THE PLANT CELL

DELLA degradation by gibberellin promotes flowering via GAF1-TPR-dependent repression of floral repressors in Arabidopsis

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Abstract

Flowering is the developmental transition from the vegetative to the reproductive phase. FLOWERING LOCUS T (FT), SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1 (SOC1), and LEAFY (LFY) are floral integrators. These genes are repressed by several floral repressors including EARLY FLOWERING3 (ELF3), SHORT VEGETATIVE PHASE (SVP), TEMPRANILLO1 (TEM1), and TEM2. Although gibberellin (GA) promotes flowering by activating the floral integrator genes, the exact molecular mechanism remains unclear. DELLAs are negative regulators in GA signaling and act as coactivators of the transcription factor GAI ASSOCIATED FACTOR 1 (GAF1). GAs convert the GAF1 complex from a transcriptional activator to a repressor. Here, we show that GAF1 functions in the GA-dependent flowering pathway by regulating FT and SOC1 expression in Arabidopsis thaliana. We identified four flowering repressors, ELF3, SVP, TEM1, and TEM2, as GAF1-target genes. In response to GAs, GAF1 forms a transcriptional repressor complex and promotes the expression of FT and SOC1 through the repression of four flowering repressor genes, ELF3, SVP, TEM1, and TEM2.

Introduction

The induction of flowering is a key developmental decision in a plant's life cycle, and its timing is controlled by a complex combination of developmental and environmental signals. In *Arabidopsis thaliana*, the autonomous, photoperiod, vernalization, thermosensory, and gibberellin (GA) pathways (Simpson and Dean, 2002; Blazquez et al., 2003; Halliday et al., 2003; Amasino, 2004) promote flowering by activating the floral integrator genes *FLOWERING LOCUS T* (*FT*), *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1* (*SOC1*), and *LEAFY* (*LFY*). FT, expressed in leaves, functions as a florigen that is transported to the shoot apex to induce flowering. Several transcription factors regulate *FT* expression. *CONSTANS* (*CO*) expression in the phloem is circadian-dependent; it induces *FT* expression under long day (LD) conditions. By contrast, *FT* expression is repressed by TEM1 and TEM2, which are RAV subfamily transcription factors containing an AP2/ERF and a B3 DNA-binding domain (Matias-Hernandez et al., 2014). Thus, CO and TEMs act as an *FT* activator and repressors, respectively. The binding sites of CO and TEM1 within the *FT* promoter are closely aligned, and their balance controls the *FT* expression level (Castillejo and Pelaz, 2008). The circadian clock component EARLY

FLOWERING3 (ELF3) and MADS-box transcription factor SHORT VEGETATIVE PHASE (SVP) are other negative regulators of flowering in *Arabidopsis* (Hartmann et al., 2000), which are involved in *FT* regulation in the leaves. In addition, SVP represses the expression of *SOC1* in the shoot apex and leaves and plays a role in the autonomous and GA pathways of flowering (Li et al., 2008). GAs promote *FT* expression in the leaves and *SOC1* expression in the shoot apex (Moon et al., 2003; Hisamatsu and King, 2008), but it is unclear how GAs regulate these genes.

GAs are critical for flowering in *Arabidopsis* under short day (SD) conditions (Wilson et al., 1992). The GA-deficient mutant *ga1-3* flowers moderately late under LD conditions but does not flower under SD conditions. Before floral initiation, the levels of bioactive GAs increase in the shoot apex via the activation of *GA20ox1*, encoding the GA-biosynthetic enzyme GA 20-oxidase, which promotes the expression of the floral integrators *SOC1* and *LFY* (Eriksson et al., 2006). DELLA proteins act as negative regulators of GA signaling (Sasaki et al., 2003; Ueguchi-Tanaka et al., 2005; Griffiths et al., 2006; Sun, 2011; Hauvermale et al., 2012). *Arabidopsis* contains five DELLAs, including GIBBERELLIN INSENSITIVE (GAI) (Sun and Gubler, 2004). DELLAs are rapidly degraded in the presence of GAs. A DELLA quadruple mutant of *Arabidopsis* flowers early under SD conditions (Cheng et al., 2004). Recently, we identified a DELLA-interacting factor, GAI-ASSOCIATED FACTOR1 (GAF1), and revealed the role of DELLAs as transcriptional coactivators (Fukazawa et al., 2014). GAF1 also interacts with corepressor TOPLESS-RELATED (TPR). DELLAs and TPR are coactivators and a corepressor of GAF1, respectively (Fukazawa et al., 2014). GAs convert the GAF1 complex from a transcriptional activator to a repressor. GAF1 is a transcription factor with zinc-finger motifs, belonging to the INDETERMINATE1 (ID1) (Colasanti et al., 1998) domain (IDD) family. In maize (*Zea mays*), an *id1* mutant flowers late (Singleton, 1946; Colasanti et al., 1998), indicating its role in floral transition in monocots. Among the *Arabidopsis* IDD family members, INDETERMINATE DOMAIN1 (IDD1)/ENHYDROUS was found to be most closely related to GAF1. In *Arabidopsis*, the *gaf1 idd1* double mutant also flowers later than the wild-type under SD conditions, whereas *GAF1* overexpressing lines flower earlier than the wild-type (Figure 1, A and previously shown [Fukazawa et al., 2014]), suggesting that the GAF1 complex is involved in the GA-mediated regulation of flowering.

In this study, we identified four flowering repressors, *ELF3*, *TEM1*, *TEM2*, and *SVP*, as GAF1-target genes. In response to GAs, GAF1 forms a transcriptional repressor complex and promotes the expression of *FT* and *SOC1* by repressing genes encoding negative regulators of flowering including ELF3, TEM1, TEM2, and SVP. We demonstrated that ELF3 represses the expression of *FT* in combination with LUX and ELF4 as part of the Evening Complex (EC). We provide a model of the GA flowering pathway in which GAs promote flowering via GAF1-TPR-dependent repression of flowering repressors.

Results

GAF1 regulates flowering via altering the expression levels of GA-controlled flowering genes

To gain insight into the role of the GAF1 complex in the regulation of flowering, we investigated the expression levels of genes encoding the floral pathway integrators FT, SOC1, and LFY in the *gaf1 idd1* double mutant and the GAF1 overexpressor under SD conditions (Figure 1, A). The expression levels of *FT*, *SOC1*, and *LFY* were increased in the GAF1 overexpressor, whereas the expression levels of *FT* and *SOC1* were decreased in the *gaf1 idd1* double mutant (Figure 1, B). The induction of *SOC1* in the GAF1 overexpressor was later, by approximately 1 week, than the induction of *FT* under SD conditions (Figure 1, B). Because GAF1 forms a transcriptional repressor complex under GA-sufficient conditions (Fukazawa et al., 2014), it cannot directly promote the expression of *FT*, *SOC1*, and *LFY* in GA-controlled flowering. Rather, the expression of *FT*, *SOC1*, and *LFY* might be induced by the GAF1-dependent downregulation of flowering repressor genes.

Identification of novel GAF1-target genes involved in the regulation of flowering

To identify GAF1-target genes involved in flowering, RNA-seq analysis was conducted using transgenic plants harboring estradiol-inducible GAF1 (Figure 2, A; Zuo et al., 2000). Flowering-related genes including *ELF3*, *ELF5*, *TEM1*, *TEM2*, *EARLY FLOWERING MYB PROTEIN* (*EFM*), and *SVP* were selected as candidates of novel GAF1-target genes among genes downregulated after the induction of GAF1 (Figure 2, B and C and Supplemental Data set S1). Their expression levels increased in the *gaf1 idd1* double mutant but the expression levels of *ELF3* and *EFM* decreased and other genes were slightly decreased in the GAF1 overexpressor (Figure 2, D).

To investigate whether the GAF1 complex was involved in the regulation of these genes, we performed a transient assay using *Arabidopsis* protoplasts. The promoter of each gene was fused with the *LUC* reporter gene (Figure 3, A). The *ELF3*, *SVP*, *TEM1*, and *TEM2* gene promoters were activated by the DELLA-GAF1 complex but repressed by the TPR4-GAF1 complex (Figure 3, B). However, the DELLA-GAF1 complex did not affect the activity of the *EFM* and *ELF5* promoters (Supplemental Figure S1). These results suggested that *ELF3*, *SVP*, *TEM1*, and *TEM2* are directly regulated by the GAF1 complex. To define the cis-acting elements for the DELLA-GAF1 complex in each promoter of the GAF1 target genes, we generated a series of 5′-deletions in each promoter—*ELF3*Δ-3000, *ELF3*Δ-1500, *ELF3*Δ-1000, *ELF3*Δ-500; *SVP*Δ-3000, *SVP*Δ-1500, *SVP*Δ-1000, *SVP*Δ-500; *TEM1*Δ-1500, *TEM1*Δ-1000, *TEM1*Δ-500; and *TEM2*Δ-1500, *TEM2*Δ-1000, *TEM2*Δ-500—fused with the *LUC* reporter gene and determined the promoter activities in a transactivation assay (Figure 3, C). The DELLA-GAF1 complex
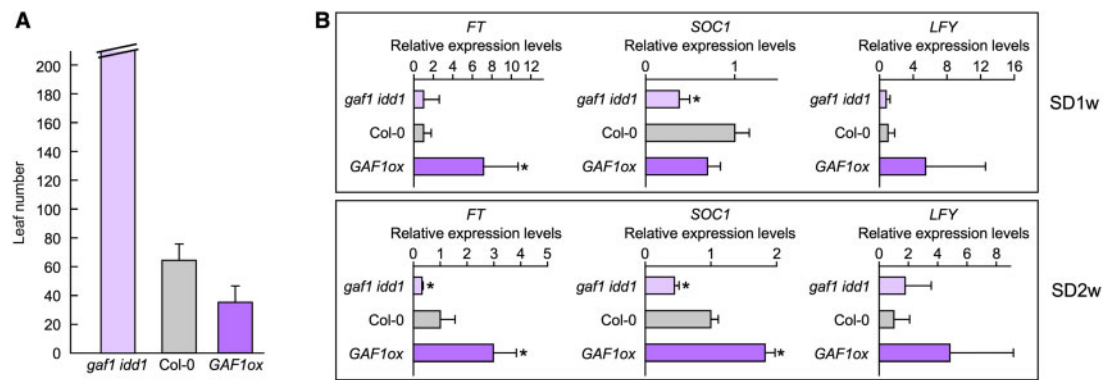
Figure 1 The GAF1 overexpressor and *gaf1 idd1* mutant exhibit early and late-flowering phenotypes, respectively, via alterations to the expression levels of flowering integrator genes. A, Flowering time analysis (rosette leaf number) under SD conditions. The *GAF1* overexpressor exhibited an early flowering phenotype under SD conditions. The *gaf1 idd1* mutant exhibited an extremely delayed flowering phenotype under SD conditions Error bars indicate SD of the mean (*n* > 8). Asterisks represent Student's *t* test significance compared with Col-0 (**P* < 0.05). B, Plants were grown under SD conditions for 1 or 2 weeks. Plants were harvested at zeitgeber (ZT) 8. Relative expression levels of *FT*, *SOC1*, and *LFY* in Col-0, *gaf1 idd1*, and *GAF1* overexpressor plants are shown. The results are shown as relative expression levels to *UBQ11*. Error bars indicate the SD of three biological replicates (*n* = 3). Asterisks represent Student's *t* test significance compared with Col-0 (**P* < 0.05).

activated *ELF3* (*ELF3Δ-1500* and *ELF3Δ-1000*), *SVP* (*SVPΔ-500*), *TEM1* (*TEM1Δ-1000*, *TEM1Δ-500*), and *TEM2* (*TEM2Δ-1500*), suggesting that each promoter contains a cis-acting element for DELLA-GAF1 in the 500-bp region between −1000 and −501 of the *ELF3* promoter, −500 to −1 of the *SVP* promoter, −500 to −1 of the *TEM1* promoter, and −1500 to −1001 of the *TEM2* promoter. The consensus binding motif for IDD family transcription factors is TTTTGTCG and GAF1 also binds to this sequence and closely related motifs (Fukazawa et al., 2014, 2017). Putative binding motifs for DELLA-GAF1 were found in each of the experimentally identified 500-bp regions of the *ELF3*, *SVP*, *TEM1*, and *TEM2* promoters. The electrophoretic mobility shift assay (EMSA) indicated that recombinant GAF1 directly binds to these sequences, which are *ELF3* (−529 to −510), *SVP* (−20 to −1), *TEM1* (−152 to −133), and *TEM2* (−1,288 to −1,269), in vitro (Figure 3, D). To investigate the binding of GAF1 to each gene promoter in vivo, we used transgenic plants expressing Myc-tagged GAF1 under the control of the CaMV 35S promoter. ChIP assays showed that GAF1 binds to each promoter (*ELF3*, −620 to −420; *SVP*, −103 to +97; *TEM1*, −241 to −41; *TEM2*, −1,382 to −1,182; Figure 3, E), indicating that GAF1 interacts directly with each target gene promoter in vivo.

Tissue-specific expression profiles and GA sensitivity of flowering integrators and GAF1 target genes

To investigate the temporal and spatial expression of flowering integrators and GAF1 target genes with or without GA, we carried out real-time PCR and histochemical analysis using transgenic plants. *FT* and *SOC1* expression levels were increased by GAs within 1 d and 7 d under SD conditions, respectively. The induction of *FT* and *SOC1* by GAs was not observed in the *gaf1 idd1* mutant, indicating that GAF1 and

IDD1 were involved in the induction of *FT* and *SOC1* by GAs under SD conditions (Figure 4, A). Histochemical analysis using transgenic plants carrying the *FT* and *SOC1* promoter fused with GUS showed that GAs promote *FT* and *SOC1* expression in the leaves and the shoot apex, respectively (Figure 4, B).

To investigate the response of the GAF1 target genes to GA, we examined the expression levels of these genes with or without GAs. We found that the expression levels of *ELF3*, *TEM1*, *TEM2*, and *SVP* were significantly reduced by GAs within 1 d (Figure 5). These reductions in *ELF3*, *TEM1*, *TEM2*, and *SVP* by GAs were not observed in the *gaf1 idd1* mutant under SD conditions, indicating that GAs promote the expression of *FT* and *SOC1* via the reduction of GAF1 target genes, including *ELF3*, *TEM1*, *TEM2*, and *SVP* (Figure 5).

To confirm GA responsibility of novel GAF1 target genes including *ELF3*, *TEM1*, *TEM2*, and *SVP*, we investigated the expression levels of these gene following shorter exposures to GA. *ELF3* and *TEM1* are decreased by GA within 3 h and *TEM2* and *SVP* was decreased by GA within 6 h (Supplemental Figure S2). Thus, GAF1 target genes respond to GA and decreased at least within 6 h.

Next, we examined the effects of GA on the tissue-specific expression patterns of GAF1 target genes using transgenic plants carrying each GAF1 target gene promoter fused with GUS. The GUS staining assay showed expression of *ELF3*, *TEM1*, and *SVP* in the leaves and *TEM1*, *TEM2*, and *SVP* in the shoot apex under both LD and SD conditions (Figure 6, A and B). Furthermore, GUS activity in all transgenic lines decreased with GA treatment in the seedling, leaf, and the shoot apex (Figure 6, C). To investigate the contribution of GAF1 and IDD1 to the spatial expression of *TEM1*, *TEM2*, *ELF3*, and *SVP*, we generated transgenic plants carrying the *TEM1*, *TEM2*, *ELF3*, and *SVP* promoters fused with *GUS* in
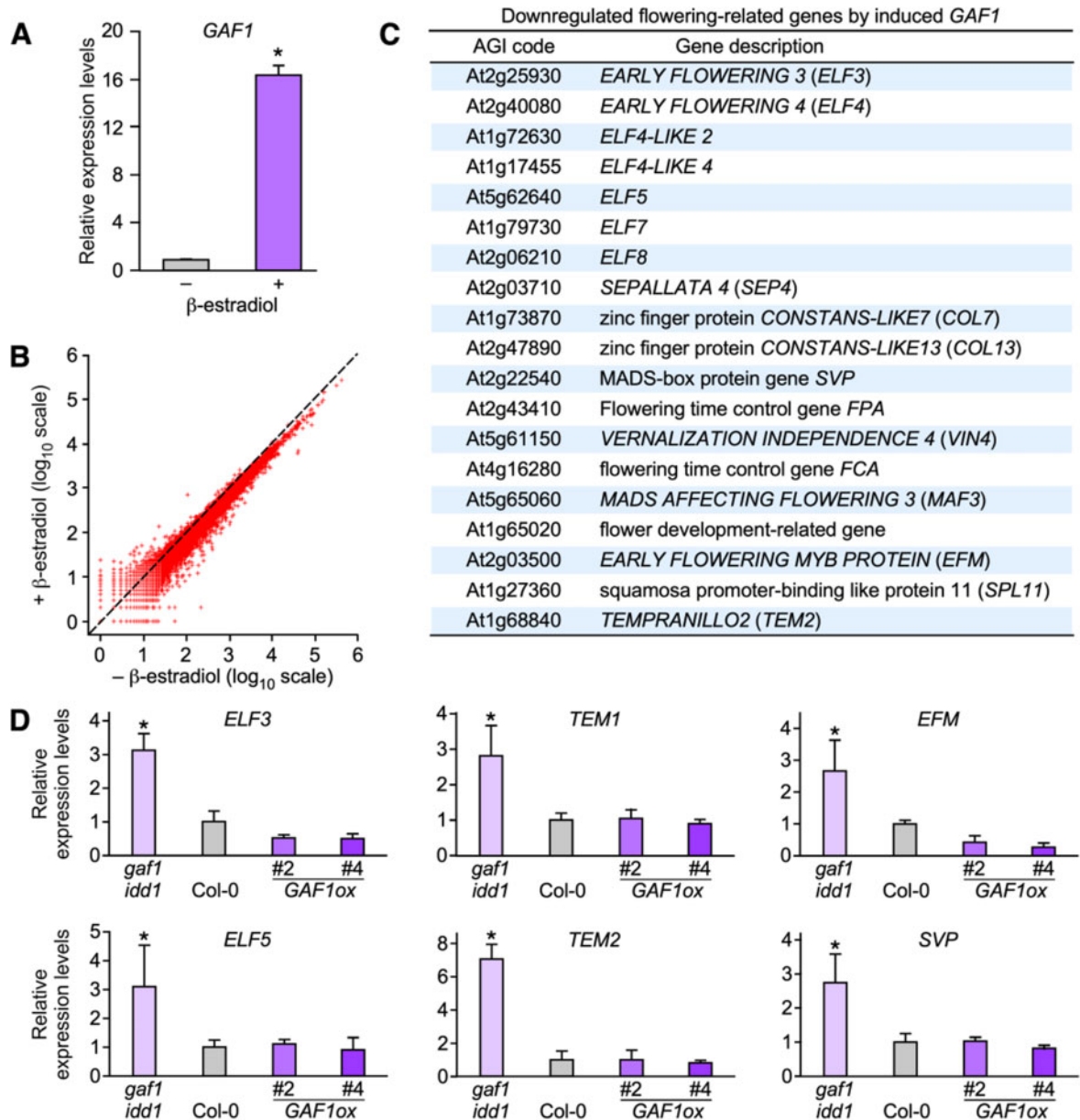
Figure 2 Search for GAF1 target genes using the inducible *GAF1* transgenic plant. A, Transgenic plants carrying pER10-GAF1 grown under LD conditions for 1 week. Whole plants were treated with or without β-estradiol for 24 h and were harvested at ZT8 under LD conditions. Relative expression levels of GAF1 of the pER10-GAF1 transgenic plant with or without β-estradiol treatment are shown. Error bars indicate the SD of three biological replicates ($n = 3$). Asterisks represent Student's *t* test significance compared with Col-0 (*$P < 0.05$). B, RNA-Seq analysis using an inducible *GAF1* transgenic line in which the XVE system induced the expression of *GAF1* in 7-d-old pER10-GAF1 *Arabidopsis* seedlings treated with β-estradiol or mock treated for 24 h. C, A list of flowering-related genes downregulated by induced GAF1. D, Plants were grown under SD conditions for 2 weeks. Whole seedlings were harvested at ZT8. Relative expression levels of *ELF3*, *TEM1*, *EFM*, *ELF5*, *TEM2*, and *SVP* in Col-0, *gaf1 idd1*, and *GAF1* overexpressor plants. Error bars indicate the SD of three biological replicates ($n = 3$). Asterisks represent Student's *t* test significance compared with Col-0 (*$P < 0.05$).

the *gaf1 idd1* mutant background. The GUS activity of the transgenic plant in the *gaf1 idd1*-background was relatively high compared with that in GUS transgenic plants in the Col-0 background (Figure 6, A and B), indicating that GAF1 and IDD1 were involved in the repression of *TEM1*, *TEM2*, *ELF3*, and *SVP*.

The expression of *FT* and *SOC1* is repressed by TEM1 and SVP

We next investigated how the floral integrator genes *SOC1* and *FT* are affected by the GAF1 target genes and if this potential effect is GA-dependent. Although LFY is also a floral integrator, it was excluded from this analysis because SOC1
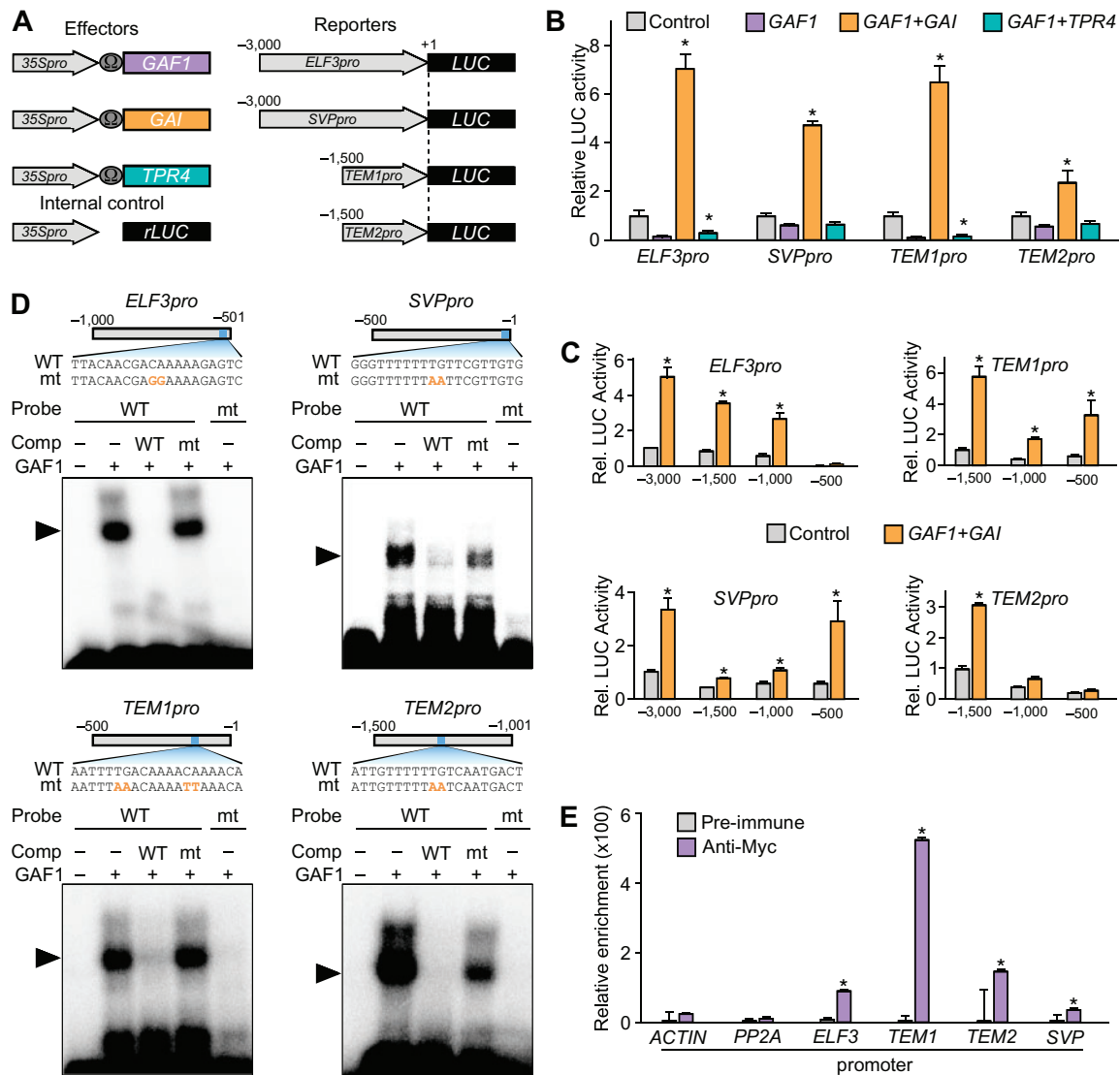
Figure 3 Identification of novel GAF1 target genes involved in the regulation of flowering. A, Schematic representation of the reporter and effector. Fragments (3,000 bp) of the ELF3 and SVP promoters and 1,500-bp fragments of the TEM1 and TEM2 promoters were fused with the LUC gene. The effector plasmid expressed full-length GAF1, GAI, or TPR4 under control of the CaMV 35S promoter with a viral translation enhancer (Ω). B, Transient expression assay showed that the DELLA-GAF1 complex activated and the TPR4-GAF1 complex repressed the ELF3, SVP, TME1, and TEM2 promoters. The reporter plasmids consisted of the 3,000-bp promoter regions of ELF3 and SVP and the 1,500-bp promoter regions of TEM1 and TEM2 fused with the LUC reporter gene. The results are shown as LUC/rLUC activity. Error bars indicate the SD of three biological replicates ($n = 3$). Asterisks represent Student's t test significance compared with mock treated control (*$P < 0.05$). C, Transactivation assay of GAF1 and GAI. The effector, reporter, and internal control constructs were co-transfected into Arabidopsis protoplasts. The transfected cells were incubated for 20 h, and Luc and rLUC activities were measured. The results are shown as LUC/rLUC activity. Error bars indicate SD of the mean ($n = 3$). Asterisks represent Student's t test significance compared with mock treated control (*$P < 0.05$). D, Identification of GAF1-binding regions in the ELF3, SVP, TEM1, and TEM2 promoters in vitro. EMSA analysis using recombinant GAF1 protein. Oligonucleotides containing ELF3pro (-529 to -510, wild-type; lanes 1–4) or mutated (mt) ELF3pro (mt; lane 5), SVPpro (-20 to -1, wild-type; lanes 1–4) or mtSVPpro (mt; lane 5), TEM1pro (-152 to -133, wild-type; lanes 1–4) or mtTEM1pro (mt; lane 5), and TEM2pro ($-1,288$ to $-1,269$, wild-type; lanes 1–4) or mtTEM2pro (mt; lane 5) were used as probes. Orange letters indicate mutated bases. Wild-type and mt indicate competition with a 100-fold to 500-fold excess of unlabeled wild-type and mutated probe, respectively. The specific GAF1–DNA complexes are indicated by arrowheads. +, Addition to the reaction mixtures; −, omission from the reaction mixtures. E, Plants were grown under SD conditions for 2 weeks. Whole seedlings were harvested at ZT8. GAF1 binds to a region of the ELF3, SVP, TEM1, and TEM2 promoters in vivo. ChIP assays were performed with pre-immune or anti-myc in CaMV 35S:myc-GAF1 transgenic plants. The co-precipitated level of each DNA fragment was quantified by real-time PCR and normalized to the input DNA. Error bars indicate SD of three technical replicates ($n = 3$). Asterisks represent Student's t test significance compared with pre-immune as control (*$P < 0.05$). Experiments were repeated twice with independently grown plants, with similar results.
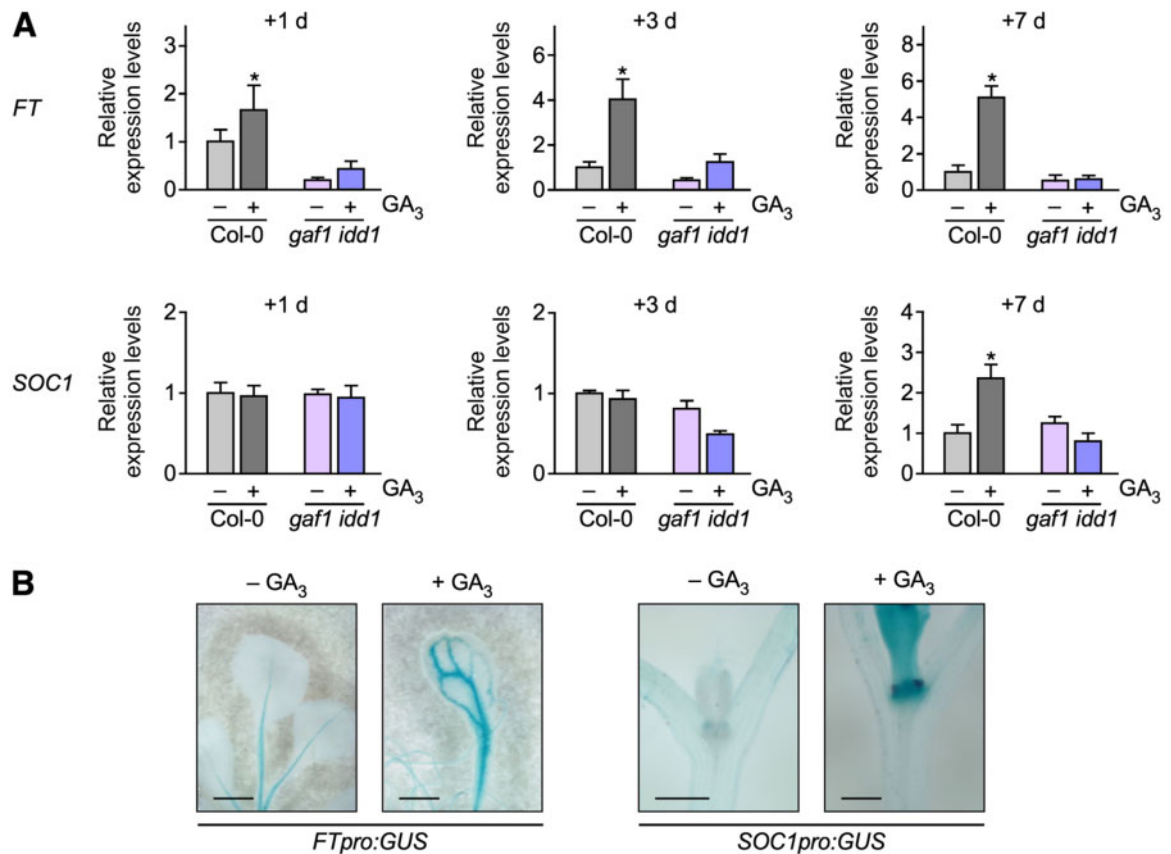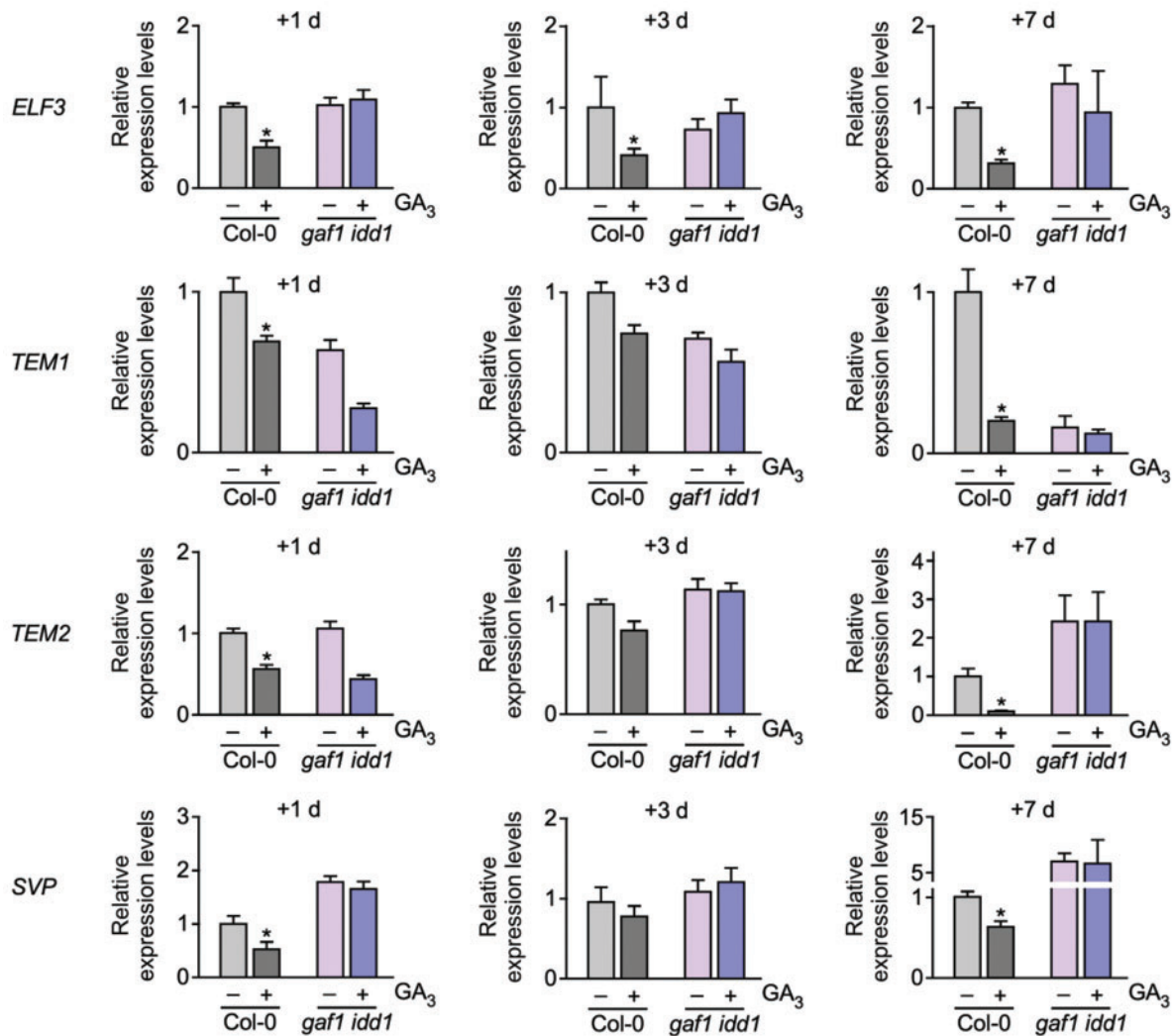
Figure 4 Tissue-specific expression profiles and GA sensitivity of *FT* and *SOC1*. A, Relative expression levels of *FT* and *SOC1* in Col-0 and *gaf1 idd1* with or without GA_3. Plants were grown under SD conditions for 1 week and were treated for 1 d, 3 d, or 1 week with or without GA_3. Whole seedlings were harvested at ZT8. Error bars indicate SD of three biological replicates ($n = 3$). Asterisks represent Student's *t* test significance compared with control (*$P < 0.05$). B, GUS expression pattern in transgenic *Arabidopsis* plants carrying *FT* promoter:*GUS* and *SOC1* promoter:*GUS*. Plants were grown under SD conditions for 1 week with or without GA_3. Scale bar indicates 1 mm.

directly activates *LFY* in the shoot apex (Lee and Lee, 2010), indicating that LFY acts downstream of SOC1 in flowering. Previous reports showed that SVP directly represses *SOC1* and *FT* by binding to their promoters (Li et al., 2008) and that TEM1 and TEM2 also act as direct *FT* repressors (Castillejo and Pelaz, 2008; Osnato et al., 2012). To confirm whether SVP, TEM1, and TEM2 regulate the expression of *SOC1* and *FT*, we conducted a transient expression assay (Figure 7, A). We observed that SVP represses the *SOC1* promoter and that TEM1 and SVP repress the *FT* promoter. TEM2 also repressed the *FT* promoter, but this repression activity of TEM2 was weaker than that of TEM1 (Figure 7, B and C).

ELF3 controls flowering through the regulation of *FT* expression

Although Boden et al. (2014) reported that the expression level of *FT* is increased in a barley (*Hordeum vulgare*) *elf3* mutant, it was unclear how ELF3 regulates flowering through the regulation of *FT* expression. We confirmed that *FT* expression is also increased in the *Arabidopsis elf3* mutant

(Figure 8, A). The phenotype of *elf3* was more pronounced than that of the other GAF1 target gene mutants. Therefore, we focused our analysis on *ELF3*. Since GAs promote the conversion of the GAF1 complex from an activator to a repressor, the GAF1-TPR complex appears to function as the repressor of *ELF3* under GA-sufficient conditions. We found that *ELF3* expression levels decreased with GAs (Figure 5). Although *gaf1* or *idd1* single mutant did not exhibit obvious phenotypes, the *gaf1 idd1* double mutant exhibited an extremely late-flowering phenotype under SD conditions (Figure 1, A; Fukazawa et al., 2014), indicating that both GAF1 and IDD1 are involved in the GA flowering pathway. ELF3 is a circadian clock gene that contributes to photoperiod-dependent flowering, and the early flowering phenotype of *elf3* was clearer under SD conditions than under LD conditions (Figure 8, D and E). We further investigated *ELF3* expression under LD and SD conditions. We found that *ELF3* expression is higher in *gaf1 idd1* plants than in Col-0 plants under SD conditions; whereas *ELF3* expression was lower in the GAF1 overexpressor than in Col-0 under SD conditions (Figure 8, B and C).

Figure 5 Tissue-specific expression profiles and GA sensitivity of GAF1 target genes. A, Relative expression levels of *ELF3*, *TEM1*, *TEM2*, and *SVP* in Col-0 and *gaf1 idd1* with or without GA$_3$ treatment. Plants were grown under SD conditions for 1 week and were treated with GA$_3$ or mock-treated for 1 d, 3 d, or 1 week. Whole seedlings were harvested at ZT8. Error bars indicate sD of three biological replicates ($n = 3$). Asterisks represent Student's *t* test significance compared with the mock-treated control (*$P < 0.05$).

## ELF3 acts downstream of GAF1 and IDD1 in flowering

The *elf3-7* mutant exhibited the early flowering phenotype, and the *gaf1 idd1* double mutant had the late-flowering phenotype under both LD and SD conditions. If ELF3 acts as a flowering repressor downstream of GAF1 and IDD1 in the GA flowering pathway, the *gaf1 idd1 elf3* triple mutant should exhibit an early flowering phenotype similar to that of the *elf3* mutant. To investigate flowering phenotype-associated epistasis between the *elf3* and *gaf1 idd1* mutations, we determined the flowering time of *elf3*, *gaf1 idd1*, and *gaf1 idd1 elf3-7*. As expected, the *gaf1 idd1 elf3-7* triple mutant flowered earlier than the wild-type Col-0 or *gaf1 idd1* mutant (Figure 8, D and E), indicating that *elf3-7* is epistatic to *gaf1 idd1*. This result suggests that ELF3 acted downstream of GAF1 and IDD1 in flowering. The *gaf1 idd1 elf3-7* triple mutant flowered slightly later than the *elf3-7* mutant (Figure 8, D and E), implying that other factors including

SVP, TEM1, and TEM2 might act downstream of GAF1 and IDD1 in flowering.

## ELF3 represses the expression of *FT* in combination with LUX and ELF4 as part of the EC

Our data indicated the possibility that ELF3 inhibits the expression of *FT* and that GAs activate *FT* via *ELF3* repression induced by the GAF1-TPR complex (Figure 9, A). However, ELF3 does not have a conventional DNA-binding domain, suggesting that a DNA-binding transcription factor was involved in the ELF3-mediated transcriptional regulation of *FT*. ELF3 is a component of the trimeric EC, formed by interactions among ELF3, EARLY FLOWERING4 (ELF4), and LUX ARRHYTHMO (LUX) (Nusinow et al., 2011). Both *elf4* and *lux* mutants also exhibit an early flowering phenotype (Doyle et al., 2002; Hazen et al., 2005). Because the EC acts as the transcriptional repressor complex that represses the expression of *PIF4* and *PIF5* (Nusinow et al., 2011), it might
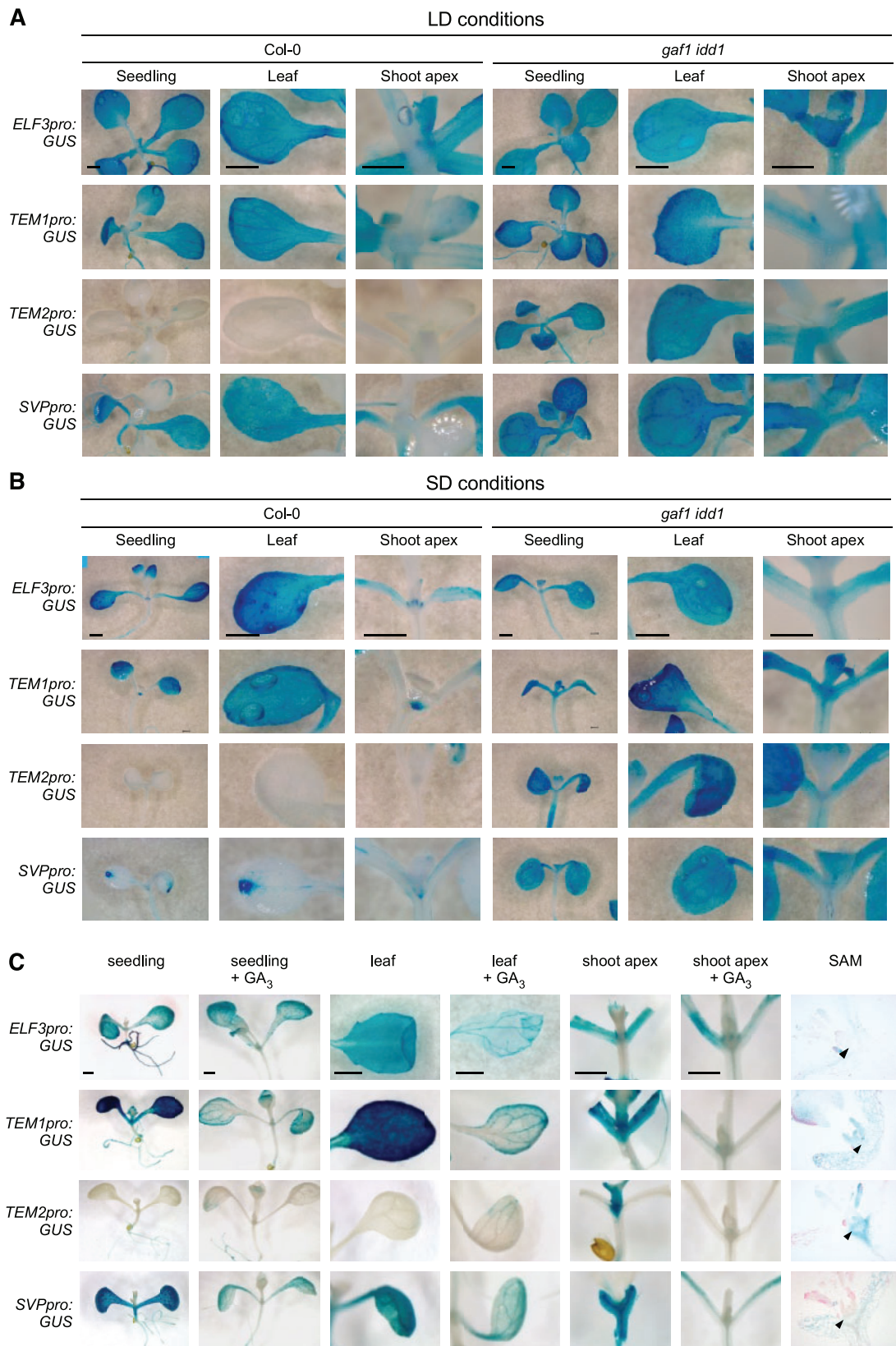
Figure 6 Spatial expression pattern and GA sensitivity of GAF1 target genes. A and B, GUS expression patterns in transgenic *Arabidopsis* plants carrying *ELF3* promoter:*GUS*, *TEM1* promoter:*GUS*, *TEM2* promoter:*GUS*, and *SVP* promoter:*GUS* in Col-0 (left side nine panels) and *gaf1 idd1* (right side nine panels). Plants were grown under LD conditions for 2 weeks (A) and SD conditions for 1 week (B). C, GUS expression patterns in transgenic *Arabidopsis* plants carrying *ELF3* promoter:*GUS*, *TEM1* promoter:*GUS*, *TEM2* promoter:*GUS*, and *SVP* promoter:*GUS*. Seedling (first and second colums), leaves (third and fourth colums) and meristematic regions (fifth and sixth colums), and SAM (arrow head in seventh colums) in transgenic plants were analyzed. Plants were grown for 1 week with or without GA_3. Scale bar indicates 1 mm.
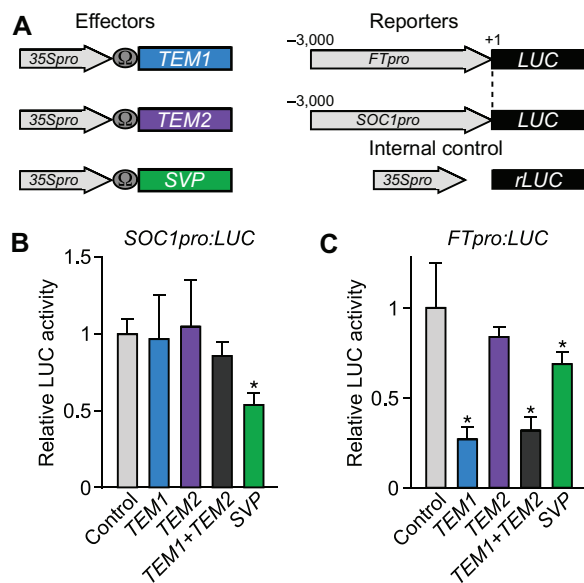
Figure 7 The expression of *FT* and *SOC1* is repressed by TEM1 and SVP. A, Schematic representation of the reporter and effector. A 3,000-bp fragment of the *FT* and *SOC1* promoter was fused with the *LUC* gene. The effector plasmid expressed the full-length TEM1, TEM2, and SVP under the control of the CaMV 35S promoter with a viral translation enhancer (Ω). B and C, The *SOC1* promoter is regulated by SVP; the *FT* promoter is regulated by TEM1. The effector, reporter, and internal control constructs were co-transfected into *Arabidopsis* protoplast cells. The transfected cells were incubated for 20 h and then Luc and rLUC activities were measured. The results are shown as LUC/rLUC activity. Error bars in (B) and (C) indicate sd of three biological replicates ($n = 3$). Asterisks represent Student's *t* test significance compared with mock treated control (*$P < 0.05$).

also repress *FT*. To test this possibility, we performed a transient expression assay using T87 *Arabidopsis* cultured cells, suggesting that the EC repressed the *FT* promoter (Figure 9, B and C). We further investigated the transcriptional repression activity of each EC protein in the transient expression assay. Only ELF3 possessed transcriptional repression activity, whereas ELF4 and LUX did not exhibit any repression activity in this assay (Figure 9, B and D). The LUX protein belongs to the MYB transcription factor family that binds to the GATACG consensus sequence (Helfer et al., 2011). EMSA showed that LUX binds to a GATACG-related motif in the *FT* promoter (−207 to −187; Figure 9, E). To determine whether the GATACG-related sequence is required for the repression of the *FT* promoter by the EC in vivo, we constructed a mutant *FT* promoter-LUC fusion, in which the LUX binding sequence was removed. The mutation in the LUX binding sequence in the *FT* promoter completely abolished transcriptional repression by the EC (Figure 9, F). The binding site of the EC including LUX is located within Block A in the proximal *FT* promoter (Figure 9, G). Since the binding site is conserved among Brassicaceae species, this *FT* promoter region might be required for the transcriptional repression of *FT* and the response to GA-dependent *FT* expression (Adrian et al., 2010). To test the binding of LUX protein to the *FT* promoter in vivo, we produced an

anti-LUX antibody and performed a ChIP assay. The ChIP assay demonstrated that LUX binds to the *FT* promoter (−300 to −100) in plants (Figure 9, G). These data indicated that ELF3 functions as a co-repressor and that the EC interacts with the *FT* promoter via the LUX transcription factor to repress *FT*. Therefore, our results suggest that GAF1 promotes the expression of *FT* via transcriptional repression of *ELF3*, a component of the EC in GA-controlled flowering.

Discussion

In *Arabidopsis*, GA deficient mutants exhibited non-flowering phenotypes under SD conditions, suggesting that the GA flowering pathway is important under SD conditions (Wilson et al., 1992). Endogenous GA levels increase substantially before floral initiation under SD conditions and promote flowering via the induction of two floral integrator genes, *FT* in the leaves and *SOC1* in the shoot apex. However, initiating the GA-dependent flowering pathway requires the suppression of several transcriptional repressors that inhibit the expression of *FT* and *SOC1*. Our study demonstrated that GA promotes the expression of *FT* and *SOC1* by suppressing a group of flowering repressors, *ELF3*, *SVP*, *TEM1*, and *TEM2*, via the GAF1-TPR complex. GUS staining analysis revealed that *ELF3*, *SVP*, and *TEM1* are expressed in the leaves and that *SVP*, *TEM1*, and *TEM2* are expressed in the shoot apex (Figure 6). The transition from SD to LD promotes the expression of *FT* by suppressing EC, TEM1, and TEM2, leading to flowering. However, to promote flowering under SD conditions, another mechanism is needed to suppress EC, TEM1, and TEM2, which maintain low *FT* expression levels. Our data indicate that GAs activate the expression of *FT* in the leaves by inducing the formation of the GAF1-TPR complex to repress *ELF3*, which encodes an EC component, along with *TEM1* and *SVP*. This process ultimately facilitates the translocation of FT from the leaves to the shoot apex. In the shoot apex, GAs decreased the expression of *SVP*, which acts as a repressor of *SOC1* (Andres et al., 2014), and promotes *SOC1* expression by mediating the translocation of FT from leaves (Figure 10).

The expression level of *ELF3* in the *gaf1 idd1* mutant is higher than that in Col-0 under SD conditions but did not differ from that in Col-0 under LD conditions (Figure 8, B and C). These results are consistent with the phenomenon that the GA-dependent flowering pathway became more apparent under SD conditions. ELF3 overexpression represses the expression of *FT* (Nieto et al., 2015). In this study, we showed that ELF3 acts as a transcriptional repressor of *FT* in association with ELF4 and LUX. The PIF4 transcription factor mediates the thermal induction of flowering via the direct activation of *FT* under SD conditions (Kumar et al., 2012). ELF3 also suppresses PIF4 activity through transcriptional repression and sequestration. The transcription of *PIF4* is repressed by EC during the early part of the night, and the binding of ELF3 to the PIF4 protein prevents PIF4-binding to the *FT* promoter (Nusinow et al., 2011; Nieto et al., 2015). Thus, ELF3 might regulate *FT* expression via the suppression
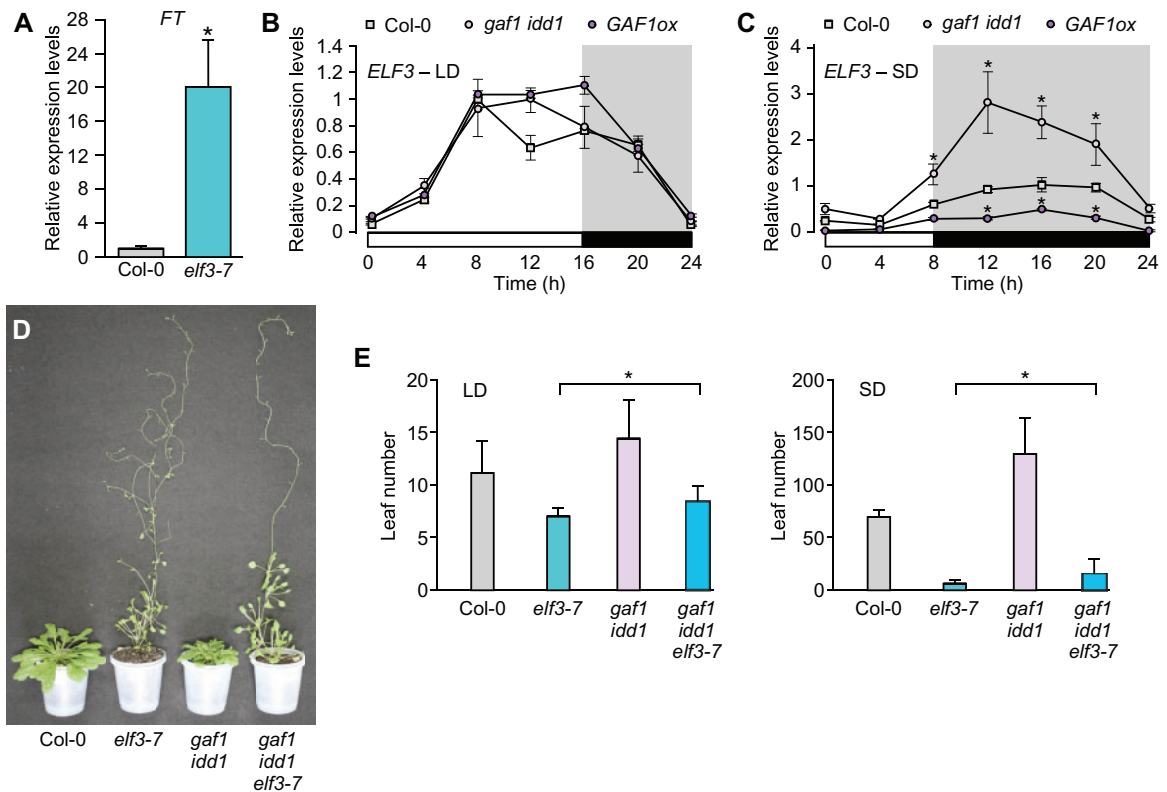
Figure 8 ELF3 acts downstream of GAF1 and IDD1 in flowering. A, Relative expression levels of *FT* in Col-0 or *elf3-7*. Plants were grown under SD conditions for 2 weeks. Whole seedlings were harvested at ZT8. Error bars indicate SD of three biological replicates (*n* = 3). Asterisks represent Student's *t* test significance compared with Col-0 as control (*P < 0.05). B, Diurnal expression of *ELF3* in Col-0, *gaf1 idd1*, and GAF1ox under LD conditions. Plants were grown under LD conditions for 2 weeks. Whole seedlings were harvested at ZT0, 4, 8, 12, 16, 20, and 24. Error bars indicate SD of three biological replicates (*n* = 3). C, Diurnal expression of *ELF3* in Col-0, *gaf1 idd1*, and GAF1ox under SD conditions. Seedlings were grown in SD conditions for 2 weeks. Whole seedlings were harvested at ZT0, 4, 8, 12, 16, 20, and 24. Error bars indicate SD of three biological replicates (*n* = 3). Asterisks represent Student's *t* test significance compared with Col-0 as control (*P < 0.05). D, Col-0, *elf3-7*, *gaf1 idd1*, and *gaf1 idd1 elf3-7* plants were analyzed at 104 d after germination under SD conditions. All mutants had a Col-0 background. E, Flowering time of Col-0, *elf3-7*, *gaf1 idd1*, and *gaf1 idd1 elf3-7* plants under LD or SD conditions. Data are means of at least eight plants. Error bars indicate the SD of total leaf number. Asterisks represent Student's *t* test significance compared between *elf3-7* and *gaf1 idd1 elf3-7* (*P < 0.05).

of PIF4 activity and EC-dependent transcriptional repression under SD conditions (Figure 9, C).

The ectopic expression of GIBBERELLIN 2 OXIDASE 7 (GA2ox7), which catabolizes active GAs, in the vascular tissue and in the shoot apices using tissue-specific promoters revealed that GA-dependent flowering was regulated in two different tissues: the leaves and the shoot apices (Porri et al., 2012). Similar results were obtained when *della-Δ17*, the GA-insensitive constitutively active DELLA protein, was ectopically expressed in leaf or shoot apex tissues (Galvao et al., 2012). They showed that the degradation of DELLA by GAs promotes expression of *FT* and *TWIN SISTER OF FT* (*TSF*) in leaves and the expression of *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE* (*SPL*) in the shoot apex. GAs also promote the expression of *SOC1* in the shoot apex through the degradation of DELLA proteins under SD conditions (Moon et al., 2003). ELF3, TEM1, and TEM2 act as repressors of *FT* and SVP acts as a repressor of *SOC1* and *SPL* (Figures 7, 9; Castillejo and Pelaz, 2008; Li et al., 2008; Andres et al., 2014). These reports support the GAF1-dependent GA flowering model in which GA-induced DELLA degradation

promotes flowering through the repression of GAF1 target genes, *TEM1*, *TEM2*, *ELF3*, and *SVP*, in leaves and/or shoot apices (Figure 10).

The *ft-1* and *soc1* mutants exhibit a low-GA sensitive phenotype rather than GA insensitive for flowering, indicating the existence of a GA-induced, FT-independent, and SOC1-independent flowering pathway (Moon et al., 2003; Hisamatsu and King, 2008). Moreover, *TSF* and *FRUITFULL* (*FUL*) are functionally redundant to *FT* and *SOC1*, respectively, in flowering. The *ft tsf soc1 ful* quadruple mutant exhibits a late-flowering phenotype, but GAs still promote flowering in this quadruple mutant, whereas the *ft tsf soc1 ful svp* quintuple mutant exhibits a GA-insensitive phenotype in flowering (Andres et al., 2014). These observations suggest that SVP regulates target genes other than *FT*, *TSF*, *FUL*, and *SOC1* in GA-dependent flowering. SVP represses the expression level of *SPL* thorough the reduction of GAs (Andres et al., 2014). Some SPL transcription factors, including *SPL3*, *SPL4*, and *SPL5*, are activated in response to GAs (Galvao et al., 2012; Jung et al., 2012; Porri et al., 2012) and SPL3 binds to the *LFY* promoter directly (Yamaguchi et al.,
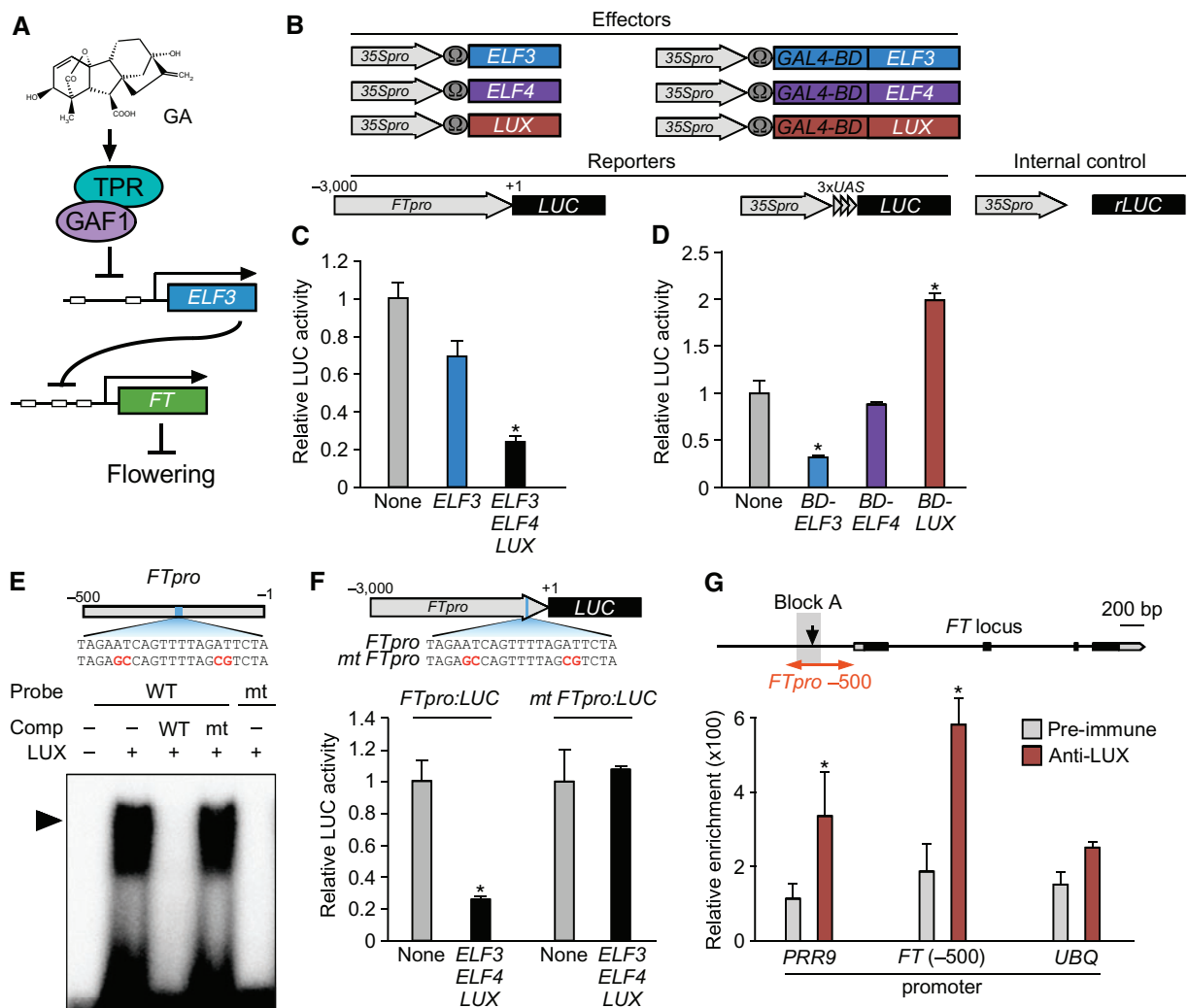
Figure 9 ELF3 represses the expression of *FT* in combination with LUX and ELF4 as part of the EC. A, GA promotes *FT* expression through repression of *ELF3* by the GAF1-TPR complex. B, Schematic representation of the reporter and effector. A 3,000-bp fragment of the *FT* promoter was fused with the *LUC* gene. The effector plasmid expressed full-length ELF3, ELF4, LUX, GAL4BD-ELF3 (GAL4 DNA-binding domain fused ELF3), GAL4BD-ELF4, and GAL4BD-LUX under the control of the CaMV 35S promoter with a viral translation enhancer (Ω). C, Transient expression assay of ELF3, ELF4, and LUX. The effector, reporter, and internal control constructs were co-transfected into *Arabidopsis* protoplasts. The transfected cells were incubated for 20 h and then Luc and rLUC activities were measured. The results are shown as LUC/rLUC activity. Error bars indicate SD of three biological replicates ($n = 3$). Asterisks represent Student's *t* test significance compared with mock treated control (*$P < 0.05$). D, Transient expression assay of GAL4BD-ELF3, GAL4BD-ELF4, and GAL4BD-LUX. The effector, reporter, and internal control constructs were co-transfected into *Arabidopsis* protoplasts. The transfected cells were incubated for 20 h and then Luc and rLUC activities were measured. The results are shown as LUC/rLUC activity. Error bars indicate SD of three biological replicates ($n = 3$). Asterisks represent Student's *t* test significance compared with mock treated control (*$P < 0.05$). E, Identification of GAF1-binding regions in the *FT* promoter in vitro. An EMSA was performed using the recombinant LUX protein. Oligonucleotides containing *FTpro* (−216 to −196, wild-type; lanes 1–4) or mutated (mt) *FTpro* (mt; lane5) were used as probes. Red letters indicate mutated bases. Wild-type and mt indicate competition with a 1,000-fold excess of the unlabeled wild-type and mutated probe, respectively. The specific GAF1-DNA complexes are indicated by an arrowhead. +, Addition to the reaction mixtures; −, omission from the reaction mixtures. F, Transient expression assay of ELF3, ELF4, and LUX. The effector, reporter, and internal control constructs were co-transfected into *Arabidopsis* protoplasts. The transfected cells were incubated for 20 h and then Luc and rLUC activities were measured. The results are shown as LUC/rLUC activity. Error bars indicate SD of three biological replicates ($n = 3$). Asterisks represent Student's *t* test significance compared with mock treated control (*$P < 0.05$). G, A triangle in the schematic map indicates the LUX-binding site in the *FT* promoter. The 200-bp region of the *FT* promoter used in the ChIP assay is indicated on the right. The *PRR9* promoter region was used as a positive control and *UBQ11* was used as a negative control. LUX binds to a region of the *FT* promoter in vivo. ChIP assays were performed with pre-immune serum or anti-LUX in Col-0. The level of each co-precipitated DNA fragment was quantified by real-time PCR and normalized to the input DNA. Error bars indicate SD of three technical replicates ($n = 3$). Asterisks represent Student's *t* test significance compared with mock treated or pre-immune control (*$P < 0.05$). Experiments were repeated twice with independently grown plants, with similar results.
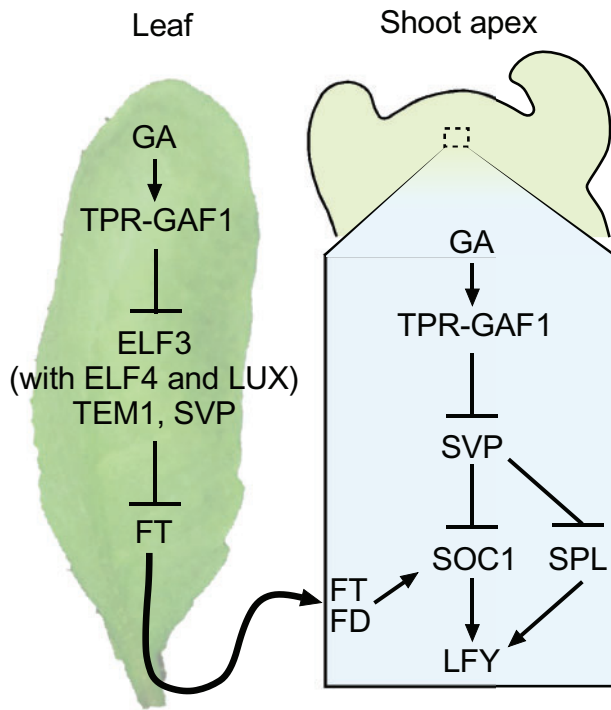
Figure 10 Regulation model of the GA-dependent flowering pathway by the GAF1 complex. GA promotes the induction of *FT* in the leaves via the suppression of ELF3, and the FT protein translocates from the leaves to the shoot apex. In the shoot apex, GA represses *SVP*, and *SOC1* expression is induced by the translocated FT protein.

2009). Our data provide a model of the GA flowering pathway by which flowering repressors are downregulated via the GAF1-TPR complex, promoting the expression of *FT* in leaves and that of *SOC1* in the shoot apex. The expression of *LFY* was also increased in the GAF1 overexpressor line; GAs could induce *LFY* by repressing *SVP* via the GAF-TPR complex in the shoot apex. Previously we reported that the DELLA-GAF1 complex involved in GA feedback regulation (Fukazawa et al., 2017). Although endogenous GA levels are controlled by feedback regulation, why endogenous GAs are highly accumulated in the shoot apex before flowering under SD conditions remains unknown. Further molecular analysis is required to establish the regulation of GA accumulation in the shoot apex before flowering.

Materials and methods

Plant material and growth conditions
All transgenic lines were derived from *A. thaliana* ecotype Col-0 (wild-type). To generate transgenic plants overexpressing myc-tagged GAF1, a 4×myc tag was amplified and cloned into the *Not*I site of pBIJ4-GAF1. To generate transgenic plants, the *FT*, *SOC1*, *ELF3*, *TEM1*, *TEM2*, and *SVP* promoters were cloned into the *Sal*I–*Bam*HI or *Hind*III–*Bam*HI site of the binary vector pBI101and pGBW3 using the Gibson Assembly system (New England Biolabs). *Agrobacterium*-mediated *Arabidopsis* transformation was carried out using the floral dip method (Clough and Bent,

1998). Kanamycin-resistant transgenic plants in the Col-0 background or Hygromycin-resistant transgenic plants in the *gaf1 idd1* background were isolated and followed by selfing to obtain fully homozygous lines. The *gaf1-1* (SALK_070916) and *idd1* (SALK_022425) mutants were obtained from the ABRC (Ohio State University). *gaf1 idd1* double mutants were generated by crossing the *gaf1* and *idd1* mutants, and the GAF1 overexpressor was described previously (Fukazawa et al., 2014). The primer sets used for cloning are listed in Supplemental Table S1. Plants were grown in a controlled growth chamber at 22°C under white light illumination (40 μmol m^{-2} s^{-1}) and LD conditions (16 h light/8 h dark) or SD conditions (8 h light/16 h dark).

RNA-Seq library construction and sequencing
Transgenic *Arabidopsis* pER10:GAF1 was used for experiments. For β-estradiol treatment, 7-d-old seedlings were grown on MS agar medium with or without β-estradiol (10 μM) for 24 h under LD condition. Each 7-d-old plant was collected and then stored in −80°C until use. All RNA samples were isolated at ZT8 under LD conditions. Total RNA was isolated using the Total RNA Extraction Kit (RBC genomics) and sent to InfoBio for RNA-seq analysis. Library construction, sequencing, and basic data analysis were carried out by InfoBio. The cDNA library was prepared using the NEBNext mRNA Library Prep Master Mix Set for Illumina (New England Biolabs) in combination with a NEBNext single read run for Illumina (New England Biolabs). The quality of the RNA and fragmentation sizes was checked by InfoBio. For sequencing, two libraries were pooled for each lane of the Illumina Chip. High-throughput sequencing was performed on an Illumina HiSeq2000 platform following the manufacturer's instructions. Mapping of the reads was performed using bowtie2 (version 2.2.2) onto the NCBI Reference sequence Database.

Short time GA treatment of plants
Fourteen-day-old seedlings of Col-0 grown on 1/2 MS agar medium were treated with 100 μM GA$_3$ or water by spraying. Plants were grown under SD conditions. Total RNA was isolated at several time points after GA or water treatment.

Expression analysis
Total RNA was isolated using the Total RNA Extraction Kit (RBC genomics) and used for reverse transcription with Reve Tra Ace (TOYOBO) according to the manufacturer's instructions. Quantitative real-time PCR was performed in triplicate on a CFX connect Real-Time PCR Detection System (BioRad) with GeneAce SYBR qPCR Mixα No Rox (NIPPON GENE). The relative expression levels were determined and normalized against *UBQ11* or *ACT2* expression. Primers used for gene expression analysis are listed in Supplemental Table S1.

GA treatment of GUS transgenic plants
Seven-day-old seedlings of transgenic plants grown on 1/2 MS agar medium were transferred to 1/2 MS agar plates

with or without 10 μM GA$_3$ to investigate GA sensitivity and grown for one additional week.

Transient transactivation assay

ELF3, *TEM1*, *TEM2*, and *SVP* promoters with 5′ deletions were cloned into the p-less LUC vector, which is a pUC18-based plasmid that contains the *LUC* gene (Takahashi et al., 1995). All primers used in this analysis are provided in Supplemental Table S1. The cDNA of *GAF1*, GAI, *TPR4*, *ELF3*, *ELF4*, *TEM1*, *TEM2*, *SVP*, and *LUX* was cloned into the pJ4 vector, which carries the CaMV 35S promoter with a viral translation enhancer, the Ω sequence (Fukazawa et al., 2000), to be used as the effector. Protoplasts were prepared from T87 *Arabidopsis* cultured cells, and protoplasts were transfected as described previously (Fukazawa et al., 2014). Relative LUC activity was calculated via normalization to rLUC activity. The data are presented as averages of three independent biological replicates.

Electrophoretic mobility shift assay

EMSA was performed following the procedure described previously (Fukazawa et al., 2000, 2010). GAF1 and *LUX* were cloned into pET30b vectors (Novagen, Madison, WI, USA). The recombinant proteins 6×His-GAF1 and 6×His-LUX were expressed and purified from *Escherichia coli* BL21 (DE3) pLysE using Ni$^+$-resin (Novagen). The nucleotide sequences of the double-stranded oligonucleotides used for EMSAs are described in Supplemental Table S1. The oligonucleotides were annealed and then labeled using (α-^{32}P) dCTP and the Klenow fragment of DNA polymerase I. Binding mixtures contained 50 fmol of the labeled probe, 1 μg of purified recombinant GAF1 or LUX, or 1 μg of control extract of *E. coli*, and 2 μg of poly (dI/dC). DNA competitor was used at a 100–500-fold excess molar concentration. The binding buffer for GAF1 consisted of 20 mM Tris–HCl pH 7.5, 3 mM MgCl$_2$, 50 mM KCl, 1 mM EDTA, 10% (v/v) glycerol, and 2 μM ZnCl$_2$. The binding buffer for LUX consisted of 10 mM Tris–HCl pH 7.0, 50 mM NaCl, 1 mM MgCl$_2$, 1 mM TCEP, 3% glycerol, 20 μg mL^{-1} BSA, 2.5% CHAPS, and 1.27 mM spermidine. Reactions were incubated at 4°C for 30 min and loaded onto 4% polyacrylamide gels containing 6.7 mM Tris–HCl, pH 7.5, 1 mM EDTA, and 3.3 mM sodium acetate.

Histochemical staining

Kanamycin-resistant transgenic plants in the Col-0 background or Hygromycin-resistant transgenic plants in the *gaf1 idd1* background were histochemically stained to detect GUS activity by immersing seedlings in a staining solution (100 mM sodium phosphate buffer, pH 7.0, with 50 mM NaCl, 1 mM potassium ferricyanide, 0.1% [v/v] Triton X-100, and 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide) overnight at 37°C. After staining, the samples were immersed in a fixing solution (5% [v/v] formaldehyde, 5% [v/v] acetic acid, 20% [v/v] ethanol) followed by dechlorophyllation in 70% (v/v) ethanol. Photographs of GUS-stained plants were taken using a DVM6 Digital Microscope (Leica). For sectioning, X-gluc-stained tissues were fixed and embedded in paraffin as described (Yamaguchi et al., 2001). Photographs of thin sections were taken using bright field microscopy (Nikon ECLIPSE Ni).

ChIP assay

The ChIP experiment was performed following the procedure described previously with some modifications (Fukazawa et al., 2010). Briefly, 2-week-old 4×myc-GAF1 transgenic or Col-0 plants were cross-linked in 1% (v/v) formaldehyde by vacuum filtration for 10 min and incubated at 4°C for 1 h. The aliquots of each protein sample were immunoprecipitated with anti-GST (Santa Cruz Biotechnology Inc. SC-138), anti-myc (MBL International Corporation 562/lot#055), and anti-LUX (generated in-house) for 12 h at 4°C. Chromatin–antibody complexes were precipitated with salmon sperm DNA/protein-G Dyna beads at 4°C for 2 h. The primers used for ChIP analysis are listed in Supplemental Table S1. The level of each co-precipitated DNA fragment was quantified by real-time PCR using specific primer sets and normalized to input DNA. The levels of co-precipitated pre-immune serum or anti-GST antibody (immunoprecipitated DNA/input DNA) were set to 1. The results are shown as relative DNA enrichment. Error bars indicate SD ranges ($n = 3$).

Statistical analysis

All data for quantification analyses are presented as means ($\pm$SD). The statistical analyses were performed by two-tailed Student's *t* test (*$P < 0.05$). Statistical results are shown in Supplemental Data set S2.

Accession numbers

The *Arabidopsis* Genome Initiative locus identifiers for the genes mentioned in this article are as follows: *GAF1* (At3g50700), *IDD1* (At5g66730), *GAI* (At1g14920), *AtGA20ox2* (At5g51810), *ACTIN2* (At3g18780), *TEM1* (At1G25560), *TEM2* (At1G68840), *ELF3* (At2g25930), *ELF5* (At5g62640), *SVP* (At2g22540), *FT* (At1g65480), *SOC1* (At2g45660), *LFY* (At5g61850), *EFM* (At2g03500), and *UBQ11* (At4g05050).

Supplemental data

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

Supplemental Figure S1. Identification of novel GAF1-target genes by transactivation assays with the DELLA-GAF1 complex.

Supplemental Figure S2. GA responsibility of GAF1 target genes by shorter time GA treatment.

Supplemental Table S1. Primer sequences used in this study

Supplemental Data set S1. RNA-seq data of GAF1-induced plant.

Supplemental Data set S2. Statistical analysis results.

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