The Plant Cell, Vol. 15, 2742–2754, November 2003, www.plantcell.org © 2003 American Society of Plant Biologists

DETERMINATE and *LATE FLOWERING* Are Two *TERMINAL FLOWER1/CENTRORADIALIS* Homologs That Control Two Distinct Phases of Flowering Initiation and Development in Pea

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Genes in the *TERMINAL FLOWER1* (*TFL1*)/*CENTRORADIALIS* family are important key regulatory genes involved in the control of flowering time and floral architecture in several different plant species. To understand the functions of *TFL1* homologs in pea, we isolated three *TFL1* homologs, which we have designated *PsTFL1a*, *PsTFL1b*, and *PsTFL1c*. By genetic mapping and sequencing of mutant alleles, we demonstrate that *PsTFL1a* corresponds to the *DETERMINATE* (*DET*) gene and *PsTFL1c* corresponds to the *LATE FLOWERING* (*LF*) gene. *DET* acts to maintain the indeterminacy of the apical meristem during flowering, and consistent with this role, *DET* expression is limited to the shoot apex after floral initiation. *LF* delays the induction of flowering by lengthening the vegetative phase, and allelic variation at the *LF* locus is an important component of natural variation for flowering time in pea. The most severe class of alleles flowers early and carries either a deletion of the entire *PsTFL1c* gene or an amino acid substitution. Other natural and induced alleles for *LF*, with an intermediate flowering time phenotype, present no changes in the PsTFL1c amino acid sequence but affect *LF* transcript level in the shoot apex: low *LF* transcript levels are correlated with early flowering, and high *LF* transcript levels are correlated with late flowering. Thus, different *TFL1* homologs control two distinct aspects of plant development in pea, whereas a single gene, *TFL1*, performs both functions in Arabidopsis. These results show that different species have evolved different strategies to control key developmental transitions and also that the genetic basis for natural variation in flowering time may differ among plant species.

INTRODUCTION

For some fundamental aspects of plant biology, the genes involved have been identified through a molecular genetics approach using the model species Arabidopsis. From this basic information, comparative studies between species can begin, in particular to understand the genetic and molecular mechanisms responsible for the large diversity in plant morphology and to identify the genes involved in adaptive evolution (Cronk, 2001). In plants, the best example of such evolutionary developmental studies is the identification and analysis of MADS box genes involved in flower development in several plant species, including gymnosperms (reviewed by Ma and De Pamphilis, 2000). Isolation of putative orthologs in different species and studies of RNA and/or protein expression patterns provide insights into the conservation and diversification of gene function in plant development (Hofer and Ellis, 2002). For instance, the *LEAFY* (*LFY*) gene of Arabidopsis, which was isolated initially as *FLORICAULA* in snapdragon, is a key gene involved in floral development (Coen et al., 1990; Weigel et al., 1992). Orthologs of *LFY* have since been studied in numerous other species, including *UNIFOLIATA* in pea (Hofer et al., 1997). In certain cases, different regulator processes or new roles can be found. For example, *UNIFOLIATA* was proposed to regulate indeterminacy during both leaf and flower development. The function of *LFY* during leaf development was not described (Hofer et al., 1997).

Flowering time is a major adaptive trait in the life strategy of flowering plants, which have to synchronize their reproduction with favorable environmental conditions. After a vegetative phase, plants undergo the floral transition. The switch from the vegetative to the reproductive stage is controlled by physiological signals and genetic networks that integrate environmental (photoperiod and temperature) and endogenous (stage of the plant) conditions (Levy and Dean, 1998; Colasanti and Sundaresan, 2000). The molecular genetics of the long-day plant Arabidopsis enable the isolation and characterization of the genes that control flowering time (reviewed by Mouradov et al., 2002). In agronomic species, most of the genetic loci that control flowering time have been identified as quantitative trait loci (in maize or

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Article, publication date, and citation information can be found at www.plantcell.org/cgi/doi/10.1105/tpc.015701.

rice; Yano et al., 2001; Salvi et al., 2002) or as mutants (in pea; Murfet and Reid, 1993). Using a combination of map-based cloning and a candidate-gene approach, two quantitative trait loci, *Hd1* and *Hd3a*, have been cloned in rice (Yano et al., 2000; Kojima et al., 2002) and one, *VRN1*, has been cloned in wheat (Yan et al., 2003). They correspond to genes that are similar to the Arabidopsis genes *CONSTANS*, *FLOWERING LOCUS T* (*FT*), and *APETALA1*, respectively.

Because pea is both a classic model species for plant development and an important crop in Europe, we used a molecular approach to study homologs of the snapdragon *CENTRORADIALIS*/Arabidopsis *TERMINAL FLOWER1* (*CEN*/*TFL1*) genes in pea. *CEN* is involved in inflorescence architecture in snapdragon (Bradley et al., 1996). The *cen* mutation leads to the conversion of the indeterminate inflorescence to a terminal flower. Orthologs of *CEN* have been found in different species: *TFL1* in Arabidopsis (Bradley et al., 1997), *SELF PRUNING* (*SP*) in tomato (Pnueli et al., 1998), *CET* in tobacco (Amaya et al., 1999), and *LpTFL1* in *Lolium perenne* (Jensen et al., 2001). In Arabidopsis, *tfl1* mutants have a terminal flower and flower earlier than the wild type (Bradley et al., 1997). This early-flowering phenotype was not observed in snapdragon. TFL1 may play a role in inflorescence meristem identity as well as in floral initiation control as a repressor of flowering. It was proposed that these two distinct roles are in fact one, with *TFL1* controlling the length of both the vegetative and reproductive phases (Ratcliffe et al., 1998).

CEN and TFL1 are similar to a family of mammalian phosphatidylethanolamine binding proteins (PEBPs) also known as Raf-1 kinase inhibitor proteins. Crystallography analysis reveals that CEN may be involved in interaction with a kinase (Banfield and Brady, 2000). In tomato, SP was shown to interact with multiple proteins and was proposed to encode a modular protein with the potential to interact with a variety of signaling pathways (Pnueli et al., 2001). Expression analysis has revealed that genes closely related to *TFL1* are expressed mainly in the shoot apical meristem in the region below the terminal meristem. *CEN* is induced during floral initiation (Bradley et al., 1996), whereas *TFL1* expression also is found during the vegetative phase; this expression could explain the role of *TFL1* in delaying flowering in Arabidopsis (Bradley et al., 1997). Analysis of mutants and sequencing of the entire Arabidopsis genome have revealed that the *TFL1* genes belong to a small family (at least six genes) with functional divergence (Mimida et al., 2001). One of them, *FT*, has a *TFL1*-antagonistic role by promoting flowering in Arabidopsis (Kardailsky et al., 1999; Kobayashi et al., 1999). Studies of *TFL1* homologs in other species may help us better understand the function and the evolution of the *TFL1* family in flowering plants.

Floral initiation and development in pea have been studied for many decades (Murfet and Reid, 1993). Based on physiological and mutational analyses, a model for flowering that involves both a floral inhibitor and a stimulus has been developed (reviewed by Reid et al., 1996; Weller et al., 1997). The stimulus is specific to flowering and is under the control of *GIGAS* (Beveridge and Murfet, 1996). The synthesis of the floral inhibitor is controlled by different genes (*STERILE NODE*, *HIGH RESPONSE*, *PHOTOPERIOD*, *DAY NEUTRAL*, and *EARLY*) and is strongly regulated by photoperiod. The integration of the signals occurs in the apex and is controlled by the *LATE FLOWERING* (*LF*) gene. *LF* determines the node of flowering in pea for a given genetic background (Figure 1). Four natural and induced classes of alleles are known—*Lf-d*, *Lf*, *lf*, and *lf-a*—that in maximal inductive conditions confer minimum nodes of flowering of 15, 11, 8, and 5, respectively. The dominance order is *Lf-d* > *Lf* > *lf* > *lf-a*, with the *lf-a* allele being recessive (Murfet, 1975). *LF* is active in the shoot, and the different alleles determine the threshold of sensitivity of the apical meristem to flowering signals. Because mutations of *LF* lead to plants with an early phenotype, *LF* may be considered a repressor of flowering.

Pea is an indeterminate-flowering plant, as is Arabidopsis. After floral initiation, the shoot apical meristem is converted to an inflorescence meristem (called I1; Figure 1). The I1 meristem grows indefinitely, and an axillary meristem in the leaf axil generates a secondary inflorescence, I2. Flowers (often two) arise laterally from I2. At the onset of senescence, the I2 meristem ceases growing and is converted to a stub, a terminal meristem with epidermal hairs (Singer et al., 1999). A pea mutant, known as *determinate* (*det*), produces a few axillary flowers and an apparent terminal flower (Reid and Murfet, 1984; Singer et al., 1990) (Figure 1). Scanning electron microscopy showed that this terminal flower actually arises from an axillary meristem and that the I1 meristem is converted to a stub as in the wild type (Singer et al., 1990) (Figure 1). A real terminal flower can be obtained in pea by crossing the *det* mutant with another mutant, *vegetative1*, which remains vegetative (Reid and Murfet, 1984; Singer et al., 1999).

Using degenerate primers, we isolated three TFL1 homologs in pea. Detailed analyses, by gene mapping, allele sequencing, and expression studies, revealed that two of these homologs correspond to important genes involved in flower initiation and development in pea: *DET* and *LF*. This study provides compar-

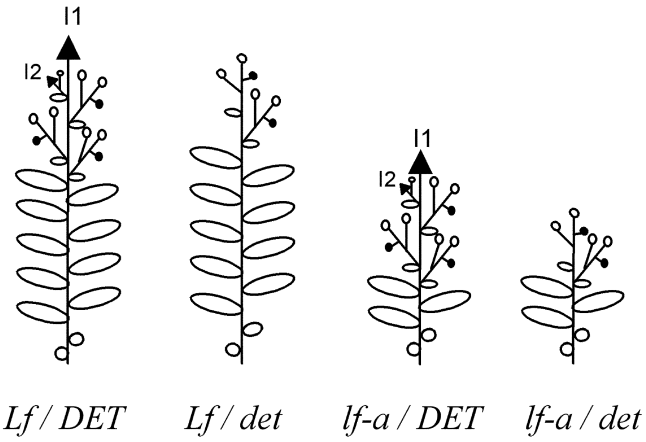

Figure 1. Scheme of the Phenotypes of *lf-a*, *det*, and *lf-a det* in Pea.

Black arrows represent shoot apical meristems, open circles represent flowers, and closed circles represent stubs (terminal meristems with epidermal hairs). I1 and I2 indicate the primary and secondary inflorescence meristems, respectively.

ative information about the function and evolution of *TFL1* genes in flowering plants.

RESULTS

Arabidopsis *TFL1* Homologs Represent a Small Gene Family in Pea

To isolate *TFL1*-related sequences in pea, we designed several different degenerate primers corresponding to conserved domains identified from the alignment of published *TFL1* homologs (Figure 2A) and other homologous EST sequences. Two different primer pairs successfully amplified fragments from pea genomic DNA. Two fragments of 450 bp (A) and 850 bp (B) obtained with the primer combination TFL1-3/TFL1-5, and a single fragment of 450 bp (C) obtained using the primer combination TFL1-1/TFL1-2, were isolated and sequenced. Each band yielded sequence similar to that of *TFL1* and *CEN*. Additional sequences for each fragment were obtained by 3′ and 5′ rapid amplification of cDNA ends PCR on seedling or flower cDNA (see Methods). Complete sequences were obtained for transcripts corresponding to fragments A and C, and these genes were designated *PsTFL1a* and *PsTFL1c*, respectively. For the third gene, designated *PsTFL1b*, only a partial sequence was obtained.

To evaluate the number of *TFL1*-related genes in pea, we made a DNA gel blot using *PsTFL1a* as a probe (Figure 3). Three hybridizing bands were seen in HindIII and EcoRV digests, and four bands were seen in digests with EcoRI. The additional band in the EcoRI digest can be explained by the presence of an EcoRI site in the *PsTFL1b* sequence. Therefore, we concluded that the three *TFL1* homologs isolated probably represent the entire *TFL1* family in pea.

PsTFL1a and *PsTFL1c* are predicted to encode proteins of 174 and 173 amino acids, respectively, according to the computer software Eugène (Schiex et al., 2000). The predicted PsTFL1a and PsTFL1c proteins show 70% amino acid identity and 72 and 65% identity with *TFL1*, respectively. The *PsTFL1b* clone, which is incomplete at the 5′ end, covers 90 amino acids and shows 73% identity with *TFL1* over this region. The protein sequence alignment in Figure 2A shows that the three predicted pea proteins contain large regions that are conserved across TFL1 homologs from other species. Intron/exon boundaries also are highly conserved across these genes (data not shown).

Previous studies have shown that despite their apparently similar functions, Arabidopsis *TFL1* and *Antirrhinum CEN* are not particularly closely related (Mimida et al., 2001). Clustering of *TFL1*-related sequences based on amino acid similarity suggested the presence of several distinct groups (Figure 2B). Both *PsTFL1a* and *PsTFL1c* cluster with *TFL1*. However, *PsTFL1b* belongs to another group of genes that includes *CEN*, *SP*, and *ATC*. Although *PsTFL1b* is a partial sequence, the same results were obtained when the analysis was performed using only the C-terminal region. Other members of the Arabidopsis *TFL1* family, such as *FT*, *TSF*, and *BFT* (Mimida et al., 2001), are more distant (Figure 2B).

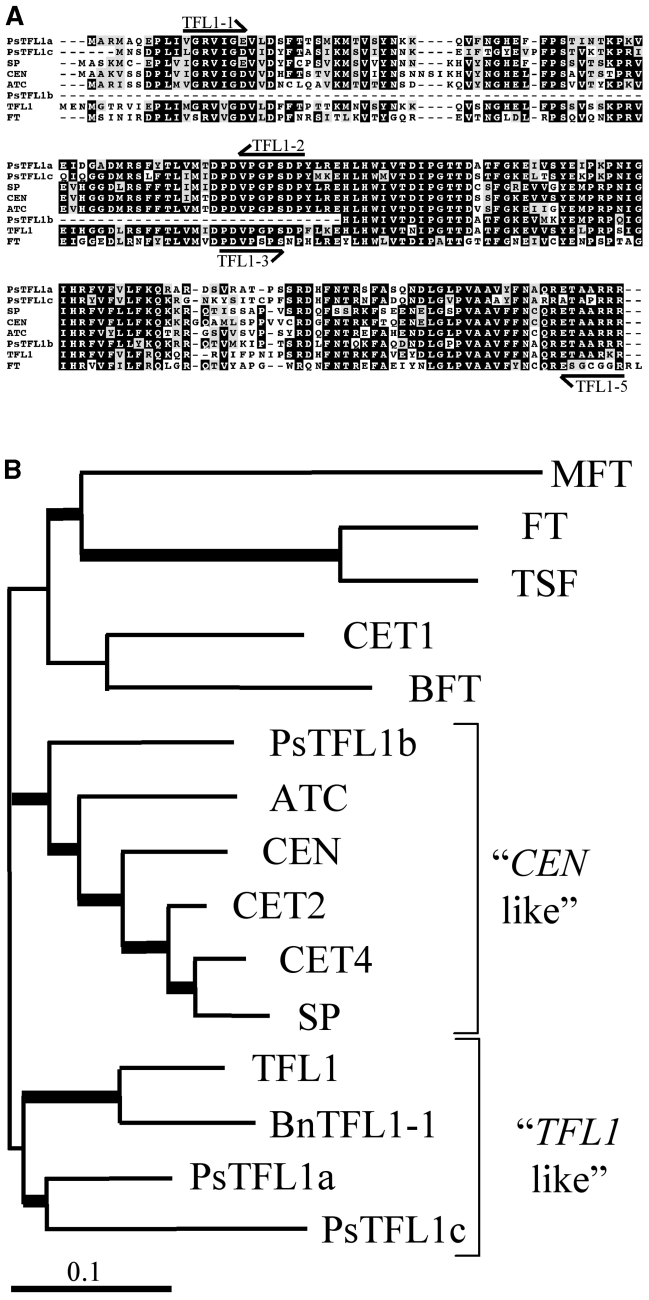

Figure 2. Comparison of Pea *TFL1* Homologs with *TFL1* Related Genes.

(A) Alignment of the predicted amino acid sequences of *PsTFL1a*, *PsTFL1b*, and *PsTFL1c*, *TFL1* (Bradley et al., 1997), *SP* (Pnueli et al., 1998), *CEN* (Bradley et al., 1996), *ATC* (Mimida et al., 2001), and *FT* (Kardailsky et al., 1999; Kobayashi et al., 1999). The alignment was performed with Multialign software (Corpet, 1988). Arrows represent the positions of the degenerate primers used to isolate the *TFL1* homologs in pea.

(B) Phylogenic tree of TFL1-related proteins constructed using the NJ method with the program CLUSTAL W. Branches with a bootstrap value of >600 (of 1000) are shown with thick lines. In addition to the proteins shown in **(A)**, TSF, BFT, MFT, CET1, CET2, and CET4 from tobacco (Amaya et al., 1999) and BnTFL1-1 from *Brassica napus* were included in the analysis.

The Map Locations of *PsTFL1a* and *PsTFL1c* Suggest That They May Be Candidate Genes for *DET* and *LF*, Respectively

To study the relationship between the *TFL1*-like sequences isolated and already known flowering loci in pea, we developed molecular markers corresponding to these sequences. We screened the parents of two different mapping populations for single nucleotide polymorphisms (SNP) and converted these to PCR-based cleaved amplified polymorphic sequence or derived cleaved amplified polymorphic sequence markers (see Methods).

PsTFL1a was mapped in the Térèse × K586 RIL population (Laucou et al., 1998) and was found to be located in group V between two RAPD markers, K3-3000 and D3-1100, a region shown previously to contain *DET* (Rameau et al., 1998). Analysis of an F2 population segregating for a *det* mutation showed no recombinant genes between *DET* and *PsTFL1a* among 120 individuals (data not shown). Thus, *PsTFL1a* is closely linked to *DET*, and in view of the phenotypic similarity between *det* mutants and Arabidopsis *tfl1* mutants, we considered it to be a good candidate for the *DET* gene.

Because of the lack of SNP for *PsTFL1b* and *PsTFL1c* between Térèse and K586, these genes were mapped in another RIL population derived from a cross between the more distant lines JI 281 and JI 399 (Ellis et al., 1992). *PsTFL1b* maps on linkage group III close to the markers PsZF18 and C7/1 (Ellis et al., 1992; Laucou et al., 1998), a region containing no obvious candidate flowering loci. By contrast, *PsTFL1c* was located in linkage group II and segregated with the B5/9 marker in a region containing *LF* (Ellis et al., 1992; Laucou et al., 1998) and an important quantitative trait loci for flowering time (our unpublished results). This finding suggests that *PsTFL1c* could be a candidate gene for *LF*. Our subsequent analyses focused on *PsTFL1a* and *PsTFL1c*, because these potentially represented genes that regulate agronomically important flowering traits in pea.

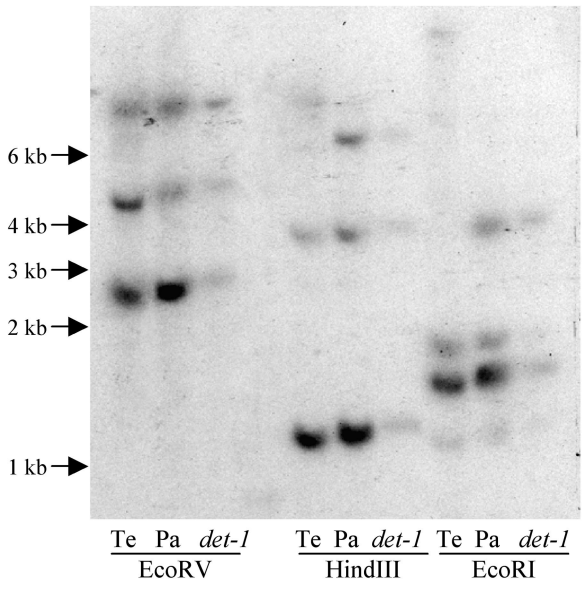

Figure 3. DNA Gel Blot of the Wild-Type Pea Lines Térèse and Paloma and the Mutant *det-1* (JI 2121).

Genomic DNA was digested with EcoRV, HindIII, and EcoRI. The blot was hybridized with a probe corresponding to *PsTFL1a*. Hybridization and washing conditions were low stringency (temperature of 60°C, 2× SSC as wash salt). Sizes of the marker fragments are represented in kilobases. Pa, Paloma; Te, Térèse.

det Mutants at the *PsTFL1a* Locus Carry Significant Mutations

To examine the relationship between *PsTFL1a* and *DET*, we next sequenced *PsTFL1a* from three different *det* mutants and their corresponding wild-type lines when available (Table 1). All three were found to contain SNP within the *PsTFL1a* gene.

In the *det-1* mutant line JI 2121 (Swiecicki, 1987), a point mutation was found at the first exon-intron junction, converting the consensus splicing donor motif TGGT to TGAT (Table 1). Using primer pairs surrounding the first and the second introns, reverse transcriptase–mediated (RT) PCR performed on root RNA from *det-1* and the Paloma wild-type line confirmed that the first intron is not spliced out in *PsTFL1a* transcripts from *det-1*, whereas the second intron is spliced out normally in both the wild type and *det-1* (Figure 4). Retention of the first intron in *det-1* is predicted to result in the introduction of a stop codon, truncating PsTFL1a at amino acid 72.

The *det-2* line JI 1358 arises as a spontaneous mutant, for which no progenitor line is available (J. Hofer, personal communication). We found five SNP in *PsTFL1a* between the *det-2* line and wild-type cv Térèse, some of which may represent natural polymorphism (Table 1). Of these, three are predicted to direct changes in the protein sequence. Two of these changes (Met-4 to Ile and Thr-47 to Pro) affect residues that are not notably conserved across the TFL1 family, whereas the Gln residue substituted for in the third change (Gln-127 to Arg) is invariant across all TFL1 sequences and is conserved even in more distantly related proteins such as FT and animal PEBPs. The high degree of conservation implies that mutation of this Gln-127 likely affects TFL1 function and could account for the *det-2* phenotype.

The *det-3* mutant was obtained by mutation of the SG line (Berdnikov et al., 1999), but this line has since been lost. Thus, as in the case of *det-2*, no progenitor line was available for *det-3*. Sequencing revealed five SNP between *det-3* and wild-type cv Térèse (Table 1). Three of these are predicted to direct changes in the protein sequence. The first is Met-4 to Ile, which it shares in common with *det-2*. The second is Thr-66 to Ile. This residue is conserved perfectly across all known TFL1 sequences. Furthermore, the corresponding substitution in Arabidopsis TFL1 is the basis for the *tfl1-14* mutant phenotype (Ohshima et al., 1997) and was shown to suppress interactions with putative partners (Pnueli et al., 2001). The third change occurs at position 104, where a conserved Glu is replaced by Lys.

Additional evidence for the importance of the Gln-127 and Thr-66 residues comes from a detailed analysis of the CEN (Banfield and Brady, 2000) and TFL1 (our unpublished data) protein structures. Thr-66 resides in the core of the protein, located on the central β-sheet F (as labeled for human PEBP; Banfield et al., 1998). The Thr side chain forms a hydrogen

Table 1. Sequence Analysis of *PsTFL1a* in the *det* Mutants

Mutant Line	Position	Nucleotide Substitution	Major Effect
det-1	+202 (junction exon 1/intron 1)	TG**G**T → TG**A**T	**Intron 1 is not spliced**
det-2	+12 (exon 1)	AT**G** → AT**C**	Met-4 → Ile
	+139 (exon 1)	**A**CC → **C**CC	Thr-47 → Pro
	+401 (intron 2)	AA**C**CA → AA**T**CA	Silent
	+655 (exon 4)	C**A**A → C**G**A	**Gln-127 → Arg**
	+749 (exon 4)	GT**T** → GT**G**	Silent
det-3	+12 (exon 1)	AT**G** → AT**C**	Met-4 → Ile
	+197 (exon 1)	A**C**A → A**T**A	**Thr-66 → Ile**
	+401 (intron 2)	AA**C**CA → AA**T**CA	Silent
	+584 (exon 4)	**G**AG → **A**AG	Glu-104→ Lys
	+749 (exon 4)	GT**T** → GT**G**	Silent

The positions of the substitutions are labeled from the +1 of translation. For *det-1*, the sequence is compared with the progenitor line, Paloma, whereas for *det-2* and *det-3*, the sequence is compared with the wild-type line Térèse. "Silent" indicates that the substitution has no effect at the amino acid sequence of the protein. Mutations in boldface are proposed to be responsible for the *det* phenotype (see details in the text).

bond to the side chain of Gln-127. Therefore, these two residues (Thr-66 and Gln-127) map to the same region of the structure. Interestingly, two of the *det* mutants, *det-2* and *det-3*, represent mutations in these residues. These mutations (Thr-66 to Ile in *det-3* and Gln-127 to Arg in *det-2*) would disrupt the hydrogen bonding interaction between the two residues and likely would affect the structural integrity of this region. Because mutations that affect protein function have been mapped to this region in both Arabidopsis TFL1 and PsTFL1a, it follows that this must be an important part of the structure. However, this importance is likely to be structural (maintenance of protein fold) rather than directly functional (e.g., interaction with other proteins/small molecules).

Because three different *det* mutant alleles carry a substitution in the predicted amino acid sequence of *PsTFL1a* that is likely to have a significant effect on PsTFL1a function, we conclude that *PsTFL1a* corresponds to *DET*.

PsTFL1c Is Another Homolog of *TFL1* and Corresponds to *LF*

We also examined the relationship between *PsTFL1c* and *LF* by sequencing *PsTFL1c* from a range of natural variant alleles and induced mutants at the *LF* locus (Table 2). A relatively large number of *LF* alleles are known and have been grouped into four phenotypic classes (Taylor and Murfet, 1993; Weller et al., 1997). Mutants in the *lf-a* class present the strongest phenotype and can flower as early as node 5. In four of six independent *lf-a* lines—HL7, XVIII/17, Wt11796, and K2—*PsTFL1c* could not be amplified from genomic DNA (data not shown), and a band corresponding to *PsTFL1c* was absent from these lines in DNA gel blot analyses (Figure 5). These lines were obtained from fast-neutron or γ-ray mutagenesis (Taylor and Murfet, 1993) and therefore likely carry large deletions in the region of *PsTFL1c*.

In the remaining two *lf-a* mutants (HL76 and Wt11795), *PsTFL1c* was amplified and sequenced (Table 2, Figure 6). Compared with its isogenic *Lf* progenitor HL75, the *lf-a* line HL76 was found to carry a 6-bp deletion in the *PsTFL1c* coding region that is predicted to direct the replacement of three amino acids (Phe-Ala-Asp) at positions 147 to 149 with a single Tyr (haplotype B in Figure 6). This deletion maps to the α-C helix (as labeled for the human PEBP [Banfield et al., 1998]), and in the mutated form, it is highly unlikely that this helix would be able to form. Loss of this helix would have a seriously destabilizing effect on the protein's structure. Interestingly, this helix maps to the same region of the structure as the Thr-66/Gln-127 pair, again suggesting the importance of this area in protein stability and/or function. In the *lf-a* line Wt11795, a C-to-T substitution at position 1625 is responsible for the replacement of Pro-109 by Ser (haplotype E in Figure 6). This Pro is conserved in all TFL1-related plant sequences (Figure 2A) and forms an integral part of the proposed "putative ligand binding site" for these proteins (Banfield et al., 1998). Consequently, this mutation could affect protein function severely.

Our analysis of six independent *lf-a* mutants has shown in each case an important modification in the *PsTFL1c* sequence, either a large deletion or a functionally significant substitution mutation. Therefore, we conclude that *PsTFL1c* corresponds to *LF*.

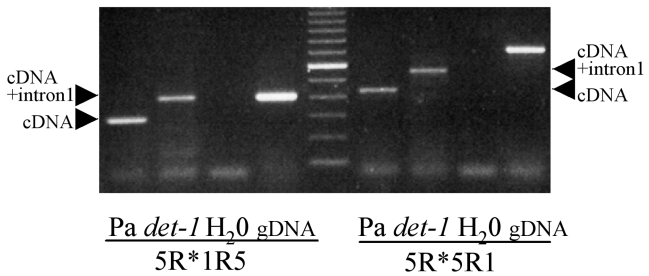

Figure 4. Analysis of the Nonsplicing of the First Intron in the *det-1* Line.

PCR was performed on root cDNA from Paloma (Pa) and *det-1* (JI 2121) with water (H_2O) and genomic DNA (gDNA) as controls. The primer pairs 5R*1R5 and 5R*5R1 surrounded intron 1 and introns 1 and 2, respectively. Arrowheads indicate the size expected for the cDNA if the first intron is spliced (cDNA) or not spliced (cDNA+intron 1). A 100-bp size marker lane is shown in the center of the gel.

Table 2. Results of Sequence Analysis of *PsTFL1c* in Several Pea Lines and Their Corresponding Mutants for *LF*

Line	*LF* Class	Haplotype	Derived Mutant Line	*LF* Class	Haplotype
Vesna	*Lf-d*	A	XVIII/17	*lf-a*	No
Torsdag	*Lf*	A	K319	*lf*	C
			K2	*lf-a*	No
Wt4042	*Lf*	A	Wt11796	*lf-a*	No
HL75	*Lf*	A	HL76	*lf-a*	B
Porta	*Lf*	A	Wt11790	*lf*	C
			Wt11791	*lf*	C
Paloma	*Lf*	A	Wt11795	*lf-a*	E
WL1771	*Lf-d*	D	WL1770	*Lf*	D
			WL1769	*lf*	D
Champagne	*Lf-d*	A			
Térèse	*Lf*	A			
			HL7	*lf-a*	No

Mutants and their progenitor lines are described by Taylor and Murfet (1993). The *PsTFL1c* sequences were grouped in five haplotypes (A to E) as described in Figure 6. "No" indicates that *PsTFL1c* could not be amplified by PCR and was not detected on a DNA gel blot (Figure 5). HL7 is a natural mutant; therefore, no progenitor line is available.

The Three *PsTFL1* Genes Present Different Patterns of Expression

We next examined the presence/absence of transcript for each of the *PsTFL1* genes by RT-PCR in different tissues at different stages. Specific intron-spanning primers were designed for each gene to control for contaminating genomic DNA. We analyzed a number of different tissues during the vegetative and reproductive phases in plants grown under either short-day or long-day conditions (Figure 7). The three genes showed distinct patterns of expression. *PsTFL1a* was expressed mainly in roots, in the apex after the floral transition, and in flower buds and flowers. Expression in roots is common for flowering genes such as *LpTFL1* (Jensen et al., 2001), *LUMINIDEPENDENS* (Aukerman and Amasino, 1996), and *GIGANTEA* (Fowler et al., 1999). No function was proposed for the expression of these genes in roots. No expression of *PsTFL1a* was detected in the shoot apex before the floral transition. In both short-day and long-day conditions, a signal was detected in the apex after the floral transition (stage A2) and remained during the reproductive phase (stage A3). For *PsTFL1b*, expression was found in the apex during the vegetative and reproductive phases (Figure 7). Expression was found in roots and dormant nodes. No expression was detectable in flowers. As with *PsTFL1a*, no difference was observed between short-day and long-day conditions. *PsTFL1c* transcripts were present in all tissues studied (Figure 7), and no change in the expression pattern was detected during the flowering process.

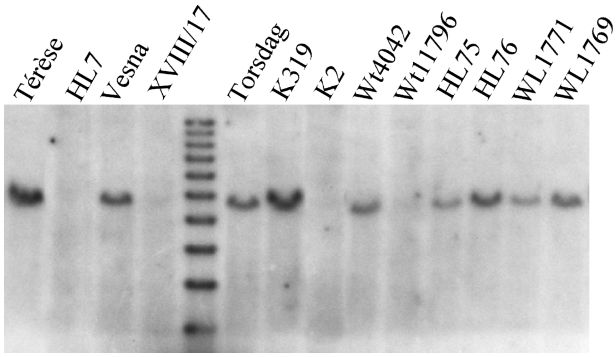

Figure 5. DNA Gel Blot of Different Wild-Type and Corresponding Mutant Lines for *LF*.

Mutant lines are described in Table 2. The genomic DNA was digested with EcoRI, and the blot was hybridized with a *PsTFL1c* probe under high stringency.

Intermediate Mutant Lines for *LF* Present Variations of the *PsTFL1c* Transcript Level

As shown previously, the stronger *LF* alleles (*lf-a*) carry deletions or substitutions of the *PsTFL1c* gene. Other alleles (*lf*, *Lf*, and *Lf-d*) were studied (Table 2). DNA gel blot analysis revealed no detectable structural change for some of these lines (K319 and WL1769; Figure 5). Sequence analysis showed no differences in the coding region. Only two silent substitutions in the introns were detected: one between *lf* mutants K319, Wt11790, and Wt11791 (haplotype C) and their wild-type progenitors (haplotype A) and another between the wild-type line WL1771, its derived mutant lines (haplotype D), and the other wild-type lines (haplotype A) (Table 2, Figure 6). Therefore, plants containing the *Lf-d*, *Lf*, or *lf* allele produce the same PsTFL1c protein.

To determine whether differences between *LF* alleles occurred at the transcriptional level, *PsTFL1c* transcripts were analyzed by real-time PCR in the apices of plants with five expanded leaves (Figure 8). At this stage, plants have not initiated flowering, except those bearing the strong *lf-a* allele. In the mutant series WL1770 (*Lf*) and WL1769 (*lf*), derived from the WL1771 (*Lf-d*) progenitor line (Table 2), *PsTFL1c* was expressed sequentially higher. Between *Lf-d* and *lf*, there was a

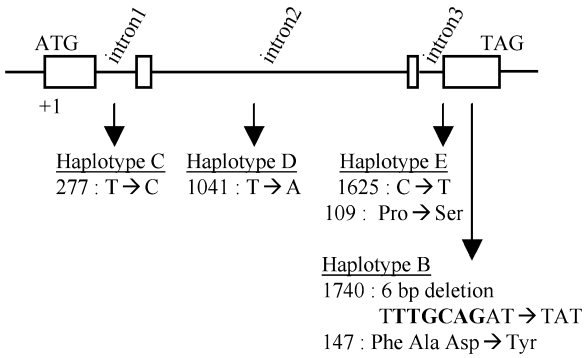

Figure 6. Description of the Different Haplotypes Identified from the Sequence Analysis of *PsTFL1c* in Several Pea Lines and Their Corresponding Mutants for *LF*.

Mutant lines are described in Table 2. Scheme of the *PsTFL1c* genomic sequence. Boxes represent the coding sequence. Arrows indicate the positions of the substitutions relative to haplotype A. For each haplotype, the position and the nature of each substitution are described and the predicted effect on the amino acid sequence is given.

10-fold difference (Figure 8). Therefore, for a given genetic background, there was a correlation between the expression of *PsTFL1c* and the node of flowering. A similar correlation was found in mutants K319 (*lf*) and K2 (*lf-a*) in the Torsdag (*Lf*) background. *PsTFL1c* transcript levels were threefold higher in Torsdag than in K319 (*lf*), whereas no significant expression was detected in K2, which carries a large deletion spanning the *TFL1* gene (Figure 6).

These results suggested that the low level of *PsTFL1c* transcription was associated with early flowering. This correlation seemed to be contradicted by the analysis of HL76 (*lf-a*), in which a relatively high level of *PsTFL1c* transcript was associated with early flowering. The *PsTFL1c* transcript level was not significantly different between HL75 (*Lf*) and HL76 (*lf-a*) (Figure 8). This contradiction can be explained by the presence of a mutation in the predicted PsTFL1c protein of HL76 (Figure 6). For this line, protein sequence, not mRNA level, was the cause of the early-flowering phenotype. Because no change was detected in *PsTFL1c* transcript level between HL75 and HL76, this finding suggests that there is no feedback regulation for *LF*.

We noticed that within the same class of *LF* allele, there was variation in the level of *PsTFL1c* transcript. For example, for the *Lf* allele, the relative expression level varied from 67 (Térèse line) to 151 (Torsdag line). This variation could be a consequence of modulation resulting from different genetic backgrounds. To test this hypothesis, we introduced *LF* alleles in new genetic backgrounds by successive backcrosses. The *Lf-d* allele, carried by the HL66 line, was introduced into the Térèse background by seven successive backcrosses. In this new genetic background, the level of *PsTFL1c* transcript in *Lf-d* plants was 40% lower than that in HL66 but still significantly higher (threefold) than that in the nearly isogenic *Lf* line Térèse (Figure 8). From these results, we conclude that *PsTFL1c* transcription is determined by the *LF* allele but also by different genetic backgrounds.

DISCUSSION

The genetics and physiology of flowering in pea have been studied in detail (Reid et al., 1996; Weller et al., 1997). We used a candidate-gene approach to study the role of *TFL1* homologs in the flowering process. Two of these homologs, *PsTFL1a* and *PsTFL1c*, were found to correspond to the pea genes *DET* and *LF*, respectively. Evidence for these correspondences and the conservation of *TFL1* homolog gene function between Arabidopsis and pea are discussed below.

DET, an Arabidopsis *TFL1* Homolog, Acts to Maintain the Fate of the Inflorescence Meristem in Pea

PsTFL1a mapped in the vicinity of *DET*. Sequencing of *PsTFL1a* in three independent *det* lines revealed mutations that would significantly modify PsTFL1a structure, leading to a nonfunctional protein. These results strongly indicate that *PsTFL1a* corresponds to *DET*. Further evidence that *PsTFL1a* corresponds to *DET* comes from mutant phenotype comparisons and gene interactions.

The phenotype of *det* mutants in pea is similar to those of *tfl1* and *cen*. In all three species, indeterminate growth is changed to determinate growth during the reproductive phase (Singer et al., 1990; Bradley et al., 1996, 1997). This conversion was proposed to result from the acceleration of the reproductive phase and the conversion of the inflorescence to a floral meristem in Arabidopsis (Ratcliffe et al., 1998). In *det* mutants, the inflorescence meristem is converted to a stub (Singer et al., 1999). As with *cen* mutants in snapdragon, *det* mutants present no phenotype during the vegetative phase, whereas *tfl1* mutants flower earlier in Arabidopsis. The absence of a vegetative phe-

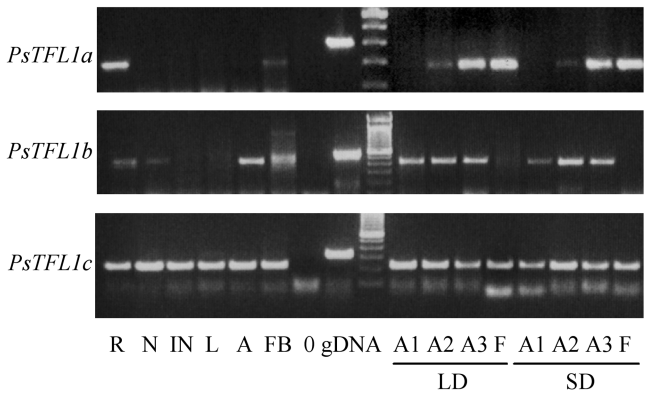

Figure 7. Expression Analysis of Pea *TFL1* Homologs by RT-PCR.

Specific primers for *PsTFL1a*, *PsTFL1b*, and *PsTFL1c* were used to amplify the cDNA (see Methods). As controls, PCR was performed on genomic DNA (gDNA) and water (0). PCR was performed on cDNA obtained from different tissues: root (R), dormant node 4 (N), internode (IN), leaf (L), vegetative apex (A), flower bud (FB), flower (F), and shoot apex during the floral transition under short-day (SD) or long-day (LD) conditions at three different stages (A1, before the floral transition; A2, after the floral transition but before flowering; and A3, after flowering). These three stages are described in detail in Methods.

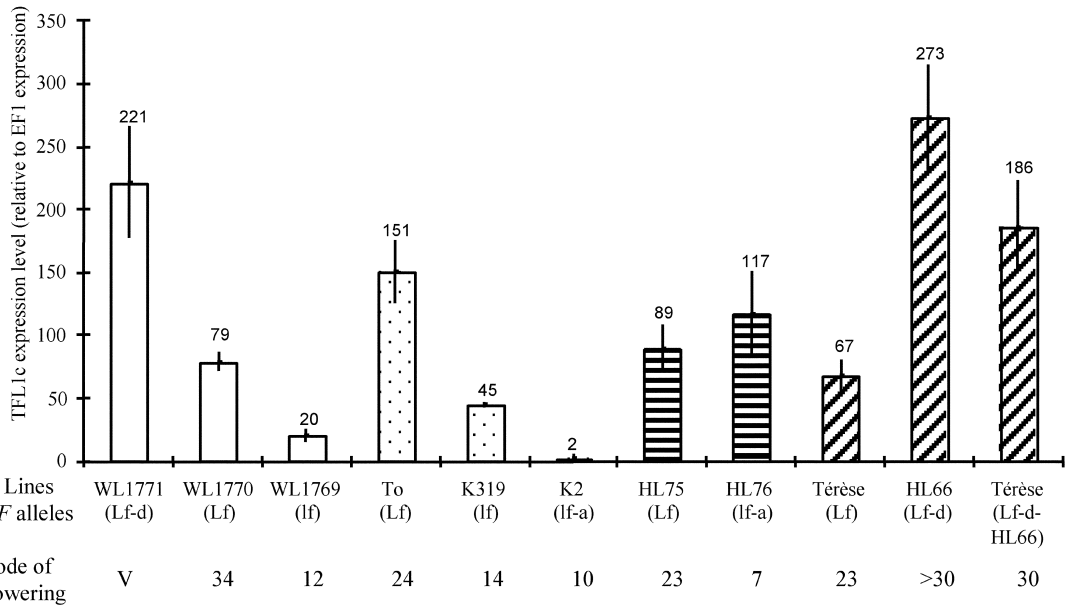

Figure 8. Analysis of the *PsTFL1c* Transcript Level in Different *LF* Lines by Real-Time PCR.

RNA was extracted from the apices of plants at the five-node stage. To compare the results, transcript levels also were evaluated for the elongation factor, *E1F*α, which is supposed to be constant in our conditions. Numbers represent the differences in expression between *PsTFL1c* and *EF1*α. The real-time PCR experiment was repeated three times. For each *LF* line, the allele at the *LF* locus and the node of flowering (average of four different plants grown in short-day conditions) are given. V indicates that the plants did not flower after 40 nodes and remained vegetative.

notype may be explained by the fact that *DET* and *CEN* (Bradley et al., 1996) are expressed only after the floral transition, whereas *TFL1* also is expressed during the vegetative phase (Bradley et al., 1997).

Interactions between *TFL1* and other important regulatory genes are conserved between some species. In Arabidopsis, *TFL1* antagonizes *LFY*, as does *CEN* with *FLORICAULA* in snapdragon. Double mutants (*tfl1 lfy* or *cen flo*) have a *lfy* or *flo* phenotype, respectively (Bradley et al., 1996, 1997). The same epistatic interaction is found in pea between *DET* and *UNIFOLIATA*, the *LFY* ortholog. The double mutant *det uni* has a *uni* phenotype (Singer et al., 1999).

LF, Another Arabidopsis *TFL1* Homolog, Is a Repressor of Flowering

The results obtained demonstrate that *PsTFL1c* corresponds to *LF*. *PsTFL1c* was mapped to linkage group II in the vicinity of *LF*. Analysis of *PsTFL1c* genomic DNA sequence in the strongest *lf-a* mutants (plants presenting the earliest flowering phenotype) showed significant modifications: four *lf-a* mutants are complete deletion mutants lacking *PsTFL1c* and can be considered null alleles; two other *lf-a* mutants contained nonsilent changes that could modify the structure or function of PsTFL1c.

Comparison of the *lf-a* and *tfl1* mutants reveals a similar early-flowering phenotype. In Arabidopsis, *TFL1* acts to maintain the apical meristem during the vegetative stage and thereby control the length of the vegetative phase (Bradley et al., 1997; Ratcliffe et al., 1998). In pea, *LF* also can be considered a regulator of the length of the vegetative phase. Mutant lines for this gene have a lower flowering node and early flowering time (Taylor and Murfet, 1993). *LF*, like *TFL1*, acts as a floral repressor by lengthening the vegetative phase. In contrast to *tfl1*, no major floral phenotype was detected for mutants at the *LF* locus.

Natural and induced mutant alleles for *LF* have been grouped into four classes: *Lf-d*, *Lf*, *lf*, and *lf-a* (Taylor and Murfet, 1993). The strongest *lf-a* phenotype is the result of mutation or deletion of *PsTFL1c*. This loss of function is consistent with *lf-a* being a recessive mutation. Sequencing of the *Lf-d*, *Lf*, and *lf* alleles revealed no change in the predicted amino acid sequence of PsTFL1c (Table 2, Figure 6). We demonstrated that the flowering phenotype in these alleles could be explained by differences in *PsTFL1c* transcript levels during the vegetative phase in the shoot apex. When comparing series of alleles in the same genetic background, *LF* transcript levels were correlated with the flowering node in pea. Plants having the late-flowering *Lf-d* allele had a high level of *LF* transcript. The *Lf-d* plants behaved like 35S-*TFL1* Arabidopsis plants, in which the upregulation of *TFL1* is responsible for a delay in flowering and a highly branched architecture (Ratcliffe et al., 1998). In pea, the influence of flowering genes on branching is well known (Floyd and Murfet, 1986; Beveridge et al., 2003). In particular, the *Lf-d* allele, by delaying flower initiation, results in plants with a high number of aerial lateral branches. The high transcript levels in *Lf-d* plants are consistent with the dominance of this allele. Furthermore, a transcript dose effect also would explain the inter-

mediate flowering node seen in heterozygous individuals (Murfet, 1975).

One hypothesis to explain the differences in transcript levels between the *LF* alleles is that important *cis*-regulatory elements are mutated. We have sequenced the untranslated region (200 bp 5′ and 260 bp 3′) and the introns in a series of different *LF* alleles (Table 2), and only two changes were detected. One substitution (haplotype D; Figure 6) seems to result from natural variation with no effect on flowering, because it is found in WL1771 (*Lf-d*), WL1770 (*Lf*), and WL1769 (*lf*) (Table 2). More interesting is the substitution in intron 1 (haplotype C; Figure 6), which is found in independent *lf* plants derived from different wild-type lines. *lf* lines Wt11790 and Wt11791 were obtained from the *Lf* line Porta (Murfet, 1991), whereas *lf* line K319 was obtained from the *Lf* line Torsdag (Uzhintseva and Sidorova, 1988). It is intriguing that identical alleles appeared independently in different mutagenesis programs. Important *cis* elements can be present in the introns, as was shown in the second intron of *AGAMOUS* (Lohmann et al., 2001). Modification of the expression of genes between different alleles has been demonstrated for important traits. In tomato, the *fw2.2* alleles regulate fruit size through changes in transcript regulation rather than in the FW2.2 protein itself (Cong et al., 2002). In rice, *Hd3*, a *FT* homolog, shows different levels and timing of gene expression between different alleles (Kojima et al., 2002). In both cases, SNP between the alleles have been detected in putative regulatory regions. Further analysis will be required to prove the association between SNP and expression changes in the *lf* alleles.

Different Regulation between *TFL1* Homologs in Pea and Arabidopsis

In pea, two *TFL1* homologs have two distinct functions: *LF* is involved in the control of the vegetative phase by delaying floral initiation, the transition from the vegetative to the I1 inflorescence meristem, and *DET* is involved in the control of the floral phase by preventing the transition from the I1 inflorescence meristem to the flower (Figure 9). This regulation is different from that in Arabidopsis, in which only one *TFL1* gene controls the length of both the vegetative and floral phases (Figure 9). To obtain the *tfl1* phenotype in pea (early flowering and determinate growth), a *det lf* double mutant is necessary (Murfet, 1989). *LF* and *DET* are homologs and may derive from a common ancestor by duplication. In pea, the two genes may have evolved separately and become specialized for two distinct functions. The *det lf-a* double mutant has only additive effects and no extra phenotype (Murfet, 1989), which suggests nonredundant functions for *DET* and *LF*. It has been argued that subfunctionalization of duplicated genes is a mechanism whereby degenerative mutations can lead to the preservation of duplicated genes (Force et al., 1999; Lynch and Force, 2000). *DET* and *LF* may provide an example in which a gene that was expressed originally during both the vegetative and reproductive phases (such as *TFL1* in Arabidopsis) diverged into two copies with a partitioning of gene expression patterns as predicted by the duplication/degeneration/complementation model. Like *DET*, *CEN* has only one function during the vegetative phase in snapdragon (Bradley et al., 1996), so it will be interesting to determine whether another *TFL1* homolog exists in snapdragon that functions during the vegetative phase in a manner similar to *LF*.

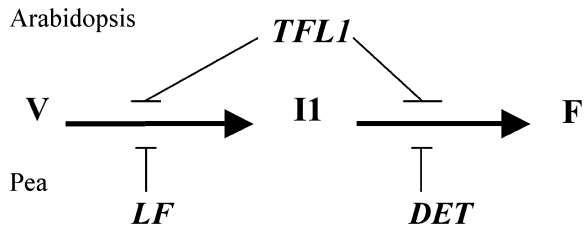

Figure 9. Model for the Control of Floral Initiation and Development by *TFL1* Genes in Arabidopsis and Pea.

F, flower; I1, inflorescence meristem; V, vegetative phase.

In this study, we have demonstrated the important role played by a *TFL1* homolog, *LF*, in the control of flowering time in pea. Detailed genetic analyses have enabled the identification of a few genes involved in the natural variation in flowering time among pea cultivars under controlled environmental conditions (Murfet, 1971b; Weller et al., 1997). *LF* was identified as a major contributor to natural variability, but other genes involved in the photoperiodic pathways also were shown to influence flowering time in pea, such as *STERILE NODE* and *HIGH RESPONSE*. These genes are implicated in the synthesis of a graft-transmissible floral inhibitor, which is perceived in the apex by *LF* (Murfet, 1971a). As a long-day flowering plant, pea has developed a strategy to control flowering in response to photoperiod and vernalization, with a central role for *LF*. Vernalization acts quantitatively by reducing the flowering node in two ways: by decreasing inhibitor production and by rendering the apex more sensitive to the flowering signal (Murfet and Reid, 1974; Reid and Murfet, 1975).

The strategy developed by Arabidopsis seems to be different, because *TFL1* plays a minor role in the control of flowering time between ecotypes. In Arabidopsis, *FLOWERING LOCUS C* (*FLC*) and *FRIGIDA* (*FRI*) are central elements and are key components of the response to vernalization (Michaels and Amasino, 1999; Sheldon et al., 1999; Johanson et al., 2000). For instance, in natural Japanese Arabidopsis populations, the digenic interaction between *FRI* and *FLC* was the major genetic system controlling flowering time (Shibaike et al., 1999). *FLC* is a floral repressor, which must be repressed by vernalization for floral initiation to occur. *FRI* acts synergistically by maintaining high levels of *FLC*. Early-flowering ecotypes of Arabidopsis carry nonfunctional *FRI* and/or *FLC* alleles (Johanson et al., 2000) and consequently have low levels of *FLC* transcript, whereas late-flowering ecotypes have high levels of *FLC* expression (Rouse et al., 2002). This important role for *FLC* also is found in other Brassicaceae species (Tadege et al., 2001; Schranz et al., 2002). Thus, pea and Arabidopsis have developed different strategies to control flowering time, which may represent different strategies to respond to environmental conditions. In natural Arabidopsis populations, flowering time variation is explained mainly by response to vernalization, whereas in pea it seems to be explained mostly by response to photo-

period. Interestingly, in both cases, the transcript level of the repressor, FLC or LF, determines flowering time. Different alleles exist at the *LF* locus and are responsible for different *LF* transcript levels. The same results were found in Arabidopsis at the *FLC* locus, where different alleles confer different *FLC* transcript levels (Schlappi, 2001).

In conclusion, we have shown that *TFL1* homologs in pea play an important role in floral initiation and development. *LF* is a major gene that controls flowering time by integrating different environmental and endogenous signals. Understanding the regulation of *LF* by genes involved in photoperiod response is an important next step for dissecting the molecular basis of flowering control in pea.

METHODS

Plant Material and Growth Conditions

det lines of pea (*Pisum sativum*) were obtained from the John Innes Pisum Germplasm collection. *det-1* corresponds to JI 2121 and was obtained by mutation of cv Paloma (Swiecicki, 1987). *det-2* (JI 1358) is a spontaneous mutant, and *det-3* (JI 3100) was obtained by mutation of line SG (Berdnikov et al., 1999). Other pea cultivars were Térèse and Torsdag. The F2 and RI lines used for genetic mapping are described below. *LF* lines were provided by Ian Murfet (University of Tasmania, Hobart, Australia). Relevant information concerning the mutants and their progenitors is described by Taylor and Murfet (1993).

For the floral initiation experiments, the plants were grown in cabinets at 20°C during the day and 15°C during the night with illumination by mercury vapor lamps (135 $mE \cdot m^{-2} \cdot s^{-1}$) in a 1:1 mixture of sphagnum: clay under short-day (12 h of light) or long-day (18 h of light) conditions. For the long-day conditions, plants received 12 h of light from the mercury vapor lamps extended by 6 h of light from a series of 40-W incandescent/fluorescent bulbs. The apices were harvested at three different stages. The A1 stage was collected at least 10 nodes before plants flower. At this stage, the apices are still vegetative (Isabelle Lejeune, personal communication). The A2 stage was harvested just three nodes before flowering. At the A2 stage, the floral initiation has occurred but flowers are not opened. The A3 stage corresponds to the apices just after flowers open.

Cloning and Isolation of Genes

The *TFL1* homologs were isolated in cv Térèse using degenerate primers. The PCR conditions were 35 cycles at 94°C for 60 s, 55°C for 60 s, and 72°C for 2 min. The primer combination TFL1-1 (5′-ATGGGGAGAGTGATA/T/CGGG/AGAA/TG-3′) and TFL1-2 (5′-TCACTAGGA/GCCA/TGGAACATCA/TGG-3′) gives a 450-bp band corresponding to *PsTFL1c*. Using the primer combination TFL1-3 (5′-GATGTTCCA/TGGA/TCCTAGTGAC/TCC-3′) and TFL1-5 (5′-CTTGCAGCA/GGTC/TC/TTCC/TCTC/TTG-3′), two bands of 400 and 800 bp were obtained corresponding to *PsTFL1a* and *PsTFL1b*, respectively.

The sequences of the genes were extended by 3′ rapid amplification of cDNA ends (RACE) PCR using the kit and the recommendations of the supplier (Life Technologies, Rockville, MD) on cDNA from seedlings and flowers. The following primers were used as gene-specific primers and nested gene-specific primers, respectively, for *PsTFL1a* (TFL1R1 [5′-TCAAACAAAGAGCGAGAGATTCAG-3′] and TFL1R2 [5′-AGACCATTTCAACACTCGTAG-3′]), for *PsTFL1b* (Tb31 [5′-GACAGATATACCAGGCACAAC-3′] and Tb32 [5′-GAAATATGAAATGCCACGTCC-3′]), and for *PsTFL1c* (TFL1-1 and TFL5R3 [5′-AGCCAAGGATTCAGATTCAAGG-3′]). The 5′ part of *PsTFL1a* was obtained by 5′ RACE PCR using the kit and the recommendations of the supplier (Life Technologies) on cDNA from flowers. The gene-specific primers used were TFLR3 (5′-CTACTTTGATACACACACGACAC-3′) and TFL5R1 (3′-GAATAGAACAAACACAAACCT-3′). The 5′ part of *PsTFL1c* was recovered by PCR walking on genomic DNA (Devic et al., 1997). Pea genomic DNA was digested by DraI, and adaptors were ligated to the digested DNA as described by Devic et al. (1997). The following specific primers were used to amplify the gene: TFL1cR8 (5′-TAACTGTAGAAGGAAAGGGTA-3′) and TFL1cR9 (5′-ATGCTTGCGGTAAAATAATCA-3′).

Mapping and Marker Development

Mapping was performed on two different mapping populations (recombinant inbred lines) obtained from the crosses Térèse × K586 (Laucou et al., 1998) and JI 281 × JI 399 (Ellis et al., 1992). Polymorphisms were sought between the parents in the mapping population to develop PCR markers such as cleaved amplified polymorphism sequence (CAPS) markers. For *PsTFL1a*, a single nucleotide polymorphism was detected between Térèse and K586. A derived CAPS (dCAPS) marker (Neff et al., 1998) was developed using the following primers (TFL2, 5′-GAACACTTGCACTGGTAAATATAATAGA-3′; TFLR, 5′-TGTAGCATCTGTTGTTCCTGG-3′) and the HinfI enzyme. For *PsTFL1b* and *PsTFL1c*, polymorphisms were found between JI 281 and JI 399 and CAPS markers were developed for *PsTFL1b* (primer pairs TFLb32 [5′-GAAATATGAAATGCCACGTCC-3′] and TFL1bR1 [5′-ACAAACTAGAACAACAACAACCC-3′] and restriction enzyme HhaI) and for *PsTFL1c* (primer pairs TFL1-1 and TFL1-2 and restriction enzyme HpyF44III).

DNA Extraction and Gel Blots

DNA was extracted from leaves according to the protocol described by Laucou et al. (1998). Ten micrograms of genomic DNA was digested with restriction enzymes and loaded on a 0.7% agarose gel. Blotting was performed according to the recommendation of the membrane supplier (Biotrans Nylon Membrane; ICN, Costa Mesa, CA). Hybridization was performed in Church and Gilbert (1984) buffer at 65°C overnight. Washing was done at 65°C according to Sambrook et al. (1989) (2× SSC [1× SSC is 0.15 M NaCl and 0.015 M sodium citrate] and 0.1% SDS for 30 min, 1× SSC and 0.1% SDS for 15 min, and 0.2× SSC and 0.1% SDS for 15 min).

RNA Extraction and Expression Analysis

RNA was extracted using the RNeasy Plant Mini Kit with a DNase treatment on an RNeasy column (Qiagen, Valencia, CA). Five micrograms of total RNA was used for cDNA synthesis. Before reverse transcription, total RNA was treated with amplification-grade DNase I (Life Technologies) according to the manufacturer's protocol. Reverse transcription was performed using 200 units of Superscript II RNase H− reverse transcriptase (Invitrogen, Carlsbad, CA) in the presence of 40 units of recombinant ribonuclease inhibitor (Life Technologies) with the AP primer. For PCR, cDNAs were resuspended in 100 μL of water, and 1 μL was used per reaction. Reverse transcriptase–mediated PCR was performed using specific primers for *PsTFL1a* (TFL5RACE [5′-TGAGTTGTACTCTTAAGTTCTTC-3′] and TFLaR5 [5′-AGGGCCAGGAACATCAGGGTC-3′]), *PsTFL1b* (TFL1bR1 and TFL1b31 [5′-GACAGATATACCAGGCACAACAG-3′]), and *PsTFL1c* (TFLcR2 [5′-AAATAAGCAGCAGCAACAGGG-3′] and TFLcR3 [5′-CAGACATTCCAGGGACAACAG-3′]) and the following program (40 cycles at 94°C for 30 s, 60°C for 60 s, and 72°C for 30 s). The PCR product was loaded on a 2% (w/v) agarose gel.

Real-time PCR was performed on a Roche Lightcycler using the FastStart DNA Master SYBR Green I kit (Mannheim, Germany) according to the manufacturer's protocol. Specific primers suitable for quantitative

reverse transcriptase–mediated PCR were designed using LC Probe Design Report software (Roche) for *EF1α* (E1FF1 [5′-GATGCACCTGGACATCGTGACT-3′] and E1FR1 [5′-CTTAGGGGTGGTAGCATCCATCT-3′]) and *PsTFL1c* (cF3 [5′-CCACATTTGGAAAAGAGTTGACAAGC-3′] and cR3 [5′-GCGTCTTCTAGGAGCCGTTGC-3′]). The PCR program consisted of a first step of denaturation and Taq activation (95°C for 8 min) followed by 45 cycles of denaturation (94°C for 10 s), annealing (58°C for 7 s), and extension (72°C for 10 s). At the end, amplified products were denatured (95°C), renatured (65°C), and progressively denatured (step from 65 to 95°C over 30 min or 0.1°C/s for the fusion curve analysis). Both primers combinations (cR3/cF3 and E1FR1/E1FE1) were tested (fusion curve, linearity, and efficiency of the primers). The primer combinations for *E1F* and *PsTFL1c* have a PCR efficiency of 86 and 80%, respectively.

For PCR, cDNA was diluted 50 times and 5 μL was used as a template in a 20-μL reaction mix. *PsTFL1c* transcript level was estimated based on the level of the constitutive *EF1α* gene (Nesi et al., 2000). For each condition, the number of cycles necessary to reach a certain level (exit position) of fluorescence was evaluated for *EF1α* ($n_{EF1\alpha}$) and *PsTFL1c* ($n_{PsTFL1c}$). The difference between the exit points of the two genes was calculated ($d1 = n_{PsTFL1c} - n_{EF1\alpha}$). The value 2^{d1} represents the difference of copy number between *PsTFL1c* and the constitutively expressed gene, *EF1α*. Because *EF1α* was expressed at a higher level than *PsTFL1c*, the final ratio was calculated as follows: PsTFL1c level = $(1/2^{dl}) \times 100{,}000\%$ EF1α.

Upon request, materials integral to the findings presented in this publication will be made available in a timely manner to all investigators on similar terms for noncommercial research purposes. To obtain materials, please contact Catherine Rameau, rameau@versaille.inra.fr.

Accession Numbers

The sequence data described herein have been submitted to GenBank with accession numbers AY340579 for *PsTFL1a*, AY340580 for *PsTFL1b*, and AY343326 for *PsTFL1c*. Accession numbers for the sequences shown in Figure 2 are as follows: AB027506 (TSF), AB016880 (BFT), AF147721 (MFT), and BAA33415 (*BnTFL1-1* from *Brassica napus*).

ACKNOWLEDGMENTS

We thank Mike Ambrose, K. Sidorova, and Ian Murfet for providing the mutant lines. Thanks also are due to Bertrand Dubreucq for help and suggestions with the real-time PCR experiments, to Magali Goussot, Karine Haurogné, Xenie Johnson, and Nathalie Mansion for technical assistance, and to Patrick Grillot for his excellent management of the greenhouse. We also particularly thank Julie Hofer and Jim Weller for their critical comments and corrections on the manuscript and for their suggestions. Finally, we dedicate this article to Ian Murfet for his great contribution to the understanding of flowering genetics in pea. This work was supported by Genoplante (Project PEA-A) and the Human Frontiers in Science Program. M.J.B. is a Royal Society (UK) University Research Fellow.

Received July 24, 2003; accepted September 12, 2003.

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