TITLE PAGE

Title: Abscisic acid and GIGANTEA signalling converge to regulate the recruitment of CONSTANS to the *FT* promoter and activate floral transition

Running title: ABA and GIGANTEA control CO recruitment to the *FT* promoter

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Highlight:

ABA and GIGANTEA signalling promote *FLOWERING LOCUS T* (*FT*) transcriptional activation by regulating the binding of the transcription factor CONSTANS to the proximal *FT* promoter.

Abstract

Plants align flowering with optimal seasonal conditions to increase reproductive success. This process depends on modulating signalling pathways that respond to diverse environmental and hormonal inputs, thereby regulating the transition to flowering at the shoot apical meristem. In *Arabidopsis*, long-day photoperiods (LDs) stimulate the transcription of *FLOWERING LOCUS T* (*FT*), encoding the main florigenic signal. *FT* activation is mediated by the transcriptional regulator CONSTANS (CO), which binds to the CO responsive elements (COREs) located in the proximal *FT* promoter region. The phytohormone abscisic acid also (ABA) contributes to *FT* activation together with GIGANTEA (GI) to regulate drought escape (DE). Whether CO is a target of ABA and GI actions for the regulation of *FT* is, however, unknown. Here we report that ABA and its signalling components promote CO recruitment to the COREs, without causing clear effects on the diel pattern of CO protein accumulation. We also found that GI promotes CO recruitment to the COREs region, and that CO recruitment is required for the accumulation of RNAPol II at the *TRANSCRIPTION START SITE* of *FT*. Finally, we show that GI and ABA signalling pathways are largely epistatic in the control of flowering time, suggesting their involvement in the same molecular process. Taken together, these observations suggest that varying water deficit conditions modulate CO recruitment and *FT* expression, thus dictating DE strategies in *Arabidopsis*.

Keywords: flowering time regulation, environmental and stress responses, florigen signalling, regulation of gene expression

INTRODUCTION

During floral transition plants undergo a major developmental shift from vegetative to reproductive growth. This complex process is orchestrated by a combination of internal (e.g., age) and external (environmental) signals that ultimately cause transcriptional reprogramming and changes of cell fates in young primordia located at the shoot apical meristem. At the molecular level, these signals are relayed through a multitude of molecular pathways, hormonal signalling cascades and transcriptional events that unfold at different temporal and tissue scales. Variations in daylengths (photoperiod) play a key role in flowering time regulation in *Arabidopsis* and its measurement occurs in the vascular tissue of leaves (Song *et al.*, 2015; Gendron and Staiger, 2023). Following perception of an inductive photoperiod condition, a specialised protein signal referred to as florigen is produced and exported systemically to activate floral transition at the shoot apical meristem (Andrés and Coupland, 2012; Zhu *et al.*, 2021). In *Arabidopsis* the photoperiodic cascade is activated under long day conditions (LDs) and requires the activity of three key genes including *GIGANTEA* (*GI*), *CONSTANS* (*CO*) and *FLOWERING LOCUS T* (*FT*) (Song *et al.*, 2015). GI is an apical component of the network and encodes a plant-specific protein with unclear molecular functions. GI forms an oscillatory (diel regulated) complex with blue light receptors to set the stage for the transcriptional activation of *CO* during late afternoon in LDs (Imaizumi *et al.*, 2005; Sawa *et al.*, 2007; Fornara *et al.*, 2009; Ito *et al.*, 2012). Light-stabilized CO protein promotes the transcriptional activation of *FT*, encoding the main florigen of *Arabidopsis*, so that peak *FT* transcript accumulation occurs at dusk under standard growth conditions (Kardailsky *et al.*, 1999; Kobayashi *et al.*, 1999; Valverde *et al.*, 2004; Corbesier *et al.*, 2007; Song *et al.*, 2018).

The molecular mechanisms that lead to the stabilization of CO, its post-translational modifications, binding to specific DNA cis-elements, and interactions with a diverse array of co-regulators are collectively important to finely tune the expression of *FT* (Takagi *et al.*,

2023). CO binds to *FT* regulatory regions at the CO response elements (*CORE*s) and *P1/2* sites. These are 4-bp TGTG core motifs located in the proximal promoter of *FT* (-285bp from ATG), broadly corresponding to Block A regulatory region (Adrian *et al.*, 2010; Tiwari *et al.*, 2010; Gnesutta *et al.*, 2017; Lv *et al.*, 2021). CO binding to DNA occurs through the positively charged CCT domain, located at the C terminus, which is evolutionarily related to the DNA binding domain observed in NUCLEAR FACTOR-Y subunit A (NF–YA) encoding genes (Wenkel *et al.*, 2006). CO forms a trimeric complex with NF–YB and NF–YC subunits to bind to DNA (Ben-Naim *et al.*, 2006; Tiwari *et al.*, 2010; Gnesutta *et al.*, 2017). At the same time, through the N terminal B-BOX domain CO can form multimers, each one being able to form NF–YB/YC complexes via the CTT for high affinity binding to the TGTG core motifs (Lv *et al.*, 2021; Zeng *et al.*, 2022). Besides NF-YB/C, other mechanisms regulate CO recruitment to chromatin (Bu *et al.*, 2014; Wang *et al.*, 2016). All in all, these factors together with CO participate in forming local chromatin looping and initiate *FT* transcription (Adrian *et al.*, 2010; Tiwari *et al.*, 2010; Farrona *et al.*, 2011; Cao *et al.*, 2014; Tian *et al.*, 2021).

Several environmental and hormonal pathways influence CO function and, ultimately *FT* transcriptional activation (Pin and Nilsson, 2012; Conti, 2017). The phytohormone abscisic acid (ABA) is emerging as an important regulator of flowering time, influencing the expression multiple floral targets (Martignago *et al.*, 2020). Much of our knowledge about the impact of ABA in flowering time derives from the study of the drought escape response (DE), an acceleration of flowering triggered by water deficit. Under these conditions, ABA promotes the expression of *FT* and the flowering time regulator *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1* (*SOC1*) in leaves, likely through different mechanisms (Riboni *et al.*, 2013; Hwang *et al.*, 2019). A set of ABA-regulated bZIPs (ABRE binding factors, ABFs) in association with NF-YC subunits activate *SOC1* (Hwang *et al.*,

2019), whereas little is known about the regulatory role of ABA on CO upstream of *FT*. Recently, the SNF1-related protein kinase 2 (SnRK2) substrate 1 (SNS1) was shown to promote DE by inducing *FT*. SNS1 activity is regulated by phosphorylation and subsequent stabilization by ABA stimulated SnRK2 protein kinases (Katagiri *et al.*, 2024). Impairing ABA signalling causes a reduction in *FT* expression with relatively weaker changes in *CO* transcript accumulation suggesting that ABA signalling may act at least in part downstream of *CO* transcriptional activation (Riboni *et al.*, 2016). In this process, GI function is key for the ABA - dependent activation of *FT* and DE (Riboni *et al.*, 2016). GI might act as a general regulator of ABA signals for its involvement in modulation of physiological traits related to gas exchange (which are also ABA regulated) as well as ABA production (Ando *et al.*, 2013; Baek *et al.*, 2020; Simon *et al.*, 2020). However, *gi* plants also display a general hyper activation of ABA signalling responses, suggesting that the mode of action of GI in DE regulation may be separate (and more general) from its role in regulating ABA signals (Siemiatkowska *et al.*, 2022).

In this study tests were made to understand how changes in ABA accumulation could alter *FT* expression through CO. We reveal that CO recruitment to the *FT* promoter is ABA regulated which directly impacts the recruitment of the RNAPol II. Surprisingly GI is also required for proper CO recruitment to the *FT* promoter, indicating that GI and ABA may operate in the same genetic pathway. Overall, we hypothesise that GI could be an important factor bridging photoperiodic stimuli with ABA signalling to relay water deficit information and regulate flowering time.

MATERIAL AND METHODS

Plant material, genotyping, and growing conditions

Arabidopsis thaliana plants used in this study are of ecotype Columbia (Col-0) or Landsberg *erecta* (L.*er*). Mutant and transgenic lines of *aba1-6* (Niyogi *et al.*, 1998), *aba2-1*

(Leon-Kloosterziel *et al.*, 1996), *nced3/5* (Frey *et al.*, 2012), *abi1-1* (Bertauche *et al.*, 1996), *SUC2::HA:CO* (Jang *et al.*, 2009), *co-10* (Laubinger *et al.*, 2006), *gi-2* (Rédei, 1962), *gi-100* (Huq *et al.*, 2000) were previously described. Seeds were stratified and plants grown under long day conditions (LDs, 16 h light / 8 h dark), under controlled-environment cabinets, as previously described (Riboni *et al.*, 2016). Temperature range in the growth chamber fluctuated between 24 °C during the day and 21 °C in the night and air humidity was about 60%. For *in vitro Arabidopsis* growth Murashige and Skoog (MS) medium was prepared by dissolving the MS salt mix (Duchefa) in distilled water. After adjusting the pH solution to 5.8, agar (Duchefa) was added to a final concentration of 0.8% w/v. Sterilized seeds (70% v/v ethanol and 1% w/v Sodium Dodecyl Sulphate, SDS for 10 minutes) were spread onto solidified agar plates, stratified for 2-3 days (4°C and dark) and then moved to the growth chamber. ABA application experiments on soil were performed following the procedure detailed previously (Riboni *et al.*, 2016). A mock solution (0.025% v/v ethanol) was used as a mock control. Mutant combinations were selected via PCR of genomic DNA with primers described previously (Kaiserli *et al.*, 2015; Riboni *et al.*, 2016) or in Supplementary Table 1 and by exploiting markers encoded by the different transgenes; *gi-100, SUC2::CO:CITRINE* (Basta resistance), *SUC2::HA:CO* (GFP expression in seeds).

Molecular cloning

Cloning was done with Gateway and Multi-Site Gateway (three-fragment vector) cloning technology (Invitrogen). To generate entry clones (pDONR207, Invitrogen), the *AttB1*/*AttB2*-containing sequences were fused to the *CONSTANS* specific primers (Supplementary Table 2). The Phusion High Fidelity DNA polymerase (New England Biolabs) was used for all the PCR reactions. The 5' and 3' elements and destination vectors were previously described; *SUC2 promoter / pDONR221 P4-P1r* (Marquès-Bueno *et al.*, 2016), *mCITRINE / pDONR221 P2r-P3* (Jaillais *et al.*, 2011) and *pB7m34GW* (Karimi

et al., 2005). All the recombinant destination vectors were transformed into *Agrobacterium* cells, strain GV3101 (Koncz and Schell, 1986), for *Arabidopsis* stable transformation.

The reporter plasmid used for monitoring luciferase (Luc) activity carries a 5' *FT* regulatory region (1666 bp upstream of ATG). The sequence was custom synthetized (Officinae Bio, Italy) and cloned in the reporter plasmid *pGreenII 0800-LUC* (Hellens *et al.,* 2005). This plasmid also contains the *35S:Renilla* (Ren) cassette. The effector construct (*35S:CO*) was built by recombining a CO entry vector (which includes the stop codon) into the Gateway destination vector *p2GW7* (Karimi *et al.,* 2002).

Plants transformation and BASTA selection

Destination vectors *SUC2::CO:CITRINE* and *pSUC2::GW* (here used as a control for Basta resistance) (An *et al.,* 2004), were introduced into *Agrobacterium* by electroporation. Transformed *Agrobacterium* were used to generate *Arabidopsis* transgenic plants via the floral dip technique (Clough and Bent, 1998). Transgenic plants were selected on soil after continuous Basta applications (12 mg/l dilution, Agrevo) and single insertion events were selected based on a Mendellian 3:1 ratio in T_2 generation. T_3 homozygous lines were selected on soil or MS+Basta plates, according to the absence of Basta resistance segregation. Transgenic *SUC2* plants propagated up to the 5th generation by selfing did not show obvious signs of silencing.

Flowering time measurement

Flowering time was measured by scoring the number of rosette leaves, excluding cotyledons. When indicated, flowering was expressed in terms of days to bolting (days between sowing and a visible emergence of the primary inflorescence). Data regarding cauline leaf number (I1 phase) were also recorded for each genotype. To avoid flowering time alterations associated to soil transfer from plates, Basta resistance selection in T1 generation was performed directly on soil.

RNA extraction and Real-Time qPCR

Total RNA was extracted with QIAzol reagent (Invitrogen) and suspended in RNase-free milliQ dH_2O. RNA concentration was measured with a UV spectrophotometer and 1000 ng aliquots were used for cDNA synthesis with the Maxima Reverse Transcriptase kit (Thermofisher). Quantitative Real-Time PCR (qPCR) was performed as previously detailed including primers to amplify *CO*, *FT*, *ACT2* and *IPP2* were described (Kaiserli *et al.,* 2015; Riboni *et al.,* 2016). Primers to amplify *CITRINE* are listed in Supplementary Table 1.

Nuclei isolation and CO protein detection in *Arabidopsis*

Approximately 100 mg of *Arabidopsis* seedlings were harvested and immediately frozen in liquid nitrogen. Tissue samples were ground by shaking with glass beads in a TissueLyser II (Qiagen, 2 pulses of 30 s each at 28Hz shaking). Leaf powder was suspended in 1.2 ml of cold nuclear isolation buffer (20 mM Tris-HCl, pH 8.8, 25 mM NaCl, 5 mM $MgCl_2$, 30% (v/v) glycerol, 5% (w/v) sucrose, 0.5% (v/v) Triton X-100, 0.08% (v/v) β-mercaptoethanol, 0.2% (v/v) SIGMA plant protease inhibitor, 1mM DTT, 1.3 mM PMSF) (Hayama *et al.,* 2017). The samples were filtrated trough two layers of Miracloth (Millipore) and centrifuged at *5,000 x g*, at 4 °C, for 10 minutes. The pellet was washed four times with 1 ml of nuclear isolation buffer and, after each wash, pelleted at 4 °C at decreasing speed: *5,000 x g*, *2,700 x g*, *2,200 x g* and *2,200 x g*, 8 minutes each time. Nuclei were suspended in 30 µl of nuclear isolation buffer, mixed with 10 µl of Laemmli Buffer and heated 10 minutes at 95 °C. Samples were centrifuged for 1 minute at *3,000 x g* to pellet all nuclear membranes, and the supernatants (enriched in soluble nuclear proteins) were recovered and 20 µl were loaded onto a 10% SDS-PAGE gel. For Western blot analysis, anti-HA-Peroxidase, High Affinity antibody (Roche) was used to detect HA:CO protein whereas Histone H3 detection (anti H3, Agrisera) was used for loading control. Chemiluminescent signals were detected

through a ChemiDoc Touch Imaging System (Biorad) and quantified with the Image Lab software (Biorad).

ChIP experiments

Chromatin immunoprecipitation assays were performed as described previously with minor modification, starting from 1-2 grams (FW) of seedlings (Perrella *et al.,* 2018, 2023). A bioruptor sonicator (Diagenode, B01020001) was used to shear the chromatin using the following settings: 40 Cycles, 30 sec ON, 30 sec OFF at high power. With these conditions the size of sheared DNA is below 100 bp. An anti-RNAPol II antibody (AbCam Ab5408) or GFP-Trap magnetic nanobodies (Chromotek) were used. qPCR was performed at the following cycles: 95°C x 3 min; 95°C x 10 sec, 59.5°C x 30 sec (45 cycles); 65°C x 0.05 sec and 95°C x 30 sec (Melting curve). Relative enrichment was calculated as described previously (Kaiserli *et al.,* 2015; Perrella *et al.,* 2018). Reactions were performed on four technical replicates and two independent biological experiments. Amplicons of the *CBS*, *CORE1/2*, *CORE3*, *CORE4* and *CORE5* regions of *FT* were obtained with primers previously described (Hayama *et al.,* 2017), whereas the *TSS* regions of *FT*, *YUCCA8* and *ACTIN2* were amplified with primers lcm217/lcm218, *YUC8 FW*/*RV* and *ACT2 FW*/*RV*, respectively (Supplementary Table 1).

Protoplast Isolation and transactivation Assays:

Leaf protoplasts were isolated from 3-week-old *Arabidopsis* plants as previously described (Iacopino *et al*., 2019). Luc and Ren activities were measured by providing the specific substrates (Dual-Luciferase reporter assay system, Promega) and luminescence was measured with the EnSight plate reader (Perkin Elmer). In each assay, data were expressed as Luc activity relative to Ren.

Statistical analysis

Analysis of variance (ANOVA) was conducted with linear models where flowering time/gene expression was predicted by genotype according to the formula: flowering time (gene expression) ~ genotype. Here, “flowering time” was expressed as either one of: leaf number at bolting, days to bolting, or number of l1 nodes. “genotype” refers to the allelic status at *ABA1*/*2* (WT, *aba1-6*, *aba2-1*) or *GI* (WT, *gi-2*, *gi-100*) loci, and different isogenic lines for the various mutants. When *aba1* mutants were in different backgrounds (WT, *SUC2::CO:CIT*, *SUC2::HA:CO*), the same model was run separately for the different backgrounds and the models were qualitatively compared. In Fig. 2A, the model used was gene expression ~ genotype*timepoint. If the interaction was not statistically significant, only the additive model (genotype + timepoint) was considered for post-hoc tests.

Before running the ANOVAs, the condition of homogeneity of variances between groups was checked with a Bartlett’s test in base R (R Core Team 2023). If group variances were not homogeneous, a log transformation was applied to the data. All ANOVA models were conducted with the R function lm (R Core Team 2023) using sum contrasts and significance of predictors was assessed with the ANOVA function from the car package (Fox and Weisberg, 2018). Post-hoc Tukey tests to assess differences between each pair of genotypes were conducted with the R packages multcomp (Hothorn *et al.*, 2008) and emmeans (Lenth, 2023). Even for models including timepoint as predictor, post-hoc tests were conducted exclusively to assess differences between genotypes within timepoints.

RESULTS

CO function is sensitive to ABA signalling status in the phloem companion cells

To evaluate the contribution of ABA accumulation in regulating CO function and *FT* expression we generated transgenic plants constitutively expressing *CO* in different ABA-impaired/defective backgrounds. Since CO regulates *FT* in the phloem companion cells (An *et al.,* 2004) we engineered a *CO:CITRINE* (*CO:CIT*) translational fusion gene under the control of the phloem companion cell-specific *SUCROSE-PROTON SYMPORTER 2* (*SUC2*) promoter (Truernit and Sauer, 1995). Basta resistant T1 plants of wild-type (Col-0) and two different ABA-deficient backgrounds (*aba1-6* and *aba2-1*, impaired at different steps of the ABA biosynthetic pathway) (Nambara and Marion-Poll, 2005) displayed a clear early flowering phenotype compared with the corresponding genotypes transformed with an empty vector (Fig. 1A). *SUC2::CO:CIT aba1-6* and *aba2-1* plants showed a mild but significant delay in flowering (p = 6.1E-07 and 1.2E-14, respectively) compared with the wild type transformed with *SUC2::CO:CIT*. Ruling out an indirect effect of reduced ABA production on *SUC2* promoter activity, we detected similar levels of *CIT* transcript accumulation across randomly chosen T1 plants (Supplementary Figure S1). This suggests a role for ABA production downstream of *CO* transcriptional activation. In support of this view, we observed similarly reduced level of *FT* accumulation in *SUC2::CO:CIT* in either *aba1-6 or aba2-1* independent T1 transgenic events compared with *SUC2::CO:CIT* transgenics events in the wild-type background (p = 0.047 and 0.026, respectively, Supplementary Figure S1). *FT* transcript levels detected in *SUC2::CO:CIT aba2-1/aba1-6* plants were, however, slightly increased compared with the wild type transformed with an empty vector, consistent with their earlier flowering.

To confirm the observed flowering time phenotypes we crossed two homozygous T3 *SUC2::CO:CIT* plants with *aba1-6* to generate isogenic lines (#5 and #18). We also performed genetic crosses between *SUC2::HA:CO* plants (Jang *et al.,* 2009) and *aba1-6* to

obtain *SUC2::HA:CO aba1-6*. *SUC2::HA:CO* plants were extremely early flowering, similar to *SUC2::CO:CIT* plants. However, in the *aba1-6* background, the early flowering phenotype conferred by *SUC2::HA:CO* or *SUC2::CO:CIT* was significantly alleviated (p < 4.6E-06, Fig. 1B and 1C). Collectively, our findings suggest that ABA may promote CO function and *FT* transcript accumulation to activate flowering.

Reduced ABA levels impair *FT* transcript accumulation

We next aimed to evaluate potential modes of regulations of CO function controlled by ABA. Using stable transgenic isogenic lines expressing *SUC2::HA:CO* or *SUC2::CO:CIT #5* in the wild-type or *aba1-6* backgrounds, we confirmed that *FT* transcript accumulation was reduced in *aba1-6* plants compared with the corresponding ABA producing WT background (p < 3.33E-03, Fig. 2A) irrespective of the time point analysed (ZT12 and 16 under LDs). In contrast, *CO* levels remained not affected in *SUC2::HA:CO aba1-6* plants or even increased (p = 1.82E-03) in *SUC2::CO:CIT#5 aba1-6* compared with the corresponding *SUC2::CO:CIT #5*. Interestingly, we also detected reduced levels of *FT* transcript accumulation in *aba1-6* plants compared with the wild type (p = 1.92E-04) at ZT16 when we also observed a marginal reduction (p = 0.06) in *CO* transcript levels. To investigate further the role of ABA production in *FT* transcript accumulation we analysed 9-day-old seedlings of wild type and strong ABA deficient mutant backgrounds, including *aba1-6*, *aba2-1* and double mutants of *9-cis-epoxycarotenoid dioxygenase* (*nced*) *3* and *5* (Frey *et al.,* 2012). Compared with the wild type, at ZT16 we observed reduced *FT* accumulation in all ABA deficient mutants (p < 6.09E-06) and correspondingly reduced levels of *CO* transcript (p < .01) (Fig. 2B). Collectively, our data indicate a role for ABA in promoting *FT* accumulation with ABA affecting both *CO* expression and its function.

To gain further insights into the potential mode of ABA-dependent stimulation of CO we analysed HA:CO protein levels derived from *SUC2::HA:CO* and *SUC2::HA:CO aba1-6*

plants. Interestingly, HA:CO protein accumulation was similar between the two genotypes at ZT12 and ZT16, suggesting that reduced ABA accumulation impairs HA:CO protein function but not HA:CO accumulation (Fig. 2C). To independently test for potential effects of ABA on HA:CO protein abundance we exogenously applied three different concentrations of ABA (0.25 -2.5 -25 µM) on a daily basis for 12 days (Riboni *et al.,* 2016). Tissue from ABA-treated plants was collected at ZT4 and ZT12 and HA:CO protein was detected from nuclear extracts. We did not observe obvious changes in HA:CO protein levels at any of the concentrations and time points assayed (Supplementary Figure S2). In addition, we did not observe obvious changes in the pattern of HA:CO protein gel mobility in the ABA deficient background *aba1-6* (Supplementary Figure S2), which are determined by its phosphorylation status (Sarid-Krebs *et al.,* 2015). In summary, our data indicate that CO function might be stimulated by ABA in the cells where *FT* is transcriptionally activated without clear changes at the post translational level.

ABA stimulates the recruiting of CO to the *FT* promoter region

We next investigated what other aspects of CO function could be influenced by ABA status. We first set up a transient reporter expression assay in *Arabidopsis* mesophyll protoplasts, by fusing a 1666 bp promoter region of *FT* upstream of the *LUCIFERASE*-encoding sequence (hereafter FT_{pro}*::LUC*). This promoter region encompasses several cis elements that are critical for the *FT* transcriptional activation, including the well characterised CO responsive element (*CORE*)*1/2* (Tiwari *et al.,* 2010). Co-transformation of FT_{pro}*::LUC* with *35S::CO* vectors resulted in a strong activation of LUC activity, indicating that the fused promoter can respond to CO binding (Supplementary Figure S3). Co-incubation of FT_{pro}*::LUC/35S::CO* transformed protoplasts in presence of ABA (10 and 100 nM), however, did not produce significant changes in LUC activity suggesting that

exogenous ABA does not directly influence CO-mediated stimulation of the *FT* promoter, at least in the absence of a native chromatin context.

We therefore evaluated CO recruitment to the *FT* promoter through a Chromatin Immunoprecipitation (ChIP) assay by comparing CO binding in isogenic transgenic seedlings of *SUC2::CO:CIT* #5 (wild type and *aba1-6* backgrounds) under LDs. Consistent with previous reports (Cao *et al.,* 2014; Song *et al.,* 2016; Hayama *et al.,* 2017), at ZT12 we observed a strong enrichment of the CO:CIT protein at the *CORE1/2 FT* promoter regions (Fig. 3A and 3B). In contrast, such CO:CIT enrichment was much decreased (approximately 4X) in the *aba1-6* background*,* suggesting that ABA promotes CO recruitment to the *CORE1/2* region (Fig 3B and Supplementary Figure S4). A weaker CO:CIT binding was also observed at the *CORE3* position (Hayama *et al.,* 2017), located in the second intron of *FT* and the *CBS* (*CAAT Binding Site*) region, located at 1.8kb upstream of the ATG start codon - Block B enhancer - (Adrian *et al.,* 2010). These enrichment signals were also reduced in in the *aba1-6* background.

ABA promotes RNAPol II recruitment to the *FT* promoter

To define the role of ABA in regulating the early transcriptional events at the *FT* promoter we monitored the RNA Polymerase II (RNAPol II) recruitment. We assayed the Ser5 phosphorylated pool of RNAPol II, which in Arabidopsis is distributed over the transcribed region of regulated genes (Obermeyer *et al.,* 2023). In the wild type at ZT12 we could detect a peak of RNAPol II enrichment around the transcription start site (TSS) of *FT* with a general reduction in the gene body (Fig. 3C and Supplementary Figure S5). In the *co* and the *gi* mutants, RNAPol II occupancy was much reduced compared with the wild type, indicating that this assay is a sensitive readout for the CO-mediated recruitment of RNAPol II to the *FT* promoter (Fig. 3C). Remarkably, we observed a similar strong reduction in RNAPol II occupancy at the TSS in ABA deficient mutants *aba1-6* and *aba2-1* (Fig. 3C).

Ruling out a general defect in RNAPol II recruitment at actively transcribed genes in ABA deficient or other mutants utilised in these assays, we observed no clear changes in RNAPol II occupancy at the TSS of *ACTIN2* and *YUCCA8* genes in the same panel of genotypes (Supplementary Figure S6). Therefore, the reduced occupancy of RNAPol II could depend on the lower levels of CO recruitment to the *CORE1/2* region observed in ABA deficient mutants (Fig. 3B). ABA signals promote the transcriptional activation of *CO* in *Arabidopsis* and other *Brassicaceae* (Yoshida *et al.,* 2010; Koops *et al.,* 2011; Zhang *et al.,* 2022) and we observed reduced CO transcript levels in ABA deficient backgrounds, albeit only at the peak of *FT* accumulation (ZT16) (Fig. 2B). To verify further the post-transcriptional control of CO mediated by ABA and its impact on RNAPol II recruitment, we examined the same *FT* regions in *abscisic acid insensitive 1-1* (*abi1-1*) mutants. *abi1-1* encodes a dominant allele of the ABI1 type 2C protein phosphatase (PP2C) which severely impairs ABA signalling (Bertauche *et al.,* 1996). *abi1-1* plants show similar levels of *CO* transcript accumulation during the day, but diminished levels of *FT* accumulation (Riboni *et al.,* 2016). Consistent with this observation, RNAPol II occupancy was reduced in *abi1-1* mutants compared with the wild type (Fig. 4A and Supplementary Figure S7). Interestingly, RNAPol II recruitment in L.*er* (unlike Col-0) was reproducibly enriched primarily towards the *CORE1/2* regions. These results suggest that impairing ABA signalling may also cause similar reductions in CO occupancy at the *FT* promoter which impacts correct RNAPol II recruitment, despite RNAPol II positioning is subject to unknown ecotype-specific controls.

Finally, we assessed the role of ABA on RNAPol II recruitment to the *FT* promoter in the presence of constitutive CO accumulation, thus independently of ABA-mediated transcriptional effects on *CO*. We observed a clear decrease in RNAPol II enrichment at the TSS of *FT* in *SUC2::HA:CO aba1-6* compared to *SUC2::HA:CO*, which correlates with the reduced levels of CO occupancy at the *FT* promoter detected in previous ChIP assays

(Fig. 4B and Supplementary Figure S5). Notably, RNAPol II was more strongly enriched in *SUC2::HA:CO* plants compared with the wild type, further supporting the limiting role of CO accumulation in RNAPol II recruitment.

ABA and GI largely act in the same pathway to control CO recruitment to the *FT* promoter and promote floral transition

Previously we reported the key role for GI in mediating flowering under water deficit conditions (Riboni *et al.,* 2016). *gi* mutants are defective in *FT* upregulation under control (Song *et al.,* 2014) or water deficit conditions (Riboni *et al.,* 2016) even in genetic backgrounds overexpressing *CO*. Consistent with these findings, we observed a strong reduction in RNAPol II recruitment to the *TSS* of *FT* in *SUC2::HA:CO gi-100* lines, suggesting that GI might also be important for CO recruitment (Fig. 4B and Supplementary Figure S5). A ChIP assay using *SUC2::CO:CIT gi-2* #5 further revealed reduced levels of CO:CIT recruitment to the *CORE1/2* region compared to *SUC2::CO:CIT* #5 (Fig 5A). However, flowering time of *SUC2::CO:CIT gi-2* #5 plants was similar to *SUC2::CO:CIT* #5 indicating that an excess of transgene-derived CO:CIT expression could ultimately compensate for its reduced recruitment observed at the *FT* promoter in *gi* mutants (Supplementary Figure S8).

Because both ABA and GI mediate CO recruitment to the *FT* promoter, we tested whether they acted in the same genetic pathway. We thus compared flowering time (bolting time and leaves counts) of *SUC2::HA:CO aba1-6* and *SUC2::HA:CO gi-100* with triple mutants of *SUC2::HA:CO aba1-6 gi-100* under LDs. As expected, flowering time of *SUC2::HA:CO aba1-6* was significantly delayed compared to *SUC2::HA:CO* plants both in terms of number of rosette leaves (p = .01) or bolting time (p = 1.78E-11) (Fig. 5B and Supplementary Figure S9). We did not detect a delay of flowering in any *SUC2::HA:CO gi* mutants combination (i.e., *gi-2, gi-100*) compared to *SUC2::HA:CO* in terms of leaf

numbers, but we observed a significant delay in days to flowering (p = 1.95E-05 for *SUC2::HA:CO gi-2*). Finally, we compared the impact of combined *aba1-6* and *gi-100* mutant alleles in the *SUC2::HA:CO* background. This showed a minor but significant (p < 0.01) aggravation of flowering time of *SUC2::HA:CO aba1-6 gi-100* compared with *SUC2::HA:CO aba1-6* in terms of rosette leaves but no consistent effects in days to flowering (Fig. 5B and Supplementary Figure S9), suggesting a partially epistatic relationship between GI and ABA signalling in this process. We also scored the number of I1 coflorescences (i.e., nodes presenting side shoots derived from the main inflorescence, Supplementary Figure S9). I1 nodes were similarly reduced in *SUC2::HA:CO* plants and *SUC2::HA:CO gi* backgrounds. We observed a significant increase in I1 nodes in *SUC2::HA:CO aba1-6* (p = .002) but no further aggravation of this phenotype in *SUC2::HA:CO aba1-6 gi-100* mutants lines. Therefore, GI and ABA signalling action is largely non additive to regulate flowering time and during inflorescence development in genetic background that overexpress *CO*.

DISCUSSION

Our study describes a yet uncharacterised role for ABA on CO function. Since ABA biosynthesis and signalling is enriched in the phloem companion cells of the leaf (Mustilli *et al.,* 2002; Kuromori *et al.,* 2014), endogenous CO function might be directly subject to varying ABA concentration, which is usually related to water deficit status. Our experiments support the involvement of ABA in regulating the recruitment of CO at the *CORE FT* promoter region, subsequently leading to RNAPol II engagement at the *FT 5' UTR*.

Recent evidence supports the role of GI as a potential target of ABA action during DE. First, we previously highlighted the key role of GI in enhancing *FT* expression in response to ABA, and that GI function was sensitive to the ABA signalling status (Riboni *et al.,* 2016).

Second, the epistatic interaction between *gi* and *aba1* mutants in the regulation of flowering time (bolting time in particular) of *SUC2::HA:CO* plants aligns with a model where ABA and GI could act in the same pathway to regulate CO recruitment to the *FT* promoter, although the regulatory role of ABA on GI function remains unknown. GI was shown to stimulate ABA production which might suggest an indirect effect on CO recruitment exerted by GI (Baek *et al.*, 2020). However, we reported an increased levels of ABA sensitivity in *gi* plants under controlled water deficit conditions (Siemiatkowska *et al.*, 2022), suggesting that GI might be crucial for relaying ABA signalling upstream of *FT*. GI exerts a direct influence on *FT* activation by associating to its promoter (Sawa and Kay, 2011). To further define the sites of GI binding onto the *FT* regulatory region, we analysed GI ChiPseq data describing the genome-wide occupancy of GI (Nohales *et al.*, 2019). GI binding regions on the *FT* promoter (Fig. 5C) closely overlap with two DNAse accessible chromatin locations (Zhang *et al.*, 2012). These regions were functionally defined as block C and E enhancers (Adrian *et al.*, 2010; Liu *et al.*, 2014; Zicola *et al.*, 2019). Block C contains NF-Y binding sites, which facilitate looping with the *CORE* region and consequent *FT* transcriptional activation (Cao *et al.*, 2014; Siriwardana *et al.*, 2016). Block E sequence exhibits binding sites for different transcriptional regulators, including the *FT* repressor SCHLAFMUTZE (SMZ), which has been shown to bind to GI (Mathieu *et al.*, 2009; Sawa and Kay, 2011). While GI is unlikely to directly bind DNA and despite its molecular function is still unclear, it is required to control transcriptional events through direct interaction with specific transcriptional regulators (Sawa and Kay, 2011; Song *et al.*, 2014; Kubota *et al.*, 2017), possibly by acting as a general chaperone-like protein (Cha *et al.*, 2017). These observations could point to a model whereby GI controls CO recruitment through binding to Block E and C enhancers, perhaps by affecting local chromatin conformation or CO protein complex accessibility (Fig.5C).

Different NF-Y encoding genes act as regulators of ABA transcriptional responses (Kumimoto *et al.*, 2013; Song *et al.*, 2016) and *nf-yb2* mutants display an exaggerated delay in flowering time compared to wild-type controls under osmotic stress conditions (Chen *et al.*, 2007). Considering the mild flowering time defects of *nf-yb2* mutants under normal growth conditions (Kumimoto *et al.*, 2008), this observation may point to a specific requirement for *NF-YB2* subunit in promoting flowering under osmotic stress, which is ABA regulated. A similar observation about the requirement for NF-Y in mediating ABA-dependent flowering derives from the lack of DE of *nf-yc3 yc4 yc9* mutants (Hwang *et al.*, 2019). Because NF-YB/C can form complexes with both CO and NF-YA subunits, their contribution in relaying ABA signals may depend on either CO, NF-YA or both functions. Indeed, the canonical NF-YA/B/C trimer also binds to the *FT* promoter, but shows a different sequence specificity (CCAAT, imposed by NF-YA) (Cao *et al.*, 2014; Siriwardana *et al.*, 2016), and binding distribution in the *FT* promoter (Block B, C and E) (Adrian *et al.*, 2010; Zicola *et al.*, 2019). Moreover, NF-YC subunits interact with the ABFs bZIP transcription factors (Kumimoto *et al.*, 2013; Hwang *et al.*, 2019), and an overrepresentation of G-box/ABRE elements (which are bound by bZIPs) was observed within promoters containing *CCAAT* boxes. This observation suggests that NF-Ys-ABFs modulate ABA-regulated gene expression by specifically binding to closely positioned cis elements (Kumimoto *et al.*, 2013). Whether this model of interplay between ABFs and NF-YA/B/C/NF-YCO/B/C can be applied to the *FT* promoter regulation is unclear. Intriguingly, recent data support the physical interaction between CO and ABF1/2/3/4 and their antagonistic roles at salinity-responsive genes promoters (Du *et al.*, 2023). The ABFs do not, however, display binding to the *FT* chromatin region (Song *et al.*, 2016; Hwang *et al.*, 2019). Considering the existence of ABF-like genes (Dröge-Laser *et al.*, 2018), further investigations will be required.

Our work establishes a role for ABA and its signalling components in influencing *FT* promoter accessibility to CO. Previous reports have described direct links between ABA signalling and function of chromatin regulatory complexes that affect *FT* expression (Farrona *et al.*, 2004; Peirats-Llobet *et al.*, 2016; Katagiri *et al.*, 2024). Here, genetic, and molecular evidence suggests that ABA could also act through GI-mediated recruitment of CO to regulate *FT* expression. Therefore, our data and experimental approaches offer new tools for investigating the ABA-dependent level of regulation of photoperiodic flowering.

FIGURES LEGENDS

Figure 1. ABA promotes CO function in the phloem companion cells.

(A) Boxplot of flowering time (right axis) and number of plants displaying a given number of rosette leaves (left axis) in Basta resistant T1 plants of the indicated genotypes. An ANOVA test to assess the impact of *aba1/2* mutations was run separately for *SUC2::CO:CIT* and empty vector - transformed plants (vertical bar). For both groups (empty vector or *SUC2::HA:CO* transformed), the genotype was a statistically significant predictor of leaf number at bolting ($p < .001$). Letters at the top of boxplots indicate statistically significant differences ($p < .05$) according to a Tukey post-hoc test. (B) Boxplot of flowering time of the indicated genotypes and isogenic lines derived from the introgression of different *SUC2::CO* transgenes (wild type Col-0) into *aba1-6*. An ANOVA test to assess the impact of *aba1-6* mutation was run separately for transgenic (*SUC2::CO:CIT/SUC2::HA:CO*) and non-transgenic controls (vertical bar). For both groups, the *aba1-6* genotype was a statistically significant predictor of leaf number at bolting ($p < .001$). Letters at the top of boxplots indicate if genotypes showed statistically significant differences ($p < .05$) according to a Tukey post-hoc test. The number of

samples analysed for each genotype is shown at the bottom of the graph (C) Representative plants of the indicated genotypes pictured 27 days after sowing.

Figure 2. ABA regulates CO function without altering its accumulation in *Arabidopsis*.

(A) Real-Time qPCR of *CO* (upper panels) and *FT* (lower panels) transcripts in seedlings of the indicated genotypes grown on plates for 13 days and harvested at the indicated ZT time points in the diurnal phase. Horizontal Lines represent the mean of the three biological replicates (shown as dots). Each replicate comprises approximately 50 mg of seedlings. Different letters denote differences ($p < .05$) between genotypes within each ZT according to post-hoc tests. (B) Same as A, from seedlings grown for 9 days under LDs and harvested at ZT16. Different letters denote different ($p < .05$) groups according to post-hoc tests. In A and B, the plot background colours denote the ABA producing vs the ABA deficient genotype (C) Immunoblot detection of HA:CO protein from nuclear fractions of seedlings grown on plates for 12 days under LDs. WT, non-transgenic Col-0 plants were used as negative controls whereas asterisks denote non-specific bands detected by the antibody and used as loading controls. Two exposures of the same blots are shown. Numbers refer to molecular mass according to the migration of a protein ladder. detected in seedlings pooled from one MS plate).

Figure 3. ABA promotes CO recruitment to the *FT* promoter and subsequent assembly of RNAPol II.

(A) Schematic representation of the *FT* locus and the regions amplified in the Real-Time qPCR assays. (B) Relative enrichment of CO:CIT on the *FT* promoter in isogenic lines *SUC2::CO:CIT #5* (WT and *aba1-6* mutant backgrounds). Non transgenic, WT Col-0 was used as negative control. Bars are the mean ± SE (n = 4 technical replicates). An

independent experiment is shown in the supplementary material. (C) RNAPol II deposition on the *FT* locus in the indicated genotypes described as % of Input. Each data point represents the mean ± SE (n = 4 technical replicates). Seedlings in B and C were grown for 12 days under LDs and harvested at ZT12.

Figure 4. ABA production and signalling are required for proper RNA Pol II recruitment at the TSS of *FT*

A and B, RNA Pol II deposition (expressed as % of input) on the FT locus in the WT (L.er) and abi1-1 mutant, A and WT (Col-0), SUC::CO:HA (in the WT, aba1-6 and gi-100 backgrounds), B. In A, no antibody denotes a negative control for the ChIP assay. In B the WT Col-0 data are the same as in Figure 3C as samples derived from the same experiment. Each point represents the mean ± SE (n = 4 technical replicates). Seedlings were grown under LDs for 12 days and harvested at ZT12. An independent experiment is shown in the supplementary material.

Figure 5. ABA signalling and *GI* act together to promoter CO recruitment to the *FT* promoter and regulate flowering time.

A Relative enrichment of CO:CIT in isogenic lines *SUC2::CO:CIT #5* (WT and and *gi-2* mutant) on the *FT* locus. Non transgenic, WT (Col-0) was used as a negative control. Bars represent the mean ± SE (n = 4 technical replicates). An independent experiment is shown in the supplementary material. B Boxplot of flowering time of the indicated genotypes and isogenic lines derived from the introgression of *SUC2::HA:CO* transgenes into *aba1-6* or *gi* mutant backgrounds. Note that for the *SUC2::HA:CO aba1-6 gi-100* combination, two sibling lines were analysed. An ANOVA test to assess the impact of mutations at the *ABA1* and *GI* loci on leaf number at bolting was run separately for transgenic (*SUC2::HA:CO*) and control plants (vertical bar). For both groups, genotype was a statistically significant

predictor of leaf number at bolting ($p < .001$). Letters at the top of boxplots indicate if genotypes showed statistically significant differences ($p < .05$) according to a Tukey post-hoc test. The number of samples analysed for each genotype is shown at the bottom of the graph. C GI ChiPseq peaks on the *FT* region coincide with DNAase accessible chromatin (referred to as enhancer blocks C and E). CO recruitment to the CORE region is regulated by ABA and GI (thick arrows). ABA and GI might act independently to promote CO binding to the CORE region. ABA might also influence the accessibility of GI to Block C/E (dotted arrows), thus indirectly controlling CO recruitment.

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Author contribution

ART, GP, LC: conceptualization and investigation; AS, SC, PKK, GC, EV, DM: investigation; BL: formal analysis; GP, ART, LC: writing - original draft; EK, MG, CT, LC: writing - review & editing; LC: supervision; LC: funding acquisition

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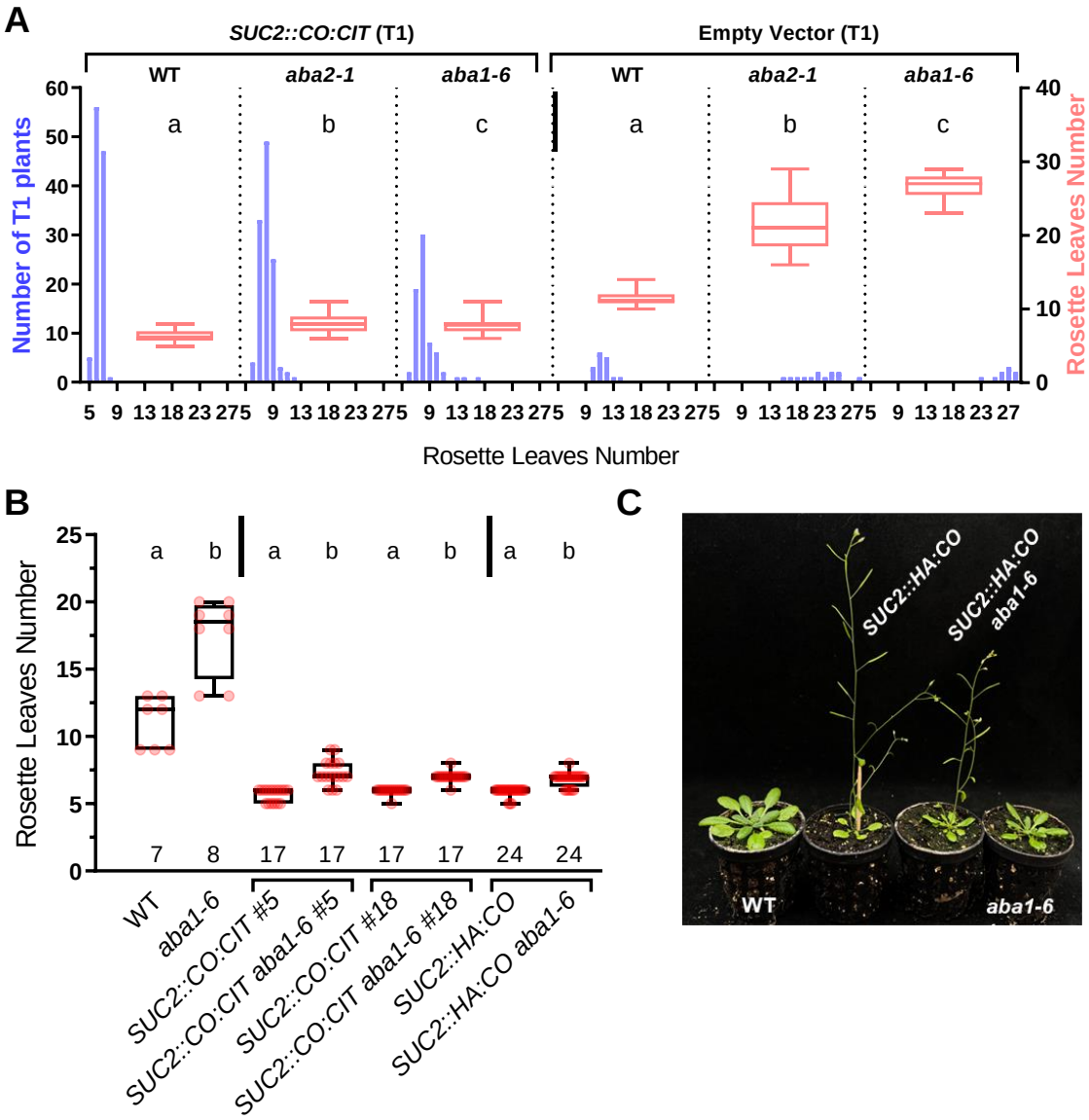

Figure 1. ABA promotes CO function in the phloem companion cells.

(A) Boxplot of flowering time (right axis) and number of plants displaying a given number of rosette leaves (left axis) in Basta resistant T1 plants of the indicated genotypes. An ANOVA test to assess the impact of *aba1/2* mutations was run separately for *SUC2::CO:CIT* and empty vector - transformed plants (vertical bar). For both groups (empty vector or *SUC2::HA:CO* transformed), the genotype was a statistically significant predictor of leaf number at bolting ($p < .001$). Letters at the top of boxplots indicate statistically significant differences ($p < .05$) according to a Tukey post-hoc test. (B) Boxplot of flowering time of the indicated genotypes and isogenic lines derived from the introgression of different *SUC2::CO* transgenes (wild type Col-0) into *aba1-6*. An ANOVA test to assess the impact of *aba1-6* mutation was run separately for transgenic (*SUC2::CO:CIT/SUC2::HA:CO*) and non-transgenic controls (vertical bar). For both groups, the *aba1-6* genotype was a statistically significant predictor of leaf number at bolting ($p < .001$). Letters at the top of boxplots indicate if genotypes showed statistically significant differences ($p < .05$) according to a Tukey post-hoc test. The number of samples analysed for each genotype is shown at the bottom of the graph (C) Representative plants of the indicated genotypes pictured 27 days after sowing.

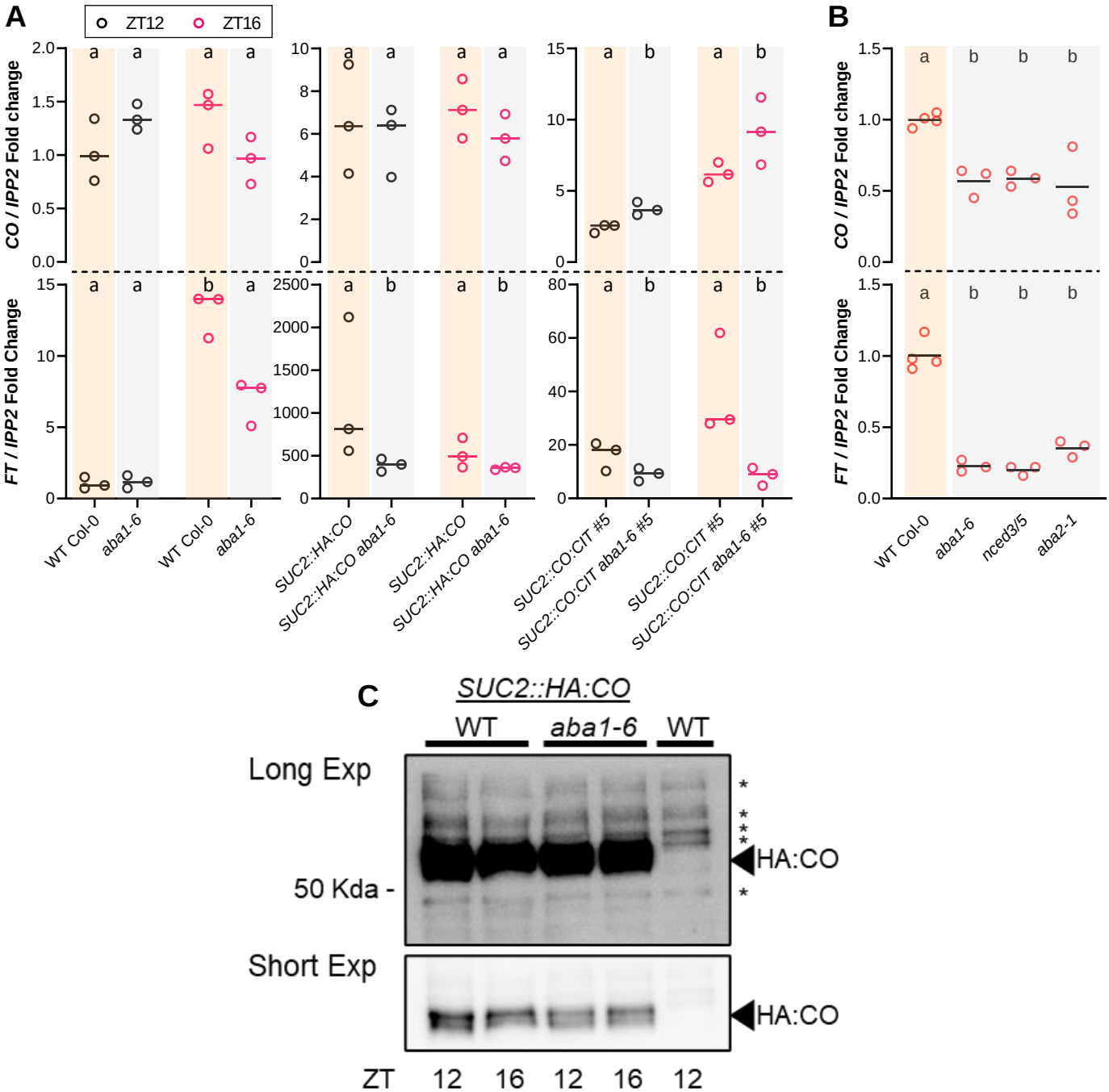

Figure 2. ABA regulates CO function without altering its accumulation in *Arabidopsis*.
(A) Real-Time qPCR of *CO* (upper panels) and *FT* (lower panels) transcripts in seedlings of the indicated genotypes grown on plates for 13 days and harvested at the indicated ZT time points in the diurnal phase. Horizontal Lines represent the mean of the three biological replicates (shown as dots). Each replicate comprises approximately 50 mg of seedlings. Different letters denote differences ($p < .05$) between genotypes within each ZT according to post-hoc tests. (B) Same as A, from seedlings grown for 9 days under LDs and harvested at ZT16. Different letters denote different ($p < .05$) groups according to post-hoc tests. In A and B, the plot background colours denote the ABA producing vs the ABA deficient genotype (C) Immunoblot detection of HA:CO protein from nuclear fractions of seedlings grown on plates for 12 days under LDs. WT, non-transgenic Col-0 plants were used as negative controls whereas asterisks denote non-specific bands detected by the antibody and used as loading controls. Two exposures of the same blots are shown. Numbers refer to molecular mass according to the migration of a protein ladder. detected in seedlings pooled from one MS plate).

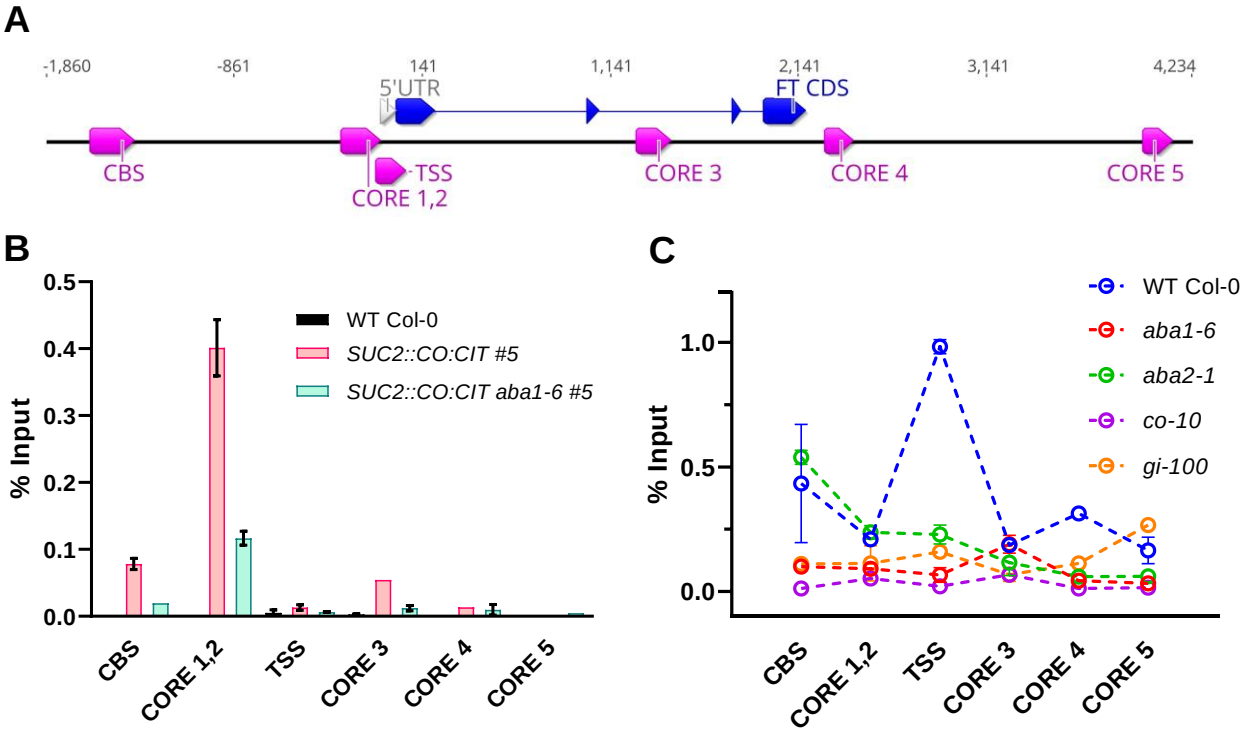

Figure 3. ABA promotes CO recruitment to the *FT* promoter and subsequent assembly of RNAPol II.

(A) Schematic representation of the *FT* locus and the regions amplified in the Real-Time qPCR assays. (B) Relative enrichment of CO:CIT on the *FT* promoter in isogenic lines *SUC2::CO:CIT #5* (WT and *aba1-6* mutant backgrounds). Non transgenic, WT Col-0 was used as negative control. Bars are the mean ± SE (n = 4 technical replicates). An independent experiment is shown in the supplementary material. (C) RNAPol II deposition on the *FT* locus in the indicated genotypes described as % of Input. Each data point represents the mean ± SE (n = 4 technical replicates). Seedlings in B and C were grown for 12 days under LDs and harvested at ZT12.

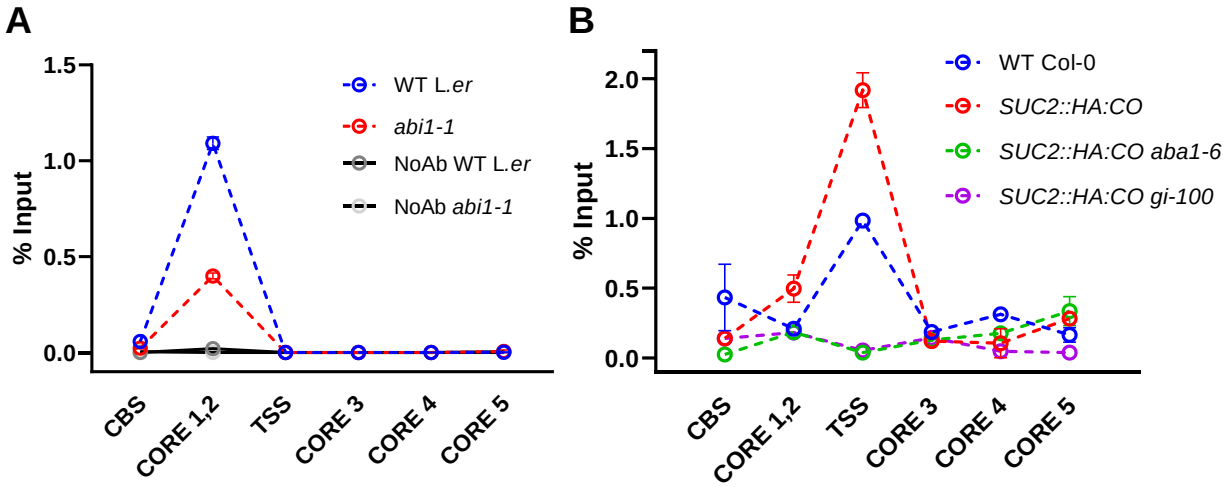

Figure 4. ABA production and signalling are required for proper RNA Pol II recruitment at the TSS of *FT*

A and B, RNA Pol II deposition (expressed as % of input) on the *FT* locus in the WT (L.*er*) and *abi1-1* mutant, A and WT (Col-0), *SUC::CO:HA* (in the WT, *aba1-6* and *gi-100* backgrounds), B. In A, no antibody denotes a negative control for the ChIP assay. In B the WT Col-0 data are the same as in Figure 3C as samples derived from the same experiment. Each point represents the mean ± SE (n = 4 technical replicates). Seedlings were grown under LDs for 12 days and harvested at ZT12. An independent experiment is shown in the supplementary material.

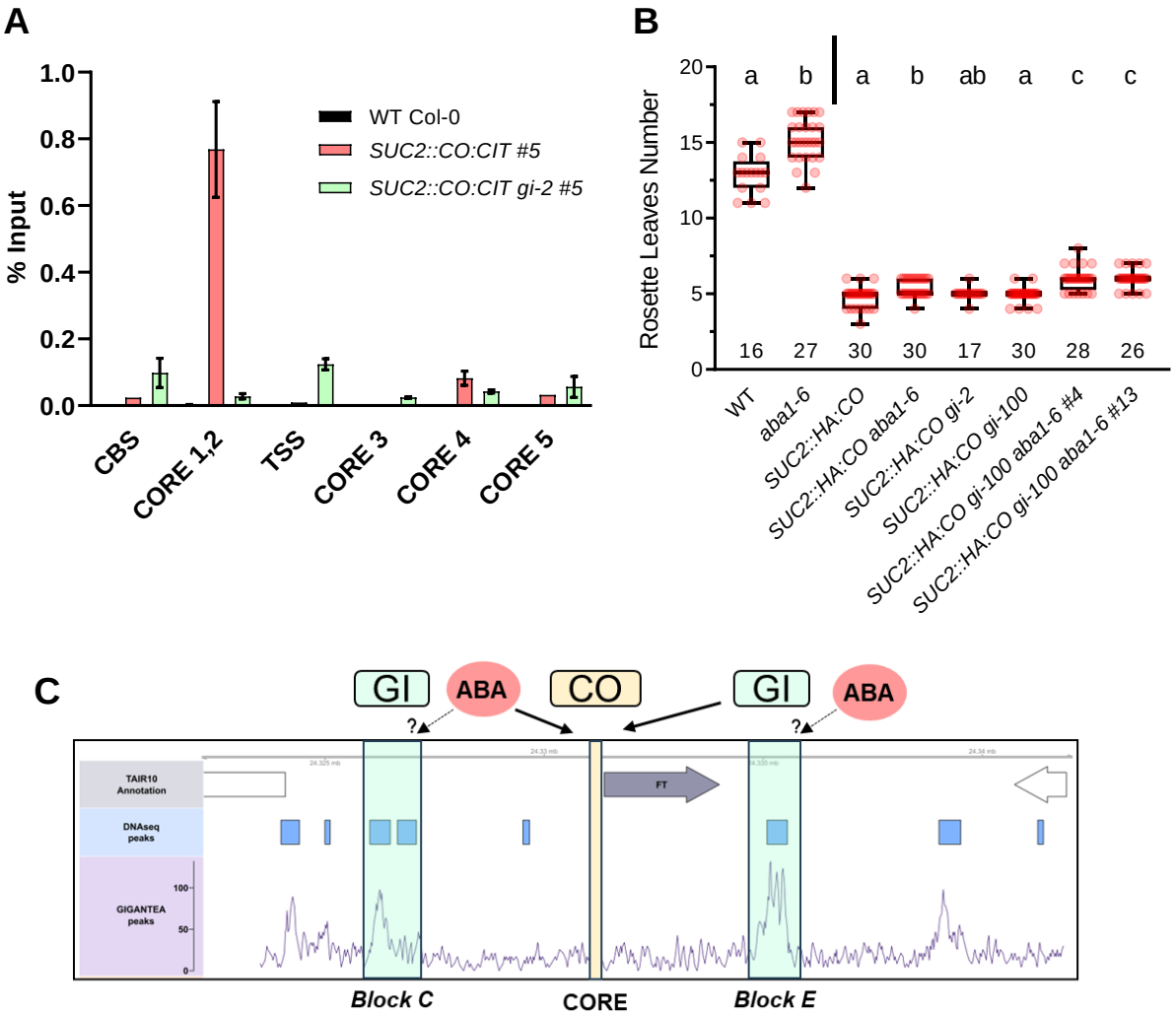

Figure 5. ABA signalling and *GI* act together to promoter CO recruitment to the *FT* promoter and regulate flowering time.

A Relative enrichment of CO:CIT in isogenic lines *SUC2::CO:CIT #5* (WT and and *gi-2* mutant) on the *FT* locus. Non transgenic, WT (Col-0) was used as a negative control. Bars represent the mean ± SE (n = 4 technical replicates). An independent experiment is shown in the supplementary material. B Boxplot of flowering time of the indicated genotypes and isogenic lines derived from the introgression of *SUC2::HA:CO* transgenes into *aba1-6* or *gi* mutant backgrounds. Note that for the *SUC2::HA:CO aba1-6 gi-100* combination, two sibling lines were analysed. An ANOVA test to assess the impact of mutations at the *ABA1* and *GI* loci on leaf number at bolting was run separately for transgenic (*SUC2::HA:CO*) and control plants (vertical bar). For both groups, genotype was a statistically significant predictor of leaf number at bolting ($p < .001$). Letters at the top of boxplots indicate if genotypes showed statistically significant differences ($p < .05$) according to a Tukey post-hoc test. The number of samples analysed for each genotype is shown at the bottom of the graph. C GI ChiPseq peaks on the *FT* region coincide with DNAase accessible chromatin (referred to as enhancer blocks C and E). CO recruitment to the CORE region is regulated by ABA and GI (thick arrows). ABA and GI might act independently to promote CO binding to the CORE region. ABA might also influence the accessibility of GI to Block C/E (dotted arrows), thus indirectly controlling CO recruitment.