 20°C. Values are normalized to the transcript level of the *ACTIN* reference gene. Each sample consisted of pooled material from two plants, and each data point represents the mean ± SE for $n = 2$ to 6. Light and dark periods are represented by white and black bars, respectively. Asterisks indicate differences between wild-type and mutant values where $P < 0.05$.

position in the F2 of a cross between the *ppd-3* mutant and cv Térèse, delimiting *PPD* to a region between markers *PepTrans* and *ThiolP* (Bordat et al., 2011). In the closely syntenic region of *Medicago truncatula* chromosome 1, these markers correspond to gene models Medtr1g009200 and Medtr1g018840 and define an interval of around 4.3 Mb containing ~900 genes. Within this region we identified several genes potentially related to flowering time control, including two miR156 genes, the *CONSTANS*-like gene *COLb* (Wong et al., 2014), and a gene with strong similarity to the previously described *HR/ELF3* gene (Weller et al., 2012), which we termed *ELF3b*. In view of the circadian clock-related role of *PPD*, we considered only the last two sequences as plausible candidates. We found that all three *ppd* alleles carried significant mutations in the *ELF3b* gene relative to their parental lines, whereas initial sequencing of *COLb* failed to identify any coding region polymorphisms and it was therefore dismissed as a candidate gene.

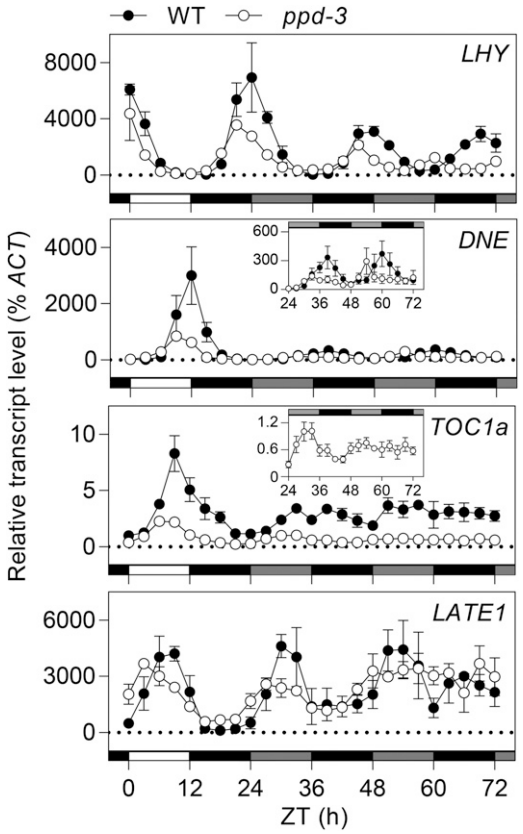


**Figure 4.** *PPD* affects expression rhythms of clock-related genes in constant darkness. Transcript levels were determined in the uppermost fully expanded leaf of 3-week-old NGB5839 (WT) and *ppd-3* plants entrained in SDs (12L:12D) at 20°C for 21 d before transfer to continuous darkness at Zeitgeber time (ZT) 24. Values are normalized to the transcript level of the *ACTIN* reference gene. Each sample consisted of pooled material from two plants, and each data point represents the mean ± SE for $n = 2$ to 3. Light and dark are represented by white and black bars, respectively. The gray bars indicate the periods of subjective day during the period of continuous darkness. Zeitgeber time refers to the time since lights-on of the last full entraining cycle. Statistical analyses are presented in Supplemental Figure S2.

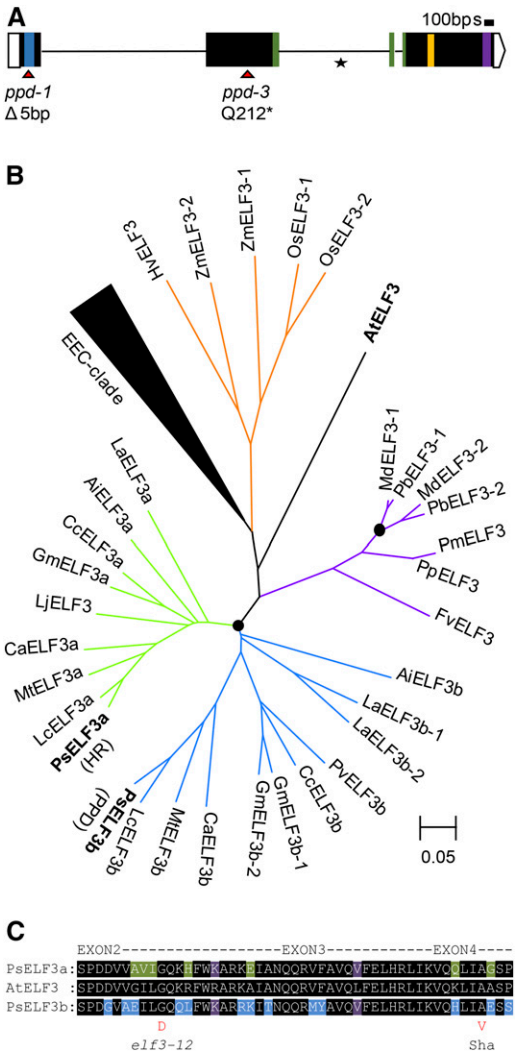


**Figure 5.** Multiple independent *ppd* mutants carry mutations in an *ELF3*-like gene. A, Diagram of the pea *ELF3b* gene showing details of mutations in the *ppd-1* and *ppd-3* mutants. Colored regions of the coding sequence represent conserved blocks as defined by Liu et al. (2001) and shown in Supplemental Figure S4. The asterisk indicates the site of a single polymorphism (a simple sequence repeat) between the two parental cultivars Borek and NGB5839. B, Phylogram of ELF3-like protein sequences from legumes and other selected species. The two clades of legume proteins are shown in green (ELF3a) and blue (ELF3b). ELF3 proteins from species in the family Rosaceae are shown in purple and those from grasses in orange. The black triangle represents a clade containing EEC-like sequences from the legume and Rosaceae species examined. Likely independent *ELF3* duplications are indicated by black circles. All major clades have at least 75% bootstrap support (from 10,000 replications). Species abbreviations are as follows: Ai, *Arachis ipaensis*; Ca, *Cicer arietinum*; Cc, *Cajanus cajan*; Gm, *Glycine max*; La, *Lupinus angustifolius*; Lc, *Lens culinaris*; Lj, *Lotus japonicus*; Mt, *Medicago truncatula*; Ps, *Pisum sativum*; Pv, *Phaseolus vulgaris*; Fv, *Fragaria vesca*; Md, *Malus domestica*; Pb, *Pyrus bretschneideri*; Pm, *Prunus mume*; Pp, *Prunus persica*; At, *Arabidopsis thaliana*; Hv, *Hordeum vulgare*; Os, *Oryza sativa*; Zm, *Zea mays*. C, Sequence differences between PsELF3a (HR), AtELF3, and PsELF3b (PPD) in the conserved block II region. Location and nature of substitutions known to affect AtELF3 function are indicated. Sha, Location of substitution in ecotype Shakdara.

As shown in Figure 5A, the *ppd-1* mutant carried a 5-bp deletion in exon 1 of *ELF3b* that results in a frame shift at codon 26 and a truncation of the protein after seven additional missense amino acids, while the *ppd-3* mutant carried a nonsense mutation in exon 2 (C2383T), resulting in replacement of residue Q212 by a stop codon. Attempts to amplify *ELF3b* genomic and cDNA sequence from the *ppd-2* mutant indicated the presence of a substantial rearrangement/insertion affecting the coding sequence, which we did not investigate further. The presence of deleterious mutations in each of three independent mutant alleles provides strong evidence that *PPD* is in fact equivalent to *ELF3b*.

The existence of a second *ELF3* homolog prompted us to examine the nature of *ELF3*-like genes in other legumes and related species. In Arabidopsis, *ELF3* is a single-copy gene, and the next most closely related gene has been termed *ESSENCE OF ELF3 CONSENSUS* (*EEC*; Liu et al., 2001). Sequence searches in legumes

and related Rosid taxa clearly identified homologs of *ELF3* and of *EEC*. Figure 5B shows that in addition to an *EEC* homolog, most legumes contained two *ELF3* homologs that represented two distinct clades: an *ELF3a* clade containing the previously described pea and lentil *ELF3* genes (Weller et al., 2012) and a second clade containing the *PPD/ELF3b* gene. The two pea ELF3 proteins show ~50% similarity to each other and 30% similarity to pea EEC. This duplication is present in both the major crop legume groups and in the more basal genistoid species narrow-leafed lupin (*Lupinus angustifolius*) and the dahlbergoid species wild peanut (*Arachis ipaensis*), but not in members of the Rosaceae, suggesting it is a relatively ancient, legume-specific feature. Evidence for *ELF3* duplication was also present in the Rosaceae, but phylogenetic analysis clearly shows that this was independent from the legume event and much more recent (Fig. 5B). Within the legumes, certain species deviated from the expected standard complement of two genes, with *Lotus japonicus* and common bean (*Phaseolus vulgaris*) databases only providing evidence of one. However, the clear identity of these genes as *ELF3a* and *ELF3b* genes suggests that the second gene has either been lost from these species or is simply not represented in current genome builds. Soybean (*Glycine max*) also appears to have lost one of two *ELF3a* homoeologs, while narrow-leafed lupin has two copies of *ELF3b* (Fig. 5B).

*ELF3* genes are defined by four short, highly conserved domains that have been designated blocks I to IV (Liu et al., 2001). The functional relevance of these domains remain largely unknown, but recent evidence suggests that block II is required for interaction with the ELF4 protein (Herrero et al., 2012; Saini et al., 2013), and mutations within block II have been shown to alter ELF3 function and cellular distribution (Anwer et al., 2014; Kolmos et al., 2011). We also assembled a more extensive collection of legume ELF3 sequences and examined the resulting alignment (Supplemental Fig. S4) for evidence of significant divergence in the region of block II. We found no differences perfectly distinguishing the ELF3a and ELF3b clades but found that ELF3b proteins from the temperate legume clade show a number of nonconservative amino acid substitutions relative to other legume ELF3 proteins and Arabidopsis ELF3 (Fig. 5C; Supplemental Fig. S4). Interestingly, these are clustered near highly conserved residues that are known to influence ELF3 function in Arabidopsis. Sequencing of the entire *ELF3b* coding sequence from selected diverse accessions of cultivated *P. sativum* var *sativum* and wild *Pisum* (Supplemental Table S1) showed no polymorphism in block II, suggesting that this region of *ELF3b*, although distinct from *ELF3a*, is highly conserved in pea germplasm.

### *PPD* Only Affects Flowering in the Absence of Functional *HR*

All three *ppd* mutant alleles were isolated in genetic backgrounds containing the *hr* mutation and therefore lack functional copies of both *ELF3* paralogs. This indicates that loss of all *ELF3*-related activity also completely eliminates the ability of the plant to respond to photoperiod and shows that the partial photoperiod response of the *hr* mutant can be attributed to presence of the *PPD* gene. To examine the effect of the *ppd* mutation in isolation, we selected the *HR ppd-3* genotype and compared it with an *HR PPD* isoline and also with near-isolines carrying single *sn*, *dne*, and *hr* mutations. Figure 6 and Supplemental Figure S5 show that in contrast to the clear promotion of flowering under SDs conferred by the *ppd-3* mutation on an *hr* genetic background (Figs. 1 and 6A), the *ppd-3* mutation alone has no effect in the presence of *HR* in either SDs or LDs ($P > 0.05$). This suggests that *ELF3b* plays a minor role that is subsidiary to *ELF3a/HR*, a difference that could conceivably be due to differences in protein structure and/or regulation. The *ppd-3* mutation therefore shows a genetic interaction with *HR* similar to that of the *dne-1* mutation, which also has minimal effect in the presence of the functional *HR* gene. These interactions contrast that of the *sn-4* mutation, which is essentially epistatic to *HR* for flowering time (Liew et al., 2014; Fig. 6; Supplemental Fig. S4).

### *PPD* and *HR* Have Similar Expression Patterns

In order to examine whether differences in *ppd* and *hr* mutant phenotypes might reflect differences in regulation of *PPD* and *HR* genes, we examined how

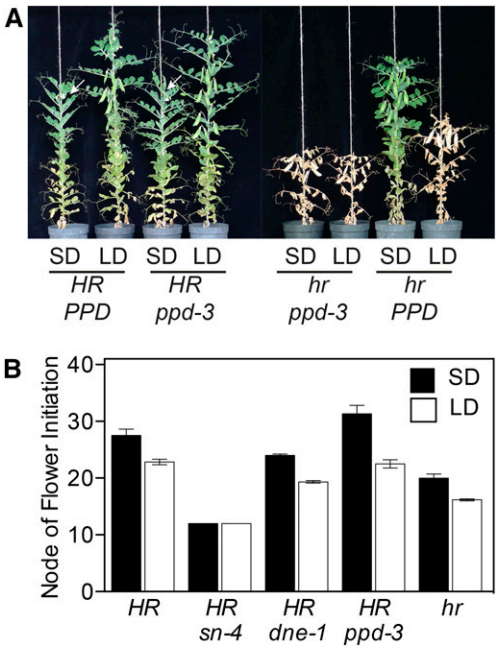


**Figure 6.** Genetic interaction of *PPD* and *HR*. A, Representative 11-week-old *PPD HR* plants, single *ppd* and *hr* mutants, and double mutant *ppd hr* plants. Arrows represent node of flower initiation where pods are not yet clearly visible. B, Comparison of photoperiod response for initiation of flowering in single *sn*, *dne*, *ppd*, and *hr* mutants. Data represent mean ± SE for $n = 5$ to 6 plants. All plants were grown in the phytotron under SD (8L:16D) or LD (16L:8D) conditions.

transcript levels of both genes varied under different circumstances and conditions. Our previous results showed that *ELF3a* transcript is expressed at a relatively low level in leaf tissue and does not show a discernible diurnal rhythm (Liew et al., 2014). Supplemental Figure S6 shows that like *ELF3a*, *ELF3b* also appears to lack a clear diurnal rhythm in expression. Comparison of different tissue types and two different developmental time series indicated a higher expression level for *ELF3b* than *ELF3a* in leaf, apex, and stem tissues in certain experiments. but overall there was no major, consistent difference in expression level or pattern between the two *ELF3* paralogs (Supplemental Fig. S6). *ELF3a* and *ELF3b* expression profiles from the pea gene expression atlas (Alves-Carvalho et al., 2015) also suggested no substantial differences. These observations indicate that the more dominant role of the *HR* gene relative to *PPD* does not simply reflect a higher expression level.

### *PPD* Does Not Substantially Affect Stem Elongation

In Arabidopsis, the evening complex genes *ELF3*, *ELF4*, and *LUX* all affect seedling vegetative development in addition to photoperiodic flowering, with each of the single mutants showing a significant increase in hypocotyl elongation compared with the wild type under white light (Zagotta et al., 1996; Doyle et al., 2002; Hazen et al., 2005). Similar seedling phenotypes are also shown by *elf3* mutants in barley, which have elongated coleoptiles and leaves and decreased chlorophyll content relative to *ELF3* plants (Boden et al., 2014). We therefore examined whether the pea EC mutants displayed similar phenotypes. When grown under long- or short-day conditions, none of the four single mutants showed obvious elongation phenotypes at the seedling stage. Figure 7A shows that in plants grown under white light, length of the first two internodes was not affected in the *sn* and *ppd* mutants in a functional *HR* genetic background and only showed a small (12%) increase in *ppd-3* under LDs. Several later internodes were actually slightly but significantly shorter relative to the wild type, in *sn-4* under SDs and both *sn-4* and *dne-1* under LDs (Fig. 7A). We also examined their effect in the original (*hr*) genetic background, where all three mutants have clear effects on flowering time (Fig. 1), but again, no clear differences were observed (Fig. 7A) except for an increase in *sn-4* over internodes 5 and 6 and *dne-1* at internode 2 only in LDs. It is probable that this increase reflects the slightly earlier induction of *FT* genes of in *sn-4* than other genotypes, as an increase in internode length after initiation of flowering is well documented in pea (Weller et al., 1997). We also examined the effect of the *ppd-3* mutation on deetiolation phenotypes under monochromatic light to exclude the possibility that an effect on elongation under white light might be masked by its spectral complexity or the relatively high irradiance used. However, the results in Figure 7B show that

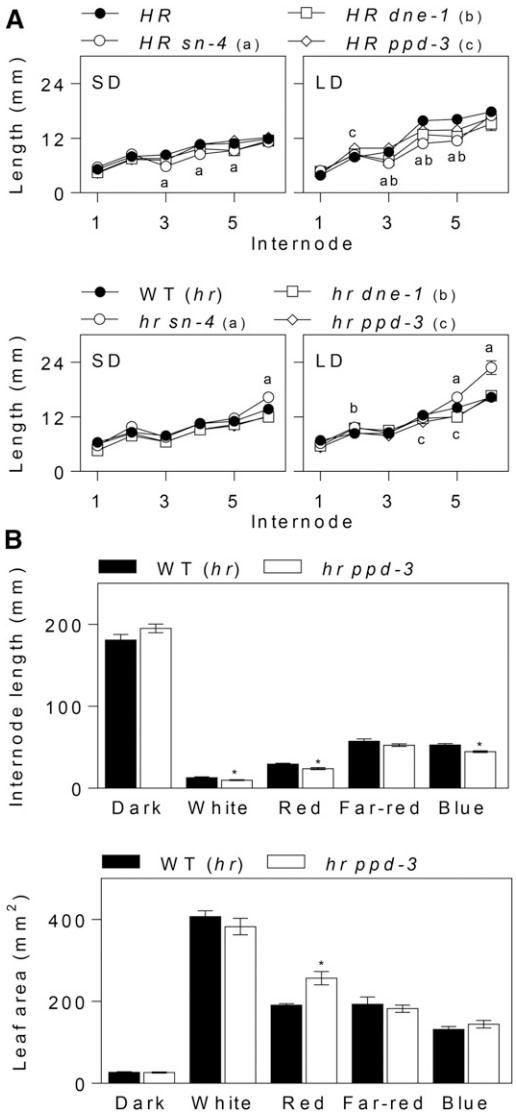


**Figure 7.** *PPD* and other EC genes do not affect stem elongation or photomorphogenesis. A, Comparison of genotypes differing at the *SN*, *DNE*, *PPD*, and *HR* loci for elongation of early stem internodes in plants grown under white light in SD (8L:16D) or LD (16L:8D). Data represent mean ± SE for $n = 5$ to 6 plants. Values significantly different from the wild type ($P < 0.05$) are indicated by letters representing different genotypes. B, Effect of the *ppd* mutation on deetiolation phenotypes. NGB5839 (WT) and *ppd-3* seedlings were grown for 14 d from sowing under continuous light or darkness. Internode length was measured as the length between nodes 1 and 3. Leaf area was estimated as the product of the length and width of a single leaflet from a leaf at node 3. Data represent mean ± SE for $n = 5$ to 6 plants. Asterisks indicate differences between wild-type and mutant values where $P < 0.05$. All plants were grown in growth cabinets, in darkness, or under continuous white (100 $\mu$mol m$^{-2}$ s$^{-1}$), red, far-red, or blue light (15 $\mu$mol m$^{-2}$ s$^{-1}$).

under white light or lower irradiance monochromatic red, far-red, or blue light, certain early internodes of *ppd-3* mutant plants were slightly shorter than the wild type, consistent with previous reports for *sn-4* and *dne-1* (Hecht et al., 2007; Liew et al., 2009). Regardless of

these small differences, the overall similarity of the pea mutants shows that there is no substantial effect equivalent to the reduction in hypocotyl growth inhibition observed in Arabidopsis EC mutants.

## DISCUSSION

It is becoming increasingly clear that the EC genes *ELF3*, *ELF4*, and *LUX* not only play an important role in circadian clock function but also provide an important component of adaptation in a number of crop species (Huang and Nusinow, 2016). In pea, an important model for temperate legume crops, a mutation in the pea *ELF3* ortholog *HR* confers early flowering and a reduction in photoperiod response that is likely to have provided a key prehistoric adaptation to shorter growing seasons (Weller et al., 2012). A similar physiological function has been demonstrated for two other EC gene orthologs in pea, *SN/LUX* and *DNE/ELF4*, through analysis of naturally occurring and induced mutants (Liew et al., 2009, 2014). The *sn* and *dne* mutations effectively eliminate the residual response to photoperiod in an *hr* genetic background, and the same is true for mutations at a third locus, *PPD* (Arumingtyas and Murfet, 1994; Taylor and Murfet, 1996; Fig. 1). Our investigations show that, like *sn* and *dne*, *ppd* mutants do not affect photomorphogenesis but exhibit mild defects in rhythmic gene expression, suggesting a role specifically related to circadian clock function (Figs. 3, 4, and 7). Genetic mapping of *PPD* together with the analysis of multiple independent *ppd* mutant alleles (Fig. 5) has subsequently provided strong evidence that *PPD* is equivalent to *ELF3b*, a paralog of the *HR* gene *ELF3a*.

This observation gives a new perspective on the effect of the *hr* mutation. It has long been clear that the single known *hr* mutant allele reduces but does not eliminate the photoperiod response (Murfet, 1973), and the identification of *hr* as an apparent null mutation has implied a degree of redundancy must exist for *ELF3* function (Weller et al., 2012; Liew et al., 2014). At the genomic level, this has been borne out by the identification of two groups of *ELF3* genes in pea and other legumes, which are both distinct from a more distantly related clade containing orthologs of the Arabidopsis gene *EEC* (Fig. 5). Evidence for the *ELF3* duplication is present in all of the crop legume genomes queried, indicating that it is relatively old, but it is not found in species in the closely related Rosaceae family, suggesting that it is most likely unique to the legume lineage. More specifically, the presence of the duplication in three distinct early branching Papilionoid legume groups (genistoid, dahlbergoid, and hologaleginoid clades) is consistent with an origin in the whole-genome duplication that occurred around 58 million years ago close to the base of this major legume clade (Pfeil et al., 2005; Lavin et al., 2005; Cannon et al., 2010; Young et al., 2011). A preliminary scan of other nonpapilionoid legume genomic resources suggests that this duplication is not present in the caesalpinoid legume *Cercis* (redbud) or the mimosoid *Acacia*, but a more comprehensive analysis will be needed to pinpoint its phylogenetic position more precisely.

Regardless of exactly how these two *ELF3* genes first arose, the genetic interaction between the *HR* and *PPD* genes confirms their functional redundancy showing that the residual response in the *hr* mutant is eliminated by the *ppd* mutation (Fig. 6). In contrast, the effect of the *ppd* mutation is minimal when functional *HR* is present, implying that *HR* has the more substantial role (Fig. 6). Comparison of expression patterns revealed no major differences in tissue-specific, developmental, or diurnal expression between *HR* and *PPD* (Supplemental Fig. S6), suggesting that their difference in function could reflect an inherent difference in the activity of the two proteins. Alternatively, it is also possible that these broad expression patterns in whole leaves may not accurately reflect localized differences in expression in specific leaf tissues critical for their influence on flowering, as recently observed for a number of evening genes in the Arabidopsis clock (Endo et al., 2014).

Previous characterizations of pea EC mutants also identified differences in severity of apparent null *sn* and *dne* mutant phenotypes and raised the possibility that this might also reflect differences in redundancy (Liew et al., 2014). Interestingly, in both of these cases, the single mutant phenotypes are consistent with the structure of the gene family in question. For example, in Arabidopsis, *LUX* has a paralog *NOX* that can contribute to recruit the EC proteins to target promoters when *LUX* activity is absent (Dai et al., 2011; Helfer et al., 2011; Nusinow et al., 2011). In legumes, however, *NOX* genes are not present, and a null mutant for the single *LUX* ortholog in pea, *SN*, has a very strong early flowering phenotype and is completely insensitive to photoperiod (Liew et al., 2014), similar to the double *ELF3* mutant *hr ppd*. Arabidopsis *ELF4* is a member of a small gene family (Khanna et al., 2003) in which the closest paralog, *ELF4-LIKE1* (*EFL1*), appears also to have *ELF4* activity but three other *ELF4-like* genes do not (Kolmos et al., 2009). In legumes, *ELF4* is also represented by small gene family (Liew et al., 2009), and the fact that in pea an apparent null mutant for the *ELF4* ortholog *DNE* has only a minor effect on flowering when the other EC genes are intact (Liew et al., 2014) again implies a probable redundancy with one or more other members of the family.

While the question of redundancy may not have much importance for how the clock functions, it may be relevant to an understanding of the importance of EC genes in a natural setting. It is notable that *ELF3*a mutations are known to have provided adaptation in three different crop legumes (Weller et al., 2012; Lu et al., 2017) as well as in the ornamental species *Lathyrus odoratus* (V. Rajandran and J.L. Weller, unpublished data), and conserved quantitative trait loci positions suggest this could be true for several other legume species (Weller and Ortega, 2015). In contrast, *ELF4* has not been implicated in natural variation for flowering time in any legume, and for *LUX*, natural mutants are only known in pea, have appeared only recently, and have a strong phenotype with limited value for large-scale production. It is

reasonable to assume that the difference in prominence of EC mutants for adaptation mainly reflects the relative severity of the single mutant phenotypes. It also suggests that the simple duplication of the *ELF3* gene may have provided a mechanism for achieving a partial loss of photoperiod responsiveness and an optimal compromise between generation of sufficient vegetative biomass to support yield and early completion of the life cycle in short season environments. However, it is also notable that *ELF3* mutations provide adaptation in diploid cereals such as barley and einkorn wheat (Faure et al., 2012; Zakhrabekova et al., 2012; Alvarez et al., 2016) where *ELF3* is single copy. This suggests that there could be another component to the adaptive advantage of *elf3* mutations over other EC mutants, which could reflect unique roles of *ELF3* that are conserved in these different crop groups. Broader physiological comparisons of EC mutants will in future be valuable in testing this idea.

Turning from questions of redundancy to the specific roles of EC genes, two output phenotypes are worth a brief consideration. In Arabidopsis, elongated hypocotyls under white light are a conspicuous feature of EC mutants and the EC has been shown to play an important role in photoperiod regulation of hypocotyl elongation, acting to repress transcription of growth-inhibiting PIF transcription factors in the evening (Nusinow et al., 2011). In contrast, none of the pea EC mutants or mutant combinations have substantial effect on elongation of early stem internodes under white or monochromatic light (Fig. 7). The fact that *HR* and *DNE* can complement the Arabidopsis *elf3* and *elf4* mutant elongation phenotypes, respectively (Liew et al., 2009; Weller et al., 2012), indicates that it is not due to an inherent difference in the structure of the pea and Arabidopsis proteins. It could instead result from a difference in the way that light signals regulating seedling stem elongation are gated in the two species and/or the activity of PIF genes in control of elongation. However, it is perhaps equally likely to reflect fundamental differences between Arabidopsis and pea in growth habit (rosette versus caulescent), germination mode (hypogeal versus epigeal), and seed size (small versus large), all of which might conceivably contribute to a reduced need for sensitive seedling elongation responses in pea versus Arabidopsis.

Early flowering under noninductive (SD) photoperiods remains the most striking overt phenotypic consequence of EC gene dysfunction in both Arabidopsis and pea, but the mechanisms through which EC genes influence flowering are surprisingly unclear. In Arabidopsis, photoperiod responsiveness depends significantly on the transcriptional rhythm of the *CONSTANS* (*CO*) gene and coincidence of *CO* expression with light under LD but not under SD conditions. Mutations in circadian clock genes such as *TOC1* and *LHY/CCA1* are considered to confer early flowering in SD through a shift in the phase of the *CO* expression rhythm to coincide with light and allow stabilization of the CO protein (Yanovsky and Kay, 2002; Mizoguchi et al., 2005). *ELF3* is also reported to influence the daily transcriptional rhythm of *CO* in a similar manner, through protein-level regulation of the indirect *CO* activator GI (Yu et al., 2008). Regulation through GI could also potentially explain *CO*-independent effects of ELF3 (Lu et al., 2012). Whether similar mechanisms could also explain the early flowering of *elf4* and *lux* mutants has not been addressed. However, *ELF4* has been reported to influence the activity of GI by sequestering it to regions of the nucleus where it is unable to access the *CO* promoter (Kim et al., 2013). These results suggest that despite acting in some circumstances as part of a complex, EC components may have other roles and influence GI activity and flowering in distinct ways. Under SDs, the pea EC mutations confer early and elevated expression of several *FT* genes known to promote flowering, in a generally similar manner (Fig. 2). The lack of effect of *ppd* on expression on the *CO* ortholog *COLa* (Supplemental Fig. S7) is consistent with results from the *dne* mutant (Liew et al., 2009) and other recent reports indicating that *COL* genes are unlikely to participate in the photoperiod response mechanism in temperate legumes (Wong et al., 2014; Ridge et al., 2016). The *GI* ortholog *LATE1* is also a key component of the photoperiod response pathway and is necessary for normal induction of *FT* genes (Hecht et al., 2007, 2011), and it is thus possible that EC genes may act to limit *LATE1* function in some way. Some evidence for this is already apparent from analysis of gene expression rhythms, which have shown that *GI* transcript levels are significantly elevated during the nighttime trough phase by the *dne*, *sn*, and *ppd* mutations (Liew et al., 2009, 2014; Fig. 3). In future it will be interesting to learn how this elevated expression may be linked to elevated *FT* expression and whether other aspects of GI regulation are influenced by the *SN*, *DNE*, and *HR*/*PPD* genes.

## MATERIALS AND METHODS

### Plant Material

Origins of the *ppd-1*, *ppd-2*, *sn-4*, and *dne-1* mutations have been described previously (King and Murfet, 1985; Arumingtyas and Murfet, 1994; Hecht et al., 2007). The *ppd-3* mutant was obtained from EMS mutagenesis of line NGB5839 (Hecht et al., 2007), which also carries the *hr* mutation (Weller et al., 2012). Generation of *HR*, *sn-4 HR*, and *dne-1 HR* lines was described previously (Liew et al., 2014). The novel *ppd-3 HR* genotype was selected from a cross between *ppd-3* and a near-isogenic line of NGB5839 carrying a functional *HR* allele (Weller et al., 2012), and its identity was verified in advanced generations by molecular genotyping using markers detailed in Supplemental Table S2.

### General Growth Conditions

All plants were grown in a 1:1 mixture of dolerite chips and vermiculite topped with potting mix and received nutrient solution weekly. Plants for gene expression experiments (Figs. 2–4; Supplemental Figs. S6 and S7) were grown in growth cabinets at 20°C under 200 $\mu$mol $m^{-2}$ $s^{-1}$ white light from cool-white fluorescent tubes. Segregating progenies and plants for flowering and stem elongation experiments (Figs. 1, 6, and 7A) were grown in the phytotron and received an 8-h photoperiod of natural daylight either with (LD) or without (SD) an 8-h extension with white light (10 $\mu$mol $m^{-2}$ $s^{-1}$) from a mixture of fluorescent and incandescent sources (Hecht et al., 2007). Branches were periodically removed for the plants in the flowering experiment. Examination of

deetiolation phenotypes (Fig. 7B) was conducted in growth chambers under light sources described previously (Hecht et al., 2007).

## Mapping, Linkage, and Basic Genetic Analysis

Several markers used for linkage analysis were modified from gene-based markers described by Aubert et al. (2006) and Bordat et al. (2011). These markers were supplemented by newly designed markers targeted to introns of appropriate genes identified in the relevant interval of the *Medicago truncatula* genome (v4.0; www.jcvi.org/medicago/) and also present in pea sequence databases in GenBank (www.ncbi.nlm.nih.gov). Details of these markers and their method of detection are provided in Supplemental Table S2. The marker used to detect the *hr* allele has been described previously (Liew et al., 2009; Weller et al., 2012), and details of the marker used to follow the *ppd-3* mutation are also provided in Supplemental Table S2.

## Sequence and Expression Analysis

*ELF3a* and *ELF3b* genes in legumes were identified by BLAST searches of various sequence databases (bios.dijon.inra.fr/FATAL/cgi/pscam.cgi, www.jcvi.org/medicago/, www.phytozome.net, www.kazusa.or.jp/lotus, www.ncbi.nlm.nih.gov, legumeinfo.org/blast; knowpulse2.usask.ca/portal/blast) using the full-length amino acid sequence of Arabidopsis (*Arabidopsis thaliana*) ELF3 and *M. truncatula* ELF3a and ELF3b. All alignments were constructed with the MAFFT algorithm FFT-NS-ix 1000 in Geneious 8.1.8 using default settings. The neighbor-joining tree shown in Figure 5B was constructed from the alignment shown in Supplemental Figure S3 using ClustalX 2.1 (Thompson et al., 1997) with default settings and 10,000 bootstraps.

For all circadian and diurnal expression experiments (Figs. 2–4; Supplemental Figs. S5 and S6), plants were 3 weeks old at harvest, and harvested tissue consisted of both leaflets from the uppermost expanded leaf. Tissue harvests for the *FT* expression experiment presented in Figure 2 were performed as described by Hecht et al. (2011). RNA extraction, reverse transcription, and real-time PCR analysis were performed as described previously (Liew et al., 2009). Primers for expression analysis are presented in Supplemental Table S2.

## Statistical Analysis

Significance of specified genotype effects were analyzed using a two-tailed Student's *t* test, with the assumption of equal variance.

Estimates of rhythmic parameters in diurnal and circadian expression data for Figures 3 and 4 (presented in Supplemental Fig. S2) were obtained from the Biological Rhythms Analysis Software System (BRASS version 3.0; http://millar.bio.ed.ac.uk/PEBrown/BRASS/BrassPage.htm), which utilizes fast Fourier transform-nonlinear least squares analysis. Period and relative amplitude error analysis was conducted using default settings with the period range set from 10 to 35 h and a confidence interval of 75%. Phase analysis was conducted with FFT-NLL and the additional program Mfourfit using default settings with a spline curve option.

## Supplemental Data

The following supplemental materials are available.

**Supplemental Figure S1.** Phenotypic comparison of photoperiod-insensitive early flowering pea mutants.

**Supplemental Figure S2.** Circadian gene rhythms analysis

**Supplemental Figure S3.** Alignment of selected ELF3-like protein sequences used to generate phylogenetic tree in Figure 5B.

**Supplemental Figure S4.** Alignment of ELF3-like protein sequences from 20 legume species with Arabidopsis ELF3.

**Supplemental Figure S5.** Phenotypic comparison of mutants for pea "evening complex" genes.

**Supplemental Figure S6.** Comparison of pea *ELF3a* and *ELF3b* gene expression.

**Supplemental Figure S7.** Diurnal regulation of group Ia *CO-like* genes in the *ppd-3* mutant.

**Supplemental Table S1.** SNPs in *PsELF3b* coding sequence in selected *P. sativum* lines.

**Supplemental Table S2.** Primer and marker details.

**Supplemental Table S3.** Sequence details.

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