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# A Distal *CCAAT*/NUCLEAR FACTOR Y Complex Promotes Chromatin Looping at the *FLOWERING LOCUS T* Promoter and Regulates the Timing of Flowering in *Arabidopsis*[W][OPEN]

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**For many plant species, reproductive success relies on the proper timing of flowering, and photoperiod provides a key environmental input. Photoperiod-dependent flowering depends on timely expression of *FLOWERING LOCUS T* (*FT*); however, the coordination of various *cis*-regulatory elements in the *FT* promoter is not well understood. Here, we provide evidence that long-distance chromatin loops bring distal enhancer elements into close association with the proximal promoter elements bound by CONSTANS (CO). Additionally, we show that NUCLEAR FACTOR Y (NF-Y) binds a *CCAAT* box in the distal enhancer element and that *CCAAT* disruption dramatically reduces *FT* promoter activity. Thus, we propose the recruitment model of photoperiod-dependent flowering where NF-Y complexes, bound at the *FT* distal enhancer element, help recruit CO to proximal *cis*-regulatory elements and initiate the transition to reproductive growth.**

## INTRODUCTION

To properly time flowering and reproduction, plants often use seasonally changing daylength signals (or photoperiod). Mechanistic descriptions of photoperiod-dependent flowering are important for understanding phenology in changing global environments and for intelligently modifying agricultural crops. During photoperiod-dependent flowering, plants integrate photoperiod with the production of mobile florigenic hormones, or florigens. A key florigen, the protein product of *FLOWERING LOCUS T* (*FT*), integrates signals from several flowering time pathways (e.g., vernalization and photoperiod; reviewed in Imaizumi and Kay, 2006). Under inductive conditions, which vary among plant species, FT protein is produced in the vascular tissue of leaves, loaded into the phloem, and translocated to the shoot apical meristem (Corbesier et al., 2007; Jaeger and Wigge, 2007; Mathieu et al., 2007; Tamaki et al., 2007). In the shoot apical meristem, FT putatively interacts with transcriptional activators, such as the bZIP protein FD (Wigge et al., 2005), driving the expression of floral promoting genes and initiating the conversion of vegetative meristems to reproductive meristems. Thus, understanding the regulation of *FT* has important implications in plant developmental biology.

An important transcriptional activator of *FT* and photoperiod-dependent flowering is encoded by *CONSTANS* (*CO*) (Putterill et al., 1995; Kobayashi et al., 1999; Samach et al., 2000). In *Arabidopsis thaliana*, CO accumulates late in the day but is rapidly degraded by the proteasome in the dark. Thus, *Arabidopsis* is a facultative long-day (LD) flowering plant, meaning that sufficiently long days (longer than ~12 h) are necessary for CO to accumulate and positively affect *FT* expression (Suárez-López et al., 2001; Valverde et al., 2004; Jang et al., 2008; L.J. Liu et al., 2008). In 2010, Tiwari and colleagues identified two CO-responsive elements (CORE1 and 2) in the *FT* promoter (*pFT*) with the consensus sequence *TGTG(N2-3)ATG* (Tiwari et al., 2010). CO directly bound CORE2 in gel shift assays, and a mutation in this site eliminated both CO binding and synthetic promoter activation. Simultaneously, Adrian et al. (2010) examined flowering time relative to various *pFT* mutations and truncations using an in vivo methodology that relied on rescuing late flowering *ft-10* mutants (*Arabidopsis* ecotype Columbia-0 [Col-0]). While *pFT:FT* constructs efficiently rescued late flowering, a mutation in the CORE1 region resulted in late flowering and suppressed *FT* expression (CORE1 was called S2 in this article; hereafter, CORE1). The CORE1 and CORE2 sites are located at −220 and −161 bp relative to the start codon, respectively. However, experiments using *pFT* truncations and a combination of flowering time and β-glucuronidase (GUS) reporter assays defined the minimal functional *FT* promoter as ~5.7 kb in length (Adrian et al., 2010). Therefore, while CO is clearly a key regulator of *FT* expression during photoperiod-dependent flowering and the proximal CORE sites appear to play an important role, the functionally defined promoter suggests that additional distal elements are required.

CO and CO-Like proteins physically associate with NUCLEAR FACTOR Y (NF-Y) transcription factors, but the nature of these interactions remain unclear (Ben-Naim et al., 2006; Wenkel et al., 2006). NF-Y complexes bind DNA with the central pentamer *CCAAT* and are composed of three unique subunits called NF-YA, NF-YB, and NF-YC (Dolfini et al., 2012). Each subunit is encoded by a 10-member gene family in *Arabidopsis* (with similar family sizes in other plant lineages) (Petroni et al., 2012; Laloum et al.,

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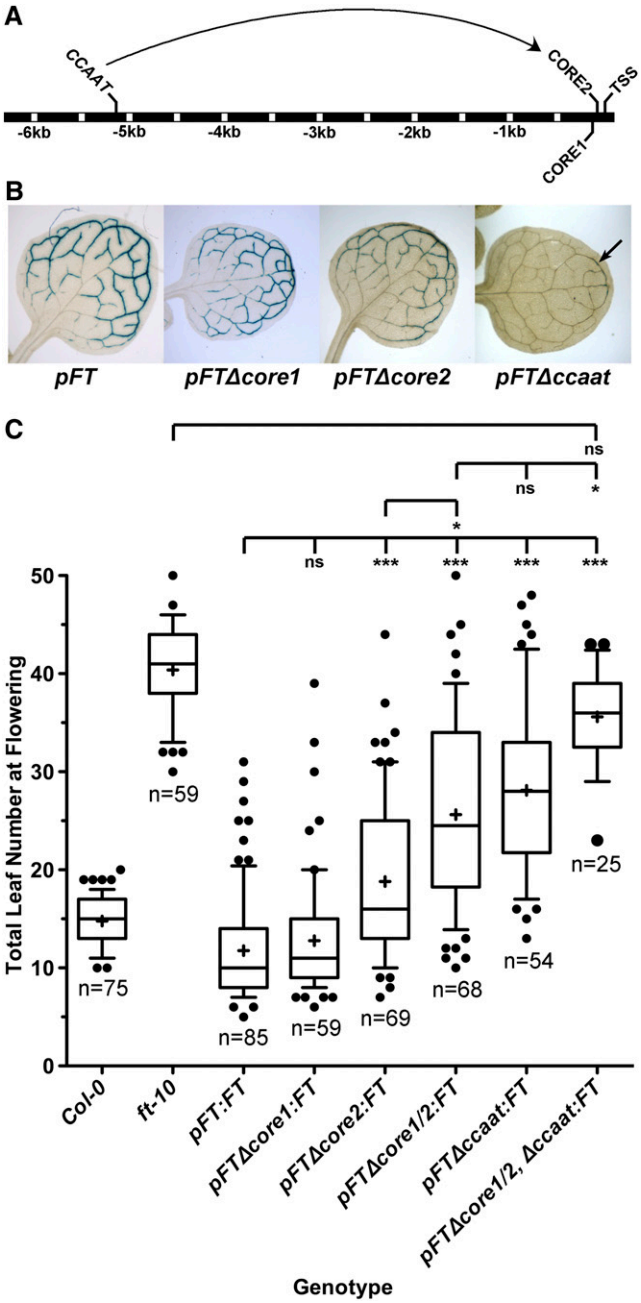


**Figure 1.** *CCAAT* and CORE Sites Are Required for Proper *FT* Expression and Flowering.

**(A)** Simplified diagram of *pFT* showing locations of hypothesized interacting *CCAAT* and CORE sites (Adrian et al., 2010; Tiwari et al., 2010).
**(B)** *GUS* expression patterns for *pFT:FTGUS*, *pFTΔcore1:FT/GUS*, *pFTΔcore2:FT/GUS*, and *pFTΔccaat:FT/GUS*. Arrow designates distal expression in *pFTΔccaat* leaf. GUS pictures represent the subjective average GUS expression pattern for multiple (~10) independent lines for each construct. Results were relatively consistent between independent lines, with the exception of *pFTΔcore1*, which showed more variable expression.
**(C)** Box and whisker plots of flowering times for *ft-10* lines transgenic for various *FT* rescue constructs. Box represents 25th/75th percentiles, whiskers are 10th/90th percentiles, horizontal line is median, + is mean, and black dots are outliers. Statistical comparison of populations done by nonparametric Kruskal Wallis test ($P < 0.0001$) and Dunn's multiple comparison test (*$P < 0.05$ and ***$P < 0.001$; ns, not significant).

2013). Higher order *nf-y* mutants appear phenotypically identical to *co* mutants (e.g., *nf-yb2 nf-yb3* double mutants have very low *FT* expression and strongly delayed flowering under normally inductive LD photoperiods) (Kumimoto et al., 2008). Furthermore, overexpression of *CO* normally drives very early flowering, except in appropriate *nf-yb* (*nf-yb2 nf-yb3*) and *nf-yc* (*nf-yc3 nf-yc4 nf-yc9*) mutant backgrounds (Kumimoto et al., 2010; Tiwari et al., 2010). Despite the direct NF-Y/CO physical interaction and prediction that they function together to drive *FT* expression, there are no canonical *CCAAT* sites located immediately adjacent to the CORE domains in *pFT* (Figure 1A). However, there are at least eight potential NF-Y binding sites in the functionally defined *FT* promoter. In particular, a *CCAAT* site at approximately −5.3 kb from the ATG is in a region that is highly conserved in the *FT* promoters of several examined Brassicaceae species (Adrian et al., 2010). This −5.3-kb region is also largely free of LIKE HETEROCHROMATIN PROTEIN1 (LHP1) interactions, which likely suppress transcription factor binding in much of *pFT* (Adrian et al., 2010). Collectively, these findings led to the hypothesis that CO binds near the transcriptional start site (TSS) of *pFT* and makes long distance genetic interactions with an activator complex bound in the −5.3-kb region (Adrian et al., 2010; reviewed in Pin and Nilsson, 2012). Here we specifically test this hypothesis, focusing on the role of the single *CCAAT* site at −5.3 kb and its interactions with the CORE1 and 2 sites to regulate the flowering transition.

## RESULTS

### A Distal *CCAAT* Box and Proximal CORE Domains Are Required for Proper Expression of *FT* and Floral Initiation

To initially explore the relative contributions of the two CORE sites and the −5.3-kb *CCAAT* site in *FT* expression, we generated various *pFT:FT/GUS* reporter lines and examined the GUS expression patterns in 2-week-old plants. As previously demonstrated for similar constructs, an ~6.5-kb *pFT* fusion to an *FT/GUS* chimeric reporter gene (*pFT:FT/GUS*) was strongly expressed in leaf vascular tissue, especially in the peripheral regions (Notaguchi et al., 2008; Adrian et al., 2010) (Figure 1B). Plants expressing constructs with mutations in either CORE sequence (*pFTΔcore1:FT/GUS* and *pFTΔcore2:FT/GUS*) had vascular expression in essentially the same regions as *pFT:FT/GUS*; however, the *Δcore2* mutation resulted in an overall reduction in the strength of reporter gene expression. A mutation in the −5.3-kb *CCAAT* sequence (*pFTΔccaat:FT/GUS* lines) consistently resulted in much weaker reporter expression, largely confined to the vascular tissue at leaf tips (see arrow in Figure 1B).

We next tested whether these apparent *FT* expression reductions translated to alterations in flowering time using *ft-10* rescue experiments. Rescue constructs included *FT* genomic sequence from −6.5 kb through ~1.5 kb of 3′untranslated region (UTR) sequence (using *gFT:GUS* from Notaguchi et al.

[2008] as template; see Methods). We chose to measure flowering time in randomly selected, glufosinate (BASTA)–treated T1 plants because this removed the biases inherent in plant selection and gave a better picture of the overall response to the individual transgenes. *ft-10* plants transgenic for *pFT:FT* (control) readily recovered photoperiod-dependent flowering and flowered even earlier than the wild-type Col-0 ecotype (Figure 1C). We note that multiple independent experiments confirmed this earlier flowering, suggesting that additional negative regulatory elements may be missing from the *pFT:FT* constructs. Consistent with the reporter gene assays, *pFTΔcore1:FT* plants flowered with similar timing to *pFT:FT* (12.8 ± 6.3 versus 11.8 ± 5.4 total leaves, respectively). However, the ability of *pFTΔcore2:FT* to rescue the *ft-10* mutation was significantly reduced (18.8 ± 7.9). A double promoter mutant, *pFTΔcore1/2:FT* (25.6 ± 9.7), flowered later than *pFTΔcore2:FT*, suggesting that the CORE1 site does have a role, albeit weaker, in photoperiod-dependent flowering. As predicted from the reporter gene assays, mutations in the −5.3-kb *CCAAT* site (*pFTΔccaat:FT*) strongly affected flowering time (28.1 ± 8.7). Finally, *pFTΔcore1/2,Δccaat:FT* triple promoter mutants flowered only slightly earlier than *ft-10* plants (35.6 ± 4.8 versus 40.4 ± 4.4), suggesting that much of the positive, photoperiod-dependent regulation of *FT* expression can be accounted for by these three *cis*-regulatory elements.

### NF-YB2 Can Physically Associate with the −5.3-kb *CCAAT* Box in the *FT* Promoter

Previous electrophoretic mobility shift assays (EMSAs; Tiwari et al., 2010) and chromatin immunoprecipitation (ChIP) assays (Song et al., 2012) confirmed in vitro CO binding to the CORE2 domain and in vivo CO binding to the *FT* proximal promoter, respectively. To determine if NF-Y complexes also bind *pFT*, we performed ChIP using stable plant lines that constitutively overexpressed *NF-YB2* (from the *35S* cauliflower mosaic virus promoter) with a yellow florescent protein (YFP) and HA epitope tag. NF-YB2 is a known component of the NF-Y complexes that positively regulate *FT* expression and photoperiod-dependent flowering and is therefore expected to be bound to a subset of the *CCAAT* sites in *pFT*. Interestingly, while there was always modest enrichment for amplicons near the −5.3-kb *CCAAT* site and at ~2 kb upstream of the TSS ($P < 0.1$), we also measured consistent enrichment for binding near the CORE1/2 elements, where canonical *CCAAT* sites are absent ($P < 0.05$; Figure 2A). In two additional independent experiments, the strength of binding to the −5.3-kb site was typically ~2-fold enriched but varied in statistical significance ($P = 0.16$ and $P = 0.0002$; Supplemental Figure 1). Therefore, we additionally performed EMSA to test NF-Y complexes for direct biochemical association with the −5.3-kb *CCAAT* site, as well as potential interaction with the CORE1/2 sites. The tested NF-Y complex consisted of NF-YB2 and NF-YC3, two known components of *FT*-regulating complexes, and mouse NF-YA (mNF-YA). mNF-YA has previously been used as a substitute for plant NF-YA and was chosen here because it remains unclear which of the 10 *Arabidopsis* NF-YA subunits are involved in flowering. Initially we demonstrated that mNF-YA/NF-YB2/NF-YC3 can bind a 31-bp oligonucleotide from the −5.3-kb site (Figure 2B). Binding of this site is *CCAAT* specific, since it could be competed with an unlabeled oligonucleotide, but not with a mutant

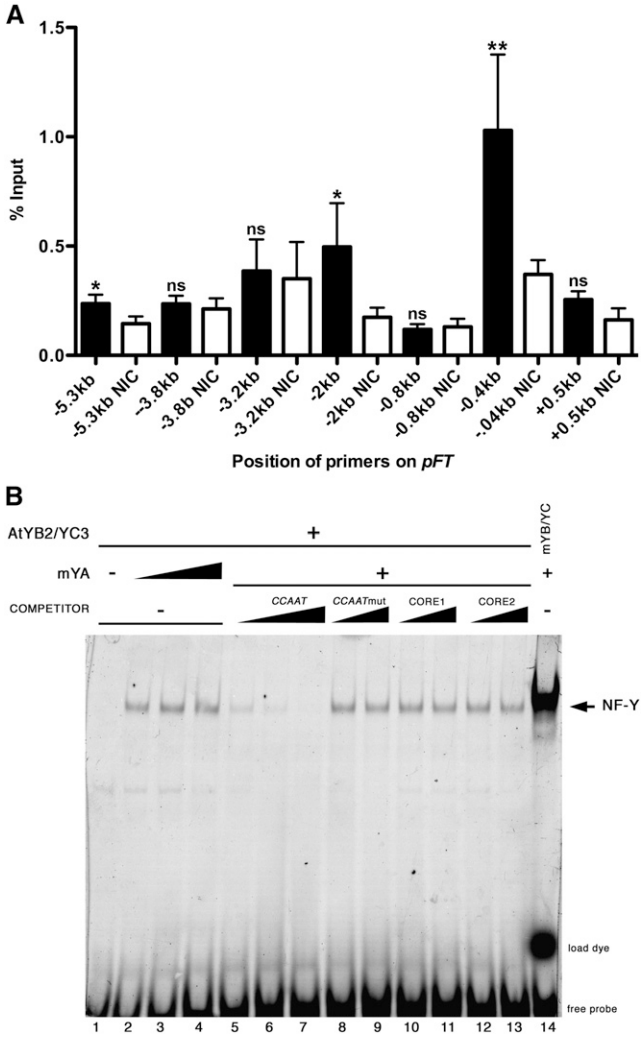


**Figure 2.** NF-Y Binding Was Enriched in *pFT* and Could Specifically Bind at *CCAAT* Sequences.

**(A)** ChIP experiment showing NF-YB2 enrichment at several locations in *pFT*. NF-YB2/GFP immunoprecipitated using anti-GFP antibodies linked to magnetic beads. NIC, (white bars) nonimmune controls where anti-Myc antibodies were substituted. Statistical comparison done by one-tailed *t* tests; *$P < 0.1$, **$P < 0.05$, $n = 9$ biological replicates, mean + SE. ChIP experiments were repeated twice (Supplemental Figure 1).

**(B)** EMSA showing −5.3-kb *CCAAT*-specific binding. Unlabeled oligonucleotide competitors were *CCAAT* (identical to 31-bp Cy5-labeled version); *CCAATmut* (*CCAAT* sequence changed to *gtcAg*); and CORE1 and 2 (CORE sequences as competitors). Oligo and primer sequences, including specific locations of ChIP amplicons, are in Supplemental Table 1.

version (*CCAAT*mut, same mutation as Figures 1B and 1C above). Importantly, we additionally demonstrated that oligonucleotides containing the CORE1 and CORE2 sites could not compete away binding, indicating no significant intrinsic affinity of the NF-Y trimer to the CORE1/2 sites. Coupled with the known protein–protein interactions between NF-Y subunits and CO, these data suggested that hypothesized genetic interactions between the −5.3-kb

*CCAAT* and CORE1/2 regions of *pFT* (Figure 1B; Adrian et al., 2010) might also represent direct physical interactions resulting in chromatin loops.

### CO- and NF-Y–Dependent Chromatin Loops Form in a Time-Dependent Manner in the *FT* Promoter

Using the chromatin conformational capture (3C) technique (Hagège et al., 2007; Louwers et al., 2009a, 2009b), we addressed whether DNA loops bring the CORE1/2 and −5.3-kb *CCAAT* sites within close proximity. With CORE1/2 acting as the anchor point, we sequentially queried cross-linking interactions between this region and various *Dpn*II restriction enzyme–released, *CCAAT*-containing DNA fragments in the functionally defined ~6.5-kb *FT* promoter (Figure 3A). These experiments were performed on chromatin extracted from LD grown plants at ~16 h after lights on, for example, during a time of peak *FT* expression. Two clear peaks were consistently measured, corresponding to interactions with *Dpn*II-released fragments IV and VII (Figure 3B; note that fragment VII corresponds to the −5.3-kb *CCAAT* site). We also inconsistently measured an interaction with fragment III, but this is likely due to linkage and incomplete digestion as previously described (Louwers et al., 2009a, 2009b). To address whether or not these putative DNA loops depended on CO or an NF-Y complex, we repeated the 3C experiment using the low *FT*-expressing, late-flowering, strong hypomorphic mutant *co-9* (also referred to as *co-sail*; Laubinger et al., 2006) and *nfyb2 nf-yb3*. In the *co-9* mutant, the peaks corresponding to fragments IV and VII were both significantly reduced but not eliminated. However, only peak VII was significantly reduced in *nf-yb2 nf-yb3* (Figure 3B). We additionally examined the late flowering *nf-yc3 nf-yc4 nf-yc9* triple mutant and it also showed a reduction in peak VII, but no change in peak IV (Supplemental Figure 2). This data suggested that several DNA loops formed in *pFT* that were

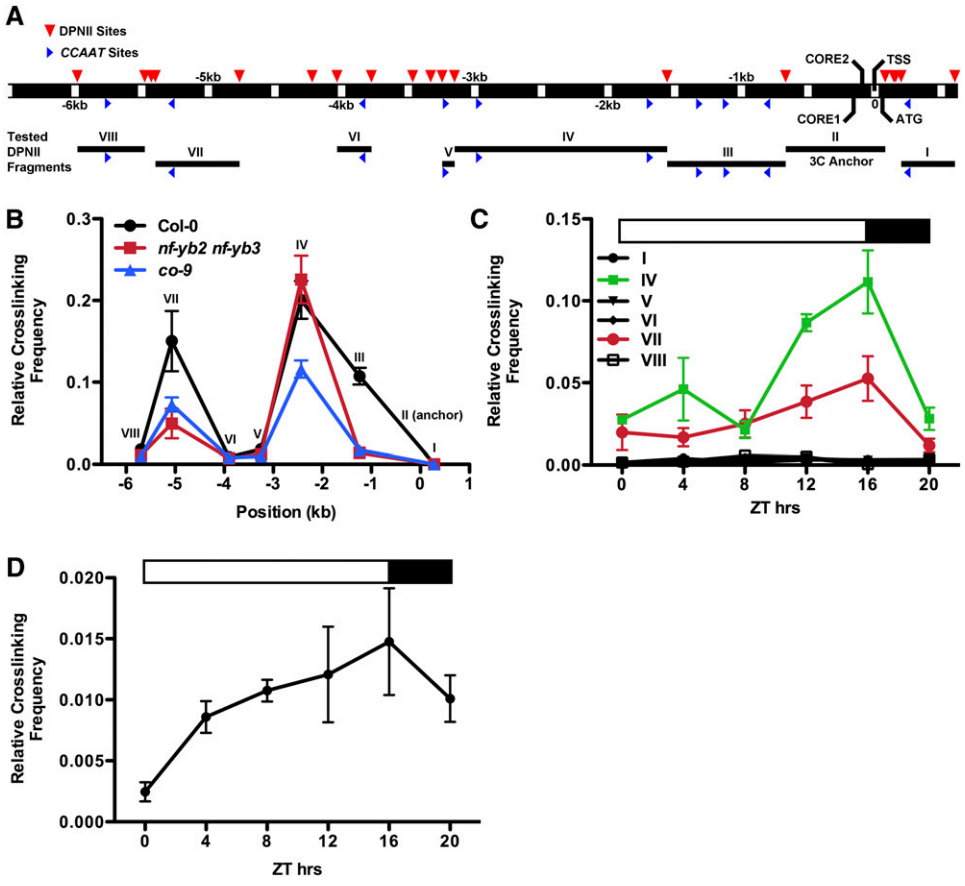


**Figure 3.** 3C Experiments Suggested Time-Dependent Loops Form between *CCAAT*-Containing and CORE1/2 Regions of *pFT*.

**(A)** Scale diagram of the *FT* promoter showing the positions of all *Dpn*II restriction sites, CCAAT boxes, and the fragments tested for interaction with the CORE1/2 region.

**(B)** Wild-type and mutant 3C analysis. Mean (±SE) for three biological replicates for each genotype. Note that apparent, but inconsistent, linkage to fragment III is likely related to incomplete digestion and not looping.

**(C)** Time-course 3C analysis. Mean (±SE) for three biological replicates for each time point. White/black bar above graph represents light and dark. All 3C experiments repeated with similar results.

**(D)** 3C using fragment VII (−5.3-kb *CCAAT* site) as anchor region revealed a similar diurnal looping pattern as when the CORE1/2 region was used as the anchor probe.

linked to the CORE sites and were partly, but not completely, dependent on CO and NF-Y.

Under inductive LD conditions (16 h light/8 h dark), *FT* expression is low early in the day, increases near the end of the day, and rapidly drops off in the dark. Using time-course 3C, we examined if the CORE-linked DNA loops followed a similar diurnal rhythm. Samples were collected every 4 h from ZT0 to ZT20. Cross-linking between fragment IV and VII with the CORE domain was strikingly similar to *FT* mRNA accumulation (i.e., relatively weak cross-linking early in the day with peak linkage late in the day and rapid drops in the dark) (Figure 3C). If region VII was used as the 3C anchor point (i.e., the probe was located at this position instead of the CORE1/2 region), we measured the same diurnal pattern for the linkage between regions II (CORE domains) and VII (−5.3-kb *CCAAT* site; Figure 3D). Therefore, cross-linking between the −5.3-kb *CCAAT* and CORE regions was sensitive to time of day, as well as CO and NF-Y availability.

## DISCUSSION

Collectively, our data are strongly supportive of an essential role for NF-Y complexes in the previously hypothesized, distal *FT* activator complex (Figure 1A). Blackman and Michaels (2010) proposed a model where CO interacts with *pFT* directly at the CORE elements and separately through distinct interactions with NF-Y complexes that are already bound to *CCAAT* boxes (an extension of the "docking" model proposed in Siefers et al. [2009]). If the putative DNA loops shown here are incorporated, this model can be updated to include NF-Y complexes in very close proximity to the CORE sites. We propose a "recruitment" model where (1) chromatin loops maintain NF-Y complexes in close proximity to the CORE sites, especially late in the day, (2) CO binding at CORE sites can be stabilized via direct NF-Y interactions, and (3) CO provides the *FT* transcriptional activation potential in this expression system (Figure 4). Thus, we hypothesize that chromatin loops do not activate *FT* expression per se, but create a time-of-day-dependent, poised environment where recruitment of CO (the key transcriptional activator of the system) is more likely and/or more stable.

Accordingly, our data could explain why moderate *CO* overexpression is ineffective at driving early flowering in various *nf-y* mutant backgrounds but that this *NF-Y* requirement can be partially overcome at very high levels of *CO* overexpression (Kumimoto et al., 2010; Tiwari et al., 2010). We hypothesize that, at sufficiently high levels, some CO effectively binds at CORE sites without NF-Y assistance. Furthermore, the partial, but not complete, requirement for NF-Y complexes and CO to form the long-distance loops suggests that other, currently undefined, *pFT*-bound proteins provide loop stability in the absence of an NF-Y/CO interaction.

It is currently unknown what roles well-known negative regulators of *FT* expression, as well as repressive chromatin marks, might play in the formation or maintenance of these chromatin loops. It is worth noting that in previous studies, reductions in LHP1 binding largely followed, but did not precede, *FT* activation. Also, localized increases or decreases in normally activating or repressing chromatin marks appeared to occur after *FT* activation, suggesting that the measured changes were part of a "fine-tuning" mechanism as opposed to activators of expression (Adrian et al., 2010). Of particular interest will be future investigations of the *FT*

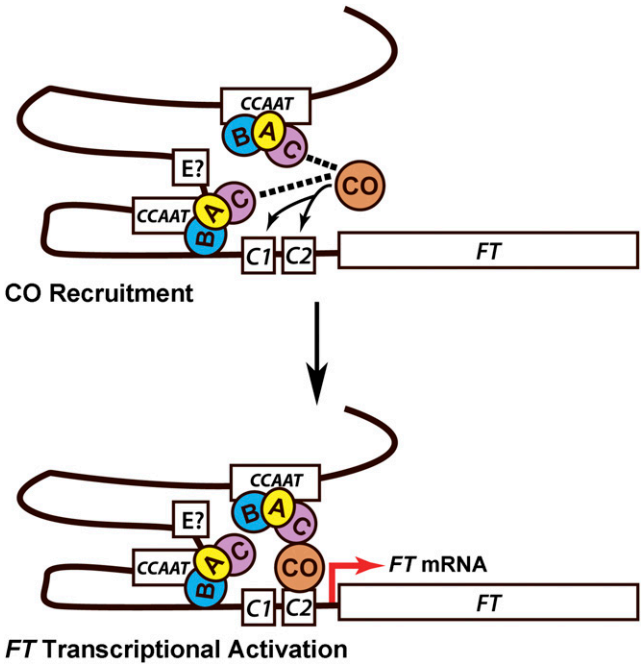


**Figure 4.** Recruitment Model of *FT* Activation.

3C-measured loops are visualized as bringing *CCAAT*-bound NF-Y complexes into close proximity with the two CORE (C) sites (Figure 3). This putatively allows for improved recruitment/stabilization of CO through the combined effects of physical interactions with both its DNA binding site (Tiwari et al., 2010) and bound NF-Y complexes (dashed lines; Wenkel et al., 2006; Kumimoto et al., 2010). A second loop with a region that contains an NF-Y–bound *CCAAT* box (Figure 2A) and a putative E-box (E?; Adrian et al., 2010) is also shown.

repressors *TEMPRANILLO1* (*TEM1*) and *TEM2*. Accumulation of *TEM1/2* is thought to be negatively correlated with *CO* accumulation, such that their *FT* repressive and activating functions are developmentally balanced (Castillejo and Pelaz, 2008; Osnato et al., 2012). Two putative TEM1/2 binding sites are located ~130 to 190 bp from the CORE1 and CORE2 sites; thus, it is certainly possible that they have a role in the formation/stability of the described *FT* chromatin loops. Alternatively, the putative TEM1/2 binding sites are located between the TSS and start codon of *FT* (Castillejo and Pelaz, 2008), suggesting that TEM1/2 binding may simply physically interfere with transcription.

It was previously suggested that CO protein competes with NF-YA for occupancy in NF-YB/NF-YC dimers to regulate *FT* expression ("replacement" model; Wenkel et al., 2006; Siefers et al., 2009; Li et al., 2011). However, the data presented here show that *CCAAT* boxes are important for the regulation of *FT* expression, and it is unclear how a CO/NF-YB/NF-YC trimer would effectively bind *CCAAT* sequences because the NF-YA component of the trimer makes almost all of the essential *CCAAT* contacts (Siefers et al., 2009; Kumimoto et al., 2010; Nardini et al., 2013). While an NF-Y crystal structure does show limited identity between NF-YA and CO in amino acids necessary for the *CCAAT* interaction, CO is also missing several key residues that are functionally required for *CCAAT* binding (Xing et al., 1993; Nardini et al., 2013). Furthermore,

from monocots to dicots, these residues are completely conserved in plant NF-YAs (Cao et al., 2011). Finally, the need for *CO* to drive flowering can be completely eliminated by overexpressing NF-YB2 fused to a strong transcriptional activation domain (Tiwari et al., 2012). Collectively, this data cannot absolutely prove a requirement for NF-YA or disprove the replacement model. However, without functional NF-YA, it is difficult to imagine how NF-YB2 fused to a transcriptional activation domain could activate *FT* expression in a *co* mutant and, simultaneously, why proper *FT* expression requires at least one *CCAAT* box. Regardless of the need for NF-YA, it is important to note that the existence of both canonical NF-Y complexes and CO/NF-YB/NF-YC complexes on *pFT* remains possible.

Although not the focal interaction of this study, we also measured the consistent formation of a DNA loop between the CORE sites region and DpnII fragment IV (Figures 3A to 3C). Due to its large size (1569 bp), fragment IV provides poor resolution for potential *cis*-regulatory elements, but it does contain two *CCAAT* sequences (located at −1743 and −3004 relative to the ATG) and a conserved E-box (−1902; Adrian et al., 2010). ChIP data confirm NF-Y binding in the region of the first *CCAAT* sequence ("−2kb" ChIP primers in Figure 2B amplify the −1789 to −2141 region); however, loop formation with fragment IV appears unaffected in *nf-y* mutant plants. No binding partner has yet been identified for the conserved E-box, although it was previously suggested that CRY2-INTERACTING bHLH1 (CIB1) is an appealing possibility because of its ability to bind E-box *cis*-regulatory elements and enhance flowering time (H. Liu et al., 2008; Adrian et al., 2010). Nevertheless, recent data suggest that while multiple CIB proteins act redundantly to interact with the *FT* promoter and promote flowering, no interaction is reported at the −1902 E-box (Liu et al., 2013). Further research is therefore required regarding the importance of putative *cis*-regulatory elements and their potential binding partners in the −2-kb region of *pFT*.

While the study of chromatin conformation in relation to gene transcriptional activity is still in its infancy, it's increasingly clear that chromatin looping is important for bringing distant enhancer elements into close association with their promoters (Harmston and Lenhard, 2013). Prior to the advent of 3C and related technologies, *CCAAT cis*-elements were shown to act as enhancers and proposed to be involved in DNA looping (Koch et al., 1989). Examining genome-wide NF-Y binding in animal cell lines revealed that only 22% of bound *CCAAT*s were located in proximal promoters; surprisingly, another 25% were located in distal enhancer elements (Fleming et al., 2013). Accordingly, NF-Y may represent a unique category of transcriptional activator because (1) binding at increasing distances from the TSS is correlated with increasing, not decreasing, regulatory control of transcription, and (2) it effectively saturates available *CCAAT* sites regardless of localized positive or repressive chromatin modifications. This latter feature suggests it is a "pioneer" transcription factor, capable of binding DNA in normally nonconducive environments and assisting with transcriptional activation through the recruitment of additional, essential regulatory transcription factors (Donati et al., 2008; Fleming et al., 2013). Considering the potential combinatorial diversity generated by the many possible plant trimeric NF-Y complexes, as well as their increasingly clear associations with other large transcription factor families (e.g., bZIPs; Yamamoto et al., 2009; Liu and Howell, 2010; Kumimoto et al., 2013), understanding how and when they act in structured chromatin conformations represents a key next step in describing their many functions.

## METHODS

### Plasmid Construction

All *FT* complementation constructs contain 6.5 kb upstream of the *ATG*, the genomic coding sequence, and 1.5 kb of downstream sequence, including the entire predicted 3′UTR. Primers used in the cloning reactions are provided in Supplemental Table 1. The *FT* promoter was amplified from Col-0 genomic DNA. The PCR products were introduced into pDONRP4-P1r via a BP Clonase reaction (Invitrogen). Point mutations of the −5.3-kb *CCAAT* box and CORE1/2 sites within the *FT* promoter were introduced by PCR and recombined into DONRP4-P1r via BP Clonase reaction. CORE1 and 2 mutations were from TGTG(N2-3)ATG to TaTa(N2-3)ATG, a mutation previously demonstrated to eliminate both CO binding and synthetic promoter activation in protoplast assays (Tiwari et al., 2010). The *CCAAT* site was mutated to *gtcAg*. The genomic coding region of *FT*, including the 3′UTR, with a translational fusion to the *GUS* gene, was amplified from a previously described *gFT:GUS* construct (Notaguchi et al., 2008) and inserted into pDONR207 via BP Clonase reaction. Multisite Gateway reactions between each version of the *FT* promoter and genomic coding region of *FT* (*FT:GUS:3′UTR*) into the destination vector R4pGWB613 (Nakamura et al., 2010) were performed via LR Clonase (Invitrogen). However, the presence of the Gateway site between the *FT* promoter and coding region resulted in nonfunctional constructs. Therefore, the Gateway site was subsequently removed using PCR and restriction digest approaches. Although the GUS-tagged FT protein is properly expressed in the leaf vasculature, this translational fusion is unable to rescue the *ft-10* late-flowering phenotype. Therefore, the *GUS* gene was removed from the above constructs for flowering time assays. The CORE1/CORE2 double mutant was created by restriction digest and ligation between the two vectors containing the single mutations using standard procedures.

All of destination plasmids based on pGWB613 were introduced into *Agrobacterium tumefaciens* strain GV3101 containing the helper plasmid pSOUP (Koncz and Schell, 1986). Plasmids were transformed into the *ft-10* mutant in the Col-0 background (Yoo et al., 2005) by the floral dip method (Clough and Bent, 1998). In the T1 generation, plants carrying a pGWB613 plasmid were identified on the basis of BASTA resistance. Construction of 35S:NF-YB2-YFP/HA was previously described (Kumimoto et al., 2010).

### GUS Staining and Flowering Time Measurement

All plants were grown at 22°C under standard LD conditions (16 h light/8 h dark) in Conviron growth chambers (model ATC13) or a custom walk-in chamber. For flowering time experiments, plants were grown in soil media containing equal parts Farfard C2 Mix and Metromix 200 supplemented with 40 g Marathon pesticide and dilute Peters fertilizer (NPK 20:20:20). Plants were watered throughout with dilute fertilizer (Peters NPK 20:20:20 at one-tenth of the recommended feeding levels). Flowering time was quantified by counting rosette and cauline leaves on the primary axis. Graphs and statistical analyses were all performed using the Prism software package (GraphPad). *nf-yb2 nf-yb3* and *nf-yc triple* mutant lines were originally described (Kumimoto et al., 2008, 2010). *ft-10* and *co-9* (*co-sail*) mutant lines were previously described (Yoo et al., 2005; Laubinger et al., 2006).

For GUS staining experiments, seeds were surfaced sterilized, sown on plates containing Gamborg B5 media, and grown for 10 d in standard LD conditions. Whole plants were harvested into GUS staining solution (50 mM $NaHPO_4$, pH 7.2, 0.2% Triton X-100, and 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid) and vacuum-infiltrated until tissue became translucent. Tissue was incubated overnight at 37°C in the GUS

staining solution. The plant material was cleared and maintained in 70% ethanol. Pictures were taken with an Axiocam mounted on a Zeiss Stemi 2000-C stereoscope with Axiovision software version 4.8. Representative images of the subjective average staining are shown.

### ChIP

Aboveground tissue of 10-d-old, LD-grown *p35S:NF-YB2-YFP/HA* was harvested at 14 h after lights on. The nuclei extraction and chromatin cross-linking was performed using a protocol from the lab of Eric Lam (Rutgers). Young leaves (1 to 3 g) were ground in liquid nitrogen and immediately transferred into 30 mL of nuclear isolation buffer (10 mM HEPES, pH 7.6, 400 mM Suc, 5 mM KCl, 5 mM $MgCl_2$, 5 mM EDTA, 1% formaldehyde, 14 mM 2-mercaptoethanol, 0.6% Triton X-100, and 0.4 mM phenylmethylsulfonyl fluoride) for 10 min at room temperature. Next, 2 mL of 2 M Gly was added to stop the cross-linking for 5 min with gentle mixing. The lysate was then filtered (Miracloth on top of a piece of Sefar Nitex mesh) and nuclei were pelleted at 2800*g* (4000 rpm with 15-cm-diameter rotor) for 10 min. Chromatin shearing was performed using a Bioruptor UCD300 per the manufacturer's instructions (Diagenode). Immunoprecipitations were performed using μMACS GFP-tagged microbeads. μMACS cMyc-tagged microbeads were used as mock ChIP controls (nonimmune controls). The immunoprecipitation procedure follows the ChIP protocol described by Miltenyi Biotec. Quantitative PCR (qPCR) was performed on a Bio-Rad CFX Connect real-time system with Maxima SYBR Green/ROX qPCR Master Mix (Fermentas). The qPCR profile was 2 min at 50°C and 10 min at 95°C, 45 cycles of 10 s at 95°C, and 1 min at 55°C followed by the default dissociation step for melt curve. Primers are listed in Supplemental Table 1. ChIP efficiency was calculated as percent of input as previously described (Haring et al., 2007).

### Recombinant Protein Purification and EMSA

Mouse NF-YA, NF-YB, and NF-YC recombinant proteins were produced and purified from *Escherichia coli* as described (Liberati et al., 1999, 2001). *Arabidopsis thaliana* NF-YB2 and NF-YC3 expression and purification with pET32 vectors were described by Calvenzani et al. (2012). To obtain NF-YB/NF-YC dimers, $Ni^{2+}$-purified soluble NF-YB2 and NF-YC3 from inclusion bodies (IB) were mixed in denaturing IB buffer (20 mM Tris, pH 8.0, 100 mM KCl, 6 M GnCl, and 2 mM DTT) in a 1:4 ratio. Refolding was obtained by 2-h dialysis steps against BC300 buffer (20 mM Tris, pH 7.8, 300 mM KCl, 10% glycerol, and 1 mM DTT) containing urea at decreasing concentrations (2.5/1.25/0.625 M), followed by overnight dialysis against BC100 (BC300 containing 100 mM KCl). Unfolded insoluble proteins were removed by centrifugation (18,000*g* × 20'), and the soluble fraction containing refolded dimers was recovered. Protein concentration was determined by Bradford protein assay.

For nonradioactive EMSAs (Figure 2B), 5′ Cy5-labeled double-stranded DNA oligo Cy5-*CCAAT* probe (20 nM) (Sigma-Aldrich; see Supplemental Table 1 for sequence) was incubated with NF-YB/NF-YC refolded dimers (240 nM) the presence or absence of NF-YA. NF-YA was added at 80 nM (lanes 2 and 3), 120 nM (lane 14), or 240 nM (lanes 4 to 13). Reactions were incubated in NF-Y binding buffer (12 mM Tris, pH 8, 50 mM KCl, 50 mM NaCl, 5 mM $MgCl_2$, 0.5 mM EDTA, 12% glycerol, and 0.2 mg/mL BSA) including 0.5 μg polydA:dT (P0883; Sigma-Aldrich) per reaction, and unlabeled competitor oligos, or TE, as indicated. Unlabeled competitor was present in molar excess with respect to the labeled probe (20 nM) as follows: 2X, 5X, and 20X for *CCAAT*; 5X and 20X for *CCAAT*mut and CORE1/2 (lanes 5 to 7, 8 and 9, 10 and 11, and 12 and 13, respectively). Binding reactions were incubated for 30 min at 25°C and then separated by electrophoresis on 4% acrylamide gels in 0.25× TBE. Fluorescence gel images were obtained with a Typhoon 8610 Variable Mode Imager (Molecular Dynamics).

### 3C-qPCR

3C experiments were performed according to the method described for maize (*Zea mays*) with minor alterations (Louwers et al., 2009a). In short, leaves from 2-week-old LD-grown *Arabidopsis* were cross-linked using formaldehyde and nuclei were isolated. Isolated chromatin was digested using *Dpn*II (New England Biolabs) followed by ligation (T4 DNA ligase; Fermentas) in a large volume and reverse cross-linking overnight. Ligated DNA was phenol-chloroform extracted and precipitated with 1 volume sterile MilliQ water, 1 volume 7.5 M NH4OAc, 40 μL glycogen (20 mg/mL), and 4 volumes 100% ethanol at −80°C overnight. Real-time PCR quantification of ligation products was performed on an Applied Biosystems 7500 real-time PCR system using JumpStart Taq ReadyMix (Sigma-Aldrich) and dual-labeled oligonucleotides (5′ 6-FAM and 3′ TAMRA) probes (Sigma-Aldrich). Primer and probe sequences are listed in Supplemental Table 1. PCR program was 2 min at 95°C, 45 cycles of 15 s at 95°C, and 1 min at 60°C.

To correct for differences in quality and quantity of the template, an internal control was required. To this end, the ligation product of an unrelated locus was used in the normalization of the qPCR data. *EF-1*α was identified to fulfill the required criteria (Czechowski et al., 2005; Louwers et al., 2009a). To control for differences in primer set efficiency during PCR amplification, a control template was required that contains all possible ligation products of the loci of interest (*FT* and *EF1*α) in equimolar amounts. The *FT* template was obtained using the above-mentioned *FT* construct (6.5-kb *FT* promoter plus *FT* genomic coding region in pDONR207). Five micrograms of *pFT:FT* construct was digested for 6 h with *Dpn*II (New England Biolabs), extracted with phenol-chloroform, and precipitated. Isolated DNA was ligated for 1 h at room temperature, followed by 5 h at 16°C (Fermentas; 100 μL 10× ligation buffer, 5 μL ligase [5 units/μL], and 895 μL water). The ligated DNA was purified by phenol-chloroform extraction, precipitated, washed, and dissolved in water. The *EF1*α control template was amplified from one of our 3C samples using primers EF1a-F and EF1a-3C-R2 (see Supplemental Table 1 for primers). The amplified fragment was gel purified and subjected to digestion, ligation, extraction, and precipitation as with *pFT:FT*. Serial dilutions of the resulting *EF1a* DNA was used to obtain standard curves that covered the same range of qPCR signals as obtained with the concentrations of the ligation products in the 3C samples, and then the standard curves were used to calculate fold difference of 3C samples according to different primer pairs. Data were normalized to interaction frequencies measured at the *EF1*α locus using the relative standard curve method (Cao et al., 2011). For each 3C experiment, qPCR was performed with three biological replicates each containing 40 to 50 plants. For each normalized data point, the mean and SE were calculated using the Prism software package. Each experiment was replicated at least two times.

### Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: AT1G65480 (FT), AT5G15840 (CO), At5g47640 (NF-YB2), At4g14540 (NF-YB3), At1g54830 (NF-YC3), At5g63470 (NF-YC4), At1g08970 (NF-YC9), and At5g60390 (EF-1α).

### Supplemental Data

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

**Supplemental Figure 1.** Additional ChIP Assays for NF-Y Binding at *pFT*.

**Supplemental Figure 2.** The Triple Mutant *nf-yc3 nf-yc4 nf-yc9* Was Similarly Affected in Chromatin Loop Formation as the *nf-yb2 nf-yb3* Double Mutant.

**Supplemental Table 1.** Primers, Oligos, and Probes Used in the Course of Study.

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## AUTHOR CONTRIBUTIONS

B.F.H., S.C., and R.W.K. principally conceived and designed experiments. B.F.H. and R.M. supervised the study. S.C., R.W.K., N.G., and A.M.C. performed experiments. B.F.H. wrote the article with assistance from all of the authors.

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