Cloning and Sequence Analysis of SVP and AGL24 Genes in Chrysanthemum

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Abstract The SVP (SHORT VEGETATIVE PHASE) AGL24 (AGAMOUS-LIKE 24) gene is a member of the MADS-box family and plays an important role in the transformation of plants from vegetative to reproductive growth. In order to understand the mechanism of flowering transformation in Chrysanthemum morifolium, two SVP/AGL24 homologous genes named CmSVP1 and CmAGL24 were cloned from C. morifolium 'linbudiao' using RT-PCR. Biological information indicated that the coding regions of CmSVP1 and CmAGL24 were 678 bp and 468 bp in length, encoding 225 and 154 amino acids respectively. The subcellular localization prediction of CmSVP1 and CmAGL24 proteins were both in the nucleus. They were hydrophilic proteins, and the α-helix was the largest structural component in the protein secondary structure. The analysis of transmembrane regions suggested that these proteins may not have transmembrane regions. Further proteins homology comparisons showed that the CmSVP1 and TcSVP were clustered into one group, while CmAGL24 was recently related to AaAGL24. This study provides a theoretical basis for analyzing the molecular mechanism of chrysanthemum flowering regulation and improving flowering by molecular means.

Keywords Chrysanthemum morifolium; SVP/AGL24 gene; Clone; Biological information analysis

The transition of meristem from vegetative to reproductive growth is an important developmental switch in the life cycle of higher plants. It is regulated by internal and external factors. These factors form a set of complex gene regulatory network in the long-term evolution and development of plants, which makes plants bloom at the best time. In recent years, a large number of studies on the floral regulation mechanism of model plants Arabidopsis and rice have found that there are six floral pathways: vernalization pathway, photoperiod pathway, thermosensitive pathway, gibberellin pathway, autonomous pathway and age pathway. In addition, FT (FLOWING LOCUS T), SOC1 (SUPPLIER OF OVER-EXPRESSION OF CONSTANTS 1) and many other key factors were isolated and identified (Blümel et al., 2015). The inhibitors of flowering also play an important role in the induction of flowering, and the silencing of the inhibitors will also accelerate the flowering. Among them, SVP gene is an important inhibitor, which is the core regulatory factor in the whole floral regulatory network. Therefore, the research on SVP gene can accelerate the improvement of flowering period of ornamental plants.

SVP gene is an important negative regulator of flowering in the STMADS11 subfamily of MADS box family. It belongs to type II MADS box protein, which has a typical MICK domain, and is highly conserved in the evolution. AGL24, another STMADS11 subfamily gene, is highly similar to SVP, but their functions are quite opposite. AGL24 is a flowering promoting factor (Michaels et al., 2003). SVP homologous genes are widely found in various plants, include many ornamental plants, and their functions are differentiated. Including kiwifruit (Wu et al., 2014), Prunus mume (Li et al., 2017), Phyllostachys violacens (Liu et al., 2016), Chrysanthemum morifolium (Gao et al., 2017), Pharbitis nil (Kikuchi et al., 2008), Paeonia suffruticosa (Wang et al., 2014), Narcissus tazetta (Li et al., 2015), Malus × domestica (Wang et al., 2019), Osmanthus fragrans (Zhu et al., 2019), eudicots (Liu et al., 2018), etc.

As a traditional Chinese flower, Chrysanthemum has a high ornamental value. Most chrysanthemum varieties are affected by short-light, with short florescence and relative concentration. Compared with other chrysanthemum varieties, day-neutral chrysanthemum with a longer flowering duration is not affected by photoperiod. It is an
ideal plant to study the mechanism of chrysanthemum flowering (Gao et al., 2019). The floral transition of
chrysanthemum is an important part of the study on the mechanism of day-neutral chrysanthemum. The results of
transcriptome sequencing (NCBI No. srp109613) showed that the expression of CmSVP and its homologous genes
was significantly different in different photoperiods. FT gene family is an important gene regulating flowering
day-neutral plants, while SVP gene inhibits flowering by down regulating FT expression (chaurasia et al., 2017;
Chen et al., 2017). At present, there are FT, CO, LFY and other genes related to flowering stage in chrysanth-
emum. These genes are all positive to regulate flowering stage and promote flower bud differentiation, but few are
negative to regulate flowering stage. If we identify and analyze the key genes of negative regulation to inhibit
flowering transformation, we can use CRISPR/Cas9 technology with simple operation and low cost, combined
with genetic transformation technology to knock out the important negative regulation factors of chrysanthemum
flowering, which is of great significance to cultivate early flowering varieties and continuous flowering varieties.

1 Results and Analysis
1.1 Cloning of SVP and AGL24 genes from Chrysanthemum morifolium
Two specific bands of about 700 bp and 500 bp were cloned from C. morifolium ‘Jinbudiao’ using RT-PCR
method (Figure 1A; Figure 1B). The two sequences showed high similarity to the SVP and AGL24 gene of various
plants using BLAST, and named CmSVP1 and CmAGL24 respectively. The ORF of CmSVP1 was 678 bp,
encoding 225 amino acids (Figure 2A), and that of CmAGL24 was 465 bp, encoding 154 amino acids (Figure 2B).
The CmSVP1 gene has the highest similarity to the SVP in Cynara cardunculus (83.16%), and 79.21% to 81.67%
similarity to the SVP gene in Helianthus annuus and Lactuca sativa. The CmAGL24 gene was 77.62% similar to
JOINTLESS-like gene in Cucurbita maxima. Further comparison with BlastP showed that CmAGL24 was 93.84%
and 83.1 similar to the AGL24 protein in Artemisia annua and Helianthus. These results indicated that the two
genes belonged to the SVP/AGL24 family.

1.2 Analysis of basic physical and chemical properties of CmSVP1 and CmAGL24
The physical and chemical properties of the CmSVP1 and CmAGL24 genes of chrysanthemum were analyzed
using ExPASy online analysis software. The results showed that the relative molecular weights of CmSVP1 and
CmAGL24 protein were 25.787 kD and 17.557 kD, and the theoretical isoelectric points were 9.54 and 9.26,
respectively. In the analysis of polar amino acids, the total number of negatively charged amino acid residues
(Asp+Glu) of CmSVP1 was 27, and the total number of positively charged amino acid residues (Arg+Lys) was 36,
with more positively charged than negatively charged. CmAGL24 has a total of 21 negatively charged amino acid
residues (Asp+Glu) and a total of 27 positively charged amino acid residues (Arg+Lys), with more positively
charged than negatively charged. The instability coefficients of both proteins are shown as unstable proteins.

1.3 Analysis of hydrophilicity and subcellular localization of CmSVP1 and CmAGL24 protein
The subcellular localization of CmSVP1 and CmAGL24 protein was predicted online using WoLF PSORT. The
results showed that the score of CmSVP1 in nucleus was 13, that of CmAGL24 in nucleus was 8, and the
cytoplasmic score was 2. It can be seen that CmSVP1 and CmAGL24 proteins were located at nucleus.

Figure 1 PCR products of CmSVP1 and CmAGL24
Note: A: 1~4: CmSVP1 gene; B: 1~6: CmAGL24 gene; M: DL2000 DNA marker
Figure 2 The sequences and deduced amino acid sequences of CmSVP1 and CmAGL24

Note: A: CmSVP1 gene; B: CmAGL24 gene

The ProtScale online software was used to predict the hydrophobicity of the protein. The larger the positive value stands for the more hydrophobic, and the larger the negative value means the more hydrophilic. The average hydrophilicity coefficient (CRAVY) of CmSVP1 and CmAGL24 proteins were -0.593 and -0.335, which indicated that these proteins have a certain degree of hydrophilicity.

1.4 Analysis of secondary structure and protein transmembrane of CmSVP1 and CmAGL24

The analysis of the secondary structure of CmSVP1 and CmAGL24 protein was used SOPMA online tools. The results showed that the secondary structure of the two proteins mainly consisted of 4 forms: α-helix (Alpha helix), β-turn (β-turn), extended chain (Extended strand) and random coil (Random coil) (Table 1). Among them, the α-helix was the most structural element in the secondary structure of these two proteins, and the remaining elements were scattered in the protein.

The TMH-MM Server2.0 tool was used to predict the transmembrane region of the two proteins. The results indicated that neither protein had a transmembrane helix region, but the CmSVP1 protein acted outside the membrane and CmAGL24 acted inside the membrane.

Table 1 Bioinformation analysis of secondary structure of CmSVP1 and CmAGL24

| Protein | Alpha helix (%) | Beta turn (%) | Extended strand (%) | Random coil (%) |
|---------|----------------|---------------|---------------------|-----------------|
| CmSVP1  | 59.11          | 3.56          | 9.78                | 27.56           |
| CmAGL24 | 62.58          | 5.16          | 14.84               | 17.42           |

1.5 Homology and phylogenetic analysis of CmSVP1 and CmAGL24

The homology of CmSVP1, CmAGL24 and other SVP/AGL24 proteins from different plants with high similarity were compared using Clustalw. The results showed that both proteins had a strongly conserved MADS-box region and a K-box region, which belonged to Type II type MADS-box protein (Figure 3).
The phylogenetic tree of CmSVP1, CmAGL24 and SVP/AGL24 homologous proteins of 13 other plants was constructed using MEGA6.0. The results showed that CmSVP1 was clustered with SVP proteins of many other species. CmSVP1 had the closest evolutionary relationship with TcSVP, and the close relationship between the SVP family members of herbs and woody plants was close, indicating that SVP genes were highly conservative in plants. It was worth noting that the CmSVP1 gene has a long homology with another CmSVP in chrysanthemum, and may be functionally differentiated. CmAGL24 and AaAGL24 were grouped together, and were closely related to other species AGL24 (Figure 4). Although the CmSVP1 gene has a high similarity with the CmAGL24 gene, the evolutionary tree showed that the two genes were grouped into different groups, indicating that they were far from each other with different functions.

Figure 3 Multiple alignment analysis of SVP and AGL24 protein
Note: The box indicates the MADs-box conservative region, and the straight line indicates the K-box conservative region.
2 Discussion

MADs-box family genes which are divided into Type I and Type II are an important class of eukaryotic transcriptional regulators. The Type II genes are composed of four domains with different degrees of conservation: MADs-box, I, K, and C domains, so they are also known as MIKC-type MADs-box genes that have been the most widely studied in recent years (Pačeniová et al., 2003). In flowering plants, the MADs-box family genes play an important role, and the MIKC-type gene mainly regulates floral organ development and flowering time. The *SVP* gene is an important member of the MIKC-type gene family and has been verified to participate in flowering regulation, flower development, dormancy and other processes in various plants (Brill and Watson, 2004; Lee et al., 2007; Jaudal et al., 2014; Yamagishi et al., 2016). *SVP* and *AGL24* have the closest genetic relationship and the highest homology, but they have opposite roles in regulating flowering. However, not all *SVP* genes have a negative regulatory effect, and some also promote flowering. The *SVP* homologous genes in *Phyllostachys violascens* and soybean promote flowering (Liu Shinan, 2016; Zhang et al., 2016), and some *SVP* genes are not involved in the regulation of flowering time, such as ryegrass *LpMADS10*, alfalfa *MtSVP1* (Petersen et al., 2006; Jaudal et al., 2014). Therefore, isolating *SVP* homologous genes play an important role in elucidating the regulation of chrysanthemum flowering.

In this study, two *SVP* homologous genes named *CmSVP1* and *CmAGL24* were isolated in chrysanthemum by RT-PCR method. The physical and chemical properties, hydrophobicity, transmembranecity, subcellular localization and protein secondary structure of *CmSVP1* and *CmAGL24* were analyzed using a series of bioinformatics methods. The results showed that both genes belonged to the MADs-box gene of the MIKC type and have highly conserved MADs-box and K-box domains. The same hydrophobicity, transmembrane domain, and subcellular localization of two genes also verified their homology. Phylogenetic trees further verify that *CmSVP1* and *CmAGL24* were grouped into different groups and may have different functions. The *SVP* gene participates in plant autonomous pathways, temperature-sensitive pathways, GA pathways, photoperiod pathways and vernalization pathways, and promotes flowering by inhibiting the expression of flowering promoting factors *FT* and *SOC1* genes (Chen Jinwen et al., 2017). *SVP* and *AGL24* genes can interact with each other, through the formation of complexes with *AP1*, *LUG* and *SEU* to down-regulate the expression of promoting factors, thereby inhibiting the floral transformation. Therefore, based on the basis of this research, the function and mode of action
of CmSVP1 and CmAGL24 would be further clarified. It will lay a foundation for the study of the chrysanthemum flowering mechanism.

3 Materials and Methods

3.1 Materials

The experiment used the typical day-neutral C. morifolium 'jinbudiao' (the natural flowering period lasts from May to November) as the research material. They were planted in the greenhouse of Beijing Forestry University, and when they grew to 7 true leaves, the stem tips were taken and immediately placed Quick-frozen in liquid nitrogen and stored in the refrigerator at -80°C. The RNA extraction kit was purchased from Beijing Huayueyang Biological Company, the PCR high-fidelity enzyme, cDNA synthesis kit, pEASY-Blunt vector, and EH5α were all purchased from TransGen Biotech.

3.2 Cloning of two SVP homologous genes in Chrysanthemum

Using RNA (polysaccharide polyphenol plant total RNA) extraction kit, the preserved stem tips were extracted from the plant total RNA according to the operating instructions.

The RNA was used 0.8% agarose gel electrophoresis and NanoDrop 2000 ultra-micro ultraviolet-visible spectrophotometer to detect the concentration, purity and integrity. The first strand of cDNA was synthesized using a cDNA synthesis kit and stored at -20°C for gene cloning.

Based on the transcriptome data obtained earlier (NCBI accession number SRP109613), specific primers for CmSVP1 and CmAGL24 genes were designed (Table 2), and PCR amplification was performed according to the following system: 1 μL cDNA template, 1 μL upstream primer CmSVP1-F (CmAGL24-F) and downstream primers CmSVP1-R (CmAGL24-R) (10 μmol/L), 12.5 μL PCR MIX (high fidelity), ddH2O make up to 25 μL. The reaction program was 94°C for 5 min, 94°C for 30 s, 58°C for 1 min, 72°C for 30 s, 35 cycles, 72°C for 10 min, and then stored at 4°C.

The gel of the target band after electrophoresis detection was recovered and purified. The recovered DNA was connected to pEASY-Blunt vector and transformed into EH5α, and 6 monoclonals were picked and sent to BGI for sequencing.

3.3 Analysis of biological information of gene sequence and protein

The sequence after sequencing was compared by Blast program on NCBI website. ORF finder was used to predict the ORF and deduced amino acid sequence of CmSVP1 and CmAGL24 gene. The physical and chemical properties of proteins were analyzed using ExPASy online analysis software (http://web.expasy.org/protparam/). The subcellular localization of protein was predicted online using WoLF PSORT (https://www.genscript.com/wolf-psort.html). The protein hydrophilicity and hydrophobicity prediction were used ProtScale online software (https://web.expasy.org/protscale/). Protein secondary structure analysis was used SOPMA online tools (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_sopma.html). The protein transmembrane region prediction was used TMH-MM Server 2.0 tool (http://www.cbs.dtu.dk/services/TMHMM/). The SVP/AGL24 homologous protein sequences of multiple species were downloaded from GenBank (http://www.ncbi.nlm.nih.gov/), and used ClustalW to perform multi-sequence homologous alignment of proteins between different plant species. Simultaneously, the conserved sites of CmSVP1/CmAGL24 protein of chrysanthemum were analyzed. Phylogenetic trees of SVP/AGL24 proteins of different plants were constructed using MEGA6.0 software.

| Primer name | Primer sequence (5’-3’) |
|-------------|------------------------|
| CmSVP1-F    | GCTAGATGAGGATGGGTAGACAGAA |
| CmSVP1-R    | TTCTACCCAGTGTTAGACATGTG |
| CmAGL24-F   | ATCTATATATGGCGAGGAAAAAT |
| CmAGL24-R   | ACTTCAAGAAGTTTCAATTTACAC |
Authors’ contributions
Gao Yaohui is the experimental designer and the experimenter. She completed the data analysis and the writing of the first draft of the manuscript; Xiao Fengjie and Zhang Xiaomin participated in the analysis of the experimental results; Wei Guangpu and Ma Bin completed the modification of the manuscript. All authors read and agreed to the final manuscript.

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