cis-Regulatory Elements and Chromatin State Cooperatively Control Temporal and Spatial Expression of FLOWERING LOCUS T in Arabidopsis

Jessika Adrian, Sara Farrona, Julia J. Reimer, Maria C. Albani, George Coupland, and Franziska Turck
Max Planck Institute for Plant Breeding Research, 50829 Koeln, Germany

Flowering time of summer annual Arabidopsis thaliana accessions is largely determined by the timing of FLOWERING LOCUS T (FT) expression in the leaf vasculature. To understand the complex interplay between activating and repressive inputs controlling flowering through FT, cis-regulatory sequences of FT were identified in this study. A proximal and an ~5-kb upstream promoter region containing highly conserved sequence blocks were found to be essential for FT activation by CONSTANS (CO). Chromatin-associated protein complexes add another layer to FT regulation. In plants constitutively overexpressing CO, changes in chromatin status, such as a decrease in binding of LIKE HETEROCHROMATIN PROTEIN1 (LHP1) and increased acetylation of H3K9 and K14, were observed throughout the FT locus, although these changes appear to be a consequence of FT upregulation and not a prerequisite for activation. Binding of LHP1 was required to repress enhancer elements located between the CO-controlled regions. By contrast, the distal and proximal promoter sequences required for FT activation coincide with locally LHP1 and H3K27me3 depleted chromatin, indicating that chromatin status facilitates the accessibility of transcription factors to FT. Therefore, distant regulatory regions are required for FT transcription, reflecting the complexity of its control and differences in chromatin status delimit functionally important cis-regulatory regions.

INTRODUCTION

The transition to flowering is controlled by genetic pathways that integrate environmental cues and the developmental state of the plant. In Arabidopsis thaliana, several floral signals, including response to photoperiod, converge at the level of transcriptional regulation of the FLOWERING LOCUS T (FT) gene. Under inductive long-day (LD) conditions, the floral integrator FT is transcribed in the leaf vasculature (Takada and Goto, 2003; Notaguchi et al., 2008). Movement of FT protein is required to transport the LD signal to the meristem and initiate meristem identity changes (Abe et al., 2005; Wigge et al., 2005; Corbesier et al., 2007; Jaeger and Wigge, 2007; Mathieu et al., 2007). Misexpression of FT causes early flowering independent of environmental and endogenous stimuli, whereas loss of function of FT results in a severe late-flowering phenotype in LDs and has just a minor effect on flowering under noninductive short-day (SD) conditions (Koomneef et al., 1991; Kardailsky et al., 1999; Kobayashi et al., 1999).

FT belongs to a small protein family whose members show homology to mammalian phosphatidylethanolamine binding proteins, such as the Raf kinase inhibitor protein (Kardailsky et al., 1999; Kobayashi et al., 1999). The closest relative of FT, TWIN SISTER OF FT (TSF), produces a functionally equivalent protein but is expressed at much lower levels than FT (Michaels et al., 2005; Yamaguchi et al., 2005; Jang et al., 2009). By contrast, the related TERMINAL FLOWER1 (TFL1) represses the floral transition and is expressed in the shoot apical meristem (Shannon and Meekswagner, 1991; Bradley et al., 1997). FT/TSF and TFL1 define clearly separated spermatophyte-specific subfamilies that have further expanded in several species (Hedman et al., 2009).

Transcriptional activation of FT under inductive daylength conditions is directly mediated by CONSTANS (CO) (Samach et al., 2000). CO mRNA shows a circadian controlled pattern of accumulation in phloem companion cells, and CO protein is further stabilized by light, so that only in LD conditions does sufficient CO protein accumulate to induce FT (Suarez-Lopez et al., 2001; Valverde et al., 2004; Laubinger et al., 2006). CO encodes a nuclear protein containing two zinc binding B-boxes and a CCT (CONSTANS, CO-like, TIME OF CAB1) domain (Putterill et al., 1995; Robson et al., 2001). Direct binding of DNA by CO could not be demonstrated and led to the suggestion that CO requires partners to activate transcription (Suarez-Lopez et al., 2001). The CCT domain of CO is able to interact with components of the Nuclear Factor Y (NF-Y) complex (Ben-Naim et al., 2006; Wenkel et al., 2006). NF-Y proteins bind DNA as a heterotrimeric complex that recognizes CCAAT cis-elements (Mantovani, 1999; McNabb and Pinto, 2005). Genetic analysis demonstrated that members of the A. thaliana NF-Y complex are involved in flowering control and placed them in the photoperiodic pathway downstream of...
CO (Cai et al., 2007; Chen et al., 2007; Kumimoto et al., 2008). Furthermore, it has been shown that the CO B-boxes interact with a member of the TGA family of basic domain/leucine zipper transcription factors (Song et al., 2008).

Several transcriptional repressors participate directly in FT regulation. The MADS box factors FLOWERING LOCUS C (FLC) and SHORT VEGETATIVE PHASE (SVP) form a complex that associates with regions within the proximal FT promoter and the first intron that comprise GA/G boxes, although the importance of these elements for FT regulation has not yet been demonstrated (Searle et al., 2006; Li et al., 2008). In addition, members of two subfamilies of the AP2-related transcription factors physically interact with the FT locus and participate in transcriptional repression. TEMPRA-NILLO1 (TEM1) and TEM2 encode two partially redundant AP2-like proteins, and TEM1 binds to the FT 5′-untranslated region (Castillejo and Pelaz, 2008). SCHLAF-MUTZE (SMZ) belongs to a subclade of the AP2 family that is targeted by miR172 (Auwerkerme and Sakai, 2003). Overexpression of SMZ but also of other members of the subclade strongly represses FT, whereas overexpression of miR172 accelerates the transition to flowering in a partially FT-dependent manner. SMZ binding sites have been identified at the genomic level, and, surprisingly, SMZ binds several kilobases downstream of the FT coding sequence (Mathieu et al., 2009).

Chromatin-associated protein complexes add another layer of complexity to FT regulation (Farrona et al., 2008; Del Olmo et al., 2009; Jeong et al., 2009). Loss of function of LIKE HETEROCROMATIN PROTEIN1 (LHP1), also known as TFL2, causes daylength-independent early flowering mainly due to upregulation of FT expression (Kotake et al., 2003). LHP1 is expressed in meristematic tissues and young leaves, whereas expression in developing leaves becomes restricted to the petiole and the proximal side of the leaf blade, areas where cells continue to proliferate. In mature leaves, LHP1 mRNA is restricted to the vascular tissue (Kotake et al., 2003). LHP1 is a single-copy gene that encodes a protein containing a chromo domain and a chromo shadow domain. Chromo domains bind Lys-methylated proteins and have been identified in many chromatin-associated proteins, while the chromo shadow domain is a distinctive feature of the animal HETEROCHROMATIN PROTEIN1 (HP1) family. Whereas the best known member of the HP1 family is predominantly involved in the stabilization of constitutive heterochromatin, the plant homolog LHP1 is dedicated to transcriptional regulation of genes located in the euchromatin (Nakahigashi et al., 2005). Through its chromodomain, LHP1 colocalizes exclusively and extensively with genes that also possess nucleosomes with trimethylated Lys residues at position 27 of histone 3 (H3K27me3) (Turck et al., 2007; Zhang et al., 2007a; Exner et al., 2009).

Trimethylation of H3K27 is performed by the Polycomb repressive complex 2 (PRC2) that is conserved between plants and animals (Farrona et al., 2008; Schlatowski et al., 2008; Luo et al., 2009). In animals, H3K27me3 is bound by the chromo domain of the Polycomb (Pc) protein, a subunit of PRC1 (Schuettengruber et al., 2007). The idea that LHP1 may be part of a plant PRC1 and fulfill a function analogous to Pc is supported by recent results showing that LHP1 can interact with RING1A, the homolog of the animal PRC1 core component Sex Combs Extra (Xu and Shen, 2008). LHP1 and H3K27me3 histone marks distribute widely over the FT locus, indicating that this gene is regulated by the Polycomb Group (PcG) pathway (Turck et al., 2007; Zhang et al., 2007b). The early flowering, high FT expressing phenotype of several mutants in PRC2 encoding genes further corroborates this conclusion (Farrona et al., 2008).

Previous studies have demonstrated the importance of transcriptional regulation of FT to ensure flowering time control in response to environmental and endogenous factors. Several transcription factors and chromatin-associated proteins have been shown to target different regions within the FT locus, but so far the relevance of the targeted regions for FT regulation has not been firmly established. Here, we show that CO-dependent FT activation requires not only proximal but also conserved distal promoter regions that together with LHP1-mediated chromatin status orchestrate the interplay between activating and repressing inputs to FT regulation.

RESULTS

Identification of Putative FT Regulatory Sequences by Phylogenetic Shadowing

To identify candidate sequences for regulatory motifs, we aligned sequences from 7.0 kb upstream of FT from A. thaliana accession Columbia (Col) (Figure 1A) to FT sequence from A. thaliana accession Landsberg erecta (Ler) and FT homologous genes from Arabidopsis lyrata, Brassica rapa, and Arabis alpina (Aa FT1-Aa FT3). The pairwise alignment revealed three highly conserved sequence blocks (Figure 1B, highlighted in light gray). Block A, closest to the FT start codon, showed high conservation among A. thaliana, B. rapa, and Aa FT1. Expanding the alignment with homologous sequences of Sisymbrium polyceratum, Brassica oleracea, Capsella rubella, and Arabis hirsuta revealed highly conserved sequence stretches within block A of the Aa FT2 and Aa FT3 genes and in the promoter sequence of A. thaliana TFS (Figure 1C). These putative cis-regulatory elements did not contain documented transcription factor binding sites and were therefore called shadow 1, 2, 3, and 4 (S1 to S4). Furthermore, two palindromic sequences flanking S3 were identified and named P1 and P2. Block B is located around 1.8 kb upstream of FT and contains two highly conserved sequence stretches that include an E-box, which is a binding site for many basic-helix-loop-helix (bHLH) proteins like Cryptochrome-interacting bHLH1 (CIB1), which has been shown to enhance FT expression in response to blue light (Liu et al., 2008; Figure 1D). A third conserved block C, located around 5.2 kb upstream of the FT start codon, is identified by the alignment of FT promoter sequences of the Arabidopsis genus and B. rapa (Figure 1B) and is conserved in C. rubella (Figure 1E). Prediction of transcription factor binding sites revealed a conserved CCAAT box, the binding site for the NF-Y complex (Mantovani, 1999). Furthermore, a GATAA motif, called I-box, present in many light-regulated genes in monocots and dicots (Terzaghi and Cashmore, 1995), is conserved in block C, as well as an REalpha consensus sequence (AACCA4A) that has been implicated in regulation by phytochromes (Degenhardt and Tobin, 1996).
Figure 1. Conservation of FT Promoter Sequences.
The 5.7-kb Sequence Upstream of the FT Translation Start Site Contains Sufficient Regulatory Information to Mediate to Daylength Response in Col

Previously, it has been shown that a transgene consisting of 8.9-kb sequence upstream of the FT translation start site fused to the cDNA of FT complements the ft-1 late flowering phenotype (Takada and Goto, 2003). To further delimit the upstream regulatory region of FT, a set of deletion constructs was generated (Figure 2A). Complementation analysis of deletion constructs between 8.1 and 4.0 kb in length revealed that 5.7-kb sequence upstream of FT fused to the FT cDNA (Figure 2A) was sufficient to rescue the late flowering phenotype of ft-10 plants grown under inductive extended short-day (ESD) conditions (Figure 2B). Under noninductive SD conditions, transgenic complementation lines containing FT promoter fragments of 5.7 kb and longer mimicked the flowering behavior of wild-type plants, as shown for the longest fragment in Supplemental Figure 1A online. β-Glucuronidase (GUS) expression driven by a FT promoter fragment of 5.7 kb was restricted to the vasculature of cotyledons and the minor veins of the distal half of the leaf as had been previously reported for longer FT promoter fragments (Takada and Goto, 2003) (Figure 2C). GUS signal was not detected in shoot apical regions, hypocotyls, or roots and was almost absent in plants grown under SD conditions. Even the strongest expressing 8.1kbFTpro:GUS line showed only single stained cells in the vasculature of some leaves in SDs (see Supplemental Figure 2 online). No GUS signal showed in wild-type seedlings during flower development, GUS expression became visible in pollen (see Supplemental Figure 2 online). In 1.0kbFTpro:GUS Col plants, GUS signal was only observed in single phloem cells of the leaf (Figure 2C), at the base of leaf trichomes and a few mesophyll cells in hypocotyls.

In summary, FT promoter sequence 5.7 kb upstream of the translation start site contains all regulatory elements required to mediate spatial and temporal expression of FT in response to photoperiod. Since shortening of the promoter to 4.0 kb abolished the ability to mediate daylength-dependent activation, a distal promoter region containing the conserved block C seems to comprise important regulatory sequences. A 4.0-kb FT promoter is not responsive to high CO levels, whereas a 1.0-kb FT promoter is marginally induced.

Identification of cis-Acting Elements in the Proximal FT Promoter

Residual expression driven by a 1.0-kb FT promoter together with high conservation of block A indicates a regulatory function of the proximal sequence. Surprisingly, in transient expression...
studies performed by leaf bombardment, constructs with a luciferase gene under control of an 8.1-, 4.0-, or 1.0-kb FT promoter fragment showed no difference in expression. Cobombardment with 35Spro:CO increased the luciferase signal from all constructs around sixfold (Figure 3A). Reporter constructs with point mutations in block A elements were therefore tested in the context of the 1.0-kb FT promoter in transient assays (Figure 3B). Compared with the original sequence, mutations in S2 and in the two palindromic sequences P1/P2 reduced luciferase activity in response to CO by twofold. Presence of S3 had only a slight impact on CO-mediated stimulation, while mutations in S1 did not affect luciferase activity.

To study the biological relevance of S2, S3, and P1/P2 in the genomic context, mutations in these elements were introduced into the 8.1-kb FT promoter fragment and analyzed in complementation experiments using stable transformation of ft-10 plants (Figure 3C). Among 10 lines tested, seven 8.1kbFTpro-S2mut:FTcDNA ft-10 lines flowered significantly later than the 8.1kbFTpro:FTcDNA ft-10 control. The 8.1kbFTpro-S3mut:FTcDNA ft-10 plants flowered with a similar number of leaves as plants containing the nonmutated construct. Mutation of the palindromic sequence P1/P2 had a strong impact on expression since 8.1kbFTpro-P1/P2mut:FTcDNA ft-10 lines did not complement the late flowering phenotype. Flowering time data were well correlated with transcript levels generated by the transgenes (Figure 3D).

To study the biological relevance of S2, S3, and P1/P2 in the genomic context, mutations in these elements were introduced into the 8.1-kb FT promoter fragment and analyzed in complementation experiments using stable transformation of ft-10 plants (Figure 3C). Among 10 lines tested, seven 8.1kbFTpro-S2mut:FTcDNA ft-10 lines flowered significantly later than the 8.1kbFTpro:FTcDNA ft-10 control. The 8.1kbFTpro-S3mut:FTcDNA ft-10 plants flowered with a similar number of leaves as plants containing the nonmutated construct. Mutation of the palindromic sequence P1/P2 had a strong impact on expression since 8.1kbFTpro-P1/P2mut:FTcDNA ft-10 lines did not complement the late flowering phenotype. Flowering time data were well correlated with transcript levels generated by the transgenes (Figure 3D).

Taken together, two phylogenetically conserved sequences within the proximal promoter have been identified that act as positive cis-elements in FT regulation.

LHP1-Mediated Repression of FT through a Regulatory Element Located between 1.0 and 4.0 kb Upstream of FT

The spatial expression pattern driven by FT promoter fragments of different length was analyzed in the lhp1 background (Figure 4). As already reported for a slightly longer construct (Takada and Goto, 2003), the expression pattern of 8.1kbFTpro:GUS in lhp1 mutants was extended to the middle vein as well to minor veins of
Figure 3. Proximal FT Promoter Sequences Are Crucial for Mediating Daylength Response.

(A) A transient expression assay was performed using a luciferase gene (LUC) under control of FT promoter fragments of 8.1, 4.0, and 1.0 kb in length. CO-dependent transcriptional activation was analyzed by cobombardment of 35Spro:CO. Light emission per leaf was normalized to the fluorescence signal obtained from a cobombardment of 35Spro:GFP construct. Fold stimulation of the promoter by CO is indicated within the light-gray bar for each construct. Data from three independent experiments are shown as mean ± SE.

(B) The 1.0kbFTpro:GreenLUC constructs carrying mutations in different putative cis-elements of the promoter were tested in a transient expression assay. Resulting green light emission was normalized to light emission of a cobombarded red light emitting luciferase (RedLUC). CO-dependent transcriptional activation was analyzed by cobombardment of 35Spro:CO. Fold stimulation of the promoter by CO is indicated within the light-gray bar for each construct. Mean ± SE is based on at least three independent experiments.

(C) Flowering time of ft-10 plants carrying transgenic constructs driving FT cDNA by mutated versions of the 8.1-kb FT promoter fragment. Several independent transformants are shown for each construct; wild-type plants, 8.1kbFTpro:FTcDNA ft-10 carrying no mutation (labeled as C in the graph), and ft-10 mutants were analyzed as controls. Plants were grown in ESD conditions. The experiment was repeated two times with similar results. Differences in total leaf number compared with 8.1kbFTpro:FTcDNA ft-10 plants were analyzed with a two-way analysis of variance. Asterisks indicate significant differences with P values < 0.0001. Number of rosette and cauline leaves of a representative experiment are shown as the mean ± SE.
the proximal part of the first true leaves (Figure 4). Interestingly, the 4.0-kb FT promoter fragment that was not able to drive expression in either wild-type or 3SSpro:CO plants became active in lhp1 seedlings. GUS signal was observed in the major vein and the proximal vascular tissue in leaves of 4.0kbFTpro:GUS lhp1 plants. Although transgenic 1.0kbFTpro:GUS Col plants did not show expression when grown on soil (Figure 2B), seedlings grown on agar occasionally showed GUS expression in single phloem cells of the distal leaf vasculature (Figure 4, magnification). In the lhp1 mutant background, GUS expression driven by a 1.0-kb FT promoter was detectable in all transgenic lines, although the observed pattern differed. Most lines showed GUS signal in the tip and the hydathodes of leaves (Figure 4) and the Y junction of the vasculature close to the meristem. One 1.0kbFTpro:GUS lhp1 line showed additional GUS signal in the distal minor veins of leaves (Figure 4) and in the entire vasculature of the hypocotyl. Among five lines tested, a single 1.0kbFTpro:GUS lhp1 line showed GUS activity in the vasculature of roots, hypocotyls, and petioles and in the middle vein of the basal part of leaves. The differing GUS pattern among these lines could be explained by differences in the genomic context, indicating that this promoter fragment alone does not induce expression but depends on the recruitment of adjacent regulatory sequences.

In summary, LHP1 mediates repression of FT in the middle vein by neutralizing an enhancer element located between 1.0 and 4.0 kb upstream of the start codon. Furthermore, expression driven by a 1.0-kb proximal promoter fragment was enhanced in the lhp1 mutant but probably also dependent on the recruitment of additional regulatory elements.

**FT Chromatin Changes Correlate with Its Transcriptional State**

To investigate how transcriptional activation of FT by CO is reflected by changes in the chromatin, chromatin immunoprecipitation (ChIP) experiments were performed with chromatin obtained from 3SSpro:LHP1:HA and 3SSpro:LHP1:HA 3SSpro:CO seedlings. Since binding of LHP1 has been shown to colocalize with the H3K27me3 histone mark (Turck et al., 2007; Zhang et al., 2007a), occurrence of LHP1:HA and H3K27me3 were analyzed along the FT locus (Figure 5A). Plants were grown in SDs so that only 3SSpro:CO seedlings expressed FT. In these plants, LHP1:HA binding was reduced along the FT structural gene and the proximal promoter compared with nonexpressing wild-type background (Figure 5B). By contrast, signal strength for the H3K27me3 histone mark did not significantly differ along the FT locus in seedlings differentially expressing FT (Figure 5C).

Histone acetylation of regulatory regions often positively correlates with gene expression either because transcription factors act through the recruitment of histone acetyl-transferases or because of histone acetyl-transferase activity that is recruited during the transcriptional process (Choi and Howe, 2009). ChIP experiments were performed with an antibody that detected acetylation of H3K9 and K14 (H3K9K14ac). Indeed, acetylation of H3 was increased in the proximal promoter and the transcribed region of FT in 3SSpro:CO plants (Figure 5D). Furthermore, H3K9K14ac increased in a distal FT promoter region that overlapped with the conserved block C. Acetylation at the distal region could be due to transcriptional upregulation of the closely located FASCIATA1 (FAS1) gene, but levels of FAS1 are not altered in 3SSpro:CO plants (see Supplemental Figure 3 online). Therefore, increased H3K9K14ac levels are likely to be linked to FT expression changes.

Taken together, expression of FT correlates with a depletion of LHP1 from FT without a corresponding change in the H3K27me3 modification pattern. In plants actively transcribing FT, H3K9K14ac was increased in distal and proximal regulatory regions and at the structural gene.

**Analysis of Changes of FT Chromatin Immediately upon Transcriptional Activation**

Use of plants constitutively overexpressing CO cannot distinguish between chromatin changes being the cause or consequence of FT expression. Therefore, we introduced the 3SSpro-LHP1:HA reporter into a 3SSpro:CO:GR line. Treatment of these plants with dexamethasone (Dex) causes nuclear import of cytosolic CO:GR fusion protein within 15 min (see Supplemental Figure 4A online) and leads to a steep increase in FT expression after a lag phase of 1 h and 30 min and flowering (Figure 6A; see Supplemental Figure 4B online). Samples for ChIP experiments were taken every 30 min for up to 3 h after Dex treatment. As expected, H3K27me3 levels at the FT locus did not change during the induction (Figure 6C). LHP1:HA was only slightly reduced at the proximal promoter and the structural gene at the later time points when FT expression was already significantly increased (Figure 6B). Furthermore, no changes in acetylation levels that anticipated the increase in expression of FT could be observed at the proximal and distal regulatory regions (Figure 6D).

In conclusion, decrease in LHP1 binding and increased acetylation of H3K9K14 at FT that were observed in plants expressing the FT activator CO constitutively seem to be a consequence of prolonged FT transcription but are not a prerequisite for early FT activation.

**High-Resolution Mapping of H3K27me3 and LHP1:HA at FT Identifies a Locally Depleted Region**

Recruitment of LHP1-associated repressive complexes by H3K27me3 are believed to lead to a more condensed chromatin state that is inaccessible for cis-regulatory element binding during the transcriptional process (Choi and Howe, 2009).
transcription factors (Farrona et al., 2008). Since FT transcriptional activation does not require the prior removal of the chromatin-mediated repressor, other mechanisms must be in place to allow transcriptional activator access to FT. High-resolution mapping of the chromatin at the FT locus by ChIP-chip experiments with high-density microarrays revealed that the conserved block C coincides with a locally H3K27me3 and LHP1 poor region (Figure 7A; see Supplemental Data Set 6 online). In addition, the proximal promoter was partially depleted in both repressive chromatin marks.

DISCUSSION

FT Promoter Comprises a Complete Set of FT Regulatory Elements

Previous studies analyzed spatial expression of a GUS reporter gene under control of an 8.9-kb FT promoter or integrated into a genomic fragment covering 7.2 kb upstream and 1.5 kb downstream of FT and found that these did not differ in their expression patterns (Notaguchi et al., 2008). These data supported the idea that CO-responsive elements are located upstream of FT. Our data demonstrate that an 8.1-kb FT promoter also drives expression in the same temporal pattern and quantitative range as the endogenous FT gene, under inductive and noninductive conditions (Figure 2; see Supplemental Figure 1C online). This result is somewhat surprising as a regulatory role has been postulated for the first intron of FT, which encompasses a CArG box as putative binding site for the interacting transcriptional repressors FLC and SVP (Searle et al., 2006; Li et al., 2008). However, a CArG box as binding site for SVP has also been identified in the proximal FT promoter, and so it is plausible that the two CArG boxes are redundant in summer annual A. thaliana plants expressing low levels of FLC.

As shown in this (Figure 2) and a previous study (Notaguchi et al., 2008) FT expression is restricted to the vasculature despite a ubiquitous ectopic expression of CO. CO activity is dependent on the stabilization of the protein by light perceived at the end of long days (Valverde et al., 2004; Turck et al., 2008), and a lack of CO accumulation in other tissues could explain the phloem-specific expression pattern of FT in 35Spro:CO. However, analysis of plants expressing a 35Spro:CO:GFP fusion in A. thaliana demonstrated that CO protein is stable outside of the vasculature, as green fluorescent protein (GFP) signal was detectable in nuclei of epidermal cells (Valverde et al., 2004). Hence, the observations support the idea that CO requires an unidentified protein partner or complex to activate transcription and suggest that at least one factor of this CO activator complex is specific to the vasculature.

Our data show that 5.7-kb sequence upstream of the translation start site of FT encodes sufficient cis-regulatory elements to mediate tissue-specific and CO-responsive expression, whereas shortening the FT promoter to 4.0 kb upstream of the ATG disrupts the ability of CO to drive FT expression (Figure 2). Therefore, sequences between 4.0 and 5.7 kb upstream of FT ATG seem to contain crucial regulatory elements required for the response to CO. A phylogenetic shadowing approach identified a conserved region (block C) located around 5.2 kb upstream of FT (Figures 1B and 1E). Sequence conservation of regulatory regions is likely to reflect the constraint to maintain gene regulation during evolution. The underlying model is that mutations have been counterselected in sites recognized by transcription factors, thereby imposing a slower rate of divergence than in surrounding sequences (Vavouri and Elgar, 2005). Notably, the evolutionary conserved regions block C and block B included two putative cis-regulatory elements that are recognized by transcription factors with a suggested role in FT regulation (Ben-Naim et al., 2006; Wenkel et al., 2006; Liu et al., 2008).
The phylogenetic shadowing approach further identified a promoter region just upstream of the \( FT \) transcription start site (block A, Figure 1B) that is highly conserved between different Brassicaceae plants (Figure 1C). Complementation and expression analysis confirmed the relevance of shadow \( S2 \), whose mutation results in reduced \( FT \) expression, and of the palindromic sequence \( P1/P2 \), apparently essential for \( FT \) expression (Figures 3C and 3D). So far, there are no candidate transcription factors that recognize the DNA sequences \( S2 \) and \( P1/P2 \).

Although histochemical GUS localization assays showed that a 1.0-kb \( FT \) promoter alone was barely able to drive expression in Col, this residual expression was enhanced under highly inductive conditions (Figures 2 and 4). Moreover, in transient expression assays, the proximal 1.0-kb \( FT \) promoter was inducible by CO. We propose a model where CO acts through the proximal promoter but requires interaction with an activator complex that associates with the distal regions of the \( FT \) promoter (Figure 7B). Interaction with a protein partner could enhance affinity of CO to DNA and thereby lead to binding of the proximal promoter region. In transient assays, extremely high amounts of DNA are introduced into bombarded cells, and interaction with a coactivator might not be required. On the other hand, expression driven by a 1.0-kb \( FT \) promoter in the transient reporter assay may well be due to general differences between bombarded and integrated DNA. While the transgene is integrated into the genomic context in stably transformed plants, genes encoded on plasmids lack certain aspects of regulation mediated through chromatin (Hebbar and Archer, 2007). Therefore, the role of factors binding to the distal region could be to increase the accessibility of the proximal region, an action not required if the controlled region is already accessible.

**Chromatin Changes at \( FT \) Play a Permissive Role**

It has been proposed that LHP1 counteracts the activity of CO on \( FT \) to ensure daylength-dependent flowering (Takada and Goto, 2003). As CO and LHP1 are expressed in an inverted gradient along the leaf, \( FT \) transcription in the distal veins was proposed to occur as a result of CO overcoming the repressive effect of decreasingly expressed LHP1. Introducing \( FT \) promoter deletion
constructs in a lhp1 mutant background revealed that repression of FT in the middle vein and the proximal half of the leaf by LHP1 is mediated through promoter sequences 1.0 to 4.0 kb upstream of FT. As a 4.0-kb FT promoter cannot be induced by high levels of CO, FT repression in the midvein by LHP1 seems to be needed to neutralize one or several unknown activators of FT in this tissue (see model in Figure 7B). The FT locus is widely covered by the repressive H3K27me3 mark and LHP1 (Turck et al., 2007; Zhang et al., 2007b), but the conserved block C that is crucial for CO responsiveness coincides with a locally H3K27me3 and LHP1 poor region that may be part of a regulatory mechanism maintaining a window of constitutively open chromatin accessible to regulatory factors (Figure 7A).

Going along with the idea that LHP1 binding and presence of the repressive H3K27me3 mark interferes with the access of transcription factors to the FT promoter, it is remarkable that LHP1 binding is reduced in 3SSpro:CO plants throughout the locus (Figure 5B). The loss of LHP1, together with the increased H3K9K14ac (Figure 5D), could result in a more permissive chromatin structure that facilitates the access of other transcriptional regulators to elements that are usually repressed and contribute to high FT expression in the midvein in 3SSpro:CO plants (Figure 7B). In contrast with LHP1, the H3K27me3 mark does not differ in the same conditions (Figure 5C). Several published results show that transcriptional activity does not always correlate with changes in H3K27me3. In plants carrying an AGAMOUSpro:GUS transgene, GUS signal is obtained even when the transgene is enriched for H3K27me3 (Schubert et al., 2006). Two cold-responsive genes targeted by H3K27me3, COR15A and ATGOLS3, lose H3K27me3 with delayed kinetics compared with transcriptional induction in the cold. In addition, upon return to warmer ambient temperature, the expression of these genes is fully repressed before recovery of H3K27me3 (Kwon et al., 2009).

The widely studied FLC locus represents an emblematic example of epigenetically maintained gene repression via a PcG-dependent mechanism. However, the structural FLC locus is always enriched in H3K27me3 (Turck et al., 2007; Zhang et al., 2007b), and only an increased spreading of the histone mark and presence of LHP1 after vernalization prevents FLC expression (Mylne et al., 2006; Sung et al., 2006; Finnegan and Dennis, 2007). The precise mechanism by which the spreading of H3K27me3 stabilizes the repression of FLC is not elucidated and could depend on the de novo condensation of a restricted regulatory region, in analogy to the situation observed...
for the regulatory regions in the 4.0-kb promoter of FT (Figure 4). Clearly, transcription can take place in the presence of repressive marks, as has been previously proposed in plants and animals (Schubert et al., 2006; Schwartz and Pirrotta, 2007).

Histone acetylation has been involved with transcription initiation and elongation (Loidl, 2004; Chen and Tian, 2007; Choi and Howe, 2009) and is mostly found at promoters and the 5' -ends of transcribed units, peaking around the core promoter (Choi and Howe, 2009). In A. thaliana, TOC1 and FLC are examples of transcriptional regulation mediated by the acetylation status (Perales and Mas, 2007; Farrona et al., 2008), and the PetE gene from pea (Pisum sativum) is hyperacetylated at cis-regulatory regions upstream of the transcriptional start site when highly expressed (Chua et al., 2003). We found two hyperacetylated regions at the FT distal and proximal FT promoter when this gene is constitutively highly expressed (Figure 5D), indicating that those regions might have a similar chromatin structure that facilitates FT expression.

Nonetheless, chromatin changes in response to FT induction seem to be a delayed response and not a prerequisite for FT

Figure 7. A Model of FT Transcriptional Activation Mediated through Interaction of Two Conserved and LHP1-Depleted Regions.
(A) Schematic diagram of the distribution of H3K27me3 chromatin mark and LHP1:HA protein in 35Spro:LHP1:HA Ler plants. ChiP-chip material was generated from 10-d-old seedlings grown on GM medium under LD conditions. Enrichment was calculated as the log2 ratio of ChIP sample versus input sample. Two Col-specific insertions that are not present in Ler are depicted in gray. Light-gray areas highlight the conserved blocks upstream of FT. (B) The model shows a constitutively LHP1 poor region that coincides with block C and might enable accessibility of transcription factors required for CO-dependent activation of FT. This interaction of CO with a protein partner or an activator complex might enhance CO binding DNA affinity to block A located in the proximal promoter. The middle part of the promoter contains response elements for one or several unknown activating factors "Y," which are specifically expressed in the midvein. The middle part of the promoter is accessible after a prolonged high expression of FT or when LHP1 is genetically deleted.
transcription because no changes were observed preceding FT expression in an inducible experimental setup (Figure 6). In A. thaliana, there are only a few reports on short-term chromatin modifications upon transcriptional activation. Using an estrogen receptor-based inducible system, Ng et al. (2006) showed that acetylation of H3K9 coincides with activation of the phases promoter. However, again, the results indicate that epigenetic changes are more the consequence of gene expression rather than the cause. Similar data were obtained in the analysis of the chromatin changes induced by the circadian clock in TOC1 expression (Perales and Mas, 2007).

In conclusion, the role of chromatin-mediated repression at FT is more one of expression tuning than a means to switch from a transcriptional off to an on state and vice versa. Induction of FT depends on an interplay between the proximal promoter and a conserved distal regulatory region that has a more accessible chromatin environment. Strong and persistent activation of FT results in a loss of repressive chromatin from the entire FT promoter. As a result, new access to chromatin-repressed cis-elements results in an extended expression domain for FT within the phloem and ultimately to an accelerated flowering response.

METHODS

Plant Material and Plant Growth Conditions

Arabidopsis thaliana plants of the accessions Col and Ler were used as genetic background. Plants were grown at 20°C under LDs (16 h light/8 h dark), ESDs (8 h light/8 h incandescent light/8 h dark), or SDs (8 h light/16 h dark) conditions after stratification at 4°C for 2 to 4 d. Light was provided by fluorescent tubes complemented by incandescent bulbs to increase the proportion of far red light. Since high-intensity light is required for photosynthesis, plants grown in ESDs were not developmentally advanced when compared with SD-grown seedlings. Nonetheless, reduced light quantity in ESD conditions compared with LD conditions did not affect floral transition. Plants on plates were grown on GM medium supplemented with 1% (w/v) sucrose under LDs.

Flowering Time Measurement

Seeds were stratified for 3 d at 4°C on wet filter paper and then sown on soil in ESD or SDs. Flowering time was measured by scoring the number of rosette and cauline leaves on the main stem of at least 12 individuals. Data are expressed as mean ± se. For statistical analysis, two-way analyses of variance were calculated using a general linear model with the SAS software package version 9.1.

Plasmid Constructions

FT promoter sequences were amplified from Col DNA with oligonucleotides indicated in Supplemental Table 1 online. PCR products were introduced into the GATEWAY pDONR207 vector (Invitrogen), and absence of PCR induced mutations in the constructs was confirmed by DNA sequencing. Overlapping primer pairs were designed to introduce point mutations into the proximal FT promoter sequence (see Supplemental Table 1 online, mutations depicted in bold). A BamHI and PstI fragment from 8.1kbFTpro:pDONR207 was replaced with the corresponding fragment containing the mutations.

To generate the binary destination vectors GW:GUS, GW:LUC, and GW:GreenLUC, GUS coding sequences from pBT10-GUS (Sprenger-Haussels and Weisshaar, 2000) and LUC coding sequences from pGEM-luc+ and pCBG68luc vector (both Promega) were cloned into the multiple cloning site of the GW-MCS-NOS-pGREEN vector (Corbesier et al., 2007). The binary destination vector GW:FTcDNA was described by Corbesier et al. (2007). To construct control vectors for transient bombardments, the GreenLUC and RedLUC genes were excised from the Promega pCBG68luc and CBRluc vectors and introduced into pJAN. The 3SSpro:gCO-pBluescript construct was described by Onouchi et al. (2000).

Plant Transformation and Transgenic Lines

All plasmids based on pGREEN0229 were introduced into Agrobacterium tumefaciens strain GV3101 containing the helper plasmid pSOUP (Koncz and Schell, 1986). Plasmids were transformed into A. thaliana plants by the floral dip method (Clough and Bent, 1998). For GUS expression analysis, constructs were introduced into wild-type Col and 3SSpro:CO Col plants (Onouchi et al., 2000) and thp-1 mutant background (Larsson et al., 1998). The T-DNA insertion line 60 of the Col background (Yoo et al., 2005) was used for complementation analysis.

In the T1 generation, plants carrying a pGREEN0229 plasmid were identified on the basis of BASTA resistance. The next generation was tested for single locus insertion of the transgene based on a 3:1 segregation on GM medium containing 12 μg/mL phosphonitricin. The selected plants were segregated to obtain T3 homozygous lines for GUS expression and flowering time experiments.

For FTpro:GUS 3SSpro:CO double transgenic lines, 8.1kbFTpro:GUS, 4.0kbFTpro:GUS, and 1.0kbFTpro:GUS Col lines were crossed with 3SSpro:CO Col plants. Transgenic lines carrying 5.7kbFTpro:GUS in Col and 3SSpro:CO background are based on independent transformations. The 3SSpro:LHP1:HA line was previously obtained (Tuck et al., 2007). The same line was crossed with a 3SSpro:CO co-2 tt-4 and a 3SSpro:CO:GR co-2 tt-4 line (Onouchi et al., 2000).

Expression Analysis

The aerial part of soil-grown plants was collected to extract total RNA with the RNeasy mini kit (Qiagen). Five micrograms of RNA was DNase treated using the DNA-free kit (Ambion) to cDNA synthesis. Quantitative real-time RT-PCR was performed using a BioRad iQ apparatus and SYBR Green II detection. A dilution series of a specific plasmid was used as standard for each primer pair during quantitative PCR and allowed calculation of molar ratios. Actin was used as a housekeeping gene. Primer sets can be found in Supplemental Table 1 online.

For GUS staining, seedlings were incubated for 30 min in 90% (v/v) acetone on ice, rinsed with 50 mM sodium phosphate buffer, pH 7.0, and incubated for 17 h at 37°C in staining solution (0.5 mg × mL −1 X-Gluc [5-bromo-4-chloro-3-indolyl-β-D-glucuronide], 50 mM sodium phosphate buffer, pH 7.0, 0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide, and 0.1% (v/v) Triton X-100). After staining, samples were washed with 50 mM sodium phosphate buffer, pH 7.0, and cleared in 70% (v/v) ethanol. The GUS histochemical staining was visualized under a light stereomicroscope (MZ 16 FA; Leica).

Phylogenetic Analysis and Transcription Factor Binding Site Identification

FT sequences from Arabidopsis lyrata, Capsella rubella, and Brassica oleracea were assembled using shotgun sequences available from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). A BAC clone from Brassica rapa containing a FT-like gene was obtained from the Multinational Brassica Genome Project (http://www.brasica.info). Arabis alpina sequences were obtained after sequencing of Aa FT1, Aa FT2, and Aa FT3 containing BACs using the Sanger method.
(M.C. Albani and G. Coupland, unpublished data). Short sequence stretches of the promoters of FT-like genes from *Sisymbrium polyceratum* and *Arabis hirsuta* were amplified using PCR primers that were based on the *A. thaliana* proximal FT promoter sequence 5′-TGCGTACT-CAAGTTGGGAGAT-3′ and 5′-TAAACTCGGATTGGTAGGAAATC-3′. mVista Shuffle-LAGAN was used to create pair-wise alignments of long FT promoter sequences from *A. thaliana*, *A. lyrata*, *B. rapa*, and the Aa FT1 gene (http://genome.lab.go.jp/vista) (Brudno et al., 2003; Frazer et al., 2004). Sequences of conserved regions were analyzed with ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2) (Larkin et al., 2007). Plant cis-acting regulatory DNA elements were searched through the PLACE database with a cutoff of >4 bp (http://www.dna.afric.go.jp/PLACE) (Higo et al., 1999). Sequences and FASTA output files of mVista and ClustalW alignments can be found in Supplemental Data Set 1 online.

**Transient Assays**

For transient assays, 5 μg of each 35Spro:GFP-pBluescript and FTpro: LUC-pGREEN or 35Spro:RedLUC-pJAN and indicated FTpro:GreenLUC-pGREEN were mixed with 5 μg of an empty vector (pK5) or 35Spro:rcGO-pBluescript according to standard procedures and bombardered 5 to 10 mm long *A. thaliana* leaves of SD-grown Col plants using the Biolistic particle delivery system (PDS-1000/HE: Bio-Rad). After incubation overnight in LDs, transformed leaves were sprayed with 1 mM luciferin, and luciferase activity was detected using a Hamamatsu photon counting system. Activity of the LUC luciferase was normalized by GFP signal obtained in five different leaves. Activities of the GreenLUC and RedLUC luciferases were measured through different optical filters (LEE Filters; filter #139 primary green and filter #126 bright red). To calculate the amount of RedLUC activity measured through the green filter and vice versa, one bombardment with 35Spro:RedLUC-pJAN and one bombardment with 35Spro:GreenLUC-pJAN were only performed. The ratios of signals were calculated by a formula provided by Promega.

**ChIP**

For ChIP experiments performed in the 35Spro:CO background, 10-d-old seedlings grown on GM media under SD conditions were harvested. For the experiments in the 35Spro:CO:GR background, plants were grown on soil under SD conditions until day 10 when they were sprayed with a solution of 1 mM Dex and harvested just before the treatment (time 0) or every 30 min until 3 h after the treatment. ChIP experiments were performed as previously described (Searle et al., 2006) with anti-HA antibodies (H6908; Sigma-Aldrich), anti-H3K27me3 (07-449; Millipore), anti-H3K914ac antibodies (06-599; Millipore). A very low signal was detected in mock antibody precipitations and was considered as background. A small aliquot of untreated sonicated chromatin was used as the total input DNA. Normalization of LHP1:HA binding and H3K27me3 data was performed using the signal obtained at At4g24640, a Polycomb and LHP1-regulated gene that is not affected by photoperiod (Turck et al., 2007). H3K914ac data were normalized with the signal obtained at At1g67090, which is highly expressed and is not influenced by photoperiod. Quantitative PCR data are shown as fold enrichment over the control genes and are means of three technical replicates. At least two independent biological replicates were performed for each experiment and a representative one is shown. Primers used for ChIP-PCR are described in Supplemental Table 1 online.

**ChIP-chip**

Chromatin was obtained from 35Spro:LHP1:HA Ler seedlings grown for 10 d on GM medium under LDs and harvested at ZT0 when FT is not expressed. Chromatin was precipitated using anti-H3K27me3 and anti-HA antibodies. DNA was amplified by linker-mediated PRC and hybridized to two-color microarrays from Roche; input samples were hybridized as reference. The data were normalized by LOESS and averages of two biological replicates uploaded to the TAIR genome browser for visualization (see Supplemental Data Set 6 online). For a detailed description of ChIP-chip sample generation and data analysis, see Göbel et al. (2010) and Reimer and Turck (2010).

**Accession Numbers**

Sequence data from this article can be found in the Arabidopsis Genome Initiative data library under the following accession numbers: AG (At4g18960), ATGOLS3 (At1g09350), CIB1 (At4g34530), CO (At5g15840), COR15A (At2g42540), FAST1 (At1g65470), FLC (At5g10140), FT (At1g65480), LHP1 (At5g17690), NF-YB2 (At5g47640), NF-YB3 (At4g14540), SMZ (At3g54990), SVP (At2g22540), TEM1 (At1g25560), TEM2 (At1g68840), TFL1 (At5g03840), TOC1 (At5g61380), TSF (At4g20370), A. alpina FT1pro (FN813298), B. rapa FT1pro (FN813299), A. lyrata FT1pro (FN813300), S. polyceratium FT1pro block A (FN813301), C. rubella FT1pro block A (FN813302), B. oleracea FT1pro block A (FN813303), A. hirsuta FT1pro block A (FN813304), A. alpina FT2pro block A (FN813305), A. alpina FT3pro block A (FN813306), and C. rubella FT1pro block C (FN813307).

**Supplemental Data**

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

**Supplemental Figure 1.** Response to Daylength Mediated by the 8.1-kb FT Promoter.

**Supplemental Figure 2.** Spatial FT Expression at Different Developmental Stages.

**Supplemental Figure 3.** FAS1 Expression Does Not Change in 35Spro:CO Plants.

**Supplemental Figure 4.** Nuclear Import of CO upon Dexamethasone Treatment of 35Spro:LHP1:HA; 35Spro:CO:GR Ler Plants Triggers Flowering.

**Supplemental Table 1.** List of Primers Used.

**Supplemental Data Set 1.** mVista Alignments of Col FTpro and Ler FTpro Sequences (FASTA Format).

**Supplemental Data Set 2.** mVista Alignments of Col FTpro and Arabidopsis lyrata FTpro Sequences (FASTA Format).

**Supplemental Data Set 3.** mVista Alignments of Col FTpro and Brassica rapa FTpro Sequences (FASTA Format).

**Supplemental Data Set 4.** mVista Alignments of Col FTpro and Arabis alpina FT1pro Sequences (FASTA Format).

**Supplemental Data Set 5.** ClustalW Alignments of FTpro Block A, B, and C Sequences (FASTA Format).

**Supplemental Data Set 6.** ChIP-chip Data for the FT Locus (GFF Format).

**ACKNOWLEDGMENTS**

We thank Markus Berns for his assistance with phylogenetic analysis, Jarod Rollins for help with statistical analysis, Doris Wagner for critical reading of the manuscript, and Anna Zakrisson-Plogander and Valentina Strizhova for the screening of the *A. alpina* BAC library and excellent technical help, respectively. We acknowledge financial support from...
European Molecular Biology Organization, the Deutsche Forschungs-
gemeinschaft, and the Max Planck Society.

Received February 17, 2010; revised April 16, 2010; accepted May 3, 2010; published May 14, 2010.

REFERENCES


