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RESEARCH ARTICLE

Construction of RNAi vector for flower-related gene and verifications of the mutant in *Arabidopsis thaliana*

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Abstract **The timing of floral transition is tightly controlled by a combination of endogenous and environmental signals. One early flowering mutant plant was screened from *Arabidopsis* library of T-DNA insertion to accelerate flowering under short-day condition, and a related-gene *EFS1* (AT4G36680.1) was isolated and identified as a novel flowering-time gene of *Arabidopsis* in our preliminary studies. To investigate the function and the specific mechanism of *EFS1* in the flower process control, the RNAi expression vector containing *EFS1* gene-specific sequences in the sense and antisense orientations was constructed and transferred into *Arabidopsis* by using the floral-dip method, with 11 transgenic plants obtained through hygromycin B screening and PCR assays. The results showed that the expression level of *EFS1* in transgenic lines was significantly lower than that in wild type and *efs1* mutant. The flowering time of the *efs1* mutant and RNAi transgenic plants was much earlier than that of wild-type plants. This result further verified that the *EFS1* gene played an important role in flowering, and its specific mechanisms need further study. These work provided a foundation to further regulatory mechanisms of *EFS1* in the control of floral transition.**

Keywords *Arabidopsis thaliana*, RNAi, *EFS1* gene, early flowering

Introduction

The timing of the floral transition is a crucial choice for most plants to ensure successful reproductive development. Therefore, the timing of floral transition is tightly controlled by a combination of endogenous and environmental signals (Komeda, 2004; Putterill et al., 2004). According to early reports, there are at least four pathways controlling the floral transition including photoperiod pathway, vernalization pathway, autonomous pathway and gibberellin (GA) pathway. Genes involved in photoperiod and vernalization are affected by environmental factors; however, genes involved in the other two pathways are regulated by the developmental conditions of plants. The flowering time is regulated by a network including the above four "cross-talking" pathways. The four pathways interact with each other by the main genes, *CO*, *FLC*, *FT*, *SOC1*, etc., and then promote the expression of the floral meristem identity genes *AP1* and *LFY* which enable the floral transition.

In plants, RNA interference (RNAi, also known as post-transcriptional gene silencing (PTGS) or co-suppression) is thought to be a key defense against viruses, as well as a way of regulating endogenous genes (Hunter and Poethig, 2003; Kidner and Martienssen, 2003). One key feature of RNAi is the production of double-stranded RNA (dsRNA) homologous to the gene being targeted for silencing (Waterhouse et al., 1998). This dsRNA is degraded into approximately 21-nucleotide RNAs, known as 'small interfering RNAs' (siRNAs), by the enzyme dicer. These siRNAs then provide specificity to the endonuclease-containing, RNA-induced silencing complex (RISC), which targets homologous RNAs for degradation (Hannon, 2002; Tijsterman et al., 2002; Pickford and Cogoni, 2003). The effectiveness of RNAi for functional genomics has already been demonstrated in the nematode *Caenorhabditis elegans*; however, it has not yet been fully utilized in plants.

Previously, one early flowering mutant plant which exhibits 10 days accelerating of flowering under short-day condition was screened from *Arabidopsis* library of T-DNA

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insertion, and the related-gene *EFS1* (AT4G36680.1) was isolated and identified as a novel flowering-time gene of *Arabidopsis*. To clarify the function and the molecular mechanism of *EFS1* gene, the RNAi vector containing *EFS1* gene-specific sequences in the sense and antisense orientations was constructed and transferred into *Arabidopsis* by using the floral-dip method in this paper. The transformed *Arabidopsis* plants were identified by PCR. The interference effects were analyzed by RT-PCR assays. The flowering time of transgenic plants was measured by counting the number of rosette and leaves. These works will contribute to the flower process control and finally be applied in gene-modified plants to increase the yield of green parts such as oat or other vegetation as food of grazing animals.

Materials and methods

Seeds of *Arabidopsis thaliana*

Arabidopsis thaliana (Columbia) was kindly provided by Dr. Xia at the Donald Danforth Plant Science Center. The bacterial strain DH5α was provided by the Molecular Plant Pathology Laboratory of Agricultural University of Hebei. pCAMBIA1300 binary vector was kindly provided by Professor Ma at the National Institute of Biological Sciences, Beijing. *Arabidopsis* plants were grown in soil under long-day (16 h light/8 h dark) and short-day (8 h light/16 h dark) at 22°C with 80% RH and fluorescent light 150 μmol/m^2 per second. Flowering time was assayed by counting the rosette leaf number.

Extraction of genome DNA in *Arabidopsis thaliana*

The genome DNA of *Arabidopsis* was extracted by the method of CTAB. The quality and quantity of DNA were measured by agarose gel electrophoresis and ultraviolet spectrophotometer.

Primers

Oligonucleotide (Table 1) used for specific PCR and RT-PCR was designed based on the specific sequence of genes and vectors. Primers were synthesized by Sangon Biotech Company.

Cloning of the sense and antisense fragments of *EFS1* gene

Taking genomic DNA of *Columbia* (wild type) as template, PCR amplifications were performed to obtain the sense and antisense fragments of *EFS1* gene by using specific primers of *EFS1* ($AT4L^+$, $AT4R^+$, $AT4L^-$, and $AT4R^-$). 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. The PCR amplifications were performed for 35 cycles (at 94°C for 30 s, 52°C for 30 s and 72°C for 2 min) followed by the final extension at 72°C for 10 min. The specific fragment was cloned and sequenced.

Construction and transformation of RNAi expression vector

The cloned sense and antisense fragments of *EFS1* gene were digested with *Bam*HI/*Sac*I and *Xba*I/*Bam*HI, respectively, and inserted into the *Bam*HI/*Sac*I and *Xba*I/*Bam*HI sites of pCAMBIA 1300 binary vector that contains the cauliflower mosaic virus 35S promoter and the NOS terminator. The strategy of *EFS1* gene's RNAi expression vector construction was showed in Fig. 1. The recombinant plasmid was introduced into Col-0 plants by using the floral-dip method.

Table 1 Primers used for specific PCR and RT-PCR

Category	Primers	Primer sequence (5′–3′)
RNAi specific primers	$AT4L^+$	5′-CGCTCTAGAATAATGAGTGCTCAAAAGGAA-3′
	$AT4R^+$	5′-TAAGGATCCCCAACAACAAACCGTCAA-3′
	$AT4L^-$	5′-TGTGAGCTCATAATGAGTGCTCAAAAGGAA-3′
	$AT4R^-$	5′-TAGGGATCCAGCCCGAGTTCCTCCTC-3′
M13 primers pMD-19 vector for PCR	M13F	5′-CGCCAGGGTTTTCCCAGTCACGAC-3′
	M13R	5′-AGCGGATAACAATTTCACACAGGA-3′
Specific primers of *EFS* gene for PCR	EFSF	5′-TACGACCACCGCTCCCT-3′
	EFSR	5′- CTCAAAATGGTGGTGAAAGCA-3′
Hyg primers	HYGF	5′-AAGCCTGAACTCACCGCGAC-3′
	HYGR	5′-CTATTTCTTTGCCCTCGGAC-3′
RT-PCR	EFS-1	5′-ATAATGAGTGCTCAAAAGGAA-3′
	EFS-2	5′-CCAACAACAAACCGTCAA-3′
	18S-1	5′-GTTGCAGTTAAAAAGCTCGT-3′
	18S-2	5′-TTGATTTCTCATAAGGTGCC-3′

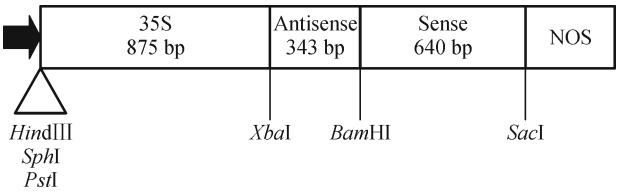

Figure 1 Illustration for construction of RNAi plant expression vector of *EFS1* gene.

Screening and PCR identification of transformed *Arabidopsis* plants

The transformed seeds were sterilized and placed on 1/2 Murashige and Skoog (MS) media containing 50 mg/L hygromycin and 1% agar. The transformed *Arabidopsis* plants with green leaves were transferred to soil for 2 to 3 weeks. Genomic DNA and RNA were extracted from transgenic plants and Col-0. The PCR amplification was performed to identify transgenic plants with *Hyg* primers *HYGF* and *HYGR*.

RT-PCR analysis of *EFS1* gene expression

Total RNA of Col-0 and transgenic plants was isolated from 4-week-old plants in short days with Trizol kit (Tiangen, China) and then used to synthesize single-strand cDNA with the Reverse Transcriptase M-MLV kit (Takara, Japan) according to the manufacturers' instructions. Using the equal aliquots of cDNA as template, the *EFS1* transcript level was measured by RT-PCR and at the same time, 18S rRNA was used for equal loading.

Phenotypic analysis of transgenic plants

Seeds of transgenic plants, Col-0 and *efs1* mutant were sterilized and germinated on MS plates at the same time. Three weeks later, seedlings were planted into soil and cultivated in the greenhouse under short-day (SD) conditions at an 8-h photoperiod and a day/night temperature of 23°C/18°C in a growth chamber. Timing of floral initiation in *Arabidopsis* plants was measured by counting the days until the inflorescence stem became visible or until the first flower starts blooming. Furthermore, the number of rosette leaves formed at the time of bolting and the time of anthesis was counted.

Results

Construction of RNAi expression vector

A fragment of 640 bp cDNA sequence was cloned as sense of *EFS1* digested with *Xba*I/*Bam*HI, and another fragment of 434 bp cDNA sequence was cloned as antisense of *EFS1* digested with *Bam*HI/*Sac*I, leaving a 206-bp region of the *EFS* gene as a spacer. PMD19 was used to connect the sense and antisense fragments separately. Then the constructed vector was transformed to DH5α in order to detect PCR by M13 primers. Then we obtained the corresponding fragments. The pCAMBIA1300 binary vector was digested with *Xba*I/*Sac*I. Then T4 polymerase was used to connect the fragment and the vector. The constructed vector was verified by sequencing. The expressive vector was constructed and confirmed by restriction enzyme digestion and DNA sequencing analysis. All PCR products were amplified using the high-fidelity polymerase Triplemaster (Fig. 2).

Screening and PCR identification of transformed *Arabidopsis* plants

The hygromycin-resistant seedlings were selected on MS media containing 50 mg/L hygromycin in this experiment (Fig. 3). Eleven independent hygromycin-resistant T_0 plants were selected. The PCR amplification was performed to identify transgenic plants using *HYG* gene primers. Ampli-

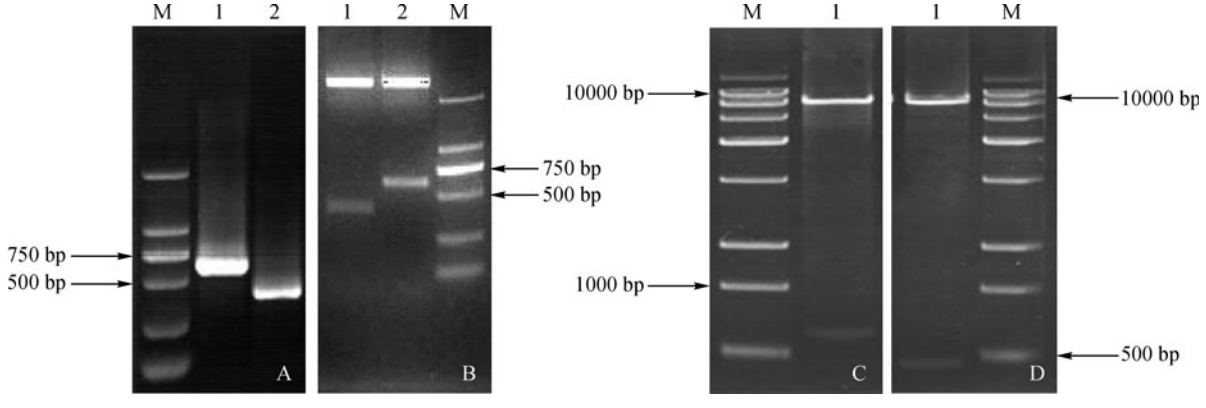

Figure 2 Construction of RNAi expression vector of *EFS1*. Note: A is PCR identification sense and antisense fragments of *EFS1* genes. B is restriction enzyme digestion analysis of T19-*EFS1*F and T19-*EFS1*R. C and D are restriction enzyme digestion analysis of RNAi expression vector of *EFS1*.

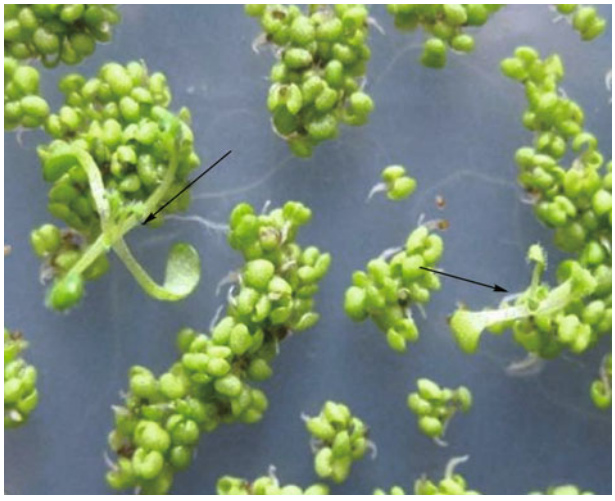

Figure 3 Phenotype of transgenic plants on Petri dish. Note: Arrows represent transgenic plants.

fication result showed that all T_0 plants had the positive band while the wild-type plants did not have the positive result (Fig. 4). The result demonstrated that the hygromycin gene had been integrated into the genome of *Arabidopsis*.

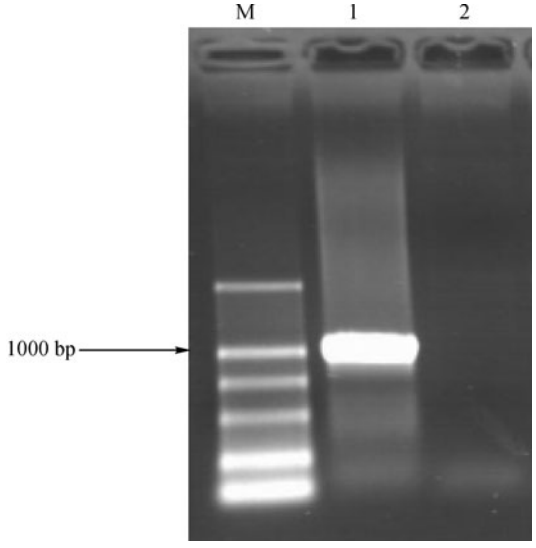

Figure 4 PCR identification of transgenic line. Note: M is DL2000 DNA Marker. 1 and 2 represent RNAi transformant and Col-0.

RT-PCR analysis of *EFS1* gene expression

The expression level of *EFS1* in transgenic plants was analyzed by RT-PCR with EFS-1 and EFS-2 primers. The results showed that the expression level of *EFS1* in RNAi transformant was significantly lower than that of the wild type and *efs1* mutant (Fig. 5), revealing that the RNAi vector was inserted with the genome of *Arabidopsis* and induced the decrease of *EFS1* gene expression.

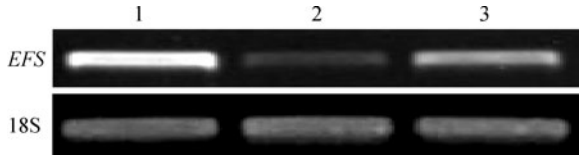

Figure 5 The expression of *EFS* was examined by RT-PCR analysis. Note: 1–3 represent Col-0, *EFS1* RNAi plants and *efs1* mutant, respectively.

Phenotypic analysis of transgenic plants

Under short-day condition, the flowering time of the *efs1* mutant and RNAi transgenic plants was much earlier than that of the wild-type plants consistent with the expression level of *EFS1* (Fig. 6). In addition, the *efs1* mutants and RNAi transgenic plants had a decreased number of rosette leaves (18/15 leaves as compared with 22 for wild-type plants at bolting) compared with wild-type plants (Fig. 7). This result verified further that the *EFS1* gene plays an important role in flowering.

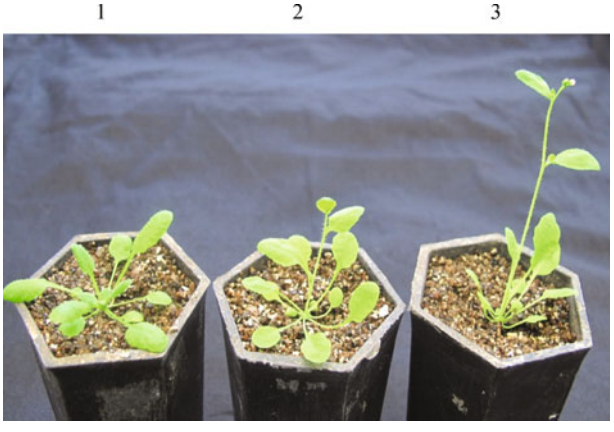

Figure 6 Flowering times of Col-0, *esf1* mutants and *EFS1* RNAi plant under SD. Note: 1–3 represent Col-0, *efs1* mutant and *EFS1* RNAi plants, respectively.

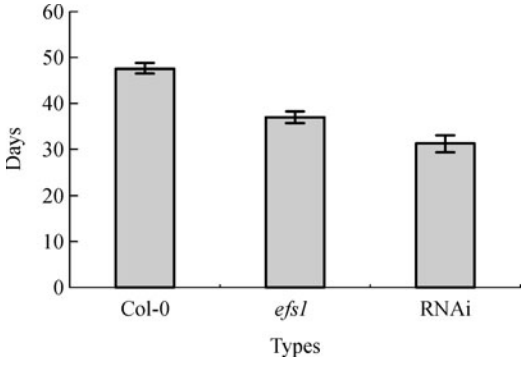

Figure 7 Flowering time of the wild-type (Col-0) and the mutant (*efs1*) (RNAi)

Discussion

Flowering is the central link from the vegetative growth to the reproductive growth in plant development (Simpson and Dean, 2002). Genes involved in the control of flowering have been grouped into genetic pathways with assigned functions based on physiological experiments (Komeda, 2004). The photoperiod, the vernalization and the autonomous pathways are not understood in details (Boss et al., 2004). In the model plant *Arabidopsis thaliana*, the major flowering-time pathways converge to regulate the expression of at least three genes that promote flowering: the pathway 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). Genetic analysis of a large number of *Arabidopsis* flowering time mutants has made the function of the related genes clear.

RNA silencing approaches have been rapidly developed and employed in plants and animals as a tool for exploring gene function. The discovery by Fire et al. (1998) that dsRNA is a primary and essential trigger for this mechanism has led to an exponential increase in the number of publications on this subject (Frantz, 2003). The silencing mechanism has provided powerful reverse genetic tools for functional genomics in several eukaryotic organisms, including *Drosophila melanogaster* and *Caenorhabditis elegans* (Kamath et al., 2003; Boutros et al., 2004). Therefore, RNA silencing offers a tool for inactivating a precisely targeted gene with less effort.

In our research, the *efs1* mutant caused by the T-DNA inserted exhibited 10 days promoting of flowering faster than the wild type. As a result of this phenomenon, the RNAi expression vector of *EFS1* was constructed to obtain the loss of function mutant. The loss of function mutant exhibited the earliest flowering time compared with *efs1* mutant and Col-0. Consistent with this phenotype, the expression of *EFS1* was the least in the RNAi transgenic plants compared with *efs1* mutant and Col-0. The bioinformatics analysis of this gene suggested that the *EFS1* encodes a PPR containing protein that plays a pivotal function in flowering time regulation. PPR proteins are composed of tandem repeats of degenerate 35 amino acid motifs. Most PPR proteins are predicted to be transported to mitochondria and/or plastids, where they participate in various mRNA maturation steps. It is verified that the *EFS1* gene plays an important role in flowering, and its specific mechanism needs further study.

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