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RESEARCH ARTICLE

Identification of a gene responsible for the 60-day delay in flowering time of *Arabidopsis*

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Abstract **Identification of genes related to flowering-time in *Arabidopsis* is very important and meaningful contribution to the flowering process control. One late flowering mutant plant, which exhibits 60-day delay in flowering, was screened from *Arabidopsis* library of T-DNA insertion. Southern blotting was used to confirm the single copy of exogenetic T-DNA in the genome of the mutant. The flanking sequence of T-DNA insert was obtained by TAIL-PCR and then analyzed by BLAST to confirm that the insertion site locates at the sixth exon of AT2G19520.1 (*FVE* gene). *FVE* is considered as a classical flowering time gene in *Arabidopsis*. It is a component of the autonomous pathway that encodes AtMSI4, which is a putative retinoblastoma-associated protein. The late-flowering mutant is named as *fve-4*, which is similar to *fve-3* of Columbia and allelic with *fve-1* and *fve-2* of *Landsberg erecta*. The *fve-4* mutant's delay of flowering was longer than that of *fve-3* mutant, whose T-DNA insertion is located at the first exon of *FVE* gene, suggesting that the sixth exon of *FVE* gene may play a more important role in the control of floral transition.**

Keywords delay of flowering, *FVE* gene, *Arabidopsis*, gene identification

Introduction

Flowering is the central link from the vegetative growth to the reproductive growth in plant development (Simpson and Deam, 2002). According to earlier reports, there are at least four pathways controlling the floral transition: photoperiod, vernalization, autonomous, and gibberellin. Genes involved in photoperiod and vernalization are affected by environmental factors, but genes involved in the two other ways are regulated by the developmental conditions of plants (Schwartz et al., 2009). The flowering control is regulated by a network including the above four "cross-talking" pathways. The pathways regulating vernalization and autonomous act on the *FLC* and its downstream genes. The pathways regulating photoperiod change the expression of *CO* and its downstream genes (Jang et al., 2008). Some genes, such as *SOCI*, *FT*, and *LFY*, are involved in all of the four pathways, and their expression can delay or prolong the flowering time (Yan et al., 2010).

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Although plant flowering requires external (environmental) conditions, internal (developmental) factors are also very important. When the genes of autonomous pathway are mutated, mutations cause delayed flowering in long and short days. The *FLC* gene is very important during vernalization, and the expression level of *FLC* is much higher than either the wild type or mutants in photoperiod and gibberellin (Simpson, 2004). When *FVE* gene is mutated, the expression of *FLC* is perturbed (Kim et al., 2004). *LD* is the first cloned gene in the autonomous pathway, which encodes a homologous allotype domain protein; the structure of *LD* gene is similar to the promoter in animals; the transcription production of *LD* spreads over in the plant, which masses up the highest in the shoot of bud and root (Lee et al., 1994), but the mechanism of *LD* regulating the *FLC* is still unclear. *FCA*, *FPA*, and *FLK* encode RNA binding proteins, overexpressed *FCA* can reverse the effect of *FRI* promotive to the *FLC*, and it can induce the flowering (Quesada et al., 2003). *FPA* can regulate the *FLC* more significantly than the *FCA* in developing tissues. *FLK* is another gene that is independent from autonomous pathway, and its expression is not affected by the mutations of *FCA* and *FPA*. In addition, the mutation of *FLK* does not influence the *FCA* and *FPA* expressions. It shows that *FLK* may be an epistatic factor in an autonomous

pathway. *FY* may participate in the splicing of mRNA 3′ end and interact with *FCA* to regulate the expression of *FLC* (Simpson et al., 2003).

In this study, we described the isolation and molecular characterization of *fve-4*, a late flowering mutant displaying 60-day delay in flowering time from the *Arabidopsis* library of T-DNA insertion (genetics background is Colombia) (Zhang et al., 2005). These results suggested that the sixth exon of *FVE* gene may play a more important role in the control of floral transition.

Materials and methods

Seeds of *Arabidopsis thaliana*

Arabidopsis thaliana (Columbia, Col-0) was kindly provided by Dr. Xia (Donald Danforth Plant Science Center, St. Louis, Missouri 63132). The *Arabidopsis* IGDB-XVE mutant seeds were kindly provided by Dr. Jianru Zuo (the Institute of Genetics and Developmental Biology, Chinese Academy of Sciences). Plants were grown in soil under long day (16 h light/8 h dark) at 22°C and 150 μmol/(m^2·s). Flowering time was assayed by counting rosette leaf number.

Primers

Oligonucleotides (Table 1) used for probe of Southern blotting, TAIL-PCR, PCR, and RT-PCR were designed based on the specific sequence of genes and vectors. Primers were synthesized by Sangon Biotech Company.

Southern blotting

The mutant was T-DNA inserted mutant induced by chemical to activate XVE (LexA-VP16-ER) system. The genomic DNA isolated by CTAB was digested with the restriction endonuclease *Xho*I, *Hin*dIII, and *Eco*RV. The digested DNA was electrophoresed on a 0.8% agarose gel and transferred to a nylon membrane. The transferred DNA was cross-linked to the filter by ultraviolet irradiation, and hybridized with DIG (Roche Applied Science, Germany)-labeled DNA probe at 42°C overnight, and then, the immunological detection was run for more than 16 h. Hybridization, washes, and hybrid detection were done according to the instructions provided with DIG DNA labeling and detection kit (Roche).

Thermal asymmetric interlaced PCR

Flanking sequence of T-DNA insert was obtained by thermal asymmetric interlaced PCR (TAIL-PCR) (Liu et al., 1995). Taking the genomics DNA of mutant as template, PCR product was amplified by the primers combination LexA2/LexA4/LexA5 and AD1. PCR products of the second and third time were tested on 1.0% agarose gel. PCR products were resolved on 1.0% TAE-agarose gel and purified from an agarose gel slice using the UNIQ-10 Gel Extraction Kit (Sangon Biotech Company, China). The purified PCR products were cloned into the plasmid pMD-18 vector (Takara, Japan) following the manufacturer's instructions. M13 primers were used to generate single pass partial sequences of the plasmid with differential band inserts. The positive clones were sequenced using an automated sequencer. Sequences were processed to remove vector and identified it on the basis of sequence similarity using the program BLAST.

Cloning of late flowering-related gene

Taking genomic DNA of mutant and Columbia (wild type) as template, respectively, PCR amplifications were performed by using specific primers of *FVE* gene (*FVEL* and *FVER*). PCR reactions were carried out using 20 ng/μL DNA, 1 × PCR buffer, 2.5 mmol/L of dNTPs, 10 μmol/L of each primer, and 0.5 U of *Taq* DNA polymerase in a total volume of 25 μL.

Table 1 Primers used for probe of Southern blotting, TAIL-PCR, PCR, and RT-PCR

Purpose	Primers	Primer sequence
Probe preparation	N1	5′-TCGGCTATGACTGGGCACAACAGA-3′
	N2	5′-AAGAAGGCGATAGAAGGCGATGCG-3′
	LexA2	5′-CTAATCGCATTATCATCCCCTCG-3′
TAIL-PCR	LexA4	5′-CTGGTTTTATATACAGCAGTCGACG-3′
	LexA5	5′-AGTCGAGGTAAGATTAGATATGG-3′
	AD1	5′-(AGCT)TCGA(G/C)T(A/T)T(G/C)G(A/T)GTT-3′
PCR identification	M13F	5′-CGCCAGGGTTTTCCCAGTCACGAC-3′
	M13R	5′-AGCGGATAACAATTTCACACAGGA-3′
Cloning of *FVE*	FVEL	5′-GAAGAGAGAGAGATATAG-3′
	FVER	5′-GCACAGAGAAGGAATCATTAGG-3′
RT-PCR	FVE-1	5′-GCTCGGTTTGGTAACAAG-3′
	FVE-2	5′-GTTTCCTCGCTGTAAATC-3′
	18S-1	5′-GTTGCAGTTAAAAAGCTCGT-3′
	18S-2	5′-TTGATTTCTCATAAGGTGCC-3′

PCR amplification was performed for 35 cycles (at 94°C for 30 s, 52°C for 30 s, and 72°C for 2 min), followed by final extension at 72°C for 10 min. The specific fragment was cloned and sequenced. The sequence was identified with the TAIR-BLAST network server.

FVE gene expression analysis

Total RNA was isolated from 4-week-old plants in long day with Trizol kit (Tiangen, China) and then used to synthesize single-stranded cDNA with the Reverse Transcriptase M-MLV kit (Takara, Japan), following the manufacturers instructions. Using the equal aliquots of cDNA as template, transcript level of *FVE* was measured by RT-PCR; at the same time, 18S rRNA (At3G41768) was used for equal loading.

Bioinformatics analysis

The bioinformatics character of *FVE* gene was analyzed by BLAST through the website www.Arabidopsis.org. and www.ncbi.nih.gov. The conserved domain of *FVE* was analyzed by ScanPro site. Bioinformatics analysis of *FVE* gene provided the basis for further study on its function.

Results

Isolation and phenotype characteristics of late flowering mutant

The flowering time mutants were screened from the T-DNA mutant library in Columbia genetic background. Among the isolated mutants, one late flowering mutant (designated 221-1) was chosen for molecular genetic analysis. When flowering occurred, the numbers of rosette leaf were measured. This result showed that the mutants had a luxuriant vegetative growth with more primary rosettes than wild type plant. The flowering time of mutant was 60 d later than the wild type (Table 2). The shape of mutant at 90 d was similar with wild type at 30 d (Fig. 1). This indicated that the mutation was primarily related to the flowering time control.

Table 2 The numbers of rosette leaf and flowering days under long-day condition

Plant	No. of rosette leaf	Days of flowering
Mutant 221-1	50±0.3 A	86±0.3 A
Col-0 (wild type)	12±0.2 B	27±0.2 B

Different letters in the same column display significant difference ($P = 0.01$).

Thermal asymmetric interlaced PCR and analysis of the gene's homology

Taking genomics DNA of mutant as template, PCR product was amplified by the primers combination LexA2/LexA4/LexA5 and AD1. A 502-bp flanking sequence was obtained by the third PCR amplification of TAIL-PCR (Fig. 2). The PCR products were purified and cloned into the plasmid pMD-18 vector. The positive clones were sequenced. Comparison of the sequence of the TAIL-PCR product with the *Arabidopsis* genomic sequence indicated that the sequence was homologous with AT2G19520.1 gene (*FVE*), which had 15 extrons in *Arabidopsis*, and the T-DNA inserted just the sixth exton of the *FVE* gene (Fig. 3). Structural analyses using NCBI revealed that the late-flowering mutant was an allele with *fve-1* and *fve-2* of *Landsberg erecta* L*er* induced by EMS and similar to *fve-3* in different locus of the same gene induced by fast neutron (Fig. 3). Therefore, the late-flowering mutant was named *fve-4*. The conserved domain of *FVE* was analyzed by ScanPro site, which indicated that the primary structure of *FVE* contained WD40 repeat-like-containing domain (Fig. 4).

A copy of T-DNA sequence insert genomic DNA of *Arabidopsis*

The number of T-DNA insert was identified by Southern blotting analysis using *npt*II as the probe. Genomic DNA of mutant was digested with *Xho*I, *Hin*dIII, and *Eco*RV. The result of hybridization with single band indicated that T-DNA only had a single copy *in vivo* (Fig. 5).

Cloning of late flowering-related gene

Length of 3590-bp sequence was obtained from the Columbia (wide type) using specific primers of *FVE* gene (*FVEL* and *FVER*) and identified as the full-length DNA complete sequence of *FVE* gene, and no PCR product was amplified from the mutant (Fig. 6). This result also confirmed that *FVE* gene was inserted by a T-DNA in the mutant.

The expression of *FVE* was examined by RT-PCR analysis. The expressed level of *FVE* mRNA appeared in Columbia but not in the mutant (Fig. 7). This result showed that the late-flowering mutant was due to loss of *FVE*.

Discussion

Deciding when to flower is a crucial choice for plants to ensure successful reproductive development. The transition to flowering is tightly controlled by both endogenous programs and environmental signals (Komeda, 2004). Genes involved in the control of flowering have been grouped into genetic pathways with assigned functions based on physiologic experiments (Fig. 8) (Komeda, 2004). The photoperiod, the vernalization, and the autonomous pathways are not understood in detail (Boss et al., 2004; Simpson, 2004; Waters et al., 2006). In the model plant, *Arabidopsis*, the major flowering-time pathways converge to regulate the expression of at least three genes that promote flowering: the pathway

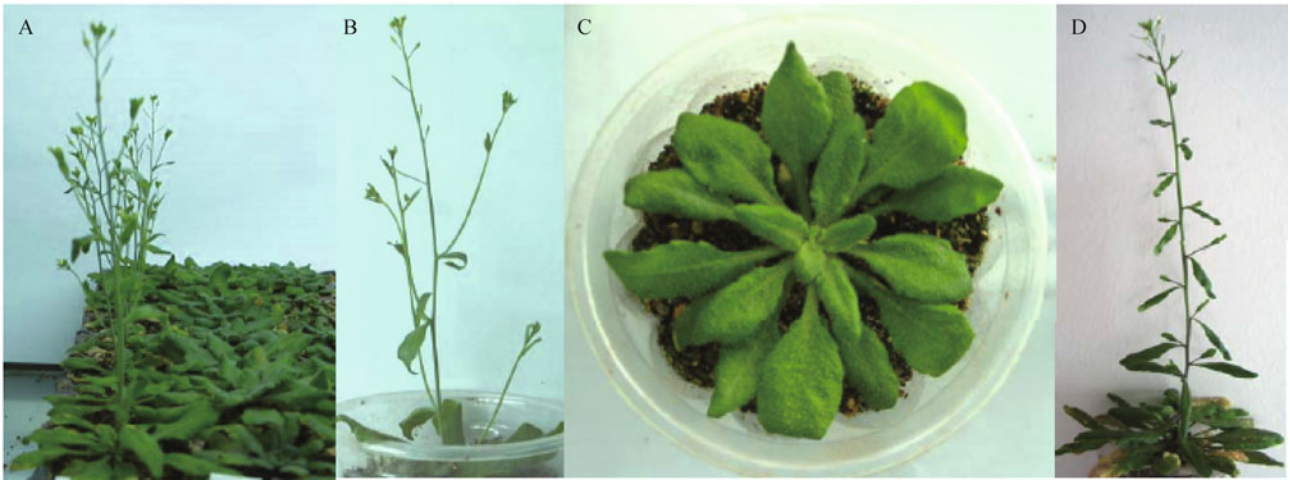

Figure 1 Phenotype of the wild type (Col-0) and the mutant. A is the mutants screened flower later than Col-0 plants in long days; B is the plant of Col-0 (30 days after sowing); C is the plant of the mutant (30 days after sowing); D is the plant of the mutant (90 days after sowing).

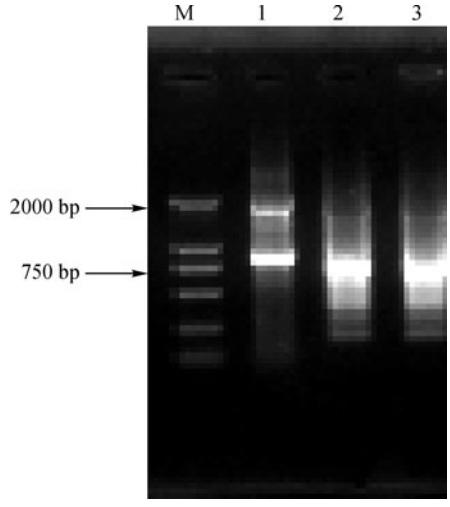

Figure 2 Clone of late flowering-related gene. M: DL2000; 1: the second gene amplification; 2, 3: the third gene amplifications.

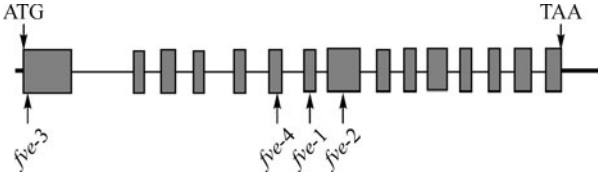

Figure 3 Structure of *five-4* mutant and mutations of the *FVE* alleles. Boxes denote exons, and lines denote introns.

integrators SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1 (SOC1, or AGL20), FLOWERING LOCUS T (FT) and LEAFY (LFY) (Weigel et al., 1992; Kardailsky et al., 1999; Lee et al., 2000; Onouchi et al., 2000; Nyathi et al., 2010).

FVE plays a key role during the flowering in autonomous pathway, and it is homologous with *MSI1* involved with the complex of histone deacetylation. It is required for histone deacetylation of FLC chromosome. When *FLC* has a histone deacetylation, its activity is lose, and then, the plants start to flower. We obtained a mutant with genetics background of Columbia, which was the allele with *five-1* and *five-2* with genetics background of Landsberg erecta (L*er*), and it is a similar mutant with *five-3* in different locus of the same gene (Kole et al., 2001; Tadege et al., 2001; Kim et al., 2006). We named it as *five-4*. All of the four mutants were mutated in *FVE*, but they were different in the locus of mutations (Fig. 3). Compared with type of L*er*, the flowering time of *five-1* was late for 12 d (Kole et al., 2001), *five-2* was late for 20 d (Tadege et al., 2001), and *five-4* was almost 60 d later than the type of Col-0. Therefore, we predicted that the sixth exon of *FVE* gene may play an important role in controlling floral transition.

Our investigation showed that the flowering-related gene of autonomous pathway reacts on the chromatin posttranscription in *Arabidopsis*. *FVE* encodes an *MSI1* homologous gene, *FLD* encodes a Lysine demethylase *LSD1* homologous gene, and both *FVE* and *FLD* are related to the deacetyl combination of histone (He et al., 2003; Ausin et al., 2004),

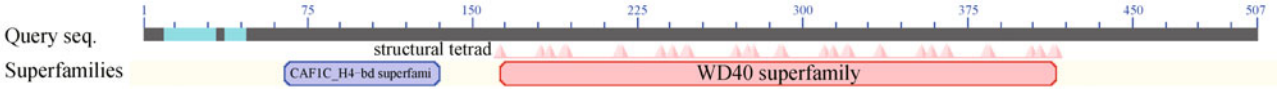

Figure 4 Conserved domain analysis of FVE.

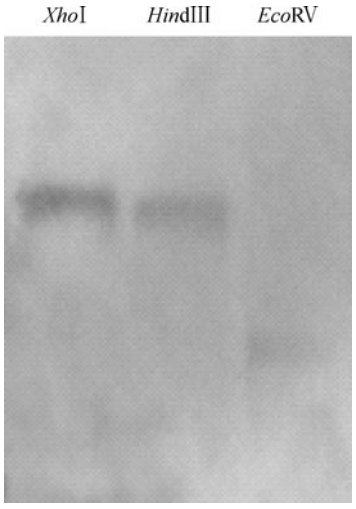

Figure 5 DNA blot hybridization analysis of the mutant.

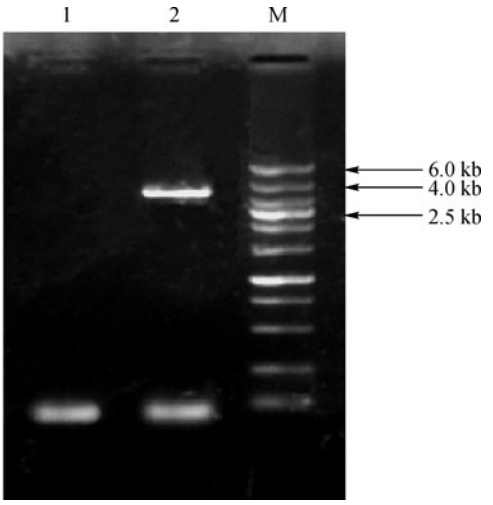

Figure 6 The result of PCR of *FVE* gene. 1: the mutant; 2: wide type; M: Wide Range DNA Marker (100–6000 bp).

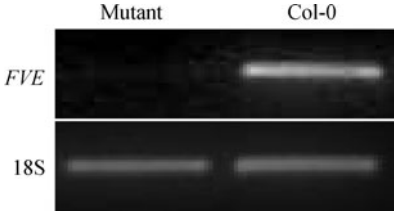

Figure 7 The expression of *FVE* was examined by RT-PCR analysis.

which are necessary to *FLC* chromosome histone deacetyl. When *FLC* chromosome histone deacetyl is done, the condition of activation of *FLC* turns out to be nonactivated, which leads to initiation of flowering. The above investigation infers that autonomous pathway works with vernalization pathway to regulate the *FLC* expression by modulating chromosome structure.

In our research, the *fve-4* mutant caused by the T-DNA inserted on the sixth exon of *FVE* exhibits 60-day delay in flowering as compared to the wild type. Different mutant sites in the *FVE* result in different flowering time of *Arabidopsis*. The regulated mechanism of flowering caused by different regions of *FVE* is still unclear. The *fve-4* mutant exhibits the latest flowering time as compared with *fve-1*, *fve-2*, and *fve-3*. The results showed that the sixth exon of *FVE* gene may be the most important regions of *FVE* in controlling floral

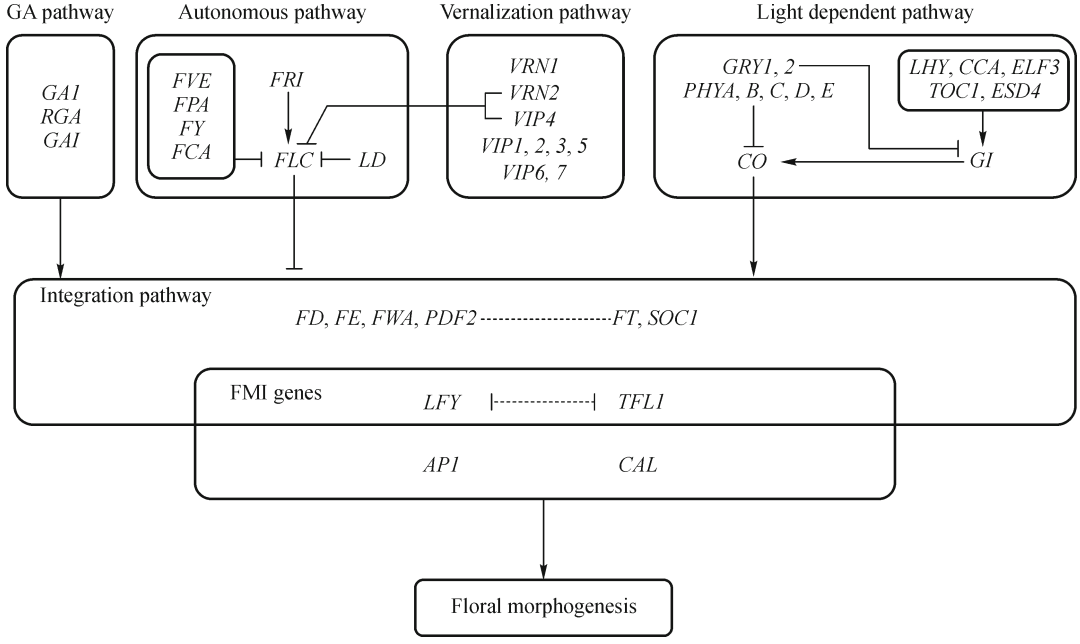

Figure 8 The genetic pathways of flowering in *Arabidopsis*. Positive (arrows) and negative (T-lines) interactions are described. Dotted lines show undescribed interaction (Komeda, 2004).

transition. Research on the regulated mechanism of *FVE* gene will be needed. The work will contribute the flowering process control and finally be applied in gene-modified plants to increase the yield of green parts, such as oat or other vegetation for grazing animals.

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