TRANSCRIPTIONAL CONTROL OF *FLOWERING LOCUS T* IN ARABIDOPSIS

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Abstract

The transition to flowering is controlled by genetic pathways which integrate environmental cues and the developmental state of the plant. In *Arabidopsis thaliana*, the photoperiod, vernalization, and autonomous pathways converge at the level of transcriptional regulation of the floral integrator gene *FLOWERING LOCUS T (FT)*. Only under inductive long-day (LD) conditions CONSTANS (CO) protein accumulates in the leaf vasculature and activates *FT* expression. As part of the systemic flowering signal, FT protein moves through the phloem to the shoot apex where it initiates meristem identity changes.

To understand the molecular mechanism of flowering time regulation mediated by *FT*, *cis*-regulatory sequences of *FT* were identified in the present study. A *FT* promoter region between 4.0 and 5.7 kb upstream of the start codon was found to be essential for *FT* expression. This region contains a sequence stretch of 430 bp (block A) that is highly conserved within *Brassicacea*. The *FT* locus is associated with the transcriptional repressor TERMINAL FLOWER 2 (TFL2) but the conserved block A in the promoter coincides with a locally TFL2-depleted region. Expression analysis of *FT* promoter deletion constructs in *tfl2* background revealed that TFL2 mediates *FT* repression via sequences 1.0 to 4.0 kb upstream of *FT*.

The proximal promoter of *FT* contains a 360 bp region that is highly conserved within *Brassicacea* (block D). Mutational analysis of short conserved "shadows" within this region suggested a role in the CO-mediated activation of *FT* based on transient expression studies. Analysis of the mutated elements in the context of the full-length *FT* promoter in stably transformed plants confirmed that a 6 bp motif (named *S1*) is essential for *FT* expression.

Endogenous signals and vernalization promote flowering through repression of *FLOWERING LOCUS C (FLC)*. FLC has been proposed to repress *FT* by binding to a region of intron 1 of *FT*. Analysis of transgenes either containing or lacking the first intron of *FT* in high *FLC* expressing plants, revealed that FLC can repress *FT* through the promoter sequences also.

Interestingly, a genomic *FT* construct containing the full-length *FT* promoter and the genomic region with all introns but lacking the 3'-untranslated region is not expressed and cannot complement the *ft* mutant phenotype. These data demonstrate a negative regulatory role conferred by the structural *FT* gene and indicate that positive regulatory regions are present downstream of *FT*.

Zusammenfassung

Die Regulation der Blütenbildung unterliegt verschiedenen genetischen Signalwegen, die den Wechsel von vegetativem zu reproduktivem Wachstum an Unweltbedingungen als auch an das Entwicklungsstadium der Pflanze anpassen. In *Arabidopsis thaliana* laufen der photoperiodische, der vernalisationsabhängige und der autonome Signalweg auf der Ebene der transkriptionellen Regulation des Blühzeitpunktgens *FLOWERING LOCUS T (FT)* zusammen. Nur unter induktiven Langtagbedingungen akkumuliert CONSTANS (CO)-Protein in den Leitgefäßen der Blätter und aktiviert die Expression von *FT*. Als Komponente eines blühteninduzierenden Signals wandert das FT-Protein durch das Phloem in das Sproßmeristem und induziert dort die Blütenbildung.

Um ein besseres Verständnis zu erlangen, wie die Blühinduktion auf der Ebene des *FT*-Gens übermittelt wird, wurden in der vorliegenden Studie regulatorische Sequenzen von *FT* identifiziert. Dabei stellte sich ein Sequenzbereich 4.0 bis 5.7 kb oberhalb des Startkodons als essentiell für die Expression von *FT* heraus. Sequenzvergleich homologer *FT*-Gene anderer *Brassicacea* ergab, dass dieser Bereich eine 430 bp lange hoch konservierte Region (Block A) enthält. Obwohl der transkriptionelle Repressor TERMINAL FLOWER 2 (TFL2) fast den gesamten *FT*-Genlokus bindet, fällt Block A mit einer lokalen TFL2-armen Region zusammen. Expressionsanalyse mit *FT*-Promoterdeletionskonstrukten in *tfl2*-Pflanzen zeigte, dass TFL2 die Repression der *FT*-Transkription durch einen Sequenzbereich 1.0 bis 4.0 kb oberhalb des Startkodons übermittelt.

Die phylogenetische Analyse zeigte zudem, dass eine 360 bp lange Region (Block D) im proximalen Promoterbereich von *FT* hoch konserviert ist. Analyse von proximalen *FT*-Promoteren mit Mutationen in konservierten Elementen in einem transienten Expressionsversuch, ließ auf eine mögliche Funktion in der CO-abhängigen Aktivierung schließen. Untersuchungen der mutierten konservierten Elemente in transgenen Pflanzen, zeigten einen Einfluss des 6 bp langen Motivs *(S1)* auf die Regulation von *FT*.

Vernalisation und der autonome Signalweg fördern die Blütenbildung durch Repression von *FLOWERING LOCUS C (FLC)*. FLC unterdrückt die Expression von *FT* durch direktes Binden an Intron 1. Expressionsanalysen mit verschiedenen Transgenen zeigten, dass FLC *FT* zudem durch Sequenzen im Promoter hemmen kann.

Interessanter Weise, ist ein genomisches *FT*-Konstrukt, das auch die Intronsequenzen beinhaltet nicht aber die 3'-untranslatierte Region, nicht in der Lage *FT* zu exprimieren und den *ft*-Phänotypen zu komplementieren. Diese Beobachtung weist der Sequenz des *FT*-

Strukturgenes eine negative regulatorische Funktion zu und deutet an, dass es positive regulatorische Sequenzen unterhalb von *FT* gibt.

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1. Introduction

1.1. Flowering time control

The transition from vegetative to reproductive development is tightly regulated in order to synchronise flowering with favourable conditions and therefore to maximize the reproductive success of a plant. Flowering is controlled by genetic pathways which integrate environmental stimuli like temperature, day length and the developmental state of the plant. In *Arabidopsis thaliana* (Arabidopsis) floral promotion pathways such as photoperiod and gibberellin (GA) pathway ultimately increase the expression levels of a small set of genes, called the floral integrators, such as *FLOWERING LOCUS T (FT), TWIN SISTER OF FT (TSF), SUPPRESSOR OF CONSTANS 1 (SOC1), AGAMOUS-LIKE 24 (AGL24)* and *LEAFY (LFY)*, whereas enabling pathways such as vernalization and autonomous pathway regulate the expression of floral repressors, such as *FLOWERING LOCUS C (FLC)*, *SHORT VEGETATIVE PHASE (SVP)* and *TERMINAL FLOWER 1 (TFL1)* and thus define the competence of the plant to flower under inductive conditions (Boss et al., 2004; Li et al., 2008; Turck et al., 2008).

Day length is perceived in the leaves and only under inductive long-day (LD) conditions are the floral integrator genes *FT* and *TSF* transcribed in the leaf vasculature (Takada and Goto, 2003; Yamaguchi et al., 2005). It has been shown that movement of FT protein is required to transport the LD signal to the meristem and initiate meristem identity changes so that the meristem gives rise to flowers rather than leaves (Abe et al., 2005; Wigge et al., 2005; Corbesier et al., 2007; Jaeger and Wigge, 2007; Mathieu et al., 2007). Mis-expression of *FT* causes early flowering independent of environmental and endogenous stimuli, whereas loss-of-function of *FT* results in a severe late-flowering phenotype under LD conditions (Samach et al., 2000; An et al., 2004). Loss-of-function of *TSF* has a minor effect on timing of floral transition and enhances the late flowering phenotype of *ft* in LDs, while overexpression of *TSF* causes early flowering independent of the day length (Yamaguchi et al., 2005). Therefore, regulation of spatial and temporal expression of the floral integrator genes *FT* and *TSF* plays a crucial role in mediating flowering initiation in response to day length.

1.2. Floral enabling pathways

The MADS-box transcription factor *FLC* is a key repressor of the floral enabling pathways in Arabidopsis (Michaels and Amasino, 1999). High levels of *FLC* render the plant incapable to initiate flowering even under inductive photoperiods, whereas vernalization and the autonomous pathway promote flowering predominantly by transcriptional repression of *FLC* (Sheldon et al., 2000). Components of the vernalization pathway repress *FLC* during prolonged exposure to cold temperatures and also maintain this repression after a subsequent increase in ambient temperature (Michaels and Amasino, 1999; Sheldon et al., 1999). In contrast, the autonomous pathway results in *FLC* repression in response to internal cues at late developmental stages. The autonomous pathway is not a linear genetic pathway but rather a collection of genetic components targeting *FLC* regulation at different levels (Koornneef et al., 1998). In fact, since several genes implicated in the autonomous pathway encode for putative RNA-binding proteins, it is conceivable that *FLC* mRNA is post-transcriptional regulated by the autonomous pathway (Simpson, 2004).

FLC is widely expressed in leaves and the shoot apical meristem (SAM) and acts as a direct repressor of floral promoting genes in both tissues to prevent response to inductive signals (Searle et al., 2006). By repression of *FT* and *TSF* in the leaves, FLC blocks the transmission of the floral promoting LD signal from the leaves to the meristem (Yamaguchi et al., 2005; Helliwell et al., 2006; Searle et al., 2006). Moreover, FLC impairs the competence of the meristem to respond to flowering signals by inhibiting the expression of *SOC1* and the FT co-factor *FD* (Helliwell et al., 2006; Searle et al., 2006). Expression of the floral integrator *SOC1* in the meristem is the earliest marker of floral transition of the meristem. The expression of this MADS-box transcription factor shows a sharp increase in the apex upon floral induction even before any physiological changes in the meristem architecture can be observed (Borner et al., 2000; Lee et al., 2000; Samach et al., 2000). The early activation of *SOC1* by inductive LD conditions is dependent on *FT* and *FD* (Searle et al., 2006).

Recently, it has been shown that the MADS-box transcription factor *SVP* mediates ambient temperature signalling within a thermo-sensory pathway and signalling from the autonomous pathway by direct repression of *FT* and *SOC1* (Lee et al., 2007; Li et al., 2008). SVP binds to a CArG sequence in the proximal *FT* promoter, as well to the non-canonical CArG-box containing region of intron 1 as it has been shown for FLC (Searle et al., 2006; Lee et al., 2007). While *FT* expression in the leaves is slightly modulated by SVP, direct binding of SVP at *SOC1* promoter regions that are also targeted by FLC plays a crucial role in controlling *SOC1* transcription in the shoot apex (Helliwell et al., 2006; Searle et al., 2006; Li et al.,

2008). Since FLC and SVP interact physically and regulate flowering in a mutually dependent way, these transcription factors may bind as a complex and modulate expression of at least two floral integrator genes in response to endogenous and environmental conditions (Li et al., 2008).

The impact of *FLC* on plant development is subject of natural variation in Arabidopsis. Winter annual Arabidopsis accessions have high levels of *FLC* and therefore require vernalization, while summer annuals have only low levels and flower without exposure to cold temperatures. This natural variation depends on the *FLC* locus itself as well as on the upstream activator *FRIGIDA (FRI)* which is non-functional in many summer annuals (Johanson et al., 2000; Shindo et al., 2005; Werner et al., 2005).

1.3. Floral promotion signals

Repression of *FLC* is not sufficient to induce flowering but rather confers competence to the plant to respond to flower promoting signals such as photoperiod and gibberellins. Floral initiation at later developmental stages is meditated by GA and induces transcription of the floral integrator genes *SOC1* and *LFY* in the meristem, while flowering in response to day length is mediated via *FT* and *TSF* in leaves (Blazquez and Weigel, 2000; Samach et al., 2000; Moon et al., 2003a; Yamaguchi et al., 2005). Transcriptional and post-transcriptional regulation of *CONSTANS (CO)* is crucial for measurement of day length. *CO* expression is circadian-controlled and rises around ten to twelve hours after dawn but drops rapidly at the beginning of the day. Only under LD conditions does *CO* mRNA expression coincide with light and CO protein accumulates. Cryptochrome 1 (Cry1), Cry2 and Phytochrome A (PhyA) stabilize CO protein and prevent its proteasome-dependent degradation at the end of LDs (Valverde et al., 2004). CO degradation during the night is dependent on SUPRESSOR OF PHYA-105-1 (SPA1), SPA3 and SPA4 which act in concert with the ubiquitin-ligase CONSTITUTIVE PHOTOMORPHOGENESIS 1 (COP1) (Laubinger et al., 2006; Jang et al., 2008). Negative regulation of CO abundance in the morning is mediated by Phytochrome B (PhyB) (Valverde et al., 2004; Laubinger et al., 2006).

Experiments with Arabidopsis plants that express inducible CO indicate that CO acts as direct activator of *FT* and *TSF* (Samach et al., 2000; Yamaguchi et al., 2005). In wild type plants shifted from non-inducible short-day (SD) conditions to inductive photoperiods, expression of *FT* and *TSF* is induced immediately upon experience of LDs. The mRNAs of the two floral integrator genes follow a diurnal pattern that is driven by CO and therefore expression peaks towards the end of the day, when CO protein accumulates (Yamaguchi et al., 2005; Corbesier

et al., 2007). However, biphasic *FT* expression patterns have also been observed and show a second *FT* peak in the morning which is dependent on *CO* (Corbesier et al., 2007). *FT* expression in the morning might be a CO-mediated light quality effect. Plants grown in far-red enriched light (FREL) contain higher levels of *CO* transcript at the beginning of the day and it has been shown that FREL can increase CO protein levels independent of transcription (Valverde et al., 2004; Kim et al., 2008). Enhanced accumulation of CO might result in *FT* expression in the morning in plants grown in FREL but not in white light (Kim et al., 2008). Nonetheless, expression of *FT* in the morning can only be observed under inductive LD conditions (Corbesier et al., 2007).

Light quality seems to affect flowering time by direct regulation of *FT* expression in a CO-independent manner as well. The transcription factor *Cryptochrome-interacting bHLH 1 (CIB1)* promotes flowering by positive regulation of *FT*. In ChIP experiments, CIB1 binds to the 5'-end of the transcribed region and in intron 2 of *FT*. *In vitro*, CIB1 has been shown to interact with E-box (CANNTG) elements that are present at the *FT* locus. *CIB1* is expressed in the vasculature of whole seedlings and at low levels in all other tissues. Stimulation of *FT* by CIB1 is dependent on *CRY2* which has been shown to interact with CIB1 in a blue light dependent manner (Liu et al., 2008).

1.3.1. Transcriptional activation of *FT* and *TSF* by CO

CO is expressed in the phloem companion cells of leaves and is most abundant in cells of the distal minor veins. Although CO mRNA is detectable in the shoot apical region above the protophloem, studies with *CO:GFP* fusions under control of the native *CO* promoter indicate that CO protein is restricted to the phloem companion cells (An et al., 2004). Also limited to the phloem companion cells of leaves, *FT* expression seems to occur mainly in the minor veins of the distal half of the leaf. Because of difficulties to detect the endogenous transcript by *in situ* hybridisation, most studies of the spatial mRNA pattern of *FT* are based on promoter reporter gene studies (Takada and Goto, 2003). Recently, it has been shown that there is no difference in spatial expression between a *GUS* reporter gene under control of the *FT* promoter or integrated into a genomic *FT* fragment in wild type plants (Notaguchi et al., 2008). These data are further supported by *in situ* hybridisations that show transcript accumulation in the phloem of a mutant with induced *FT* expression (Takada and Goto, 2003). Expression analysis of *TSF* with a *GUS* reporter integrated into a genomic *TSF* fragment revealed that the spatial patterns of *TSF* and *FT* mainly do not overlap, although both genes are both direct targets of CO. *TSF* is expressed in the vascular tissue of hypocotyls, petioles and the basal part of

cotyledons, as well as close to the SAM. Very little expression is detectable in the most-distal phloem of first true leaves (Yamaguchi et al., 2005).

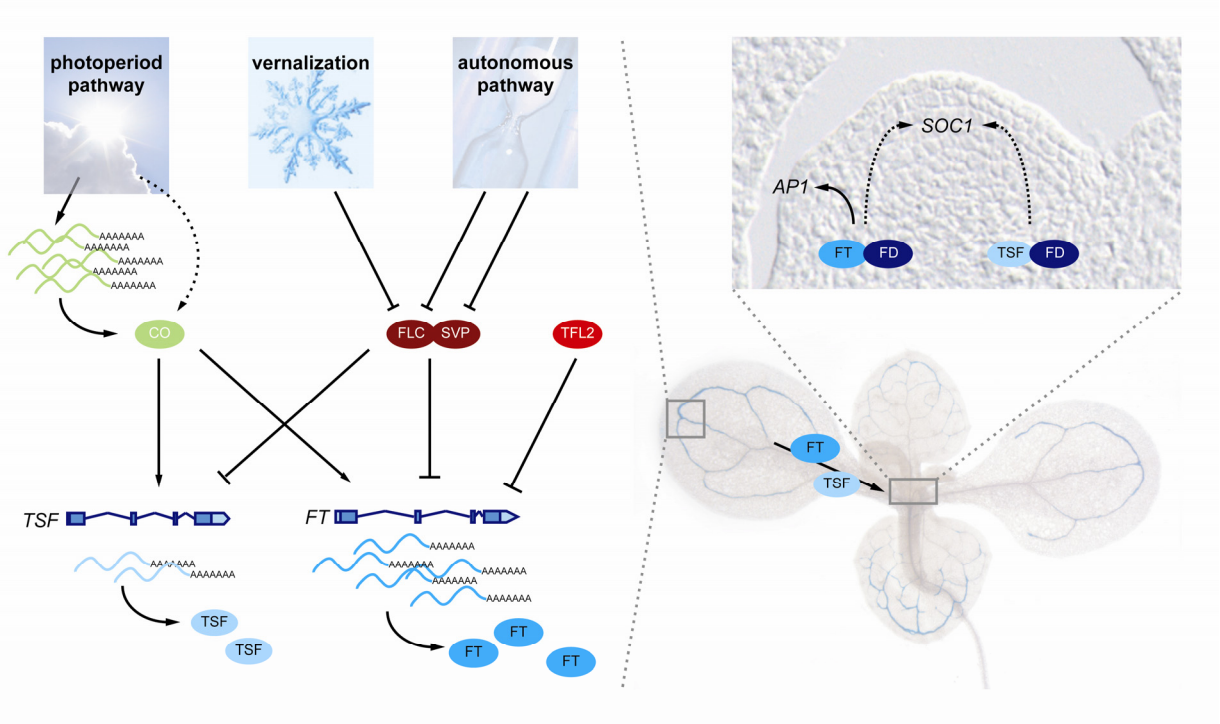

Figure 1. Flowering time control mediated by the floral integrator genes *FT* and *TSF*.
In Arabidopsis, cues from several floral transition pathways, such as the photoperiod response, the vernalization dependent and the autonomous pathway, are integrated at the transcriptional regulation of *FT* and *TSF*. Vernalization and autonomous pathway promote flowering via transcriptional repression of *FLC*, while high *FLC* levels cause repression of *FT* and *TSF* and antagonize floral induction by inductive photoperiods. Since it has been shown that FLC can physically interact with SVP, a MADS-box transcription factor regulated by the thermo-sensory, autonomous and GA pathways, the two proteins may represses transcription of *FT* as a complex. Flowering in response to inductive photoperiods is mediated via direct activation of *FT* and *TSF* by CO. Only under LD conditions CO protein is stabilized in the second half of the day (dashed line) and can induce *FT* and *TSF* transcription in the leaf vasculature (Wild type seedling grown for 10 days under inductive LDs shows spatial *GUS* expression driven by an 8.1 kb *FT* promoter). *FT* and *TSF* have been show to act redundantly; nonetheless, loss-of-function of *FT* has a stronger effect on flowering time control. As part of the systemic signal, FT and probably TSF proteins move through the phloem to the apex.
Upon interaction with the bZIP transcription factor FD, FT and TSF mediate changes in gene expression that lead to re-programming of the meristem identity. *SOC1* expression is the earliest molecular marker for floral transition. Although activation of *SOC1* by inductive photoperiods is dependent on *FT* and *FD*, it is not known if the activation of *SOC1* is directly mediated by FT/FD (dashed line). In floral primordia, the FT/FD dimer directly activates expression of the meristem identity gene *AP1* (Apex section shows a wild type meristem undergoing floral transition; Stefano Torti, MPIZ, Cologne).

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). Zinc-binding B-boxes are present in many animal transcription factors. Often one or two B-boxes are associated with a RING domain and a coiled-coiled domain and form a motif which has been implicated in protein-protein interaction rather than DNA binding (Torok and Etkin, 2001). The amino terminal B-box of CO belongs to a class of plant-specific B-box variants which

occur as a single domain or in a duplicated version. In Arabidopsis, CO and sixteen CO-like (COL) proteins carry at least one B-box in combination with a CCT domain (Robson et al., 2001; Griffiths et al., 2003). The CCT domain is plant-specific and has been shown to confer nuclear localization to the protein. Analysis of different *CO* loss-of-function alleles demonstrated that the B-boxes and the CCT domain are required for CO function (Robson et al., 2001).

Although CO features some properties of a transcription factor, direct DNA binding could not be demonstrated and led to the suggestion that CO requires an unidentified protein partner to activate transcription (Suarez-Lopez et al., 2001). Since an artificial fusion protein of the CO B-box domains and a yeast GAL4 DNA binding domain is able to act as a transcriptional activator in yeast, CO might add a trans-activating function to a protein or protein complex (Ben-Naim et al., 2006). Furthermore, it has been shown that the CO B-boxes are able interact with TGA4 in yeast. TGA4 is a member of the TGA family of basic domain/ leucin zipper transcription factors, which have been implicated in salicylic acid (SA) dependent activation of *PATHOGENESIS-RELATED (PR)* genes. Interestingly, TGA4 can bind proximal *FT* promoter pieces *in vitro*. Nevertheless, *in vivo* experiments and genetic analyses demonstrating if TGA4 has an impact on CO-mediated flowering control have not been reported (Song et al., 2008).

It may be that interaction with other proteins enhances a low DNA-binding affinity of the CCT domain. This idea is based on the observation that the CCT domain of CO is able to interact with subunits of the eukaryotic CCAAT-box binding complex and also shows similarities to the DNA binding domain of one member of this complex (Ben-Naim et al., 2006; Wenkel et al., 2006). The CCAAT binding complex is well conserved in a wide range of organisms from yeast to humans and known as HEME ACTIVATOR PROTEIN (HAP) complex, Nuclear Factor Y (NF-Y) complex and CCAAT Box Factor (CBF) complex. The trimeric complex consists of HAP2 (NF-YA or CBF-B), HAP3 (NF-YB or CBF-A) and HAP5 (NF-YC or CBF-C). In mammals, the complex forms through initial dimerisation of HAP3 and HAP5 which then associate with the structurally unrelated HAP2, while the yeast trimeric HAP complex is formed in a single-step mechanism. None of the units alone is able to stably bind to DNA but the preassembled hetero-trimeric complex binds to a CCAAT-box with a very high specificity and affinity (Mantovani, 1999; McNabb and Pinto, 2005). CCAAT *cis*-acting elements are present in promoter regions of approximately 30% of all eukaryotic genes and are commonly located in reverse orientation 60-100 bp upstream of the transcription start site (Mantovani, 1999). In yeast, the assembled trimeric HAP complex has no transcriptional potential and

requires a fourth subunit, HAP4, that mediates transcriptional activation (McNabb and Pinto, 2005).

In contrast to other organisms, each subunit of the Arabidopsis HAP (AtHAP) complex is encoded by ten or more genes (Edwards et al., 1998; Gusmaroli et al., 2002). Although redundancy is expected, genetic analysis demonstrated that members of the AtHAP complex are involved in flowering control and placed them in the photoperiodic pathway downstream of *CO*. AtHAP3b and AtHAP3c have been shown to play a role in promotion of flowering under inductive LD conditions by regulation of *FT* transcript levels (Cai et al., 2007; Chen et al., 2007; Kumimoto et al., 2008). In combination with yeast HAP2 and HAP5, AtHAP3b and AtHAP3c are able to bind to a CCAAT-box containing *FT* promoter region *in vitro* (Kumimoto et al., 2008). CO has been shown to interact with AtHAP3a and AtHAP5a and the CCT domains of CO and COL proteins show structural similarities to the DNA binding domain of HAP2. Amino residues of HAP2, required for interaction with the HAP3/HAP5 dimer and CCAAT sequence recognition, are well conserved in the CCT domains of CO and COL proteins (Ben-Naim et al., 2006; Wenkel et al., 2006). Furthermore, some mutations of *CO* that delay flowering affect these highly conserved residues (Wenkel et al., 2006). It is possible that CO replaces AtHAP2 in the trimeric AtHAP complex. The late flowering phenotype of plants overexpressing HAP2 and HAP3a might be due to stoichiometrical changes of complex formation. This effect can be counterbalanced by increasing *CO* levels (Wenkel et al., 2006).

1.3.2. TEM1 – a repressor of *FT*

Recently it has been proposed that *TEMPRANILLO 1 (TEM1)* acts as a direct repressor of *FT* to avoid precocious flowering (Castillejo and Pelaz, 2008). *TEM* genes belong to the RAV subfamily of transcription factors which contain two plant-specific DNA-binding domains, an AP2/ERF and a B3 DNA-binding domain. The sequences CAACA and CACCTG have been identified as the DNA recognition sites of RAV1 (Kagaya et al., 1999). These motifs are present in the 5'-untranslated region of the *FT* gene and it has been demonstrated that TEM1 can bind to this region *in vivo* (Castillejo and Pelaz, 2008). *TEM1* is expressed in all vasculature and mesophyll tissues. Before floral transition *TEM1* mRNA levels start to decrease. During the floral transition phase expression patterns of *TEM1* and *FT* overlap in the outer part of the leaves. Since *TEM1* abundance is low during the day and peaks at dusk of LDs it might play a counterpart to CO (Castillejo and Pelaz, 2008). Nevertheless genetic

evidence that *TEM* genes mediate flowering through the photoperiod pathway has not been demonstrated.

1.4. Epigenetic mechanisms of flowering control

1.4.1. Gene regulation by Polycomb repressive complexes

Chromatin modifications have been demonstrated to play an important role in floral transition in Arabidopsis. For example, loss-of-function of genes encoding Polycomb group (PcG) components causes severe flowering defects and changes in floral organ formation (Farrona et al., 2008; Schatlowski et al., 2008). In *Drosophila melanogaster* (Drosophila) PcG proteins are involved in stable repression of key developmental genes through chromatin modifications. The Polycomb Repressive Complex 2 (PRC2) includes the catalytic activity to tri-methylate lysine 27 (H3K27me3). The catalytic core is the SET-domain protein *Enhancer of Zeste* (E(Z)). The complex also includes the zinc-finger protein *Suppressor of Zeste 12* (SU(Z)12), the WD40-domain protein *Extra sex combs* (ESC), and *Multicopy suppressor of Ira* (MSI) (Schwartz and Pirrotta, 2007). In Arabidopsis, most components of the complex are encoded by small gene families and the composition might differ dependent on developmental states and tissues. *MEDEA (MEA), CURLY LEAF (CLF)* and *SWINGER (SWN)* are homologs of E(Z). *FERTILISATION INDEPENDENT SEED 2 (FIS2), VERNALIZATION 2 (VRN2)* and *EMBRYONIC FLOWER 2 (EMF2)* encode SU(Z)12 related genes. The only homologous gene of ESC in Arabidopsis is *FERTALISATION INDEPENDENT ENDOSPERM (FIE)*. A family with 5 members encodes MSI-like genes *(MSI1 –MSI5)*. CLF, SWN and FIE probably take part in many complexes, while SU(Z)12 homologues proteins apply a specific role to the complex (Farrona et al., 2008; Schatlowski et al., 2008).

In animals, gene repression through H3K27me3 histone marks requires binding of the Polycomb Repressive Complex 1 (PRC1). The chromodomain protein *Polycomb* (Pc) is member of the large multi-protein complex and specifically binds to H3K27me3 (Schwartz and Pirrotta, 2007). In plants, many components of the PRC1 are lacking and are possibly replaced by functionally similar but unrelated proteins (Schubert et al., 2005). In Arabidopsis, *TERMINAL FLOWER 2 (TFL2)/ LIKE HETERCHROMATIN PROTEIN 1 (LHP1)* encodes a chromodomain protein which has been shown to co-localize exclusively and extensively with H3K27me3 chromatin *in vivo* (Turck et al., 2007; Zhang et al., 2007a). Recently is has been shown that TFL2 and CLF can interact with two Arabidopsis RING domain proteins (AtRING1a and AtRING1b) which show high homology to *Sex combs extra* (Sce) which is another component of the PRC1 in animals (Sanchez-Pulido et al., 2008; Xu and Shen, 2008).

Loss-of-function of AtRING1a and AtRING1b greatly enhances the developmental phenotypes observed in *clf* and *lhp1* single mutant plants (Xu and Shen, 2008).

1.4.2. *FLC* – a classical example of epigenetic repression

Transcriptional repression of *FLC* during vernalization is one of the best studied epigenetic mechanisms in plants. In a first phase, starting from a steady state of high *FLC* expression such as observed in winter annual accessions or mutants of the autonomous pathway, transcriptional repression of *FLC* is induced as an acute response to cold. During this phase the plant homeodomain (PHD) finger proteins VERNALIZATION INSENSITIVE 3 (VIN3) and VERNALIZATION 5 (VRN5)/ VIN3-like 1 (VIL1) associate with a region near the 5'-end of intron 1 of *FLC* (De Lucia et al., 2008). *VIN3* expression is up-regulated after exposure to cold and therefore *VIN3* is one of the early components of the vernalization pathway (Sung and Amasino, 2004). VRN5 association with *FLC* is dependent on the presence of VIN3 (De Lucia et al., 2008). During the first *FLC*-repressive phase, chromatin modifications observed at the *FLC* locus change, so that those generally associated with highly expressed genes decrease, whereas others that are correlated with low gene expression levels increase. For instance, tri-methylation of lysine 4 and di-methylation of lysine 36 of histone 3 (H3K4me3 and H3K36me2) decrease at the 5'-end of the transcribed region and the first intron of *FLC*, respectively (Zhao et al., 2005; Finnegan and Dennis, 2007; Xu et al., 2008).

After initial *FLC* repression during cold exposure, a second phase of vernalization is marked by a gradual increase in repressive chromatin modifications such as di-methylation and tri-methylation of lysine 27 of histone 3 (H3K27me2 and H3K27me3) and di-methylation of H3 lysine 9 (H3K9me2) at the *FLC* locus (Bastow et al., 2004; Sung and Amasino, 2004; De Lucia et al., 2008). During this phase *FLC* repression becomes irreversible so that it persists even after a return to warmer ambient temperatures. Recently it has been reported that VRN5 and VIN3 together with their homolog VERNALIZATION5/VIN3-like 1 (VEL1)/ VIN3-like 2 (VIL2) form a complex with proteins of the PRC2 (Wood et al., 2006; De Lucia et al., 2008). The PRC2 complex mediating vernalization in Arabidopsis probably contains VRN2, CLF, SWN, FIE and MSI1 (Wood et al., 2006; De Lucia et al., 2008; Schatlowski et al., 2008). It has been shown that VRN2-PRC2 is constitutively associated with the *FLC* locus at sites where it correlates with the H3K27me3 chromatin mark (De Lucia et al., 2008). The current model proposes that upon cold perception, association of the PHD finger protein complex VRN5-VIN3-VEL1 with VRN2-PRC2 increases H3K27me3 methylation activity of the resulting PHD-VRN2-PRC2 super-complex. The effect of this stimulation is an increase in

H3K27me3 levels and a broader spreading of the histone mark over the *FLC* locus that largely takes place after exposure to cold when the temperature has increased (Finnegan and Dennis, 2007; De Lucia et al., 2008). Accordingly, loss-of-function of *VRN2*, *VRN5* or *VIN3* inhibits accumulation of tri-methylated H3K27 in the cold and attenuates the down-regulation of *FLC* during vernalization. Although a local slight increase in H3K27me3 is still observed in *vrn5*, *vin3* and *vrn2* plants, *FLC* repression is unstable after vernalization and *FLC* transcription leads to a late flowering phenotype of these mutant plants (Greb et al., 2007; De Lucia et al., 2008).

The increased and expanded H3K27me3 marks require further repressors which assist in the maintenance of stable *FLC* repression. The increase of H3K27me3 at the *FLC* locus during vernalization correlates with increased TFL2 association and *tfl2* mutants fail to maintain *FLC* repression (Mylne et al., 2006; Sung et al., 2006).

1.4.3. *FT* – targeted by epigenetic mechanisms

In Arabidopsis plants grown under SD conditions, a transient shift for three days to LDs is sufficient to irreversibly commit the plants to flower (Corbesier et al., 2007). Since *FT* mRNA levels decrease to pre-induced levels if plants are shifted back from inductive LD conditions to SDs, the *FT* locus is unlikely to be directly involved in the maintenance of floral commitment (Corbesier et al., 2007). Interestingly, although *FT* does not show a memory of its expression state, *FT* is targeted by epigenetic mechanisms. TFL2 and H3K27me3 histone marks distribute widely over the *FT* locus (Turck et al., 2007; Zhang et al., 2007b). Loss-of-function of *TFL2* causes day length-independent early flowering which is mainly due to up-regulation of *FT* expression (Kotake et al., 2003). In contrast to *FT, TSF* levels seem not to be increased in *tfl2* plants, although H3K27me3 marks and TFL2 association could be also detected at the promoter region of the *TSF* locus (Yamaguchi et al., 2005; Turck et al., 2007). *TFL2* transcript accumulates in proliferating cells in the meristematic tissues throughout development. It is expressed in young leaves, whereas expression in older leaves becomes restricted to the petiole and the proximal side of the leaf blade, areas where cells continue to proliferate. This expression disappears in mature leaves and *TFL2* mRNA is restricted to the vascular tissue (Figure 2A) (Kotake et al., 2003).

Genomic analysis revealed that in comparison to a random set of genes, H3K27me3 target genes are more tissue-specifically expressed (Turck et al., 2007). However, analysis of *FT* expression in the *tfl2* mutant background revealed that the expression of *FT* was increased but still restricted to the *CO*-expression domain (Takada and Goto, 2003). It has been proposed

that TFL2 counteracts the activity of the activator CO on *FT* to ensure day length dependent flowering. *CO* and *TFL2* are expressed in an inverted gradient along the leaf (Figure 2A). *FT* expression in the proximal half of the leaf may be repressed due to high expressed *TFL2*. In the distal veins of the leaves, *FT* transcription occurs as a result of CO overcoming the repressive effect of decreasingly expressed *TFL2* (Figure 2B).

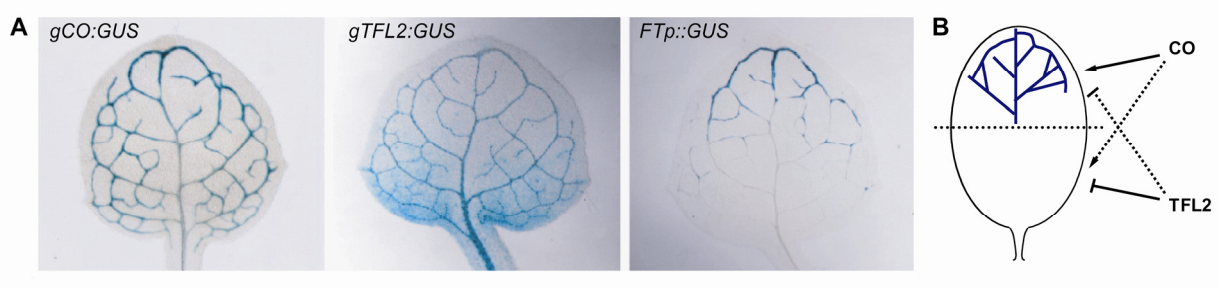

Figure 2. Spatial *FT* expression.
(**A**) Analysis of *CO*, *TFL2* and *FT* expression in the first true leaves of 8-day-old seedlings (Takada and Goto, 2003). Expression is visualized by a *GUS* reporter gene integrated into a genomic fragment of *CO* and *TFL2* respectively. Localisation of spatial *FT* expression is based on a promoter *GUS* fusion construct.
(**B**) Model of the regulation of spatial *FT* expression by CO and TFL2 (Sara Farrona, MPIZ, Cologne).

Lack of the PRC2 components *EMF2, CLF* and *FIE* causes high expression of *FT* independent of photoperiod and therefore early flowering as well (Moon et al., 2003b; Barrero et al., 2007; Jiang et al., 2008). Whether the spatial expression of *FT* is effected by loss-of-function of *EMF2, CLF* and *FIE,* has to be elucidated. As *FT* expression reaches levels sufficient for floral induction even in the presence of *TFL2, EMF2, CLF* and *FIE*, a possible model is that *FT* regulation by PcG proteins creates a threshold for activation that ensures that *FT* is not induced under low inductive conditions (Moon et al., 2003b; Takada and Goto, 2003; Barrero et al., 2007; Jiang et al., 2008).

In addition to PRC2 components, the putative chromatin factors *EARLY BOLTING IN SHORT DAYS (EBS)* has been shown to be involved in *FT* and *TSF* repression under non-inductive conditions. Loss-of-function of *EBS* results in *FT* and *TSF* expression in SDs and therefore accelerates flowering. *EBS* encodes for a ubiquitous expressed nuclear protein with a bromoadjacent homology (BAH) domain and a PHD zinc finger. Both domains seem to mediate protein-protein interactions of transcriptional regulators involved in chromatin re-modelling. However, whether the repressing effect of EBS on *FT* and *TSF* under non-inductive conditions is direct or indirect has yet to be elucidated (Pineiro et al., 2003; Yamaguchi et al., 2005).

1.5. Floral induction at the meristem

Movement of FT protein through the phloem into the apex is required for floral promotion (Corbesier et al., 2007; Jaeger and Wigge, 2007; Mathieu et al., 2007). The FT protein belongs to a small family of six genes which encode proteins that contain a phosphatidyl ethanolamine binding domain (PEBP) (Ahn et al., 2006). Based on studies in animals PEBP proteins are proposed to act in signalling cascades via protein-protein interactions (Chardon and Damerval, 2005). Several reports suggest that in the SAM, FT interacts with the bZIP transcription factor FD and directly regulate gene expression. Direct activation by FT/FD has been shown for the meristem identity gene *APETALA1 (AP1)* (Abe et al., 2005; Wigge et al., 2005). In addition, early activation of *SOC1* is dependent on *FT* and *FD*, but whether the activation of *SOC1* by FT/FD is direct has still to be investigated (Searle et al., 2006). Upon interaction with the floral integrator AGL24, SOC1 directly induces expression *LFY* (Yu et al., 2002). *LFY* is already expressed in leaf primordia, but upon floral transition its abundance in the apex rises (Blazquez et al., 1997; Hempel et al., 1997). It is still unclear whether FT regulates *LFY* directly or indirectly through *SOC1* (Schmid et al., 2003). Since *TSF* has been shown to act redundantly with *FT* it is likely that TSF mediates floral promotion via interaction with FD and other transcription factors as well.

Interestingly, another member of the *FT*-family, *TFL1* is involved in regulation of floral transition in an antagonistic manner to *FT*. *TFL1* acts as a floral repressor and confers shoot identity to the meristem (Shannon and Meekswagner, 1991; Bradley et al., 1997). In wild type plants the expression of *TFL1* occurs in the centre of the SAM, just below the inflorescence meristem, where it prevents *LFY* and *AP1* expression (Bradley et al., 1997). Similar to FT, TFL1 protein is mobile and spreads through the meristem beyond the region where *TFL1* mRNA is transcribed (Conti and Bradley, 2007). Interestingly, a single amino acid exchange between FT and TFL1 is sufficient to reverse the function of these proteins (Ahn et al., 2006). It has been proposed that TFL1 might compete with FT for FD binding or interact with another bZIP transcription factor, similar to the FT/FD module (Abe et al., 2005; Wigge et al., 2005; Ahn et al., 2006).

2. Aim of the Study

Previous studies have demonstrated the importance of transcriptional regulation of *FT* to ensure flowering time control in response to environmental and endogenous factors. The aim of the present study was to identify regulatory sequences that are required for *FT* expression. Characterization of *cis*-regulatory regions is the prerequisite for understanding the complex interaction between positive and negative regulators like CO, TFL2 and FLC.

Complementation and expression analyses of promoter deletion constructs in transgenic plants were used to identify regulatory regions of *FT*. To reveal the impact of CO, TFL2 and FLC on these regulatory regions, reporter gene constructs were analysed in corresponding genetic backgrounds. Based on a phylogenetic shadowing approach we aimed to identify *cis* elements in these regulatory regions. To analyse if the putative *cis*-regulatory elements are involved in *FT* regulation and mediation of CO response, we tested their role in a transient dual-luciferase reporter assay. Mutational complementation and expression analyses revealed their biological relevance *in planta*.

3. Material and Methods

Plasmid constructions

All PCR products were amplified from Arabidopsis DNA of Columbia (Col) ecotype. For amplification of promoter sequences specific primers with GATEWAY™ tails were used. The forward primers contained the attB1 extension (5'-GGGGACAAGTTTGTACAAAAAAGC AGGCT-3'); reverse primers contained the attB2 tail (5'-GGGGACCACTTTGTACAAGAA AGCTGGGT-3'). Specific sequences for each promoter construct were:

8.1kbFTp

5'-(attB1)GATTACCTCCCAGCACCAAA-3'

J14: 5'-(attB2)CTTTGATCTTGAACAAACAGGT-3'

7.2kbFTp

J47: 5'-(attB1)TATATAAATAGAGACTAGAA-3'

J14: 5'-(attB2)CTTTGATCTTGAACAAACAGGT-3'

6.7kbFTp

5'-(attB1)TTGCGGCATTGTACTAAACG-3'

J14: 5'-(attB2)CTTTGATCTTGAACAAACAGGT-3'

5.7kbFTp

5'-(attB1)CATTTGCTGAACAAAAATCT-3'

J14: 5'-(attB2)CTTTGATCTTGAACAAACAGGT-3'

4.0kbFTp

5'-(attB1)CAAGCTTTTGTTGGACATTCAGT-3'

J14: 5'-(attB2)CTTTGATCTTGAACAAACAGGT-3'

1.0kbFTp

J13: 5'-(attB1)ATAATATGGCCGCTTGTTTATA-3'

J14: 5'-(attB2)CTTTGATCTTGAACAAACAGGT-3'

1.5kbTSFp

J43: 5'-(attB1)TATGCTAATTAAATATGAAT-3'

J44: 5'-(attB2)AATTTATCTTGGATCTCAA-3'

Long PCR fragments were amplified with *TaKaRa Ex Taq*™ polymerase (Takara Bio Inc.), while for shorter ones the Expand High Fidelity PCR system (Roche) was used. To generate the promoter entry clones, PCR products were introduced into the GATEWAY™ pDONR207 vector (Invitrogen) through BP reactions. *35S, SUC2* and *8.1kbFT* promoter entry clones used

in this study were previously described (An et al., 2004; Corbesier et al., 2007). 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:

1.0kbFTp-S1mut

J07: 5'-CAATG**GTCG**GTACGTAGAATCAGTTTTAG-3'

J08: 5'-ACGTAC**CGAC**CATTGTCGTCTTATTTCATA-3'

1.0kbFTp-S2mut

J09: 5'-AGTTT**GCTCG**TCTAGTACATCAATAGACAAG-3'

J10: 5'-CTAGA**CGAGC**AAACTGATTCTACGTACATC-3'

1.0kbFTp-S3mut

J11: 5'-TGGGT**GGTTCC**TACCACAAACAGAAATAAAAAG-3'

J12: 5'-TGGTA**GGAACC**ACCCACCACACTAATACACTG-3'

1.0kbFTp-S4mut

J45: 5'-**GCGCCGGGTTCCGCG**TTCCAGTGTATTAGT-3'

J46: 5'-**CGCGGAACCCGGCGC**GGTGAACCATCGGTG-3'

1.0kbFTp-P1/P2mut

5'-TAG**GTGTTG**GGGTTTGGAAT**CAACAC**AACAGAAATAAAAAG-3'

5'-ATTCCAAACCC**ACCACAC**TAATACACTGGA-3'

Forward and reverse primers carrying the point mutations (depicted in bold) were used in combination with primer J14 and primer J13 respectively. PCR amplification with J13 and J14 on the basis of the two overlapping PCR fragments resulted in mutated versions of *1.0kbFTp*. From the *1.0kbFTp-pDONR207* a *BamHI* and *PstI* fragment containing the mutations was introduced into the *8.1kbFTp-pDONR207* construct to generate the mutated 8.1 kb *FT* promoter version.

As previously described the GATEWAY™ vector conversion fragment *rfA* was inserted in the multiple cloning site which is followed by a nopaline synthase terminator of vector pGREEN0229 *(GW-MCS-NOS-pGREEN)* (Corbesier et al., 2007). To generate the binary destination vectors *GW::FTcDNA, GW::GUS* and *GW::GreenLUC, FT* cDNA (Corbesier et al., 2007), *GUS* coding sequences amplified from pBT10-GUS (Sprenger-Haussels and Weisshaar, 2000) and *GreenLUC* amplified from pCBG68luc vector (Promega) were cloned into the multiple cloning site of the *GW-MCS-NOS-pGREEN* vector. The *GW::FTgDNA* binary destination vector was created as follows. The genomic *FT* sequence was amplified from the BAC clone F5I14 using primers 5'-TCTAGAAAGTCTTCTTCCTCCGCA-3' and

5'-AAGCTTATGTCTATAAATATAAGAGACCCTC-3', the same ones used for generation of *GW::FTcDNA*. The PCR fragment was digested using restriction enzymes *XbaI* and *HindIII* and cloned into the multiple cloning site of the *GW-MCS-NOS-pGREEN* binary vector cut by *SpeI* and *HindIII*. The destination vector *GW::FTE$_1$I$_1$E$_2$:GUS* was generated in three steps (Franziska Turck, MPIZ, Cologne). First, an overlap extension PCR was carried out using the primer pair 5'-AAGCTTAAGATGTCTATAAATATAAGA-3' and 5'-CTTTTCGCGTTT CACCATGGCAGGACTTGGAACATCTGGATC-3' for the amplification of a *FT* fragment, and the primer pair 5'-GCCATGGTGAAACGCGAAAAGAACGTG-3' and 5'-ACTAGTCT CGAGCTAGCCGCCAGCTTTTTCGAG-3' for amplification of the *GreenLUC* gene from pCBG68luc vector (Promega). The fragments were fused in a second PCR step using the outer flanking primers that contained *HindIII*, *SpeI* and *XhoI* recognition sites, respectively. The generated *FTE$_1$I$_1$E$_2$:GreenLUC* fragment was introduced into a TOPO™ pCRII vector (Invitrogen) and the luciferase gene was excised by restricting with *NcoI* and *SpeI*. Ligation with a *GUS* gene amplified from pBT10-GUS (Sprenger-Haussels and Weisshaar, 2000) using 5'-CCATGGTACGTCCTGTAGAAACC-3' and 5'-ACTAGTCTCGAGTTGCAGCAGAAA AGCCGC-3' and digested with *NcoI* and *SpeI,* resulted in the *FTE$_1$I$_1$E$_2$:GUS-pCRII* intermediate. From this vector, *FTE$_1$I$_1$E$_2$:GUS* was excised by restriction digestion with *HindIII* and *SpeI* and introduced to *GW-MCS-NOS-pGREEN* vector that had been prepared using the same restriction endonucleases. The different promoter fusions were made by LR reactions.

Genomic *FT* fragments used for complementation assays were cloned by homologous recombination (Warming et al., 2005). Sequence was sub-cloned from the *FT* containing BAC clone F5I14 into the pGAP-Km vector (Csaba Koncz, MPIZ, Cologne). PCR fragments of 600 bp length (FLANKs) were amplified and inserted into the pGAP-Km as sites for homologous recombination. FLANK1 cleaved with *BamHI* and *SalI,* FLANK2/FLANK3 cleaved with *SalI* and *EcoRI* were ligated to the pGAP-Km vector cut by *BamHI* and *EcoRI*.

FLANK1

J74: 5'-GGGAAGAAGGGATCCGATTACCTCCCAGCACCAAAGAC-3'

J76: 5'-GGGGTGTTGGTCGACCCATCTTCCCACTCCCTTCT-3'

FLANK2

J77a: 5'-GGGAAGAAGGTCGACCGGACAGTCTTGTCAAACCA-3'

J78: 5'-GGGGTGTTGGAATTCGCGCCCATTAAAATAAGTTCC-3'

FLANK3

J79a: 5'-GGGAAGAAGGTCGACGTGCTCTTTGGGTCAGCTTC-3'

J80: 5'-GGGGTGTTGGAATTCAAACTGCCACTTGACCATCA-3'

The FLANK1-FLANK2-pGAP-Km and FLANK1-FLANK3-pGAP-Km vectors were introduced into SW102 cells containing BAC clone F5I14 and recombination was induced. The resulting pGAP-Km vectors contained 12.6 kb and 24.0 kb *FT* genomic fragments encompassing 8.1 kb upstream of the start codon to 2.3 kb and 13.7 kb downstream of the stop codon respectively.

For the transient bombardment assay a green light and a red light emitting luciferase was used. The sequences for the *GreenLUC* and *RedLUC* genes were excised from the Promega vectors pCBG68luc and pCBRluc respectively using the endonucleases *XhoI* and *XbaI* (Branchini et al., 2005). The fragments were cloned into pJAN using the restriction sites *XhoI* and *SpeI*. The *35S::gCO-pBluescript* construct is described in Onouchi et al. (2000).

Plant transformation

All plasmids based on pGREEN0229 were introduced into *Agrobacterium tumefaciens* strain GV3101 (pSOUP), while pGAP-Km constructs generated by homologous recombination were introduced into GV3101 (pPMRK) strain (Koncz and Schell, 1986). Plasmids were transformed into Arabidopsis plants by the floral dip method (Clough and Bent, 1998). For *GUS* expression analysis constructs were introduced into Col wild type and Col plants carrying a functional *FRI* allele from Arabidopsis ecotype San Feliu-2, referred to as *FRI* (Searle et al., 2006). Furthermore, *GUS* constructs were transformed to *35S::CO* Col plants (Onouchi et al., 2000) and *tfl2-1* mutant background (Larsson et al., 1998). The T-DNA insertion line in Col background *ft-10* (Yoo et al., 2005) was used for complementation analysis.

Transgenic lines

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 ½ strength Murashige and Skoog (GM) medium supplemented with 1% sucrose and containing 12 µg/ml Phosphinotricin (PPT). Only lines that showed a segregation ratio between 2:1 and 4:1 for the transgene were tested for *GUS* expression and flowering time. Homozygous lines were identified in the T3 generation.

To obtain *FTp::GUS 35S::CO* double transgenic lines, two lines of each *FTp::GUS* construct in Col background were crossed with *35S::CO* Col plants. In the F2 progeny, seedlings were

identified on the basis of PPT resistance and the early flowering *35S::CO* phenotype. Double homozygous F4 plants were used for final analysis.

Plant growth conditions

Plants were grown in controlled environment at 20°C under LD (16-hours light/ 8-hours dark), ESD (8-hours light/ 8-hours day extension/ 8-hours dark) or SD (8-hours light/ 16-hours dark) conditions after stratification at 4°C for 2-4 days to synchronize germination. Light was provided by fluorescent tubes complemented by incandescent bulbs to increase the proportion of far red light. In ESD conditions the 16-hours light period was provided by fluorescent tubes and incandescent bulbs for 8 hours (ZT 0 to ZT 8) and by incandescent bulbs for the subsequent 8 hours (ZT 8 to ZT 16). Plants on plates were grown on GM medium supplemented with 1% sucrose under LDs (16-hours light/ 8-hours dark).

For vernalization experiments, stratified seeds on soil were kept for 28 days at 4°C in SD conditions and were afterwards transferred to inductive conditions. In the shift experiment, 15-day-old plants were transferred from SDs to ESDs. SD-controls remained continuously in SD conditions.

Flowering time measurement

For flowering time analysis seeds were stratified for three days 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. In case of hemizygous lines, presence of the transgene was verified by PCR. Data are expressed as mean ± SE.

Preliminary flowering time experiments were performed with the T2 generation. After analysis for 3:1 segregation on PPT containing GM medium, 10-day-old seedling were transferred to soil and grown in LD climate controlled glasshouses (20°C).

Histochemical analysis of GUS expression

For GUS staining, seedlings were incubated for 30 minutes in 90% Acetone on ice, rinsed with 50 mM sodium phosphate buffer (pH 7.0) and incubated for 17 hours 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, 0.1% Triton X-100]. After staining, samples were washed with 50 mM sodium phosphate buffer (pH 7.0) and cleared in 70% Ethanol. The GUS histochemical staining was visualized under a light stereomicroscope (MZ 16 FA, Leica).

Preliminary histochemical expression analysis was performed with 10-day-old seedlings of the T2 generation which were grown on PPT containing GM medium for 3:1 segregation analysis.

GUS Activity Measurement

For quantitative measurements, we used a fluorimetric assay based on the GUS substrate MUG (4-methylumbelliferone-β-D-glucuronide), as previously described (Jefferson et al., 1987). 15 10-day-old transgenic *GUS* seedlings grown in LDs were used for each protein extraction. Proteins were extracted in 120 µl extraction buffer containing 50 mM $NaPO_4$ (pH 7.0), 10 mM DTT, 1 mM Na_2EDTA (pH 8.0), 0.1% Sodium Lauryl Sacrisine, 0.1% Triton X-100. Protein concentrations were quantified using a bicinchoninic acid (BCA) assay kit (Pierce). For quantitative analysis of GUS reporter activity 16 µl of protein extract for each sample was incubated at 37°C in 134 µl extraction buffer containing 1 mM MUG. Aliquots of 16 µl were removed after 5, 10, 20, 30, 60, 120, 180, and 270 minutes of incubation and added to 184 µl stop buffer (0.2 M $NaCO_3$) in a 96-well micro plate. The fluorescence was analysed using a micro plate fluorescence reader (Synergy 4, Bio-Tek Instruments Inc., excitation wavelength 365 nm and emmition wavelength 455 nm). Resulting fluorescence was subtracted from background signal detected in extracts made from Col seedling and GUS activity was calculated using a standard curve of MU (4-methylumbelliferone). Values are MU concentration [pM] normalized using amount of protein [mg/ml] and incubation time [min]. Data are represented as mean of different time points ± SE.

mRNA abundance determination

The aerial part of soil-grown plants was collected for mRNA measurement. Total RNA was extracted with the RNeasy™ Mini Kit (Qiagen) and 5 µg RNA was DNase-treated using the DNA-*free*™ kit (Ambion). cDNA synthesis was performed using dT18 primer and the Superscript II reverse transcriptase enzyme (Roche). cDNA was diluted to 150 µl with water, and 2 µl of diluted cDNA was used for quantitative real-time RT-PCR using a BioRad iQ5 apparatus and SYBR Green I detection. A dilution series of a specific plasmid was used as standard for each primer pair and allowed calculation of molar ratios. Actin was used as a control to normalize the varying amounts of cDNA between samples. For the quantification of gene expression the following sets of primers were used:

FT-3'UTR

J59: 5'-CGAGTAACGAACGGTGATGA-3'

J60: 5'-CGCATCACACACTATATAAGTAAAACA-3'

FT-cDNA

J116: 5'-GGTGGAGAAGACCTCAGGAA-3'

J117: 5'-ACCCTGGTGCATACACTGTT-3'

GUS

J90: 5'-TTCGATGCGGTCACTCATTA-3'

J91: 5'-TAGAGCATTACGCTGCGATG-3'

FLC

J114: 5'-ACAAAAGTAGCCGACAAGTCACCT-3'

J115: 5'-GGAAGATTGTCGGAGATTTGTCCA-3'

ACTIN

J112: 5'-GGTGATGGTGTGTCT-3'

J113: 5'-ACTGAGCACAATGTTAC-3'

FTE_1-GUS

J88: 5'-TGGCCAAAGAGAGGTGACTAA-3'

J87: 5'-ACAGTTTTCGCGATCCAGAC-3'

FTI_1-GUS

J89: 5'- TCTGATATTCAAGCCAGCCTTT-3'

J87: 5'-ACAGTTTTCGCGATCCAGAC-3'

FTE_1-FTE_2

J88: 5'-TGGCCAAAGAGAGGTGACTAA-3'

J96: 5'- TGGAGATATTCTCGGAGGTG-3'

FTI_1- FTE_2

J89: 5'- TCTGATATTCAAGCCAGCCTTT-3'

J96: 5'- TGGAGATATTCTCGGAGGTG-3'

Phylogenetic analysis

FT sequences from *Arabidopsis lyrata* were assembled using shot gun sequences available on NCBI (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 (M*B*GP, http://www.brassica.info). *Arabis alpina* sequences were kindly provided by Maria Albani (MPIZ, Cologne). 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 Arabidopsis proximal *FT* promoter sequence 5'-GTGGCTACCAAGTGGGAG AT-3' and 5'- TAACTCGGGTCGGTGAAATC-3' (Franziska Turck, MPIZ, Cologne).

The tool mVista was used to create pair wise alignments of long sequences (http://genome.lbl.gov/vista) (Brudno et al., 2003; Frazer et al., 2004). Sequences of conserved regions were taken and analysed with the multiple alignment tool ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2) (Larkin et al., 2007). PLACE, a database of nucleotide sequence motifs found in plant *cis*-acting regulatory DNA elements was used to analyse highly conserved sequences (http://www.dna.affrc.go.jp/PLACE) (Higo et al., 1999). Weblogos were generated with a tool from the UC Berkeley (http://weblogo.berkeley.edu) (Crooks et al., 2004).

Transformation of Arabidopsis leaves by particle bombardment

For ten bombardments 30 µg of gold (size 1.0 micron) was washed with 1 ml of 70% ethanol for 15 minutes while shaking. The gold-ethanol mixture was spun down for a few seconds in a microcentrifuge and washed three times with sterile water. Finally the gold particles were resuspended in 500 µl of sterile 50% glycerol.

For each bombardment experiment 15 µg of plasmid DNA was used. Gold beads were coated with 5 µg *35S::RedLUC-pJAN*, 5 µg *1kbFTp::GreenLUC-pGREEN* and 5 µg of an empty vector (pKS) or *35S::gCO-pBluescript*. To each DNA-mix 50 µl of the gold-glycerol mix was added under constant shaking, followed by the addition of 50 µl of 2.5 M $CaCl_2$ and 20 µl 0.1 M spermidine. The mixtures were incubated for another three minutes shaking and spun down in a microcentrifuge. After two washes, first with 140 µl of 70% ethanol, second with 140 µl of 100% ethanol, the DNA-gold mixture was resuspended in 50 µl of 100% ethanol. 20 µl of the DNA-gold mixture was used for each bombardment. All bombardments were carried out using the Biolistic™ Particle Delivery System (PDS-1000/HE, BIO-RAD)

5 to 10 mm long Arabidopsis leaves of SD grown Col plants were transformed by particle bombardment using rupture disks that burst at 900 psi. After incubation over night in a LD chamber (Percival), transformed leaves were sprayed with 1 mM Luciferin and luciferase activity was detected using a Hamamatsu photon counting system. Activities of the two different luciferases were measured through different optical filters (LEE Filters; filter #126 “bright red” and filter #139 “primary green”). Luciferase signals at five different spots were quantified using the Hamamatsu photonics device control program HPD-LIS. To calculate the amount of RedLUC activity measured through the green filter and vice versa, one bombardment with *35S::RedLUC-pJAN* and one bombardment with *35S::GreenLUC-pJAN* only were performed. The ratios of signals were calculated by a formula provided from Promega (www.promega.com/chromacalc/Chroma-Luc_Technology_calculator.xls).

4. Results

4.1. *FT* promoter-mediated response to day length

To identify regulatory sequences required for proper expression of the *FT* gene, different promoter fragments fused to *FT* were tested for their ability to complement the *ft* mutant phenotype (Figure 3A). An 8.1 kb promoter fragment (-8095 to -1) covered the entire region from the start codon of *FT* to the next upstream gene *FASCIATA 1 (FAS1)* as well as the first intron and part of the second intron of *FAS1*. *FT* cDNA driven by the 8.1 kb promoter was able to rescue the late flowering phenotype of *ft-10* plants grown under inductive extended short-day (ESD) conditions. Although plants experienced 16 hours of light under this condition, high-intensity fluorescent light was provided during just the first half of the day from zeitgeber time (ZT) 0 to ZT 8. Since high-intensity light is required for photosynthesis, plants grown in ESD were not developmentally advanced when compared with SD grown seedlings. Nonetheless, reduced light quantity in ESD conditions compared to LD conditions did not affect floral transition (data not shown). Under non-inductive SD conditions, *8.1kbFTp::FTcDNA ft-10* lines mainly mimicked the flowering behaviour of wild type plants. Among ten lines segregating 3:1 for the transgene, only one line flowered early under SD conditions (data not shown) which might be explained by a positional effect of the transgene. Constructs driving the *FT* cDNA under control of a 4.0 kb promoter (-3986 to -1) were not able to complement the *ft-10* mutant (Figure 3A). Therefore, an 8.1 kb *FT* promoter region contains all regulatory elements required for day length response in Col plants, whereas a 4.0 kb long promoter sequence is not sufficient.

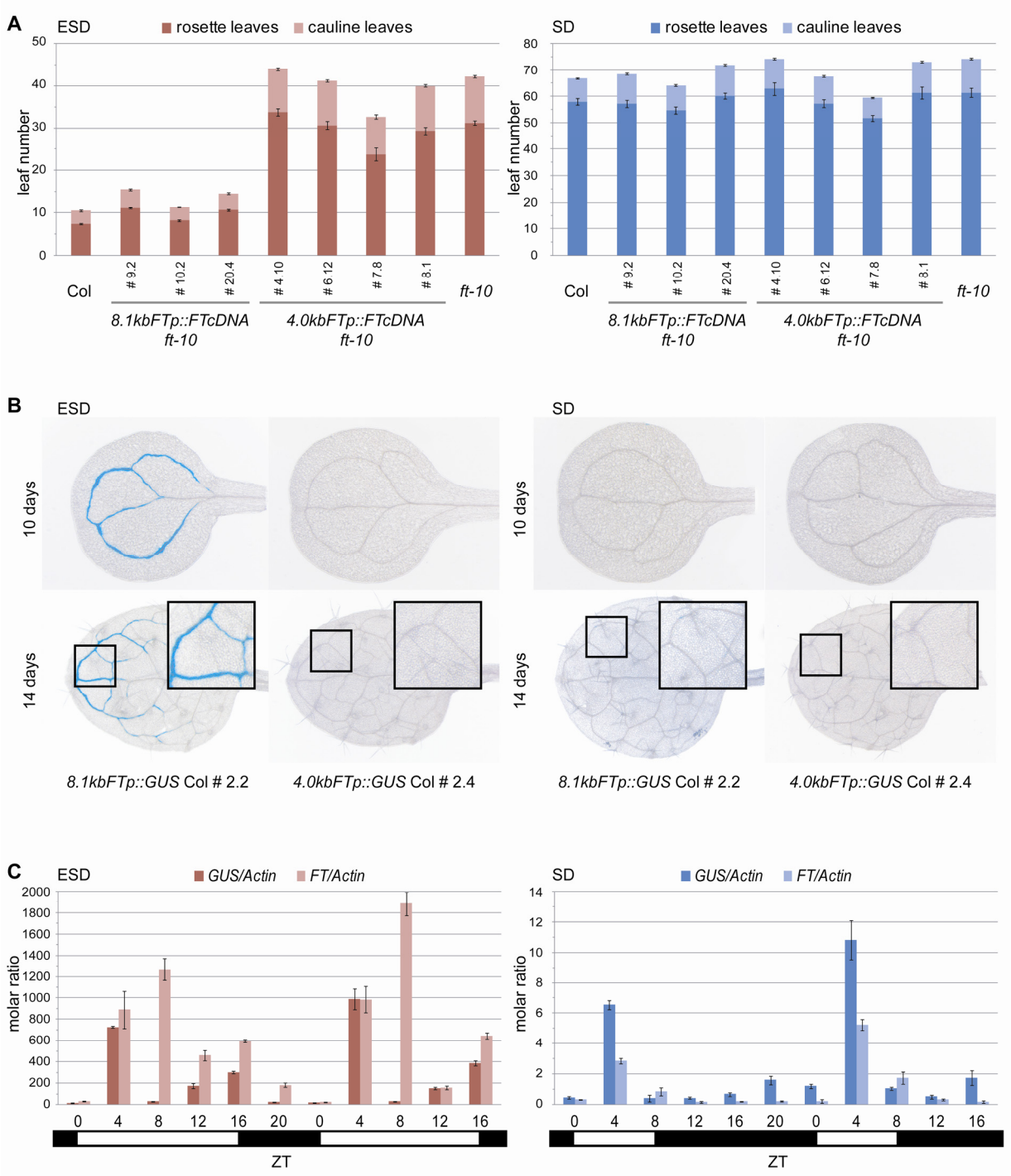

Figure 3. Promoter-mediated response to day length.
(**A**) Flowering time of *ft-10* plants carrying transgenic constructs driving *FT* cDNA by an 8.1 kb and a 4.0 kb *FT* promoter fragment. Wild type plants and *ft-10* mutants were analysed as control. Plants were grown either in ESD or SD conditions. The experiment was repeated three times with similar results. Number of rosette and cauline leaves of a representative example are shown as the mean ± standard error (SE).
(**B**) Histochemical localisation of GUS activity in *8.1kbFTp::GUS* and *4.0kbFTp::GUS* Col plants grown on soil in ESD and SD. Cotyledons from 10-day-old and first true leaves from 14-day-old seedlings are shown. Insert show a higher magnification of an area of the distal half of the leaves.
(**C**) Expression levels of *GUS* mRNA and endogenous *FT* mRNA during day 10 and 11 in *8.1kbFTp::GUS* Col #2.2 seedlings grown in ESD and SD conditions. RNA was sampled every 4 hours at the indicated ZT. Total amount of mRNA was calculated in pmol, normalized by Actin [pmol] and presented as mean ± SE of the molar ratios. Data are based on one experiment. White bars illustrate duration of day, black bars duration of night.

To analyse the spatial expression pattern, a *GUS* reporter gene was expressed in Col plants under control of the 8.1 kb and 4.0 kb *FT* promoter fragments (Figure 3B). Consistent with published results (Takada and Goto, 2003), expression driven by a long *FT* promoter fragment *(8.1kbFTp::GUS)* was observed in the vasculature of cotyledons and leaves of plants grown under inductive photoperiods. Expression in the leaves was restricted to minor veins of the distal half of the leaf. No expression was detected in shoot apical regions, hypocotyls, or roots. While published data showed reduced but clearly detectable *GUS* expression under SD conditions (Takada and Goto, 2003), GUS signal was almost absent in plants grown in SDs. Even in the strongest expressing line (*8.1kbFTp::GUS* Col #2.2) only single stained cells of the vasculature in some leaves were obtained (Figure 3B, magnification). *GUS* expression could not be detected in 10-day-old *4.0kbFTp::GUS* Col plants either in ESD or in SD conditions. Nevertheless, GUS signal could be obtained in 12-day-old *4.0kbFTp::GUS* Col seedlings grown in LDs in the Y junction of the hypocotyl vasculature close to the meristem (Figure 4A). Expression in a few hypocotyl cells below the meristem however did not seem to be sufficient to trigger flowering. *GUS* expression could be obtained at the base of carpels and siliques and along the septum (Figure 4A) which suggests that the *4.0kbFTp::GUS* construct is functional. From microarray studies it is known that *FT* is highly expressed at later developmental stages, especially in siliques and seeds (Figure 4B).

A RNA time course experiment was performed with the *8.1kbFTp::GUS* Col line #2.2 to explore the temporal expression pattern of *GUS* in comparison to the endogenous *FT* gene (Figure 3C). Expression was analysed every four hours at day 10 and 11 of plants grown under inductive and non-inductive conditions. In ESDs both genes, *GUS* and *FT* showed a diurnal expression pattern with the first peak in the morning and a second peak around dusk. The expression pattern and quantity of both genes were similar except at ZT 8. While morning accumulation of *GUS* was restricted to ZT 4, *FT* mRNA was detectable at ZT 4 and ZT 8. In control plants grown under SD conditions, *GUS* and *FT* mRNA remained low. Nevertheless, at ZT 4 a slight accumulation of *GUS* and *FT* transcript could be detected. In summary, a *GUS* reporter gene driven by an 8.1 kb *FT* promoter largely mirrors the temporal expression pattern of *FT* and therefore might contain all the regulatory elements needed to mediate correct temporal expression in response to photoperiod.

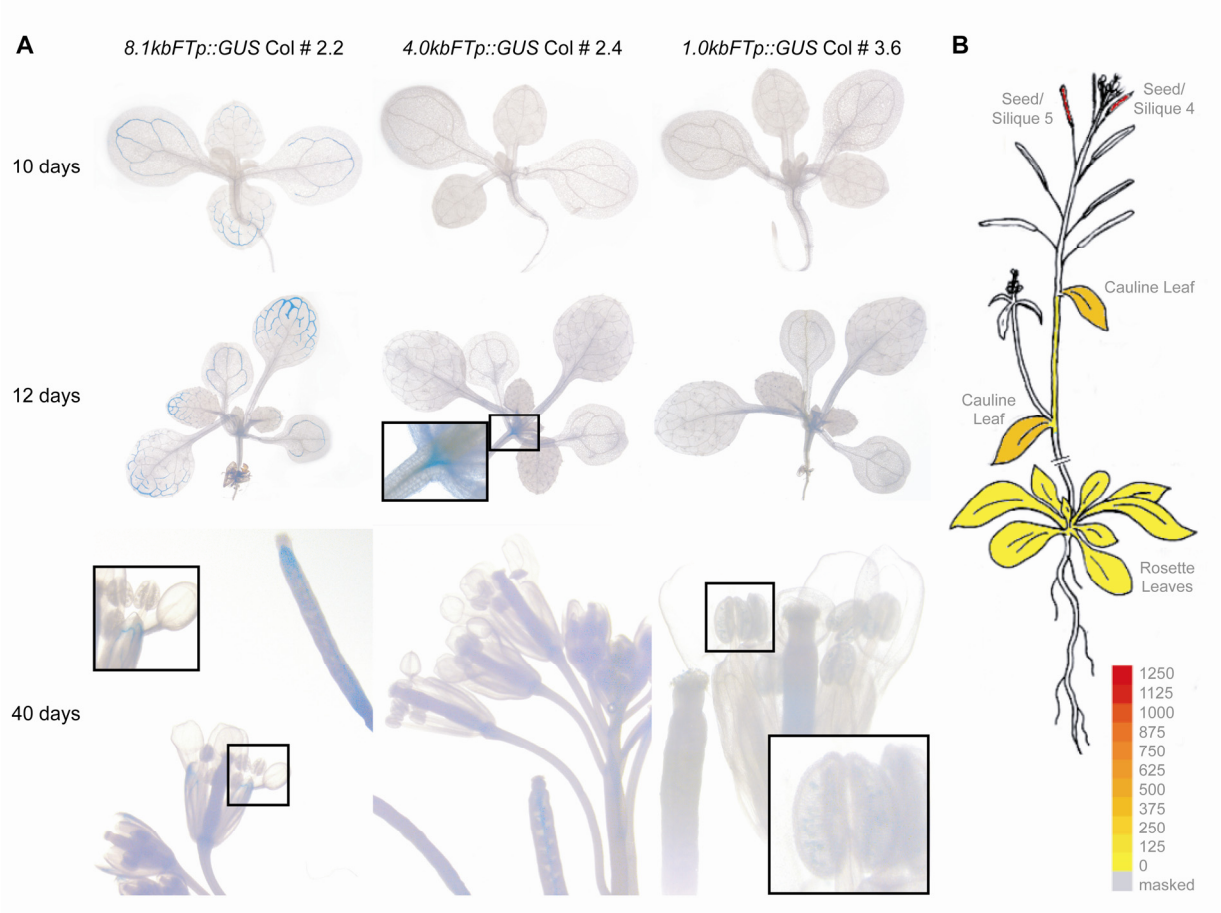

Figure 4. Spatial *FT* expression at different developmental stages.
(**A**) Localisation of GUS activity in *8.1kbFTp::GUS*, *4.0kbFTp::GUS* and *1.0kbFTp::GUS* Col plants of different age grown in LD conditions. 10-days-old seedlings were grown on GM medium, 12- and 40-day-old plants on soil. Inserts show higher magnifications.
(**B**) Arabidopsis eFP Browser view for absolute *FT* expression based on microarray data (Winter et al., 2007). Plant tissues are coloured according to measured expression levels of *FT* after MAS5.0 normalization. The average expression level of each microarray was scaled to 200.

4.2. CO and TFL2 mediated *FT* regulation through different *FT* promoter regions

Since it has been shown that *FT* expression pattern is altered in plants with increased expression of the activator *CO (35S::CO)* and mutants lacking the repressor *TFL2* (Takada and Goto, 2003), the spatial expression pattern of three different *FT* promoter *GUS* reporter constructs was analysed in *35S::CO* and *tfl2* background (Figure 5A). In addition to *8.1kbFTp::GUS* and *4.0kbFTp::GUS*, another construct with a proximal 1.0 kb promoter fragment (-1000 to -1) was used for expression analysis *(1.0kbFTp::GUS)*. No GUS signal could be obtained in wild type seedlings carrying a *GUS* reporter gene under control of the proximal 1.0 kb *FT* promoter (Figure 4A and Figure 5A). Only during flower development did *GUS* expression become visible in pollen (Figure 4A, magnification). Furthermore, a

1.0kbFTp::FTcDNA construct was not able to complement the late flowering phenotype of a *ft-7* mutant (Laurent Corbesier, MPIZ, Cologne, personal communication).

In plants with increased CO levels, *GUS* activity driven by the 8.1 kb *FT* promoter was enhanced around 3-fold (Figure 5B). The expression pattern extended to all major veins of the leaves (Figure 5A). Interestingly, staining was mainly restricted to the phloem tissue in leaves. Positively correlated to the strength of expression of *8.1kbFTp::GUS* in wild type and the age of the plant, GUS signal tended to occur also outside of leaf vasculature in mesophyll cells in *35S::CO* background. In general, *GUS* expression was less restricted to phloem cells in cotyledons and could also be observed at the base of trichomes of leaves and in the mesophyll cells of hypocotyls. GUS staining in the root vasculature only occurred if plants were grown on plates (data not shown). Nonetheless, the tissue specific expression pattern is in contrast to published result that shows that ectopic *CO* expression leads to ubiquitous GUS signal in the whole plant (Takada and Goto, 2003). The *4.0kbFTp::GUS* construct was not inducible by CO (Figure 5A and B). Similar to 12-day-old wild type plants, *GUS* expression was obtained in the Y junction of the vasculature just below the meristem (Figure 4) and in the Y junction of the vasculature of the inflorescence stems in *4.0kbFTp::GUS 35S::CO* plants (data not shown). In *1.0kbFTp::GUS 35S::CO* seedlings GUS signal was rarely detectable (Figure 5A and B). *GUS* expression could be observed in single phloem cells of the leaf, at the base of leaf trichomes (Figure 5A, magnification) and a few mesophyll cells in hypocotyls.

In comparison to wild type plants the expression pattern of *8.1kbFTp::GUS* in *tfl2* mutants was extended to the middle vein as well to minor veins of the proximal part of first true leaves as expected (Takada and Goto, 2003). GUS signal in the major vein and the proximal vascular tissue was also observed in leaves of *4.0kbFTp::GUS tfl2* plants. In contrast, the 1.0 kb *FT* promoter did not become active in leaves of *tfl2* seedlings. *GUS* expression in *1.0kbFTp::GUS tfl2* plants was only observed in the Y junction of the vasculature close to the meristem (data not shown) which was similar to seedlings containing the *4kbFTp::GUS.*

Altogether, the expression pattern of different promoter fragments in different genetic backgrounds demonstrated that more than 4.0 kb upstream sequence of *FT* is required to respond to CO-mediated stimulation of *FT* expression. *FT* expression was still restricted to the vasculature of plants ubiquitously expressing *CO*. One explanation for this is that a factor acting in concert with CO and whose expression is restricted to the vascular tissue, may be required for CO-dependent induction of *FT*. In contrast, TFL2 mediates repression of *FT* through a 4.0 kb *FT* promoter fragment. Lack of this transcriptional repressor leads to expression in the middle vein and the proximal vasculature of the leaf. Nevertheless,

expression in the distal minor veins seems to require promoter elements which mediate CO response. The 1.0 kb proximal promoter fragment could not drive expression in any genetic background tested.

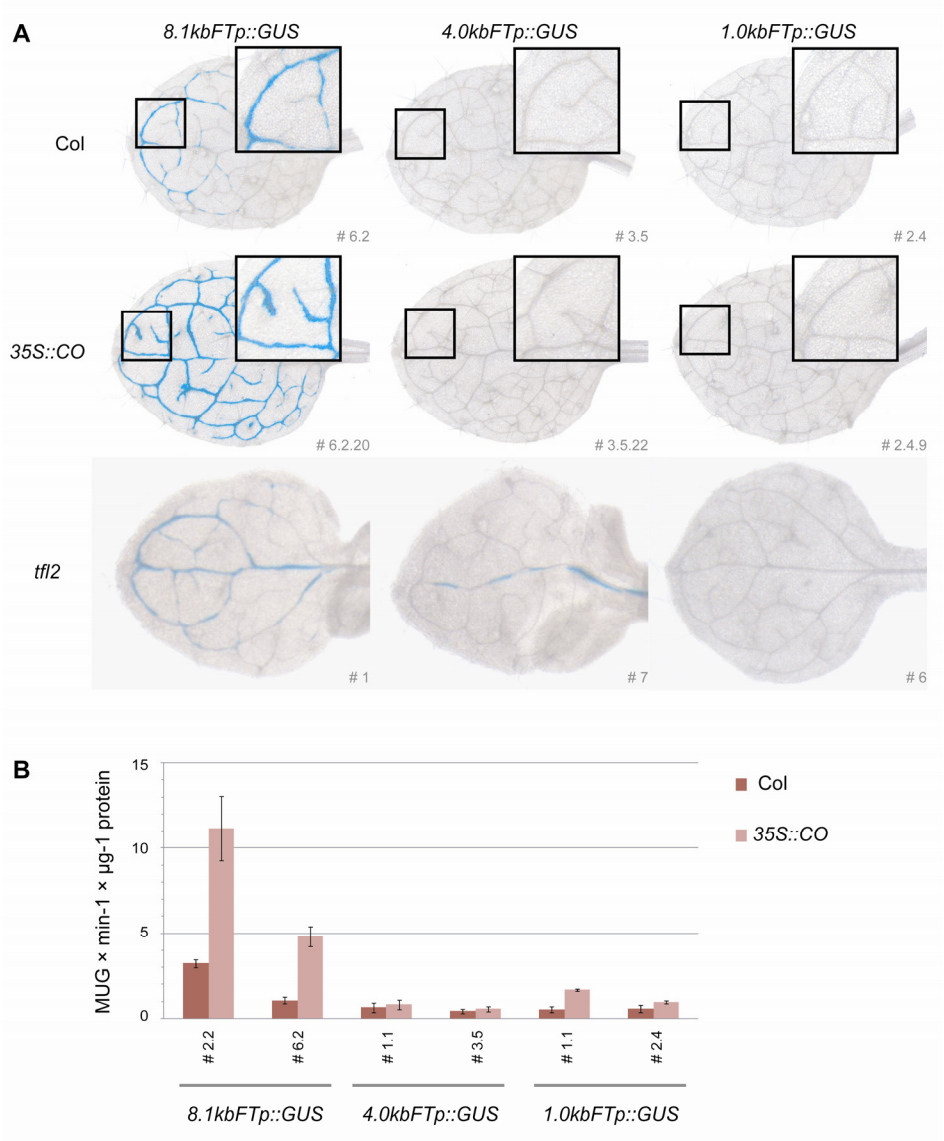

Figure 5. CO and TFL2 mediate *FT* transcriptional regulation through different promoter regions.
(**A**) Spatial *GUS* expression pattern in first true leaves of *8.1kbFTp::GUS, 4.0kbFTp::GUS* and *1.0kbFTp::GUS* plants. Transgenic plants in Col and *35S::CO* background were grown for 10 LDs on soil, while *tfl2* plants were grown for 10 LDs on GM medium.
(**B**) Quantitative *GUS* expression analysis of different *FTp::GUS* constructs in Col and *35S::CO* plants. GUS activity is shown as the mean ± SE of MUG × min^{-1} × μg^{-1} protein based on one experiment. Protein extracts were made from the same plants shown in (A).

4.3. 5.7 kb *FT* promoter sequence is sufficient to drive *FT* expression

To identify the sequence responsible for the drastic difference in expression driven by the 8.1 kb and the 4.0 kb *FT* promoter, three fragments of intermediate length were generated. The resulting *FT* promoters of 7.2 kb (-7201 to -1), 6.7 kb (-6735 to -1) and 5.7 kb (-5722 to -1) in length were applied in complementation and expression analyses. All fragments were able to drive expression similar to the 8.1 kb *FT* promoter. *5.7kbFTp::FTcDNA ft-10* plants flowered like wild type and *8.1kbFTp::FTcDNA ft-10* #9.2 plants under ESD conditions (Figure 6A). Also, the expression pattern between *5.7kbFTp::GUS* and *8.1kbFTp::GUS* in Col and *35S::CO* background did not show any differences (Figure 6B).

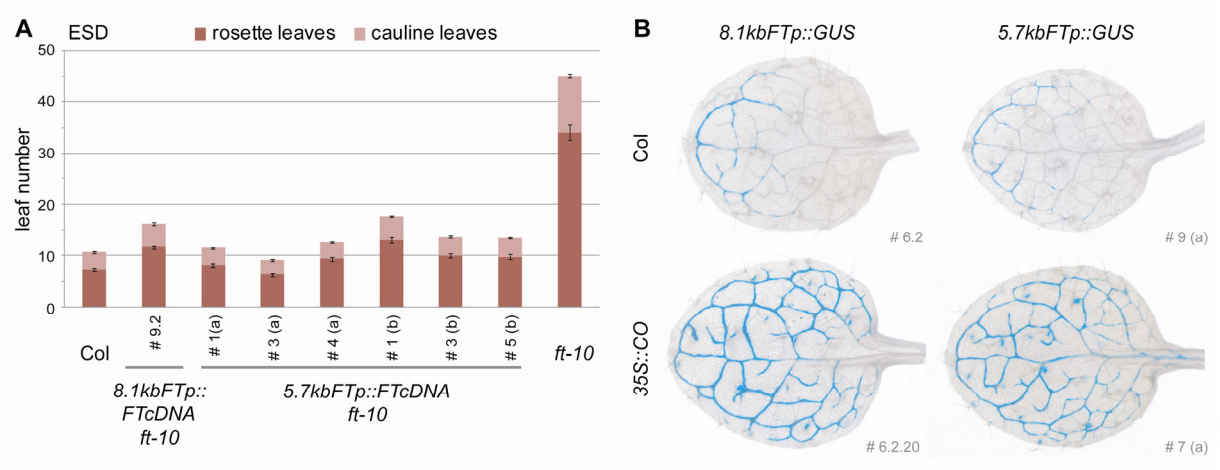

Figure 6. 5.7 kb *FT* promoter sequence is sufficient to drive *FT* expression.
(**A**) Flowering time of *5.7kbFTp::FTcDNA ft-10* plants in comparison to wild type, *8.1kbFTp:: FTcDNA ft-10* #9.2 and *ft-10* plants grown in ESDs. The experiment was repeated twice with similar results. Number of rosette and cauline leaves of a representative example are shown as the mean ± SE. Two different versions of the 5.7 kb *FT* promoter (labelled a and b) were used, as both sequences contain a point mutation at different positions.
(**B**) Histochemical localisation of GUS activity of *8.1kbFTp::GUS* and *5.7kbFTp::GUS* in Col and *35S::CO* plants grown on soil under LD conditions. Expression pattern in the first true leaves of 10-day-old seedlings are shown. Transgenic lines carrying *5.7kbFTp::GUS* in Col and *35S::CO* background are based on independent transformations, while *8.1kbFTp::GUS* lines were generated by crosses.

Since the 5.7 kb sequence upstream of *FT* was sufficient to drive *FT* expression and mediate induction by CO, the 1.7 kb sequence difference between the 5.7 kb and the 4.0 kb *FT* promoter (Figure 7, highlighted in gray) may contain important CO-responsive elements. Interestingly, although the *FT* locus is widely covered with the repressive H3K27me3 chromatin mark (Figure 7B), the region between the 5.7 kb and the 4.0 kb promoter fragments includes a locally H3K27me3 and TFL2 depleted region (Figure 7B, highlighted in violet). Furthermore, alignment of upstream sequences of *FT* homologous genes from *Arabidopsis lyrata* and *Brassica rapa* to *FT* promoters of *Arabidopsis thaliana* accessions Col and Landsberg *erecta* (L*er*) revealed that exactly this region, named block A, is highly conserved

(Figure 7C and D). The conservation of regulatory regions is likely to reflect the constraint to maintain gene regulation during evolution. The underlying model is that mutations have been counter-selected in sites recognized by transcription factors, thereby imposing a slower rate of divergence than in surrounding non-coding sequences. Identification of *cis*-regulatory elements based on conservation of promoter sequences is therefore called phylogenetic shadowing or phylogenetic footprinting. Interestingly, block A contains a conserved CCAAT-box, a binding site for the HAP complex (Figure 7D). 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 A, as well as a REalpha consensus sequence (AACCAA) which has been implicated in regulation by phytochromes (Degenhardt and Tobin, 1996).

Strikingly, only the proximal promoter of around 500 bp length (Figure 13; see below) and some short stretches (block B and C, highlighted in violet) around one to two kilobase pair upstream of *FT* seemed to be conserved between the *Arabidopsis* species, *Brassica rapa* and the promoter of the *Arabis alpina FT1* gene. While sequence alignment of block C did not reveal any regions with three or more conserved nucleotides, Figure 7E shows two highly conserved sequence stretches within block B which contains an E-box binding site for many basic-helix-loop-helix (bHLH) proteins like CIB1.

Figure 7. S*ee following page.*
(**A**) Genome browser view of the *FT* locus on chromosome 1. Exons of *FT* and the flanking gene *FAS1* are represented as dark blue boxes, untranslated regions in light blue. Arrows indicate the direction of transcription. Promoter constructs used for analyses are represented by turquoise boxes.
(**B**) Schematic diagram of the distribution of H3K27me3 chromatin mark and TFL2:HA protein in *35S::TFL2:HA* L*er* plants. ChIP-chip material was generated from 10-day-old seedlings grown on GM medium under LD conditions. Enrichment was calculated as the log2 ratio of ChIP sample versus Input sample (Julia J. Reimer, MPIZ, Cologne, personal communication). Gray areas highlight the region between the 4.0 kb and 5.7 kb *FT* promoter constructs, as well the 500 bp long proximal promoter sequence.
(**C**) Pair wise alignment of *FT* promoter sequences from different species to 7.0 kb *FT* promoter sequence of Arabidopsis Col using mVISTA. Graphical output shows base pair identity in a sliding window of 75 bp in a range of 50% to 100%. Violet areas highlight conserved blocks which were further analysed with ClustalW.
(**D**) ClustalW alignment of a region with high conservation (-5640 bp to -5209 bp), named block A and depicted in violet in (C). Intensity of the colour corresponds with the degree of conservation.
(**E**) Sequence alignment of bock B (-2031 bp to -1794 bp) with ClustalW. Intensity of the colour corresponds with the degree of conservation.

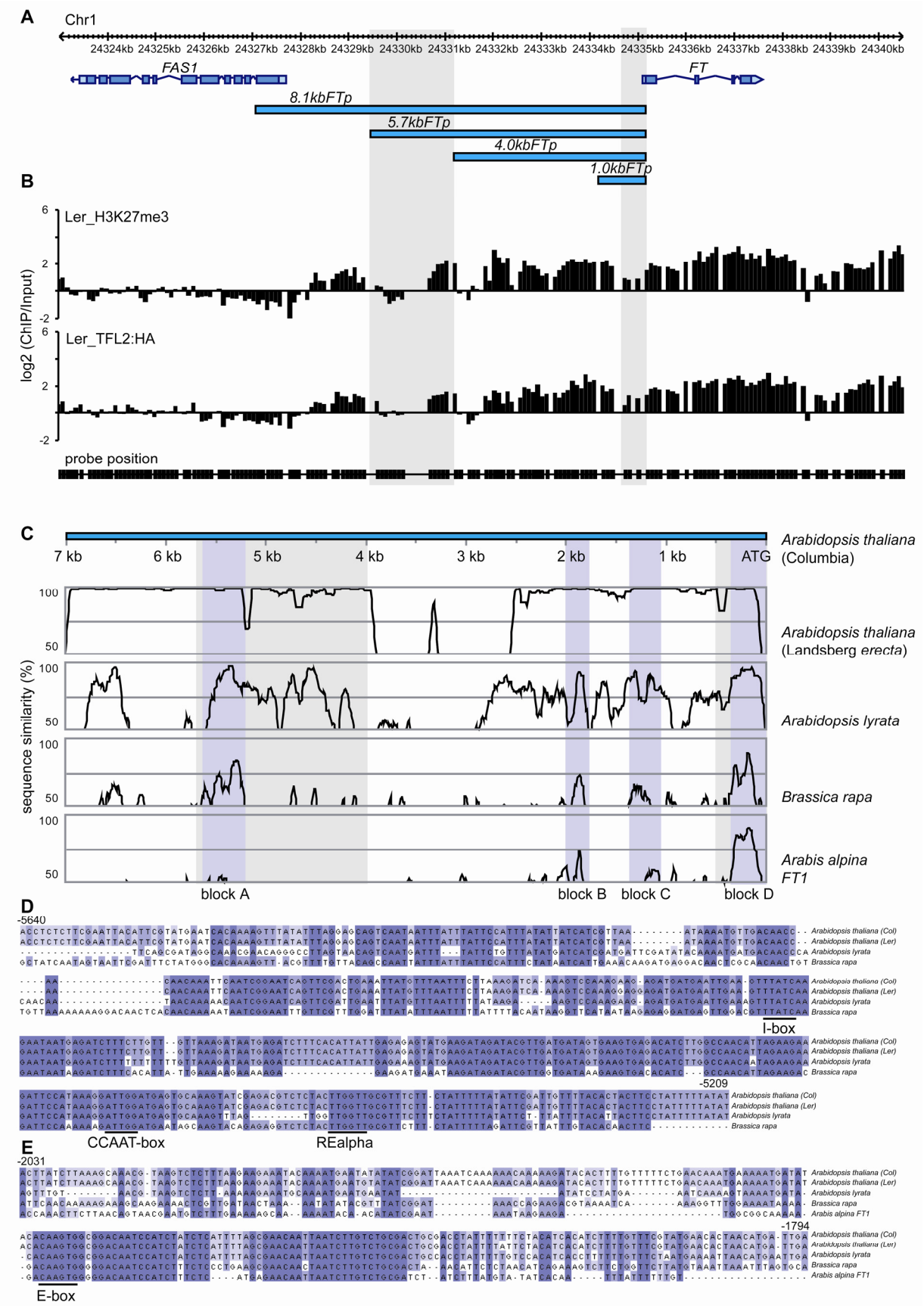

Figure 7. The *FT* locus.

4.4. Impact of T-DNA insertions in *FT* regulatory sequences

Since both conserved sequence regions in the promoter and the chromatin context seem to play a role in *FT* regulation, one may assume that insertions of foreign DNA into *FT* regulatory sequences might influence gene expression. Therefore, flowering time of plants with T-DNA insertions in different regions of the *FT* promoter were analysed (Figure 8). While most lines were not affected in timing of flowering, line #5 (SALK_038707) flowered after forming around 10 leaves more than wild type plants. Line #5 carries a T-DNA insertion around 3550 bp upstream of the start codon. Since the insertion site is not highly conserved (Figure 7C), further work will be directed towards the analysis if the T-DNA has an impact on H3K27me3 distribution and TFL2 binding. Interestingly, line #4 has apparently no effect on flowering although it is inserted in a similar chromatin environment and also separates the proximal promoter from the distal regulative region. The T-DNA flanking sequence on both sides of the insertions and the size of the insertion will be confirmed. It is possible that multiple T-DNA insertions in line #5 affect the spacing of important *cis*-regulatory elements.

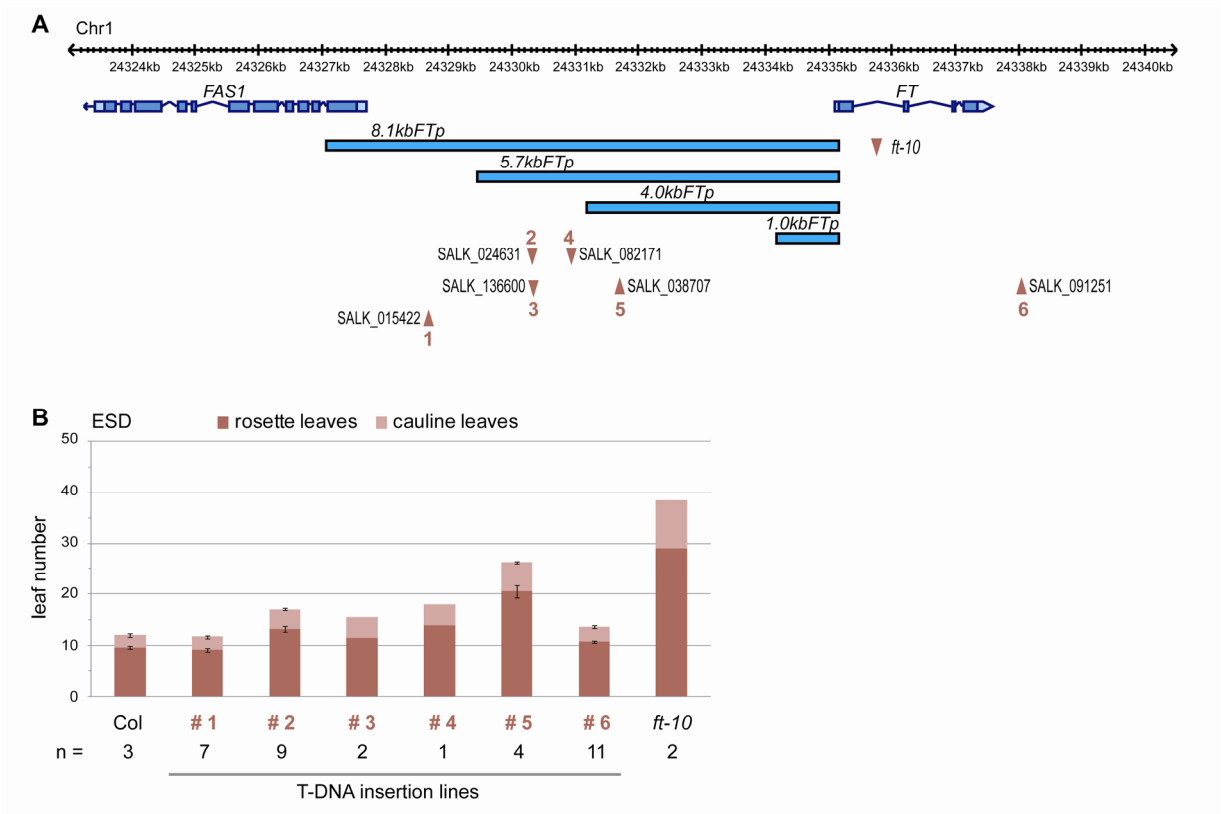

Figure 8. T-DNA insertion lines at the *FT* locus.
(**A**) Schematic diagram of the *FT* locus and the locus of T-DNA insertions in plants from different T-DNA plant collections. Exons of *FT* and the flanking gene *FAS1* are represented as dark blue boxes, untranslated regions in light blue. Arrows indicate the direction of transcription. Promoter constructs used for analyses are represented by turquoise boxes; T-DNA insertions are represented as red triangles.

Figure 8. ***See previous page.***
(**B**) Flowering time of plants carrying a homozygous T-DNA insertion in the *FT* locus. Plants were grown in climate controlled glasshouses under LD conditions. Number of rosette and cauline leaves are depicted as the mean ± SE from a varying number of homozygous lines (n).

4.5. Impact of *FLC* levels on *FT* expression

Based on studies showing that negative regulators like FLC and SVP bind to regions of the intron 1 of *FT* (Helliwell et al., 2006; Searle et al., 2006; Lee et al., 2007), it might be likely, that at least the first intron has regulatory functions. To study the impact of intron 1 on *FT* regulation the proximal part of *FT*, from the ATG to the middle of the second exon, was fused with the *GUS* gene *($FTE_1I_1E_2$:GUS)*. The *$FTE_1I_1E_2$:GUS* fusion under the control of the 8.1 kb *FT* promoter was introduced into low *FLC* expressing Col plants as well into *FRI* plants (Col ecotype) which contain high levels of *FLC* as they contain a functional *FRI* allele introduced from a winter-annual accession (Michaels et al., 2005; Helliwell et al., 2006; Searle et al., 2006). After four weeks of vernalization under non-inductive SD conditions, plants were grown for 10 days in LD conditions. The non-vernalized samples were taken from 10-day-old seedlings grown in LDs without cold treatment. Expression was analysed by histochemical localisation of GUS activity (Figure 9A) and quantitative real-time PCR of *GUS* transcripts (Figure 9B).

As expected, *FLC* levels were low in Col wild type plants, but nonetheless vernalization led to further reduction. *FT* mRNA accumulation was slightly increased in the vernalized sample. This accumulation might be an effect of reduction of *FLC* or because during the vernalization under SD conditions seeds started to germinate and were therefore slightly ahead in development. In two samples (*8.1kbFTp::$FTE_1I_1E_2$:GUS* Col #6 and #7), *FT* was expressed before vernalization, but levels were lower when compared to other Col samples. Expression of *8.1kbFTp::GUS* could be detected in vernalized and non-vernalized Col plants. Since the transgene in the lines used was segregating, the possible impact of vernalization expression was not clear. From the histochemical localisation of GUS, it seemed that expression of *8.1kbFTp::GUS* Col was slightly higher after vernalization which might be again caused by differences in the developmental state (Figure 9A).

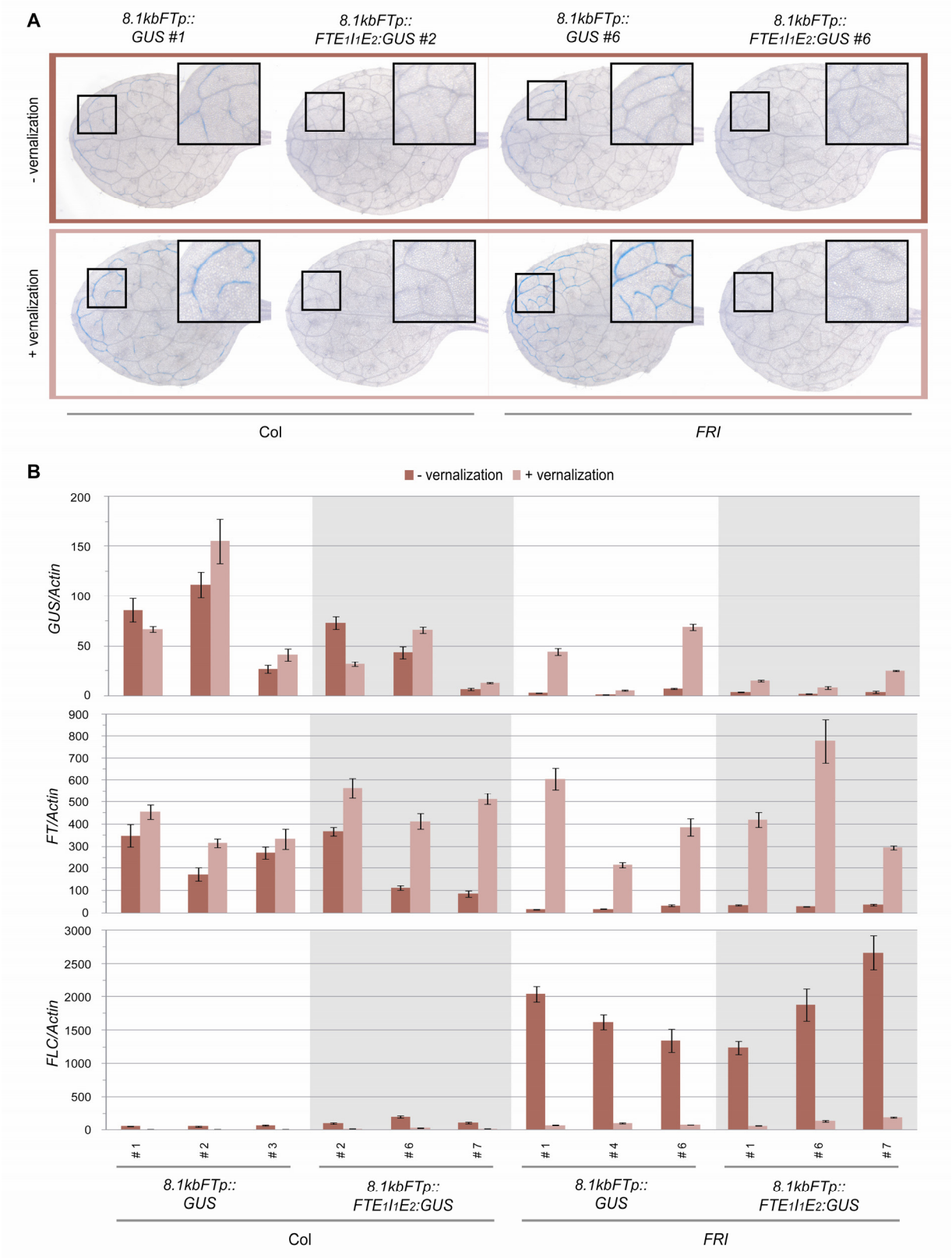

Figure 9. Impact of *FLC* levels on *FT* expression.
(**A**) Whole-mount analyses of *GUS* expression patterns of *8.1kbFTp::GUS* and *8.1kbFTp::$E_1I_1E_2$:GUS* in Col and *FRI* background. Seedlings were grown on soil in LD conditions for 10 days without vernalization or after four weeks of vernalization. Inserts show higher magnifications of areas of the distal half of leaves.
(**B**) Quantitative real-time PCR of *GUS, FT* and *FLC* expression in Col and *FRI* seedlings carrying *8.1kbFTp::GUS* and *8.1kbFTp::$E_1I_1E_2$:GUS* (hemizygous T2 generation). Plant material was harvested at ZT 16 on LD 10 from the same plants shown in (A). Molarity of mRNA [pmol] was calculated and normalized by Actin [pmol]. Error bars represent SE of the mean. Data are based on one experiment.

Strikingly, no GUS signal could be obtained in plants containing *8.1kbFTp::FTE$_1$I$_1$E$_2$:GUS* (Figure 9A), although mRNA analysis revealed that *FTE$_1$I$_1$E$_2$:GUS* was expressed in Col plants in line #2 and #6 (Figure 9B). As the levels of *FTE$_1$I$_1$E$_2$:GUS* expression reached amounts comparable to those obtained in *8.1kbFTp::GUS* Col #1 and #3, GUS activity should have been detectable in the histochemical GUS assay. Further, expression analysis with different primer combinations (Figure 10B) did not reveal any mis-splicing of the *FTE$_1$I$_1$E$_2$:GUS* fusion transcript. Amplification with a primer pair binding to the first exon of *FT* and the *GUS* reporter gene generated a PCR product whose size was consistent with the spliced form of the *FTE$_1$I$_1$E$_2$:GUS* transcript (Figure 10A). Amplification with a primer that anneals in the first intron of *FT* detected unspliced transcript generated by the transgene. However, similar amounts of unspliced transcript were also detected for endogenous *FT* mRNA and therefore inefficient splicing can not explain the discrepancy in GUS activity detection per se (Figure 10A).

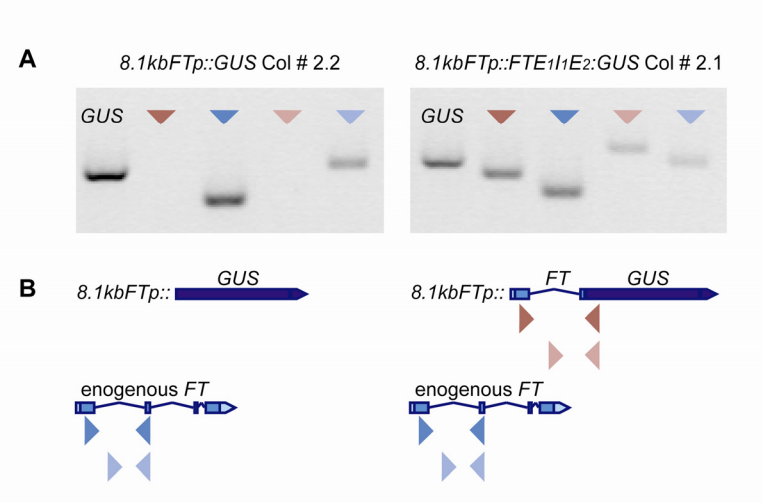

Figure 10. Transcript analysis of *8.1kbFTp::GUS* and *8.1kbFTp::FTE$_1$I$_1$E$_2$:GUS* Col plants.
(**A**) Semi-quantitative RT-PCR (35 cycles) of 10-day old seedlings grown in LD conditions without vernalization. Plant material was harvested at ZT 16. Expression was analysed using a *GUS* specific primer pair and primers depicted in (B).
(**B**) Binding sites of primers used for expression analysis. Red indicated primer pairs bind to transcript of *FTE$_1$I$_1$E$_2$:GUS* fusion while blue colored primer bind to endogenous *FT* mRNA.

While *FLC* expression was high in non-vernalized *FRI* plants, expression levels decreased in vernalized plants and reached levels of non-vernalized Col plants. Conversely, *FT* levels were low before vernalization and high after. The same pattern was detected in *8.1kbFTp::GUS* Col lines #1 and #6; *GUS* expression was low in non-vernalized plants and increased after cold treatment (Figure 9A and B). In contrast, *FTE$_1$I$_1$E$_2$:GUS* expression levels remained low in vernalized plants (Figure 9B). Therefore, expression of both *8.1kbFTp::GUS* and *8.1kbFTp::FTE$_1$I$_1$E$_2$:GUS* was repressed in plants with high levels of *FLC*. Repression could be released in *8.1kbFTp::GUS FRI* plants by vernalization, but cold treatment had just a slight effect on *8.1kbFTp::FTE$_1$I$_1$E$_2$:GUS FRI* plants.

4.6. Regulatory function of intragenic *FT* sequences

In order to identify all important regulatory elements including those located in the intragenic region of *FT*, we cloned a genomic *FT* fragment containing the coding region of the *FT* gene with all introns, but the 3'-downstream sequence was replaced with the nopaline synthase terminator. The *8.1kbFTp::FTgDNA* construct was transformed into *ft-10* mutant plants. Surprisingly, plants containing this transgene could not rescue the late flowering phenotype and flowered like *ft-10* mutants (Figure 11A). Expression analysis via quantitative real-time PCR showed that the genomic transgene was not expressed. In contrast expression of the *FT* cDNA driven by an identical promoter was detected. Even vernalization did not lead to detectable expression (Figure 11B). Re-sequencing of the transgene did not reveal any sequence mistakes. Another reason could be that the genomic transcript is post-transcriptionally silenced *in planta* because repressive elements downstream of *FT* are required to prevent production of an anti-sense transcript.

To test if a factor acting in *trans* is responsible for silencing of the transgene, an *8.1kbFTp::gDNA ft-10* line was crossed to wild type plants. If silencing would be caused in *trans*, one would expect that endogenous functional *FT* from wild type would be silenced as well. Flowering time analysis showed that crossed plants flowered like wild type plants and the control cross of Col with *ft-10* (Figure 11C). Therefore, we can rule out that the transgene is silenced by a factor acting in *trans*. Another explanation might be a missing binding site for a *cis*-acting regulator of *FT*. It could be that repression mediated by the intragenic region has to be overcome by an activator binding to downstream sequences. Since strong and ubiquitous expression of *FT* gDNA by a *35S* and *SUC2* promoter did not lead to at least partial complementation (data not shown), the construct per se is probably not functional. An 12.6 kb genomic fragment (*12.6kbFTgDNA*) spanning the 8.1 kb promoter sequence, the gene coding region, and the 2.3 kb downstream sequence (-8095 to +2297) was cloned via homologous recombination. Since it has been shown that a 11.8 kb genomic fragment of *FT* covering 8.9 kb upstream and 700 bp downstream sequences was able to complement the late flowering phenotype of an *ft-101* mutant (Takada and Goto, 2003), we expect to rescue the late flowering phenotype with that fragment. Nevertheless, a larger construct of 24.0 kb (-8095 to +13731) has been generated and transformed to *ft-10* plants.

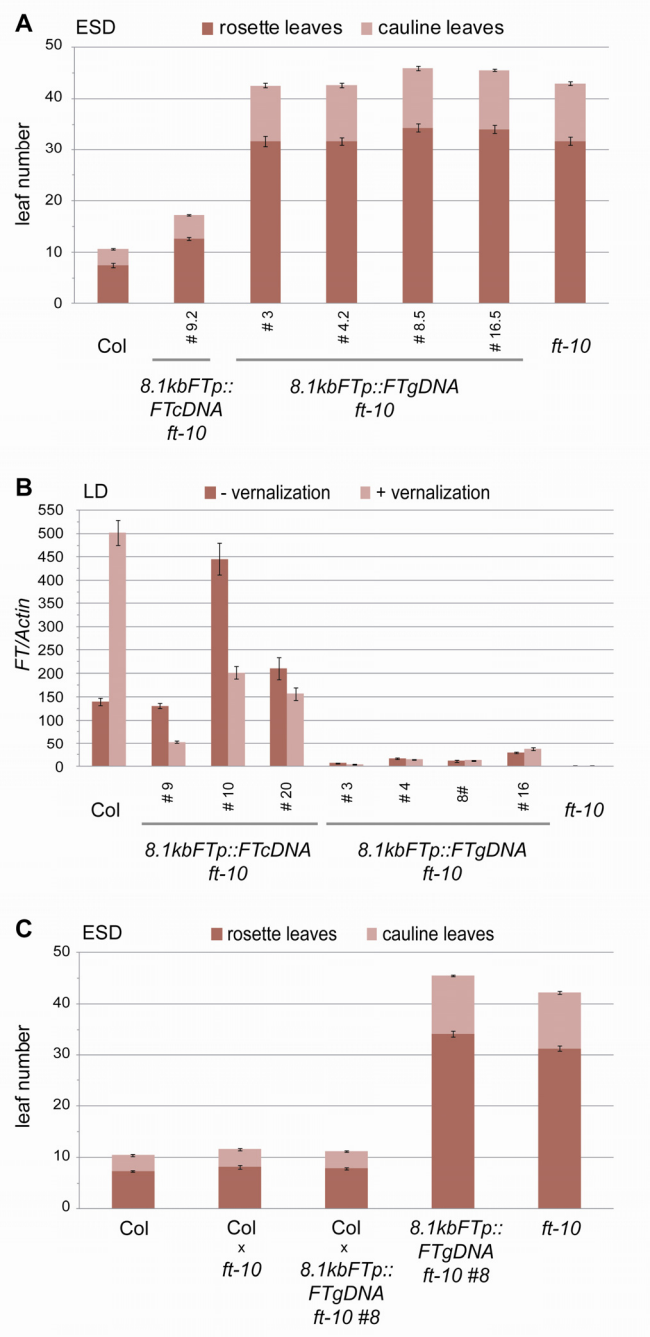

Figure 11. Regulatory function of intragenic *FT* sequences.
(**A**) Flowering time of *ft-10* mutants carrying the *FT* cDNA or the corresponding genomic sequence of *FT (FTgDNA)* under control of an 8.1 kb *FT* promoter fragment. Wild type plants and *ft-10* mutants were analysed as control. Plants were grown on soil under ESD conditions. The experiment was repeated three times with similar results. Number of rosette and cauline leaves of a representative example are shown as the mean ± SE.
(**B**) Quantitative *FT* expression in *8.1kbFTp::FTcDNA ft-10* and *8.1kbFTp::FTgDNA ft-10* plants at ZT 16 on day 10. Seedlings were grown on soil in LD conditions without vernalization or after four weeks of vernalization. Total amount of mRNA was calculated in pmol, normalized by Actin [pmol] and presented as mean ± SE. Data are based on one experiment.
(**C**) Flowering time of F1 plants from a cross of *8.1kbFTp::FTgDNA ft-10* to Col and *ft-10* to Col wild type. For comparison wild type plants, a hemizygous *8.1kbFTp::FTgDNA ft-10* line and *ft-10* mutants were analysed as well. Plants were grown on soil under ESD conditions. The experiment was performed once. Number of rosette and cauline leaves are shown as the mean ± SE.

4.7. Identification of putative *cis*-acting elements in the proximal *FT* promoter

The promoter region close to the transcription start site of *FT* is highly conserved between different *Brassicacea* plants (Figure 12A). Prediction of putative *cis*-regulatory elements by phylogenetic shadowing was utilized for the proximal *FT* promoter. A region of around 360 bp upstream of the start codon showed an especially high conservation and was named block D (Figure 12A, highlighted in violet). The alignment of the promoter sequences revealed highly conserved short stretches which did not contain any known transcription factor binding site

(Figure 12B). Blocks of 5-15 bp length were identified on the basis of previous alignments and called shadow 1, 2, 3 and 4 *(S1-S4)* (Figure 12B). Two palindromic sequences flanking *S3* were named *P1* and *P2*. To analyse their importance we generated *FT* promoter constructs with point mutations in these elements (Figure 14A).

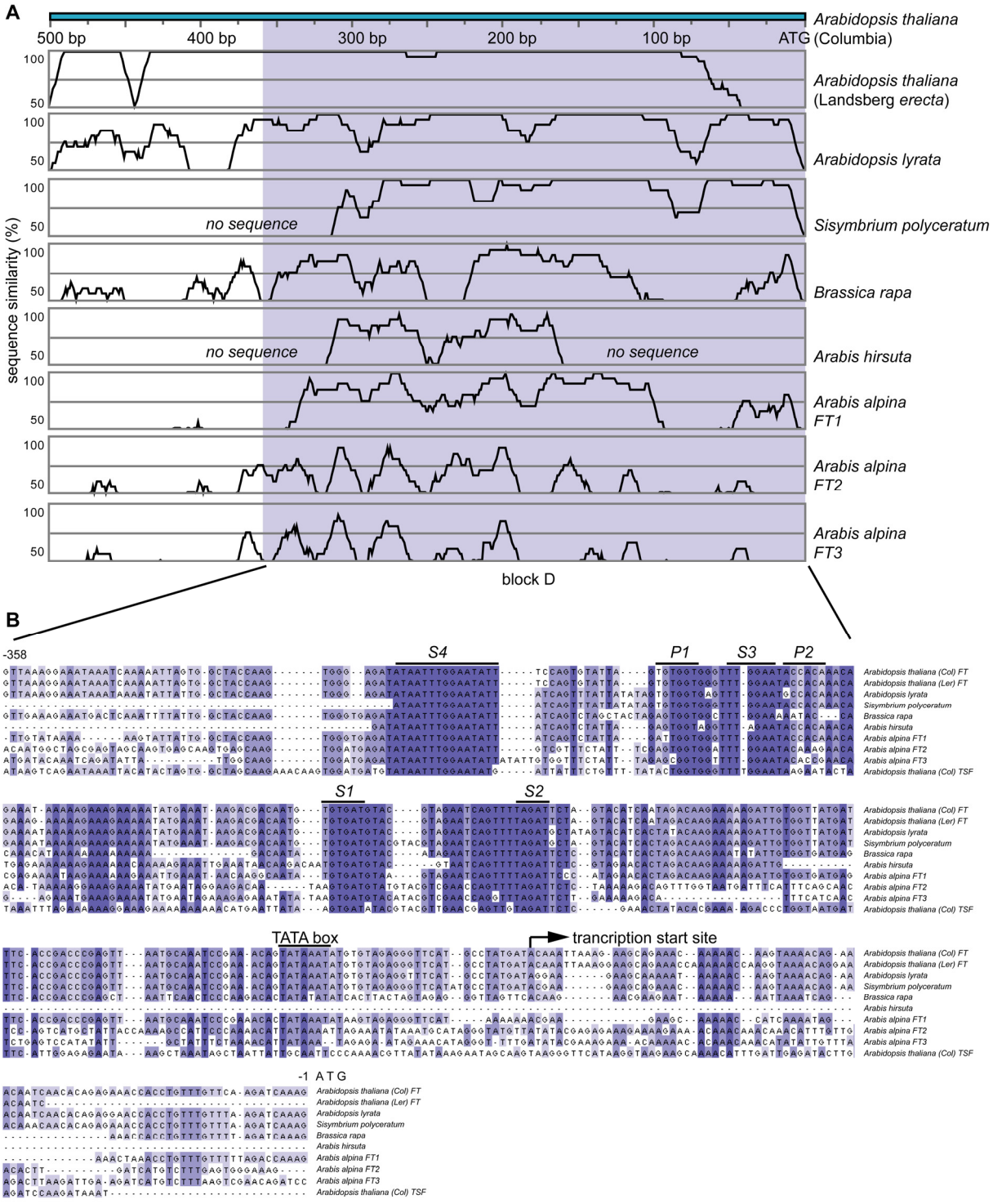

Figure 12. Identification of putative *cis*-regulatory elements in the proximal *FT* promoter.

Figure 12. S*ee previous page.*
(**A**) Pair wise alignment of *FT* promoter sequences from different species to a 500 bp *FT* promoter sequence of Arabidopsis Col using mVISTA. Graphical output shows base pair identity in a sliding window of 20 bp in a range of 50% to 100% identity. Violet area highlights block D, a region which was used for ClustalW alignment shown in (B).
(**B**) ClustalW alignment of the proximal *FT* promoter (-358 bp to -1 bp from the ATG, block D). Intensity of the colour corresponds with the degree of conservation. Based on previous alignments four conserved blocks were identified and called shadow 1 to 4 *(S1-S4)*. A palindromic sequence flanking *S3* is labelled with *P1* and *P2*. Furthermore, the putative TATA-box and the transcription start side are indicated.

To get a preliminary idea if the identified conserved elements might be involved in *FT* regulation and especially mediation of CO induction, we tested their role in a transient dual-luciferase reporter assay. Arabidopsis leaves were co-bombarded with two different forms of a firefly luciferase. A red light-emitting luciferase *(RedLUC)* driven by a *35S* promoter was used as an internal standard for bombardment efficiency while the green light-emitting luciferase gene *(GreenLUC)* was used to assess *FT* promoter activity. The activities of the enzymes were measured with the same *in vivo* substrate but through different optical filters. The relative specificity was calculated by dividing the activity of the green light-emitting luciferase by the red light emitting-luciferase activity. To prove if one can measure a reduction in promoter activity with the assay, we mixed dilutions of *35S::GreenLUC* plasmid with constant amount of control plasmid *(35S::RedLUC)* (Figure 13). The linear slope of GreenLUC activity showed that the transient dual-luciferase reporter system is suitable to quantify promoter activities.

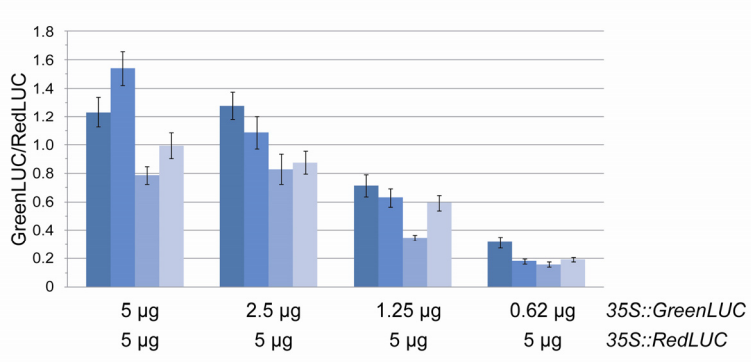

Figure 13. Transient dual-luciferase reporter assay.
Col leaves of plants grown in SD were bombarded with different amounts of *35S::GreenLUC* and a constant amount of *35S::RedLUC* constructs. Light emission of the green and red light emitting luciferases was measured through corresponding filters. Light emission of GreenLUC was normalized with light emission of RedLUC. Data from four independent experiments are shown as the mean ± SE.

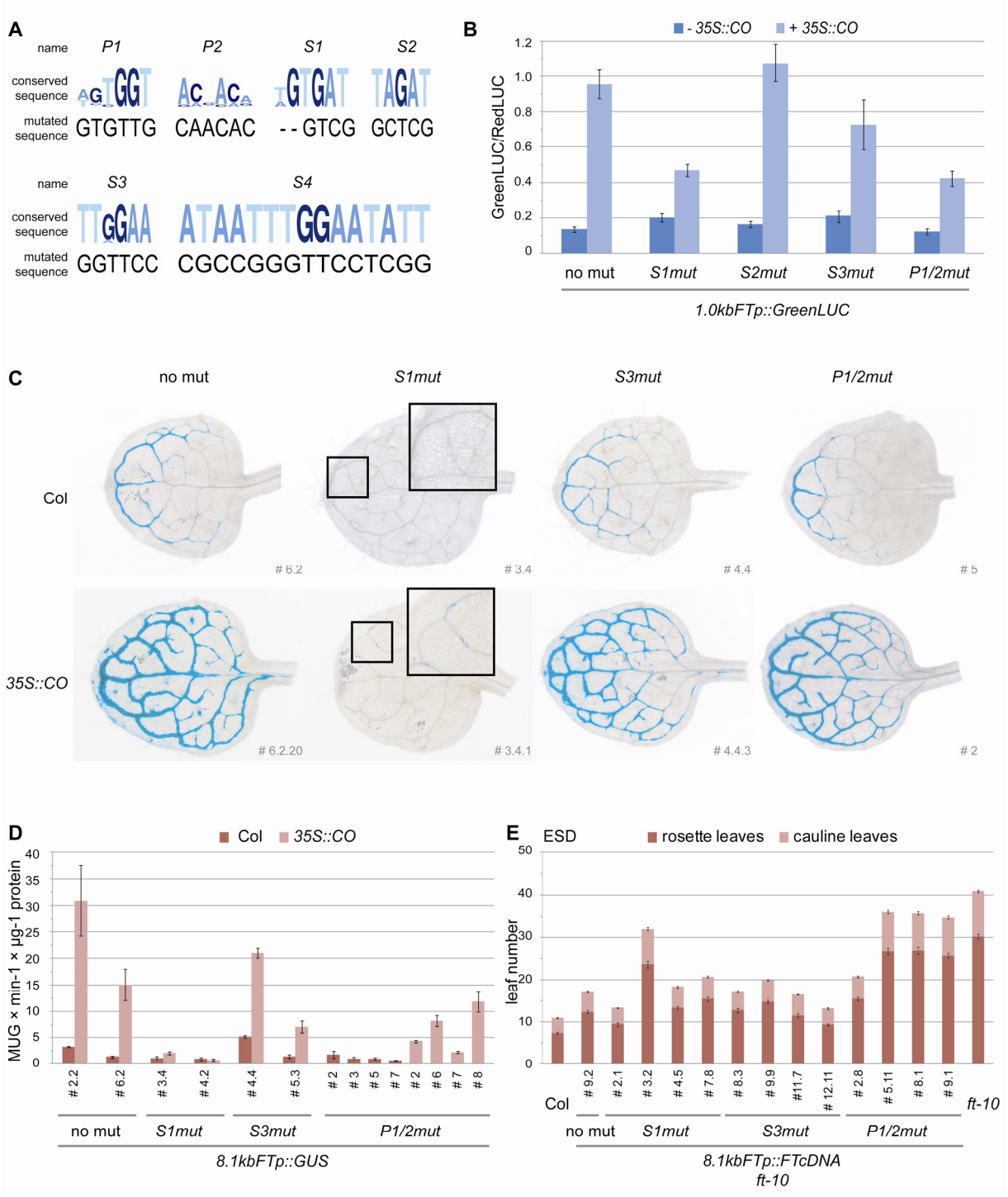

Figure 14. Analyses of putative *cis* elements in the proximal *FT* promoter.
(**A**) Name and sequence of analysed putative *cis* elements. Degree of conservation is visualized by WebLogo. Sequence changes made in mutated promoter versions are shown below the original sequence.
(**B**) *1.0kbFTp::GreenLUC* constructs carrying mutations in the different putative *cis* elements of the promoter were used for a transient expression assay. Resulting light emission was normalized to light emission of a co-bombarded RedLUC. CO-dependent transcriptional activation was analysed by co-bombardment of *35S::CO*. Mean ± SE is based on at least three independent experiments.
(**C**) Spatial *GUS* expression pattern in first true leaves of Col and *35S::CO* plants carrying mutated versions of the *8.1kbFTp::GUS* construct. Transgenic lines carrying *8.1kbFTp-P1/P2mut::GUS* in Col and *35S::CO* background are based on independent transformations, while other lines were generated by crosses. Transgenic plants were grown for 10 days on GM medium under LD conditions.

Figure 14. S*ee previous page.*
(**D**) Quantitative *GUS* expression analysis of *8.1kbFTp::GUS* constructs with different mutations in Col and *35S::CO* plants. GUS activity is shown as the mean ± SE of MUG × min^{-1} × μg^{-1} protein based on one experiment. Protein extracts were made from the same plants shown in (C).
(**E**) Flowering time of *ft-10* plants carrying transgenic constructs driving *FT* cDNA by mutated versions of the 8.1 kb *FT* promoter fragment. Wild type plants and *ft-10* mutants were analysed as control. Plants were grown in ESD conditions. The experiment was repeated two times with similar results. Number of rosette and cauline leaves of a representative example are shown as the mean ± SE.

Although we know from complementation studies that only *FT* promoter fragments of 5.7 kb or longer are able to drive proper *FT* expression, no difference in luciferase activity was detectable in leaves bombarded with a luciferase gene under control of an 8.1 kb or 1.0 kb *FT* promoter fragment or constructs of intermediate length (Franziska Turck, MPIZ, Cologne, personal communication). For further analysis a *1.0kbFTp::GreenLUC* construct was applied in the transient dual-luciferase reporter assay (Figure 14B). Arabidopsis leaves of SD grown Col plants bombarded with *1.0kbFTp::GreenLUC* gave a weak signal, which increased around 6-fold upon co-bombardment with *35S::CO*. Introduction of point mutations in conserved elements of the 1.0 kb *FT* promoter generally did not alter GreenLUC activity, whereas the enhanced response with CO was reduced in some cases. Compared to the original sequence promoter, mutations in *S1* and in the two palindromic sequences *P1/P2* reduced GreenLUC signal in response to CO induction by 2-fold. Presence of *S3* might have a slight impact on CO-mediated stimulation, while mutations in *S2* did not affect GreenLUC activity. Analysis of *S4* is in progress. Although the transient bombardment assay only allows analysis of a subset of the complete *FT* regulation, the assay reveals which conserved blocks of the proximal *FT* promoter might be crucial for CO-mediated stimulation.

To study the biological relevance of *S1, S3* and *P1/P2* in the proper expression context, the mutations were introduced to the 8.1kb *FT* promoter fragment and applied in spatial expression and complementation analyses using stable transformation (Figure 14C, D and E). In plants carrying the *8.1kbFTp-S3mut::GUS* construct, no difference in GUS staining could be observed compared to plants carrying the non-mutated construct either in Col or in *35S::CO* background (Figure 14C and D). Furthermore, introducing mutations in *P1/P2* did not affect the spatial expression pattern and measurement of GUS activity in these plants revealed similar levels of expression compared to the *8.1kbFTp::GUS* control. In contrast, no staining could be obtained in 10-day-old *8.1kbFTp-S1mut::GUS* Col plants (Figure 14C) and only a few cells of the distal leaf vasculature in 12-day-old seedlings showed staining (data not shown). In *35S::CO* background little *GUS* expression was detectable in the distal vasculature of 10-day-old seedlings (Figure 12C). Levels of GUS activity in Col plants were similar to

those of *8.1kbFTp::GUS* Col plants, but expression was not stimulated in *35S::CO* background.

For flowering time analyses, *ft-10* plants were transformed with *8.1kbFTp::FTcDNA* constructs containing the different mutations (Figure 14E). As expected, mutations in *S3* did not have an impact on flowering time. Although expression seemed to be decreased, *8.1kbFTp-S1mut::cDNA ft-10* plants flowered similar to *8.1kbFTp::cDNA ft-10* #9.2 control. Among eight lines tested in total, only two line flowered slightly later. Surprisingly, out of five lines four *8.1kbFTp-P1/P2mut::cDNA ft-10* lines did not complement the late flowering phenotype.

4.8. Response mediated by *FT* and *TSF* promoter regions

TSF responds to CO induction followed by a temporal expression pattern which is very similar to *FT* (Yamaguchi et al., 2005). Nevertheless, the genomic loci of the two genes are very different. While the distance from *FT* to the next upstream gene *FAS1* is unusually large for an Arabidopsis gene, the intergenic region at the *TSF* locus is only 1.5 kb long and therefore more similar to that of a typical Arabidopsis gene (Figure 15A) (The Arabidopsis Genome Initiative, 2000). Since most of the conserved elements observed in the proximal *FT* promoter are also present in the *TSF* promoter (Figure 12B), it might be that day-length responsive elements are present in upstream regulatory sequences of *TSF*. Therefore, we tested if a 1.5 kb *TSF* promoter (-1500 to -1) is sufficient to drive *FT* cDNA expression and complement the *ft-10* mutant phenotype (Figure 15B). Flowering time analysis showed that the *TSF* promoter was not able to express *FT* cDNA sufficiently to complement the late flowering phenotype. Only in one out of thirteen 3:1 segregating lines was flowering accelerated compared to *ft-10*, but the plants flowered still later than wild type.

Among eight Col plants containing a *1.5kbTSFp::GUS* transgene, GUS activity could only be detected in three lines. Expression was visible at the base of petioles close to the meristem, but no expression could be detected in the vasculature of the plants. A shift of 15-day-old seedlings from non-inductive SDs to inductive ESD conditions induced transcription of constructs driven by the 8.1 kb *FT* promoter, but no signal could be detected in leaves of *1.5kbTSFp::GUS* Col (Figure 15C). Since GUS signal in the mesophyll cells of the petiole could be observed under non-inductive SD conditions and in inductive ESDs, day-length responsive expression driven by the 1.5 kb *TSF* promoter could not be detected. In conclusion, the promoter sequence alone was not sufficient to drive expression above the detection limit and to confer a response to photoperiod.

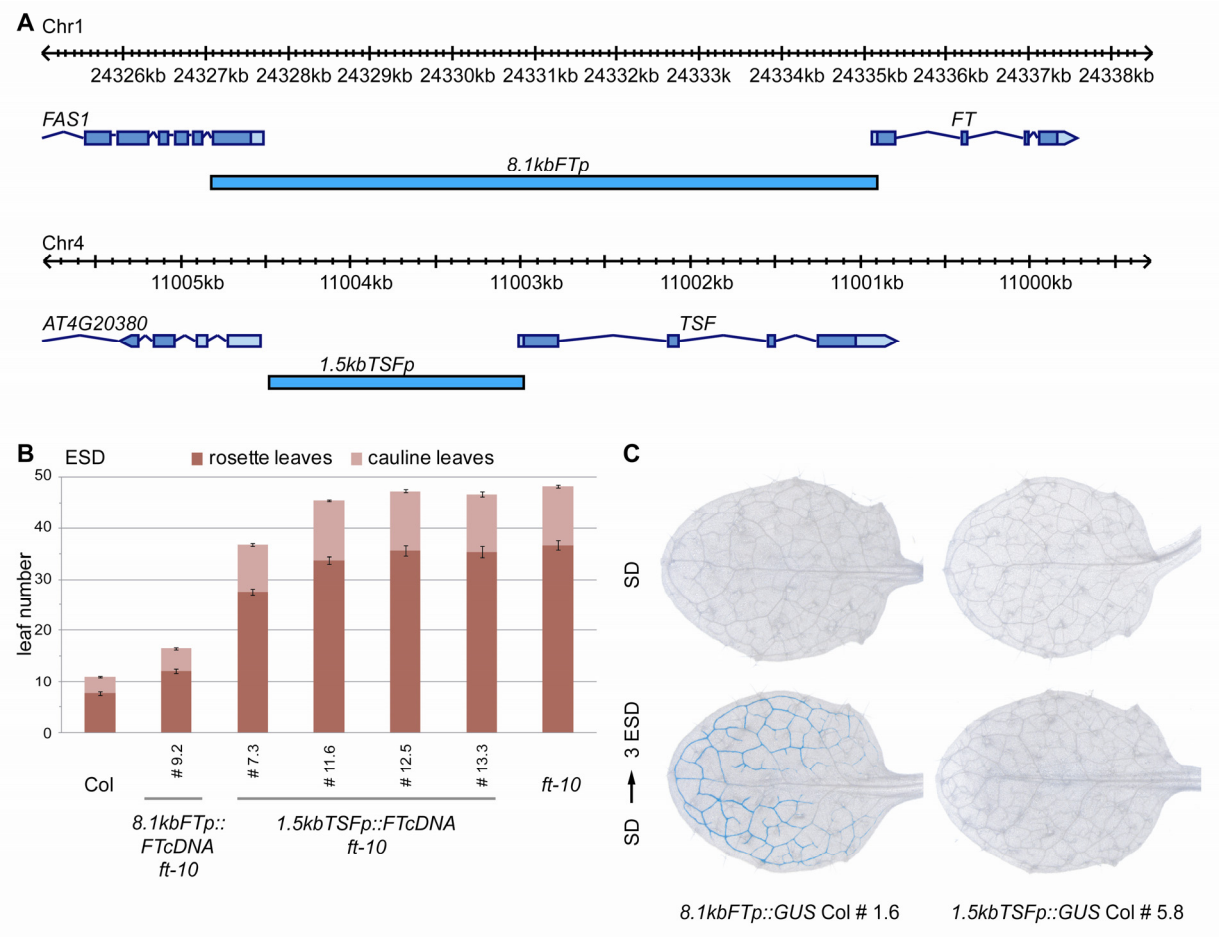

Figure 15. *TSF* promoter regions in comparison to *FT*.
(**A**) Schematic diagram of the *TSF* locus in comparison to *FT*. Exons of *TSF, FT* and their flanking genes are represented as dark blue boxes, untranslated regions in light blue. Arrows indicate the direction of transcription. Promoter constructs used for analyses are represented by turquoise boxes.
(**B**) Flowering time of *ft-10* mutants carrying the *FT* cDNA under control of a 1.5 kb *TSF* promoter fragment. Wild type plants, one *8.1kbFTp::FTcDNA ft-10* line and *ft-10* mutants were analysed as control. Plants were grown on soil under ESD conditions. The experiment was repeated twice with similar results. Number of rosette and cauline leaves of a representative example are shown as the mean ± SE.
(**C**) Histochemical localisation of GUS activity of *8.1kbFTp::GUS* and *1.5kbTSFp::GUS* in Col background. Grown on soil for 15 days under SD conditions, plants were shifted to ESD for 3 days while control plants remained in SDs. The experiment was done two times with similar results.

5. Discussion

5.1. *FT* expression in response to day length

Complementation experiments demonstrated that an 8.1 kb *FT* promoter fused to the *FT* cDNA was able to mediate response to day length. Transgenic *8.1kbFTp::FTcDNA ft-10* plants flowered like wild type plants under inductive and non-inductive conditions. Furthermore, expression of a *GUS* reporter gene driven by an 8.1 kb *FT* promoter followed the temporal pattern of the endogenous *FT* gene in Col background. In plants grown under inductive ESD conditions both genes showed a diurnal expression pattern with a peak of expression in the morning and in the evening. Calculation of absolute transcript levels revealed that the *GUS* transgene and *FT* were expressed at almost equal molecules per cell and differences only were observed at ZT 8. While *GUS* mRNA accumulation reached a maximum at ZT 4, transcription of *FT* was highest at ZT 8. Morning expression of *FT* can be explained by light quality affecting *CO* levels. Far-red light has been shown to cause *CO* transcript accumulation at the beginning of the day and to enhance CO protein stability independent of transcription (Valverde et al., 2004; Kim et al., 2008). In the study of Kim et al. (2008), *FT* mRNA accumulated in the morning and in the evening of plants grown under far-red enriched light (FREL) while the *FT* gene was only transcribed in the second half of the day in plants grown under white light. In our experiments light provided by florescent tubes was supplemented with light from incandescent bulbs which enrich the proportion of far-red light. Therefore, strong morning expression of *FT* might be a result of enhanced accumulation of *CO* mRNA in the morning going along with enhanced protein stability in FREL. Although morning expression might due to CO protein abundance, it could also be mediated by another LD-induced factor.

As expected *GUS* and *FT* expression was almost not detectable in SD grown plants. Interestingly, very low expression levels could be obtained in the morning at ZT 4. It seems that signals to induce the *FT* promoter are present during this time of the day but need to be enhanced in the second half of LDs to drive expression at higher levels on the following day. Since it has been demonstrated that COP1 and the SPA proteins mediate CO protein degradation efficiently during the night, it is unlikely, that the morning expression is a result of residual CO protein from the previous day (Laubinger et al., 2006; Jang et al., 2008). Nonetheless, CO abundance in the evening might cause changes on the *FT* locus which make the regulatory sequence more susceptible for expression on the following morning.

Histochemical localisation of the *GUS* reporter gene under control of an 8.1 kb *FT* promoter revealed expression in the vasculature of cotyledons and leaves of plants grown under inductive photoperiods. GUS signal was restricted to minor veins of the distal half of the leaf, while no expression was detected in shoot apical regions, hypocotyls, or roots. The expression pattern driven by an 8.1 kb *FT* promoter is consistent with published results which are based on an 8.9 kb *FT* promoter construct and a genomic fragment covering 7.2 kb upstream and 1.5 kb downstream of *FT* (Takada and Goto, 2003; Notaguchi et al., 2008). Under non-inductive SD conditions *GUS* expression in *8.1kbFTp::GUS* Col seedlings was almost absent. Staining could be detected in just some single phloem cells in a few leaves. In contrast the published *8.9kbFTp::GUS* line showed reduced but clearly detectable *GUS* expression under SD conditions (Takada and Goto, 2003). Since we demonstrated that the transgenic line *8.1kbFTp::GUS* Col #2.2 expressed *GUS* in the same quantitative range and the same temporal pattern as the endogenous *FT* gene, we believe that spatial expression of this line mimics the actual *FT* expression pattern accurately. Expression of a *FTp::GUS* line in SDs might be due to a position effect of the transgene, as one out of ten *8.1kbFTp::FTcDNA ft-10* complementation lines flowered early under non-inductive conditions as well.

In plants with increased *CO* levels expression of the *GUS* reporter gene under control of the 8.1 kb *FT* promoter was extended to all major veins of the leaves. Although GUS signal tended to occur outside the vasculature in the strongest lines and was also observed at the base of trichomes of leaves and in the mesophyll cells of hypocotyls, the expression pattern is clearly in contrast to published results that show that ectopic *CO* expression leads to ubiquitous GUS signal in *8.9kbFTp::GUS* plants (Takada and Goto, 2003). On the other hand the same *8.9kbFTp::GUS 35S::CO* line when studied by the Araki group showed that *GUS* expression was restricted to the vasculature in spite of ubiquitous *CO* activation (Yamaguchi et al., 2005). One may argue that the tissue specific expression pattern is due to the fact that CO protein is not stable outside the vasculature. However, analysis of plants overexpressing a CO:GFP fusion in Arabidopsis demonstrated that CO protein is stable as GFP signal was detectable in nuclei of cells outside the phloem tissue (Kishore Panigrahi, MPIZ, Cologne, personal communication). The observed vascular specific expression of *FT* supports the idea that CO requires an unidentified protein partner to activate transcription and leads to the suggestion that this co-activator is specifically expressed in the vasculature. Nonetheless, it is also possible that expression of *FT* is restricted to the veins, because transcription is efficiently repressed outside the vasculature.

Interestingly, it has been hypothesised that, apart from transcriptional control mediated by specific transcription factors and their respective *cis*-regulatory promoter binding site, higher-level spatial and temporal chromosome structure plays an important role in regulation of spatiotemporal expression (Tetko et al., 2006). Around 300 bp long AT-rich sequences, named scaffold/ matrix attachment regions (S/MARs), have been proposed to be essential for structural organization of the chromatin within the nucleus and serve as anchor of chromatin loop domains. On the basis of genome-wide *in silico* analysis, presence of intragenic S/MARs not only correlates with low expression levels of genes but targeted genes also show a pronounced specificity for tissues, organs and developmental phases (Rudd et al., 2004; Tetko et al., 2006). Interestingly, for the *FT* locus a S/MAR is predicted for a region covering the end of intron 1, the second exon and the beginning of the following intron (Rudd et al., 2004). Nonetheless, the mechanism of gene regulation based on intragenic S/MARs has yet to be elucidated.

5.2. Identification of sequences required for *FT* expression in response to day length

Shortening of the *FT* promoter to 4.0 kb upstream of the ATG, disrupted the ability to drive *FT* cDNA expression and therefore to complement the late flowering phenotype of *ft-10* plants. Consistent with these data, GUS signal was not be detected in the leaves of *4.0kbFTp::GUS* Col plants. Although GUS activity could be detected in the Y junction of the vasculature below the meristem, expression at this position did not seem to trigger flowering. Since *GUS* staining could be detected in siliques, expression of *FT* might be regulated differently at later developmental stages or in other tissues.

We raised the question which regulatory sequences were missing in a 4.0 kb *FT* promoter that are necessary to mediate CO response in the leaves and therefore to drive proper *FT* expression under inductive conditions. Appling deletion constructs between 8.1 kb and 4.0 kb length in the complementation analysis, revealed that 5.7 kb upstream of the ATG fused to the *FT* cDNA was sufficient to rescue *ft-10* mutant phenotype. Furthermore, the promoter was able to drive the same expression pattern in wild type and *35S::CO* background as *8.1kbFTp::GUS* plants. Therefore, regulatory elements mediating *FT* activation in response to day length are encoded within the 1.7 kb long sequence which is covered by the 5.7 kb *FT* promoter construct, but is missing in the 4.0 kb *FT* promoter. The *FT* locus is widely covered with the repressive H3K27me3 mark (Turck et al., 2007; Zhang et al., 2007b). Presence of this

chromatin mark might interfere with the access of transcription factors to the *FT* promoter. Interestingly, the 1.7 kb long sequence includes a locally H3K27me3 and TFL2 depleted region that could be more open to *trans*-acting factors. Moreover, alignment of *FT* promoter sequences from *Arabidopsis thaliana, Arabidopsis lyrata* and *Brassica rapa* revealed that the H3K27me3 and TFL2 depleted region coincides within a sequence stretch of around 430 bp (referred as block A) that is highly conserved. Therefore, it is likely that transcriptional regulation of *FT* in response to inductive photoperiod is mediated through *cis* elements possibly located in the sequence of block A (Figure 16A).

Since it has been proposed that CO might activate transcription as a component of the AtHAP complex, it is remarkable that block A encodes a CCAAT-box that is the binding site for the HAP complex. CCAAT-box elements are commonly located 60-100 bp upstream of the transcription start site (Mantovani, 1999). The proximal *FT* promoter region does not encode a CCAAT-box and the closest CCAAT sequence is located around 850 bp upstream of the start codon. As the *FT* promoter is likely to be under complex control functional CCAAT-box elements might be located beyond the proximal promoter region. Mutational analysis of the CCAAT element in sequence block A will elucidate if it acts as a CO-responsive element mediating day length response.

The prediction of individual transcription factor binding sites can be a helpful tool to understand transcriptional regulation of gene expression. However, it has to be considered that just presence of a conserved binding side does not imply that the element has a function on gene expression. The use of evolutionary conservation of sequence in putative regulatory elements can be helpful in narrowing down the search space and increasing the significance of some sites (Vavouri and Elgar, 2005). Nevertheless, important transcription factor binding sites might be also located in non-conserved regions. Studies on the conservation and turnover of binding sites in regulatory elements in Drosophila species have shown that between one- and two-thirds of identified transcription factor binding sites are not conserved even between relatively closely related species (Costas et al., 2003; Emberly et al., 2003). Based on binding site enrichment in a set of co-expressed genes, a I-box and a REalpha consensus sequence have been implicated in light-dependent gene regulation (Terzaghi and Cashmore, 1995; Degenhardt and Tobin, 1996). So far, no transcriptional regulator could be assigned to the predicted binding sites which could be identified in the conserved region of block A. Although it has been mainly demonstrated that light quality affects flowering time via *CO* transcript and CO protein levels (Valverde et al., 2004; Kim et al., 2008), it is also possible that light quality affects *FT* transcription in a CO-independent way. The transcription factor CIB1 has been

proposed to regulate *FT* expression in a blue light dependent manner through binding to an E-box element in the 5'-end of the transcribed region and in intron 2 of *FT* (Liu et al., 2008). Interestingly, a short conserved sequence stretch around two kilobase pair upstream of *FT* (block B) contains an E-box consensus sequences that is a potential recognition site for many basic-helix-loop-helix (bHLH) proteins including CIB1. However, the published ChIP experiment does not cover regulatory sequences beyond 1.5 kb upstream of the start codon of *FT* and therefore does not include block B (Liu et al., 2008).

5.3. Analysis of the proximal *FT* promoter region

Besides the conserved region located around 5.3 kb upstream of *FT* (block A), alignment of *FT* promoter sequences from different *Brassicacea* plants revealed that a 360 bp long region in the proximal promoter, named block D, is highly conserved during evolution. Interestingly, in the transient bombardment assays expression of *1.0kbFTp::GreenLUC* was detectable and could be stimulated through co-bombardment of *35S::CO*. Based on the idea that shortening of the 4.0 kb *FT* promoter will result in loss of repressive elements and therefore lead to expression, we applied a proximal *FT* promoter of 1.0 kb length in expression analyses *in planta*. Complementation and histochemical *GUS* localisation assays showed that a 1.0 kb *FT* promoter was not able to drive expression. Even under high inductive conditions like in *35S::CO* background, no expression could be measured. In the genomic context, CO might act through the proximal promoter, but requires interaction with a co-activator which binds more upstream regions of the *FT* promoter (Figure 16A and B). Interaction with a protein partner might enhance the DNA affinity of CO which then leads to binding in the proximal promoter region. Because of the extreme high amounts of DNA introduced into bombarded cells, interaction with a co-activator might not be required, because the low DNA-binding affinity of CO is sufficient to induce expression marginally. Induction by CO in the transient expression assay is maximally 6-fold, which is a fraction of the approximately 100-fold stimulation observed in transgenic *35S::CO* plants (Franziska Turck, MPIZ, Cologne, personal communication). Nonetheless, expression driven by a 1.0 kb *FT* promoter in the transient reporter assay might be due to a general difference between bombarded and integrated DNA. While in stable transformed plants the transgene is integrated into the genomic context, genes encoded on plasmids probably lack most regulation mediated through chromatin.

The importance of proximal *FT* promoter regions was further confirmed by mutational analysis of conserved elements. Putative *cis*-regulatory motifs were identified based on conservation of the proximal promoter sequences. Using the transient dual-luciferase reporter

assay, mutation of shadow 1 and 3 *(S1* and *S3)* and the palindromic sequence flanking *S3 (P1* and *P2)* seemed to affect CO-dependent induction of *FT*. Introduction of point mutations to the 8.1 kb *FT* promoter fragment allowed to apply their function in spatial expression and complementation analyses *in planta*. Based on the spatial expression analysis, *S1* is likely to be crucial for *FT* induction. Expression under control of an *8.1kbFTp-S1mut* promoter was clearly decreased in wild type and *35S::CO* plants. However, in the complementation analysis *8.1kbFTp-S1mut::FTcDNA ft-10* plants appear to flower similar to the non-mutated control. Therefore, expression levels driven by an *8.1kbFTp-S1mut* promoter seem to be sufficient to induce flowering. Surprisingly, *8.1kbFTp-P1/P2mut::FTcDNA ft-10* plants were not able to complement the late flowering phenotype although mutations in *P1* and *P2* did not affect the spatial expression.

Going along with the idea that distal and proximal promoter regions of *FT* plays an important role mediating CO response, it is interesting that *TSF* expression seems to require less upstream sequence. Previous studies demonstrated that a *GUS* reporter gene integrated into a genomic construct covering 1.5 kb sequence upstream of *TSF* and 1.36 kb downstream sequence is expressed in the vascular tissue of hypocotyls, petioles and the basal part of cotyledons, as well very little expression is detectable in the most-apical phloem of first true leaves. Insertion of transposable elements in the 3'-region around 150 bp downstream of *TSF* in many Arabidopsis ecotypes, such as Col, did not seem to affect transcription and mRNA stability (Yamaguchi et al., 2005). As the proximal promoter sequences of *FT* and *TSF* are well conserved (block D) and both genes are supposed to be direct targets of CO (Yamaguchi et al., 2005), we tested the possibility that the *TSF* promoter might be sufficient to drive *FT* expression. Complementation analysis revealed that the *ft-10* mutant phenotype could not be rescued by the *FT* cDNA under control of the 1.5 kb *TSF* promoter. As in most *1.5kbTSFp::GUS* Col lines no GUS signal could be observed, the promoter might not be able to drive expression at all or at a sufficient level. Similar to *FT*, proximal promoter elements of *TSF* might be important for expression of the gene and CO-mediated response, but enhancer sequences are required to reach detectable expression levels. While enhancer sequences might be located more than 4.0 kb upstream of the transcription start site in the case of *FT*, at the *TSF* locus they are more likely located in the intragenic region or downstream of the gene. On the other hand, low expression of *TSF* compared to *FT* might be explained by the lack of an enhancing *cis*-element. Stronger expression of *FT* compared to *TSF* might be the consequence of a more complex way of gene regulation in which factors binding different sequence stretches act synergistically in transcriptional activation (Figure 16B).

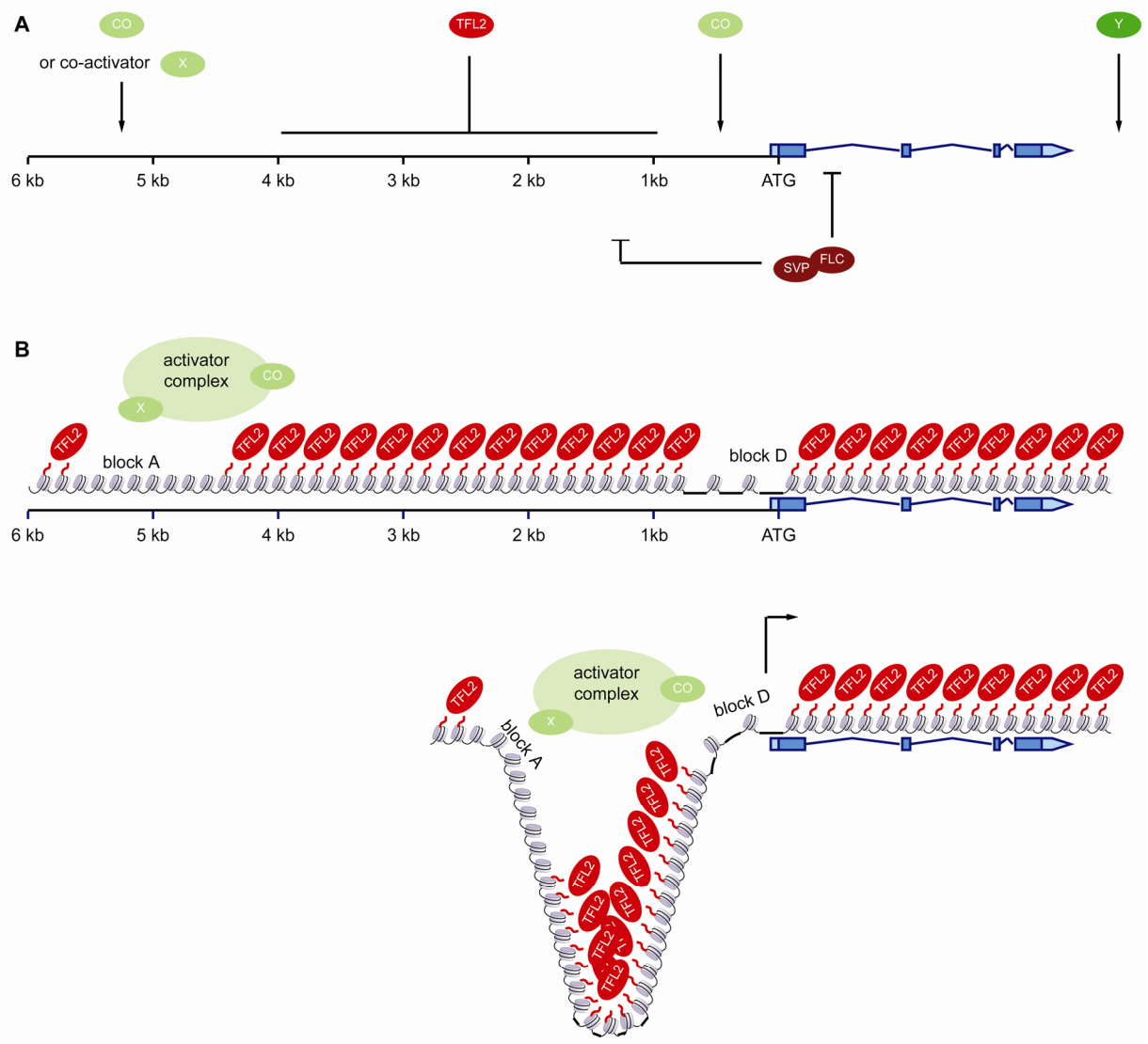

Figure 16. Identification of *cis*-regulatory sequence involved in transcriptional control of *FT*.
(**A**) We demonstrated that 5.7 kb regulatory sequence upstream of *FT* was sufficient to drive expression. A shorter 4.0 kb *FT* promoter fragment lost the ability to respond to CO. Block A is highly conserved and might be crucial for mediating day length response. CO or a CO-dependent co-activator might bind to block A and activate transcription. Nevertheless CO might also act through the proximal *FT* promoter since a 1.0 kb promoter fragment was inducible by CO in a transient expression assay. TFL2 has been shown to mediate repression through a promoter region 1.0 to 4.0 kb upstream of the ATG.
The first intron has been proposed to mediate repression by FLC. Spatial expression analysis revealed that in high *FLC* expressing plants, FLC can repress *FT* through the promoter region as well. Since FLC interact physically with SVP and both genes regulate flowering in a mutually dependent way, these transcription factors may bind as a complex and modulate *FT* expression. Since complementation of the *ft-10* phenotype could not be achieved with constructs containing *FT* intragenic sequence, it might be that *cis*-regulatory elements downstream of *FT* are important for positive regulation through factor Y.
(**B**) Block A coincides with a TFL2 depleted region which might enable accessibility of transcription factors such as an unknown CO-interacting factor. Interaction of CO with a protein partner or a complex might enhance CO-binding DNA affinity. Since the density of histone 3 is reduced in the proximal region (block D) (Sara Farrona, MPIZ, Cologne, personal communication) "activated" CO can bind to elements in the proximal promoter and initiate *FT* transcription.

For mammalian cells and in flies a model of structured protein–DNA complexes has been proposed. *Cis*-regulatory DNA enhancer sequences contain multiple binding sites for distinct transcription factors that co-assemble into higher order multi-component complexes. Fully assembled enhancer complexes, called "enhanceosomes", modify the local chromatin architecture and recruit the RNA polymerase II machinery to the promoter (Panne, 2008). Furthermore, it has been proposed that gene expression can be regulated via long-range chromatin interactions (Tiwari et al., 2008). In this model, chromatin loops form a spatial unit of regulatory DNA and allow the appropriate physical interaction between an enhancer and a promoter (de Laat and Grosveld, 2003; Noordermeer and de Laat, 2008). These structures have been termed active chromatin hubs (ACH). Although complexes, such as enhanceosomes and ACH, have been identified and dissected, it is unclear if and how much of transcription regulation is mediated by those structures in plants.

5.4. TFL2 dependent repression of *FT* expression

TFL2 has been proposed to be involved in creating a threshold for activation of *FT*. Under LD conditions TFL2 might counteract the activity of CO on *FT* expression. The *FT*-specific expression pattern can be explained as the result of spatial expression of *CO* and *TFL2* (Takada and Goto, 2003). TFL2 is strongest expressed in the proximal part of the leaf where it prevents *FT* activation. While TFL2 is able to repress *FT* in the distal vasculature under low inductive conditions only, strong *CO* expression in the distal vasculature of leaves might overcome TFL2-dependent repression and leads to flowering under high inductive conditions.

Since TFL2 co-localizes with H3K27me3 chromatin marks over the whole *FT* locus (Turck et al., 2007), we raised the question how loss of *TFL2* affects expression of the different *FTp::GUS* constructs. As expected from published results (Takada and Goto, 2003), spatial expression observed in the distal veins of leaves of *8.1kbFTp::GUS* Col plants was extended to the middle vein as well to minor veins of the proximal part of first true leaves in *tfl2* mutants. In plants carrying a *4.0kbFTp::GUS* construct loss of *TFL2* resulted in expression in the middle vein, but not in the distal vasculature. Since expression in the leaf tip seemed to require the more upstream *FT* promoter elements which mediate CO response, it is questionable if expression in the middle vein is a CO-dependent effect. Since TFL2 mediates *FT* repression throughout development and loss-of-function of *TFL2* causes *FT* transcription under SD conditions (Takada and Goto, 2003), expression in the middle vein might be an effect that is independent from day length and visible under SDs or in *co* mutant background. It has been published that a *8.9kbFTp::GUS* construct in *tfl2 co* double mutant background is

expressed in the main vein and the proximal vasculature (Takada and Goto, 2003). These observations suggest that the expression of *FT* in the distal part of the leaves requires the activity of CO and sequences mediating CO response. *FT* expression in the middle vein and the proximal half of the leaf is repressed by TFL2 and loss of the repressor might enable an unknown factor to activate *FT* in this tissue. As no expression could be detected in *1.0kbFTp::GUS tfl2* plants, TFL2 seems to mediate repression of *FT* through sequences 1.0 to 4.0 kb upstream of the ATG (Figure 16A).

5.5. Effect of insertions in *FT* regulatory regions

Based on the idea that insertion of foreign DNA into *FT* regulatory sequences can affect expression, we measured flowering time of plants with T-DNA insertions in different regions of the *FT* promoter. The T-DNA line #5 which carries a T-DNA insertion around 3550 bp upstream of the start codon of *FT* flowered later than the wild type control. Since the insertion site is not highly conserved, it is less likely that the T-DNA disrupted a *cis*-regulatory element or unit. Introduction of the T-DNA sequence may impair the spatial relationship between the important regulatory regions block A (5.7 kb *FT* promoter) and block D (proximal *FT* promoter). As several lines analysed carry a T-DNA between block A and block D and only line #5 was affected in flowering time, spatial distance between the enhancer and promoter sequences might be not crucial for transcription. In fact, the data may suggest that a *FT* promoter construct of 4.2 kb length that contains all the sequence downstream of line #4 is sufficient to drive *FT* expression. Nonetheless, insertion of multiple T-DNAs in line #5 might change the spatial relationship between the regulatory elements significantly as compared to the other lines. Insertion of foreign DNA, in particular of tandem repeats might also have an impact on the chromatin context of the *FT* locus. Further analysis of line #5 in comparison to other promoter T-DNA lines might reveal changes at the *FT* locus that will give new insights in regulation of *FT*.

5.6. *FT* regulation by intragenic sequences and role of FLC

To study the regulatory function of the first intron of *FT*, the *GUS* reporter gene was fused to the first exon, first intron and part of the second exon of *FT (FTE$_1$I$_1$E$_2$:GUS)*. Expressed under control of the 8.1 kb *FT* promoter in Col plants, *FTE$_1$I$_1$E$_2$:GUS* mRNA reached levels comparable to those of *GUS* in *8.1kbFTp::GUS* Col plants. The Col ecotype of Arabidopsis has no functional *FRI* allele and therefore *FLC* expression is low even without vernalization

(Johanson et al., 2000). Nonetheless cold treatment could further reduce *FLC* transcription. The slight decrease of *FLC* mRNA in vernalized Col seedlings did not seem to have an impact on the expression levels of the transgenes. Srikingly, although *FTE$_1$I$_1$E$_2$:GUS* was expressed in Col plants, no GUS staining could not be observed. Protein fusions with GUS have been shown in other studies and did not affect GUS function (Yamaguchi et al., 2005). Nonetheless fusion of GUS with the proximal part of FT might have caused the fusion protein to be more unstable and hence undetectable.

Based on ChIP experiments it has been proposed that FLC mediates repression of *FT* via direct binding to the first intron (Helliwell et al., 2006; Searle et al., 2006). Interestingly, expression level analysis of *8.1kbFTp::GUS* in high *FLC* expressing plants revealed that FLC can repress *FT* transcription through the promoter region as well. Yu and colleagues suggested that FLC may act in concert with SVP to suppress *FT* expression (Li et al., 2008). Since it has been shown that SVP binds to a CArG-box containing region in the *FT* promoter (Lee et al., 2007), the interacting FLC and SVP proteins might target *FT* at two different CArG-box elements, one in the promoter and one in the first intron (Figure 16A). As FLC and SVP function is supposed to be mutually dependent (Li et al., 2008), reduction of *FLC* levels by cold treatment might release the repressive effect mediated through the *FT* promoter and *GUS* expression could increase. Strikingly, the repression of *FTE$_1$I$_1$E$_2$:GUS* in non-vernalized *FRI* plants could not be released by cold treatment. The intragenic *FT* region might recruit FLC or a FLC-repressor complex that mediates repression which once initiated is stably maintained even after vernalization. Therefore, a positive regulator is needed that counteracts the repressive effect, but this activator requires regulatory sequences which the *8.1kbFTp::FTE$_1$I$_1$E$_2$:GUS* construct does lack.

Strikingly, a genomic *FT* fragment under control of the 8.1 kb promoter was not able to rescue the *ft-10* mutant phenotype. The genomic *FT* construct contained the coding region of the *FT* gene with all introns, but the 3'-untranslated region was replaced with the nopaline synthase terminator. Sequences downstream of *FT* might encode a *cis*-regulatory binding site for a positive regulator which is required to overcome repression mediated by the intragenic sequence of *FT*. On the other hand lack of a repressor or a repressive structure in the 3'-region of *FT* might lead to post-transcriptional silencing by production of an anti-sense transcript. However, repression of *FT* mediated in *trans* could be excluded by crossing the transgenic *8.1kbFTp::FTgDNA ft-10* line to wild type. Therefore, it is likely that an activator binding to a *cis*-regulatory element downstream of *FT* is necessary to achieve expression (Figure 16A, factor Y). The effect of *trans*-acting factors binding to downstream regulatory sequences of *FT*

could further explain the differentially expression of the *8.1kbFTp::GUS* transgene and the endogenous *FT* at ZT 8. Complementation analysis with a genomic fragment cloned by homologous recombination with a *FT* containing BAC covering the 8.1 kb promoter sequence, the gene coding region, and 2.3 kb downstream sequence is in progress. Since it has been shown that a 11.8 kb genomic fragment of *FT* covering 8.9 kb upstream and 700 bp downstream sequences was able to complement the late flowering phenotype of an *ft-101* mutant (Takada and Goto, 2003), we expect to rescue the late flowering phenotype of *ft-10* with the fragment.

6. Conclusions and Perspectives

After the first induction of *FT* in the leaves, expression changes of meristem identity genes can be detected in the SAM within one day. So far, the first marker upon floral induction at the apex is the expression of *SOC1*. After three days, macroscopic changes are observed at the SAM and the plants are stably committed to flower. This observation indicates that flowering time of summer annual Arabidopsis accessions is largely determined by the timing of *FT* expression in the leaves.

Identification of *cis*-regulatory regions and *cis* elements sets the basis for a better understanding of *FT* regulation by various factors. During this work, a region between 4.0 and 5.7 kb upstream of the *FT* start codon was identified as essential for *FT* expression. Currently, a screen for *trans*-acting factors that bind to the 1.7 kb long promoter region is in progress (in collaboration with Jiang Zhang, MPIZ, Cologne). Candidate transcription factors will be overexpressed in the phloem to validate their potential impact on flowering. To perform this experiment, we can make use of an Arabidopsis transformant collection overexpressing approximately 600 transcription factors under control of the *SUC2* promoter (generated by Lionel Gissot, MPIZ, Cologne).

The phylogenetic shadowing approach suggests focusing on a 430 bp long sequence within the identified 1.7 kb *FT* promoter region that is highly conserved and coincides with a TFL2-depleted region. Prediction of transcription factor binding sites revealed a CCAAT-box element. Although a possible connection between CO mode-of-action and the CCAAT-box binding complex has been demonstrated previously, no CCAAT-box with a regulatory role has been identified at the proximal *FT* promoter. However, the fact that the identified CCAAT-box is highly conserved in contrast to several other CCAAT-boxes that are encoded in the upstream regulatory sequence of *FT* increases the significance of this potential *cis*-regulatory element. Mutational analysis will elucidate if this element is crucial for a CO-mediated response.

Alignment of proximal *FT* promoter sequences from homologous *Brassicacea* genes revealed that a 360 bp long proximal promoter region is highly conserved. In the course of this work it was shown that the proximal promoter can drive *FT* expression in transient expression assays but not in stably transformed plants. However, mutational analysis of conserved proximal elements (shadows) in the context of the full-length promoter in stably transformed plants confirmed that the motif *S1* is crucial for *FT* expression. A yeast one hybrid screen was performed to identify *trans*-interacting factors that bind to the proximal promoter region.

Several candidate transcription factors were tested in transgenic plants and some showed a flowering effect caused by overexpression in the phloem. Currently, further analysis of the mis-expression lines for these factors has been initiated (collaboration with Jian Zhang). The candidate transcription factors will also be analysed for their binding affinity to *S1*. *Trans*-*cis*-interaction at *S1* would in turn validate the importance of the *trans*-acting factor in *FT* regulation in the natural context. We aim to evaluate the relevance of the *trans*-acting factor by the analysis of T-DNA insertion lines that cause loss-of-function. However, since most candidate transcription factors are part of large gene family, the genetic analysis can become very complex.

Our current working model proposes that *FT* expression is mediated by an enhancer region located 5.3 kb upstream of the *FT* transcriptional start and by regulatory regions that are found at the proximal promoter. A synergistic effect between the distal enhancer sequences and the proximal promoter region is required for *FT* expression. However, complementation with the full-length promoter driving the genomic *FT* sequence rather than the *FT* cDNA resulted in a lack of expression. Therefore, *FT* genomic regions, most likely found in regulatory introns seem to recruit a repressor or repressive complex. Since the cDNA and genomic constructs do not contain the 3'-untranslated region, we deduce that regulatory sequences downstream of *FT* are required to counteract the repression mediated through the intragenic region. Given the complexity of the locus, it is a long-term goal to understand how and how many *cis*-regulatory elements at the *FT* locus communicate with each other and achieve the spatial and temporal expression pattern of *FT*.

FT regulation is also affected by the surrounding chromatin since lack of the chromatin associated transcriptional repressor TFL2 increases *FT* levels and thereby causes a loss of photoperiod control. TFL2 is mechanistically linked to PcG-mediated gene repression and loss of the PRC2 components *EMF2, CLF* and *FIE* also causes increased expression of *FT*. In collaboration with Sara Farrona (MPIZ, Cologne), the available *FTp::GUS* constructs were introduced into different PcG-mutant backgrounds. Spatial expression analysis will reveal if other PcG proteins are involved in creating a threshold for activation of *FT* in the phloem as it has been proposed for TFL2 or if they have a more general role in repressing *FT* in other parts of the plants. These *FT* expression studies may also extend our knowlwdge of the different layers of transcriptional regulation mediated by PcG.

7. Literature

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8. Abbreviations

General abbreviations

:	fused to (in the context of gene fusion constructs)
::	under the control of (in the context of promoter-gene constructs)
-	minus, not present
%	percentage
°C	degrees Celsius
3’	three prime end of DNA fragment
35S	promoter of the Cauliflower Mosaic virus
5’	five prime end of DNA fragment
µ	micro
A	Adenine
ACH	active chromatin hub
Arabidopsis	*Arabidopsis thaliana*
BAH	bromoadjacent homology
BCA	bicinchoninic acid
bHLH	basic helix loop helix
bp	base pair
C	Cytosine
C-	carboxy-terminal
cDNA	complementary DNA
Col	*Arabidopsis thaliana* ecotype Columbia-0
DNA	desoxyribonucleic acid
dNTP	deoxyribonucleic triphosphate
Drosohila	*Drosophila melanogaster*
E. coli	*Escherichia coli*
et al.	*et alii* / *et aliae* [Lat.] and others
F1, F2, F3…	first, second, third... filial generation after a cross
FREL	far-red enriched light
G	Guanine
g	gram
GA	gibberellic acid
GM	½ strength Murashige and Skoog medium

h	hour
H3K4me3	tri-methylated lysine 4 at histone 3
H3K27me3	tri-methylated lysine 27 at histone 3
H3K36me2	di-methylated lysine 36 at histone 3
k	kilo
kb	kilobase pair
l	liter
LD	long-day
L*er*	Landsberg *erecta*
M	molar (mol/l)
m	milli
min	minute
mol	mole
mRNA	messenger RNA
MU	4-methylumbelliferone
MUG	4-methylumbelliferone-β-D-glucuronide
n	nano
nt	nucleotide
N-	amino-terminal
p	pico
PCR	polymerase chain reaction
PEBP	phosphatidyl ethanolamine binding domain protein
pH	negative logarithm of proton concentration
PHD	plant homeodomain
PPT	Phosphinotricin
PRC	Polycomb repressive complex
RNA	ribonucleic acid
RNase	ribonuclease
RT-PCR	reverse transcription PCR
S/MAR	scaffold/ matrix attachment region
SA	salicylic acid
SAM	shoot apical meristem
SD	short-day
SE	standard error

SUC2	promoter of the plasma-membrane sucrose-H^+ symporter gene *SUC2* from *Arabidopsis thaliana*
T	Thymine
T1, T2, T3…	first, second, third... filial generation after transformation
T-DNA	transferred DNA
UTR	untranslated region
wt	wild type
x	crossed to (crosses are always indicated in the order: female x male)
X-Gluc	5-bromo-4-chloro-3-indolyl-β-D-glucuronide
ZT	zeitgeber time

Abbreviations of gene and protein names

The nomenclature for plant genes follows the Arabidopsis standard: *GENES* are written in upper case italics, while mutant *genes* are indicated in lower case italics. PROTEINS appear in upper case regular letters, mutant proteins in lower case regular letters.

AGL24	AGAMOUS-LIKE 24
AP1	APETALA 1
AtHAP	HEME ASSOCIATED PROTEIN from *Arabidopsis thaliana*
CBF	CCAAT-BINDING FACTOR
CIB1	Cryptochrome-interacting bHLH 1
CLF	CURLY LEAF
CO	CONSTANS
COL	CO-LIKE
COP1	CONSTITUTIVE PHOTOMORPHOGENESIS 1
Cry1	Cryptochrome 1
Cry2	Cryptochrome 2
E(Z)	Enhancer of Zeste *(Drosophila melanogaster)*
EBS	EARLY BOLTING IN SHORT DAYS
EMF2	EMBRYONIC FLOWER 2
ESC	Extra sex combs *(Drosophila melanogaster)*
FD	- (Traditionally this gene/protein has not been given a full name.)
FIE	FERTLIZATION INDEPENDENT ENDOSPERM
FIS2	FERTLIZATION INDEPENDENT SEED 2

FLC	FLOWERING LOCUS C
FRI	FRIGIDA
FT	FLOWERING LOCUS T
GUS	β-glucuronidase
HAP	HEME ASSOCIATED PROTEIN
LFY	LEAFY
LHP1	LIKE HETEROCHOMATON PROTEIN1 (also known as TFL2)
MEA	MEDEA
MSI	Multicopy suppressor of Ira *(Drosophila melanogaster)*
NF-Y	NUCLEAR FACTOR-Y
PcG	Polycomb group genes
PhyA	Phytochrome A
PhyB	Phytochrome B
PR	PATHOGENESIS-RELATED
Sce	Sex combs extra *(Drosophila melanogaster)*
SOC1	SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1
SU(Z)12	Supressor of Zeste *(Drosophila melanogaster)*
SVP	SHORT VEGETATIVE PHASE
SWN	SWINGER
TEM1	TEMPPANILLO 1
TFL1	TERMINAL FLOWER 1
TFL2	TERMINAL FLOWER 2 (also known as LHP1)
TSF	TWIN SISTER OF FT
VEL1	VERNALISATION5/VIN3-like 1 (also known as VIL2)
VIL1	VIN3-like 1 (also known as VRN5)
VIL2	VIN3-like 2 (also known as VEL1)
VIN3	VERNALISATION INSENSITIVE 3
VRN2	VERNALIZATION 2
VRN5	VERNALIZATION 5 (also known as VIL1)

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Erklärung

Ich versichere, dass ich die von mir vorgelegte Dissertation selbständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit - einschließlich Tabellen, Karten und Abbildungen -, die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie - abgesehen von unten angegebenen Teilpublikationen - noch nicht veröffentlicht worden ist sowie, dass ich eine solche Veröffentlichung vor Abschluss des Promotionsverfahrens nicht vornehmen werde. Die Bestimmungen dieser Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von Prof. Dr. George Coupland betreut worden.

Köln, den 22. März 2008 _____

Jessika Adrian

Teilpublikationen

Es liegen keine Teilpublikationen vor.