AGAMOUS-LIKE 24, a dosage-dependent mediator of the flowering signals

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The most dramatic phase change in plants is the transition from vegetative to reproductive growth. This flowering process is regulated by several interacting pathways that monitor both the developmental state of the plants and environmental cues such as light and temperature. The flowering-time genes *FLOWERING LOCUS T* (*FT*) and *SUPPRESSOR OF OVEREXPRESSION OF CO1* (*SOC1*), together with the floral meristem identity gene *LEAFY* (*LFY*), are three essential regulators integrating floral signals from multiple pathways in *Arabidopsis thaliana*. Part of the crosstalk among these genes is mediated by a putative transcription factor, *AGAMOUS-LIKE 24* (*AGL24*). This gene is gradually activated in shoot apical meristems during the floral transition and later located in the whole zone of both inflorescence and floral meristems. Loss and reduction of *AGL24* activity by double-stranded RNA-mediated interference result in late flowering, whereas constitutive overexpression of *AGL24* causes precocious flowering. The correlation between the level of *AGL24* accumulation and the alteration of flowering time suggests that *AGL24* is a dosage-dependent flowering promoter. Analysis of *AGL24* expression in various flowering-time mutants shows that it is regulated in several floral inductive pathways. Further genetic analyses of epistasis indicate that *AGL24* may act downstream of *SOC1* and upstream of *LFY*.

Multiple genetic pathways in response to developmental and environmental signals coordinate the transition from vegetative to reproductive development in *Arabidopsis thaliana* (1, 2). The autonomous pathway responds to endogenous signals from specific developmental states, whereas the photoperiod and vernalization pathways monitor environmental conditions, such as light and temperature. The pathway mediated by gibberellic acid (GA) plays a particularly promotive role in flowering under noninductive photoperiods, especially in *Arabidopsis*. Analysis of flowering mutants and natural variation in different ecotypes in *Arabidopsis* has revealed >80 loci that are related to the control of flowering time. To date, at least 20 genes that affect flowering time have been identified and assigned to distinct genetic pathways by the investigation of mutant phenotypes and epistatic relationships (3–5).

Recent striking advances have shown that the flowering-time genes *FLOWERING LOCUS T* (*FT*) and *SUPPRESSOR OF OVEREXPRESSION OF CO1* (*SOC1*), in parallel with the meristem identity gene *LEAFY* (*LFY*), are important regulators integrating floral inductive signals from multiple promotion pathways (6, 7). *FT* and *LFY* mostly regulate independently of each other downstream from *CONSTANS* (*CO*), a critical gene that promotes flowering in response to long days (7–10). Although several pathways may simultaneously play roles in the regulation of *FT* and *LFY*, it is clear that *FT*, but not *LFY*, is one of the early targets of *CO* in the photoperiod pathway (7). *SOC1* is another essential integrator positively regulated by not only the redundant vernalization and autonomous pathways but also the photoperiod pathway (7, 11). It has been demonstrated that *SOC1* is also one early target of *CO* with possible interactions with *FT* and *LFY* (7, 11). In the suggested scenario (11), *LFY* acts at least in part downstream of *SOC1*, whereas *FT* plays a role in the regulation of *SOC1*. So far, substantial gaps still remain in our understanding of the contexts in which these basic regulators integrate floral signals and crosstalk to act synergistically in the control of flowering time.

Double-stranded RNA (dsRNA)-mediated interference, which is the approach that simultaneously expresses both antisense and sense fragments of a specific gene in transgenic organisms, has provided consistent and efficient suppression of target genes in plants (12–14). Compared with other classical methods for reverse genetic screening of mutants, it is possible to generate a series of specific mutants with gradually reduced gene expression by the application of RNA interference (RNAi). Furthermore, genetic interference by RNAi in plants was shown to be stably heritable (14), thus facilitating the further investigation of gene functions and interactions by applying the RNAi materials with genetic methods.

By the generation of dsRNA-mediated interference with expression of the *AGL24* gene, we created both *agl24* loss- and reduction-of-function mutants. Molecular genetic studies of *AGL24* in this study suggest that *AGL24* is a basic dosage-dependent promoter acting downstream of *SOC1* in the regulation of flowering time in *Arabidopsis*.

Materials and Methods

Plant Materials. *A. thaliana* ecotype Columbia was grown in long days (LD, 16 h light/8 h dark) or short days (SD, 16 h light/8 h dark), at 23 ± 2°C. The late-flowering mutants *co-1*, *gi-1*, *ft-1*, *fve-3*, and *soc1* are in the Columbia background, and *co-2*, *gi-3*, *ft-1*, *fve-1*, *fpa-1*, *fca-1*, and *soc1* are in the Landsberg *erecta* background. *soc1* introgressed into L*er* and *ft-1* into Columbia was provided by I. Lee (Seoul National University, Seoul, South Korea) and D. Weigel (Salk Institute, San Diego), respectively. *FRI FLC* line is a Columbia near-isogenic line described previously (15). *agl24* is an En transposon line in the Columbia background, which was provided by M. Yanofsky (University of California, San Diego). *agl24 LFY::GUS* was generated by crossing the Landsberg *erecta* LFY::GUS line (DW 150-304) with the L*er* near-isogenic *agl24* line (HY17), which was obtained by three backcrosses of the *agl24* Columbia line into Ler.

Analysis of *AGL24* Expression. For GA treatment, exogenous GA (100 μM) was sprayed onto wild-type Columbia plants grown under SDs with visible floral buds for two consecutive days. The inflorescence apices were harvested with and without GA treatment for the RT-PCR analysis. For vernalization treatment, seeds were sown on Murashige and Skoog (Life Technologies, Grand Island, NY) agar plates and incubated at 4°C under low-light levels for 6 weeks. Although no obvious circadian modulation of *AGL24* RNA accumulation was found, all of the samples were harvested at the same time of day. The amplified RT-PCR products were fractionated on agarose gels, transferred onto nylon membranes (Roche Molecular Biochemicals), and hybridized with the appropriate digoxigenin-labeled DNA probes (Roche Molecular Biochemicals). Signal intensity was

Abbreviations: dsRNA, double-stranded RNA; RNAi, RNA interference; GA, gibberellic acid; GUS, β-glucuronidase; LD, long day; SD, short day.

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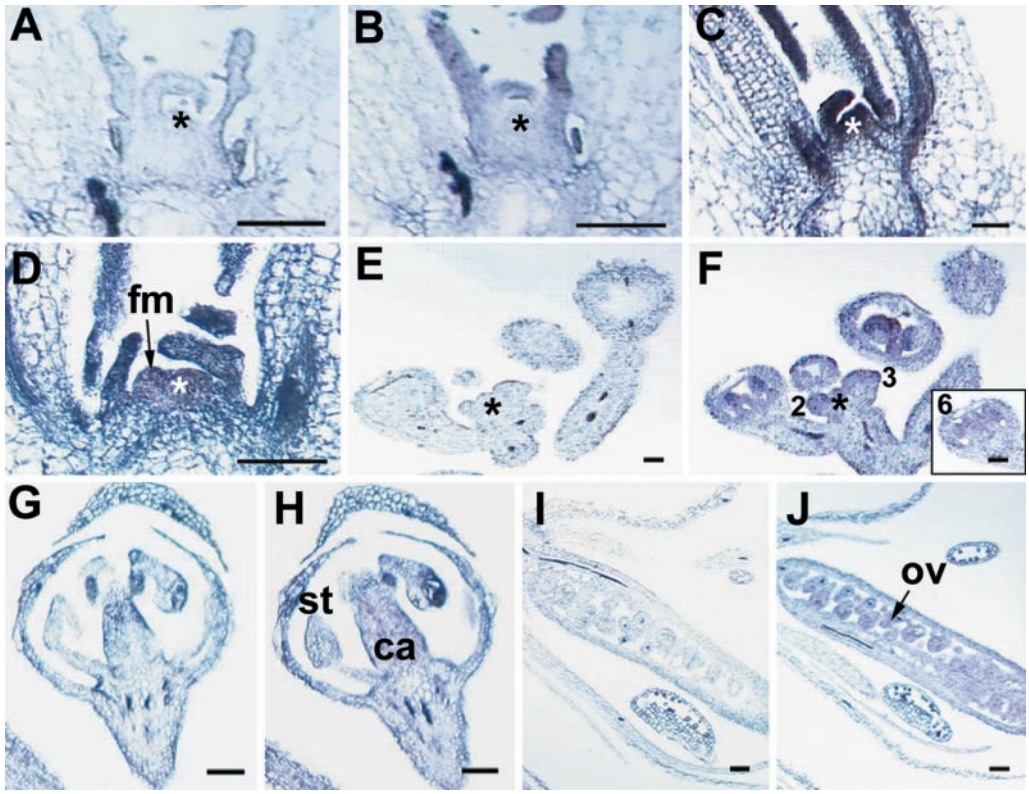

Fig. 1. *In situ* localization of *AGL24* expression in wild-type plants. Sections (*B–D*, *F*, *H*, and *J*) are hybridized with the antisense probe, and control sections (*A*, *E*, *G*, and *I*) are hybridized with the sense probe. Asterisks indicate shoot apical meristem. (*A* and *B*) An 8-day-old seedling. (*C*) A 12-day-old seedling. (*D*) A 16-day-old seedling. (*E* and *F*) An inflorescence apex and a stage 6 flower (*Inset*). (*G* and *H*) A stage 8 flower. (*I* and *J*) A stage 15 flower. fm, floral meristem; st, stamen; ca, carpel; ov, ovule. (Bars = 100 μm.)

detected by autoradiography and determined with Gel-Pro image analyzer (Media Cybernetics, Silver Spring, MD).

Primers used for RT-PCR were as follows: AGL24-SP1 (5′-GGATGAGAATAAGAGACTGAGGGATAAAC-3′) and AGL24-SP2 (5′-GACCCAATAACACGTACAATATCT-GAAA-C-3′) for *AGL24*; AP1-P1 (5′-GCACCTGAGTC-CGACGTC-3′) and AP1-P2 (5′-GCGGCGAAGCAGCCA-AGG-3′) for *APETALA1* (*AP1*); FLC-P1 (5′-GAGAAGCCA-TGGGAAGAAAAAAACTAG-3′) and FLC-P2 (5′-TTAAG-GTGGCTAATTAAGTAGTGGGAG-3′) for *FLC*; LFY-P1 (5′-TGAAGGACGAGGAGCTT-3′) and LFY-P2 (5′-TTGC-CACGTGCCACTTC-3′) for *LEAFY* (*LFY*); and TUB-P1 (5′-ATCCGTGAAGAGTACCCAGAT-3′) and TUB-P2 (5′-TCACCTTCTTCATCCGCAGTT-3′) for β-tubulin (*TUB2*). RT-PCR analysis was repeated three times using samples that were collected separately.

For Northern blot analysis, total RNA was fractionated on 1% glyoxal-agarose gels and transferred to positively charged nylon membranes (Roche Molecular Biochemicals) by capillary blotting. RNA gel blots were hybridized and detected as described (16).

Construction of *35S*::*AGL24*. The entire *AGL24* cDNA was amplified by RT-PCR on the total RNA extracted from Columbia inflorescence stems and cloned into pGEM-T Easy Vector (Promega, Madison, WI) to yield pHY1. The primers used were AGL24-G1 (5′-AGAACAGTAGTGAAGGAGAGATCTGG-TAA-3′) and AGL24-G2 (5′-ATTTGTGGGCTTCCATC-GAAGTCAACTCT-3′). The *AGL24* cDNA with blunt ends was subsequently obtained from the pHY1 by PCR amplification with Vent DNA polymerase (New England Biolabs) and AGL24-G1 and SP6 primers. The resulting PCR products were cut with *Sac*I to produce 3′ cohesive end and cloned into the *Sma*I and *Sac*I sites of pBI121 binary vector (CLONTECH) downstream of the cauliflower mosaic virus 35S promoter in the place of β-glucuronidase gene (*GUS*). The construct was sequenced to eliminate selection of PCR-introduced mutations.

Construction of *AGL24* dsRNA Interference (*AGL24-RNAi*) Plasmid. For the construction of *AGL24-RNAi* plasmid, the *GUS* fragment containing nucleotides 787–1,809 was used as a loop linker between the *AGL24* 3′ end-specific fragments in the antisense and sense orientations (14). The 540-bp *AGL24* gene-specific region was produced by PCR amplification with the primers AGL24-SP2 and AGL24-SP3 (5′-GTCGAAGACAAAAC-CAAGCAGCTACG-3′). The *GUS* loop was amplified by the primers GUS1 (5′-GATATCTACCCGCTTCGC-3′) and the GUS2-Sense linker (5′-CTTGGTTTTGTCTTCGACTCATT-GTTTGCCTCCCT-3′). The linked *GUS*::*Sense* fragment was subsequently generated by PCR amplification on both the templates of the *AGL24* gene-specific region and *GUS* loop using the primers GUS1 and AGL24-SP2. This fragment was then cloned into the *Sma*I and *Sac*I sites of pBI121 vector (CLONTECH) downstream of the cauliflower mosaic virus 35S promoter and instead of *GUS* gene (pHY5). *AGL24* gene-specific sequence in the antisense orientation with *Xba*I and *Bam*HI sites on respective ends was created by PCR mutations, digested with the corresponding enzymes, and cloned into the *Xba*I and *Bam*HI sites of the pHY5 vector upstream of the *GUS*::*Sense* insert. The sequence of this *AGL24-RNAi* plasmid was also confirmed to eliminate selection of possible mutations introduced by PCR.

Plant Transformation. The binary vectors, harboring the cassettes of *35S*::*AGL24* and *AGL24-RNAi*, were introduced into

Agrobacterium tumefaciens LBA4404 by triparental mating. *Arabidopsis* ecotype Columbia was transformed by using the floral dip method (17). Transformants were subsequently selected on half-strength Murashige and Skoog medium containing 50 mg/liter kanamycin.

***In Situ* Hybridization.** For synthesis of the antisense and sense *AGL24* RNA probes, the 3′ end gene-specific region was amplified with AGL24-SP1 and AGL24-SP2 primers, introduced into the pGEM-T Easy vector (Promega), and transcribed *in vitro* using the digoxygenin (DIG) RNA Labeling kit (Roche Molecular Biochemicals). Tissues were fixed in a solution of formaldehyde/acetic acid/ethanol (3:5:60 vol/vol) at 4°C overnight. The fixed materials were dehydrated, cleared, and embedded in paraffin. Microtome sections (8 μm thick) were mounted on Superfrost Plus slides (Fisher Scientific). *In situ* hybridization and immunological detection were performed as described by Yu *et al.* (18).

GUS Staining. *In situ* localization of GUS activity was performed according to the method of Sieburth and Meyerowitz (19). Tissues were prefixed in 90% acetone on ice for 20 min and stained at 37°C overnight in a solution containing 50 mM sodium phosphate (pH 7.0), 10 mM EDTA, 2 mM 5-bromo-4-chloro-3-indoyl glucuronide, 1 mM potassium ferricyanide, and 1 mM potassium ferrocyanide. Stained tissues were cleared of chlorophyll in an ethanol series. Except for tissues used for direct observation, other tissues were fixed, and embedded in paraffin. Ten-micrometer sections were made, briefly incubated in xylene and photographed on a microscope (TMS-F, Nikon).

Results and Discussion

AGL24 encodes a typical MADS-domain protein containing the conserved MADS-box at its N-terminal end and the relatively conserved K-box located between residues 88 and 156 (Fig. 5, which is published as supporting information on the PNAS web site, www.pnas.org). Sequence alignment showed that in *Arabidopsis*, *AGL24* has the highest similarity to the *SHORT VEGETATIVE PHASE* (*SVP*) gene, which is a negative regulator of the floral transition in *Arabidopsis* (20). *In situ* localization of *AGL24* transcripts showed that *AGL24* was expressed in the whole zone of the vegetative shoot apical meristem and emerging leaf primordia, as well as the provascular strands of relatively old leaves (Fig. 1 *A–C*). During floral transition, high levels of *AGL24* mRNA were detected in both the shoot apex and the emerging floral meristem (Fig. 1*D*). At a later stage, *AGL24* mRNA was distributed throughout the inflorescence meristem and the stage 2–3 flowers (Fig. 1 *E* and *F*). In the stage 6 flower, the *AGL24* gene expression was mostly confined to the carpel and stamen primordia (Fig. 1*F*). The accumulation of *AGL24* mRNA was obvious in the carpel (Fig. 1 *G* and *H*) and ovules (Fig. 1 *I* and *J*) in the developing flower. These expression patterns suggest that *AGL24* might play roles in the regulation of flowering time and the development of floral organs.

***AGL24* Is a Dosage-Dependent Promoter of Flowering.** To investigate the function of the *AGL24* gene, we introduced *AGL24* dsRNA-expressing constructs into *Arabidopsis* with *Agrobacterium*-mediated transformation. We isolated a total of 36 transformants, among which 30 plants showed different degrees of late-flowering phenotype. These plants were grouped into two categories: reduction- and loss-of function mutants. The *AGL24* dsRNA interference (*AGL24-RNAi*) strong mutants show typical late flowering under both LD and SD conditions, which is comparable to *agl24* loss-of-function mutants (Table 1; Fig. 2*C*, *D*, *G*, and *H*; and R. M. Amasino, personal communication). It is noteworthy that the *AGL24-RNAi* weak mutants show intermediate reduction-of-function phenotypes between wild-type and loss-of-function plants corresponding to the endogenous levels of *AGL24* mRNA (Figs. 2 *A–H* and 3*D*; Table 2, which is published as supporting information on the PNAS web site), which is similar to the semidominant nature of *agl24* mutants (Table 1). These data demonstrate that *AGL24* is a dosage-dependent promoter of flowering. This suggestion is further supported by the results of constitutive expression of *AGL24* in transgenic *Arabidopsis* plants. Among 47 *35S*::*AGL24* transgenic plants, 35 plants demonstrated early flowering under both LDs and SDs (Table 1; Fig. 2 *A–H*). Also, we observed that the up-regulated level of *AGL24* transcripts was closely related to the degree of precocious flowering (Fig. 3*D*; Table 3, which is published as supporting information on the PNAS web site). Simultaneously, although we have not observed obvious defects in flower development in RNAi mutant lines, overexpression of *AGL24* caused some phenotypic aberrations in flower shape and size, which indicates its potential function in flower development as revealed by its expression patterns.

Table 1. Comparison of flowering times of transgenic and mutant plants

Genotype*	Rosette leaves†	*n*
LD‡		
Columbia wild type	16.3 ± 0.9	35
35S::*AGL24* (no. 3-6)	14.7 ± 1.0	18
35S::*AGL24* (no. 8-4)	11.9 ± 0.8	32
AGL24-RNAi (no. 18-3)	19.2 ± 0.7	27
AGL24-RNAi (no. 35-17)	23.2 ± 1.4	29
agl24	23.4 ± 1.5	37
agl24/+	18.7 ± 1.7	9
35S::*SOC1*	4.4 ± 0.3	32
35S::*SOC1 agl24*	9.8 ± 0.6	35
soc1	29.8 ± 1.7	19
35S::*AGL24* (no. 8-4) *soc1*	18.8 ± 0.9	18
35S::*FT*	3.7 ± 0.6	34
35S::*FT agl24*	5.6 ± 0.7	29
35S::*LFY*	12.3 ± 1.2	19
35S::*LFY agl24*	13.2 ± 1.5	27
SD‡		
Columbia wild type	45.6 ± 2.3	45
35S::*AGL24* (no. 3-6)	36.6 ± 1.7	23
35S::*AGL24* (no. 8-4)	25.7 ± 1.5	33
AGL24-RNAi (no. 18-3)	52.3 ± 1.1	26
AGL24-RNAi (no. 35-17)	65.0 ± 0.8	43
agl24	64.7 ± 0.9	46
soc1	75.6 ± 0.7	33

*All of the plants are of the same Columbia background.
†Flowering time is presented as the number of rosette leaves on the main shoot when the inflorescence was ≈3 cm in length.
‡LD, 16 h light/8 h dark; SD, 8 h light/16 h dark.

***AGL24* Acts in Part Downstream of *CONSTANS* (*CO*) and *SOC1*.** *AGL24* mRNA is present in all of the tissues with the strongest expression in stems (Fig. 3*A*). The expression of *AGL24* gradually increases under both LDs and SDs, with the overall levels delayed and reduced levels in SDs (Fig. 3*B*; Fig. 6, which is published as supporting information on the PNAS web site). Simultaneously, the elevation of *AGL24* expression was obvious upon the treatment of GA and vernalization (Fig. 3*B*). Therefore, it is most likely that *AGL24* functions downstream of several floral promotion pathways, including the autonomous, vernalization, photoperiod, and GA pathways (4, 21). This scenario was confirmed by the observation of the relatively reduced expressions of *AGL24* in various loss-of-function mutant backgrounds in the key genes of the different promotion pathways (Fig. 3*C*). However, the only exception is the almost

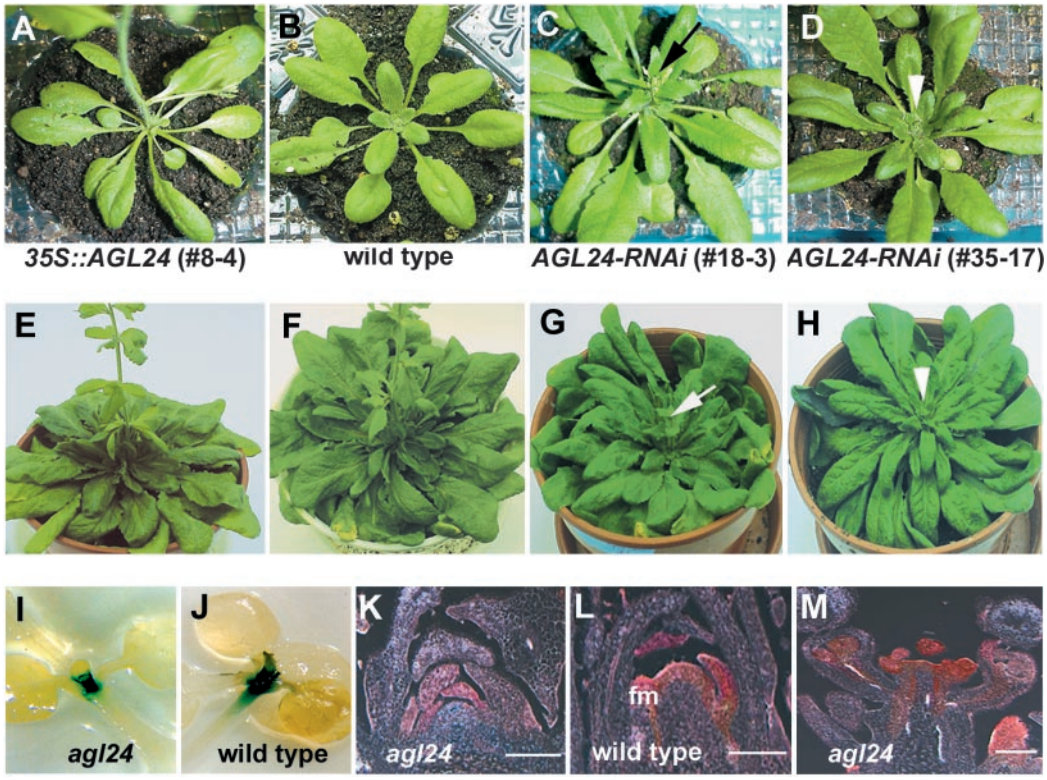

Fig. 2. Phenotypes of transgenic plants in Columbia background. (*A–D*) Transgenic plants under long days. At 30 days after germination, an early flowering *35S*::*AGL24* line (*A*) is compared with a wild-type plant (*B*), which has not bolted. At 40 days, a bolting (black arrow) *AGL24-RNAi* weak line (*C*) is compared with an *AGL24-RNAi* strong line (*D*), whose inflorescence apex is only visible (arrowhead). (*E–H*) Transgenic plants under short days. At 75 days, a *35S*::*AGL24* transgenic line (*E*) and a wild-type plant (*F*) are flowering with 32 and 45 rosette leaves, respectively. Simultaneously, a weak line (*G*) is just bolting (arrow) and the inflorescence apex in a strong line (*H*) is only visible (arrowhead). (*I–M*) Localization of *LFY*::*GUS* activity in Landsberg *erecta* plants. In 9-day-old *agl24* (*I*) and wild-type (*J*) plants at the similar developmental stage under long days, there is a stronger GUS expression in the leaf primordia surrounding the shoot apical meristem in a wild-type plant than in *agl24*. At 12 days, strong *LFY*::*GUS* expression is observed in both the shoot apex undergoing floral transition and the emerging floral meristem (fm) in a wild-type plant (*L*), whereas relatively weak GUS expression is present in the leaf primordia surrounding the shoot apex in *agl24* (*K*). *LFY*::*GUS* expression is detectable in the inflorescence meristem and young flowers (stages 2 and 3) in *agl24* (*M*), which is comparable to its expression in a wild-type plant. (Bars = 100 μm.)

unchanged *AGL24* expression in *ft-1* mutant. Because *CO*, *FT*, and *SOC1* are three essential components in the photoperiod promotion pathway, we examined the details of the *AGL24* accumulation in their loss-of-function mutants. *AGL24* expression is significantly reduced and delayed in both *co-1* and *soc1* seedlings, whereas its expression profiles are similar in wild-type and *ft-1* plants under LDs (Figs. 6 and 7, which are published as supporting information on the PNAS web site). Because *FT* and *SOC1* act in independent pathways downstream of *CO* (6–9, 11), our results indicated that *AGL24* might function in part downstream of *CO* and *SOC1*. This suggestion of the epistatic relation between *SOC1* and *AGL24* is consistent with the reduced *AGL24* expression in most of the mutants examined (Fig. 3*C*), because *SOC1* is an essential regulator downstream of *CO* integrating several promotion pathways (7, 11).

Our conclusions regarding the contribution of *AGL24* in the flowering promotion pathways were supported by several lines of genetic evidence (Table 1). First, *35S*::*AGL24* could partially, though not totally, rescue the late-flowering phenotype of *soc1*, which is consistent with the suggested epistatic relation between *SOC1* and *AGL24*. Second, whereas loss of *AGL24* could significantly suppress the precocious flowering in *35S*::*SOC1* plants, constitutive expression of *SOC1* in *agl24* still caused earlier flowering than in the wild type. Thus, it is likely that *AGL24* is not the only downstream effector of *SOC1*. It is also clear that both *AGL24* gain- and loss-of function plants demonstrate less severe alterations of flowering time than those of *SOC1* (Table 1). In addition, *in situ* hybridization revealed that *AGL24* and *SOC1* had especially different expression patterns in the emerging floral primordia (Fig. 1 *E* and *F*) (7, 11). Together, these results support a hierarchy from *SOC1* to *AGL24* with the involvement of other unknown flowering-time regulators in parallel with and upstream of *AGL24*. Third, although *agl24* could slightly attenuate the precocious flowering phenotype in *35S*::*FT* plants, constitutive expression of *FT* almost completely suppressed the *agl24* phenotype. This means that the photoperiod pathway via *FT* could compensate for the effect of loss of *AGL24* function. Strikingly, the mutants with the loss and gain of *SOC1* or *AGL24* functions were still sensitive to photoperiod (Table 1) (7, 11, 22), which is in sharp contrast to the phenomena demonstrated by the plants with the altered activities of *CO* and *FT* (7–9, 21). Therefore, despite *FT* and *SOC1* functions in concert downstream of *CO*, *FT* should have a much greater contribution to the photoperiod pathway than the gene cascade from *SOC1* to *AGL24*. Nevertheless, the incomplete suppression of *agl24* phenotype by *35S*::*FT* is consistent with the previous suggestion that *FT* also plays a role in the regulation of *SOC1* (11), thus influencing the *AGL24* activity, though *FT* may not directly or mainly regulate *AGL24*.

***AGL24* Acts Upstream of *LFY*.** *LFY* plays an important role in promoting the floral transition (23, 24). To determine the possible relation between *AGL24* and *LFY*, we introduced the *LFY*::*β-glucuronidase* (*GUS*) transgene into *agl24* mutants and

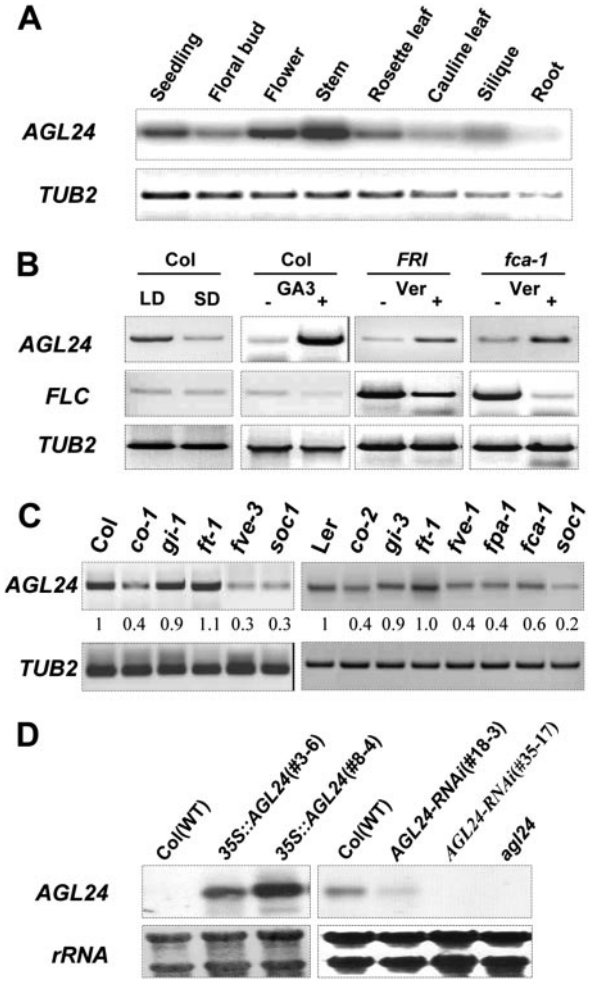

Fig. 3. Expression of *AGL24*. (*A–C*) RT-PCR analysis of *AGL24* expression. The β-tubulin gene (*TUB2*) was amplified as a quantitative control. (*A*) *AGL24* expression in different organs. (*B*) Effect of photoperiod, GA, and vernalization on the expression of *AGL24* and *FLC*. (*C*) *AGL24* expression in late-flowering mutants in Columbia (*Left*) and Landsberg *erecta* (*Right*) backgrounds. The numbers indicate the relative expression levels of *AGL24*. (*D*) Northern blot analysis of *AGL24* expression in transgenic plants. For *35S*::*AGL24* lines, total RNA was isolated from roots. *35S*::*AGL24* transgenic lines 3-6 and 8-4 represent weak and strong overexpression of *AGL24*, respectively. For *AGL24* double-stranded interference (*AGL24-RNAi*) mutants, total RNA was isolated from stems. *AGL24-RNAi* 18-3 and 35-17 represent weak and strong mutant lines, respectively. The rRNAs stained by methylene blue indicate the amount of total RNA loaded in each lane.

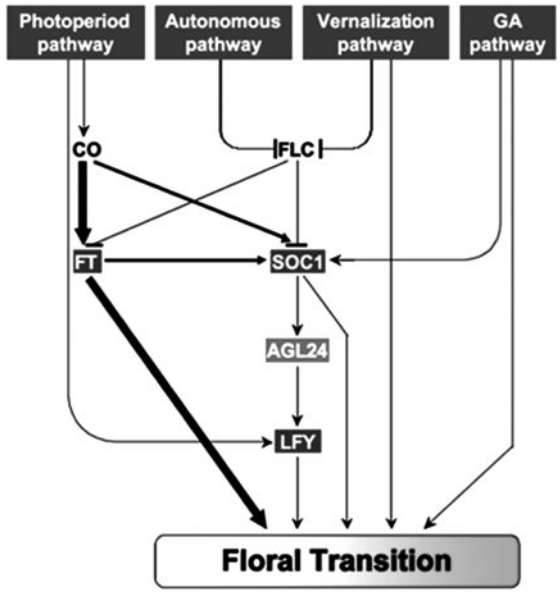

Fig. 4. Integration of floral signals is mediated by *AGL24*. Floral transition in *Arabidopsis* is regulated by multiple promotion pathways. Two flowering-time genes, *FT* and *SOC1*, along with the floral meristem identity gene *LFY*, are three essential regulators integrating floral signals from different pathways. *AGL24* acts downstream of *SOC1* and upstream of *LFY*. Arrows and T bars represent promotion and repression effects, respectively. The thickness of arrows indicates the relative contribution of *CO* and *FT* to the regulation of downstream genes.

compared the mimic *LFY* expression in *agl24* and wild-type backgrounds (25).

Our results demonstrated that the delay of the floral transition in *agl24* is coupled with the delayed and reduced profile of *LFY* expression. At the similar developmental stage, there is a much stronger *LFY*::*GUS* activity in wild-type plants than in *agl24* (Fig. 2 *I* and *J*; Fig. 8*A*, which is published as supporting information on the PNAS web site). Particularly, the up-regulation of *LFY*::*GUS* was delayed during floral transition in *agl24* (Fig. 2 *K* and *L*; Fig. 8*A*). These results are in agreement with the totally reduced *LFY* expression in *agl24* examined by RT-PCR during floral commitment (Fig. 8*B*), suggesting that *AGL24* affects the transcriptional induction of *LFY*. This was supported strongly by the genetic evidence showing the almost complete compensation of the late-flowering phenotype of *agl24* by *35S*::*LFY* (Table 1). Therefore, *LFY* functions at least in part downstream of *AGL24*. It has been shown that *FT* and *LFY* may act independently of each other to regulate in concert the flowering process (9, 24, 26). Meanwhile, *LFY* is a possible regulator in part downstream of *SOC1* (11). Together, this evidence indicates that *SOC1* partially regulates the *LFY* activity via *AGL24*, which is independent of *FT*. Also, it has been clear that GA promotes flowering by activating the *LFY* gene (25). Because *SOC1* and *AGL24* are possible regulators in the GA promotion pathway (Fig. 3*B*) (27), and the up-regulation of *AGL24* expression is dramatically reduced upon GA treatment in *soc1* background (data not shown), the transcriptional regulation from *SOC1* to *LFY* may integrate floral signals from multiple pathways including the GA promotion pathway (Figs. 4 and 8).

To test if loss of *AGL24* activity affects the function of *LFY* in the establishment of floral meristem fate and the activation of floral homeotic genes (28), we monitored *LFY*::*GUS* activity in the inflorescence meristem in *agl24* (Fig. 2*M*). The results showed that the activity of *LFY*::*GUS* in the inflorescence meristem and young floral primordia is comparable to what is observed in a wild-type plant (24). Therefore, although *AGL24* regulates the induction of *LFY* expression during floral transition, loss of *AGL24* activity does not influence the expression of *LFY* in the subsequent stages of flower development.

In conclusion, *AGL24* is a dosage-dependent promoter in regulating floral transition in *Arabidopsis*. In support of the suggestion of the presence of substantial interaction among three important integrators *LFY*, *FT*, and *SOC1* (6, 11), our study reveals that *AGL24* is a possible mediator acting in the regulatory cascade from *SOC1* to *LFY*, which is only slightly affected by *FT*. The genetic branch via *FT* has a greater contribution to the LD photoperiod promotion pathway than the genetic pathway from *SOC1* to *AGL24*. It is obvious that the level of *AGL24* accumulation still gradually increases in *soc1* mutants in response to the age of plants (Fig. 6), indicating that regulation of *AGL24* is partially independent of *SOC1*. Meanwhile, we have

suggested that genetically redundant gene(s) may act parallel to *AGL24* downstream of *SOC1*. Thus, the linear hierarchy from *SOC1* to *AGL24* may only represent one typical regulatory pathway among their related networks leading to the control of flowering time. With the linear pathway clarified here, we will further elucidate if there are direct regulatory relationships among *SOC1*, *AGL24*, and *LFY*.

It is interesting to note that the MADS-box gene family, which is divided into repressors (*FLC*, *FLM/MAF1*, and *SVP*) and promoters (*FUL*, *SOC1*, and *AGL24*), has been one of the major classes of transcription factors mediating the antagonism between the promotive and repressive pathways during floral transition (7, 11, 15, 20, 29–32). The transcriptional cascades among MADS-box genes, just like the genetic pathway from *FLC* to *SOC1* to *AGL24*, which is reminiscent of the proposed subtle regulatory hierarchy of MADS-box genes controlling flower development (33, 34), may represent one of the essential characteristics in the complex regulatory networks in the flowering-time control.

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