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Cis-regulatory architecture downstream of *FLOWERING LOCUS T* underlies quantitative control of flowering in *Arabidopsis thaliana*

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Abstract

Background: The *FLOWERING LOCUS T* (*FT*) gene is a central integrator of floral induction in *Arabidopsis thaliana*, with its precise expression controlled by complex transcriptional networks. While upstream regulatory regions are well-studied, the role of downstream *cis*-regulatory elements in modulating *FT* expression remains poorly characterized.

Results: Systematic dissection of the *FT* downstream region in its native chromosomal context using CRISPR/Cas9-mediated genome editing provides genetic evidence that a 2.3-kb sequence, encompassing the Block E enhancer immediately adjacent to the *FT* coding sequence, is essential for proper *FT* expression and timely flowering. Fine-scale deletions within Block E reveal that a 63-bp sequence containing one CCAAT-box and one G-box, both closely spaced, forms a core functional module, whereas other conserved motifs contribute modestly in a context-dependent manner. Strikingly, a cryptic CCAAT-box module downstream of Block E that becomes active when repositioned. This coincides with increased transcription factor occupancy and local chromatin accessibility.

Conclusions: Our work reveals that quantitative *FT* expression and flowering time are governed by the spatial organization and chromatin context of downstream *cis*-regulatory elements. The positional sensitivity and modular logic of these elements provide framework for understanding and engineering quantitative gene regulation through targeted *cis*-regulatory design, a concept broadly applicable across diverse developmental systems.

Keywords: *FLOWERING LOCUS T* (*FT*), *Cis*-regulatory elements, Downstream enhancer, CCAAT-box, CRISPR/Cas9, Flowering time regulation, *Arabidopsis thaliana*

Background

Flowering time in *Arabidopsis* is triggered by the mobile florigen encoded by *FLOWERING LOCUS T* (*FT*), whose transcription is restricted to phloem companion cells of leaves before flowering and tightly tuned by environmental conditions and



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developmental stage [1–5]. Under long days (LD), *FT* transcripts peak at dusk, and in natural conditions show a bimodal pattern (morning and dusk), underscoring strong temporal control [6, 7]. Understanding how *FT* integrates external with developmental cues is therefore central to explaining seasonal flowering.

Multiple flowering pathways converge at the *FT* locus through activators and repressors acting on defined cis-elements [8–10]. The key activator, CONSTANS (CO), tightly controlled by the circadian clock and light, promotes *FT* through CO-responsive elements (COREs) in its proximal promoter and acts in phloem companion cells overlapping *FT* [3–5, 11]. PHYTOCHROME INTERACTING FACTOR 4 (PIF4) and PIF7 activate *FT* under warm or shaded conditions by binding promoter and downstream sites [12–14]. MADS-box transcription factors, including FLOWERING LOCUS C (FLC), SHORT VEGETATIVE PHASE (SVP), and AGAMOUS-LIKE 15 (AGL15), repress *FT* via CArG-box motifs in the promoter and first intron, with AGL15 additionally binding downstream [15–17]. AP2-like repressors, such as SCHLAFMÜTZE (SMZ) and TARGET OF EAT1 (TOE1), suppress *FT* either directly, by binding promoter and downstream regulatory regions [18–20], or indirectly, by interfering with CO function [21]. Together, these regulators act through cis-elements distributed across the *FT* locus to ensure tight spatiotemporal control.

Our previous work identified conserved promoter elements, including Blocks A, B, and C, that contribute strongly to *FT* regulation [4, 22, 23]. Beyond promoter regions, cis-regulatory elements can also occur downstream, where they function as transcriptional enhancers that integrate developmental and environmental cues [24–26]. Such downstream enhancers are increasingly recognized across eukaryotes, including plants, animals, and fungi, and can act over large genomic distances [27–31]. We recently identified Block E, an accessible region ~ 1.4 kb downstream of *FT*, as a novel enhancer [32]. Block E is conserved across Brassicaceae and harbors multiple conserved transcription factor binding motifs: two CCAAT-boxes, a G-box, an I-box, a RE- α , and a TBS-like motif [20, 23, 32]. It overlaps with binding sites of both activators (PIF4, PIF7) and repressors (AGL15, SMZ, TOE1) [17–20, 33, 34]. The CCAAT-box is one of the most frequently occurring cis-elements in eukaryotic promoters and enhancers, recognized by the conserved NF-Y heterotrimeric transcription factor complex [35–37]. The G-box is probably the direct binding site for activators PIF4 and PIF7, while the TBS-like motif is bound by AP2-like repressors TOE1 [20]. Yet it remains unclear whether additional essential elements lie downstream, which Block E motifs are required in vivo, and whether Block E acts as a multifunctional platform that integrates both activating and repressive signals.

Here, using CRISPR/Cas9 editing at the native locus, we show that a 2.3-kb region immediately downstream of *FT* (including Block E) is indispensable for *FT* expression and flowering, whereas more distal sequences are largely dispensable. Fine dissection identifies a 63-bp core in Block E, comprising a unit of one closely spaced CCAAT- and G-box, as necessary for enhancer activity; other motifs contribute modestly and context-dependently. We also uncover a latent downstream CCAAT-box module that becomes active when repositioned closer to Block E. Our findings reveal a modular, position-sensitive downstream architecture of cis-regulatory code that confers robustness and plasticity to *FT* regulation.

Results

Identification of potential regulatory elements including expressed long non-coding RNAs downstream of *FT*

To investigate the presence of additional *cis*-regulatory elements in the downstream region, we analyzed a 14.3-kb sequence downstream of *FT* that contains only two small peptide-coding genes, *AT1G65481* and *AT1G65483* (Fig. 1a). Comparative genomic analysis of *A. thaliana* and five related species revealed high conservation in one coding gene (*AT1G65481*) and four non-coding regions: Block E, *AT1NC09061*, *AT1G08757*, and *AT1G08763* (Fig. 1a). *AT1G08757* and *AT1G08763* are annotated as long non-coding RNAs (lncRNAs) in Araport11, while *AT1NC09061*, although not annotated, was identified as a lncRNA in a large-scale tiling array analysis [38]. Despite the entire 14.3-kb region being enriched in H3K27me3, two prominent open chromatin regions were detected in leaf [39]: one encompassing Block E and another at the transcription start site of *AT1G08757* (Fig. 1a). Given the potential involvement of lncRNAs in gene regulation, these three conserved lncRNAs (*AT1NC09061*, *AT1G08757*, and *AT1G08763*) were considered candidate regulatory elements of *FT*.

We then assessed the diurnal expression patterns of *FT* and these three lncRNAs in *Arabidopsis* Col-0 wild type grown under long-day (LD) conditions. As previously reported [7, 40–42], *FT* displayed a clear diurnal rhythm with a small morning peak and a larger dusk peak (Additional file 1: Fig. S1a). Expression of *AT1NC09061* was undetectable in our experiments, suggesting very low or silenced expression, possibly due to its proximity to a transposon (Fig. 1a). Interestingly, *AT1G08757* also exhibited a clear diurnal expression pattern, with a morning trough and a flattened evening peak (Additional file 1: Fig. S1b), whereas *AT1G08763* showed no discernible diurnal rhythm (Additional file 1: Fig. S1c).

The spatial expression pattern of *AT1G08757* was further examined using GUS staining (Additional file 1: Fig. S1d). Transgenic lines harboring the *AT1G08757p-GUS* construct showed GUS activity in the vasculature of cotyledons and the distal regions of the first true leaves, resembling the *FT* promoter-driven expression pattern, with additional signals at leaf hydathodes.

Together, these analyses show that three conserved lncRNAs reside within the 14.3-kb downstream region of *FT*, of which *AT1G08757* displays spatial and temporal expression patterns overlapping with *FT*.

The immediate 2.3-Kb downstream region of *FT* is essential for its expression and floral transition

To assess the functional significance of the downstream lncRNAs and broader regulatory region, we generated CRISPR/Cas9 deletions across the 14.3-kb interval downstream of *FT*. Seven sgRNAs were assembled into a Cas9 expression vector and transformed into Col-0 (Fig. 1b). Deletion of the annotated *AT1G08757* (*d1*, *d2*) had no effect on flowering time or *FT* expression under LD conditions (Fig. 1c, d), indicating that *AT1G08757* is dispensable for *FT* regulation in these conditions.

We then generated larger deletions spanning 2.5–11 kb (*d3–d6*; Fig. 1b). Under LD conditions, only *d3* and *d4* mutants, which removed Block E, showed delayed flowering

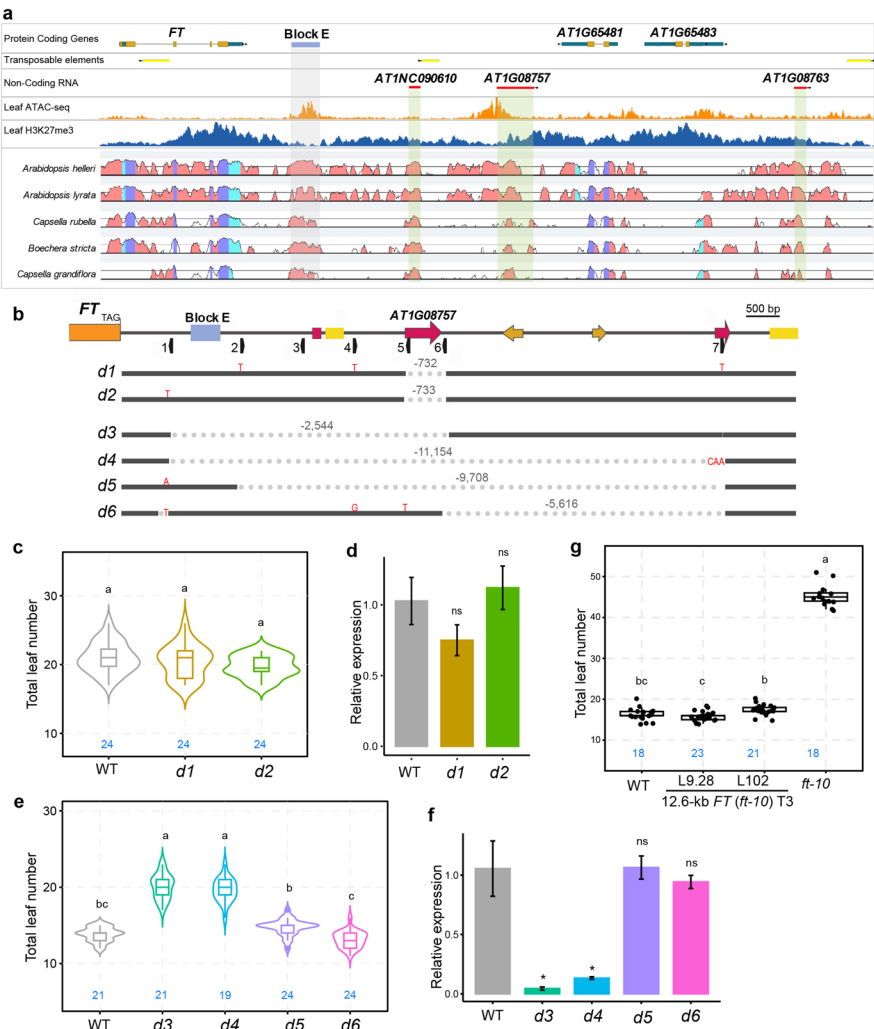


Fig. 1 The immediate 2.3-Kb downstream region of *FT* is essential for proper expression and flowering. **a** Genomic context of the *FT* locus and 14-kb downstream region. Protein coding genes, transposable elements, and non-coding RNAs are shown according to the Araport11 annotation. The *FT* downstream enhancer Block E is indicated by a blue box and gray shading. ATAC-seq and H3K27me3 ChIP-seq coverage tracks depict open chromatin and Polycomb-targeted regions, respectively [39]. The VISTA plot shows percent identity to syntenic regions in other Brassicaceae species. **b** Scheme of ~14 Kb *FT* downstream region and nature of 6 deletion variants (*d1*–*d6*) generated by CRISPR/Cas9 genome editing. Genomic features are indicated by boxes: Block E (blue), non-coding transcripts (red), transposable elements (yellow), and annotated coding transcripts (orange). **c** Flowering time measured as leaves produced at the main shoot until flowering in wild type (WT) and two independent deletion mutants (*d1*, *d2*) of non-coding transcript *AT1G08757*. Blue numbers within the plot indicate the number of plants analyzed for flowering time. Statistical analysis by ANOVA with posthoc Tukey HSD testing; different letters denote significant differences. **d** *FT* transcript accumulation in genotypes as in **c** measured by RT-qPCR for samples collected at ZT16 from 12-day old seedlings grown under long days. Relative expression was calculated using the $2^{-\Delta\Delta Cq}$ method with *PP2A* as reference. Significance by Welch's t-test (ns $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). **e** Analysis of flowering time as in **c** for two deletions encompassing Block E (*d3*, *d4*) and two deletions downstream of Block E (*d5*, *d6*). **f** *FT* transcript accumulation measured as in **d** for genotypes used in **e**. **g** Complementation test showing flowering time of two independent single-copy transgenic lines carrying a 12.6-kb genomic fragment of *FT* (Chr1:24,323,415–24,335,986) encompassing all regulatory regions

and reduced *FT* expression, whereas *d5* and *d6*, which retained Block E but eliminated downstream lncRNAs, were indistinguishable from wild type (Fig. 1e, f). These results confirm Block E is essential for *FT* expression and floral induction, while excluding a functional role for the lncRNAs.

To further test the sufficiency of the downstream interval, we performed complementation assay using a 12.6-kb *FT* genomic fragment encompassing the 8.1-kb promoter, coding region, and 2.3-kb downstream sequence (Additional file 1: Fig. S2a). T2 populations of five independent lines segregated into wild-type-like and *ft-10*-like flowering, reflecting transgene presence (Additional file 1: Fig. S2b). Early flowering in L6 and L7 correlated with multiple transgene copies, whereas single-copy lines L8 and L9 flowered near WT, with L9 showing minimal variation (Additional file 1: Fig. S2b, c). Homozygous T3 plants from two independent lines flowered uniformly at WT timing under LD conditions (Fig. 1g), demonstrating full phenotypic complementation of the *ft-10* mutant by a single-copy 12.6-kb *FT* genomic fragment. Consistent with this complementation, *FT* transcript levels in the two transgenic lines closely matched the wild-type photoperiod-dependent expression pattern in LD and SD growth conditions (Additional file 1: Fig. S2d).

A 63-bp sequence containing CCAAT-box within Block E is crucial for Block E's enhancer activity

To explore the function of the 2.3 kb downstream region of *FT*, we generated a series of mutants using CRISPR/Cas9 and guide RNAs targeting Block E and its surrounding sequences (Fig. 2a). Comparative sequence analysis across six Brassicaceae species revealed strong conservation of the I-box, the first CCAAT-box, and the RE- α motif (Additional file 1: Fig. S3). The G-box and TBS-like motifs were also highly conserved but contained a single substitution in the two *Capsella* species. The second CCAAT-box was restricted to *Arabidopsis* and *Boechera*, while the PBE-box was unique to *A. thaliana*. A fully conserved TATA-box further suggested that Block E carries promoter-like features.

Mutant *e1*, lacking the PBE-box, second CCAAT-box, RE- α , and TBS-like motifs, flowered slightly earlier than wild type in some experiments, or showed a weak early-flowering tendency in others (Fig. 2b and Additional file 1: Fig. S4a). *FT* expression analysis over a 24-h time course showed a marginal, non-significant increase at ZT4 in *e1* seedlings (Fig. 2c), suggesting a role for these motifs in repression, potentially through AP2-like factors acting at the TBS-like site. In contrast, *e2*, which carries deletions similar to *e1* plus an additional 63-bp deletion encompassing the first CCAAT-box and G-box within Block E (Fig. 2a), flowered significantly later than wild type though earlier than *ft-10*, with strongly reduced *FT* expression across the day (Fig. 2b, c, d). The phenotypic contrast between *e1* and *e2* highlights the contribution of the 63-bp sequence to Block E enhancer activity.

Additional late-flowering Block E mutants (*e3*–*e5*) were identified. Deletion of the entire Block E (*e3*, *e4*) or almost all of it while retaining only 13 bp of the critical 63-bp region (*e5*) consistently reduced *FT* expression and delayed flowering, but did not alter its diurnal rhythm (Fig. 2c, d), indicating Block E controls expression amplitude rather than rhythmicity and the Block E G-box alone is insufficient for full enhancer activity.

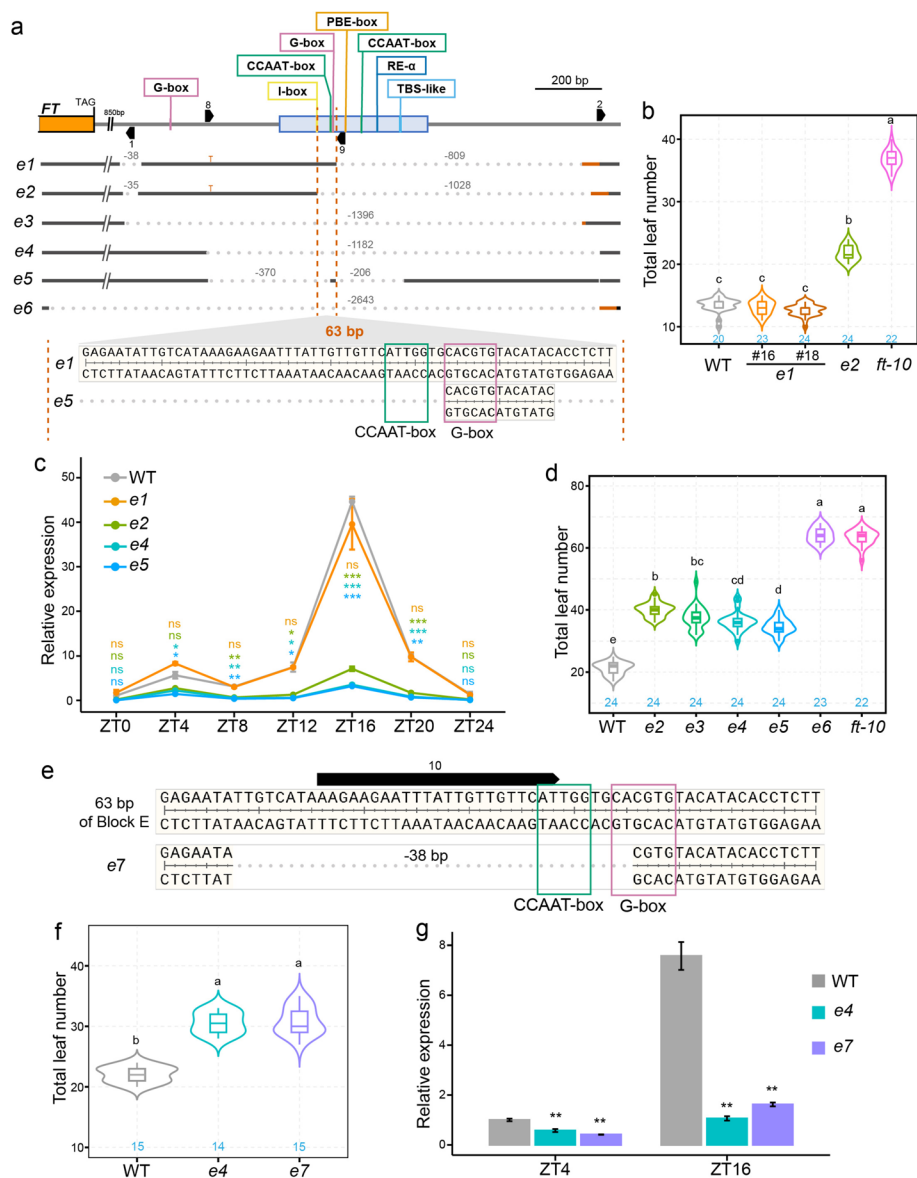


Fig. 2 CCAAT-box and G-box are crucial for Block E in promoting *FT* expression and floral transition. **a** Scheme of 2.5 Kb of *FT* down-stream region and nature of 6 Block E deletion variants (*e1-e6*) generated by CRISPR/Cas9 genome editing. The locations of Block E are indicated by a blue box, the locations of relevant *cis*-motifs are indicated by colored lines. The positions of sgRNAs used for genome editing are indicated by black arrowed boxes. **b** Flowering time measured as leaves produced at the main shoot until flowering in wild type (WT), two independent segregants of *e1*, *e2*, and *ft-10* grown in the greenhouse. Blue numbers within the plot indicate the number of plants analyzed for flowering time. Statistical analysis by ANOVA with posthoc Tukey HSD testing; different letters denote significant differences. **c** *FT* transcript accumulation from *e1*, *e2*, *e4*, *e5*, and WT measured by RT-qPCR for samples collected at 4 h intervals during a period of 24 h from 12-day old seedlings grown under long days. Relative expression was calculated using the $2^{-\Delta\Delta Cq}$ method with *PP2A* as reference. Statistical analysis by Welch's t-test, significant differences indicated by stars (ns $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). **d** Flowering time analyzed as in **b** for deletions (*e2-e5*) that included a 63 bp region of Block E which contains a CCAAT-box and a G-box. *ft-10* was used as loss-of-function control, deletion *e6* includes a partial deletion of the *FT* coding region. **e**, Detailed view of the 63 bp sequence of Block E in WT and the 38 bp deletion line *e7* that lack the CCAAT-box and the first two base pairs of the G-box. The position of sgRNA 10 is indicated above with a black arrowed box. **f** Flowering time analyzed as in **b** for deletions *e4* and *e7*. **g** *FT* transcript accumulation from genotypes as in **f** measured as in **d**.

Additionally, the presence of G-box in immediately upstream of Block E did not compensate for enhancer loss, as *e2*, *e3*, and *e4* phenotypes were comparable (Fig. 2d). As a control, *e6*, disrupting the last exon of *FT*, was indistinguishable from *ft-10* (Fig. 2d).

Public PIF7 ChIP-seq data showed strong enrichment at Block E specifically under low red/far-red (R/FR) light [34], consistent with shade-induced *FT* activation (Additional file 1: Fig. S4b, c) [13, 14]. In agreement, *FT* expression was reduced in the *pif457* triple mutant only under far-red light (WL + FR), whereas Block E mutants *e3*, *e4*, and *e5* exhibited reduced *FT* expression under both control (WL) and far-red light conditions (Additional file 1: Fig. S4c). Although far-red light elevated *FT* expression across all genotypes, *FT* levels in *e3*, *e4*, and *e5* remained consistently lower than wild type, with no significant differences among the three mutants (Additional file 1: Fig. S4c). Similar to *pif457*, the induction ratio of *FT* by far-red light (WL + FR/WL) was significantly reduced in the Block E mutants compared to wild type (Additional file 1: Fig. S4c). Together, these results demonstrate Block E is required for maintaining *FT* expression under both normal and shade conditions.

To specifically test the importance of the first CCAAT-box and G-box, we generated mutant *e7* with a 38-bp deletion within the critical 63-bp region of Block E, disrupting both motifs (Fig. 2e). *e7* flowered as late as *e4* and had similarly reduced *FT* expression at ZT4 and ZT16 (Fig. 2f, g), confirming that both motifs contribute to Block E enhancer function, though their individual roles remain unresolved. Collectively, these results establish Block E as a critical enhancer of *FT* expression, with the 63-bp region containing the first CCAAT-box as central to its regulatory function.

Minor contributions of the second CCAAT-box, RE-alpha, and TBS-like motifs in Block E to *FT* expression and flowering

To further dissect the contribution of individual transcription factor binding sites within Block E, we generated CRISPR/Cas9 mutants targeting the second CCAAT-box (*e8*), RE-alpha (*e9*), and TBS-like (*e10*) motifs. In *e8*, 27-bp encompassing the second CCAAT-box was replaced by 5 bp; In *e9*, a 67-bp fragment containing RE-alpha was deleted; and in *e10*, a 46-bp deletion removed the TBS-like motif (Fig. 3a). In contrast to the first CCAAT-box and G-box, which are essential for Block E enhancer function (Fig. 2), deletion of these individual sites had limited effects on flowering time and *FT* expression.

Plants carrying the *e8* mutation showed a slight but non-significant trend toward earlier flowering compared to wild type (Fig. 3b). *FT* expression was consistently elevated in 6-day-old seedlings but indistinguishable from wild type by day 13. Notably, the deleted sequence overlaps with a previously defined AGL15-binding region B3 (Fig. 3a), suggesting a potential role in modulating MADS-domain protein enrichment at Block E.

By contrast, the *e9* and *e10* mutants showed delayed flowering relative to WT, with *e9* exhibiting a stronger delay and *e10* an intermediate phenotype (Fig. 3b). In *e9*, *FT* expression was elevated in 6-day-old seedlings, but in 13-day-old seedlings expression was similar to wild type at ZT4 and not significantly reduced at ZT16 (Fig. 3c). In *e10*, *FT* expression remained largely unchanged except for a significant reduction at ZT16 in 13-day-old seedlings (Fig. 3c).

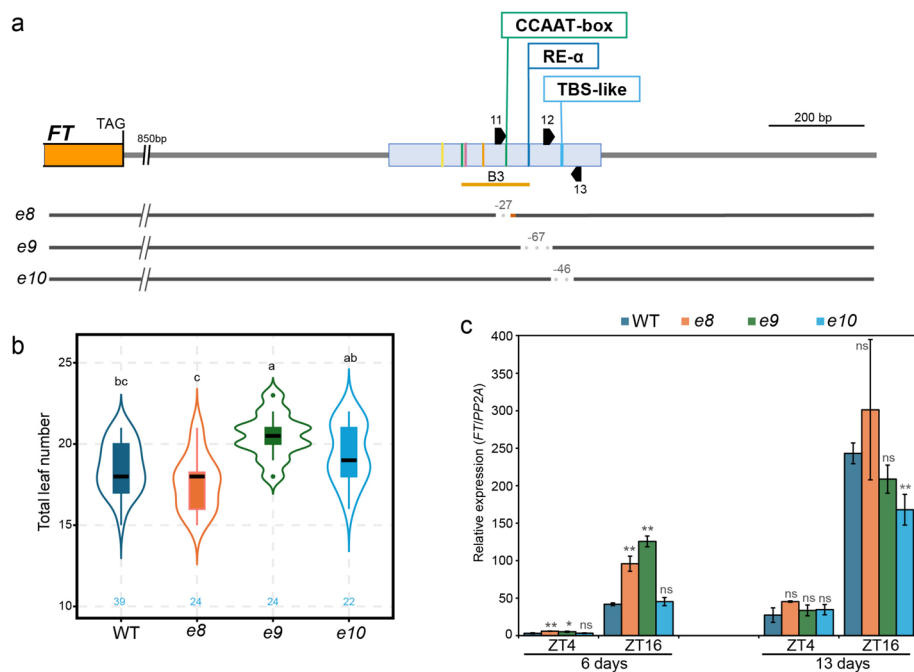


Fig. 3 Deletion of the second CCAAT-box, RE-alpha, or TBS-like motifs minimally affects *FT* expression and flowering. **a** Schematic representation of the 2.5 kb downstream region of *FT* and the CRISPR/Cas9-generated Block E mutants *e8*, *e9*, and *e10*. Block E is indicated by a blue box. Colored lines within the blue box denote the positions of specific motifs, and the second CCAAT-box, RE-alpha, and TBS-like motifs are labeled above as named boxes. Black arrowheads indicate the positions of sgRNAs used for genome editing. The orange underline marks the B3 region associated with AGL15 binding. **b** Flowering time, measured as the total number of leaves produced at the main shoot until flowering, in wild type (WT) and the three mutants (*e8*, *e9*, and *e10*), each targeting a different motif within Block E. Blue numbers within the plot indicate the number of plants analyzed for flowering time. Statistical analysis by ANOVA with posthoc Tukey HSD testing; different letters denote significant differences. **c** *FT* transcript accumulation from genotypes as in **b** measured by RT-qPCR for samples collected at ZT4 and ZT16 from 6- and 13-day-old seedlings grown under long days. Relative expression was calculated using the $2^{-\Delta\Delta Cq}$ method with *PP2A* as reference. Statistical analysis by Welch's *t*-test, significant differences indicated by stars (ns $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

These findings suggest that the second CCAAT-box, RE-alpha and TBS-like motifs each contribute modestly to Block E activity but are not individually required for robust *FT* induction or timely flowering under LD conditions. This limited impact may reflect context-dependent or stage-specific roles for these motifs as indicated by the age-dependent changes in *FT* expression.

A downstream CCAAT-box module may enhance or partially compensate for Block E activity

The extension of the *e1* to *e4* deletions beyond Block E prompted us to investigate the regulatory role of the immediate downstream sequence. We used six CRISPR/Cas9 guide RNAs targeting both Block E and its immediate downstream region, generating mutants *e11* to *e16* (Fig. 4a).

Mutants *e11* and *e12* had deletions largely confined to Block E. All known Block E motifs (including the first CCAAT-box and G-box) were removed in both mutants, with only the second CCAAT-box and RE-alpha motif retained in *e11* (Fig. 4a). Consistent

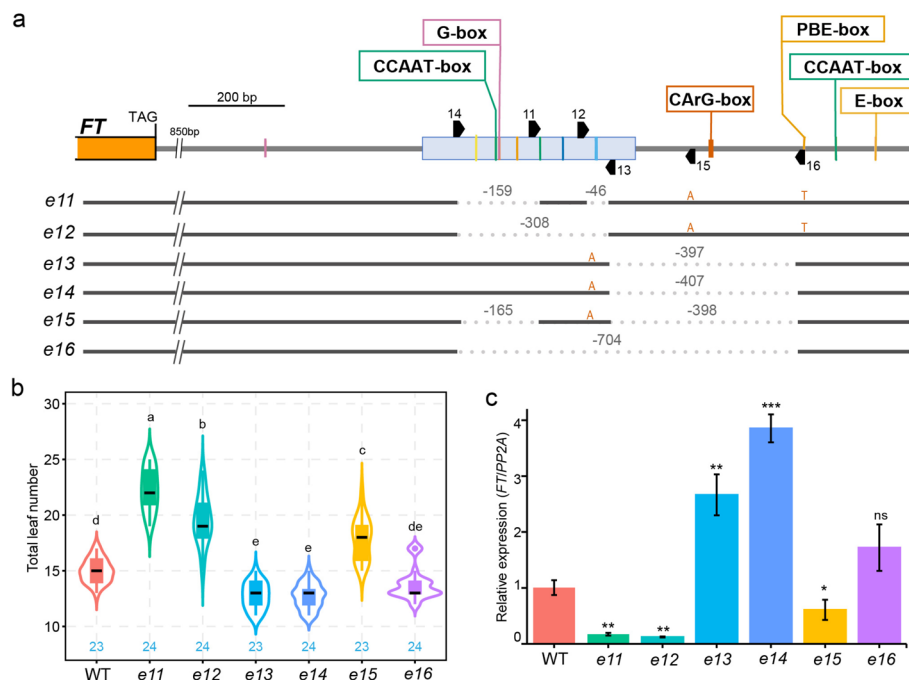


Fig. 4 Deletions of the Block E core and its immediate downstream region differentially affect *FT* expression and flowering. **a** Schematic of the 2.5 kb *FT* downstream region and CRISPR/Cas9-generated deletion variants (*e11–e16*). Block E is indicated by a blue box. Relevant *cis*-regulatory motifs within and downstream of Block E are shown as colored lines, with selected motifs labeled above. sgRNA target sites are marked by black arrowheads. **b** Flowering time measured as leaves produced at the main shoot until flowering in wild type (WT) and deletion lines *e11–e16*. Blue numbers within the plot indicate the number of plants analyzed for flowering time. Statistical analysis by ANOVA with posthoc Tukey HSD testing; different letters denote significant differences. **c** *FT* transcript accumulation in genotypes as in **b** measured by RT-qPCR using RNA from 12-day-old long-day seedlings harvested at ZT16. Relative expression was calculated using the $2^{-\Delta\Delta Cq}$ method with *PP2A* as reference. Significance by Welch's t-test (ns $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

with the essential roles of the first CCAAT-box and G-box, both *e11* and *e12* exhibited delayed flowering and markedly reduced *FT* expression compared to wild type (Fig. 4b, c). Surprisingly, mutants *e13* and *e14*, which carried ~400-bp deletions immediately downstream of Block E, flowered significantly earlier and displayed remarkably higher *FT* transcript levels than wild type (Fig. 4a, b, c). This early-flowering phenotype depended on Block E integrity, as *e15* (harboring the same downstream deletion as *e13/e14* but lacking the first CCAAT-box and G-box of Block E) flowered later than wild type, albeit earlier than *e11* and *e12*, and showed intermediate *FT* expression levels (Fig. 4a, b, c). Mutant *e16*, which deleted both Block E and the adjacent downstream region, had minimal impact on flowering or *FT* expression (Fig. 4a, b, c), suggesting a neutralization of the individual effects of Block E and the immediate downstream sequence when both are deleted.

These contrasting outcomes suggested that the downstream region normally exerts a repressive effect and that its removal alters the functional output of Block E. Sequence analysis revealed a potentially repressive CARG-box motif within the downstream regions deleted in *e13* to *e16* (Fig. 4a). Intriguingly, a PBE-box, CCAAT-box, and E-box module resides immediately downstream of these deleted regions (Fig. 4a). We therefore hypothesized that loss of the CARG-box, coupled with repositioning of these

downstream *cis*-elements, could reshape transcription factor binding at Block E and the downstream module, thereby enhancing or compensating *FT* activation.

To test this hypothesis, we performed Micrococcal Nuclease-defined cistrome Occupancy Analysis (MOA-seq) in wild type, *e12*, *e14*, and *e16* (Fig. 5; Additional file 1: Fig. S5; Additional file 2: Table S1; Additional file 3: Table S2). In wild type, strong transcription factor (TF) binding was detected at the distal enhancers Block C and Block E, whereas little or no occupancy was observed at the proximal promoter (Block A) (Fig. 5a). TF occupancy at Block C, Block A, and their conserved motifs (including the CCAAT-box in Block C) remained unchanged in all Block E mutants (Fig. 5a–d; Additional file 1: Fig. S5a), indicating that deletions surrounding Block E do not globally disrupt the *FT* regulatory landscape.

By contrast, pronounced and genotype-specific changes were confined to CCAAT-boxes within and immediately downstream of Block E. In wild type, strong TF (likely NF-Y complexes) binding was detected at the first CCAAT-box within Block E, with weaker occupancy at the second CCAAT-box and no clear binding at the downstream (third) CCAAT-box (Fig. 5a, e; Additional file 1: Fig. S5b). In *e12*, deletion of Block E eliminated TF binding across the region (Fig. 5a, b; Additional file 1: Fig. S5b), indicating that Block E is essential for maintaining local chromatin accessibility. In *e14*, deletion between Block E and the third CCAAT-box repositioned the three CCAAT-boxes in closer proximity, resulting in strong binding at both Block E CCAAT-boxes and novel binding at the third CCAAT-box (Fig. 5a, c, e; Additional file 1: Fig. S5b), indicating enhanced TFs (NF-Ys) recruitment and correlating with elevated *FT* expression and early flowering. In *e16*, removal of both Block E and its immediate downstream sequences placed the third CCAAT-box at the former location of the first CCAAT-box, allowing TFs (NF-Ys) binding and remaining of chromatin accessibility (Fig. 5a, d, e; Additional file 1: Fig. S5b) and leading to a wild type-like flowering time. TF occupancy at other predicted motifs in and around Block E was weak or undetectable and was largely consistent across genotypes wild type, *e14*, *e16* (Fig. 5e). Together, these results identify CCAAT-boxes within and downstream of Block E as the primary *cis*-elements whose altered occupancy underlies the flowering phenotypes of Block E-associated mutants.

Our combined genetic and MOA-seq analyses support a model in which the activity of the CCAAT-box module downstream of *FT* is determined not only by motif identity but also by their spatial arrangement and chromatin context (Fig. 5f). In wild type, the first CCAAT/G-box module in Block E is essential and dominant, while the downstream CCAAT-box remains largely inactive, likely constrained by the intervening CArG-containing region and/or an unfavorable chromatin context. In late-flowering *e12* mutants, deletion of Block E CCAAT motifs abolished TF-binding and reduced *FT* expression. By contrast, early-flowering *e13* and *e14* retained Block E but lost the putative repressive barrier, repositioning the downstream CCAAT-box module next to Block E and boosting enhancer activity. In *e16*, deletion of both Block E and the barrier relocated the downstream module to the Block E position, enabling TF binding and restoring wild-type flowering. Together, these findings reveal that the downstream CCAAT-box module acts as a latent enhancer, activated only when placed in a favorable chromatin and spatial context.

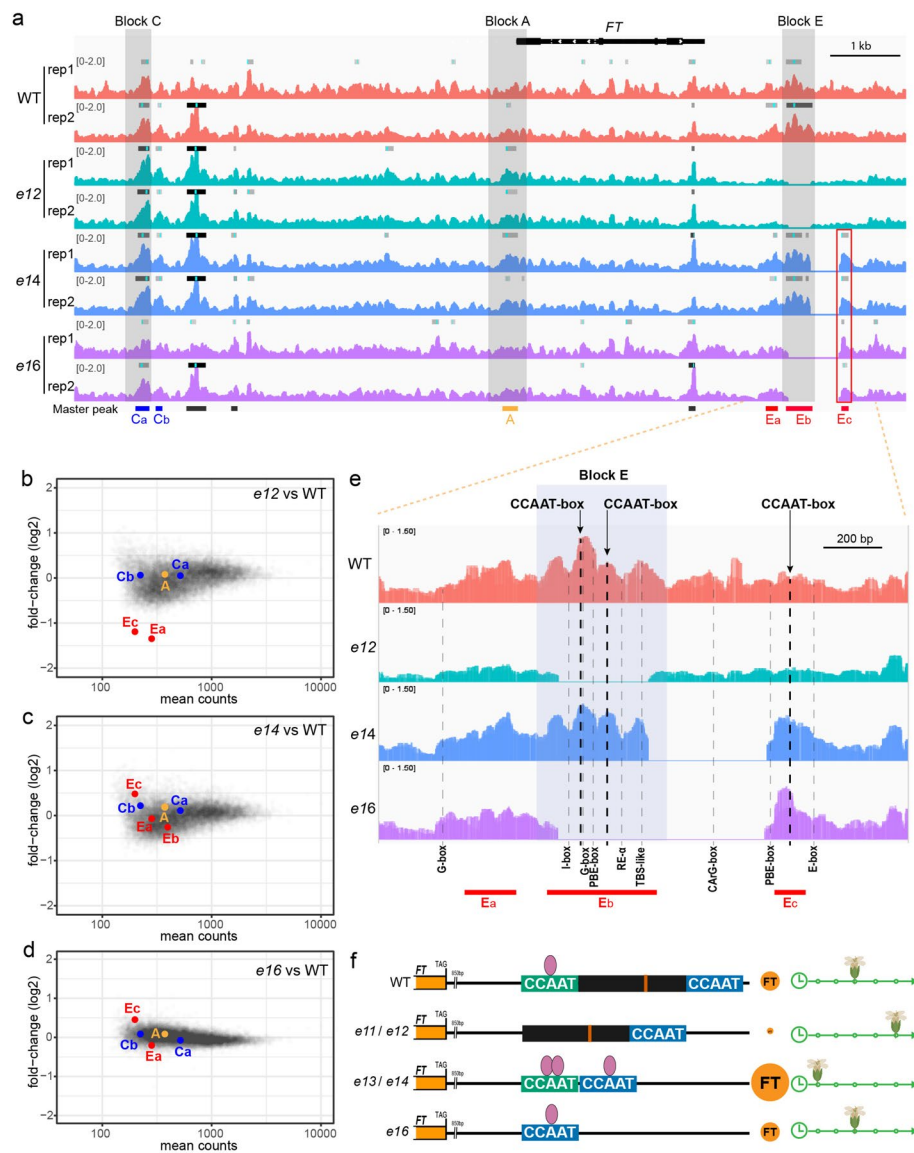


Fig. 5 Chromatin occupancy analysis reveals activation of a latent downstream CCAAT-box module after genomic rearrangement. **a** MOA-seq read coverage from two biological replicates (rep1 and rep2) across the *FT* locus, including upstream and downstream regions (~10 kb total), in wild type (WT; red), *e12* (cyan), *e14* (blue), and *e16* (purple). The *FT* gene structure is shown above the tracks, and conserved regulatory regions Block C, Block A, and Block E are indicated by gray shading across the tracks. Peaks (gray to black boxes, darker colors indicating higher scores) and peak summits (blue lines) detected as enriched by MACS3 are indicated above the coverage tracks for each replicate. A master peak list with peaks detected in at least two genotypes is shown at the bottom, with the Block C peaks (Ca, Cb), Block A peak (A), and Block E-associated peaks (Ea, Eb, Ec) colored in blue, orange, and red, respectively. The red frame highlights downstream Ec peaks that appear specifically in *e14* and *e16*. **b–d** MA-plots showing log-fold change and base mean of count data as determined by DESeq2 after dispersion correction at all peaks of chromosome 1 for *e12*-vs-WT (**b**), *e14*-vs-WT (**c**) and *e16*-vs-WT (**d**). Peaks located at *FT* are indicated by colors and labels as in (**a**). **e** Overlay of MOA-seq read coverage from two biological replicates around the Block E region (1.8 kb) in WT, *e12*, *e14*, and *e16*. The positions of indicated DNA motifs are shown by dashed lines across the tracks. **f** Model illustrating the positional and contextual effects of the downstream CCAAT-box on *FT* expression and flowering time. The downstream CCAAT-box module (blue) can compensate for the loss of the Block E CCAAT-box module (green) and enhance expression when repositioned adjacent to it. A black box represents a regulatory barrier, with the embedded red line indicating the *CArG*-box. Purple ovals represent transcription factors (e.g., NF-Y). Orange circles labeled “FT” indicate *FT* expression, with circle size reflecting relative expression levels. Clocks with arrows and flowers depict flowering time, with flower position along the arrow indicating earlier or later flowering.

Discussion

Our study uncovers the critical and previously underappreciated role of the 2.3-kb region downstream of *FT*, particularly the distal enhancer Block E, in sustaining full *FT* transcription and ensuring timely flowering under LD conditions. Genetic deletion of this region, or of key motifs within Block E, caused significant *FT* downregulation and delayed flowering. These findings challenge the historical focus on upstream regulatory sequences and demonstrate that downstream elements can be equally essential. We recommend that future genomic complementation of *ft* mutants, and broader studies of the *FT* regulatory landscape, should include both upstream and downstream sequences to achieve faithful expression with single-copy insertions.

By contrast, deletion of three conserved lncRNAs in the *FT* downstream region had no detectable effect on *FT* expression or flowering time under standard LD conditions. One of these, *AT1G08757*, shows diurnal expression and hydathode enrichment, features associated with stress or developmental regulation. Although dispensable under our conditions, such lncRNAs may act in specific environmental contexts, such as abiotic stress or pathogen challenge.

Dissection of Block E revealed that the first CCAAT-box and adjacent G-box, separated by only 2 bp, form an essential composite module. This extreme proximity, makes it challenging to selectively disrupt one motif without affecting the other by CRISPR/Cas9, but likely facilitates cooperative action between NF-Y and G-box-binding factors such as PIFs. This interpretation is supported by ChIP-seq studies showing that NF-Y binding sites are frequently enriched not only for CCAAT motifs but also for G-box-containing elements [43], suggesting NF-Y may also associate with G-boxes either directly or indirectly, potentially through interactions with bZIP or PIF partners. We therefore propose that the first CCAAT-box and adjacent G-box together constitute a core module within Block E enabling NF-Y-mediated transcriptional activation of *FT*. Future work using more precise gene-editing strategies will be required to dissect the individual contributions of these two closely spaced motifs.

Not all conserved motifs in accessible chromatin were essential. The second CCAAT-box, RE- α , and a TBS-like motif within Block E made modest or context-dependent contributions. Their effects often differed between developmental stages or time points, consistent with conditional activity. RE- α deletion transiently increased *FT* expression early in development, in line with its proposed role in repression during darkness [44]. Instead of promoting flowering, the TBS-like motif deletion caused mild late flowering, suggesting it contributes to balancing repressor activity rather than acting as a strict silencer.

A downstream CCAAT-box located beyond Block E was largely inactive in wild type, showing little TF binding by MOA-seq. This inactivity is likely due to an unfavorable chromatin context or to interference from the intervening CArG-box containing region, which may recruit repressors or influence nucleosome positioning. Strikingly, deletion of this barrier in *e13/e14* repositioned the downstream module adjacent to Block E, enabling TF binding and elevated *FT* expression. In *e16*, relocation of this module to the Block E position supported near wild-type flowering. Thus, the downstream CCAAT-box can function as a latent enhancer when placed in a favorable spatial and chromatin context. Analogous to findings in *Drosophila* where Zelda- or

Grainyhead-biased sequences can prime genomic regions to act or evolve as enhancers, our results suggest that the CCAAT-box represents a plant-specific manifestation of this general principle [45].

Together, these findings highlight a modular regulatory architecture in which enhancer activity depends not only on motif identity but also on spatial arrangement and chromatin context. This modularity provides a mechanistic explanation for how regulatory sequences can buffer genetic variation: critical motifs in dominant modules (e.g., Block E) ensure robust *FT* induction, whereas downstream latent modules can compensate under altered genomic contexts. More broadly, plant *cis*-regulatory elements may frequently harbor latent enhancer activity that is constrained in their native genomic contexts. Such latent modules could serve as evolutionary “reserve” elements, capable of being activated by sequence rearrangements, deletions, or chromatin remodeling, potentially contributing to phenotypic plasticity and adaptive evolution. Future work will be needed to determine how widespread such context-dependent enhancer activation is across plant genomes and whether similar principles govern other developmental regulators.

Conclusions

The *FT* downstream regulatory landscape is highly modular and position-sensitive, integrating multiple activators and repressors within a dynamic chromatin environment. This architecture may enable evolutionary flexibility in flowering-time control while preserving core regulatory logic. The principles uncovered here— particularly the positional activation of latent enhancer modules—may inform strategies to engineer precise gene expression in diverse plant species and potentially beyond the plant kingdom.

Methods

Plant material and growth conditions

The Columbia (Col-0) ecotype of *Arabidopsis thaliana* was used as the wild type and for CRISPR/Cas9 mutagenesis. The *ft-10* mutant was described previously [46]. For flowering time analysis, seeds were stratified in the dark at 4 °C for two days and then grown on soil in a greenhouse under LD conditions (16 h light/8 h dark, 20–24 °C). For gene expression analysis and MOA-seq, surface-sterilized seeds were sown on MS medium (4.4 g/L MS salts, 0.5 g/L MES, 10 g/L sucrose, 9 g/L PhytoAgar, pH 5.7). After two days of stratification at 4 °C, plates were transferred to a growth chamber (Percival) under LD conditions (16 h light/8 h dark, 120–150 $\mu\text{mol m}^{-2} \text{s}^{-1}$, 21 °C).

Comparative genomic analysis using VISTA-Point

To identify conserved regions downstream of *FT* across *Arabidopsis* accessions and relative species, the VISTA-Point (<http://genome.lbl.gov/vista/>) was used with pre-computed genome datasets available in its database. The *A. thaliana* genome was selected as the base genome. Conservation thresholds were set to 70% sequence identity across a 100 bp window. The tool visualized sequence conservation within and downstream the *FT* locus, with a focus on non-coding sequences to infer potential regulatory elements. The motifs within *A. thaliana* Block E (Chr1:24,335,009–24,335,622), as well as in the

upstream and downstream regions flanking Block E, were identified using the New PLACE database (<https://www.dna.affrc.go.jp/PLACE/?action=newplace>) [47].

Generation of mutants by CRISPR/Cas9 technology

CRISPR/Cas9 constructs were generated using the genome editing toolkit as described previously [48]. In brief, sgRNAs were designed with CRISPR-P 2.0 [49], annealed oligonucleotides were assembled into shuttle vectors via simultaneous restriction/ligation with *BbsI*-HF (NEB), and subsequently cloned into the dicot genome-editing recipient vector pDGE347 by Golden Gate assembly with *BsaI*-HF (NEB). The resulting CRISPR/Cas9 constructs, carrying sgRNAs targeting the *FT* downstream region, were transformed into *Agrobacterium* strain GV3010(*pSoup*) and introduced into Col-0 plants by floral dip in *Agrobacterium* suspension (5% sucrose, 0.02% Silwet L-77) [50].

Large-deletion mutants (*d3*, *d4*, *d5*) and the small-deletion mutant (*e7*) were generated by crossing primary T1 transformants with wild type, following a previously described strategy [51]. Indels were detected in F1 plants by PCR using primers flanking the target sites and confirmed by Sanger sequencing. CRISPR/Cas9-free homozygous mutants were obtained in the F2 or F3 generation and used for further experiments. For other mutants, indels were identified in T1 or T2 plants, and CRISPR/Cas9-free homozygous lines were selected in the T3 or T4 generation. PCR primers and sgRNA oligonucleotides were synthesized at SIGMA listed in Additional file 4: Table S3.

GUS reporter construct and staining

To generate the binary vector *AT1G08757p-GUS*, a 1.5-kb promoter fragment together with 222 bp of the *AT1G08757* were amplified from Col-0 genomic DNA and cloned into the previously developed *GW-GUS-pGREEN* vector [4] using NotI and HindIII restriction sites. Primer sequences used for amplification are listed in Additional file 4: Table S3. GUS staining was performed as previously described [4].

Generation of complementation constructs and transgenic plants

A 12.6 kb *FT* genomic fragment (spanning 8.1 kb upstream of the start codon to 2.3 kb downstream of the stop codon) was subcloned from the BAC clone F5114 into the *pGAP-Km* vector by homologous recombination as described previously [52]. To enable homologous recombination, two ~600 bp PCR fragments (FLANK1 and FLANK2) were amplified and inserted into *pGAP-Km*: FLANK1 cleaved with *Bam*HI and *Sal*I, FLANK2 cleaved with *Sal*I and *Eco*RI, and the vector cleaved with *Bam*HI and *Eco*RI. The resulting FLANK1–FLANK2–*pGAP-Km* construct was introduced into *E. coli* SW102 cells harboring BAC F5114, and recombination was induced. PCR primers used for FLANK amplification are listed in Additional file 4: Table S3.

The final construct, *pGAP-12.6kFT*, was transformed into *Agrobacterium* strain GV3010(*pMP90RK*) and introduced into *ft-10* plants by floral dipping [50].

Copy number testing

A plasmid containing fragments of *PP2A* and *KanR* cloned in single copy into a pBlue-script KSII backbone was diluted to generate standard curves for absolute quantification of qPCR reactions. The ratios between *KanR* and *PP2A* signals were determined for

standard reactions and for those using DNA prepared from selected transgenic T2 or T3 lines as template. Primers are listed in Additional file 4: Table S3.

Flowering time analysis

Flowering time was assessed by counting the total number of rosette and cauline leaves (including cotyledons) at bolting. Leaf numbers were averaged from at least 11 plants per genotype.

Total RNA extraction and gene expression analysis

Seedlings (12–14 days old) grown under LD conditions were harvested either every 4 h from ZT0 to ZT24 or at defined time points (ZT4 and/or ZT16), as described in the figure legends. Total RNA was extracted using TRIzol™ Reagent (Ambion, Life Technologies), and genomic DNA was removed with the DNA-free™ DNA Removal Kit (Invitrogen, AM1906).

For temporal expression profiling of *FT* and non-coding RNAs, one-step RT-qPCR was conducted with 500 ng of DNA-free RNA in a 10 µL reaction using the iTaq™ Universal SYBR® Green One-Step Kit (Bio-Rad) on a CFX384 Real-Time System (Bio-Rad). For comparative analysis of *FT* expression in wild type and mutants, 2 µg of DNA-free RNA was reverse transcribed into cDNA in a 20 µL reaction using oligo(dT) primers and SuperScript IV Reverse Transcriptase (Invitrogen, Thermo Fisher Scientific). Quantitative PCR was then performed with 1 µL of cDNA in a 10 µL reaction using iQ™ SYBR® Green Supermix (Bio-Rad) on the CFX384 Real-Time System (Bio-Rad), with *PP2A* as an internal control for normalization. Primers are listed in Additional file 4: Table S3.

MOA-seq and data analysis

MOA-seq was performed as described previously [53], with minor modifications. Briefly, ~80 mg of frozen, N₂-ground 12-day-old seedlings were fixed in a freshly prepared solution of 1% paraformaldehyde in a fixation buffer (18 mM PIPES, 80 mM KCl, 20 mM NaCl, 2 mM EDTA, 0.5 mM EGTA, 384 mM sorbitol, 1.2 mM DTT, 0.18 mM spermine, 0.6 mM spermidine, 0.2 mM phenanthroline, 0.4 mM PMSE, and 2 µg/ml each of aprotinin and pepstatin A) for 10 min, quenched with glycine, and nuclei were isolated in MDB buffer (50 mM HEPES, 12.5% glycerol, 25 mM KCl, 4 mM MgCl₂, 1 mM CaCl₂, pH 7.6) by homogenization and filtration. Nuclei were resuspended in 70 µl MDB supplemented with 1% Triton X-100 and digested with MNase (50 or 100 U/ml; buffer only for control) at 37 °C for 15 min. Reactions were stopped with EGTA and RNase A, followed by Proteinase K decrosslinking at 65 °C overnight. DNA was purified using the NEB Monarch PCR & DNA Cleanup Kit, quantified with the Qubit dsDNA HS Assay Kit, and checked on agarose gels.

Libraries were prepared with the NEBNext® Ultra II DNA Library Prep Kit (Illumina®) following the same modifications as Engelhorn et al. [53]. Sequencing was performed on an Illumina NovaSeq 6000 S4 (Col-0 Rep1, *e16* Rep1) or on the DNBSEQ G400/PE85 platform (BGI; all other replicates) in paired-end mode.

Adapters were trimmed with SeqPurge [54] using default settings, ensuring a minimum read length of 20 bp. Paired-end reads were merged with FLASH [55]. Reads were aligned to the *A. thaliana* TAIR10 genome using STAR [56], with maximum

permissible intron size restricted to 1 (`-alignIntronMax 1`) and multimapping tolerance set to 1 (`-outFilterMultimapNmax`), allowing only uniquely mapping reads. Alignments were converted to coordinate-sorted BAM files and coverage tracks generated with deepTools `bamCoverage` [57] using CPM normalization, 1-bp bins, maximum fragment length of 80 bp, and minimum mapping quality of 255. Coverage was visualized using IGV [58]. MOA-signal enriched peaks were identified with the following settings: `-q 0.02 -g 119,667,750 -s 45 -min-length 45 -max-gap 45 -extsize 45 -nomodel -keep-dup all` using MACS3 [59]. Peaks identified in two biological replicates were merged with the irreproducible discovery rate (idr) tool using the following settings: `-peak-merge-method min -only-merge-peaks` [60]. A peak Master list was generated using functions of bedtools [61]. First, all peaks detected in all genotypes were merged with bedtools `merge`; subsequently, the merged peak list was scored against the genotype-specific peaks using bedtools `intersect -c`. Only peaks detected in at least two genotypes were retained in the Master list. To compare signal strength at peaks in the Master list, read coverage for each sample was counted using bedtools `multicov`. The resultant count matrix of peaks was imported to DEseq2 (for Chromosome 1) to perform dispersion corrected read normalization and statistical analysis using the implemented Wald test [62]. MA-plots ($\log_2$ fold-change versus base mean of counts) were generated with the help of the R library ggplot2. Custom scripts to run the bioinformatics pipelines have been deposited in GitHub [63].

Statistical analyses

Statistical tests were performed as described in the figure legends. Pairwise comparisons were made using Welch's *t*-test (ns $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). Multiple-sample comparisons were determined by one-way ANOVA followed with post-hoc Tukey HSD, and significant differences at the $P < 0.01$ level are indicated by different lowercase letters.

All boxplots show the range from the first to the third quartile, lines within boxes indicate the median and whiskers represent 1.5-fold of the interquartile range.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s13059-026-04064-4>.

Additional file 1: Supplemental Figures. Fig S1. Spatiotemporal expression pattern of the non-coding gene AT1G08757. Fig. S2. Full complementation of the late-flowering *ft-10* phenotype by FT with upstream and downstream regulatory elements. Fig. S3. Conservation of cis-regulatory DNA motifs within Block E across Brassicaceae species. Fig. S4. Neither the upstream G-box nor the Block E G-box alone restores FT expression without the CCAAT-box. Fig. S5. MOA-seq reveals selective CCAAT-box occupancy changes around Block E in deletion mutants.

Additional file 2: Table S1. Matrix from differential count analysis.

Additional file 3: Table S2. Merged peaks from two MOA-seq libraries.

Additional file 4: Table S3. Oligonucleotide sequences used in this study.

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Peer review information

Pil Joon Seo and Wenjing She were the primary editors of this article and managed its editorial process and peer review in collaboration with the rest of the editorial team. The peer-review history is available in the online version of this article.

Author contributions

HRZ and FT designed the study, performed data analysis, and wrote the manuscript. HRZ carried out all experimental work. DTHD performed the MOA experiment, and DTHD together with TH and FT analyzed the MOA-seq data. All authors read and approved the final manuscript.

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Data availability

The raw MOA-seq data described in this paper have been deposited at NCBI SRA under accession number PRJEB97763 [64]. No other scripts and software were used other than those mentioned in the [Methods](#) section. Custom scripts to run the bioinformatics pipelines have been deposited under CC0-1.0 license in GitHub [63] and Zenodo [65].

Declarations**Ethics approval and consent to participate**

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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