1 **Potential function of *CbuSPL* and gene encoding its interacting protein during flowering in**

2 ***Catalpa bungei***

3 Zhi Wang¹, Tianqing Zhu¹, Erqin Fan^{1,3}, Nan Lu¹, Fangqun Ouyang¹, Nan Wang¹, Guijuan Yang¹,
4 Lisheng Kong², Guanzheng Qu³, Shougong Zhang¹, Wenjun Ma^{1*}, and Junhui Wang^{1*}

5 ¹State Key Laboratory of Tree Genetics and Breeding, Key Laboratory of Tree Breeding and
6 Cultivation of State Forestry Administration, Research Institute of Forestry, Chinese Academy of
7 Forestry, Beijing 100091, PR China.

8 ²Department of Biology Centre for Forest Biology, University of Victoria, Victoria 11 BC,
9 Canada.

10 ³State Key Laboratory of Tree Genetics and Breeding (Northeast Forestry University), 26 Hexing
11 Road, Harbin 150040, PR China.

12 **Running title: *SPL* during *C. bungei* flowering**

13 * To whom correspondence should be addressed. Tel. +086-01062888539.; +086-01062888864.

14 Email: wangjh808@sina.com (J.H.W); mwjlx.163@163.com (W.J.M)

Abstract

“Bairihua”, a variety of the *Catalpa bungei*, has a large amount of flowers and a long flowering period which make it an excellent material for flowering researches in trees. SPL is one of the hub genes that regulate both flowering transition and development. Here, a SPL homologues *CbuSPL9* was cloned using degenerate primers with RACE. Expression studies during flowering transition in Bairihua and ectopic expression in *Arabidopsis* showed that *CbuSPL9* was functional similarly with its *Arabidopsis* homologues. In the next step, we used Y2H to identify the proteins that could interact with *CbuSPL9*. HMGA, an architectural transcriptional factor, was identified and cloned for further research. BiFC and BLI showed that *CbuSPL9* could form a heterodimer with *CbuHMGA* in the nucleus. The expression analysis showed that *CbuHMGA* had a similar expression trend to that of *CbuSPL9* during flowering in “Bairihua”. Intriguingly, ectopic expression of *CbuHMGA* in *Arabidopsis* would lead to aberrant flowers, but did not effect flowering time. Taken together, our results implied a novel pathway that *ChuSPL9* regulated flowering development, but not flowering transition, with the participation of *ChuHMGA*. Further investments need to be done to verify the details of this pathway.

Keywords: *Catalpa bungei*; Flowering; SPL; HMGA; Architectural transcriptional factor.

Introduction

Flowers allow flowering plants to have a broader evolutionary relationship and extend their ecological niche so that they can dominate the terrestrial ecosystem. Flowering is extremely important for the development of perennial woody plants and for improving the economic value of plants. However, due to complex genomes and other objective characteristics of perennial woody plants, research on the flowering process in perennial woody plants remains limited. *Catalpa bungei* is valuable as both a timber and an ornamental tree¹. “Bairihua”, which is a natural variety of *C. bungei*, has been characterized for its especially short juvenile period, large number of flowers and long flowering period. The flowering period of “Bairihua” is approximately 15 days, and its accumulative flowering period reaches 100 days, which is very rare for woody plants (<http://www.forestry.gov.cn/>). “Bairihua” provides an excellent opportunity to evaluate the flowering process of woody plants.

Flowering is controlled by sophisticated regulatory networks²⁻⁵. Five major pathways are involved in these processes, including the aging pathway⁶, gibberellin pathway⁷⁻¹¹, photoperiod

pathway^{12–16}, vernalization pathway^{17–19} and autonomous pathway²⁰. The SQUAMOSA promoter-binding protein-LIKE (SPL) family of transcription factors (TFs) integrate multiple pathways^{21–25}. SPLs have been shown to regulate flowering time and flower organ development in both herbs and woody plants, such as *Gossypium hirsutum*²⁶, maize²⁷, birch²⁸, *Prunus mume*²⁹, and *Platanus acerifolia*³⁰. In the model plant *Arabidopsis*, AtSPLs have been shown to be a group of dominant regulators of the flowering process^{4,11,13,22,24,31–34}. The overexpression of AtSPLs leads to early flowering and abnormal inflorescence, and conversely, the inhibition of AtSPL expression delays the occurrence of floral transition^{21,35–37}. As a group of TFs, SPLs regulate the expression of other genes. Numerous downstream genes of SPLs have been identified; for example, AtSPL3 can directly upregulate the expression of *LFY*, *FUL* and *API* by binding to their promoters^{38–40}. However, in addition to protein-DNA interactions, TFs also affect plant growth and development by forming protein complexes. For example, the MYB-bHLH-WD40/WDR (MBW) complex regulates late biosynthetic genes in anthocyanin biosynthesis, impacts fruit quality in apple^{41,42}, and regulates trichome initiation in *Arabidopsis thaliana*⁴³. Two TFs, AT-HOOK MOTIF NUCLEAR LOCALIZED PROTEIN 3/4, regulate the formation of the tissue boundary between the procambium and xylem in *Arabidopsis* roots⁴⁴. Few studies have investigated whether there are other factors that interact with SPLs and affect their binding ability, especially in trees. The function of SPLs in woody plants is still in its infancy.

In an effort to study the molecular mechanism of the flowering process in “Bairihua”, we evaluated whether there were protein interactions involved in SPL regulation during the flowering process. As a first step to address this question, we isolated and characterized the *SPL9* orthologous gene from “Bairihua” and performed native in planta gene expression analysis. The putative function of this gene was then tested by ectopic expression experiments. This gene showed similar expression patterns and the ability to induce flower organ development and early flowers in *Arabidopsis*. The findings of this study indicated that *CbuSPL9* is functionally conserved. An architectural TF, CbuHMGA, was found via screening CbuSPL9-interacting proteins. CbuHMGA is involved in the floral organ development of “Bairihua”, but not in the regulation of flowering time. These results provide a molecular basis for studying the molecular mechanism of “Bairihua” flowering and provide a research direction for the study of the floral transition of perennials.

Materials and methods

Plant materials

C. bungei is a perennial tree that typically flowers over a 30-day flowering period. However, “Bairihua”, the new variety of *C. bungei*, was found in Henan Province, China, and confirmed by the National Forestry and Grassland Administration (<http://www.forestry.gov.cn/>). The flowering period of a single flower is approximately 15 days, and its accumulative flowering period reaches 100 days, which is very rare for woody plants. From January 15 to April 2, 2017, we collected the first round of axillary buds of EF and NF varieties every one to two days. Samples were collected every 10 days during the dormant and germinating periods. Since the floral transition of “Bairihua” was completed within 7 to 10 days, samples were collected every day during the floral transition period, and the samples were collected every 5 days during the reproductive growth stage. The samples used for RNA extraction were washed with distilled water, frozen immediately in liquid nitrogen, and stored at -80°C. Samples for histological analysis were fixed in a formalin:glacial acetic acid:70% ethanol (5:5:90 vol.; FAA) solution under a vacuum for at least 24 h.

Histological analysis

For histological analysis, the samples were immersed in FAA fixative and placed under vacuum at 4°C. Samples were dehydrated in gradient ethanol and then embedded in paraffin. Ten-mm-thick sections (RM2255 Fully Automated Rotary Microtome; Leica, Germany) were stained with Safranin O and fast green FCF (Sigma-Aldrich, USA). The slices were observed and photographed using a Leica DM 6000B fully automated upright microscope (Leica Microsystems GmbH, Wetzlar, Germany).

Cloning *CbuSPL9* and *CbuHMGA* sequences from “Bairihua”

RNA from “Bairihua” was extracted from the buds of the mutant using an RNA extraction kit (TaKaRa), and contaminating DNA was removed with RNase-free DNase I (TaKaRa). One or two micrograms of total mRNA template was added to Oligod (dT)18 primer and reverse-transcribed into single-stranded cDNA by M-MLV RTase (TaKaRa). The full-length cDNA of *CbuSPL9* was cloned via 3'-RACE and 5'-RACE by using the (TaKaRa) according to the manufacturer's instructions. In 3'-RACE, the *CbuSPL9* gene-specific forward primers F1/F2 were designed based on published and aligned *SPL* sequences from NCBI (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). F1

nested immediately upstream of F2. The 3'-cDNA synthesis primer was provided in a kit. The PCR products were cloned into the PMD18-T vector and sequenced. *CbuSPL9s* were identified using BLAST. In 5'-RACE, the gene-specific reverse primers R1/R2 (Supplementary Table S1) were designed based on sequences from 3'-RACE. R2 nested immediately upstream of R1. The PCR products were cloned into the PMD18-T vector and sequenced.

Yeast two-hybrid (Y2H) screening of the “Bairihua” cDNA library

A yeast library was constructed by Oebiotech (Shanghai, China) via cloning the full-length cDNA library from mRNAs in the “Bairihua” buds into the pGADT7 vector⁴⁶. *CbuSPL9* was inserted into the pGBKT7 vector. Screening of the yeast library was performed using pGBKT7-*CbuSPL9*. The corresponding primers are listed in Supplementary Table S1. Transformed cells were grown on SD medium supplemented with Trp. The transformants were screened on supplemented SD medium lacking Leu, Trp, His and Ade and supplemented with X-a-Gal and Aureobasidin A. Plates were incubated at 30°C for 48-72 h and photographed. The positive clones were verified with both pGBKT7 vector consensus primers. Positive control mating was as follows: pGADT7-T in Y2HGold and pGBK-53 in Y187. Negative control mating was as follows: pGADT7-T in Y2HGold and pGBKT7-Lam in Y187.

Subcellular localization

To validate subcellular localization, the full-length coding sequences (without the stop codon) of *CbuSPL9* and *CbuHMGA* were amplified from RNA of “Bairihua” buds by RT-PCR. The PCR products of *CbuSPL9* and *CbuHMGA* were ligated to the vector pCAMBIA1304 using the Seamless Assembly Cloning Kit (CloneSmarter, Beijing, China) to construct the *CbuSPL9/CbuHMGA*-GFP fusion genes driven by a CaMV35S promoter (Niwa, 2003). pCAMBIA1304-GFP was used as a positive control. The transient expression vectors *CbuSPL9*-GFP, *CbuHMGA*-GFP and GFP-HDEL were injected into the leaf lower epidermal cells of *Nicotiana tabacum* L. using Agrobacterium transformation as described by Chen et al. (2011). The transformed cells were incubated for 2 days. The leaves were removed, cut into squares and immersed in PBS buffer containing 1 g mL⁻¹ DAPI to stain the nuclei. The transient expression of the *CbuSPL9/CbuHMGA*-GFP fusion proteins was observed under an UltraVIEW VoX 3D Live Cell Imaging System Spinning Disk confocal laser scanning microscope (PerkinElmer, Waltham, MA, USA). The wavelength of excitation used was 488 nm for GFP and 405 nm for DAPI.

BiFC analysis

To confirm and visualize the interaction between CbuSPL9 and CbuHMGA in protoplasts from *Populus trichocarpa*, a BiFC assay was performed based on split EYFP. EYFP was fused to the C-terminus of CbuSPL9 and the N-terminus of CbuHMGA, resulting in CbuSPL: EYFP^C and CbuHMGA: EYFP^N. A positive EYFP signal indicates an interaction of EYFP^C and EYFP^N due to the heterodimerization of CbuSPL9 with CbuHMGA. CbuSPL: EYFP^C was cotransfected with CbuHMGA: EYFP^N and H2A: mCherry into protoplasts. The transient expression of the CbuSPL9/ CbuHMGA- GFP fusion proteins was observed under an UltraVIEW VoX 3D Live Cell Imaging System Spinning Disk confocal laser scanning microscope (PerkinElmer, Waltham, MA, USA).

Biolayer interferometry assay

CbuSPL9 was cloned into pGEX6P-1 as a C-terminal GST-tagged construct, and the construct was confirmed by sequencing. CbuHMGA was cloned into pET28a as an N-terminal 6His-tagged construct, and the construct was confirmed by sequencing. The protein was purified using the procedure for EMCV-3C and RV-3C as described above. Real-time interactions between CbuHMGA and CbuSPL9 were monitored with an Octet QK (Forte-Bio) that is based on BLI⁴⁷. BLI was used to determine dissociation constants (K_D) as well as the on- and off-rate (k_{on} and k_{off}) for HIS- CbuHMG binding to GST- CbuSPL1.

Bioinformatic analysis

Multiple sequence alignment and phylogenetic analysis were performed using MEGA6.0. After alignment, the evolutionary history was calculated using the neighbor-joining (NJ) method. The tree was inferred from 1000 bootstrap replicates to show the evolutionary history of the genes. The MEME online tool (<http://meme-suite.org/tools/meme>) was used to identify the motifs of the CbuSPL9 protein. MEME was run locally with the following parameters: number of repetitions = any and maximum number of motifs = 20. All candidate interacting protein sequences were examined by the domain analysis program SMART (Simple Modular Architecture Research Tool) (<http://smart.embl-heidelberg.de/>).

RNA extraction and quantitative real-time PCR

Total RNA was isolated from “Bairihua” buds at different developmental stages. The purity and quality of RNA was checked by NanoDrop8000 (Thermo Fisher Scientific, Waltham, MA, USA)

and analyzed by gel electrophoresis. First-strand cDNA synthesis was carried out with ~1 µg RNA using the SuperScript III reverse transcription kit (Invitrogen) and random primers according to the manufacturer's instructions. Primers were designed using Primer 3 online. The melting temperature of the primers was 60°C, and the amplicon lengths were 100-200 bp. All primers are listed in Supplementary Table S1. qRT-PCR was performed on a 7500 Real-Time PCR System (Applied Biosystems, CA, USA) using a SYBR Premix Ex Taq™ Kit (TaKaRa, Dalian, China) according to the manufacturer's instructions. Relative expression levels were calculated using the 2-ΔΔCt method. Actin was used as an internal control, and each reaction was conducted in triplicate. The stem expression values were set to 1.

Transient overexpression in *Arabidopsis thaliana*

Full-length CbuSPL9 and CbuHMGA were cloned into the binary vector pBI121 (BD Biosciences Clontech, USA) under the control of the cauliflower mosaic virus 35S promoter in the sense orientation. The transgenic plants were generated with the 35S:CbuSPL9 and 35S:CbuHMGA constructs via *Agrobacterium tumefaciens* GV3101 by using the floral dip method (Clough and Bent, 1998). Surface-sterilized T1 seeds were grown on a solid 0.5 × MS medium containing 30 µg mL⁻¹ hygromycin at 4°C for 2 days, which were then transferred to the greenhouse under long-day conditions (16 h light/8 h dark) at 22°C for 10 days. Subsequently, the seedlings were transplanted into soil. Phenotypes of the transgenic plants were observed in the T1 generation, and the overexpression of *CbuSPL9* and *CbuHMGA* in the transgenic plants was confirmed by PCR genotyping (Supplementary Figure S1 and S2). For each construct, at least 10 transgenic lines with similar phenotypes were observed, and 3 of them were used for detailed analysis.

Flowering time measurement

Flowering time was measured by counting the total number of rosette leaves and the number of days to flower (when the floral buds were visible). The numbers of rosette and cauline leaves of ~20 plants were counted and averaged. The presence of abaxial trichomes was used to differentiate between the juvenile and adult vegetative leaves. Data were classified with Win-Excel and analyzed via analysis of variance (ANOVA) using the SPSS (version 8.0, SPSS Inc., Chicago, IL, USA) statistical package. Comparisons between the treatment means were made using Tukey's test at a probability level of P ≤ 0.05.

Result

A flowering-related *SPL* homologous gene was isolated in “Bairihua”

The *SPL* homologous genes in *C. bungei* were isolated by using degenerate primers targeting the homeodomain. The isolated homeodomain sequences were extended by genome walking to acquire the full genomic sequences (exons and introns). One of the isolated sequences encoded a protein with a typical *SPL* protein structure, which included a highly conserved SBP-box domain bearing two zinc-binding sites and one bipartite nuclear localization signal⁴⁵ (Supplementary Table S2). The first Zn-finger-like structure (ZN-1 in Figure 1a) was C3H-type, and the second (Zn-2 in Figure 1a) was C2HC-type. The nuclear localization sequence (NLS) is a highly conserved bipartite domain located at the C-terminus of SBP. Phylogenetic analysis showed that the isolated sequence clustered with AtSPL9 and AtSPL15 (Figure 1b). Since it shared more sequence similarity with *AtSPL9*, we named the *SPL* homologous gene *CbuSPL9*.

Expression of *CbuSPL9* during the flowering process

Intensive sampling was performed to investigate the changes in *CbuSPL9* expression during the flowering process (Figure 2). T1-T3 was the dormant period during which no significant differences in morphology could be observed between the flowering buds (FBs) and the leaf buds (LBs). T4-T5 was the germination period. During this period, the internal morphology was similar between the FBs and LBs, but the external morphology of the FBs and LBs had a pale green appearance compared with that of the FBs and LBs during the dormant period. T6-T9 followed the short germination period and was the floral transition period, during which flower primordium and leaf primordium were developed in the FBs and LBs, respectively. Finally, T10-T12 was the reproductive growth period. The expression level of *CbuSPL9* was significantly higher in the FBs than in the LBs, especially during the dormant period and the reproductive growth period. Overall, the expression study supported that *CbuSPL9* was an *SPL* homologous gene and involved in flowering regulation.

The overexpression of *CbuSPL9* in Arabidopsis

As there is no available transformation system in *C. bungei*, *CbuSPL9* was overexpressed in Arabidopsis (Columbia ecotype, *col*). The flowers from *col* were tetradynamous and had four petals distributed in cross type (Figure 3aI). In contrast, the *oe-spl9* transgenic plants showed aberrant flower organs (Supplementary Table S3). The *oe-spl9* transgenic lines exhibited flower organs with altered numbers and locations, such as shrunken petals, increased stamens, and

overlapping petals (Figure 3aII-VI). In addition to the evident changes in floral organ morphology, an acceleration in flowering time was observed in the *oe-spl9* lines (Figure 3b, Supplementary Table S4). The *col* line initiated flowering when 14 rosette leaves were present (Figure 3c). However, *oe-spl9* possessed less than 9 rosette leaves at the time of bolting (Figure 3d). This result indicated that the function of *CbuSPL9* was conserved. The regulatory mechanism of *CbuSPL9* in “Bairihua” might be similar to that in *Arabidopsis*.

Screening *CbuSPL9*-interacting proteins

As a TF, SPL-DNA interaction studies have been extensively performed. However, TFs can also fine-tune specific biological processes through protein interactions. We constructed a *C. bungei* yeast two-hybrid cDNA library to explore the proteins that interact with *CbuSPL9*. A total of 809 blue colonies representing potential positive clones were obtained on QDO/Aba/X-a-Gal plates. The potential positive clones were subsequently tested by PCR for the library plasmid, and 406 clones were positive. The resulting PCR products were sequenced, and the sequences were aligned using the NCBI BLASTp search function. Finally, 12 interacting protein candidates were identified (Table 1). These predicted proteins included HMGA, aquaporins, bHLH48, GATA-related protein, PHD finger protein ALFIN-LIKE 4, heavy metal-associated protein, etc.

Cloning the *HMGA* gene

Among these candidates, HMGA, which is an AT-hook rich protein, belongs to the most abundant nonhistone protein family in the nucleus. We cloned the full-length *HMGA* homologous gene via rapid amplification of cDNA ends. Phylogenetic analysis revealed that this protein has a close relationship with AtHMGA. Therefore, this protein was renamed CbuHMGA. *CbuHMGA* encoded a H15 domain and 4 AT-hook motifs (Figure 4a). The expression analysis showed that the expression trend of *CbuHMGA* was similar to that of *CbuSPL9* in the four periods in “Bairihua” (Figure 4b). The expression of *CbuHMGA* was elevated in the dormant period, and the highest expression level was detected in the reproductive growth period. However, the increase in the expression of *CbuHMGA* before the reproductive growth period occurred slightly earlier than that of *CbuSPL9*.

Localization of the *CbuSPL9* and *CbuHMGA* proteins

We fused GFP at the C-terminus of *CbuSPL9* and *CbuHMGA* and transformed them into *Nicotiana benthamiana* leaf epidermal cells to determine the localization of the *CbuSPL9* and

CbuHMGA proteins. 35S:GFP was used as a control. The GFP fluorescence in CbuSPL9-GFP and CbuHMGA-GFP was exclusively observed in the nucleus, whereas the fluorescence of GFP in the control was distributed throughout the entire cell (Figure 5a). These results showed that CbuSPL9 and CbuHMGA were located in the nucleus. This result was in agreement with the prediction based on the protein structure.

Protein interaction analysis

Biolayer interferometry (BLI) was used to determine dissociation constants (K_D) as well as the on- and off-rate (k_{on} and k_{off}) for HIS-CbuHMGA binding to GST-CbuSPL9 (Table 2). Five different concentrations of GST-CbuSPL9 (2381.00 nM, 1191.00 nM, 595.30 nM, 297.60 nM and 148.80 nM) were evaluated, and the K_D was 311 nM for each tested concentration. These results suggested a strong interaction between CbuSPL9 and CbuHMGA (Figure 5b).

To confirm these interactions, the full-length cDNA of *CbuSPL9* was inserted into the vector pGBKT7 (BD-CbuSPL9) as bait, and the full-length cDNA of *CbuHMGA* was inserted into the vector pGADT7 (AD-CbuHMGA) as prey. Yeast strains containing AD-CbuHMGA and BD-CbuSPL9 were positive for X- α -gal activity when grown on synthetically defined (SD)/-Trp/-Leu/-His/-Ade medium (Figure 5c). These results showed that CbuSPL9 interacted with HMGA in yeast. Finally, a bimolecular fluorescence complementation (BiFC) assay was performed. CbuSPL9:YFP^C was cotransfected with CbuHMGA:YFP^N and H2A:mCherry into protoplasts. The signal of enhanced yellow fluorescent protein (EYFP) was colocalized with mCherry (Figure 5d). Collectively, we demonstrated that CbuSPL9 formed a heterodimer with CbuHMGA in the nucleus.

The overexpression of *CbuHMGA* in Arabidopsis

To characterize the potential function of *CbuHMGA*, we overexpressed *CbuHMGA* in Arabidopsis. The *oe-hmga* transgenic lines developed aberrant flowers with abnormal petals and stamens (Figure 6aI-VI). This mutant phenotype was similar to the phenotype of floral organs when *CbuSPL9* was overexpressed (Supplementary Table S5). However, flowering time was not affected in the *oe-hmga* transgenic lines compared to that in the *col* line (Supplementary Table S6). The expression of endogenous *AtSPL9* was thoroughly studied to further monitor flower development in the transgenic lines. Within the sampling period, the expression level of *AtSPL9* continuously increased in *oe-hmga* and wild type. However, in *oe-spl9*, the expression of *AtSPL9*

reached a peak in T6 and then decreased (Figure 6b). The shift in *AtSPL9* expression in *oe-spl9*, but not in *oe-hmga*, further confirmed the observation that *CbuHMGA* cannot accelerate flower development, while *CbuSPL9* can accelerate flower development.

Discussion

Flowering is a very complex process, a qualitative change in the life history of higher plants, and a central link in plant development. “Bairihua”, a variety of the flowering perennial woody plant *C. bungei*, has a large amount of flowers and a long flowering period. “Bairihua” is an excellent material for evaluating the flowering process in trees.

SPLs play an important role in regulating flowering in many plants, most notably in Arabidopsis. In woody plants, *SPLs* are studied extensively, but limited information about flowering is known^{29,48–51}. Here, we cloned the SBP-domain-encoding gene *CbuSPL9* in *C. bungei*. Its conserved structure and flower development-related expression trends suggest that *CbuSPL9* might have conserved *SPL9* functions in *C. bungei*. Furthermore, heterogeneous overexpression of *CbuSPL9* in Arabidopsis accelerated flower development and lead to aberrant flower organs. Although we could not generate homogenous transgenic plants due to technology and time, these results are sufficient to demonstrate that *CbuSPL9* is an *SPL* homolog and participates in flower development.

TFs can affect biological processes by forming complexes with other TFs. However, most of the studies on *SPLs* have focused on their interactions with DNA motifs^{38–40}. The proteins that could directly interact with *SPLs* and participate in flower regulation are largely unknown. For further insight, we screened the yeast hybridization library for *CbuSPL9* in buds. Many flowering-related proteins were detected, for example, the GATA-related proteins^{52–57}, bHLH48⁵⁸, FLA^{59–62}, and PHD finger protein ALFIN-LIKE^{63–65}. Furthermore, a high mobility group (HMG) protein, *CbuHMGA*, was identified to interact directly with *CbuSPL9*. *CbuHMGA* contains an H15 domain and 4 AT-hook motifs. The BiFC assay and BLI assay further confirmed their interaction. The BiFC assay suggested that *CbuSPL9* and *CbuHMGA* could form protein complexes in the nucleus. HMGA proteins have a highly conserved structure^{66,67}. Unlike the HMGA proteins in the animal kingdom, which invariably contain three AT-hook motifs, most plant HMGA proteins have four AT-hook motifs^{67,68}. Additionally, the amino-terminal region of plant HMGA proteins shares remarkable homology with the DNA-binding domain of histone H1^{69,70}. A similar structural

description was confirmed in CbuHMGA, which encoded a H15 domain and four AT-hook motifs. HMGA is an architectural TF^{70–73}. It regulates gene expression in vivo by controlling the formation of multiprotein complexes on the AT-rich regions of certain gene promoters^{68,70,81,73–80}. To date, some plant HMGA proteins have been isolated, such as those in soybean⁸², rice⁸³, maize⁸⁴ and Arabidopsis^{67,73,75,80,85–87}. The Arabidopsis HMGA gene was detected in flowers, and developing siliques had the highest expression⁸⁷. However, studies of the interacting proteins and the function of HMGA in woody plants are rare. In “Bairihua”, the expression of *CbuHMGA* was elevated in the dormant period, and the highest expression level was detected in the reproductive growth period. The result was consistent with that in Arabidopsis. Although the *oe-hmga* transgenic lines did not show a flowering time phenotype, the floral organ mutation of *oe-hmga* was similar to that of the *oe-spl9* transgenic lines. The HMGA proteins have an important role in biological processes and interact with different TFs^{70,73,88}. Their intrinsic flexibility allows the HMGA proteins to participate in specific protein-DNA and protein-protein interactions that induce structural changes in chromatin and the formation of stereospecific complexes called ‘enhanceosomes’ on the promoter/enhancer regions of genes whose transcription they regulate. The chromatin structure changes affect the ability of TFs to bind with the promoter/enhancer regions^{68,74,77,78,80,85}. From the results in this study, we suggest that the interaction of CbuHMGA with CbuSPL9 might strengthen or weaken the binding ability of CbuSPL9 with the corresponding DNA sequences or downstream proteins, thus affecting the flowering process. Further experiments are needed to test this hypothesis.

Conclusion

“Bairihua” provides us with a valuable opportunity to gain a deeper understanding of the flowering process of woody plants. Our study showed that *SPLs* may have similar structures and regulatory mechanisms in perennial trees and Arabidopsis. Screening CbuSPL9-interacting proteins revealed not only additional proteins for research on the regulatory pathway of CbuSPL9 but also the CbuSPL9 interaction with CbuHMGA. *oe-hmga* showed a phenotype that affected floral organ development but did not change flowering time. This result suggests that the mechanism by which *CbuSPL9* affects the flowering process is a complex process, and *CbuHMGA* is involved in the development of flower organs but not in the regulation of flowering time.

Conflict of interest

None declared

Funding

This work was supported by Fundamental Research Funds of Chinese Academy of Forestry (CAFYBB2017ZY002); Fundamental Research Funds of Chinese Academy of Forestry (CAFYBB2017ZA001-8).

Author contributions

J.H.W., S.G.Z., G.Z.Q., L.I.S., Z.W. and W.J.M. designed the experiments. Z.W. and W.J.M. analyzed the RNA-seq data and wrote the manuscript. Z.W., and T.Q.Z. detected the expression of genes using qRT-PCR. Z.W., N.L., F.Q.O.Y., N.W. and G.J.Y. collected the samples used in the experiment. All the authors have read the paper and agreed to list their names as co-authors.

Supplementart Data

Supplementary Table S1 The list of the all Primers used in this paper.

Supplementary Figure S1 Verification of *oe-SPL9* positive Arabidopsis.

Supplementary Figure S2 Verification of *oe-HMGA* positive Arabidopsis.

Supplementary Table S2 Details of motif-sequences of CbuSPL9 were identified by MEME.

Supplementary Table S3 Statistics of mutant of floral organs in *oe-SPL9* Arabidopsis.

Supplementary Table S4 Statistics of mutant of flowering time in *oe-SPL9* Arabidopsis.

Supplementary Table S5 Statistics of mutant of floral organs in *oe-HMGA* Arabidopsis.

Supplementary Table S6 Statistics of mutant of flowering time in *oe-HMGA* Arabidopsis.

References

1. Jing, D., Xia, Y., Chen, F., et al. 2015, Ectopic expression of a *Catalpa bungei* (Bignoniaceae) PISTILLATA homologue rescues the petal and stamen identities in *Arabidopsis* pi-1 mutant. *Plant Sci.*, **231**, 40–51.
2. Flowers, J. M., Hanzawa, Y., Hall, M. C., et al. 2009, Population genomics of the *Arabidopsis thaliana* flowering time gene network. *Mol. Biol. Evol.*, **26**, 2475–86.
3. Mouradov, A., Cremer, F., and Coupland, G. 2002, Control of flowering time: interacting pathways as a basis for diversity. *Plant Cell*, **14 Suppl**, S111–30.
4. Fornara, F., de Montaigu, A., and Coupland, G. 2010, SnapShot: Control of flowering in *Arabidopsis*. *Cell*, **141**, 3–5.
5. Bouché, F., Lobet, G., Tocquin, P., et al. 2016, FLOR-ID: An interactive database of flowering-time gene networks in *Arabidopsis thaliana*. *Nucleic Acids Res.*, **44**, D1167–71.
6. Wang, J. 2014, Regulation of flowering time by the miR156-mediated age pathway. *J. Exp. Bot.*, **65**, 4723–7430.
7. Murase, K., Hirano, Y., Sun, T. P., et al. 2008, Gibberellin-induced DELLA recognition by the gibberellin receptor GID1. *Nature*, **456**, 459–63.
8. Eriksson, S. 2006, GA4 Is the Active Gibberellin in the Regulation of LEAFY Transcription and *Arabidopsis* Floral Initiation. *Plant Cell Online*, **18**, 2172–81.
9. Livne, S., Lor, V. S., Nir, I., et al. 2015, Uncovering DELLA-Independent Gibberellin Responses by Characterizing New Tomato *procera* Mutants. *Plant Cell*, **27**, 1579–94.
10. Cao, D., Cheng, H., Wu, W., et al. 2006, Gibberellin Mobilizes Distinct DELLA-Dependent Transcriptomes to Regulate Seed Germination and Floral Development in *Arabidopsis*. *Plant Physiol.*, **142**, 509–25.
11. Yu, S., Galvao, V. C., Zhang, Y.-C., et al. 2012, Gibberellin Regulates the *Arabidopsis* Floral Transition through miR156-Targeted SQUAMOSA PROMOTER BINDING-LIKE Transcription Factors. *Plant Cell*, **24**, 3320–32.
12. Matsubara, K., Ogiso-tanaka, E., Hori, K., et al. 2017, Natural Variation in Hd17 , a Homolog of *Arabidopsis* ELF3 That is Involved in Rice Photoperiodic Flowering. *Plant Cell Physiol.*, **53**, 709–16.
13. Jung, J. H., Ju, Y., Seo, P. J., et al. 2012, The SOC1-SPL module integrates photoperiod

- 397 and gibberellic acid signals to control flowering time in Arabidopsis. *Plant J.*, **69**, 577–88.
- 398 14. Minmin, S., and Youjun, H. 2018, Advances of GIGANTEA and CONSTANS, the Key
- 399 Genes of Flowering in Photoperiod Pathway. *Mol. Plant Breed.*, **16**, 5601–7.
- 400 15. Wang, H., Pan, J., Li, Y., et al. 2016, The DELLA-CONSTANS Transcription Factor
- 401 Cascade Integrates Gibberellic Acid and Photoperiod Signaling to Regulate Flowering.
- 402 *Plant Physiol.*, **172**, 479–88.
- 403 16. Cano, B., Ruiz, M. T., Romero, J. M., et al. 2014, Photoperiodic Control of Carbon
- 404 Distribution during the Floral Transition in Arabidopsis. *Plant Cell*, **26**, 565–84.
- 405 17. Huang, B., Qian, P., Gao, N., et al. 2017, Fackel interacts with gibberellic acid signaling
- 406 and vernalization to mediate flowering in Arabidopsis. *Planta*, **245**, 939–50.
- 407 18. Kim, D.-H., and Sung, S. 2013, Coordination of the Vernalization Response through a
- 408 VIN3 and FLC Gene Family Regulatory Network in Arabidopsis. *Plant Cell*, **25**, 454–69.
- 409 19. Heo, J. B., and Sung, S. 2011, Vernalization-mediated epigenetic silencing by a long
- 410 intronic noncoding RNA. *Science (80-.)*, **331**, 76.
- 411 20. Simpson, G. G. 2004, The autonomous pathway: Epigenetic and post-transcriptional gene
- 412 regulation in the control of Arabidopsis flowering time. *Curr. Opin. Plant Biol.*, **7**, 570–4.
- 413 21. Unte, U. S. 2003, SPL8, an SBP-Box Gene That Affects Pollen Sac Development in
- 414 Arabidopsis. *Plant Cell Online*, **15**, 1009–19.
- 415 22. Yu, N., Niu, Q. W., Ng, K. H., et al. 2015, The role of miR156/SPLs modules in
- 416 Arabidopsis lateral root development. *Plant J.*, **83**, 673–85.
- 417 23. Casero, D., Singh, V., Wilson, G. T., et al. 2012, Transcriptome Sequencing Identifies
- 418 SPL7-Regulated Copper Acquisition Genes FRO4/FRO5 and the Copper Dependence of
- 419 Iron Homeostasis in Arabidopsis. *Plant Cell Online*, **24**, 738–61.
- 420 24. Jung, J. H., Lee, H. J., Ryu, J. Y., et al. 2016, SPL3/4/5 Integrate Developmental Aging
- 421 and Photoperiodic Signals into the FT-FD Module in Arabidopsis Flowering. *Mol. Plant*, **9**,
- 422 1647–59.
- 423 25. Preston, J. C., and Hileman, L. C. 2013, Functional Evolution in the Plant
- 424 SQUAMOSA-PROMOTER BINDING PROTEIN-LIKE (SPL) Gene Family. *Front. Plant*
- 425 *Sci.*, **4**, 1–13.
- 426 26. Zhang, X., Dou, L., Pang, C., et al. 2015, Genomic organization , differential expression ,

427 and functional analysis of the SPL gene family in *Gossypium hirsutum*. *Mol. Genet.*
428 *Genomics*, **290**, 115–26.

429 27. Mao, H. De, Yu, L. J., Li, Z. J., et al. 2016, Genome-wide analysis of the SPL family
430 transcription factors and their responses to abiotic stresses in maize. *Plant Gene*, **6**, 1–12.

431 28. Lannenpaa, M., Janonen, I., Sopanen, T., et al. 2004, A new SBP-box gene BpSPL1 in
432 silver birch (*Betula pendula*). *Physiol. Plant.*, **120**, 491–500.

433 29. Xu, Z., Sun, L., Zhou, Y., et al. 2015, Identification and expression analysis of the
434 SQUAMOSA promoter-binding protein (SBP)-box gene family in *Prunus mume*. *Mol.*
435 *Genet. Genomics*, **290**, 1701–15.

436 30. Han, H., Liu, G., Zhang, J., et al. 2016, Four SQUAMOSA PROMOTER BINDING
437 PROTEIN-LIKE homologs from a basal eudicot tree (*Platanus acerifolia*) show diverse
438 expression pattern and ability of inducing early flowering in *Arabidopsis*. *Trees*, **30**,
439 1417–28.

440 31. Xing, S., Salinas, M., Höhmann, S., et al. 2010, miR156-Targeted and Nontargeted
441 SBP-Box Transcription Factors Act in Concert to Secure Male Fertility in *Arabidopsis*.
442 *Plant Cell*, **22**, 3935–50.

443 32. Yamasaki, H., Hayashi, M., Fukazawa, M., et al. 2009, SQUAMOSA Promoter Binding
444 Protein-Like7 Is a Central Regulator for Copper Homeostasis in *Arabidopsis*. *Plant Cell*
445 *Online*, **21**, 347–61.

446 33. Xing, S., Salinas, M., Garcia-Molina, A., et al. 2013, SPL8 and miR156-targeted SPL
447 genes redundantly regulate *Arabidopsis* gynoecium differential patterning. *Plant J.*, **75**,
448 566–77.

449 34. Yan, J., Chia, J.-C., Sheng, H., et al. 2017, *Arabidopsis* Pollen Fertility Requires the
450 Transcription Factors CIT1 and SPL7 that Regulate Copper Delivery to Anthers and
451 Jasmonic Acid Synthesis. *Plant Cell*, tpc.00363.2017.

452 35. Poethig, R. S. 2013, Vegetative phase change and shoot maturation in plants. *Curr. Top.*
453 *Dev. Biol.*, **105**, 125–52.

454 36. Yu, N., Cai, W.-J., Wang, S., et al. 2010, Temporal Control of Trichome Distribution by
455 MicroRNA156-Targeted SPL Genes in *Arabidopsis thaliana*. *Plant Cell Online*, **22**,
456 2322–35.

- 457 37. Hyun, Y., Richter, R., and Coupland, G. 2016, Competence to Flower: Age-Controlled
458 Sensitivity to Environmental Cues. *Plant Physiol.*, **173**, 36–46.
- 459 38. Wu, G. 2006, Temporal regulation of shoot development in *Arabidopsis thaliana* by
460 miR156 and its target SPL3. *Development*, **133**, 3539–47.
- 461 39. Yamaguchi, A., Wu, M. F., Yang, L., et al. 2009, The MicroRNA-Regulated SBP-Box
462 Transcription Factor SPL3 Is a Direct Upstream Activator of LEAFY, FRUITFULL, and
463 APETALA1. *Dev. Cell*, **17**, 268–78.
- 464 40. Wang, J. W., Czech, B., and Weigel, D. 2009, miR156-Regulated SPL Transcription
465 Factors Define an Endogenous Flowering Pathway in *Arabidopsis thaliana*. *Cell*, **138**,
466 738–49.
- 467 41. An, X. H., Tian, Y., Chen, K. Q., et al. 2015, MdMYB9 and MdMYB11 are involved in
468 the regulation of the ja-induced biosynthesis of anthocyanin and proanthocyanidin in
469 apples. *Plant Cell Physiol.*, **56**, 650–62.
- 470 42. Xie, X. Bin, Li, S., Zhang, R. F., et al. 2012, The bHLH transcription factor MdbHLH3
471 promotes anthocyanin accumulation and fruit colouration in response to low temperature
472 in apples. *Plant, Cell Environ.*, **35**, 1884–97.
- 473 43. Qi, T., Song, S., Ren, Q., et al. 2011, The Jasmonate-ZIM-Domain Proteins Interact with
474 the WD-Repeat/bHLH/MYB Complexes to Regulate Jasmonate-Mediated Anthocyanin
475 Accumulation and Trichome Initiation in *Arabidopsis thaliana* . *Plant Cell*, **23**, 1795–814.
- 476 44. Zhou, J., Wang, X., Lee, J.-Y. 2013, Cell-to-Cell Movement of Two Interacting AT-Hook
477 Factors in *Arabidopsis* Root Vascular Tissue Patterning . *Plant Cell*, **25**, 187–201.
- 478 45. Kropat, J., Tottey, S., Birkenbihl, R. P., et al. 2005, A regulator of nutritional copper
479 signaling in *Chlamydomonas* is an SBP domain protein that recognizes the GTAC core of
480 copper response element. *Proc. Natl. Acad. Sci.*, **102**, 18730–5.
- 481 46. Yan, T., Chen, M., Shen, Q., et al. 2017, HOMEODOMAIN PROTEIN 1 is required for
482 jasmonate-mediated glandular trichome initiation in *Artemisia annua*. *New Phytol.*, **213**,
483 1145–55.
- 484 47. Zhang, Y., Mao, D., Roswit, W. T., et al. 2015, PARP9-DTX3L ubiquitin ligase targets
485 host histone H2BJ and viral 3C protease to enhance interferon signaling and control viral
486 infection. *Nat. Immunol.*, **16**, 1215–27.

- 487 48. Castillo, M. C., Forment, J., Gadea, J., et al. 2013, Identification of transcription factors
488 potentially involved in the juvenile to adult phase transition in Citrus. *Ann. Bot.*, **112**,
489 1371–81.
- 490 49. Tewari, S., and Buonaccorsi, J. P. 2013, Impact of Early Season Apical Meristem Injury
491 by Gall Inducing Tipworm (Diptera: Cecidomyiidae) on Reproductive and Vegetative
492 Growth of Cranberry Entomology. *J. Econ. Entomol.*, **106**, 1339–48.
- 493 50. Lannenpaa, M., Janonen, I., Sopanen, T., et al. 2004, A new SBP-box gene BpSPL1 in
494 silver birch (*Betula pendula*). *Physiol. Plant.*, **120**, 491–500.
- 495 51. Lu, C. L. and S. 2014, Molecular characterization of the SPL gene family in *Populus*
496 *trichocarpa*. *BMC Plant Biol.*, **14**, 131.
- 497 52. Zhang, X., Zhou, Y., Ding, L., et al. 2013, Transcription Repressor HANABA TARANU
498 Controls Flower Development by Integrating the Actions of Multiple Hormones, Floral
499 Organ Specification Genes, and GATA3 Family Genes in Arabidopsis . *Plant Cell*, **25**,
500 83–101.
- 501 53. Ding, L., Yan, S., Jiang, L., et al. 2015, HANABA TARANU (HAN) Bridges Meristem
502 and Organ Primordia Boundaries through PINHEAD, JAGGED, BLADE-ON-PETIOLE2
503 and CYTOKININ OXIDASE 3 during Flower Development in Arabidopsis. *PLoS Genet.*,
504 **11**, 1–23.
- 505 54. Ding, L., Yan, S., Jiang, L., et al. 2015, HANABA TARANU regulates the shoot apical
506 meristem and leaf development in cucumber (*Cucumis sativus* L.). *J. Exp. Bot.*, **66**,
507 7075–87.
- 508 55. Mara, C. D., and Irish, V. F. 2008, Two GATA Transcription Factors Are Downstream
509 Effectors of Floral Homeotic Gene Action in Arabidopsis. *Plant Physiol.*, **147**, 707–18.
- 510 56. Richter, R., Bastakis, E., and Schwechheimer, C. 2013, Cross-Repressive Interactions
511 between SOC1 and the GATAs GNC and GNL/CGA1 in the Control of Greening, Cold
512 Tolerance, and Flowering Time in Arabidopsis. *Plant Physiol.*, **162**, 1992–2004.
- 513 57. Wang, L., Yin, H., Qian, Q., et al. 2009, NECK LEAF 1, a GATA type transcription factor,
514 modulates organogenesis by regulating the expression of multiple regulatory genes during
515 reproductive development in rice. *Cell Res.*, **19**, 598–611.
- 516 58. Li, Y., Wang, H., Li, X., et al. 2017, Two DELLA-interacting proteins bHLH48 and

517 bHLH60 regulate flowering under long-day conditions in *Arabidopsis thaliana*. *J. Exp.*
518 *Bot.*, **68**, 2757–67.

519 59. Li, J., Yu, M., Geng, L. L., et al. 2010, The fasciclin-like arabinogalactan protein gene,
520 FLA3, is involved in microspore development of *Arabidopsis*. *Plant J.*, **64**, 482–97.

521 60. Johnson, K. L. 2003, The Fasciclin-Like Arabinogalactan Proteins of *Arabidopsis*. A
522 Multigene Family of Putative Cell Adhesion Molecules. *Plant Physiol.*, **133**, 1911–25.

523 61. Johnson, K. L., Kibble, N. A. J., Bacic, A., et al. 2011, A fasciclin-like
524 Arabinogalactan-protein (FLA) mutant of *Arabidopsis thaliana*, *fla1*, shows defects in
525 shoot regeneration. *PLoS One*, **6**, 1–11.

526 62. Zhang, Z., Wang, C., Kong, F., et al. 2017, Fasciclin-like arabinogalactan proteins,
527 PtFLAs, play important roles in GA-mediated tension wood formation in *Populus*. *Sci.*
528 *Rep.*, **7**, 1–13.

529 63. Shu, K., Chen, Q., Wu, Y., et al. 2016, ABSCISIC ACID-INSENSITIVE 4 negatively
530 regulates flowering through directly promoting *Arabidopsis* FLOWERING LOCUS C
531 transcription. *J. Exp. Bot.*, **67**, 195–205.

532 64. Song, Y., Gao, J., Yang, F., et al. 2013, Molecular Evolutionary Analysis of the
533 Alfin-Like Protein Family in *Arabidopsis lyrata*, *Arabidopsis thaliana*, and *Thellungiella*
534 *halophila*. *PLoS One*, **8**, e66838.

535 65. Pan, W.-J., Zhang, J.-S., Tao, J.-J., et al. 2018, An Alfin-like gene from *Atriplex hortensis*
536 enhances salt and drought tolerance and abscisic acid response in transgenic *Arabidopsis*.
537 *Sci. Rep.*, **8**, 1–13.

538 66. Grasser, K. D. 2003, Chromatin-associated HMGA and HMGB proteins: Versatile
539 co-regulators of DNA-dependent processes. *Plant Mol. Biol.*, **53**, 281–95.

540 67. Klosterman, S. J., and Hadwiger, L. A. 2002, Plant HMG proteins bearing the AT-hook
541 motif. *Plant Sci.*, **162**, 855–66.

542 68. Dragan, A. I., Liggins, J. R., Crane-Robinson, C., et al. 2003, The energetics of specific
543 binding of AT-hooks from HMGA1 to target DNA. *J. Mol. Biol.*, **327**, 393–411.

544 69. Krech, A. B., Wulff, D., Grasser, K. D., et al. 1999, Plant chromosomal HMGI/Y proteins
545 and histone H1 exhibit a protein domain of common origin. *Gene*, **230**, 1–5.

546 70. Reeves, R. 2001, Molecular biology of HMGA proteins : hubs of nuclear function. *Gene*,

547 **277**, 63–81.

548 71. Tessari, M. A., Gostissa, M., Altamura, S., et al. 2003, Transcriptional Activation of the

549 Cyclin A Gene by the Architectural Transcription Factor HMGA2. *Mol. Cell. Biol.*, **23**,

550 9104–16.

551 72. Reeves, R., and Beckerbauer, L. 2001, HMGI/Y proteins:flexible regulators of transcription

552 and chromatin structure. *BBA-Biochimica Biophys. Acta*, **1519**, 13–29.

553 73. Sgarra, R., Zammiti, S., Lo Sardo, A., et al. 2010, HMGA molecular network: From

554 transcriptional regulation to chromatin remodeling. *Biochim. Biophys. Acta - Gene Regul.*

555 *Mech.*, **1799**, 37–47.

556 74. Gonzalez, N., Pauwels, L., Baekelandt, A., et al. 2015, A Repressor Protein Complex

557 Regulates Leaf Growth in Arabidopsis. *Plant Cell*, **27**, 2273–87.

558 75. Rodríguez-concepción, M., and Rodri, M. 1999, Arachidonic Acid Alters Tomato HMG

559 Expression and Fruit Growth and Induces 3- Hydroxy-3-Methylglutaryl Coenzyme. *Plant*

560 *Physiol.*, **119**, 41–8.

561 76. Reeves, R., and Beckerbauer, L. 2001, HMGI/Y proteins: Flexible regulators of

562 transcription and chromatin structure. *Biochim. Biophys. Acta - Gene Struct. Expr.*, **1519**,

563 13–29.

564 77. Richter, T., Mu, T. E., Heinkele, G., et al. 2005, Interaction between HMGA1a and the

565 origin recognition complex creates site-specific replication origins. *PNAS*, **308**, 1–24.

566 78. Norseen, J., Thomae, A., Sridharan, V., et al. 2008, RNA-dependent recruitment of the

567 origin recognition complex. *EMBO J.*, **27**, 3024–35.

568 79. Gupta, R., Webster, C. I., and Gray, J. C. 1997, The single-copy gene encoding

569 high-mobility-group protein HMG-I/Y from pea contains a single intron and is expressed

570 in all organs. *Plant Mol. Biol.*, **35**, 987–92.

571 80. Matsushita, A., Furumoto, T., Ishida, S., et al. 2007, AGF1, an AT-Hook Protein, Is

572 Necessary for the Negative Feedback of AtGA3ox1 Encoding GA 3-Oxidase. *Plant*

573 *Physiol.*, **143**, 1152–62.

574 81. Reeves, R., and Beckerbauer, L. 2001, HMGI/Y proteins: Flexible regulators of

575 transcription and chromatin structure. *Biochim. Biophys. Acta - Gene Struct. Expr.*, **1519**,

576 13–29.

577 82. Laux, T., Seurinck, J., and Goldberg, R. B. 1991, A soybean embryo cDNA encodes a
578 DNA binding protein with histone and HMG-protein-like domains. *Nucleic Acids Res.*, **19**,
579 4768.

580 83. Yamamoto, S., and Minamikawa, T. 1997, Two genes for the high mobility group protein
581 HMG-Y are present in the genome of *Canavalia gladiata* D.C. *Plant Mol. Biol.*, **33**,
582 537–44.

583 84. Zhao, J., Paul, L. K., and Grafi, G. 2009, The maize HMGA protein is localized to the
584 nucleolus and can be acetylated in vitro at its globular domain, and phosphorylation by
585 CDK reduces its binding activity to AT-rich DNA. *Biochim. Biophys. Acta - Gene Regul.*
586 *Mech.*, **1789**, 751–7.

587 85. Pierantoni, G. M., Rinaldo, C., Esposito, F., et al. 2006, High Mobility Group A1
588 (HMGA1) proteins interact with p53 and inhibit its apoptotic activity. *Cell Death Differ.*,
589 **13**, 1554–63.

590 86. Cho, J. H., Kim, M. J., Kim, K. J., et al. 2011, POZ / BTB and AT-hook-containing zinc
591 finger protein 1 (PATZ1) inhibits endothelial cell senescence through a p53 dependent
592 pathway. *Cell Death Differ.*, **19**, 703–12.

593 87. Xiao, C., Chen, F., Yu, X., et al. 2009, Over-expression of an AT-hook gene, AHL22,
594 delays flowering and inhibits the elongation of the hypocotyl in *Arabidopsis thaliana*.
595 *Plant Mol. Biol.*, **71**, 39–50.

596 88. Michela A. T., Monica Gostissa, Sandro Altamura, et al. 2004, Transcriptional Activation
597 of the Cyclin A Gene by the Architectural Transcription Factor HMGA2. *Mol. Cell. Biol.*,
598 **23**, 9104–16.

599

600 **Table 1 Details of the proteins interacting with the CbuSPL9 protein**

Annotation	Quantity
Sesamum indicum HMG-Y-related protein B-like (LOC105159884), mRNA	4
PREDICTED: Sesamum indicum aquaporin PIP2-4-like (LOC105174310), mRNA	1
PREDICTED: Sesamum indicum GATA transcription factor 8 (LOC105166606), transcript variant X2, mRNA	1
PREDICTED: Sesamum indicum heavy metal-associated isoprenylated plant protein 33-like (LOC105156967), mRNA	2
PREDICTED: Sesamum indicum probable E3 ubiquitin ligase SUD1 (LOC105169523), mRNA	4
PREDICTED: Erythranthe guttatus transcription factor bHLH148-like (LOC105966580), mRNA	1
PREDICTED: Sesamum indicum aquaporin TIP1-1-like (LOC105170758), mRNA	1
PREDICTED: Sesamum indicum probable GTP diphosphokinase RSH2, chloroplastic (LOC105157806), transcript variant X1, mRNA	1
Arabidopsis thaliana proline-rich family protein mRNA (PRPs)	1
PREDICTED: Sesamum indicum fasciclin-like arabinogalactan protein 17 (LOC105161692), mRNA	1
PREDICTED: Erythranthe guttatus MAG2-interacting protein 2 (LOC105963338), mRNA	1

601

602 **Table 2 Interaction between HIS-CbuHMGA and GST-CbuSPL9 by BLI**

Protein1	Protein2	Conc. of Protein2/nM	K_{on}/Ms	K_{off}/s	K_D/nM
HIS-CbuHMGA	GST-CbuSPL9	2381	5.40E-04	1.74E+03	311
HIS-CbuHMGA	GST-CbuSPL9	1191	5.40E-04	1.74E+03	311
HIS-CbuHMGA	GST-CbuSPL9	595.3	5.40E-04	1.74E+03	311
HIS-CbuHMGA	GST-CbuSPL9	297.6	5.40E-04	1.74E+03	311
HIS-CbuHMGA	GST-CbuSPL9	148.8	5.40E-04	1.74E+03	311

603

Figure 1 The phylogenetic relationship and motif composition analysis of the CbuSPL9 gene

in *C. bungei*. a) Multiple sequence alignment and sequence logo of the *C. bungei* SBP-box domain. Sequence alignment was performed with DNAMAN. The two conserved zinc fingers and NLS are indicated. The sequence logo was obtained from MEME online software. The overall height of the stack indicates the sequence conservation at that position. b) The phylogenetic tree was constructed with MEGA 6.0 by the neighbor-joining (NJ) method with 1000 bootstrap replicates. Bootstrap support is indicated at each node. *A. thaliana* (At), *C. bungei* (Cbu).

Figure 2 Expression profile of *CbuSPL9* in the flower buds and leaf buds during the developmental periods of *C. bungei*. T1-T3 represents the flowering buds and leaf buds collected

in the dormant period; T4-T5 represents the flowering buds and leaf buds collected in the germination period; T6-T9 represents the flowering buds and leaf buds collected in the floral transition period; and T10-T12 represents the flowering buds and leaf buds collected in the reproductive period. D1: Image of flowering buds in the dormant period; D2: Image of leaf buds in the dormant period; G1: Image of flowering buds in the germination period; G2: Image of leaf buds in the germination period; F1: Section of flowering buds in the floral transition period; note the flat generative apex (Ga); F2: Section of leaf buds in floral transition period, note the bulged vegetative apex (V); R1: Image of flowering buds in the reproductive period; and R2: Image of leaf buds in the reproductive period. Notably, even though floral transition was never observed in the leaf buds, the leaf buds collected in the period corresponding to floral transition are henceforth called F2 and R2 for convenience. Error bars indicate SD from three independent biological replicates. *Difference between flowering buds (black) and leaf buds (gray) is significant (Student's test; $p < 0.05$). **Difference between flowering buds (black) and leaf buds (gray) is highly significant (Student's test; $p < 0.01$).

Figure 3 Phenotype of overexpression mutants of *CbuSPL9*. a) The change in floral organs

occurred in the *CbuSPL9*-overexpressing transgenic *Arabidopsis* lines. Image of a normal flower from col as the control (a/I); and the floral organ mutant from *oespl9* (a/II-VI). b) Comparison of the flowering phenotype in both the col and *oespl9* transgenic plants. From top to bottom: col (control) and *oespl9* (transgenic plants). c) and d) are the statistical data of the time of flower bud emergence and the number of rosette leaves. The *oespl9* transgenic plants (black), the *oe-hmga* transgenic plants (gray) and the col plants (white). A total of 30 plants were averaged to obtain the

634 mean. Error bars indicate SD.

635 **Figure 4 The sequence and expression profile analysis of *CbuHMGA*.** a) Sequence analysis of
636 the *CbuHMGA* protein by SMART. b) The expression analysis of *CbuHMGA* between flowering
637 buds and leaf buds during the development periods. T1-T3 represents the flowering buds and leaf
638 buds collected in the dormant period; T4-T5 represents the flowering buds and leaf buds collected
639 in the germination period; T6-T9 represents the flowering buds and leaf buds collected in the
640 floral transition period; and T10-T12 represents the flowering buds and leaf buds collected in the
641 reproductive period. Notably, even though floral transition was never observed in the leaf buds,
642 the leaf buds collected in the period corresponding to floral transition are henceforth called the
643 floral transition period and reproductive period for convenience. Error bars indicate SD from three
644 independent experiments. *Difference between flowering buds (black) and leaf buds (gray) is
645 significant (Student's test; $p < 0.05$). **Difference between flowering buds (black) and leaf buds
646 (gray) is highly significant (Student's test; $p < 0.01$).

647 **Figure 5 Interaction between the two proteins *CbuSPL9* and *CbuHMGA*.** a) Nuclear
648 localization of the *CbuSPL9* protein and *CbuHMGA* protein. The GFP (control) gene,
649 *CbuSPL9*-GFP fusion gene and *CbuHMGA*-GFP fusion gene were expressed transiently in
650 *Nicotiana benthamiana* leaf epidermal cells and observed with confocal microscopy. DAPI, DAPI
651 for nuclear staining image; GFP, GFP green fluorescence image; Merge, the merged images of
652 bright-field, GFP and DAPI staining. b) Binding of GST-*CbuSPL9* to HIS-*CbuHMGA*, with
653 GST-*CbuSPL9* concentrations of 2381.00 nm, 1191.0 nm, 595.30 nm, 297.60 nm, 148.80 nm
654 assessed by real-time biolayer interferometry. c) Yeast two-hybrid assays for the interactions
655 between *CbuSPL9* and *CbuHMGA*. *CbuHMGA* (as prey) was fused with the GAL4 activation
656 domain (AD) in pGADT7, while *CbuSPL9* (as bait) was fused with the GAL DNA-binding
657 domain (BD) in pGBKT7. The positive control was as follows: pGADT7-T and pGBK-53. The
658 negative control was as follows: pGADT7-T and pGBK-Lam. Interactions are indicated by the
659 blue color on SD/-Trp/-Leu/-His/-Ade/X- α -gal medium. d) Confocal images of the BiFC analysis
660 in protoplasts from *P. trichocarpa*. *CbuSPL9* was fused with EYFP^C, and *CbuHMGA* was fused
661 with EYFP^N. EYFP signal was detected in the nucleus of the S1-21 protoplasts transfected by
662 H2A:mCherry and *CbuSPL9*:EYFP^C with (A) *CbuHMGA*:EYFP^N. Bars=50 μ m.

663 **Figure 6 Phenotype of overexpression mutants of *CbuHMGA*.** a) The change in floral organs

664 occurred in the overexpression of the CbuHMGA transgenic Arabidopsis lines. The images are the
 665 floral organ mutant from oe-hmga transgenic Arabidopsis lines (a/I-VI). b) Expression profiles of
 666 atSPL9 homologous genes in the oespl9 transgenic plants, oe-hmga transgenic plants and col.
 667 —■— represents the PCR results in oe-hmga; —■— represents the PCR results in oespl9; and —▲—
 668 represents the PCR results in col. Three independent biological replicates were performed, and
 669 each replicate was measured in triplicate. The error bars show the standard deviation of the results
 670 of three technical replicates.

Expression level of gene

6

4

2

0

T1

T2

T3

T4

T5

T6

T7

T8

T9

T10

T11

T12

Development period

Flowering buds Leaf buds

Dormant period

Floral transition period

Germination period

Reproductive growth period

a

b

c

d

a

b

a

b

Supplementary Table S1 The list of the all Primers used in this paper.

	Gene name	Primer sequence (5'-3')
Primers used in homology-basd cloning of SPL and RACE of homologous gene CbuSPL	SPLF	CAGGTGGAGGGTTGTAAGGT
	SPLR	CCAAGACTCAAGGATCGGGT
	3'RSPLF1	GCAAAGGTTTTGCCAGCAGTG
	3'RACE Out	TACCGTCGTTCCACTAGTGATTT
	3'RSPLF2	CGCCTTGCTGGCCATAACGAGCGTA
	3'RACE Inner	CGCGGATCCTCCACTAGTGATTTCACTATAGG
	5'RSPLR1	AGAAGAGAGAGAGCACCGGTG
	5'RACE Out	CATGGCTACATGCTGACAGCCTA
	5'RSPLR2	CCTGATATGGAAGTTGATACTTT
	5'RACE Inner	CGCGGATCCACAGCCTACTGATGATCAGTCGA TG
Primers used in PCR of cDNA from homologous gene CbuSPL9 and CbuHMGA	QCCbuSPLF	ATGCACTGATTGTATGAGGGAG
	QCCbuSPLR	GACACACAGAAAGCAACAACAT
	CbuSPL9F	ATGGAAAAGGGTTCTTCCTC
	CbuSPL9R	TCAGATAGAGGATTTTGTGAGTTT
Primers used in overexpression analysis	CbuHMGA F	ATGGCGAGCGAAGAAGTACA
	CbuHMGAR	CTACAATGATCCATAAATTAAAAAAC
	121CbuSPL9F	TCTAGAATGGAAAAGGGTTCTTCCTC
	121CbuSPL9R	CCTAGGTCAGATAGAGGATTTTGTGAGTTT
Primers used in analysis for the presence of the transgene	121CbuHMGA F	TCTAGAATGGCGAGCGAAGAAGTACA
	121CbuHMGAR	CCTAGGCTACAATGATCCATAAATTAAAAAAC
	CbuSPL9F	GCCATCATTGCGATAAAGGAAA
	CbuSPL9R	ATCCAGACTGAATGCCCACAGG
Primers used in subcellular localization analysis	CbuHMGA F	GCCATCATTGCGATAAAGGAAA
	CbuHMGAR	ATCCAGACTGAATGCCCACAGG
	CbuSPL9-GFPF	CCATGGATGGAAAAGGGTTCTTCCTC
	CbuSPL9-GFPR	TCTAGATCAGATAGAGGATTTTGTGAGTTT
Primers used in yeast two-hybrid	CbuHMGA-GFPF	CCATGGATGGCGAGCGAAGAAGTACA
	CbuHMGA-GFPR	TCTAGACTACAATGATCCATAAATTAAAAAAC
	BD-CbuSPL9F	CATATGATGGAAAAGGGTTCTTCCTCCT
	BD-CbuSPL9R	GTCGACGTCAGATAGAGGATTTTGTGAGTTTC CC
Primers used in prokaryotic expe	AD-CbuHMGA F	CATATGATGGCGAGCGAAGAAGTACA
	AD-CbuHMGAR	GTCGACGCTACAATGATCCATAAATTAAAAAAC C
Primers used in prokaryotic expe	CbuSPL9-6P-F	GGATCCATGGAAAAGGGTTCTTCCTC
	CbuSPL9-6P-R	CTCGAGTCAGATAGAGGATTTTGTGAGTTT

	Gene name	Primer sequence (5'-3')
ssion Primers used in BIFC	CbuHMGA-HIS-F	CGCGGATCCATGGCGAGCGAAGAAGTACAG
	CbuHMGA-HIS-R	CGCAAGCTTCTAAGCCCCTACGGGTGCGGC
	CbuSPL9F	CACCATGGAAAAGGGTTCTTCCTCCTC
	CbuSPL9R	AAGTGACCAGTGCACAGAAGAATC
	CbuHMGAF	CACCATGGCGAGCGAAGAAGTACAGG
	CbuHMGAR	AGCCCCTACGGGTGCGG
	M13F	GTTGTAAAACGACGGCCAG
	M13R	CAGGAAACAGCTATGAC
	CaMV35S-F	GACGCACAATCCCACTATCC

Supplementary Table S2 Details of motif-sequences of CbuSPL9 were identified by MEME

No.	Sequence (5'-3')	Quantity of motif site
1	YYCRHKVCGMHSKSPKVIVAGLEQRFCCQCSRFLHQLPEFD QGKRSCRRRL	16
2	RCQVEGCKVDLSDAK	16
3	AGHNERRRKP	16
4	RTGRIVFKLFGKEPNEFPIVLRGQILDWLSHSPTDMESYIRP GCIVLTIY	4
5	PAGLTPLHIAAGKDGSEDVLDALTEDPAMVGIEAWKTCRD STGFTPEDYA	3
6	LGLKL GKRTYFEDFW	7
7	AYRPAMLSMVAIAAVCVCVALLFKSCPEVLYVFQ	3
8	FFPFLVVEDDDVCSEIRILETTLEFTGTDSAKQAMDFIHEIG WLLHR	2
9	FPLIRFQWLIEFSMDREWCAVIRKLLNMFFD	3
10	QAETAWEELSDDLGFSLGKLLDLSDDPLWTTGWIYVRVQN QLAFVYNGQV	3
11	GKRSVEWDLNDWKWD	5
12	ATLSELCLLHRAVRKNSKPMVEMLLRY	5
13	EKAQFTVKGMNLRQRGTRLLCSVEGKYLIQETT	4
14	GRDSWPNTTSEGLGNQSATTGKYQLPYQGNSQNP	2
15	DHHHQSRQYMEDENTRAYDSSSHHTNW	2
16	VNFSCDMPILSGRGFMEIEDQ	2
17	FDNHSRSGGFMMDFSAY	2
18	MECNAKPPFQWELENLISFGTSTAEPVKLKPMWEI	2
19	DSNCALSLLSN	7
20	DDQTSNYMLITLLKILSNHSNQSDQ	2

Supplementary Table S3 Statistics of mutant of floral organs in *oe-SPL9* Arabidopsis

Type	overlapping petals	Change in petals number	shrunk petals
CbuSPL9-1	-	*	*
CbuSPL9-2	-	*	-
CbuSPL9-3	-	-	-
CbuSPL9-4	*	-	-
CbuSPL9-5	-	*	-
CbuSPL9-6	*	-	-
CbuSPL9-7	*	*	-
CbuSPL9-8	-	*	-
CbuSPL9-9	-	-	-
CbuSPL9-10	-	-	*
CbuSPL9-11	*	*	-
CbuSPL9-12	-	*	*
CbuSPL9-13	*	*	-
CbuSPL9-14	*	*	-
CbuSPL9-15	-	-	*
CbuSPL9-16	-	-	*
CbuSPL9-17	*	*	-
CbuSPL9-18	*	-	-
CbuSPL9-19	-	*	-
CbuSPL9-20	-	*	
CbuSPL9-21	-	-	*
CbuSPL9-22	-	-	-
CbuSPL9-23	-	*	-
CbuSPL9-24	-	-	*
CbuSPL9-25	-	*	-
CbuSPL9-26	-	*	*
CbuSPL9-27	*	-	-
CbuSPL9-28	-	*	-
CbuSPL9-29	*	*	-
CbuSPL9-30	-	*	-
CbuSPL9-31	-	-	*
CbuSPL9-32	*	-	-
CbuSPL9-33	-	*	-
CbuSPL9-34	-	-	*
CbuSPL9-35	*	*	-
CbuSPL9-36	-	*	-

Supplementary Table S4 Statistics of mutant of flowering time in *oe-SPL9* Arabidopsis

Type	DAY	Type	DAY
CbuSPL9-01	22	WT-01	27
CbuSPL9-02	17	WT-02	27
CbuSPL9-03	23	WT-03	26
CbuSPL9-04	21	WT-04	27
CbuSPL9-05	22	WT-05	25
CbuSPL9-06	16	WT-06	28
CbuSPL9-07	22	WT-07	28
CbuSPL9-08	23	WT-08	26
CbuSPL9-09	23	WT-09	25
CbuSPL9-10	22	WT-10	27
CbuSPL9-11	21	WT-11	25
CbuSPL9-12	22	WT-12	27
CbuSPL9-13	23	WT-13	27
CbuSPL9-14	22	WT-14	26
CbuSPL9-15	22	WT-15	27
CbuSPL9-16	24	WT-16	27
CbuSPL9-17	22	WT-17	25
CbuSPL9-18	18	WT-18	29
CbuSPL9-19	23	WT-19	27
CbuSPL9-20	21	WT-20	28
CbuSPL9-21	24	WT-21	27
CbuSPL9-22	23	WT-22	26
CbuSPL9-23	21	WT-23	26
CbuSPL9-24	24	WT-24	27
CbuSPL9-25	21	WT-25	27
CbuSPL9-26	23	WT-26	25
CbuSPL9-27	21	WT-27	26
CbuSPL9-28	18	WT-28	26
CbuSPL9-29	21	WT-29	27
CbuSPL9-30	20	WT-30	26

Supplementary Table S5 Statistics of mutant of floral organs in *oe-HMGA* Arabidopsis.

Type	Overlapping petals	Change in petals number	shrunk petals
CbuHMGA-1	*	*	
CbuHMGA-2			*
CbuHMGA-3		*	
CbuHMGA-4	-	-	-
CbuHMGA-5		*	*
CbuHMGA-6		*	
CbuHMGA-7	*	*	
CbuHMGA-8		*	
CbuHMGA-9			*
CbuHMGA-10	*	*	
CbuHMGA-11	-	-	-
CbuHMGA-12	*	*	
CbuHMGA-13		*	
CbuHMGA-14		*	
CbuHMGA-15			*
CbuHMGA-16		*	
CbuHMGA-17		*	*
CbuHMGA-18		*	
CbuHMGA-19	-	-	-
CbuHMGA-20	-	-	-
CbuHMGA-21		*	
CbuHMGA-22	*		
CbuHMGA-23			*
CbuHMGA-24		*	
CbuHMGA-25			*
CbuHMGA-26	*	*	
CbuHMGA-27			*
CbuHMGA-28	-	-	-
CbuHMGA-29			*
CbuHMGA-30	*	*	
CbuHMGA-31			*
CbuHMGA-32		*	
CbuHMGA-33			*
CbuHMGA-34		*	
CbuHMGA-35	-	-	-
CbuHMGA-36	*	*	

Supplementary Table S6 Statistics of mutant of flowering time in *oe-HMGA* Arabidopsis.

Type	Time	Type	Time
CbuHMGA-01	25	WT-01	27
CbuHMGA-02	25	WT-02	27
CbuHMGA-03	26	WT-03	26
CbuHMGA-04	24	WT-04	27
CbuHMGA-05	27	WT-05	25
CbuHMGA-06	28	WT-06	28
CbuHMGA-07	26	WT-07	28
CbuHMGA-08	24	WT-08	26
CbuHMGA-09	28	WT-09	25
CbuHMGA-10	27	WT-10	27
CbuHMGA-11	24	WT-11	25
CbuHMGA-12	28	WT-12	27
CbuHMGA-13	27	WT-13	27
CbuHMGA-14	26	WT-14	26
CbuHMGA-15	25	WT-15	27
CbuHMGA-16	28	WT-16	27
CbuHMGA-17	25	WT-17	25
CbuHMGA-18	24	WT-18	29
CbuHMGA-19	28	WT-19	27
CbuHMGA-20	27	WT-20	28
CbuHMGA-21	26	WT-21	27
CbuHMGA-22	26	WT-22	26
CbuHMGA-23	26	WT-23	26
CbuHMGA-24	25	WT-24	27
CbuHMGA-25	27	WT-25	27
CbuHMGA-26	25	WT-26	25
CbuHMGA-27	27	WT-27	26
CbuHMGA-28	26	WT-28	26
CbuHMGA-29	26	WT-29	27
CbuHMGA-30	28	WT-30	26