

# Regulation of flowering time in *Arabidopsis* by K homology domain proteins

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The transition from vegetative growth to reproductive development in *Arabidopsis* is regulated by multiple floral induction pathways, including the photoperiodic, the autonomous, the vernalization, and the hormonal pathways. These pathways converge to regulate the expression of a small set of genes critical for floral initiation and different signal transduction pathways can interact to govern the time to flower. One important regulator of floral initiation is the MADS-box transcription factor FLC, which acts as a negative regulator of flowering in response to both endogenous and environmental signals. In this report, we describe a study of the flowering-time gene, *FLK* [flowering locus K homology (KH) domain] that encodes a putative RNA-binding protein with three KH domains. The *flk* mutations cause delayed flowering without a significant effect on the photoperiodic or vernalization responses. *FLK* functions primarily as a repressor of *FLC* expression, although it also modestly affects expression of genes associated with the photoperiodic pathway. In addition to *FLK*, the expression of two other KH domain genes are modestly affected by the *flk* mutation, suggesting a possible involvement of more than one KH domain protein in the regulation of flowering time in *Arabidopsis*.

Floral initiation is a major developmental transition in plants and it is regulated by various environmental and physiological cues. Genes regulating flowering time in *Arabidopsis* have been grouped based on genetic analysis into four floral induction pathways, each being responsive to different internal or environmental changes. The autonomous pathway and gibberellin pathway allow plants to monitor their developmental and physiological status, whereas the photoperiodic pathway and vernalization pathways respond to light and temperature changes associated with the seasonal transition (1–5). The actions of these signaling pathways result in altered gene expression by means of mechanisms that include transcription regulation (6), RNA metabolism (7), protein turnover (8), and histone modification (9–11). It is clear that different signaling pathways ultimately converge to regulate expression of a small set of key regulatory genes and/or floral meristem identity genes that activate floral initiation (3, 4, 12). Such a signal convergence allows plants to integrate different environmental and internal cues into the control of the same developmental process. In addition to signal convergence, different floral induction pathways also interact by other means. For example, it has been reported that the blue light receptor cryptochrome 2 and the autonomous pathway gene *FCA* act synergistically in the suppression of *FLC* expression (13, 14); and that increased *FLC* expression and/or activity suppresses *CRY2* mRNA expression (15). Interaction of different signal transduction pathways before their convergence may allow a coordinated regulation of the activity of the respective pathways, but the underlying molecular mechanism remains less clear.

One example of signal convergence of multiple floral induction pathways is the control of *FLC* expression. *FLC* is a MADS-box transcription factor that acts as a negative regulator of floral initiation and an integrator of the autonomous and vernalization pathways (16, 17). Another level of signal conver-

gence for floral induction pathways is the regulation of *SOC1* and *FT* expression. *SOC1*, a MADS-box transcription factor, and *FT*, a RAF-kinase inhibitor-like protein, are positive regulators of the expression of floral meristem identity genes and floral initiation (18–22). The expression of *SOC1* and *FT* is negatively regulated by *FLC* but positively regulated by *CO*, which encodes a B-box zinc-finger transcription factor (6, 20). The autonomous and vernalization pathways both suppress the expression of *FLC*, resulting in a decreased expression of *FLC* and consequently increased expression of *SOC1* and *FT* in a later developmental stage or after a prolonged exposure of plants to low temperature (14, 22–25). The photoperiodic pathway, acting through many components, including photoreceptors, the circadian clock, and transcription regulators, positively regulates expression of *SOC1* and *FT* to stimulate flowering in *Arabidopsis* when the day length increases (4, 6, 20, 21, 26).

Transcription regulation is one mechanism underlying the control of flowering time and the interaction between different floral induction pathways. It has been shown that *FLC* binds to the *CArG* element in the promoter of *SOC1* to antagonize the positive effect of *CO* at a separate cis-element of the *SOC1* promoter (6). Another example of different signaling pathways converging at transcription regulation is in the control of *LFY* expression by the photoperiodic pathway and the gibberellin pathway. These two pathways have been shown to control *LFY* mRNA expression through different cis-elements of the *LFY* promoter (12). In addition to transcription controls, RNA processing has also been shown to play important roles in the regulation of flowering-time gene expression. Three (*FCA*, *FY*, and *FPA*) of the six (*FCA*, *FY*, *FPA*, *FLC*, *LD*, and *FVE*) classic autonomous pathway genes encode proteins that either contain RNA recognition motifs or interact with a RNA-binding protein (27–29). *FCA* expression is autoregulated by means of alternative utilization of polyadenylation sites in its premRNA. The *FCA*/*FY* complex interacts with the *FCA* premRNA to promote the selection of the promoter-proximal polyadenylation site, resulting in accumulation of the alternatively spliced *FCA*  $\beta$ -mRNA that encodes an inactive protein and inhibition of the production of *FCA*  $\gamma$ -mRNA that encodes the active *FCA* protein (29). The major function of *FCA*, *FY*, and *FPA* in floral initiation is to suppress *FLC* expression, but how these RNA-binding proteins affect *FLC* mRNA abundance remains unclear (3, 30).

In addition to the RNA recognition motif found in *FCA* and *FPA*, another widely found RNA-binding motif is the K homology (KH) domain (31). The *Arabidopsis* genome encodes at least 196 RNA recognition motif-containing proteins and 26 KH domain proteins (32). The biological function of most of the *Arabidopsis* RNA-binding proteins is not known. The only

Abbreviations: Q-PCR, quantitative PCR; KH, K homology.

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*Arabidopsis* KH domain protein with a reported function is HEN4, which plays an important role in the processing of the *AGAMOUS* premRNA and floral organ development (33). We report here a study of the flowering-time gene *FLK* (flowering locus KH domain) that positively regulates floral initiation by suppression of *FLC* expression. The *flk-4* allele described in this report is the same as the *flk1* allele reported by Lim *et al.* (34), who recently reported similar findings that also demonstrate that *FLK* is a negative regulator of *FLC*. Furthermore, we also found that mutations of *FLK* also modestly affect the expression of two other KH domain genes. These results suggest that, in addition to RNA recognition motif domain proteins reported previously, KH domain proteins also play important roles in the regulation of flowering time in *Arabidopsis*.

## Materials and Methods

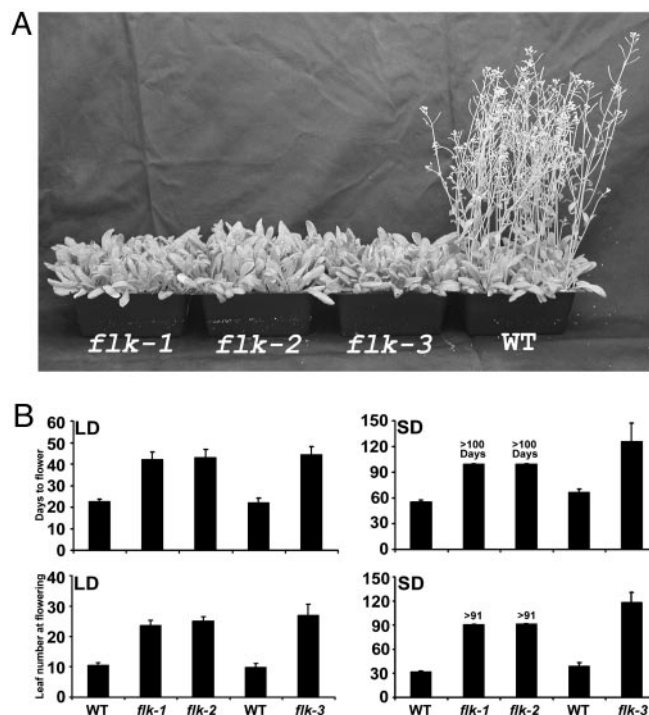
Approximately 1,100 T-DNA insertion lines (in the Col-0 background) affecting 326 putative RNA-binding proteins were selected (<http://signal.salk.edu/cgi-bin/tdnaexpress>) and the seeds of these lines were obtained from the *Arabidopsis* Biological Resource Center ([www.biosci.ohio-state.edu/~plantbio/Facilities/abrc/abrc/home.htm](http://www.biosci.ohio-state.edu/~plantbio/Facilities/abrc/abrc/home.htm)) or the Salk Institute Genomic Analysis Laboratory (<http://signal.salk.edu>) (35). These lines were grown in a green house; flowering time was scored as the rosette leaf number at bolting and days from the end of imbibition to bolting (36); and plants that showed apparent flowering-time variations from the wild type were selected for further analysis. Among the putative mutants studied, Salk\_007750 (*flk-1*), Salk\_001523 (*flk-2*), Salk\_139230 (*flk-3*), and Salk\_112850 (*flk-4*) affected the same locus (locus At3g04610, accession no. AY070475), which was referred to as *FLK* by this and a recently published report (34).

Methods used for gene expression studies, including RNA isolation, DNA microarray analysis, real-time quantitative PCR (Q-PCR), RT-PCR, and RNA stability analysis, can be found in *Supporting Materials and Methods*, which is published as supporting information on the PNAS web site.

## Results and Discussion

In an attempt to understand how *Arabidopsis* photoreceptors regulate photoperiodic flowering (26, 36, 37), we investigated whether RNA-binding proteins contribute to the photoperiodic regulation of gene expression. We screened T-DNA insertion mutations that affect genes encoding putative RNA-binding proteins for variations in flowering time. Four independent insertion mutations of the same gene (At3g04610) on chromosome 3 were found to have a similar late-flowering phenotype (Fig. 1). All four mutants flowered later than the wild type in both long days and short days. The mutant plants did not flower until >40 days (long-day) or 120 days (short-day) after germination, in contrast to wild-type plants that flowered within 30 days (long-day) or 60 days (short-day) after germination (Fig. 1). The *flk* mutant remains responsive to vernalization because the mutant plants flowered significantly earlier after a vernalization treatment (Fig. 5, which is published as supporting information on the PNAS web site). These results indicate that although the corresponding gene may not be directly involved in the photoreceptor or temperature regulation of flowering, it does play an important role in the regulation of floral initiation.

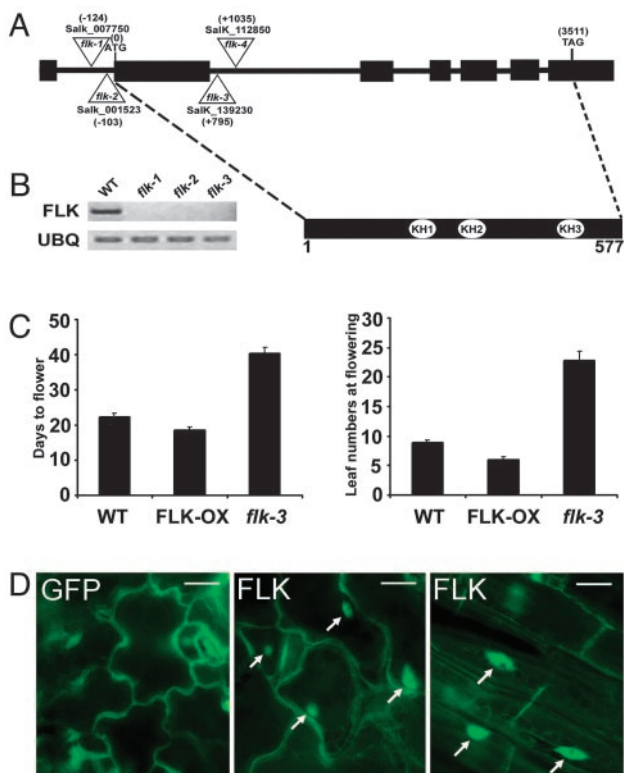
The gene corresponding to the mutations was identified based the analysis of DNA sequences flanking the T-DNA inserts. This gene encodes a 577-residue protein that contains three KH motifs (Fig. 2A and Fig. 6A, which is published as supporting information on the PNAS web site). Because none of the four mutant alleles showed readily discernable morphological changes other than delayed flowering, we reasoned that the major function of the corresponding gene is to regulate flowering time and referred to it as *FLK*. The T-DNAs of the two *flk* alleles



**Fig. 1.** *flk* is a late-flowering mutant. (A) Plants (35 days old) of the wild-type and three *flk* mutant alleles (*flk-1*, *flk-2*, and *flk-3*) grown in long-day photoperiods (LD, 18 h light/6 h dark). (B) A comparison of the flowering time of the wild type and the *flk* mutants. Results of two separate experiments are shown. In one experiment that includes *flk1* and *flk2*, the *flk* mutants failed to flower in short-day photoperiods (SD) when the experiment was terminated (>100 days after germination). In the other experiment, only *flk3* was included, which flowered eventually in short-day photoperiods.

(*flk-1* and *flk-2*) were inserted in the first intron, the T-DNAs of other two alleles (*flk-3* and *flk-4*) were inserted in the second intron (Fig. 2A). It appears that all of the *flk* alleles identified are loss-of-function or null mutations, because none of the *flk* alleles tested expressed detectable amounts of *FLK* mRNA (Fig. 2B and data not shown). In the wild-type plant, *FLK* is expressed in various tissues, including flowers, leaves, roots, and siliques, and it is relatively abundant in the young inflorescence (<http://mpss.udel.edu/at/java.html>). The *FLK* gene and *flk* mutant have also been reported recently by others (34).

The KH domain is an evolutionarily conserved RNA-binding motif found in proteins of diverse organisms, including eubacteria, archaea, and eukaryotes (31, 38). The core consensus sequence V/IIGXXGXXI/V in the middle of the KH domain (~50–70 residues) is perfectly conserved in all three KH domains of *FLK* (Fig. 6A). This core consensus sequence has been found to be important for RNA-binding activity of KH domain proteins. For example, a single amino acid substitution in the KH domain core sequence of the human FMR1 protein abolishes its RNA-binding activity and causes fragile X mental retardation syndrome (39–41). The first two KH domains of *FLK* are grouped near the N terminus, and the third KH domain is located at the C terminus (Figs. 2A and 6A). Such an overall architecture of KH domain arrangement is conserved in the PCBP (polyC-binding protein) type of KH domain RNA-binding proteins, including the founding member of the KH domain protein family, heterogeneous nuclear ribonucleoprotein K (38). The N terminus of *FLK* preceding the first KH domain is rich in glutamine (33/150 residues or 22%). This region also contains three perfect 8-residue repeats (LEPQQYEV), although these



**Fig. 2.** The *FLK* gene encodes a KH domain protein. (A) The *FLK* gene structure and the location of the T-DNA insertions in four different *flk* mutant alleles. Filled boxes represent exons of *FLK*; the numbers on the top or the bottom of the gene indicate nucleotide positions relative to the start codon ATG. (Lower) Relative positions of three KH domains the diagram depicting the FLK protein. (B) *FLK* mRNA in the wild-type and *flk* mutant alleles were analyzed by RT-PCR. (Upper) RT-PCR results by using primers specific to *FLK*. (Lower) RT-PCR results by using primers specific to *UBQ*. (C) Transgenic plants expressing the 35S::GFP-*FLK* gene flowered earlier than the wild type. (D) GFP fluorescence images showing cellular localization of the GFP-*FLK* fusion protein.

repeats are not strictly conserved in the putative rice ortholog of *FLK* (Fig. 6A).

To study the function and cellular localization of the FLK protein, we prepared transgenic plants overexpressing the GFP-FLK fusion protein under the constitutive 35S promoter (see *Supporting Materials and Methods*). The 35S::GFP-*FLK* transgenic plants flowered earlier than the wild type (Fig. 2C). Because the loss-of-function *flk* mutant flowered later and 35S::GFP-*FLK* transgenic plants flowered earlier, we concluded that FLK is a positive regulator of floral initiation. The GFP-FLK fusion protein was enriched in the nucleus, although it was also found in the cytosol (Fig. 2D). The nuclear localization of GFP-FLK is consistent with a proposition that FLK may be involved in the regulation of flowering-time genes.

We investigated whether the *flk* mutation affects gene expression by analyzing genome-wide expression profiles of three different samples: 16-day-old seedlings grown in long days and harvested 16 h after light-on (Exp. 1), 16-day-old seedlings grown in continuous light (Exp. 2), and 7-day-old seedlings grown in long days (18 h light/6 h dark) but harvested 1 h after light-on (Exp. 3). Affymetrix (Santa Clara, CA) ATH1 genechip arrays representing  $\approx 24,000$  *Arabidopsis* genes were used in all three experiments. The complete DNA microarray results of the three experiments can be found online ([www.ncbi.nlm.nih.gov/geo](http://www.ncbi.nlm.nih.gov/geo), accession no. GSE1512). We also used Q-PCR and RT-PCR to reexamine the gene expression changes detected by the DNA

microarray studies. Results of the three microarray experiments are summarized in Table 1, for which three statistical criteria (see Table 1) were used to determine a putative misexpression event in the *flk* mutant. Although a number of genes showed altered expression in at least one microarray experiment (referred to as one misexpression event and marked by asterisks in Table 1), only eight genes showed altered mRNA expression in the *flk* mutant in all three microarray experiments (Table 1, type A, and [www.ncbi.nlm.nih.gov/geo](http://www.ncbi.nlm.nih.gov/geo)). The two genes for which the expression showed the most profound change in the *flk* mutant are *FLC* and *FLK*. The level of *FLK* expression is close to the background level in the *flk* mutant as demonstrated by RT-PCR (Fig. 2B) and Q-PCR (see Fig. 4A). The mRNA level of *FLC* was  $\approx 6$ - to 10-fold higher in the *flk* mutant than in the wild type in all three microarray experiments. Increased *FLC* expression in the *flk* mutant was confirmed by both RT-PCR (Fig. 3A) and Q-PCR (see Fig. 4A). Because KH domain proteins are often associated with RNA metabolism, we examined whether the elevated *FLC* mRNA level in the *flk* mutant was due to a defect in *FLC* mRNA turnover (Fig. 3B). In this experiment, tissues were excised and incubated with transcription inhibitors (cordycepin, cycloheximide, and actinomycin D), and the *FLC* mRNA level was examined at different time points after the inhibitor treatment. Fig. 3B shows that *FLC* mRNA level decreased gradually in tissues treated with transcription inhibitors in both the wild type and *flk* mutant, but there was no obvious difference of the rate of *FLC* mRNA decay between the two genotypes. This result suggests that FLK may regulate *FLC* mRNA expression by means of a mechanism other than RNA turnover. The FLK protein expressed and purified from the *in vitro* translation system or from *Escherichia coli* also failed to bind *FLC* mRNA or polyribonucleotides in various conditions tested (X.Y. and C.L., unpublished data).

In addition to *FLK*, two other KH domain genes also showed altered expression profiles in the *flk* mutation in all three microarray experiments (Table 1). The mRNA level of one KH domain gene (At5g06770) was  $\approx 100\%$  higher in the *flk* mutant than in the wild type, whereas the other KH domain gene (At3g32940) showed  $\approx 30$ – $40\%$  decreased expression in the *flk* mutant in all three microarray experiments (Table 1, type A). In contrast, the expression of other KH domain genes, including *HEN4*, which regulates floral organ development, showed normal expression in the *flk* mutant in all microarray experiments (Table 1, type A). The modest misexpression of At5g06770 was confirmed by RT-PCR, in which this KH domain gene showed modestly elevated expression in three different *flk* mutant alleles tested (Fig. 7, which is published as supporting information on the PNAS web site). A phylogenetic analysis indicates that at least one of the two KH domain genes affected by the *flk* mutation is closely related to FLK, although the sequence similarity between these KH domain proteins are not particularly strong (Fig. 6B and C). The function of neither gene is known. Because the expression of these two KH domain genes is affected by the *flk* mutation, it is speculated that their functions may be associated with FLK. The functions of the other four genes listed in Table 1 and their relationship with *FLK* are not clear.

Results of the expression comparison for genes that play roles in the autonomous pathway controlling flowering time or genes that regulate floral development, such as floral organ identity, are summarized in Table 1, type B. The expression profiles of genes known or likely to be involved in photoperiodic control of flowering time are listed in the Table 1, type C. Table 1, type B, shows that, with few exceptions, genes associated with autonomous pathway or floral development generally were expressed normally in the different samples analyzed in all three microarray experiments. For example, two autonomous pathway genes involved in RNA metabolism and regulation of *FLC* expression, *FCA* and *FY*, showed no change of expression in the *flk* mutant

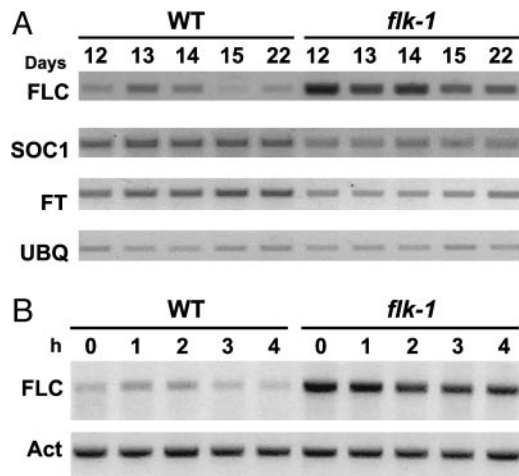
**Table 1. A summary of the results of three DNA microarray studies**

| Type      | Locus     | Gene description   | Exp. 1, <i>flk</i> /WT ( <i>P</i> ) | Exp. 2, <i>flk</i> /WT ( <i>P</i> ) | Exp. 3, <i>flk</i> /WT ( <i>P</i> ) |            |
|-----------|-----------|--|-------------------------------------|-------------------------------------|-------------------------------------|------------|
| A         | At1g13650 | Expressed protein  | 1.4 (0.01)*                         | 3.0 (0.04)*                         | 1.4 (0.03)*                         |            |
|           | At1g17440 | Transcription initiation factor IID (TFIID) subunit A family protein | 0.5 (0.00)*                         | 0.7 (0.04)*                         | 0.6 (0.01)*                         |            |
|           | At3g04610 | FLK  | 0.1 (0.00)*                         | 0.2 (0.04)*                         | 0.1 (0.00)*                         |            |
|           | At3g32940 | KH domain protein  | 0.7 (0.05)*                         | 0.6 (0.02)*                         | 0.6 (0.01)*                         |            |
|           | At3g60980 | Pentatricopeptide (PPR) repeat-containing protein                    | 0.6 (0.00)*                         | 0.6 (0.01)*                         | 0.5 (0.01)*                         |            |
|           | At5g06770 | KH domain/zinc finger protein  | 2.1 (0.01)*                         | 1.9 (0.01)*                         | 1.9 (0.02)*                         |            |
|           | At5g10140 | FLC  | 8.2 (0.00)*                         | 10.8 (0.00)*                        | 6.1 (0.00)*                         |            |
|           | At5g42670 | Agenet domain-containing protein                                     | 1.5 (0.00)*                         | 1.9 (0.02)*                         | 1.3 (0.05)*                         |            |
|           | B         | AT4G18960  | AG                                  | 1.0 (0.70)                          | 0.9 (0.10)                          | 0.9 (0.20) |
|           |           | AT1G69120  | AP1                                 | 0.8 (0.09)                          | 0.9 (0.02)                          | 1.1 (0.02) |
| AT4G36920 |           | AP2  | 1.0 (0.62)                          | 1.0 (0.83)                          | 1.0 (0.78)                          |            |
| AT3G54340 |           | AP3  | 0.9 (0.29)                          | 0.8 (0.49)                          | 1.0 (0.94)                          |            |
| AT1G75820 |           | CLV1   | 1.0 (0.43)                          | 0.8 (0.34)                          | 1.0 (0.13)                          |            |
| AT1G65380 |           | CLV2   | 1.0 (0.78)                          | 1.0 (0.82)                          | 1.0 (0.53)                          |            |
| AT2G27250 |           | CLV3   | 0.9 (0.38)                          | 0.8 (0.24)                          | 1.1 (0.10)                          |            |
| AT4G16280 |           | FCA  | 1.0 (0.67)                          | 1.0 (0.52)                          | 0.9 (0.42)                          |            |
| AT3G10390 |           | FLD  | 1.0 (0.34)                          | 1.1 (0.44)                          | 1.1 (0.23)                          |            |
| AT1G77080 |           | FLM  | 1.1 (0.69)                          | 0.8 (0.22)                          | 1.0 (0.63)                          |            |
| AT4G00650 |           | FRI  | 1.0 (0.85)                          | 0.8 (0.26)                          | 0.9 (0.10)                          |            |
| AT2G19520 |           | FVE  | 1.0 (0.77)                          | 1.2 (0.22)                          | 1.0 (0.41)                          |            |
| AT4G25530 |           | FWA  | 1.0 (0.51)                          | 0.9 (0.51)                          | 1.1 (0.31)                          |            |
| AT5G13480 |           | FY   | 1.2 (0.07)                          | 0.9 (0.16)                          | 1.0 (0.69)                          |            |
| AT5G64390 |           | HEN4   | 1.0 (0.23)                          | 1.1 (0.45)                          | 1.0 (0.55)                          |            |
| AT2G39810 |           | HOS1   | 1.1 (0.03)                          | 1.2 (0.04)                          | 1.0 (0.87)                          |            |
| AT3G12680 |           | HUA1   | 1.1 (0.10)                          | 1.1 (0.56)                          | 1.2 (0.01)                          |            |
| AT5G23150 |           | HUA2   | 0.8 (0.13)                          | 0.8 (0.28)                          | 0.9 (0.06)                          |            |
| AT4G02560 |           | LD   | 1.0 (0.75)                          | 1.5 (0.02)*                         | 1.0 (0.81)                          |            |
| AT5G61850 |           | LFY  | 1.0 (0.38)                          | 0.9 (0.18)                          | 1.0 (0.69)                          |            |
| AT5G20240 |           | PI   | 1.0 (0.38)                          | 0.8 (0.47)                          | 0.7 (0.00)*                         |            |
| AT3G23130 |           | SUP  | 0.9 (0.40)                          | 1.0 (0.97)                          | 1.0 (0.86)                          |            |
| AT5G03840 |           | TFL1   | 0.8 (0.11)                          | 1.0 (0.47)                          | 1.1 (0.44)                          |            |
| AT5G17690 |           | TFL2   | 1.1 (0.08)                          | 1.2 (0.28)                          | 1.1 (0.41)                          |            |
| AT3G18990 |           | VRN1   | 1.1 (0.43)                          | 1.2 (0.09)                          | 1.1 (0.03)                          |            |
| AT4G16845 |           | VRN2   | 0.9 (0.02)                          | 1.3 (0.38)                          | 1.1 (0.31)                          |            |
| C         |           | AT4G24540  | AGL24                               | 0.5 (0.01)*                         | 0.8 (0.02)*                         | 1.0 (0.33) |
|           |           | AT5G60100  | APRR3                               | 1.4 (0.01)*                         | 0.9 (0.65)                          | 1.0 (0.59) |
|           | AT5G24470 | APRR5  | 1.5 (0.02)*                         | 0.5 (0.02)*                         | 0.9 (0.54)                          |            |
|           | AT5G02810 | APRR7  | 1.1 (0.03)                          | 0.5 (0.01)*                         | 0.8 (0.09)                          |            |
|           | AT2G46790 | APRR9  | 1.1 (0.07)                          | 1.0 (0.95)                          | 0.8 (0.16)                          |            |
|           | AT2G46830 | CCA1   | 1.4 (0.04)                          | 1.5 (0.01)*                         | 0.9 (0.00)                          |            |
|           | AT5G15840 | CO   | 1.0 (0.20)                          | 1.0 (0.98)                          | 0.7 (0.02)*                         |            |
|           | AT1G65480 | FT   | 0.8 (0.00)                          | 0.7 (0.05)*                         | 1.1 (0.09)                          |            |
|           | AT1G22770 | GI   | 1.4 (0.00)*                         | 0.4 (0.00)*                         | 0.9 (0.46)                          |            |
|           | AT1G01060 | LHY  | 0.9 (0.19)                          | 1.6 (0.02)*                         | 0.9 (0.08)                          |            |
|           | AT5G37260 | RVE2   | 0.9 (0.24)                          | 2.6 (0.01)*                         | 1.0 (0.67)                          |            |
|           | AT2G45660 | SOC1   | 0.9 (0.14)                          | 0.3 (0.00)*                         | 0.5 (0.05)*                         |            |
|           | AT5G61380 | TOC1   | 1.1 (0.04)                          | 0.8 (0.04)                          | 1.2 (0.26)                          |            |

The *flk*/WT score represents the relative expression levels of a gene in the wild type and the *flk* mutant. A *flk*/WT score >1 indicates a higher expression of the respective gene in the *flk* mutant than in the wild type. A *flk*/WT score <1 indicates a lower expression in the *flk* mutant. A score with the false-discovery rate <20%, *P* value <0.05, and fold change  $\geq 1.3$  represents one putative misexpression event in the *flk* mutant and is denoted by an asterisk. Genes showing the FLK-dependent expression change in all three microarray experiments (i.e., with three putative misexpression events) are listed under type A. Representative genes associated with floral development or autonomous/venalization control of flowering-time are listed under type B, regardless of their expression change. Representative genes associated with photoperiod regulation of flowering are listed under type C, regardless of their expression change. Samples used in three microarray experiments are 16-day-old wild-type and *flk* mutant seedlings grown in long days and harvested 16 h after light-on (Exp. 1), 16-day-old seedlings grown in continuous light (Exp. 2), and 7-day-old seedlings grown in long days (18 h light/6 h dark) and harvested 1 h after light-on (Exp. 3). The entire array dataset can be found online ([www.ncbi.nlm.nih.gov/geo](http://www.ncbi.nlm.nih.gov/geo)) under accession no. GSE1512.

in all three microarray experiments (*FPA* is not represented on the ATH1 array). The expression of other genes that are known to regulate *FLC* expression, such as *FRI*, *FLD*, *FVE*, *VRN1*, and *VRN2*, were also not apparently affected by the *flk* mutation.

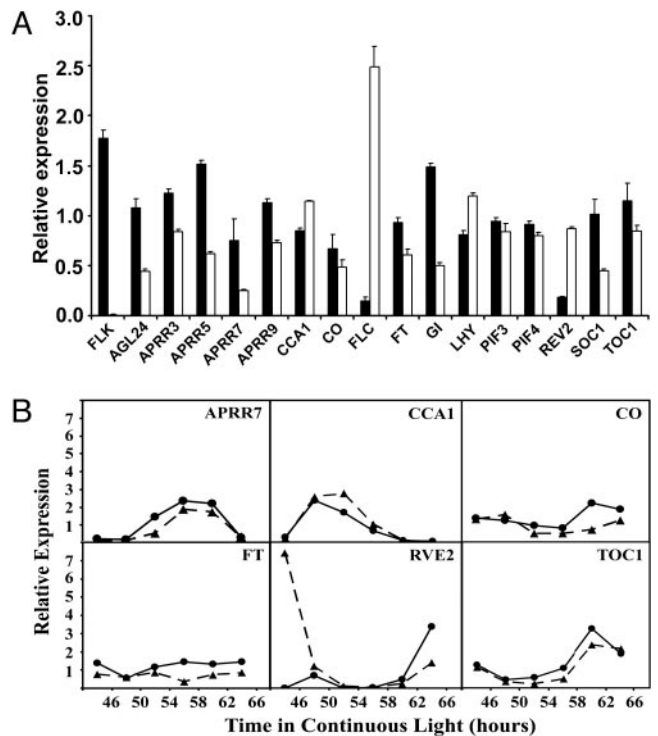
Genes known for their roles in the regulation of floral organ development, such as *AGAMOUS*, *API1*, *AP2*, *CLV1*, and *SUP*, also demonstrated normal expression in the *flk* mutant in all three microarray experiments (Table 1, type B).



**Fig. 3.** The *flk* mutation affects *FLC* expression. (A) A representative RT-PCR result showing the expression of *FLC*, *FT*, and *SOC1* in the wild type and the *flk-1* mutant. Plant tissue was harvested shortly after light-on from 12-, 13-, 14-, 15-, or 22-day-old seedlings grown in long-day photoperiods. (B) The lack of apparent effect of *flk* mutation on the mRNA stability of *FLC*. Seedlings of the wild type or *flk* mutant were treated with transcription inhibitors for up to 4 h (see *Supporting Materials and Methods*). The *FLC* mRNA level was examined by using RT-PCR at different time points (h) after incubation in transcription inhibitors.

In contrast to the genes associated with autonomous pathway or floral development (Table 1), the expression profiles of genes associated with photoperiodic regulation of flowering time showed more complicated patterns in the *flk* mutant (Table 1, type C). For example, among the 26 genes listed in Table 1, type B, only two putative misexpression events (marked by asterisks in Table 1) were detected according to the selection criteria (see Table 1). In contrast, a total of 15 putative misexpression events were detected among 13 genes listed in Table 1, type C. At least one putative misexpression event was detected for almost every gene listed in Table 1 (and 4 of 13 genes showed misexpression in two microarray experiments). Among the genes listed in Table 1, type C, *FT* and *SOC1* are known to be positively regulated by *CO* and negatively regulated by *FLC* in response to different signals (3–5). One or two putative misexpression events were detected for the *FT* or *SOC1* genes, respectively, both showing decreased expression in the *flk* mutant (Table 1, type C). The expression of *FT* and *SOC1* were reexamined with RT-PCR or Q-PCR analyses. Fig. 3A shows that *FT* and *SOC1* both were expressed at lower levels in the *flk* mutant in samples collected from 12 to 22 days after germination. A modestly decreased mRNA accumulation of *FT* and *SOC1* was also demonstrated by using a Q-PCR assay (Fig. 4A). Because *FLC* expression was significantly increased in the *flk* mutant, our results are consistent with *FLC* being a negative regulator of the mRNA expression of *FT* and *SOC1*, resulting in delayed flowering. We concluded that *FLK* is a negative regulator of *FLC* expression that suppresses *FLC* expression to derepress floral initiation.

As described previously, most genes involved in photoperiodic regulation of flowering time registered putative misexpression events in at least one of the three microarray experiments, although the expression changes were modest (Table 1, type C). It is noted that most of the putative misexpression events were detected in 16-day-old samples grown in continuous light (Table 1, type C, Exp. 2), but not in 7-day-old samples grown in long days and collected 1 h after light-on (Table 1, type C, Exp. 3). Most of the gene expression changes detected in the 16-day-old *flk* mutant seedlings were confirmed by Q-PCR experiment (Fig. 4). For example, *AGL24*, *APRR3*, *APRR5*, *APRR7*, and *CO*



**Fig. 4.** Q-PCR showing misexpression of genes in the *flk* mutant. (A) Real-time Q-PCR results showing relative expression levels of selected genes in wild-type (open bars) and *flk* mutant plants (filled bars) grown in continuous cool white fluorescent light for 16 days. Data represent the mean and standard deviation of three replicates. For each gene, the relative amount of calculated message was normalized to the level of the control gene ubiquitin (At5g15400). (B) Q-PCR results showing relative expression levels of six circadian clock-regulated genes in wild-type (filled circles with solid line) and *flk* mutant plants (filled triangles with dashed line). Wild-type (Col-0) and *flk1* plants were entrained in 12:12 (light:dark) photoperiods for 7 days and then transferred to continuous light. Plant tissue was harvested in 4-h increments from plants that had been in continuous light at the time indicated (see *Supporting Materials and Methods*). Data represent the mean of four independent biological replicates. The relative expression of the indicated genes was normalized to the level of the control gene ubiquitin (At5g15400).

genes each registered at least one decreased expression in the *flk* mutant (Table 1, type C) and similarly decreased expression of these genes were detected in the Q-PCR experiment (Fig. 4A). In contrast, *CCA1* and *LHY* showed slightly increased expression in the 16-day-old *flk* mutant in both microarray and Q-PCR experiments (Table 1, type C, and Fig. 4A). This discrepancy does not seem to be due to the developmental difference between the wild type and the *flk* mutant that flower later, because few of the 26 genes, which are associated with autonomous/vernalization control of flowering time or floral development and are also known to increase expression at later developmental stages, showed similar bias (Table 1, type B, Exp. 2). Moreover, the genes that showed decreased expression in the *flk* mutant, such as *CO*, *FT*, *SOC1*, *APRR5*, and *APRR7*, are known positive regulators of floral initiation (20, 42, 43), whereas the genes that showed increased expression in the *flk* mutant, such as *CCA1* and *LHY*, are known negative regulators of flowering (44, 45). In both cases, the function of those genes for which the expression was modestly altered by the *flk* mutation correlated with the delayed flowering phenotype of *flk*. This analysis again suggests that the misexpression of these genes in the *flk* mutant may not be due to random experimental variations. The function of *RVE2* is not known, but it is a MYB protein related to *CCA1* and *LHY*. Moreover, a recently re-

ported protein called EPR1 that shares  $\approx 57\%$  amino acid similarity to RVE2 was found to suppress flowering (46).

Most of the photoperiodic pathway genes mentioned above are regulated by the circadian clock, which may explain the different results derived from samples grown in different photoperiods and collected at different times. So we investigated whether the expression of the photoperiodic pathway genes in young seedlings may be detected under a free-running condition. In this experiment, 7-day-old wild-type and *flk* mutant seedlings were entrained in photoperiods (12 h light/12 h dark) and transferred to continuous light for 2 days of "free running"; samples were collected every 4 h for 24 h and analyzed by using real-time Q-PCR. Among the six genes tested, four (*APRR7*, *CO*, *FT*, and *TOC1*) showed lower amplitude of the circadian rhythm, whereas the other two (*CCA1* and *RVE2*) showed increased expression at some points of free running. Both results are consistent with their registered misexpression events found in the microarray (Table 1, type C) or Q-PCR studies with adult (16-day-old) plants, which indicate that the modest changes in the expression of photoperiodic pathway genes can also be detected in young (7-day-old) seedlings of the *flk* mutation by using a more sensitive method. We conclude that FLK mainly regulates the expression of *FLC*, but it may also play a minor role,

directly or indirectly, in the expression of genes associated with photoperiodic pathway. In this regard, it is interesting to note that *flc* mutations have been recently found to cause shortened periods of the circadian rhythm (47), and the increased *FLC* expression or activity may suppresses the expression of the photoreceptor gene *CRY2* (15, 48, 49). Whether the modest effect of *flk* mutation on the expression of the photoperiodic pathway genes is a consequence of the increased *FLC* activity in the *flk* mutation remains to be further investigated.

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