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# An *APETALA1*-like gene of soybean regulates flowering time and specifies floral organs

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ABSTRACT

MADS-box proteins are key transcription factors involved in plant reproductive development. *APETALA1* (*AP1*) in *Arabidopsis* is a MIKC-type MADS-box gene and plays important roles in flower development. In this research, we isolated and characterized *GmAP1*, which encoded an AP1-like protein in soybean. *GmAP1* contained eight exons and seven introns and was specifically expressed in the flower, especially in the sepal and petal. GmAP1 was a nucleus-localized transcription factor and displayed transactivation activity. It caused early flowering and alteration of floral organs when ectopically expressed in tobacco. To our knowledge, this is the first report characterizing an *AP1*-like gene from soybean.

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## Introduction

Vegetative and floral developments are the key developmental processes in flowering plants that determine the overall structure of plants. The transition between the two phases is controlled by complicated regulatory pathways that respond to different environmental and endogenous signals. In the model species *Arabidopsis thaliana*, many genes have been identified that are responsible for the transition to flowering. It was reported that several genes, namely *GIGANTEA* (*GI*), *CONSTANS* (*CO*) and *FLOWER LOCUS T* (*FT*), were circadian-regulated and promoted flowering; *FLOWERING LOCUS C* (*FLC*) is a repressor of flowering and is negatively regulated by vernalization (Li et al., 2008).

Most angiosperm flowers are composed of four types of organs arranged in concentric whorls: sepals, petals, stamens and carpels. The identities of the floral organs are specified by groups of genes. Based on analysis of homeotic floral mutants in *Arabidopsis*, *Petunia hybrida* and *Antirrhinum majus*, the ABCDE model was proposed to demonstrate how five classes of genes work together to specify floral organ identities (Theissen and Saedler, 2001). A (*APETALA1*, *AP1*; *APETALA2*, *AP2*) alone determines sepals; A and B (*APETALA3*, *AP3*; *PISTILLATA*, *PI*) together specify petals; B and C (*AGAMOUS*, *AG*) specify stamens; C alone determines carpels; D (*SHATTERPROOF1/2*, *SHP1/2*) specifies the ovule; and E class genes (*SEPALLATA1/2/3/4*, *SEP1/2/3/4*) determine the identities of all four whorls of floral organs. Except for *AP2*, all of these genes encode proteins with high similarities and belong to the MIKC-type MADS-box gene family.

MADS-box transcription factors regulate a diverse range of developmental procedures in eukaryotes. MIKC-type MADS-box genes belong to the group only found in plants and were named after four conserved domains that exist in those proteins: MADS domain, Intervening (I) domain, Keratin (K) domain and C-terminal domain (Gramzow and Theissen, 2010). The MADS domain is highly conserved and is a DNA-binding domain that binds to consensus sequences known as CArG box [$CC(A/T)_6GG$]. The I domain is more diverse in sequence and structure, while the K domain is conserved and characterized by a coiled-coil structure. These two domains are involved in protein–protein interactions. The C-terminal domain is the least conserved and likely to be involved in transactivation. MIKC-type genes are well characterized and are recognized to have roles in plant development and signal transduction.

AP1 is a MIKC-type MADS-box factor. It appears to establish floral meristem identity by regulation of genes that are related to phase transition and flower initiation (Mandel et al., 1992; Kaufmann et al., 2010). *AP1* belongs to the A class in the ABCDE model, and also orchestrates the formation of floral primordia by regulation of genes involved in organ growth and patterning (Kaufmann et al., 2010). Mutation in *AP1* of *Arabidopsis* causes partial flower transformation into inflorescence shoots with alterations in sepal and petal identities (Bowman et al., 1993). In contrast, constitutive expression of *AP1* or its orthologues in

*Abbreviations:* 3-AT, 3-amino-triazole; DAF, days after flowering; EST, expressed sequence tag; gDNA, genomic DNA; ORF, open reading frame; RT-PCR, reverse transcriptase polymerase chain reaction; SAM, shoot apical meristem.

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*Arabidopsis* usually cause early flowering and conversion of inflorescence to the terminal flower (Mandel and Yanofsky, 1995). If controlling expression of *AP1*-like genes is able to control flowering time and alter floral organ identity in other species, it might produce valuable phenotypic changes that would be used for crop improvement.

Soybean is one of the most economically important crops, as it is a major source of vegetable oil and protein. Reproductive growth directly affects seed yield and quality. Therefore, to unravel the genetic mechanisms underlying the regulation of flower development is of great significance to improve soybean production. In this study, we isolated an *AP1*-like gene, *GmAP1*, from soybean and characterized its expression pattern. As a transcription activator, GmAP1 promoted flowering time and determined floral organ formation when overexpressed in tobacco.

## Materials and methods

### *Plant materials and growth conditions*

Seeds of soybean (*Glycine max* (L.) Merr. cv. Jackson) were provided by the National Center for Soybean Improvement, China. The seeds were germinated and grown in the experimental field of Nanjing Agricultural University, Nanjing, China.

Leaves, roots and stems were collected at the third euphyll expanding stage. Seeds and pods were harvested at 25 days after flowering (DAF). Flowers at different developmental stages were collected. Four types of floral organs, namely sepals, petals, stamens and carpels, were collected from mature flowers. All samples were frozen in liquid nitrogen and stored at −80 °C until analysis.

Tobacco (*Nicotiana tabacum* cv. SamSun), both transgenic and wild-type plants, were grown in a growth room at 25 °C under a 16 h light/8 h dark photoperiod.

### *RNA isolation and first-strand cDNA synthesis*

Total RNA was isolated using an RNA Plant Extraction Kit (TIANGEN, China) and treated with RNase-free DNase I (Takara, Japan) to remove contamination by genomic DNA (gDNA). About 2 μg of purified total RNA was reverse-transcribed by AMV reverse transcriptase (Takara) using oligo$(dT)_{18}$ as primer (Takara) following the manufacturer's instructions.

### *Full-length cDNA cloning of GmAP1*

The full-length cDNA of *GmAP1* was amplified from flower cDNA with the following primers (sense: 5′-ATGGGAAGGGGTAGGGTT-3′; anti-sense: 5′-TGTCAAATGCCATACCAAAGC-3′). Similarly, the full-length gDNA of *GmAP1* was amplified from leaves. All PCR products were gel-purified (Axygen, USA) and cloned into the pGEM®-T easy vector (Promega, USA) followed by sequencing (Invitrogen, China).

### *Sequence and phylogenetic analysis*

Conserved domains were searched with SMART (http://smart.embl-heidelberg.de/). Sequences of published MADS-box genes were obtained from the NCBI databases (http://www.ncbi.nlm.nih.gov/). Multiple alignment of the sequences was conducted with Clustal X 2.0 and viewed with GeneDOC. A phylogenetic tree was constructed by MEGA 4 using the neighbor-joining method.

### *DNA gel blot analysis*

Genomic DNA was extracted from leaves using the CTAB method and digested with *Eco*RI or *Xba*I (Takara) at 37 °C for 16 h. The digested DNA was separated on a 0.8% (w/v) agarose gel and transferred onto a Hybond $N^+$ nylon membrane (Roche, Germany) by capillary transfer (Reed and Mann, 1985). A fragment encoding the C-terminus of GmAP1 was amplified and used as a probe. The probe-labeling, hybridization and detection procedures were performed using the DIG High Prime DNA Labeling and Detection Starter Kit II (Roche, Germany) according to the manufacturer's instructions.

### *Gene expression analysis*

Real-time PCR was carried out with the iQ5 real-time PCR system (BIO-RAD, USA) using the SYBR® Green Real-time PCR Master Mix (Toyobo, Japan). Relative expression levels were normalized using *tubulin* (GenBank accession no. AY907703) as an internal control. Primers used were as follows: TubF (5′-GGAGTTCACAGAGGCAGAG-3′) and TubR (5′-CACTTACGCATCACATAGCA-3′) for *tubulin*, GmAP1F (5′-ATGATTCCGAGTCACAGGGAA-3′) and GmAP1R (5′-TGTCAAATGCCATACCAAAGC-3′) for *GmAP1*. The PCR amplification protocol was 95 °C for 1 min, followed by 45 cycles at 95 °C for 15 s, 60 °C for 15 s and at 72 °C for 45 s, with a final extension at 72 °C for 10 min. The relative expression of *GmAP1* was calculated according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The threshold cycle (Ct) values were the means of two replicate independent PCRs.

RNA *in situ* hybridization was performed on longitudinal sections of the soybean apical inflorescence as previously described (Coen et al., 1990). RNA antisense and sense probes were generated from a 300 bp fragment in the 3′ region of the *GmAP1* cDNA, and were labeled with digoxigenin.

### *Subcellular localization of the GmAP1–GFP fusion protein*

The WoLF PSORT Prediction (http://wolfpsort.org) was employed to predict the subcellular localization of GmAP1. The *GmAP1* open reading frame (ORF) without the stop codon was inserted into the *BamH*I–*Xba*I sites of pBI121-GFP vector (Clontech, USA), resulting in translational GFP fusion at the C-terminus of GmAP1. This construct was transferred into onion epidermal cells by *Agrobacterium tumefaciens* strain EHA105 (Sun et al., 2007). Cells harboring the empty pBI121-GFP vector (35S:GFP) were used as a control. The GFP signals were monitored under a confocal spectral microscope (Leica CP SP2, Germany).

### *Transcriptional activities assay*

The GAL4 DNA-binding domain vector, pBD-GAL4 Cam (Stratagene, USA), was used for transcriptional activities analysis. The entire coding region of *GmAP1* was inserted into the *Eco*RI–*Sal*I sites of the vector. This construct was introduced into the yeast YRG-2 strain, which contained the *His*3 reporter gene in the genome. The transactivation activities were evaluated according to the growth status of these cells on the synthetic defined medium lacking histidine (SD/−His + 10 mM 3-AT). The pBD-GAL4 Cam empty vector (pBD-GAL4) and pGAL4 (Stratagene, USA) were transformed into yeast cells, and the transformants were streaked on plates, as negative and positive controls, respectively.

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