Proteins from the FLOWERING LOCUS T-like subclade of the PEBP family act antagonistically to regulate floral initiation in tobacco

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SUMMARY

Flowering is an important agronomic trait that often depends on the integration of photoperiod, vernalization, gibberellin and/or autonomous signaling pathways by regulatory proteins such as FLOWERING LOCUS T (FT), a member of the phosphatidylethanolamine-binding protein (PEBP) family. Six PEBP family proteins control flowering in the model plant Arabidopsis thaliana, and their regulatory functions are well established, but variation in the number and structural diversity of PEBPs in different species means their precise functions must be determined on a case-by-case basis. We isolated four novel FT-like genes from Nicotiana tabacum (tobacco), and determined their expression profiles in wild-type plants and their overexpression phenotypes in transgenic plants. We found that all four genes were expressed in leaves under short-day conditions, and at least NtFT3 expression was restricted to phloem companion cells. We also found that the NtFT1, NtFT2 and NtFT3 proteins are floral inhibitors (atypical for FT-like proteins), whereas only NtFT4 is a floral inducer. We were unable to detect the expression of these genes under long-day conditions, suggesting that all four tobacco FT-like proteins may control flowering in response to short days. Phylogenetic analysis of PEBP family proteins and their functions in different solanaceous species confirmed that gene duplication and divergence within the FT-like clade has led to the evolution of antagonistic regulators that may help to fine-tune floral initiation in response to environmental cues.

Keywords: FLOWERING LOCUS T, floral transition, floral repressor, biomass, antagonistic FTs, tobacco.
zinc-finger transcription factor CONSTANS (CO), which is stabilized by illumination (Samach et al., 2002; Valverde et al., 2004). The FT protein then enters the sieve elements and is transported by mass flow to the SAM, where it interacts with the basic leucine zipper domain (bZIP) transcription factor FD to activate downstream targets such as SUPPRESSOR OF OVEREXPRESSSION OF CONSTANS 1 (SOC1) and the floral meristem identity gene APETALA 1 (AP1) (Kardailsky et al., 1999; Kobayashi et al., 1999; Abe et al., 2005; Wigge et al., 2005; Yoo et al., 2005; Corbesier et al., 2007). The second FT-like protein in Arabidopsis (TSF) is also a direct regulatory target of CO, and acts as a floral promoter, presumably through the same regulatory mechanism (Yamaguchi et al., 2005). The MFT protein in Arabidopsis also acts as a floral promoter (Yoo et al., 2004).

FT-like proteins that promote flowering have been identified in dicotyledonous plants such as Solanum lycopersicum (tomato; Molinero-Rosales et al., 2004), Populus spp. (poplar; Böhlenius et al., 2006; Hsu et al., 2006, 2011), Malus domestica (apple; Hättasch et al., 2008; Tränkner et al., 2010), Beta vulgaris (sugar beet; Pin et al., 2010), cucurbits (Lin et al., 2007), Helianthus annuus (sunflower; Blackman et al., 2010), Pisum sativum (pea; Hecht et al., 2011), Glycine max (soybean; Kong et al., 2010) and Solanum tuberosum (potato; Navarro et al., 2011), as well as in monocotyledonous plants such as Oryza sativa (rice; Kojima et al., 2002), Hordeum vulgare (barley; Faure et al., 2007) and Zea mays (maize; Danilevskaya et al., 2008; Meng et al., 2011). Under short-day (SD) conditions, flowering in Arabidopsis is controlled by the gibberellin pathway, which activates SOC1 expression directly, with no involvement from PEBP family proteins (Moon et al., 2003). However, many other species do use FT-like proteins to regulate flowering under SD conditions, e.g. the closely related FT-like proteins Heading date 3a (Hd3a) and RICE FLOWERING LOCUS T1 (RFT1) that are essential for flowering in rice (Komiya et al., 2001; Yoo et al., 2010). FT and TFL1 (and presumably the other PEBPs) interact with FD in the shoot apex to antagonistically regulate downstream target genes (Abe et al., 2005; Wigge et al., 2005; Hanano and Goto, 2011). The substitution of specific amino acids can convert TFL1 into a floral inducer and FT into a floral repressor, suggesting that functional switching between positive and negative regulatory modes may be a straightforward evolutionary mechanism to fine-tune the regulation of flower development (Hanzawa et al., 2005; Ahn et al., 2006).

Flowering time has a strong impact on biomass production, and it is therefore necessary to understand how flowering is regulated in important crops such as Nicotiana tabacum (tobacco), where the genetic basis of flowering has received little attention. Different Nicotiana species show diverse flowering-time responses in relation to the photoperiod, e.g. some diploid species are LD plants (e.g. N. sylvestris), whereas others are SD plants (e.g. Nicotiana tomentosiformis), and most N. tabacum varieties are day-neutral, although the variety Hicks Maryland Mammoth is a prominent exception (Garner and Allard, 1920). N. tabacum is particularly interesting because this species probably arose from a tetraploidization involving the LD species N. sylvestris and a close relative of the facultative SD species N. tomentosiformis.

We identified four FT-like genes in tobacco and characterized their roles by expression analysis in wild-type plants and overexpression in transgenic plants. The resulting phenotypes ranged from precocious flowering in tissue culture to the development of giant, non-flowering plants, permanently restricted to vegetative growth. Our data suggest that although members of the FT-like clade are primarily regarded as floral promoters, the evolution of antagonistic FT-like paralogs may be a common strategy in solanaceous plants to fine-tune floral initiation in response to multiple internal and environmental cues.

RESULTS

Isolation and phylogenetic classification of tobacco FT-like genes

We searched for potential FT-related genes in tobacco using the Arabidopsis FT gene as a BLAST query against tobacco sequences in the National Center for Biotechnology Information (NCBI) tobacco expressed sequence tag (EST) and genomic survey sequences (GSS) databases. This revealed a single cDNA clone (DV999455.1) containing a complete FT open reading frame, as confirmed by alignment with Arabidopsis FT. We used this tobacco cDNA clone to design primers that were then used in different PCR-based strategies (rapid amplification of cDNA ends, RACE, and genome walking) to isolate sequences from tobacco genomic DNA and leaf cDNA, resulting in the isolation of four distinct FT-related sequences, which we named NtFT1, NtFT2, NtFT3 and NtFT4. All four genes comprised four exons with three introns at conserved positions identical to those in FT genes from other species, but the introns differed in length. The exon–intron structures of the four tobacco genes are compared with Arabidopsis FT in Figure 1a.

Sequence analysis using INTERPROSCAN revealed the presence of the characteristic PEBP domain in all four tobacco FT-related proteins. A recent phylogenetic analysis of the
plant PEBP family revealed three main clades, described as FT-like (floral promoters), TFL1-like (floral repressors) and MFT-like (floral promoters) (Chardon and Damerval, 2005). We fitted the tobacco FT-related proteins into this classification by creating a maximum-likelihood tree from an alignment of the four novel tobacco FT proteins, the key Arabidopsis regulators FT, TFL1 and MFT, the tobacco TFL1 homologs CET1, CET2 and CET4 (CENTRORADIALIS-like genes from tobacco; Amaya et al., 1999), and additional PEBP family proteins characterized by Karlgren et al. (2011). As shown in Figure 1b, the three Arabidopsis proteins (in red) represent the corresponding main clades, as expected,

**Figure 1.** Classification of the tobacco FT-like proteins NtFT1–NtFT4.
(a) The exon–intron structure of the tobacco NtFT1–NtFT4 genes resembles that of AtFT. Exons are shown as boxes and introns are shown as lines. Dashed lines indicate NtFT3 and NtFT4 introns of unknown size, only partially identified by genome walking and PCR, most probably because of the large intron sizes.
(b) Phylogenetic tree of the plant PEBP family, defined by Karlgren et al. (2011), including our novel tobacco FT-like proteins NtFT1–NtFT4 (green). Representative Arabidopsis proteins are shown in red. The tobacco CET proteins are shown in blue. Sugar beet FTs are shown in magenta. Abbreviations: ATC, Arabidopsis thaliana CENTRORADIALIS; BFT, A. thaliana BROTHER OF FT and TFL1; BvFT, Beta vulgaris FLOWERING LOCUS T; FT, A. thaliana FLOWERING LOCUS T; MFT, A. thaliana MOTHER OF FT and TFL1; NtCET1, 2, 4, CENTRORADIALIS-like genes from tobacco; NIFT1–NtFT4, Nicotiana tabacum FLOWERING LOCUS T; TFL1, A. thaliana TERMINAL FLOWER 1; TSF, A. thaliana TWIN SISTER OF FLOWERING LOCUS T.
whereas all four tobacco FT proteins (in green) cluster conspicuously within the FT-like clade, suggesting that they should act as floral promoters.

To validate the phylogenetic classification of the novel tobacco FT-like proteins, we aligned their amino acid sequences with Arabidopsis FT, and with Arabidopsis TFL1 and its tobacco homologs CET1, CET2 and CET4 using T-Coffee (EMBL-EBI) (Figure S1). The putative tobacco FT proteins were closely related to each other (ranging from ~70% identity between NtFT3 and NtFT4, to ~89% identity between NtFT1 and NtFT3), and to Arabidopsis FT (ranging from ~62% identity between AtFT and NtFT2, to ~73% identity between AtFT and NtFT4). However, they showed only ~52% identity to tobacco CETs and Arabidopsis TFL1. A detailed list of all comparative sequence identities calculated using emblucss needle (EMBL-EBI, http://www.ebi.ac.uk/) is provided in Table S1. These data show convincingly that NtFT1–NtFT4 belong to the FT-like clade, and not to the TFL1-like clade.

Tobacco FT-like genes have antagonistic functions in floral development

We investigated the functions of the four tobacco FT-like genes by overexpressing them in transgenic tobacco plants under the control of the strong and constitutive cauliflower mosaic virus (CaMV) 35S promoter. We generated up to seven independent transgenic lines expressing each FT-like gene by transformation with Agrobacterium tumefaciens, and propagated the transgenic plants in tissue culture before transferring to pots containing soil.

The ability of NtFT4 to promote flowering became apparent almost immediately, because flower-like structures and complete flowers began to form on 35S:NtFT4 transgenic shoots during the early stages of tissue culture, arresting further shoot development, preventing the formation of roots and thus precluding the regeneration of mature transgenic plants (Figure S2a,b). The phenotype of the 35S:NtFT4 transgenic plants was nearly identical to that of control plants expressing Arabidopsis FT (35S:AtFT), as shown in Figure S2c,d.

To further validate this proposed function for NtFT4, we additionally generated five independent transgenic Arabidopsis lines expressing the NtFT4 gene under the control of the CaMV 35S promoter (35S:NtFT4) by the floral-dip method. We produced 10 T2 plants from each line, and found in all cases that the transgenic plants flowered earlier than wild-type controls (line 1 is shown as an example in Figure 2a). The number of rosette leaves before flowering was also lower in the transgenic lines, ranging from an average of 7.5 in line 3 to 14.2 in line 5, compared with 35 in the wild-type control (Figure 2b). The precociousness of the early-flowering phenotype correlated with the level of NtFT4 overexpression (Figure 2c). Together, these data strongly suggest that NtFT4 is a floral promoter.

In contrast, the shoots of the 35S:NtFT1, 35S:NtFT2 and 35S:NtFT3 transgenic tobacco plants were near normal, although internode elongation was slightly inhibited in some cases. Nevertheless, plantlets transformed with each of the three constructs (and expressing the corresponding genes at high, moderate and low levels) were propagated as cuttings in order to provide two clones of each line with identical expression levels, and were cultured until the roots developed, at which point they were transferred to phytotrons with one clone cultivated under LD conditions and the other under SD conditions, along with wild-type control plants.

The control plants flowered after 4 weeks under LD conditions and after 5 weeks under SD conditions. Because N. tabacum SR1 is a day-neutral variety, the difference in LD/
SD flowering times in the control plants probably reflects the higher rate of photosynthesis and faster plant maturation under LD conditions. The transgenic plants showed a delayed flowering phenotype, the severity of which was dependent on the transgene expression level, but independent of the photoperiod. Under both LD (Figure 3a–c) and SD (Figure 3d–f) conditions, low transgene expression generated a mild phenotype in which flowering was delayed by approximately 3 days (Figure 3j–l), moderate transgene expression generated a moderate phenotype in which flowering was delayed by 1–1.5 weeks (Figure 3j–l) and there was a slight reduction in internode length (no moderate expression or phenotype was produced for 3SS:NtFT3 plants), and high transgene expression generated a severe phenotype in which flowering was prevented (Figure 3j–l) and the internodes were significantly shorter than normal.

The number of leaves on all transgenic plants remained comparable with wild-type plants, regardless of genotype and phenotype. Comprehensive quantitative RT-PCR experiments using total leaf RNA confirmed the direct correlation between the severity of the phenotype and the level of transgene expression, with the highest transcript levels
found in the most severely affected plants, regardless of the construct (Figure 3g–i).

We next set out to determine whether flower development in the severely affected plants was blocked or merely delayed. Therefore, severely affected transgenic plants representing all three constructs were cultivated under LD conditions in the glasshouse (Figure 4). We observed an increase in the internode length (hereafter described as bolting) after 6–8 weeks in all 35S:NtFT1 transgenic lines, and in one 35S:NtFT2 line, followed by flower development on plants of ~2 m in height after 11 weeks (individual lines are shown as examples in Figure 4a,b,e,f). The remaining 35S:NtFT2 lines and all 35S:NtFT3 lines produced a less compressed but still flowerless phenotype (Figure 4c,d,g,h). Quantitative RT-PCR experiments indicated that the FT transcript levels were generally higher in plants with the more severe phenotypes (Table S2), and these plants continued to grow without flowering up to the time of manuscript submission (8.5 months thus far), as shown in Figure 4i,j, reaching a maximum height of 5 m. The most severely affected plants accumulated substantially more biomass than wild-type plants, with ~210 leaves, each with a maximum length of 70 cm when mature, which is 60% more than 8-week-old wild-type plants (Figure 4k,l). The biomass of the severely affected plants under SD conditions was terminated after 6 months when plants reached the (2-m) ceiling of the phytotron, but until then their habit was similar to that of their counterparts grown under LD conditions.

All four tobacco FT-like genes have the same basal expression profile in leaves

We sought further insight into the roles of the four tobacco FT-like genes by monitoring their spatiotemporal expression profiles. We isolated total RNA from the leaf, apex, stem and root tissues of 4-week-old tobacco plants cultivated under LD and SD conditions, and compared the expression levels of NtFT1, NtFT2, NtFT3 and NtFT4 with the housekeeping genes.

Figure 4. Growth behavior of transgenic tobacco lines overexpressing NtFT1–NtFT3 with severe phenotypes.

(a–j) Time series of selected lines overexpressing NtFT1, NtFT2 or NtFT3 under long-day (LD) conditions. Pictures were taken 8, 11.5 and 29 weeks after transfer (wat) to the phytotron. The wild-type (WT) plant in (i) and (j) is 8 weeks old. Bolting in plants with severe phenotypes correlates with the overexpression levels of NtFT1–NtFT3 (Table S2), because plants already bolting at 8.5 wat (35S:NtFT1,1, and 35S:NtFT2,1) show the lowest expression level among the severe-phenotype plants. Although plants with a compressed phenotype at 8.5 wat (35S:NtFT2,2 and 35S:NtFT3,1) started bolting at ~11.5 wat, they were still growing vegetatively, and failed to flower under LD conditions. Scale bars: left, 50 cm; right, 1 m.

(k) Comparison of leaf number, height, stem diameter and biggest leaf length in cm.

(l) Comparison of apical (top), medial (middle) and basal (bottom) leaves between 35S:NtFT2,2 plants (at 28 weeks) 35S:NtFT3,1 plants (at 28 weeks) and a flowering wild-type plant (at 8 weeks). Scale bar: 10 cm.
gene NtEF1a by quantitative RT-PCR (Figure 5). NtFT1, NtFT2 and NtFT4 were expressed exclusively in leaf tissue under both LD and SD conditions, but the expression level under LD conditions was weak and near the detection threshold (Figure 5a,b). The expression level of NtFT3 was too low under both LD and SD conditions for reliable quantitation by RT-PCR.

We studied the spatial expression profile of the tobacco FT genes in more detail by expressing an ER-tagged version of green fluorescent protein (GFP<sub>ER</sub>) under the control of a 1-kb NtFT3 promoter fragment (P<sub>NtFT3</sub>). Confocal laser scanning microscopy (CLSM) showed that GFP expression was restricted to the leaf vascular bundle, as shown in the cross section of a leaf petiole in Figure 5c. The strongest signal was observed in the veins of basal leaves. The longitudinal section of the petiole confirmed that P<sub>NtFT3</sub> is active specifically in phloem companion cells, which are typically localized adjacent to sieve elements, as shown by the sieve plate staining with the callose-specific dye aniline blue in Figure 5d. P<sub>NtFT3</sub> activity therefore corresponds with the leaf-specific expression of NtFT1, NtFT2 and NtFT4, and suggests that all four FT-like proteins share a common leaf-specific expression profile, regardless of their positive or negative impact on flowering.

We investigated the temporal expression profiles of the tobacco FT mRNAs by isolating total RNA from tobacco seedlings and basal leaves harvested weekly from tobacco plants cultivated under LD as well as SD conditions, and monitoring the transcript levels by quantitative RT-PCR (Figure 5e–g). As we already noted, the level of NtFT3 expression under SD and LD conditions, and the levels of the remaining genes under LD conditions, were near the assay detection limit, and were not included in the study. NtFT1, NtFT2 and NtFT4 showed similar expression profiles under SD conditions, with low levels in seedlings but progressively higher levels in leaves, reaching a maximum during flowering (Figure 5e–g). This trend was also evident for NtFT3. Although NtFT4 was expressed at a lower level than NtFT1 and NtFT2, the 4400-fold increase in NtFT4 levels during flowering was significantly greater than that of NtFT1 (164-fold) and NtFT2 (936-fold), suggesting a significant shift in the relative levels of positive and negative regulators (Figure 5h).

Flower-repressing and flower-promoting tobacco FT proteins interact with Arabidopsis FD in the nucleus

To determine whether the flower-repressing activity of tobacco FT-like proteins is transferrable to other species, we overexpressed NtFT2 in Arabidopsis plants under the control of the CaMV 35S promoter. Arabidopsis does not possess endogenous FT-like floral repressors, instead relying on three TFL1-like proteins. Even so, the phenotypes

![Figure 5](image_url)

Figure 5. Spatial and temporal expression pattern of NtFT1–NtFT4. (a, b) NtFT1, NtFT2 and NtFT4 are expressed solely in leaves under short-day (SD, a) and long-day (LD, b) conditions. Values have been normalized to the reference gene EF1a.

(c, d) Localization of NtFT3 expression by CLSM using 1 kb of the NtFT3 promoter cloned upstream of the reporter gene GFP<sub>ER</sub> stably expressed in transgenic tobacco plants. Expression of NtFT3 can be localized to the vascular bundle (c, cross section of a petiole), and more precisely to the companion cells (d, longitudinal section of a petiole). The auto-fluorescence of the xylem (X) reflects the low expression level of NtFT3. Arrows in (c) indicate vascular bundles. The dotted line marks the longitudinal plane. Arrowheads in (d) mark sieve plates stained with aniline blue; CC, companion cell; SE, sieve element. Scale bars: (c) 200 μm; (d) 50 μm.

(e–g) The expression levels of NtFT1 (e), NtFT2 (f) and NtFT4 (g) increase gradually during development under SD conditions, with the lowest expression levels in seedlings (time point 1) and the highest levels in the leaves of flowering plants (time point 6). Transcript levels were determined in seedlings (time point 1) and basal leaves, which were harvested weekly until the opening of the first flowers (time points 2–6). Values were normalized against the reference gene EF1a. Error bars represent technical replicates. Further information can be found in Experimental procedures.

(h) Expression levels relative to time point 1. The expression of NtFT4 (encoding a floral activator) increases to a greater magnitude than either NtFT1 or NtFT2 (encoding floral repressors).
of the resulting transgenic Arabidopsis plants were similar to those of tobacco plants overexpressing NtFT2. Whereas wild-type Arabidopsis plants normally flower approximately 8 weeks after sowing under LD conditions (Figure 6a), the transgenic lines showed a dose-dependent delay in flowering, with the highest expression level causing the most severe phenotype, and delaying flowering by 1–2 weeks, with a concomitant increase in biomass (Figure 6b,c).

The conserved activity of NtFT2 in a heterologous background prompted us to investigate whether the tobacco FT-like proteins functioned in the same manner as the endogenous Arabidopsis floral inhibitor TFL1, i.e. by interacting with the transcription factor FD. This was established using bimolecular fluorescence complementation (BiFC) in N. benthamiana epidermal cells transiently expressing Arabidopsis FD, in concert with each of the tobacco FT proteins or Arabidopsis FT as a control. We initially expressed the Arabidopsis and tobacco FT proteins alone as C-terminal fusions with the fluorescent reporter protein Venus under the control of the CaMV 35S promoter, and monitored the resulting fluorescence by confocal laser scanning microscopy (CLSM). As expected, epidermal cells expressing each of the FT-Venus fusion proteins revealed fluorescence in the peripheral cytoplasm (surrounding the vacuole) and in the nucleus, indicating there was no restriction to any particular subcellular compartment. This is shown for Arabidopsis FT (Figure 6d), NtFT1 (Figure 6e), NtFT2 (Figure 6f), NtFT3 (Figure 6g) and NtFT4 (Figure 6h).

To characterize potential interactions between Arabidopsis FD and the five FT-like proteins, we expressed FD as a fusion with the C-terminal portion of monomeric red fluorescent protein (mRFP) (Jach et al., 2006; Uhrig et al., 2006). Figure 6. The repressive activity of NtFT1–NtFT3 is not species-dependent, and may be mediated by interaction with FD. (a–c) Overexpression of NtFT2 in Arabidopsis also delays flowering (a, b), and results in an increase in biomass (c). Plants were grown under inductive long-day (LD) conditions, and pictures were taken at the time point when wild-type control plants started to flower (a, c) and again 1 week later (b). (d–h) Intracellular localization of C-terminal Venus-tagged AtFT (d), NtFT1 (e), NtFT2 (f), NtFT3 (g) and NtFT4 (h) in Nicotiana benthamiana leaf epidermal cells. Scale bars: 50 \mu m.

(i–m) BiFC analysis of interactions between AtFD and AtFT (i), NtFT1 (j), NtFT2 (k), NtFT3 (l) or NtFT4 (m) in Nicotiana benthamiana leaf epidermal cells. The 35S:CaMFRP AtFD construct was co-expressed with 35S:NmrFP fusions of AtFT and NtFT1–NtFT4, respectively. Scale bars: 50 \mu m.

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2007) and each of the FT-like proteins as fusions with the N-terminal portion of mRFP. N. benthamiana epidermal cells were then co-inoculated with all five pairwise combinations of the constructs and monitored by CSLM. This revealed exclusively nuclear fluorescence for all pairwise combinations, showing that all four tobacco FT-like proteins were able to interact with Arabidopsis FD in a similar manner to Arabidopsis FT (Figure 6i–m).

**DISCUSSION**

**Evolution of FT-like proteins as floral repressors**

The plant PEBP family is divided into three major clades, with the FT-like and MFT-like clades primarily acting to promote floral development, and the TFL1-like clade primarily acting to repress floral development. Comprehensive phylogenetic analysis suggests that the FT-like and TFL1-like clades arose from a common ancestor that was a TFL1-like floral repressor, and that FT evolved as a floral promoter within the angiosperm lineage (Karlgren et al., 2011). This suggests that there were probably three PEBP family proteins in the last common ancestor of monocot and dicot plants (FT-like, TFL1-like and MFT-like), and that the evolution of antagonistic regulators controlling the transition from vegetative to reproductive development (i.e. TFL1 and FT) took place at least 150 million years ago (Chardon and Damerval, 2005).

The interactive mechanism between TFL1 and FT appears to be highly conserved, as shown in dicots such as tomato and pea, and in monocots such as rice (Kojima et al., 2002; Nakagawa et al., 2002; Foucher et al., 2003; Shalit et al., 2009; Hecht et al., 2011). Tobacco TFL1 homologs have already been identified and they appear to prolong vegetative growth (Amaya et al., 1999), which suggests that tobacco FT-like proteins should exist and should promote flowering. We isolated four tobacco FT-like genes and found that NtFT4 does indeed act as a floral promoter. However, we have provided convincing functional evidence that the other three tobacco FT-like proteins act as floral repressors, which is considered atypical. Within the FT-like clade, only one previously identified protein is known to act as a floral repressor, namely the sugar beet protein BvFT1 (Pin et al., 2010).

Arabidopsis does not possess an FT-like floral repressor, raising the question as to whether such proteins in other plants are trans-species compatible or represent independent (and functionally isolated) evolutionary events in tobacco and sugar beet. We therefore studied the phylogeny of the FT-like repressors, and a branch from the resulting tree is highlighted in Figure 7 (an extended version including AtFT and other characterized FTs is shown in Figure S3). We asked whether the last common ancestor of the known FT-like repressors (node α) was more likely to be a floral promoter or repressor, and found that if it was a repressor then at least three independent transitions were necessary to produce floral activators in the same lineage (*A1–*A3) whereas if it was an activator then only two independent transitions would be required (*R1 and *R2). The latter model is the most parsimonious, and fits better with the concept that FT-like proteins in general are floral activators, despite their derivation from a TFL1-like precursor (Karlgren et al., 2011).

![Figure 7. Evolution of FT-like floral repressors.](image-url)

(a) A branch of the phylogenetic tree from Figure 1 to visualize the evolution of FT-like floral repressors; α, last common ancestor; β, last common ancestor of tomato/potato and tobacco; *A1–*A3, transitions resulting in floral activators; *R1 and *R2, transitions resulting in floral repressors. Floral repressors are marked in red and floral activators are marked in blue.

(b) Partial amino acid alignment of FT sequences and other PEBP family proteins. Asterisks on the top row indicate amino acids essential for AtFT activity (Tyr85/Gln140) versus AtTFL1 activity (His88/Asp144) (Ahn et al., 2006). Segment B is part of exon 4 and encodes an external loop that evolved very rapidly in TFL1 homologs, but is almost invariant in FT homologs. Letters in italics show amino acids that are important for the antagonistic activities of BvFT1 and BvFT2 (Pin et al., 2010).
Interestingly, the phylogenetic branch containing the tobacco FT-like floral repressors also includes FT-like proteins from the only other solanaceous plants with completed genome sequences (tomato and potato), as well as from the more distant Solanales genus Ipomoea, from the family Convolvulaceae (Figure 7). From the available data, it remains unclear whether *R1 occurred before or after the split between tomato/potato and tobacco (node β). However, in addition to the proposal that StSP5G inhibits tuberization, which is based on its expression pattern under non-inductive LD conditions (Navarro et al., 2011), we have preliminary data indicating that StSP5G represses flowering in potato, which suggests that the transition to a repressor (*R1) took place prior to the divergence of these species. These results also suggest that tomato SP5G and perhaps potato SP5G-like should act as floral repressors. Furthermore, the closest homolog to sugar beet FT1 is the Chenopodium rubrum FT-like 2 protein, the function of which remains unclear (Cháb et al., 2008), suggesting the possibility of a second transition to a repressor (*R2) before the division of these Amaranthaceae species. The increasing evidence for a larger population of FT-like floral repressors with no clear phylogenetic distinction from FT-like floral promoters suggests that the concept of FT-like proteins representing the ‘florigen’ needs to be reconsidered, and that FT-like proteins should be recognized as mobile signals that can have either a positive or negative regulatory role in flower development.

Different motifs and amino acids specify the antagonistic activities of FT-like proteins

Floral promoters can be converted into repressors and vice versa by modifying a small number of specific residues, suggesting that key residues are more important for the regulatory activity of PEBP-family proteins than the overall sequence identity. X-ray crystallography has shown that the antagonistic Arabidopsis proteins TFL1 and FT contain two key motifs that are common to PEBP-family proteins, namely a putative ligand-binding pocket and an external loop (Banfield and Brady, 2000; Hanzawa et al., 2005; Ahn et al., 2006). Specific amino acid residues within these motifs play a decisive role in determining the regulatory activity of the proteins, e.g. Tyr85 located at the entrance of the binding pocket is required for FT activity, whereas His88 in the corresponding position in TFL1 is required for TFL1 activity. Another critical residue is found within the 14-amino-acid external loop encoded by the fourth exon (segment B), which evolved very rapidly in TFL1 orthologs, but is almost invariant in FT orthologs (Ahn et al., 2006). In TFL1, Asp144 forms a hydrogen bond with His88, whereas FT carries a glutamine residue at the corresponding position (Gln140), and this does not interact with Tyr85.

Figure 7b is a partial sequence alignment showing these critical residues in the four tobacco FT-like proteins, Arabidopsis FT and TFL1, and the other known FT-like repressor BvFT1, along with its antagonistic counterpart BvFT2. The three tobacco FT-like repressors and BvFT1 contain the critical residues in Arabidopsis FT needed for floral promotion (Tyr 85 and Gln 140), which indicates these residues do not contribute to the ability of NtFT1–3 or BvFT1 to act as floral repressors (Pin et al., 2010).

Another important sequence is the triad LYN, located in segment C, which is conserved in most FT-like floral promoters (including NtFT4), but is not present in NtFT1–NtFT3 or TFL1. However, this sequence is present in both BvFT1 and BvFT2, so it is clearly not required for the functional antagonism of these two proteins. Indeed, the only difference between BvFT1 and BvFT2 within the critical region is the sequence of three residues in segment B, which are shown in italics in Figure 7b. The substitution of these residues is sufficient to convert an activator into a repressor, and vice versa (Pin et al., 2010). Although NtFT4 and other known activators such as StSP3D and SISP3D match Arabidopsis FT and BvFT2 at these positions, the corresponding positions in the floral repressors are not conserved, although in the solanaceous species there is a conserved IID motif that may be functionally important. This may suggest that floral promotion is an active phenomenon, whereas repression occurs by default in the absence of key residues, or it may indicate that several combinations of residues are sufficient for repression, and these are taxonomically conserved, such as the IID motif mentioned above.

The expression profiles of tobacco FT-like genes suggest a role in the control of flowering exclusively under SD conditions

The expression of FT-like genes is regulated in a photoperiod-dependent manner in many species (Samach et al., 2000; Kojima et al., 2002; Hayama et al., 2003). We found that the expression of all four tobacco FT-like genes was difficult to detect under LD conditions, but increased gradually under SD conditions until flowering (Figure 5). Although N. tabacum SR1 is day-neutral with respect to flowering, the expression of the FT-like genes identified here seems to be photoperiod-dependent, and the FT-like proteins most likely regulate flowering under SD conditions. However, the molecular basis of floral induction under LD conditions remains to be determined, and may or may not involve other FT-related proteins. This phenomenon could be investigated further by comparing floral initiation in the LD plant N. sylvestris and the facultative SD plant N. tomentosiformis, which are the progenitors of tobacco. We have preliminary evidence that the N. sylvestris genome contains homologs of NtFT2–NtFT4, whereas the N. tomentosiformis genome contains homologs of NtFT7, NtFT2 and NtFT4, although their functions remain to be determined.
All four tobacco FT-like genes are expressed exclusively in leaf tissue (Figure 5), and we have presented direct and indirect evidence that all four genes are restricted to the same cell type, namely the phloem companion cells. It therefore seems likely that all four gene products are transferred into the sieve elements and subsequently translocated by mass flow to the apex, where they compete for interaction with FD to either repress flowering, and prolong vegetative growth, or promote flowering under favorable conditions. The outcome of this competition probably depends on the relative abundance of the different proteins, and therefore the modulation in FT-like gene expression during development is likely to set the timing of the developmental switch from vegetative to reproductive growth. The relative levels are likely to be more important than the absolute levels because the sole floral promoter NtFT4 is expressed at a lower level than NtFT1 and NtFT2, but the fold increase in abundance during development is much higher than the three floral repressors (Figure 5). The importance of the ratio of floral activators to repressors in the transition to flowering has already been proposed by others, e.g. local ratios of FT-like and TFL1-like proteins control the balance between determinate and indeterminate growth in tomato (Shalit et al., 2009; McGarry and Ayre, 2012). In this study this is supported by the late-flowering tobacco transgenants overexpressing repressive NFTs, where, with increasing leaf number, an accumulation of the endogenous activating NtFT4 shifts the activator/repressor ratio towards floral induction. The three FT-like floral repressors appear to have taken on the role usually played by TFL1 homologs in most other plants, which highlights the currently unclear role of the genuine TFL1 homologs (CETs) in tobacco. One possibility is that CET expression is restricted to the axillary meristem (Amaya et al., 1999), so FT-like repressors are required to fulfill the same function in the SAM. Additional research is required to compare the expression profiles of CETs and the antagonistic FT-like regulators, as well as potential interactions among tobacco CET proteins and FD homologs.

EXPERIMENTAL PROCEDURES

Plant materials and growth conditions

For transgene overexpression studies, tobacco (N. tabacum cv. SR1) plants were grown on MS medium (Murashige and Skoog, 1962) under sterile LD conditions (16-h photoperiod, 23°C, 100 μmol m⁻² s⁻¹), and were transformed with Agrobacterium tumefaciens, as described by Horsch et al. (1986). Independent transgenic lines were regenerated, and identical clones were produced from cuttings under sterile conditions, transferred into soil after rooting and grown in phytotrons under LD (16-h photoperiod) or SD (8-h photoperiod) conditions at 25/22°C day/night temperatures (200 μmol m⁻² s⁻¹). The data reported for flowering time and NtFT1–NtFT3 expression levels in transgenic plants are the mean values of all transgenic lines in the same phenotypic class. Plants cultivated under LD conditions were transferred to the glasshouse (22–25°C light, 20–25°C dark; artificial lighting switched on if natural light fell below 700 μmol m⁻² s⁻¹) when they reached 2 m in height, whereas plants cultivated under SD conditions were discarded because they reached the phytotron ceiling.

For promoter studies, we regenerated five independent transgenic lines and transferred them to soil in the glasshouse after rooting. We analyzed 4–6-week-old plants by CLSM using a Leica TCS SP5 X microscope (Leica Microsystems, http://www.leica-microsystems.com), with excitation and emission wavelengths of 488 and 500–600 nm, respectively.

For gene expression analysis, tobacco seeds were sown in soil and cultivated in phytotrons under LD or SD conditions. Spatial gene expression profiles were determined by extracting total RNA from leaf, apex, stem and root tissues, pooled from three 4-week-old plants. Temporal gene expression profiles were determined by extracting total RNA from seedlings and the basal leaves, pooled from three plants, harvested at weekly intervals until flowering.

Arabidopsis thaliana Col-0 seeds were sown in soil and cultivated under LD conditions in the phytotron (23/17°C day/night temperatures, 100 μmol m⁻² s⁻¹). Transgenic plants were generated by the floral-dip method (Clough and Bent, 1998). Seeds from transformed plants were sown in soil and sprayed with Basta after germination to select for transgenic lines.

Gene isolation

The tobacco FT-like genes NtFT1–NtFT4 were isolated from tobacco leaf cDNA and genomic DNA by rapid amplification of cDNA ends (SMARTer RACE cDNA amplification kit; Clontech, http://www.clontech.com) and genome walking (GenomeWalker Universal kit; Clontech) with primers designed according to a sequence (GenBank DQ994551/EFT10798) identified by using the Arabidopsis FT cDNA (GenBank AB027504.1) as a BlastN query against the NCBI tobacco (NCBI taxid 4097) EST/GSS database. Primers used for the identification of the tobacco FT-like genes are shown in Table S3.

Phylogenetic analysis

Protein sequences of the FT/TFL family members listed by Karlgren et al. (2011) were downloaded from GenBank, and potato FT/TFL sequences described by Navarro et al. (2011) were extracted from the potato genome sequence (The Potato Genome Sequencing Consortium., 2011; http://potatogenomics.plantbiology.msu.edu). These proteins were aligned with the tobacco FT sequences using MUSCLE (Edgar, 2004), and were masked using MALIGN and ALICUT (Kück et al., 2010; http://zfmk.de/web/Forschung/Abteilungen/AG_Wgele/Software/Utilities/index.en.html). A maximum-likelihood tree from the masked alignment was computed using FASTTREE 2 (Price et al., 2010) with the gamma option. The tree was visualized using FIGTREE (http://tree.bio.ed.ac.uk/software/figtree).

Cloning strategies

Tobacco overexpression constructs were generated by amplifying the tobacco FT-like gene coding regions using primers containing restriction sites, as shown in Table S3. PCR products were digested with the corresponding enzymes and inserted in pRT104 (Töpfer et al., 1987), and subsequently transferred to pCambia1300 or pBin19Hyg (digested with HindIII; Bevan, 1984) to yield pCambia1300.35S:NtFT1 and pBin19Hyg.35S:NtFT2–4. The coding region of Arabidopsis FT was amplified from Arabidopsis leaf cDNA, subcloned in pCR® Topo® as above, amplified with flanking restriction sites (Table S3), digested with the appropriate enzymes, and transferred to pRT104 and then pCambia1300, as above. The 35S:NtFT2 and 35S:NtFT4 constructs were also

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transferred to binary vector plab12.1 (Post et al., 2012) for overexpression in Arabidopsis. All binary vectors were introduced by electroporation into Agrobacterium tumefaciens strain LBA4404 (Hoekema et al., 1983).

The promoter-GFP<sub>EN</sub> fusion was constructed by amplifying 1 kb of the <sup>P<sub>NPT</sub></sup> sequence from tobacco genomic DNA using primers containing restriction sites, as shown in Table S3. The product was digested with appropriate enzymes and inserted upstream of the GFP<sub>EN</sub> reporter gene in vector pBSGFP<sub>EN</sub> (Noll et al., 2007). The <sup>P<sub>NPT</sub></sup>GFPER/CaMV 35S terminator cassette was amplified using primers containing flanking restriction sites and transferred to pBin19Hyg for electroporation into Agrobacterium tumefaciens strain LBA4404 (Hoekema et al., 1983).

The BiFC constructs were produced by amplifying the coding regions of NtFT1-NtFT4, Arabidopsis FT and Arabidopsis FD from cDNAs (primers with restriction sites as shown in Table S3). The amplified products were transferred to pENTR<sub>4</sub>™ (Invitrogen, http://www.invitrogen.com). The Venus and split mRFP fusions were created by introducing the NtFT1–NtFT4, FT and FD pENTR<sub>4</sub>™ constructs into the pBatTL Venus-cccB, pBatTL CaMV-RFP-cccB, pBatTL cccB-Venus, pBatTL cccB-CmRFP, pBatTL cccB-NmRFP constructs by LR recombination. Constructs were kindly provided by Dr Joachim Uhrig and Dr Guido Jach, Cologne (Müller et al., 2010), and by Dr Boje Müller (Münster, Germany).

Gene expression studies

Total RNA was extracted from frozen plant tissue using the Nucleo Spin® RNA Plant kit (Macherey-Nagel, http://www.mn-net.com), and genomic DNA was eliminated using DNAase I (NEB, http://www.neb.com), followed by phenol-chloroform extraction. The RNA was reverse transcribed with SuperScript II (Invitrogen) following the manufacturer’s instructions, and 1 μl of cDNA was used in each quantitative real-time RT-PCR reaction, which involved 45 amplification cycles followed by melting curve analysis in a CFX 96 cycler (Bio-Rad, http://www.bio-rad.com), using the IQ SYBR Green Supermix (Bio-Rad). The primers are shown in Table S3. Each sample was assayed in triplicate, whereas reference genes, NRT (not reverse transcribed control) and NTC (non-template control) were assayed in duplicate. The levels of the two potential reference transcripts, EF1α and L25 (Schmidt and Delaney, 2010), were examined in each reverse transcription sample, and EF1α was found to be the most stable and therefore the most suitable for normalization. Relative expression levels were calculated using REST-MCS (Pfaffl et al., 2002). The temporal expression profiles were determined twice (NtFT1), or once (NtFT2–NtFT4), the spatial expression profiles were determined twice and the overexpression studies were carried out three times.

BiFC analysis

The pBatTL constructs were introduced into Agrobacterium tumefaciens strain GV3101 pMP90 by electroporation. Transient expression was achieved by the simultaneous infiltration of A. tumefaciens strains GV3101 pMP90 (containing the NtFT1–NtFT4, FT or FD constructs) and C58C1, carrying the pCH32 helper plasmid, containing the RNA silencing suppressor protein p19 from <i>Tomato bushy stunt virus</i> (Voinnet et al., 2003; Walter et al., 2004), into the leaves of 3–4-week-old <i>N. benthamiana</i> plants grown under LD conditions in the glasshouse.

Transformed plants were cultivated under constant light and analyzed 4 days after infiltration by cutting leaf discs and screening abaxial epidermal cells for fluorescence. Venus and mRFP fluorescence was detected by CLSM using a Leica TCS SP5 X microscope (Leica Microsystems) with excitation and emission wavelengths of 514 and 525–600 nm, respectively (Venus), or 549 and 569–629 nm, respectively (mRFP). Bright-field images were obtained by activating the transmission photomultiplier tube.

Accession numbers

Sequence data from this article can be found in the EMBL/GenBank data libraries under accession number(s) JX679067 (NtFT1), JX679068 (NtFT2), JX679069 (NtFT3) and JX679070 (NtFT4). The accession numbers of the proteins used for phylogenetic analysis can be found in Table S4.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Protein alignment of selected members of the plant PEBP family.

Figure S2. Overexpression of NtFT4 and AtFT in tobacco generates an early-flowering phenotype.

Figure S3. Branch of the phylogenetic tree to visualize the evolution of FT-like floral repressors.

Table S1. Protein sequence percentage identities (and similarities) for selected members of the plant PEBP family.

Table S2. Expression levels determined by qRT-PCR (compared with wild-type expression levels) and growth behavior in T<sub>0</sub> plants from each independent transgenic line with a severe phenotype.

Table S3. Primer sequences.

Table S4. Accession numbers of proteins used for phylogenetic analysis.

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