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# Expression of the alfalfa CCCH-type zinc finger protein gene *MsZFN* delays flowering time in transgenic *Arabidopsis thaliana*

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ABSTRACT

Zinc finger proteins comprise a large family and function in various developmental processes. CCCH type zinc finger protein is one kind of zinc finger protein, which function is little known. *MsZFN* gene encoding a CCCH type zinc finger protein was first discovered by its elevated transcript level in a salt-induced alfalfa SSH cDNA library. The previous experiment had showed that MsZFN protein was localized to the nucleus and little is known about the function of MsZFN protein and its homologous proteins in other plants including model plant, *Arabidopsis thaliana*. In the current study, we found that *MsZFN* transcript levels increased in alfalfa under continuous dark conditions and that expression was strongest in leaves and weakest in unopened flowers under light/dark conditions. Expression of *MsZFN* in transgenic Arabidopsis plants resulted in late flowering phenotypes under long day conditions. Yeast two-hybrid and bimolecular fluorescence complementation assays indicated that MsZFN protein can interact with itself. Transcript analyses of floral regulatory genes in MsZFN$^+$ transgenic Arabidopsis showed enhanced expression of the flowering repressor *FLOWERING LOCUS C* and decreased expression of three key flowering time genes, *FLOWERING LOCUS T*, *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS* and *GIGANTEA*. These results suggest that MsZFN primarily controls flowering time by repressing flowering genes expression under long day conditions.



## 1. Introduction

Molecular genetic studies in the long day flowering species *Arabidopsis thaliana* Heynh. (in Holl. and Heynhi) have identified at least four major pathways that regulate flowering, each perceiving and processing different signals [1,2]. Transition from the vegetative state to the onset and development of flowering is regulated by both endogenous and environmental signals. Photoperiod is one of the most important factors among these various signals and is regulated both by day length and by the endogenous circadian rhythm [3,4]. Previous work revealed that light causes a large-scale reorganization of chromatin during floral transition in Arabidopsis and that the presence or absence of light results in distinct gene expression profiles during Arabidopsis seedling development [5–7].

The signaling cascades of photoperiod-dependent flowering have been extensively studied in Arabidopsis [8,9] and in short day (SD) rice (*Oryza sativa* L.) [10]. A number of signaling cascade genes have been identified and characterized in these studies. In Arabidopsis *GIGANTEA* (*GI*) integrates cellular signals from light sensory transduction and the circadian clock and activates *CONSTANS* (*CO*), which encodes a zinc-finger transcriptional activator [11,12]. CO induces *FLOWERING LOCUS T* (*FT*), which encodes a mobile flowering signal under LD conditions [13–15]. Several of these gene families, including MADS-box, *CO*, and *FLOWERING LOCUS TERMINAL FLOWER1* families, have expanded in the model legumes *Medicago truncatula* Gaertn., soybean (*Glycine max* L. Merr.), and *Lotus japonicus* (Regel) K. Larsen [16]. Others, including *FRIGIDA (FRI)* and members of the MADS box clade, *FLOWERING LOCUS C* (*FLC*), are absent, although information on gene-specific variation of flowering in *Medicago* spp. is generally limited. In alfalfa, a *CONSTANS-LIKE* gene has single nucleotide polymorphisms (SNPs) associated with stem height and flowering date [17].

Zinc finger motifs, which are classified by the arrangements of the zinc-binding amino acids, cysteine (C) and histidine (H), are present in many transcription factors and play critical roles in their

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interactions with nucleic acids, proteins, or other small molecules [18,19]. So far, only a few zinc finger (ZF) proteins are known to regulate flowering time and most are negative regulators. The INDETERMINATE protein (ID1) is a CCCH-type positive regulator of the transition to flowering in maize (*Zea mays* L.) [20]. *SUPPRESSOR OF FRIGIDA 4*, encoding a C2H2-type zinc finger protein, represses flowering by transcriptional activation of the Arabidopsis repressor, *FLC* [21]. RFI2, a red/far-red insensitive Arabidopsis RING-domain zinc finger protein, negatively regulates *CO* expression and photoperiodic flowering to cause late flowering [22]. More recently, the Arabidopsis C2H2 zinc-finger protein, LATE FLOWERING, has also been found to act as a floral repressor [23].

The *M. sativa* CCCH-type zinc finger gene *MsZFN* was initially detected several years ago in a salt-induced suppressive subtractive hybridization (SSH) cDNA library, and its transcript level is quickly and transiently elevated during salt treatment [24]. When the *MsZFN* coding region was used to transform tobacco (*Nicotiana tabacum* L.), the resultant transgenic plants showed no additional tolerance to NaCl (data was not shown). In the current study, isolation and analysis of the genomic clone, transcript analysis in alfalfa, and expression of *MsZFN* in transgenic tobacco and Arabidopsis, showed that MsZFN is a repressor of flowering time in long day (LD) plants.

## 2. Methods

### 2.1. Plant material and growth conditions

Alfalfa seeds (*Medicago sativa* cv. "Zhongmu NO.1") bred by the Chinese Academy of Agricultural Sciences (CAAS) in 1997 were obtained from CAAS. *A. thaliana* ecotype Col-0 was used either as a recipient for plant transformation or as a wild type control plant. For growth on soil, Arabidopsis seeds were sown directly into pots and incubated at 22 °C under either LD (16 h light and 8 h dark) or SD (8 h light and 16 h dark) conditions. For growth on tissue culture plates, Arabidopsis seeds were sown on ½-strength MS solid medium containing 1% sucrose and 0.8% agar and incubated at 22 °C under continuous light.

### 2.2. Cloning and sequencing of a gDNA clone for MsZFN

Genomic DNA was isolated from alfalfa using a CTAB method and used to isolate a gDNA clone for the *MsZFN* gene from transcript start to end (without a promoter) using specific primers ZFN-f and ZFN-r (Table 1). The full-length cDNA sequence of *MsZFN* (GenBank: EU624138) with 5′ and 3′ UTRs had been obtained earlier by 5′ and 3′ RACE extension of an EST isolated from a *M. sativa* SSH cDNA library induced by NaCl [24]. The gDNA of *MsZFN* was sequenced by the Beijing Genomics Institute (Beijing, China).

### 2.3. Bioinformatics analysis

DNA sequence alignments between *MsZFN* cDNA and gDNA were conducted using the DNAMan software (version: 6.0, Lynnon Biosoft, Point-Claire, Canada) to determine intron–exon borders. Amino acid sequence for MsZFN was obtained by cDNA translation using the DNAMan software (version: 6.0). Related proteins were obtained by BLAST analysis to the NCBI database. Phylogenetic analysis was conducted using MEGA version 5.0 software [25] with bootstrap values obtained from 1000 replications.

### 2.4. MsZFN expression analysis in alfalfa

In order to investigate the expression pattern of *MsZFN* in alfalfa, semi-quantitative and quantitative RT-PCR reactions were carried out in mature tissues, including roots, stems, leaves, flower buds, and opened flowers. Expression profiles of *MsZFN* were determined by semi-quantitative RT-PCR in 15-day-old alfalfa seedlings induced by a time course of 26 h of 20 μM $GA_3$ or 6 h of 20 μM ABA or grown on soil for an additional 5 h or 6 days under continuous dark conditions and compared with alfalfa seedlings grown in an 16/8 h light/dark cycle. To investigate the expression of *MsZFN* gene in germinating alfalfa seeds, seeds were submerged in water for 1, 2 and 5 days and expression patterns compared with dry seeds as a control. For one-step semi-quantitative RT-PCR, RNA extracted from alfalfa plants under the above conditions was digested with RNase-free DNase I and used as cDNA templates in PCR amplifications with forward and reverse *MsZFN*-specific primers, ZFN-1 and ZFN-2 (Table 1). Actin1 and Actin2 served as specific primers to amplify an internal cDNA loading control gene, *MsACTIN* (Table 1). RT-PCR images were captured using a Gel Image Analysis System FR-200A (Furi Science & Technology, Shanghai, China). Real time quantitative RT-qPCR was conducted on the cDNA at Tsinghua University (Beijing, China) using an ABI 7500 Real Time PCR system and SYBR Green I detection (Applied Biosystems, Life Technologies™, Carlsbad, CA, USA) with *MsZFN*-specific primers, ZFN-RT-f and ZFN-RT-r (Table 1). The *MsGAPDH* gene was used as a control in alfalfa RT-qPCR reactions to normalize the amount of cDNA (Table 1).

### 2.5. Binary plasmid construction and transgenic plants

For transgenic expression experiments, the full-MsZFN ORF was also amplified by PCR with the primer pair 1302-ZFN-f and 1302-ZFN-r designed to contain *Nco*I and *Spe*I sites (Table 1). PCR products were digested and inserted into the same enzyme sites in pCAMBIA1302 (Cambia, Canberra, Australia) to generate the green fluorescence binary fusion construct 35S::MsZFN-GFP.

To generate transgenic tobacco plants expressing MsZFN, *Agrobacterium* GV3101 transformed with plasmid *35S::MsZFN*-GFP was used to infect Arabidopsis using the floral dip method [26]. Transformed Arabidopsis seeds were selected on ½-strength MS medium containing 20 mg $L^{-1}$ hygromycin B and 0.8% agar.

### 2.6. Yeast two-hybrid and BiFC assays

For yeast two-hybrid self-interaction experiments, the coding sequence of *MsZFN* was cloned into pGBKT7 and pGADT7 vectors (Clontech, USA) to generate the DNA binding domain plasmid BD-MsZFN and the activation domain plasmid AD-MsZFN, respectively [27]. Yeast strain AH109 (Clontech, USA) harboring plasmids BD-MsZFN, or AD-MsZFN alone, or both together, were spread onto synthetic defined (SD) $Trp^-$/$Leu^-$ selection plates. Single colonies were transferred onto additional SD selection plates ($Trp^-$/$Leu^-$/$His^-$/X-α-Gal), which were placed at 30 °C for 3 days and tested for β-galactosidase activity.

To test the interaction of MsZFN with itself in living plant cells, bimolecular fluorescence complementation (BiFC) analysis was conducted according to Walter [28]. The coding sequence of *MsZFN* obtained as above was cloned into the yellow fluorescent protein (YFP) binary vectors, pSPYNE-35S and pSPYCE-35S, such that two recombinants plasmids, MsZFN-pSPYNE-35S and MsZFN-pSPYCE-35S, respectively, were obtained. The binary vectors, rc-pSPYNE-35S and rc-pSPYCE-35S containing the *GLABRA3* gene [29], were used as positive controls. As negative controls, one was pSPYNE-35S and MsZFN-pSPYCE-35S, another was pSPYCE-35S and MsZFN-pSPYNE-35S. The constructs were delivered by *A. tumefaciens* strain GV3101 into leaf cells of tobacco (*Nicotiana benthamiana* Domin.) according to Voinnet [30]. YFP fluorescence (indicating self-interaction) within the transformed tobacco leaves was imaged in the 514 nm channel using a Nikon confocal laser scanning microscope (above) after the tobacco plants were grown in continuous dark for 40–48 h.