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Transcriptomic Analysis of Alfalfa Flowering and the Dual Roles of *MsAP1* in Floral Organ Identity and Flowering Time

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Abstract: Flowering, the transition from the vegetative to the reproductive stage, is vital for reproductive success, affecting forage quality, the yield of aboveground biomass, and seed production in alfalfa. To explore the transcriptomic profile of alfalfa flowering transition, we compared gene expression between shoot apices (SAs) at the vegetative stage and flower buds (FBs) at the reproductive stage by mRNA sequencing. A total of 3,409 DEGs were identified, and based on gene ontology (GO), 42.53% of the most enriched 15 processes were associated with plant reproduction, including growth phase transition and floral organ development. For the former category, 79.1% of DEGs showed higher expression levels in SA than FB, suggesting they were sequentially turned on and off at the two test stages. For the DEGs encoding the components of circadian rhythm, sugar metabolism, phytohormone signaling, and floral organ identity genes, 60.71% showed higher abundance in FB than SA. Among them, *MsAP1*, an *APETALA1* (*AP1*) homolog of *Arabidopsis thaliana*, showed high expression in flower buds and co-expressed with genes related to flower organ development. Moreover, ectopic expression of *MsAP1* in Arabidopsis resulted in dwarfism and early flowering under long-day conditions. The *MsAP1*-overexpression plant displayed morphological abnormalities including fused whorls, enlarged pistils, determinate inflorescence, and small pods. In addition, MsAP1 is localized in the nucleus and exhibits significant transcriptional activity. These findings revealed a transcriptional regulation network of alfalfa transition from juvenile phase to flowering and provided genetic evidence of the dual role of *MsAP1* in flowering and floral organ development.

Keywords: AP1; flowering time; floral formation; alfalfa; transcriptome

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1. Introduction

The flowering process is pivotal in angiosperms, determining overall plant structure. This transformation is governed by intricate regulatory pathways responsive to diverse environments and endogenous signals. In alfalfa, flowering represents a critical agronomic trait influencing nutrient redistribution and post-blooming lignification, thereby significantly impacting forage productivity and quality [1–3]. Moreover, successful flowering in alfalfa is essential for seed production. Therefore, understanding and utilizing the regulatory mechanisms of flowering are important for alfalfa breeding.

Extensive genetic and molecular studies on model species have shown that plants have complex regulatory pathways for coordinating internal and external signals in the flowering process, including gibberellins (GAs), vernalization, autonomy, photoperiods, and temperature [4–6]. GA-mediated floral induction requirement is determined by the degradation of DELLA protein, a strong inhibitor of flowering under short-day (SD) conditions in Arabidopsis [7–9]. Recent studies have shown that in addition to gibberellins, plant hormones like auxin (AUX), cytokinin (CTK), jasmonic acid (JA), and brassinolide (BR) are involved in the transition of flowering in plants [10–15]. In addition to hormones,

the photoperiod pathway has larger effects on regulating the floral induction in alfalfa. Research has shown that alfalfa flowering is induced by long days, and short days can reverse the flowering of alfalfa [16]. Mutation in photoreceptor *PHYA* and the circadian clock components *MtPRR9* significantly altered flowering time by modulating a set of clock-related genes in *Medicago truncatula* [17,18]. Apart from the signal pathways identified previously, secondary metabolism nutrients such as sugars, including starch, sucrose, and glucose, would function as signaling substances to trigger floral transition in Arabidopsis [19].

Various signaling pathways converge to regulate floral integrator AP1, MADS-box transcription factor, which activates floral meristem induction by integrating signals from multiple pathways [20–22]. As a floral meristem identity gene, *AP1* plays a pivotal role in the transition from shoot apical meristem to floral meristem [23]. AP1 works together with heterotypic MADS transcription factors to determine the characteristics of flower organs. In the Arabidopsis ABCE model of flower development, *AP1* functions as an A-class gene involved in sepal and petal development [23,24]. The B-class genes, including *APETALA3* (*AP3*) and *PISTILLATA* (*PI*), regulate petal and stamen development, while the C-class gene *AGAMOUS* (*AG*), along with E-class genes (*SEPALLATA*s, *SEP*s), controls pistil development [25]. However, recent studies have demonstrated that *AP1* expression extends beyond sepals and petals to include vegetative and other floral organs [26–28]. In other core eudicots, conservation of the A-class gene specifically controlling the two outer whorls of floral organs appears to be lacking [29–32].

Alfalfa is a pivotal leguminous forage crop due to its role as the primary protein source for livestock. The reproductive growth phase significantly impacts both seed yield and forage quality. Thus, elucidating the genetic mechanisms regulating flower development holds substantial importance for enhancing alfalfa seed yield and quality. In this study, we employed transcriptome analysis to explore the regulatory pathways governing flowering transformation. We identified and characterized an *AP1*-like gene, *MsAP1*, from alfalfa, examining its expression patterns. *MsAP1* functions as a transcriptional activator that accelerates flowering time, determines floral organogenesis, and influences pod development upon overexpression in Arabidopsis.

2. Materials and Methods

2.1. Plant Materials and Treatment

Seeds of *Medicago sativa* (Zhongmu No. 1) were collected from the Germplasm Resources Nursery of the Chinese Academy of Agricultural Sciences (CAAS) in Langfang, Hebei, China. Seeds were sterilized and imbibed in a Petri dish at 4 °C for five days in darkness. The germinated seeds were transferred into a pot containing nutrient-rich soil (nutrient soil: vermiculite = 1:1). The plants were moved from a greenhouse to the field of the CAAS research station in Langfang, Hebei Province (39.59 °N, 116.59 °E) at the early branches phase in 2020. Plant height was measured every five days until full bloom after the first cutting in 2020. The tobacco (*Nicotiana benthamiana*) and *Arabidopsis thaliana* (Columbia-0) seeds were sown in a pot. Plants were grown in a greenhouse at 22 °C/18 °C (day/night) with a photoperiod of 16 h of light and 8 h of darkness.

2.2. RNA-Seq Library Construction and Sequencing

The alfalfa plant apical meristems on day 25 and early floral buds on day 35 were freshly collected separately with three replicates for each. The tissues were immediately frozen in liquid nitrogen and stored at −80 °C for subsequent RNA extraction. Total RNA was extracted using the Trizol reagent (Invitroge, Waltham, MA, USA) following the manufacturer's protocol. RNA quality and quantity were assessed using the nanophotometer NP80 system (Implen, Munich, Germany). RNA libraries were prepared using the NEBNext®Ultra™ RNA Library Prep Kit for Illumina® (NEB, San Diego, CA, USA) as per the manufacturer's instructions, and subjected to paired-end sequencing on an Illumina Hiseq 2000 platform (Illumina, San Diego, CA, USA). Subsequently, fastp (v0.23.4) was used to calculate the Q30 score, GC content, and adaptor deletion of the reads. The

HISAT2 R package (v2.2.1) was utilized for aligning the reads to the reference genome of XinJiangDaYe [33] (https://figshare.com/projects/whole_genome_sequencing_and_assembly_of_Medicago_sativa/66380) (accessed on 4 August 2024).

2.3. Identification of DEGs and Gene Ontology (GO) Analysis

Differentially expressed genes (DEGs) were determined using the DESeq2 package (v1.10.1) based on the criteria of $|\log2 \text{ fold change}| > 1$ and adjusted p value < 0.05. Their homologs were searched against the genome database of XinJiangDaYe. For the regulatory network of flowering, the MapMan software V3.5.1R2 (https://mapman.gabipd.org/download, 4 August 2024) was employed. The protein interaction network was built by GeneMANIA (https://genemania.org/) (accessed on 4 August 2024). Heatmaps were generated using TBtools (https://github.com/CJ-Chen/TBtools/releases) (accessed on 4 Augest 2024). For gene ontology (GO) enrichment analysis, the R package ClusterProfiler (v4.8.1) was utilized. GO terms with an adjusted p-value (p.adjust) ≤ 0.05 were considered significantly enriched.

2.4. Reverse Transcription and Quantitative PCR (RT-QPCR)

Total RNA was extracted according to the instructions of the Promega's Plant Total RNA Extraction Kit (Promega, Madison, WI, USA, LS1040) and evaluated by NanoDrop. For the first-strand cDNA synthesis, one microgram of total RNA was used via the cDNA Synthesis Kit (Takara, Dalian, China, Code No. 6210A). RT-qPCR analysis was performed using SYBR Premix Ex Taq (TaKaRa, Dalian, China) on the CFX96^{TM} System (Bio-Rad, Hercules, CA, USA). *MsACTIN2* (*MS.gene013348*) and *AtACIN2* (*At3g18780*) were used as the internal reference for *M. sativa* and Arabidopsis, respectively. The $2^{-\Delta\Delta CT}$ method [34] was used to calculate the relative expression level (primers are listed in Table S5).

2.5. Cloning and Construction and Plant Transformation

The coding sequence of *MsAP1* was amplified from cDNA reverse-transcribed from the total RNA of the tip using primer pairs MsAP1-F/R (Table S5) and cloned into a T vector for sequencing. For the construction of *MsAP1* overexpression, the sequence-verified plasmid was used as a template for amplification with primers of OE-MsAP1-F/R (Table S5). Purity PCR products were subcloned to linearized *pCAMBIA1302* (*Nco* I digested) using an In-Fusion HD Cloning Kit (Takara, Dalian, China, Code No. 639649). The *pCAMBIA1302-MsAP1-GFP* was introduced into *A. tumefaciens* (strain GV3101) using a freeze–thaw method. Arabidopsis transformation was performed via a floral dip method [35]. For transgenic plant isolation, Arabidopsis seeds were sowed in 1/2 MS solid medium supply with Glufosinate (4 mg/L).

2.6. Sequence and Phylogenetic Analysis

The MADS-box specific to the known AP1s was used as a query to search against the NCBI-conserved domain database (https://www.ncbi.nlm.nih.gov/cdd) (accessed on 4 August 2024). A total of 22 putative proteins from the representative plant species were used to build the neighbor-joining algorithm-based phylogenetic tree using the Poisson model, with uniform rates and 1000 bootstrap replicates, by MEGA 6.0. The multiple sequence alignment was performed via DNAMAN (version 8).

2.7. Subcellular Localization and Transactive Activity Assay

For subcellular localization, the epidermal cells of 5-week-old tobacco seedlings were analyzed using an inverted fluorescence microscope, as previously described [36]. To assess the transcriptional activation activity in yeast, we subcloned the full-length coding sequence of *MsAP1* into the pGBKT7 vector to fuse with the GAL4 binding domain, and then introduced it to the yeast (Y2H gold) through an electric shock system with the parameters of 1.5 KV and 200 Ω. Transformants were selected on a tryptophan-lacking SC medium (SC/-T), followed by growth on an SC medium lacking tryptophan and histidine

(SC/-T-H) with 15 mM 3-AT for 3 days to evaluate the transcriptional activation activity based on the growth status.

2.8. Statistical Analysis

Statistical analysis was conducted using SPSS 26.0 for the Student's *t*-test. Data represent the averages of three biological replicates.

3. Results

3.1. Transcriptomic Analysis Revealed a Distinct Expression Pattern of Alfalfa Shoot Apex from Floral Buds

To monitor alfalfa growth, the plant height was measured every 5 days after the initial cutting of a population consisting of 392 plants. A logistic growth pattern was observed in the first 60 days (Figure 1a). Alfalfa growth accelerated at the early stage and maximized on day 25, and plant height reached full capacity on day 45 with flower emergence on day 40 for about 10% of the population. To explore the genes involved in alfalfa transition from vegetative to reproductive development, we conducted transcriptome analysis of the shoot apical tissues (SAs) on day 25 (Figure 1b) and floral buds (FBs) on day 35, which showed round-shaped buds (about 22 mm in diameter) with the inner three whorls of floral organs enclosed in sepals (Figure 1c). Based on the sequencing results, a total of 24 Gb of clean reads were obtained after removing the adaptors, with a Q30 percentage of 92.2 and a GC percentage of 41.43 (Table S1). The clean reads were aligned to the reference genome of *M. sativa* (XinJiangDaYe), which represented 71.46–75.84% of the forage's global transcriptome. A total of 3,409 DEGs accounting for 12.96% of the detectable alfalfa transcripts were identified (Table S2). Hierarchical clustering analysis of the DEGs revealed a distinct expression pattern between SA and FB (Figure 1d) with 962 (28.22%) showing a higher abundance in SA than FB, and 2447 (71.78%) upregulated in FB relative to SA (Figure 1e). Additionally, RT-qPCR validation of 15 randomly selected DEGs showed a significantly positive correlation with the RNA sequencing results ($R^2 > 0.7$), indicating the reliability of our mRNA sequencing data (Figures 1f and S1).

3.2. Genes Related to Reproduction Processes Were Enriched

According to the gene ontology (GO) enrichment analysis, 108 GO terms ($p < 0.05$) were enriched (Table S2). In terms of the biological process (BP), the top 15 processes enriched in our analysis accounted for 11.38% of the DEGs. Among them, seven processes were categorized as plant-reproduction related, including growth phase transition (positive regulation of reproductive process and regulation of timing of transition from vegetative to reproductive phase), and floral organ development (pollen wall assembly, pollen exine formation, gynoecium development, carpel development, and ovule development) (Figure 2a). These DEGs account for 45.58% of the most enriched 15 processes. In the former category, about 79.1% of DEGs were down-regulated (FB vs. SA), i.e., higher expression level in SA on day 25 than in FB on day 35, suggesting a positive role in promoting alfalfa transition to the reproductive stage. In the latter category, about 54.9% were upregulated (FB vs. SA), meaning a higher expression level in FB than in SA (Figure 2a). The results imply that these genes are sequentially turned on and off in flowering.

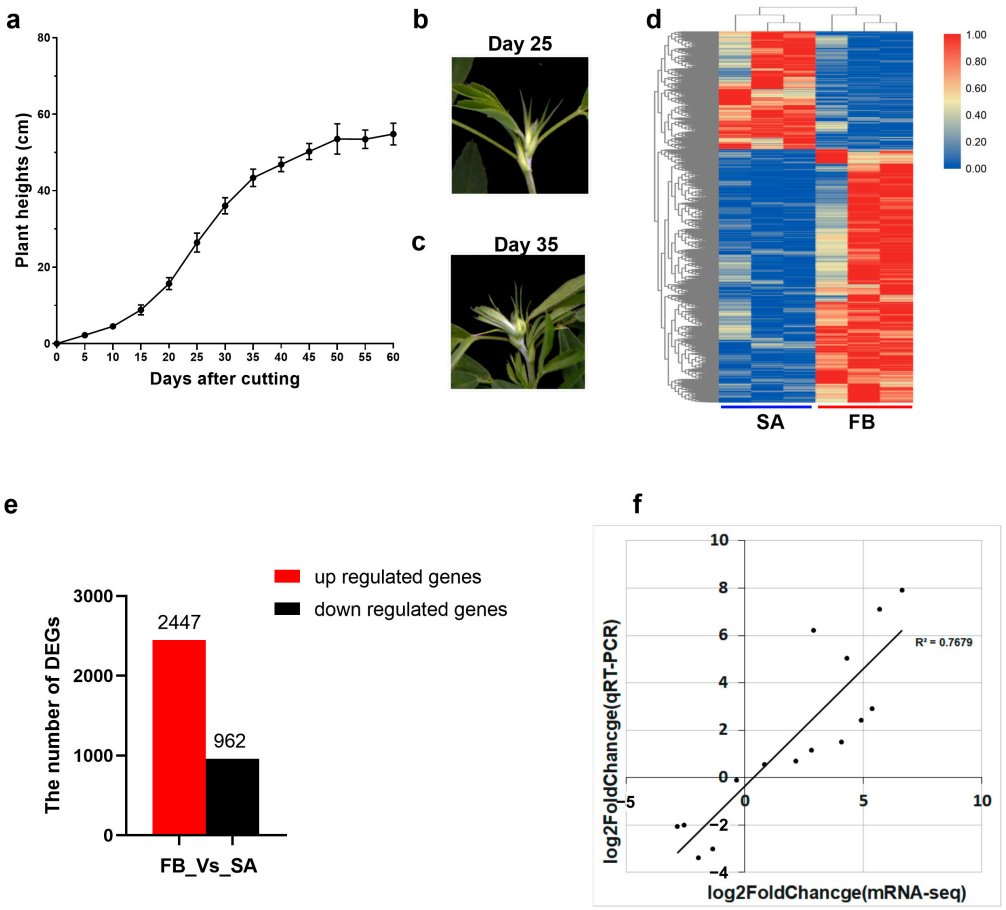


Figure 1. Transcriptome analysis of alfalfa shoot apical tissues on day 25 and the floral buds on day 35. (**a**) The kinetic growth analysis of alfalfa in terms of plant height under the normal conditions. (**b**,**c**) Image of alfalfa shoot apex (SA) and floral bud (FB) on day 25 and day 35, respectively. The tissues were used for mRNA sequencing. (**d**) Heatmap of differential gene expression profiles of SA (day 25) and FB (day 35). The heatmap was constructed using FPKM values and normalized to a range of zero to one. Red represents high FPKM values, and blue for low values. (**e**) DEGs identified in this study with cut off $|\log2Foldchange| > 1$. Red stands for the upregulated genes in FB relative to SA, and blue for the downregulated genes. (**f**) Linear regression analysis between mRNA sequencing data and the expression level test by RT-qPCR of the 15 randomly selected DEGs.

3.3. Components of Multiple Flowering-Regulation Pathways Were Involved in Alfalfa Phase Change

Based on the pathway analysis using Mapman, "transcription factors" (157) and "phytohormone action and signal" (61) were the top two of the 15 most enriched categories, which accounted for 37.26% of DEGs (Figure 2b), and "plant reproduction" (20) ranked 11th in the pathway enrichment. Among the DEGs encoding transcription factors, 17.2% were associated with reproduction-related processes, such as floral organ morphogenesis and ovule development (Figure 2b). For the "phytohormone action and signal" pathway, 41 DEGs were homologs of the known components of gibberellin, cytokinin, auxin metabolism, and signaling pathways (Figure 2b). In addition, genes involved in the sugar metabolism pathway showed a distinctly unique pattern with 73% (24 out of 33) upregulated in FB relative to SA (Figure S2a). For the DEGs homologous to the known circadian rhythm components, 64.3% (9 out of 14) were upregulated, including *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*). As expected, *TIMING OF CAB EXPRESSION 1* (*TOC1*) displayed an opposite pattern of *CCA1* (Figure S2b), which represses *TOC1* transcription by binding to its promoter [37]. Thus, alfalfa flowering is manipulated by a complex network.

3.4. MsAP1 Might Be a Central Regulator of Alfalfa Flowering

The implication of transcription factors (TFs) in plant phase transition has been shown in a variety of species [38–41]. As the harvest time for alfalfa is determined according to its flowering time, we focused on the DEGs encoding TFs. Among the 165 DEGs enriched in plant reproduction-related processes, 157 encoded transcription factors and 21 were associated with floral organ morphogenesis and ovule development (Figure 2c). Based on sequence identity, the enriched transcription factor encoding genes were named after their Arabidopsis orthologs and mapped to the flowering pathway (Figure 3a). Among them, *AP1* and *AP2*, floral homeotic genes specifying floral meristem and sepal identity, showed a significant upregulation in FB. The network interaction analysis revealed that *AP1* connected the floral organ identity genes with flowering time genes including *SVP, SOC1,* and *AGL24* (Figure 3b). Among them, the expression of genes related to flowering time was significantly reduced after flowering, whereas the expression of genes related to floral organs increased, indicating that the development of the initial flower organ inhibited the expression of flowering-related genes. However, *AP2* was predicted to be only associated with a few genes, indicating that its role is not as strong as *AP1* during the flowering process of alfalfa. To determine the expression profiles of *MsAP1*, multiple tissues from two developmental stages (on day 25 and day 35) were tested by RT-qPCR. The results revealed that *MsAP1* on day 35 exhibited high abundance levels in reproductive floral organs, particularly in the sepals, petals, buds, and pistils, while a lower express level was detected in stems and pods. In addition, the signal of *MsAP1* cannot be detected in the leaves and roots during the reproductive period (Figure 3c). At the vegetative stage, *MsAP1* was detected in shoot tips but not in other tissues (Figure 3c).

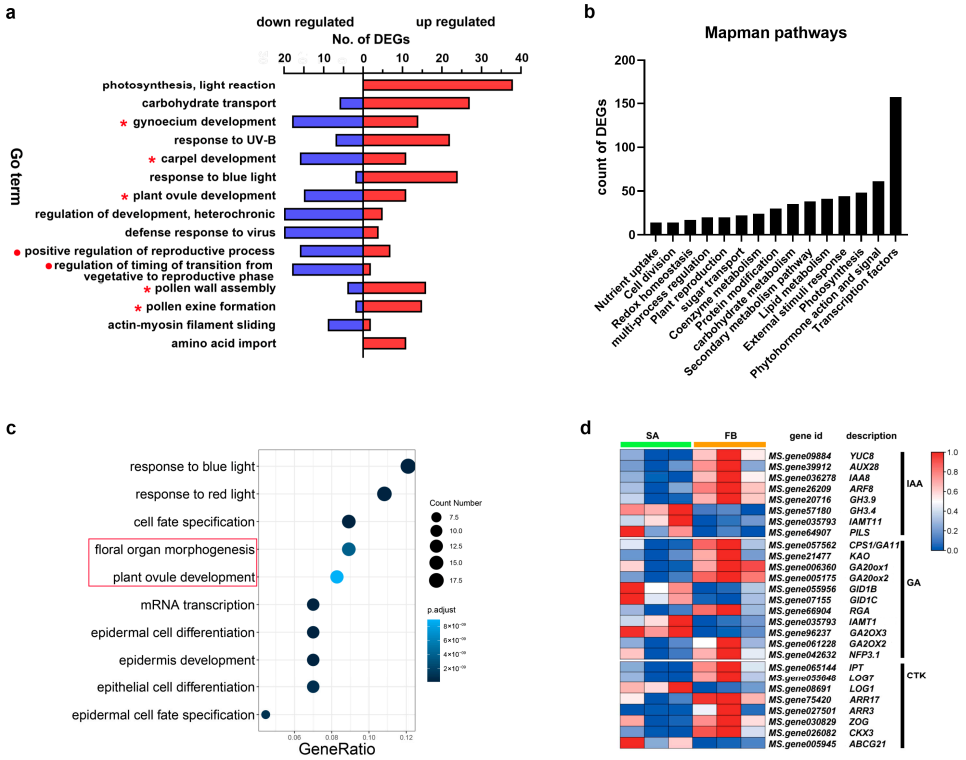


Figure 2. Classification of the DEGs enriched in terms of biological process and pathway. (**a**) Analysis of biological processes of the DEGs. GO terms for growth stage transition are marked with dots, while GO terms for flower organ development are indicated with asterisks. (**b**) Top 15 enriched pathways via Mapman. (**c**) The most enriched GO function of the putative transcription factors. (**d**) Transcript profile of the DEGs involved in phytohormone IAA, GA, and CTK signaling. The scale represents normalized FPKM for the annotated genes via sequence homology. The gradient colors from red to blue denote high and low expression, respectively.

3.5. MsAP1 Shares the Common Features of AP1

To explore the function of *MsAP1* in flower organ development, the full-length coding sequence (CDS) of *MsAP1* (*MS.gene61028*) was cloned from flower buds. *MsAP1* (723 bp) encodes 240 amino acids, and the putative MsAP1 shares a higher sequence identity with its orthologs from legumes, such as *Lotus japonica* (93.4%), soybean (94.8%), and barrel clover (99.1%), relative to the homologs from other select species (Figure S3, Table S4). Phylogenetic analysis demonstrated that *AP1* from angiosperms were grouped into two subgroups: monocots and dicots (Figure S4). Interestingly, blast against the plant kingdom showed that no hit was found in either unicellular green alga *Chlamydomonas reinhardtii* or non-vascular plant *Physcomitrium patens*, indicating *AP1* was angiosperm-specific. Multiple sequence alignment revealed that MsAP1 contained the conserved MADS-box domain (type II) located at the N-terminus (1–60 amino acids), a highly conserved K domain (75–174) for protein interaction [42]. Interestingly, AP1s from dicot species possess an euAP1 motif at the C-terminal region (Figure S3) predicted to play a crucial role in trans-activation [43].

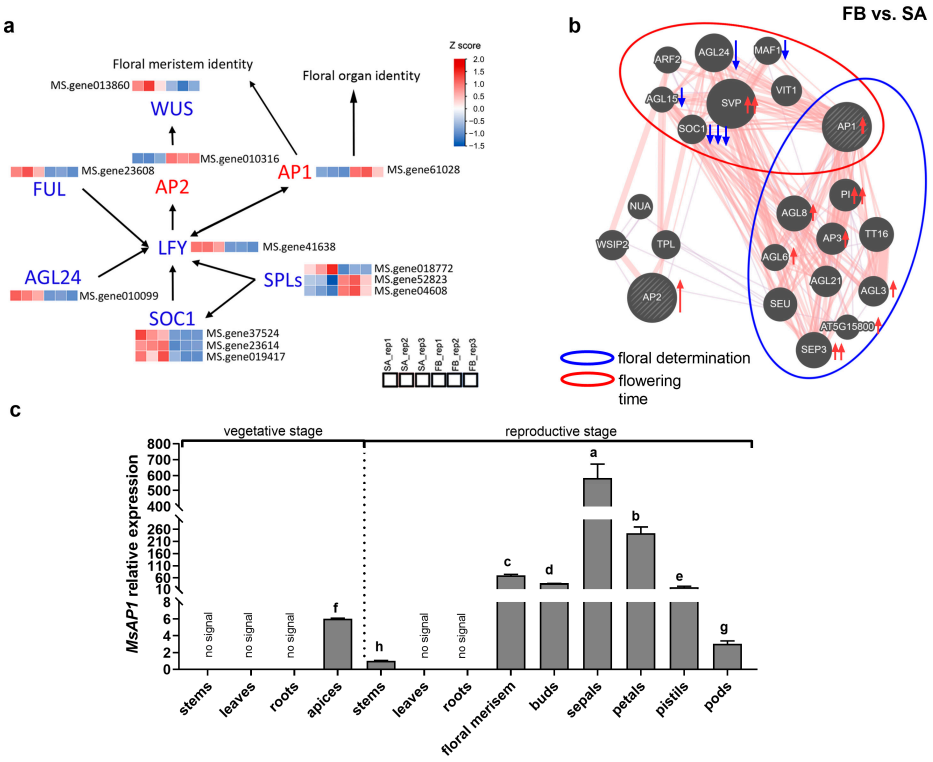


Figure 3. Analysis of floral formation-related TFs. (**a**) Gene expression profiles of DEGs related to the timing of meristematic phase transition. The color scale indicates normalized FPKM changes in gene expression levels in alfalfa shoot apical and flower bud tissue. The gradient colors from red to blue indicate the abundance of gene expression from high to low. The arrow symbol represents the activation relationship. LFY, LEAFY; FUL, FRUITFULL; WUS, WUSCHEL; SOC1, SUPPRESSOR OF CONSTANS OVEREXPRESSION 1; SPL, squamosa promoter-binding-like protein. (**b**) Network analysis of *AP1* and *AP2* and their network genes. Pale purple lines indicate co-expression network and pale red lines indicate physical interaction in Arabidopsis. The red arrow symbol represents upregulated expression of genes in flower buds, while the blue symbol represents downregulated expression. (**c**) Analysis of *MsAP1* expression pattern in different organs in vegetative and reproductive growth phase. Tissue sampling during the vegetative growth stage was performed on the 25th day after harvesting, floral meristem tissues were collected on the 30th day, and stems, leaves, flower buds, and flowers during the flowering stage were collected on day 40. Data represent mean values (with error bars indicating standard deviations from 3 biological replicates), and different letters denote significance levels < 0.01, determined by statistical analysis using one-way ANOVA.

3.6. The Nuclear Protein MsAP1 Has Transcription Activity

To determine the subcellular localization, MsAP1 tagged with GFP (green fluorescent protein) was transiently introduced into tobacco leaves. As shown in Figure 4a, the green signal of MsAP1-GFP was observed predominantly in the nucleus, while the GFP signal of the control vector was detected in both the cytoplasm and nucleus. The results suggest that MsAP1 is a nuclear protein, which is supportive of the prediction of MsAP1 as a transcription factor. We then examined the transcriptional activation activity of MsAP1 using an in vitro expression system in yeast (Figure 4b,c). The assay demonstrated that on the control medium (SC/-Trp), both yeast cells harboring pGBKT7 (BD) or pGBKT7-MsAP1 grew healthily like the positive control (pGBKT7-GAL4AD). In contrast, on histidine-deficient medium (SD/-Trp-His), the cells expressing pGBKT7-MsAP1 but not pGBKT7 (BD) grew in a way similar to the positive control, indicating the induction of the *HIS3* reporter gene by pGBKT7-MsAP1 (Figure 4c). Hence, MsAP1 could activate transcription in vitro. The findings are in agreement with the previous results from *AP1* homologous genes, which function as transcription factors in regulating flower organ formation in several species [24,32,44–46].

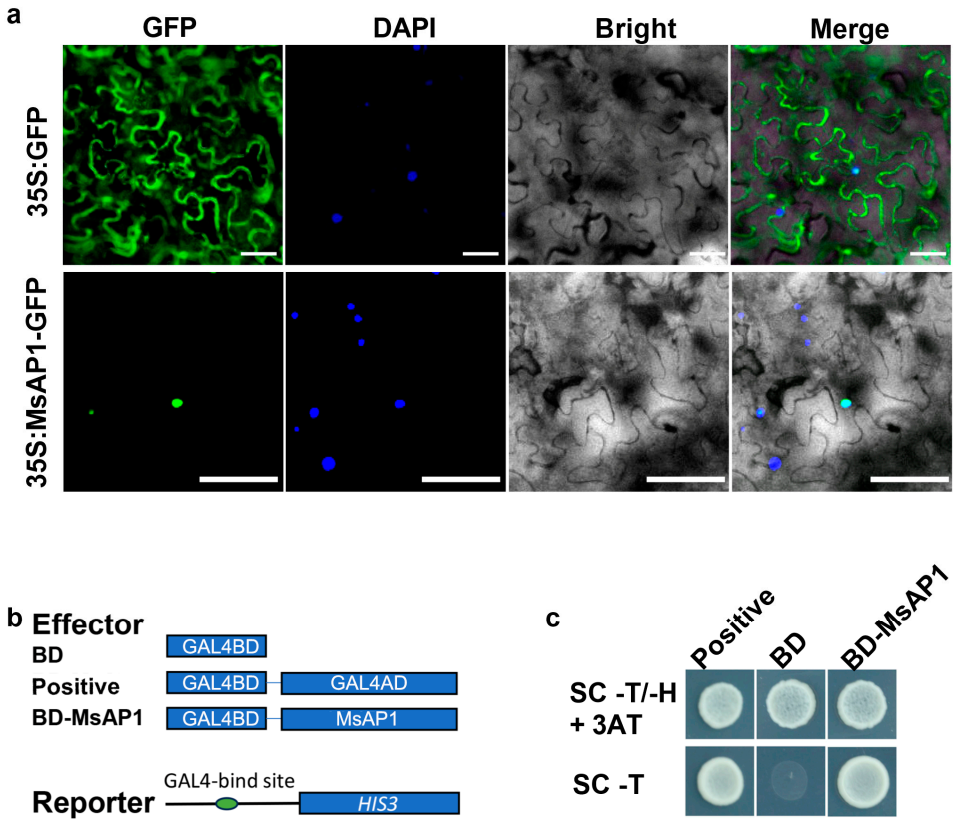


Figure 4. Transcriptional activity assay and subcellular localization investigation of MsAP1. (**a**) The subcellular localization of the MsAP1-GFP fusion protein transiently expressed in tobacco leaves. Images were captured using a confocal microscope. Label the two constructs (control upper and recombinant vector lower panel, respectively); scale bars: 100 μm. Green represents GFP fluorescence signal, and blue dots represent cell nuclei labeled with DAPI (4′,6-diamidino-2-phenylindole). (**b**) Schematic diagram of His reporter gene expression activated by MsAP1 in a yeast cell. GAL4-BD represents the binding domain of GAL4. (**c**) Assay of the transcriptional activation of MsAP1 in yeast (Y2H) cells. Yeast were transfected with pGBKT7-MsAP1 (BD-MsAP1), pGBKT7-GAL4AD (positive control), and pGBKT7 (BD, negative control), respectively. The transformed cells were streaked on SC/-T and selective medium (SC/-T-H + 15 mM 3-AT) to assess growth. SC/-T: synthetic dropout (SC) yeast growth medium lacking tryptophan, SC/-L-H: SC medium lacking tryptophan and histidine, and supply with 15 mM 3-AT.

3.7. Overexpression of MsAP1 in Arabidopsis Promoted Flowering and Altered Floral Structure

To investigate the biological roles of *MsAP1*, the *35S:MsAP1* construct was introduced into Arabidopsis, and two independent overexpression lines (OE1 & OE3) were generated. The transcriptional analysis using RT-qPCR with the *MsAP1*-specific primers showed a significant upregulation of *MsAP1* in the two OE lines compared to the wild-type plants (Figure 5a). Under long-day conditions, *MsAP1*- OE plants exhibited dwarfism with fewer rosette leaves (Figure 5b). In terms of days to bolting, both OE lines bolted on day 13–day 15, which was 7–9 days earlier than Col-0 (Day 22) (Figure 5c). At the emergence of the first flower, the rosette leaf numbers of the OE lines were about 4.8 on average, while the wild-type plants had about 9 rosette leaves at the same stage (Figure 5d). The results indicated that overexpression of *MsAP1* promoted Arabidopsis flowering. Additionally, morphological abnormalities of floral organs were observed in both *MsAP1-OE* lines (Figure 5e). For example, the expansion of the pistil development was observed in OE plants. For inflorescence, overexpression of *MsAP1* transformed the indeterminate pattern of Arabidopsis into a determinate one, and the transgenic plants displayed fused sepals and small pods (Figure 5e,f). Our expression analysis revealed that the floral organ initiation activators, such as *AtAP1*, *AtAGL6*, *AtPI*, and *AtAP3*, were significantly upregulated in the *MsAP1*-overexpression Arabidopsis (Figure 5g). For the key flowering time-regulating genes, a significant increase in *AtLFY* and decrease in *AtSOC1* and *AtTFL* in the shoot apex were detected. These findings suggested that *MsAP1* promoted flowering in Arabidopsis by upregulating *AtLFY*. Taken together, our findings indicated that *MsAP1* played dual roles in floral organ development and flowering time manipulation in Arabidopsis.

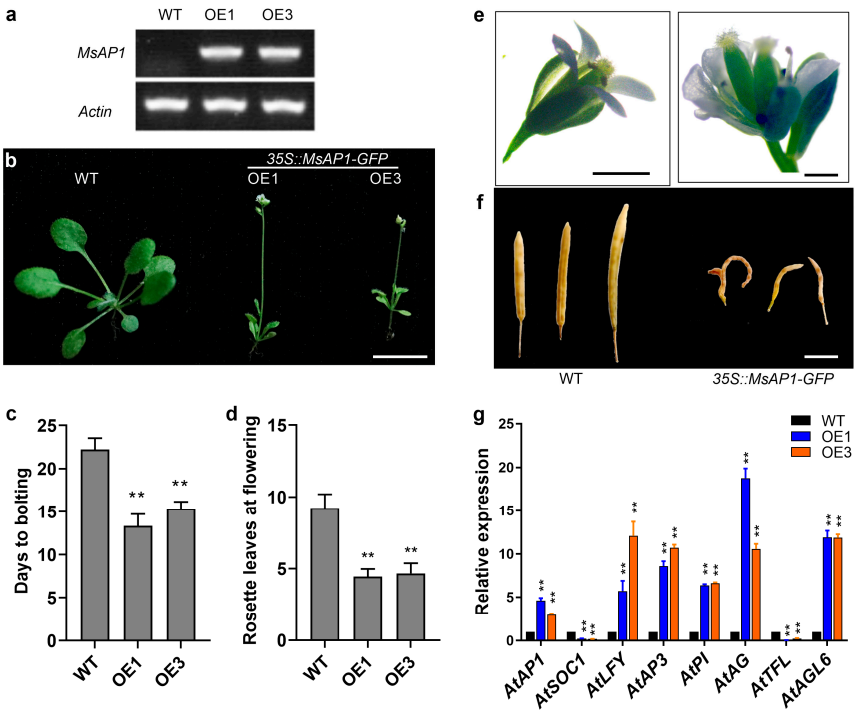


Figure 5. Overexpression of *MsAP1* promoted flowering and altered floral organ morphology in Arabidopsis. (**a**) The relative transcription level of *MsAP1*. (**b**) Image of the homozygous T2 seedlings on day 20 after germination (DAG) under the long-day conditions. WT: Col-0, OE1, and OE3 represented the two independent transgenic Arabidopsis lines (*35S::MsAP1-GFP*). Bar = 2 cm. (**c**) Flowering time analysis in terms of days to bolting under the long-day conditions. (**d**) Analysis of rosette leaf number at the emergence of the first flower under the long-day conditions. (**e**) Phenotypes of the *MsAP1* overexpressing Arabidopsis terminal flowers, bar = 3 mm. (**f**) Phenotype of the fruit of Arabidopsis *thaliana*. Bar = 5 mm. (**g**) Relative transcription levels of the key genes related to Arabidopsis floral transition. Asterisks indicate significant difference at $p < 0.01$ compared with wild type by Student's *t*-test.

4. Discussion:

4.1. Alfalfa Flowering Consists of Multiple Regulatory Pathways

Flowering is a process of morphogenesis involved in complex biological processes regulated by a large number of genes via a complex regulatory network. The well-established flowering regulatory pathways in Arabidopsis include the photoperiod pathway, gibberellin pathway, temperature pathway, autonomous pathway, vernalization pathway, and age pathway [4,5,7,9,47–49]. Plant hormones such as AUX, CTK, JA, and ABA, as well as secondary metabolic processes, are also involved in the transition to flowering in plants [10–12,15,19,50]. Our transcriptomic comparison between the two stages (pre- and post-flower initiation) of alfalfa showed that for the 12.96% differentially expressed genes, 3.66% showed higher abundance at the former stage than the latter, and the remaining 9.3% displayed an opposite pattern, suggesting the sequential turn-on of these genes before and after flower initiation in alfalfa. According to gene ontology, 42.53% of the most enriched 15 processes were associated with plant growth phase transition and floral organ development. Among them, 20.44% are orthologues of the components of sugar metabolism, light response, plant circadian rhythm, and phytohormone (Figures 2d and S2a,b).

4.2. MsAP1 May Serve as a Candidate Gene for Flower Determination and Inflorescence Development

The initiation of floral development in plants begins with the transition from inflorescence meristem to floral meristem, and this transition requires *AP1* [7,32]. Here, transcriptome pathway and network interaction analyses indicate that *MsAP1* expression levels increase during flowering, and co-expression with many floral organ genes suggests that it plays an important role in the transition to flowering in alfalfa shoot apices (Figure 3). Similar in function to the Arabidopsis *AP1* gene, our results also indicate that *MsAP1*, as a transcription factor, alters flowering time and floral organ development in Arabidopsis by regulating the expression of downstream flowering-related genes (Figures 4 and 5). Consistent with previous studies [24,28,45,51–55], these results suggest that *MsAP1* may have functional conservation as a floral initiator in alfalfa, thereby presenting a candidate gene for generating late-flowering varieties through gene editing. Moreover, overexpression of *MsAP1* suppressed inflorescence formation, leading to terminal flower formation (Figure 5e), reminiscent of observations in *tfl* mutants [56,57]. A significant reduction in *AtTFL* gene expression was confirmed in Arabidopsis overexpressing *MsAP1* (Figure 5g). This outcome suggests that *MsAP1* may inhibit inflorescence formation by downregulating *MsTFL* expression in alfalfa, offering a potential strategy to enhance seed production through *MsAP1* mutation.

4.3. MsAP1 May Be Related to Alfalfa Pistil and Fruit Development

AP1-like genes from various species are functionally characterized to induce early flowering in plants, yet their phenotypic effects on floral organs vary across species. The *AP1* gene is known to specifically regulate the development of outer whorl sepals and petals in Arabidopsis [58,59]. *MtPIM* is a functional homolog of *AP1* in *Medicago truncatula*, with mutant-altered sepals transforming into leaves and some stamen developing into petaloid extensions [22,60]. Expression of an *AP1*-like gene from apple in Arabidopsis caused abnormal floral organ development and reduced fertility [61]. Here, our research shows that the expression level of the *MsAP1* gene in alfalfa sepals and petals is significantly higher than in other tissues (Figure 3c). Overexpression of *MsAP1* induces irregularly curved petals and axillary leaves resembling sepals in Arabidopsis *thaliana* (Figure 5e). This indicates that *MsAP1* may regulate the development of sepals and petals in alfalfa. Moreover, the *MsAP1* gene was also detected to be significantly expressed in the pistil and pod. Overexpression of *MsAP1* also results in pistil enlargement in Arabidopsis similar to the role of *AG* in promoting pistil development in Arabidopsis [62]. Overexpression of *MsAP1* in Arabidopsis significantly upregulates the expression of the *AG* gene, which specifically regulates pistil development, thereby supporting the observed phenotype (Figure 5g).

In addition, the shortened and bowed pods of transgenic Arabidopsis, accompanied by a decrease in seed quantity (Figure 5f), suggest that the *MsAP1* may play a key role in the development of alfalfa pods. Based on these new phenotypes, we speculate that the function of *MsAP1* may not be limited to sepals and petals, but also includes reproductive organs and pods. However, further transgenic experiments on alfalfa are needed to confirm this speculation.

5. Conclusions

In summary, this study conducted a transcriptomic analysis of the process by which the apical meristem transitions to floral buds in alfalfa. The flowering transition was accompanied by an increase in photosynthetic activity and sugar metabolism. Plant rhythms and hormones may play important roles in regulating this transition. Additionally, differential transcription factors associated with flowering pathways were identified in the apical meristem, with *MsAP1* being significantly upregulated as a central gene involved in integrating flowering. Further functional and mechanistic analyses revealed that *MsAP1* can regulate both flowering time and floral organ development in Arabidopsis by modulating key flowering genes. Interestingly, for the first time, we discovered that *MsAP1* is involved in regulating pistil and pod development when overexpressed in Arabidopsis, which further enriches the functional repertoire of the *AP1* gene family. However, the function of *MsAP1* in alfalfa requires further investigation.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy14081741/s1, Figure S1. qRT-PCR validation of DEGs in this study. Figure S2. The transcript profiles of "sugar metabolism". Figure S3. alignment of the deduced amino acid sequence of MsAP1 and the AP1 orthologues from the indicated species. Figure S4. Phylogenetic analysis of AP1 proteins in the representative monocot and dicot species. Table S1. The results of sequencing from the individual sample. Table S2. Differential genes identified in this study. Table S3. Significant enrichment of GO terms between FB and SA. Table S4. Sequence homology of the putative AP1s in the indicated species. Table S5. Primers used in this study.

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