Trees (2016) 30:2203–2215
DOI 10.1007/s00468-016-1445-y

ORIGINAL ARTICLE

Ectopic expression of a *SOC1* homolog from *Phyllostachys violascens* alters flowering time and identity of floral organs in *Arabidopsis thaliana*

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Received: 12 January 2016 / Accepted: 19 July 2016 / Published online: 26 July 2016

Abstract

***Key message PvMADS56* may regulate the flowering time, the identity of floral organs, and the development of leaves.**

Abstract As a floral activator, *SUPPRESSOR OF OVEREXPRESSION OF CO1/AGAMOUS-LIKE 20* (*SOC1/AGL20*) gene plays a key role in the flowering pathway of *Arabidopsis*. Bamboo MADS box gene *PvMADS56*, a homolog of *SOC1/AGL20*, was cloned from *Phyllostachys violascens*. Sequence comparison and phylogenetic analysis showed that PvMADS56 was closely related to MADS56-like proteins, which are the members of SOC1-like family. *PvMADS56* was widely expressed in all the tested tissues of flowering and non-flowering bamboo plants, and its function was investigated by ectopic expression in transgenic *Arabidopsis* plants. The results showed that the overexpression promoted flowering in wild-type *Arabidopsis* and complemented the delayed flowering phenotype of *soc1 Arabidopsis*. Meanwhile the transgenic plants displayed abnormal floral organs and leaves, low fertility and dwarfism. Overexpression of *PvMADS56* in the wild-type *Arabidopsis* not only caused early flowering by upregulating *Flowering Locus T* and downregulating *Flowering Locus C* expression, but also led to abnormal floral organs by downregulating *APETALA1*, *APETALA3*, *PISTILLATA*, and *AGAMOUS*. Furthermore, PvMADS56 might be a nuclear protein, and interacted with PvAP1 and PvSEP3 from *P. violascens* in the yeast two-hybrid assay. In addition, the activity of *PvMADS56* promoter was enhanced by exogenous abscisic acid (ABA) and methyl jasmonate (MeJA). Taken together, *PvMADS56* may be a multifunctional gene that not only regulates the flowering time but also involves in the identity of floral organs in response to ABA and MeJA.

Keywords *Phyllostachys violascens* · *PvMADS56* · Flowering · Activator

Communicated by F. Canovas.

Electronic supplementary material The online version of this article (doi:10.1007/s00468-016-1445-y) contains supplementary material, which is available to authorized users.

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Introduction

Proper timing of flowering (or "heading date" in cereals) is very important for a plant to complete its life cycle, and is controlled by both environmental signals, such as temperature, day length (or photoperiod) and availability of nutrients (Brambilla and Fornara 2013; Putterill et al. 2004), and internal signals, such as flowering suppressors and activators (Jack 2004; Jarillo and Piñeiro 2011). *SUPPRESSOR OF OVEREXPRESSION OF CO1/AGAMOUS-LIKE 20* (*SOC1/AGL20*) has been identified in *Arabidopsis thaliana* through screening of the suppressor mutants that function in early flowering (Onouchi et al.

2000). As a well-studied activator, SOC1 encodes a MADS box protein (Borner et al. 2000; Lee et al. 2000) that regulates the flowering time, floral patterning, and floral meristem determinacy by integrating multiple flowering pathways derived from photoperiod, temperature, hormones, and age-related signals (Liu et al. 2009; Lee and Lee 2010; Melzer et al. 2008).

Early studies indicated that *FLOWERING LOCUC* (*FLC*) gene involves in the vernalization pathway and represses the expression of *SOC1* and *FLOWERING LOCUC T* (*FT*) by binding to their promoter regions (Helliwell et al. 2006; Hepworth et al. 2002). *FT* and *SOC1* may act in parallel pathways downstream of *CONSTANS* (*CO*), and their mutations partially suppress the early flowering phenotype of *35S::CO* plants (Samach et al. 2000). Meanwhile, activation of *SOC1* by *CO* is dependent on *FT* under long-day condition (Yoo et al. 2005). *APETALA1* (*AP1*, class A gene) is not only essential for the determination of flower meristem identity at early stage, but also for the initiation and development of floral organs at late stage (Bowman et al. 1993). *SOC1*, *AGAMOUS-LIKE 24* (*AGL24*), and *SHORT VEGETATIVE PHASE* (*SVP*), which are repressed by the binding of AP1 to their promoters, act redundantly to maintain shoot identity (Lee and Lee 2010) and prevent class B and C genes from precociously expressing in the emerging floral meristem. Nevertheless, *SEPALLATA 3* (*SEP3*) is also required for the repressions in the process (Liu et al. 2009). Taken together, *SOC1* regulates the flowering time via the mediation of *FLC*, *FT* and *CO*, and the floral development via manipulation of the class A, B and C genes.

SOC1-like genes have been widely studied in both eudicots and monocots, and found to have similar expression patterns but possibly play different functions, depending on the plant species (Lee and Lee 2010). In addition to their roles in floral development and flowering time (Ferrario et al. 2004; Lee and Lee 2010; Ruokolainen et al. 2011), *SOC1*-like genes may have special functions (Cseke et al. 2003; Kimura et al. 2015; Melzer et al. 2008). Recently, a pair of *SOC1* homologues have been found in some plant species including *Oryza sativa* (*OsMADS50* and *OsMADS56*), *Hordeum vulgare* (*HvSOC1-like1* and *HvSOC1-like2*), *Glycine max* (*GmGAL1* and *GmGAL2*), *Citrus sinensis* (*CsSL1* and *CsSL2*) and *Chrysanthemum* (*CISOC1-1* and *CISOC1-2*) (Fu et al. 2014; Papaefthimiou et al. 2012; Ryu et al. 2009; Tan and Swain 2007; Zhong et al. 2012). The pair of *SOC1* homologues usually do not show opposite functions but the pair of *OsMADS50* and *OsMADS56* from rice functions antagonistically in the regulation of LD-dependent flowering by controlling expression of *OsLFL1* and *Ehd1* (Ryu et al. 2009). Ectopic expression of *OsMADS50* causes early flowering (Lee et al. 2004; Tadege et al. 2003), but overexpression of *OsMADS56* results in delayed flowering (Ryu et al. 2009). These data indicate that individual *SOC1*-like genes may play different role in different species, even in the same species.

The majority of bamboo species have a long and unpredictable period of flowering that varies from a few years to 120 years (Janzen 1976; Sharma 1994). They flower after a long vegetative growth phase, and are often followed by the death of flowered clumps (Keeley and Bond 1999). This brings difficulties for disclosing the phenomenon of bamboo flowering. To overcome them, scientists took sequencing approach to dissect the genome of *Phyllostachys edulis* (*Phyllostachys heterocycla*) and the transcriptome of *P. edulis*, *Bambusa oldhamii*, *Bambusa edulis*, *Dendrocalamus latiflorus*, and identified numerous genes that could play important roles in bamboo flowering (Gao et al. 2014; Lin et al. 2010; Peng et al. 2013; Shih et al. 2014; Zhang et al. 2012; Zhao et al. 2014). Novel miRNAs were also identified in *P. edulis* and *D. latiflorus*, and verified to play significant regulatory roles in bamboo flowering (Gao et al. 2015; Zhao et al. 2015). Most recently, proteomic approach has been applied to reveal the veil of bamboo flowering. It turned out that the sporadic bloom of bamboo was associated with stress elements, mobile genetic elements and signal transduction cross-talk elements (Louis et al. 2015). Undoubtedly, these results provide critical starting points for evaluating the regulatory roles of the genes in bamboo flowering, but the functions have to be verified experimentally.

Here, we isolated a *SOC1* homolog from *Phyllostachys violascens* and named it as *PvMADS56*. The gene was characterized by transferring into the wild-type *A. thaliana*. The flowering time and transcriptional levels of the floral-related genes were determined. On the basis that *PvMADS56* gene promoted flowering, and regulated the development of floral organs, leaves and culms, while its promoter activity was upregulated by abscisic acid (ABA) and methyl jasmonate (MeJA), we proposed that *SOC1*-like gene *PvMADS56* may regulate bamboo flowering by responding to ABA and MeJA.

Materials and methods

Plant materials and growth conditions

Bamboo samples used for gene cloning and quantitative real-time PCR (RT-qPCR) were collected in the Bamboo Garden of Zhejiang Agriculture and Forestry University. To analyze the tissue-specific expression of *PvMADS56*, the samples including different tissues (young leaves, mature leaves, culms, bamboo shoots, rhizome roots and flowers) of the flowering and non-flowering *P. violascens* plants were collected on 12 April, 2014. *P. violascens*

usually flower from later of March to mid of April every year. Thus, the leaves and flowers of the flowering plants at different stages were harvested for the gene expression analysis every 2 weeks from 15 March to 12 April, 2014, while the leaves of non-flowering plants were also collected. The sampling time was selected according to the structural change of floral organs (Lin et al. 2012): T1, the time when the floral bud formed and switched from the vegetative phase into reproductive stage (15 March); T2, the time when the inner organs of flower began to form, which was examined by anatomy under stereomicroscope (29 March); T3, the bloom stage when the anther was outcropped from palea (12 April).

All *A. thaliana* including the wild-type and *soc1* mutant (SALK_138131C) (from ABRC) in Columbia-0 (Col-0) background were planted under long days (16 h light/8 h dark) at 22 °C.

Isolation of *PvMADS56* and its promoter

Total RNA was isolated using Trizol method. First-strand cDNA was synthesized according to the manufacturer's recommendation of Reverse Transcriptase M-MLV (TAKARA Company). The sequence of *OsMADS56* (a *SOC1* homolog from rice) ORF was blasted on the *P. edulis* (affinis species of *P. violascens*) transcriptome database (Peng et al. 2013) by BioEdit software. A sequence (ID: PH01000059G1270) with the highest identity to *OsMADS56* was identified, which was actually annotated as a homolog of *OsMADS56* in the database. Then the corresponding genomic sequence (PH01000059) was extracted from the genome database of *P. edulis* (Peng et al. 2013), and used to design the primers for amplifying *PvMADS56* gene and its promoter. A pair of primers was designed to assure the accuracy and integrity of the full-length ORF (Table S1), and a 780 bp cDNA fragment containing a 666 bp open reading frame (ORF) was thus obtained. The *PvMADS56* promoter was amplified by PCR using the primers (Table S1) designed on the basis of the 5′ flanking sequence of PH01000059. A sequence of 1880 bp in length was obtained.

Quantitative real-time PCR

The RT-qPCR primers were designed based on the full-length ORF sequence of *PvMADS56*. Here, *PheUBC18* was used as the internal control gene (Qi et al. 2013) (Table S1). PCR amplification was carried out using SYBR Premix Ex Taq II mix (Takara) and CFX96^{TM} Real-Time PCR Detection System (Bio-Rad). The amplification was 95 °C for 3 min, followed by 40 cycles of amplification (95 °C for 10 s, 60 °C for 20 s). Reactions were performed in 20 μL mixtures consisting of 10 μL 2 × SYBR Premix Ex Taq II mix, 0.5 μL each of forward or reverse primer (Table S1), and 1 μL cDNA template. The sample was made up with water to a final volume of 20 μL. Data were analyzed by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Ectopic expression of *PvMADS56* in *Arabidopsis*

To accomplish the constitutive expression of *PvMADS56*, its full-length ORF was cloned into binary vector pCAMBIA1301 under the control of the cauliflower mosaic virus (CaMV) *35S* promoter. *Arabidopsis* including wild-type and *soc1* mutant were transformed according to the floral dip method as described by Clough and Bent (1998). Transformed *Arabidopsis* seeds were selected on 1/2 MS solid medium containing 50 mg/mL kanamycin, and the survival plants were further confirmed by genomic PCR. The flowering time of *PvMADS56* overexpressing plants was measured by counting the number of rosette leaves and days when the first bolt reached to 1-cm-long inflorescence. Total RNA was extracted from the seedlings of transgenic *Arabidopsis* expressing *35S::PvMADS56* and wild type, and analyzed for the expression levels of *PvMADS56* as well as several other genes associated with flowering including *FT*, *FLC*, *AP1*, *AP3*, *PISTILLATA* (*PI*) and *AGAMOUS* (*AG*) by RT-qPCR using gene-specific primers (Table S1). A SAND family gene (TAIR ID: AT2G28390) was used as the control to normalize the amount of cDNA (Hong et al. 2010).

Yeast two-hybrid assays

To construct the plasmids for yeast two-hybrid assays, the ORFs of *PvMADS56*, *PvAP1* (also named as *PpMADS1*), and *PvSEP3* were amplified using the corresponding primer pair (Table 1). PCR fragments were ligated into the vectors pGBKT7 (BK, bait) or pGADT7 (AD, pray) provided by the Matchmaker^{TM} Gold Yeast Two-Hybrid System (Clontech). All clones were verified by sequencing. The bait and prey plasmids were transformed into yeast strains Y2H Gold and Y187, respectively, by the lithium acetate method (Gietz et al. 1992) for growth on SD/-Trp or SD/-Leu solid medium. The positive bait and prey were mated together in yeast peptone dextrose adenine (YPDA) media, and then spread out on SD/-Leu/-Trp solid medium. Finally, positive interactions were confirmed by plating on the SD/-Leu/-Trp/-His/-Ade with X-α-gal. PvMADS56, PvAP1, PvSEP3 in pGBKT7 or pGADT7 were tested for autoactivation activity. Yeast containing a vector combination of pGBKT7-53 and pGADT7-T that activates the expression of the reporter gene was served as a positive control, and that of pGBKT7-Lam and pGADT7-T as a negative control.

Table 1 Phenotypes of *35S::PvMADS56 Arabidopsis* plants

Groups	Patterning of the floral organs	Leaves	Fertility	Representatives
I	Normal floral organs (4 lines)	Rosette leaves became smaller, and cauline leaves were bigger and wider	Low fertility	Line 1
II	Some floral organs were normal; some had greenish petals, and leaf-like sepals did not enclose inner organs (3 lines)	Rosette leaves became smaller, and cauline leaves were bigger and wider	Low fertility	Line 2
III	Floral organs had greenish petals, and leaf-like sepals did not enclose inner organs (3 lines)	Rosette and cauline leaves were inflexed and became smaller	No fertility	Line 3
IV	Floral organs had greenish petals, leaf-like sepals did not enclose inner organs, and stamens did not exist (2 lines)	Rosette and cauline leaves were inflexed and became smaller	No fertility	Line 4

Subcellular localization of PvMADS56

The ORF sequence of *PvMADS56* without the stop codon was cloned into CaM *35S-gfp* vector to generate a PvMADS56-GFP fusion protein for the subcellular localization study. The particle bombardment method was adopted for the transient expression assays (Wang et al. 1988). Empty vector was used as control. The onion epidermal cells were visualized and recorded by confocal laser scanning microscopy (LSM510, Zeiss, Germany).

Agrobacterium-mediated transient expression for promoter activity assay in *efr-1 Arabidopsis* seedlings subject to exogenous ABA and MeJA treatments

A fragment of 1852 bp in the flanking region of *PvMADS56* (*PvMADS56p*-1852) before the start codon was cloned into the *pBI101* vector with gusA gene using the ClonExpress®II One-Step Cloning Kit (Vazymem™, C112-01). The sequences of the primers are listed in Table 1. The construct was transformed into *Agrobacterium tumefaciens* strain *C58C1* by electroporation. Transient expression experiment was carried out in *efr-1 Arabidopsis* seedlings by AGROBEST (*Agrobacterium*-mediated enhanced seedling transformation) method (Wu et al. 2014). Three-day-old seedlings were treated with ABA (100 μM) or MeJA (100 μM) for 24 h before *Agrobacterium* infection. Ten seedlings grown in each well were infected and three biological repeats were performed in each independent experiment.

For detection of GUS activity, *Arabidopsis* plants were vacuum-infiltrated (three times for 1–2 min each) and incubated for 24–48 h at 37 °C in X-Gluc solution (50 mM Na_2PO_4, 0.5 mM $K_3Fe(CN)_6$, 0.5 mM $K_4Fe(CN)_6{\cdot}3H_2O$, 0.1 % Triton-X-100, and 0.5 mg ml^{-1} X-Gluc) and subsequent destaining of the tissue in 95 % ethanol. RT-qPCR was used to estimate the expression of *GUS* gene using the primers (Rusconi et al. 2013), and the *ACTIN 2* (At3g18780) was used as an internal control (Wu et al. 2014) (Table S1). Three biological and technical replicates were used to assess *GUS* gene expression.

Bioinformatics and statistical analysis

Primers were designed by the Vector NTI and Premier Primer 5. Related proteins were obtained by BLAST analysis from the NCBI database. Phylogenetic analysis was conducted using neighbor-joining method of MEGA 5.0 with default parameters and a bootstrap of 1000 replicates (Tamura et al. 2011). The software ProtParam from ExPASy (http://exPasy.org) was used to analyze the physical and chemical properties of the protein. Subcellular localization of the protein was predicted with WoLF PSORT (http://www.genscript.com/psort/wolf_psort.html). *Cis-acting* regulatory elements of the promoter were predicted using PlantCARE data (Lescot et al. 2002). Statistical analysis was carried out using SPSS 11.5. Differences were analyzed with one-way ANOVA followed by Tukey's test. Significance was accepted at the level of $p < 0.05$.

Results

Isolation of *PvMADS56* and analysis of its expression pattern

The *SOC1*-like gene from *P. violascens* was successfully isolated using the specific primers (Table S1). Its ORF contained 666 bp encoding a protein of 221 aa (GenBank Accession No. KX241616) (Fig. S1). A phylogenetic tree was constructed to determine its relationship with SOC1-like proteins derived from other plant species. As shown in Fig. 1, this protein belonged to the family of SOCl homologs from monocots and was closely related to MADS56-like proteins from *O. sativa*, *Setaria italica*, *Triticum urartu* and *Brachypodium distachyon*. PvMADS56 shared high identity with OsMADS56

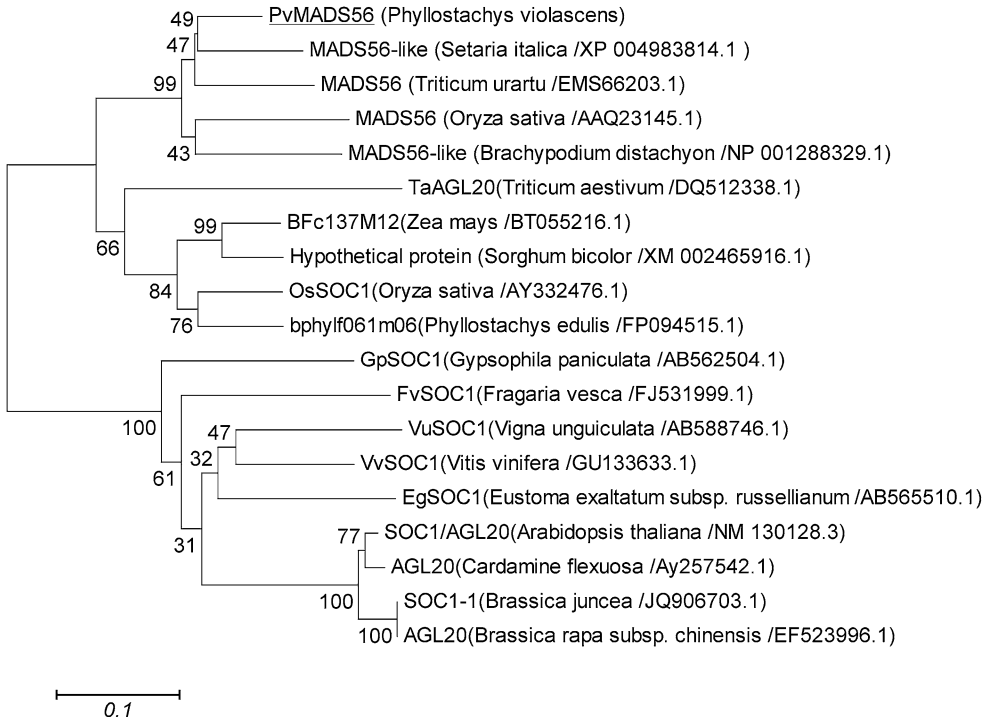

Fig. 1 Phylogenetic analysis of PvMADS56 protein. The phylogenetic tree was generated using MEGA 5.0 and displayed as a phylogram with the branch lengths proportional to the distances. Bootstrap values for 1000 replicates are provided to indicate reliability at each node. The *scale bar* indicates the branch length

(75.65 %), OsSOC1 (65.95 %) and SOC1 (55.16 %) (Fig. S2). Thereby, the name *PvMADS56* was assigned.

To determine the expression level of *PvMADS56* in different bamboo tissues in both flowering and non-flowering *P. violascens*, we performed RT-qPCR using young and mature leaves, culms, bamboo shoots, rhizome roots, and flowers. *PvMADS56* transcripts were detected in all the tested tissues (Fig. 2a). Except bamboo shoots, *PvMADS56* expression in all tissues of the non-flowering plants was higher than that in the flowering plants, especially in the young leaves and culms. Within flowering plants, the highest transcript level of *PvMADS56* was found in flowers.

The temporal expression experiment showed that *PvMADS56* expression gradually decreased in the leaves of flowering (FL) and non-flowering (VL) plants from T1 to T3 (Fig. 2b). Despite *PvMADS56* transcripts declined significantly in the leaves of flowering plants at the early stage, it remained stable in flower (FF) during the flower development (Fig. 2b).

PvMADS56 promoter activity was upregulated by ABA and MeJA in *efr-1 Arabidopsis*

A fragment of 1852 bp located at upstream of the start codon (GenBank Accession No. KX241617) in *PvMADS56* gene was obtained. Its sequence shared a high similarity (90.9 %) with the reference sequence from *P. edulis* genomic database. To explore potential regulatory elements, *PvMADS56* promoter was analyzed using PlantCARE web tool. Several putative *cis*-regulatory elements were deciphered (Table S2). Besides a typical TATA box and a CAAT-box, the promoter also contained *cis*-acting elements including 3-AF1 binding site, ACE, Box I, CAAT-box, G-Box, G-box, GT1-motif involving light responsiveness, and ABRE and CGTCA-motif responding to hormones (Fig. S3). The ABRE, and CGTCA-motif are regulated by ABA and MeJA, respectively (Fig. S3). The other types of *cis*-acting elements including CCAAT motif (an MYBHv1 binding site), GC motif (an enhancer-like element) involving anoxic induction, GCN4_motif mediating endosperm expression and O2 site regulating zein metabolism were also found in the *PvMADS56* promoter region (Table S2).

To determine the regulation of *PvMADS56* promoter activity, we used transient expression by agroinfiltrated in *Arabidopsis* seedlings. The efficiency of transient expression by infection with *C58C1* using AGROBEST was higher in the *efr-1* mutant seedlings than in the wild-type *Arabidopsis* (Col) (Wu et al. 2014). The *PvMADS56p*-1852 was fused with *GUS* reporter gene in *pBI101* vector. Then the *PvMADS56p*-1852:*GUS* chimeric construct was used to infect *Arabidopsis* seedlings by AGROBEST method, while the *pBI101* vector was served as a control. A histochemical assay showed that strong GUS activity was

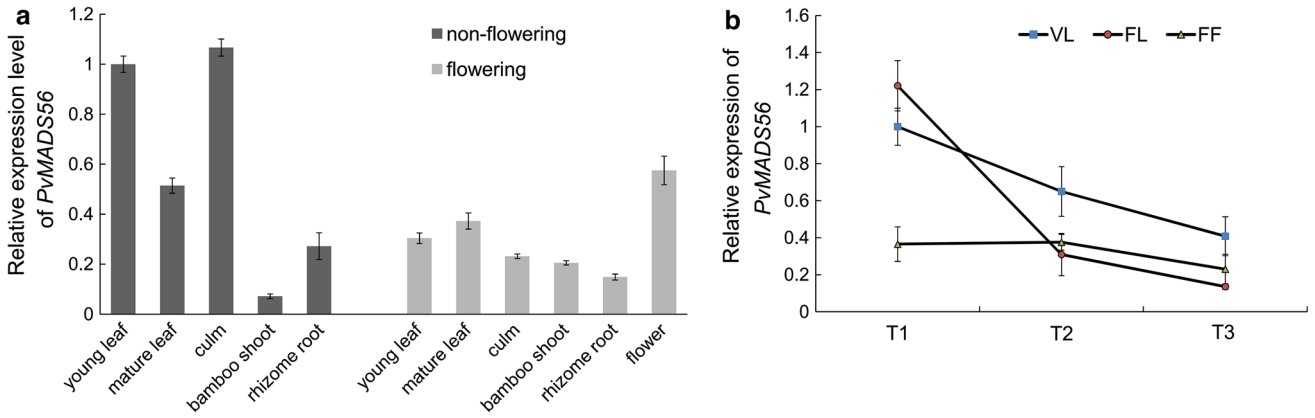

Fig. 2 Spatial and temporal expression of *PvMADS56* in the flowering and non-flowering *P. violascens*. **a** Relative expression level of *PvMADS56* was detected in different tissues of flowering and non-flowering plants. **b** Relative expression level of *PvMADS56* mRNA was detected in the leaves of non-flowering (VL), flowering plants (FL) and flowers (FF) of the flowering plants during flower development. T1, T2 and T3 represented the sampling time points. T1 the time when the floral bud formed and switched from the vegetative phase into reproductive stage (15 March); T2 the time when the inner organs of flower began to form, which was examined by anatomy under stereomicroscope (29 March); T3 the bloom stage when the anther was outcropped from palea (12 April). *Error bars* on each column indicate the standard deviations from three replicates

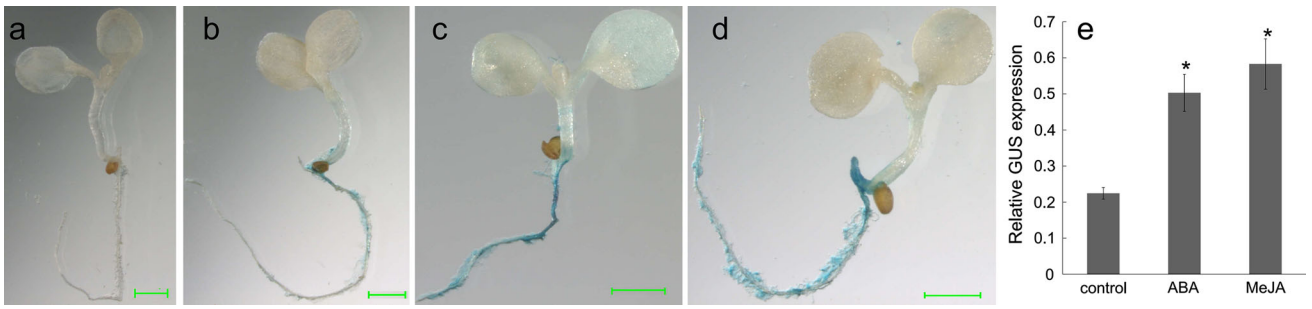

Fig. 3 *PvMADS56* promoter-*GUS* transient expression in *Arabidopsis* under treatments with ABA and MeJA. **a** Control plants carrying the *pBI101* vector. **b** Control plants carrying *PvMADS5p*-1852:*GUS* construct without treatments. **c** Plants carrying *PvMADS5p*-1852:*GUS* construct were treated with ABA (100 μM) for 24 h. **d** Plants carrying *PvMADS5p*-1852:*GUS* construct were treated with MeJA (100 μM) for 24 h. **e** RT-qPCR analysis of *GUS* expression in response to ABA (100 μM) and MeJA (100 μM). *Scale bars* represent 1 mm. Data are expressed in mean ± SD. *Error bars* on each column indicate the standard deviation from three replicates. *Asterisks* indicate significant differences (*$P < 0.05$)

mainly found in roots (Fig. 3a, b), indicating *PvMADS56p*-1852 was functional in *Arabidopsis*. Furthermore, ABA and MeJA treatments for 24 h induced GUS expression driven by *PvMADS56p*-1852, and GUS activity became stronger in the treated plants when compared with the untreated plants (Fig. 3c, d). Based on RT-qPCR, *GUS* expression in *Arabidopsis* was also significantly increased under ABA and MeJA treatments ($p < 0.05$) (Fig. 3e).

Ectopic expression of *PvMADS56* caused early flowering and abnormal floral organs in *Arabidopsis thaliana*

To examine the function of *PvMADS56* in the regulation of flowering, we generated transgenic *Arabidopsis* plants in which *PvMADS56* was overexpressed. A total of 13 independent T_1 transgenic lines were obtained based on Kanamycin selection and genomic DNA PCR. An early flowering phenotype (Fig. 4a, b) and abnormal floral organs (Fig. 5) were observed in 12 transgenic lines, whose flower sizes were all smaller than the wild-type plant. They were classified into four groups based on the abnormal severity of the floral organs with least abnormality in Group I and most abnormality in Group IV (Table 1; Fig. 5). As shown in Fig. 6 and Table 1, *35S::PvMADS56* transgenic plants also showed abnormal leaves. The rosette leaves of all the transgenic lines became smaller (Fig. 6a) and were easier to enter senescence than those in the wild-type plants. The fifth rosette leaves of the transgenic lines (Groups III and IV) began to be inflexed (Fig. 6a, h), meanwhile the inflexed phenotype was also observed in the cauline leaves (Fig. 6c). However, cauline leaves in the transgenic lines of Groups I and Group II were bigger and wider than those in the wild-type *Arabidopsis* plants

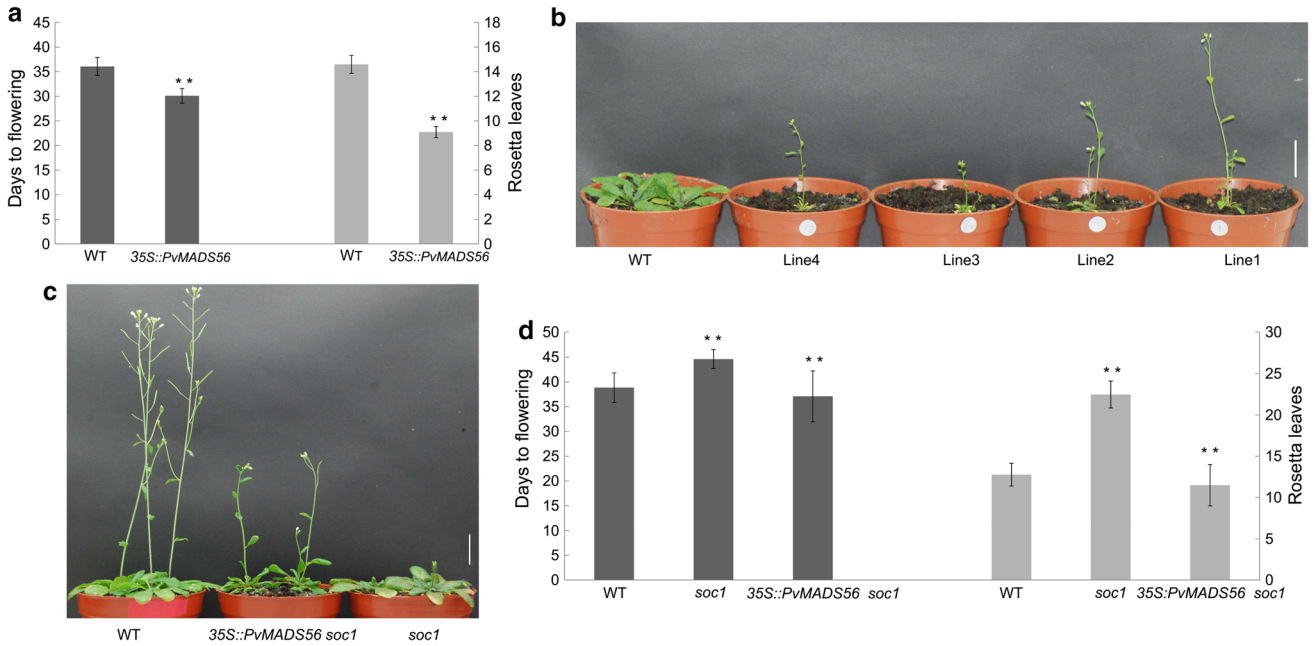

Fig. 4 Phenotypic differences between transgenic plants and WT *Arabidopsis thaliana*. Overexpression of *PvMADS56* enhanced flowering in WT *Arabidopsis*. **a** Days to flowering and the counts of rosette leaves for T_1 *35S::PvMADS56* transgenic *Arabidopsis* plants ($n = 13$); **b** Flowering phenotype of *PvMADS56* transgenic *Arabidopsis* plants under LD conditions. **c** Early flowering phenotype of *PvMADS56* transgenic *Arabidopsis* plants under LD conditions; **d** Days to flowering and rosette leaves counts for T_1 *35S::PvMADS56* transgenic *Arabidopsis* plants ($n = 15$). Data are mean ± SD. The *scale bar* represents 2 cm. *Error bars* on each column indicate standard deviations from three replicates. *Asterisks* indicate significant differences ($^{**}P < 0.01$)

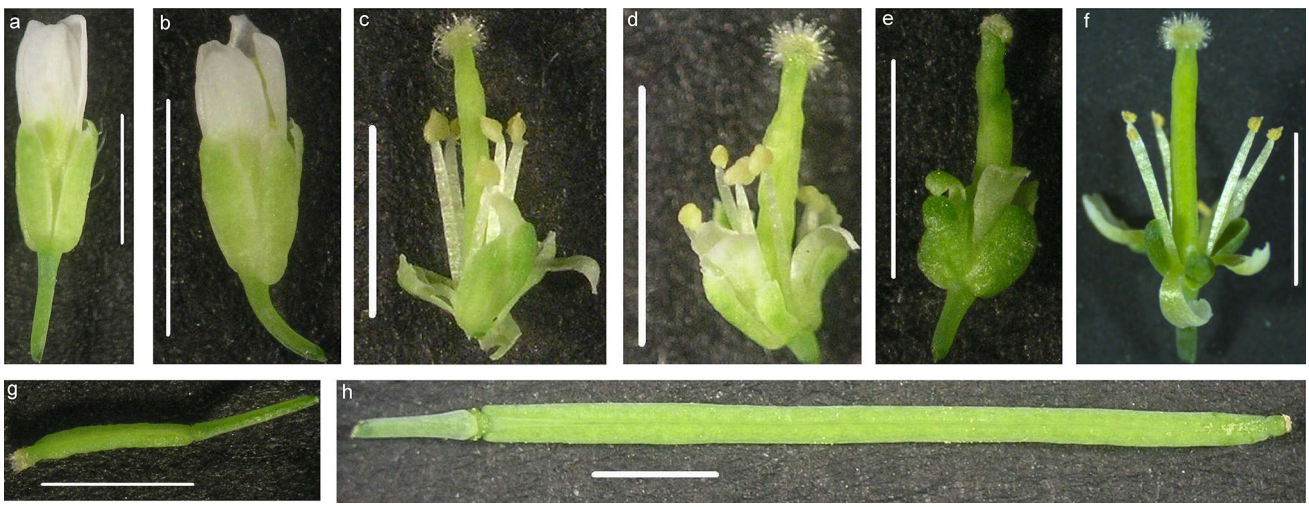

Fig. 5 Flower phenotypes of WT and *soc1 Arabidopsis* overexpressing *PvMADS56* under LD conditions. **a** Flowers of WT *Arabidopsis*. **b**, **c**, **d**, **e** Flowers of WT *Arabidopsis* overexpressing *PvMADS56*. **f** Flowers of *soc1 Arabidopsis* overexpressing *PvMADS56*. **g** Siliques of *35S::PvMADS56* transgenic *Arabidopsis*. **h** Siliques of WT *Arabidopsis*. All the *scale bars* represent 2 mm

(Fig. 6d). Besides, *35S::PvMADS56* transgenic plants became dwarfs (data not shown) and had shorter capsules (Fig. 5g) with reduced fertility (Groups I and II), or even without fertility (Groups III and IV).

To further recapitulate its function, *PvMADS56* was overexpressed in *soc1* mutant. It turned out that *PvMADS56* completely rescued the late-flowering phenotype of *soc1* mutant (Fig. 4c, d). Similar to *35S::PvMADS56* transgenic *Arabidopsis*, *35S::PvMADS56 soc1* transgenic plants also displayed abnormal floral organs (Fig. 5f) and leaves (rosette and cauline leaves) (Fig. 6e, g) and low fertility.

To explore if the abnormal phenotype of the floral organs in the transgenic plants was associated with the

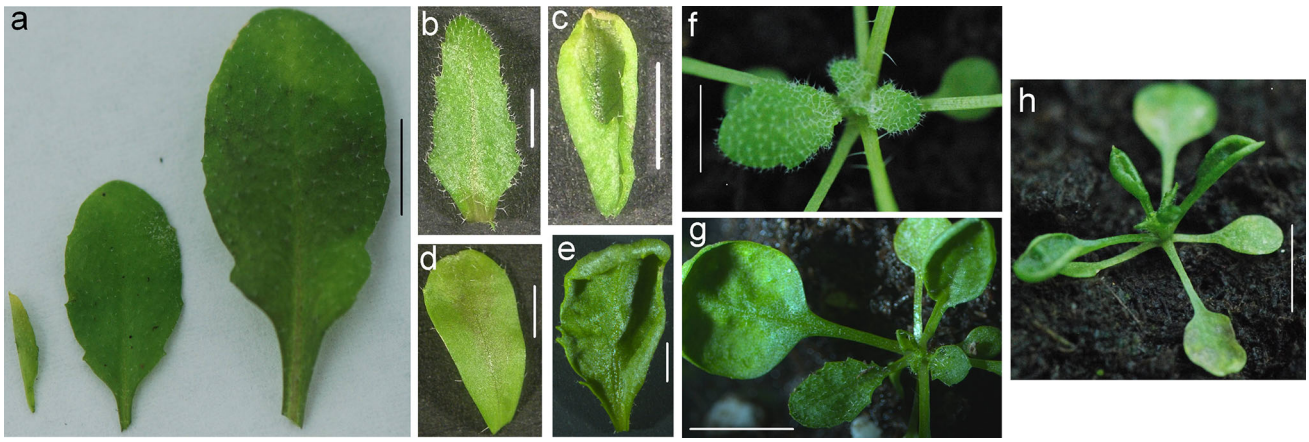

Fig. 6 Leaf phenotypes of WT and *soc1 Arabidopsis* overexpressing *PvMADS56* under LD conditions. Rosette leaf phenotypes of WT and transgenic *Arabidopsis* plants (**a**, **f**, **g**, **h**). **a** Rosette leaf phenotypes of WT (*right*) and overexpressing *PvMADS56* in WT *Arabidopsis* (*middle* and *left*) when they grew for 45 days; **f** WT *Arabidopsis*; **g** *35S::PvMADS56* transgenic plants; **h** *35S::PvMADS56 soc1* transgenic plants. Cauline leaf phenotypes of WT and transgenic *Arabidopsis* plants (**b**–**e**). **b** Cauline leaf phenotypes of WT *Arabidopsis*; **c**, **d** cauline leaf phenotypes overexpressing *PvMADS56* in WT *Arabidopsis*; **e** cauline leaf phenotypes overexpressing *PvMADS56* in *soc1 Arabidopsis*. *Scale bars* in **a**, **f**, **g**, **h** represent 5 mm, in **b**–**e** represent 2 mm

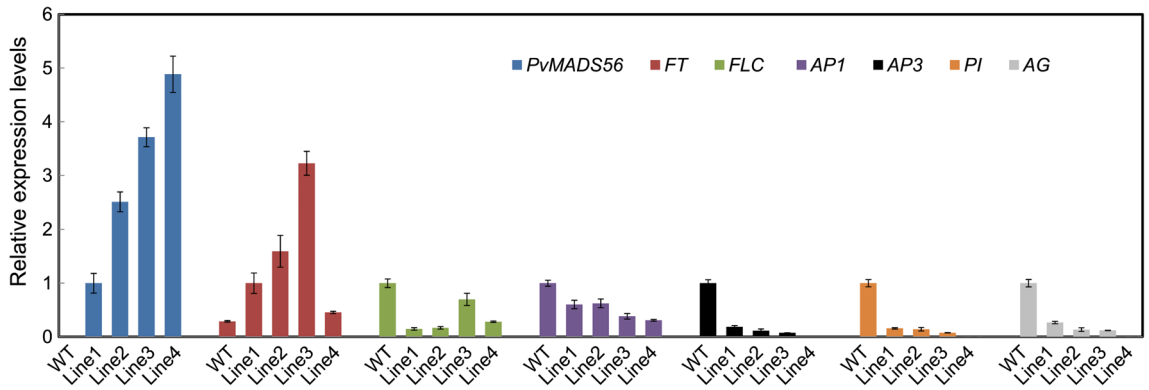

Fig. 7 Expression analysis of *PvMADS56* and some flowering-related genes in transgenic plants overexpressing *PvMADS56* in WT *Arabidopsis*. Data are mean ± SD. *Error bars* on each column indicate the standard deviations from three replicates. *Blue columns* represent *PvMADS56* gene; *red columns* are for *Flowering Locus T* (*FT*) gene; *green columns* for *Flowering Locus C* (*FLC*) gene; *purple columns* for *APETALA1* (*AP1*) gene; *black columns* for *APETALA3* (*AP3*) gene; *yellow columns* for *PISTILLATA* (*PI*) gene and *gray columns* for *AGAMOUS* (*AG*) gene

expression level of *PvMADS56*, its transcripts were tested in representative samples from the four individual groups of *35S::PvMADS56* transgenic *Arabidopsis* plants (Table 1). The RT-qPCR results showed that the expression level of *PvMADS56* was gradually increased with the abnormal severity of the floral organs (Fig. 7).

Taken together, our data indicated that *PvMADS56* gene could regulate the development of floral organs and cause early flowering.

Induction of flowering-related genes in *35S::PvMADS56* transgenic *Arabidopsis* plants

Genes such as *FLC*, *FT*, *AP1*, *AP3*, *PI* and *AG* are involved in floral initiation and development in *Arabidopsis*. We assumed that overexpression of *PvMADS56* affects the expression of these genes in transgenic *Arabidopsis* plants, resulting in early flowering and abnormal floral organs. To test this hypothesis, we determined the expression of *FLC*, *FT*, *AP1*, *AP3*, *PI*, and *AG* in transgenic plants using RT-qPCR.

In *35S::PvMADS56* transgenic *Arabidopsis* plants, the level of *FT* (an activator) was significantly increased (mean ≈5.4-folds more than that in wild-type control plants), while the level of *FLC* (a repressor) was dramatically decreased (mean ≈4.4-folds less than that in wild-type control plants) (Fig. 7). The phenotypic alterations of flowering organs in the *35S::PvMADS56* transgenic plants were also observed. According to the ABC model, the related genes such as *AP1* (A-function gene), AP3/PI (B-

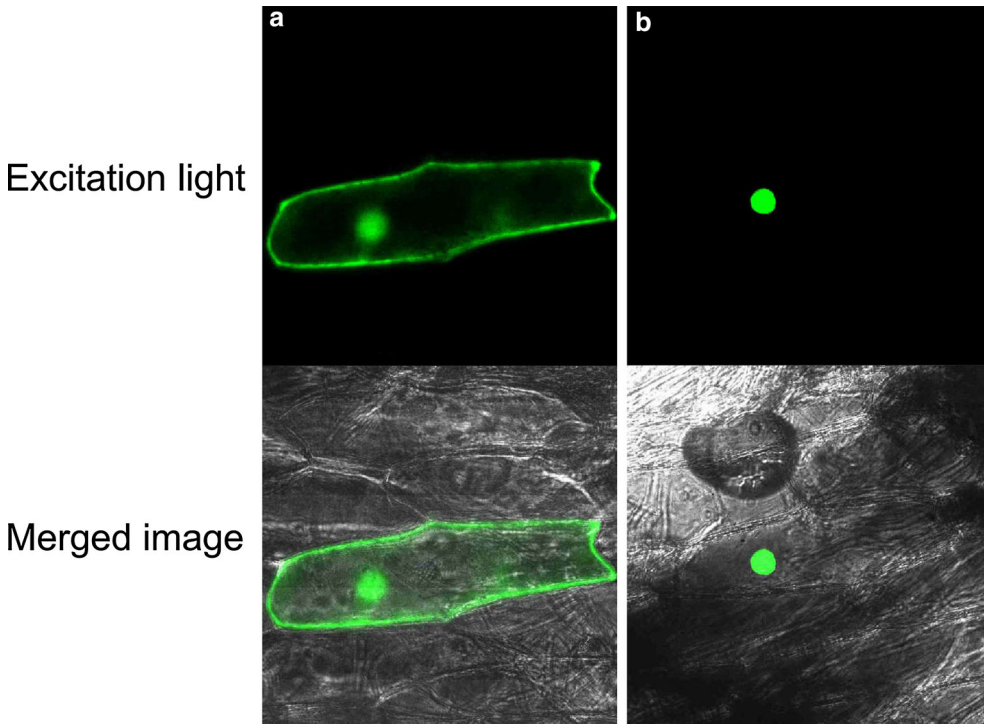

Fig. 8 Confocal images of PvMADS56 in onion epidermal cells. **a** Fluorescence of the control GFP plasmid was distributed throughout the cell; **b** fluorescence of the PvMADS56-GFP fusion plasmid was strongly detected in the nucleus. *Scale bars* represent 100 μm

function gene) and *AG* (C-function gene) were selected for analysis. The expression levels of *AP1*, *AP3*/*PI* and *AG* were downregulated in the four individual representatives from Groups I to IV and the downregulation levels correlated with the abnormal severity of the floral organs (Fig. 7). These results indicated that *PvMADS56* involves in the development of floral organs by regulating the expression of *AP1*, *AP3*/*PI* and *AG* in *Arabidopsis*.

PvMADS56 is a nuclear protein and interacts with PvAP1 and PvSEP3

To determine the subcellular location of PvMADS56 protein, we adopted particle bombardment method. Green fluorescent protein (GFP) was used as the marker in the in vivo targeting experiment. As shown in Fig. 8, the fusion protein PvMADS56-GFP was located in the nucleus of the onion epidermal cells, whereas the control GFP was uniformly distributed in the whole onion cell.

In *Arabidopsis*, *SOC1* regulates the flower development by mediating *AP1* and *SEP3*, and interacts with AP1 and SEP3 both in vitro and vivo (de Folter et al. 2005; Liu et al. 2013). To test if PvMADS56 interacts with PvAP1 and PvSEP3 in *P. violascens* (Lin et al. 2009), a yeast two-hybrid assay was performed. The fact that PvMADS56, PvAP1 and PvSEP3 in pGBKT7 or pGADT7 did not show autoactivation activity makes them suitable to test their mutual interactions (Fig. 9a). Transformants of pairs pGADT7-PvMADS56 and pGBKT7-PvAP1, pGBKT7-PvMADS56 and pGADT7-PvAP1, pGADT7-PvMADS56 and pGBKT7-PvSEP3, and pGBKT7-PvMADS56 and pGADT7-PvSEP3 grew well on the SD/-Leu/-Trp/-His/-Ade media (Fig. 9b) and demonstrated the activity of β-galactosidase, indicating that PvMADS56 could bind to PvAP1 and PvSEP3 in vitro.

Discussion

Here, we identified and characterized a *SOC1*-like gene *PvMADS56* from *P. violascens*. Amino acid sequence alignment and phylogenetic analysis showed that PvMADS56 was closely related to the OsMADS56-like SOC1 homologues in monocots. Similar to other *SOC1*-like genes (Lee and Lee 2010), *PvMADS56* was expressed in all the examined tissues in bamboo. Its overexpression caused early flowering by upregulating *FT* and downregulating *FLC* in *Arabidopsis*. Consistent with many *SOC1*-like genes (Ferrario et al. 2004; Lei et al. 2013; Zhong et al. 2012; Ding et al. 2013), *PvMADS56* complemented the delayed flowering phenotype of *soc1* mutant completely. These results suggested that *PvMADS56* plays an evolutionarily conserved role in the regulation of flowering time in bamboo. Both PvMADS56 and OsMADS56 shared a high identity in their protein sequences, but they performed opposite function on flowering time. This may be resulted

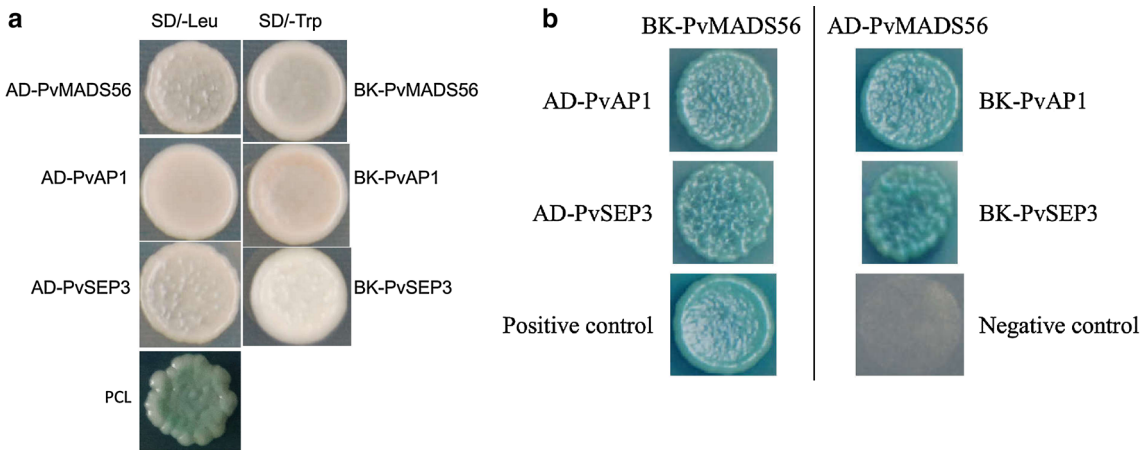

Fig. 9 PvMADS56 protein interacted with PvAP1 and PvSEP3, respectively. Interactions between these proteins were examined by yeast two-hybrid experiment. **a** The positive control (PCL) turned *blue color*, while the PvMADS56, PvAP1 or PvSEP3 remained *white* whether they were served as prey or as bait; **b** Yeast containing dual vectors of pGBKT7-53 and pGADT7-T served as the positive control, that of pGBKT7-Lam and pGADT7-T as the negative control. SD/-Leu/-Trp/-His/-Ade medium was used for clone selection purpose. Appearance of X-α-gal activity mirrors positive interaction

from evolutionary diversity of the genes, leading to the different flowering characteristics between bamboo and rice.

FLC, an upstream target of *SOC1* represses *SOC1* by directly binding to its promoter (Lee and Lee 2010). *FT* could move from leaves to the meristem and initiate flowering through activation of *SOC1* (Corbesier et al. 2007). These results indicate that *FLC* and *FT* can directly regulate *SOC1* in *Arabidopsis*. In this paper, all the four representative lines displayed an early flowering phenotype, and the expression of flowering time integrators *FLC* and *FT* was, respectively, downregulated or upregulated in *35S::PvMADS56 Arabidopsis* plants, suggesting that *FLC* and *FT* were possible targets for *PvMADS56* in transgenic plants. Although the expression levels of *FT* and *FLC* in *35S::PvMADS56* plants were affected, they did not show a correlation with *PvMADS56* expression. For example, line four displayed highest expression level for *PvMADS56* but lowest for *FT* and lower for *FLC* among these four lines. These results suggested that *PvMADS56* promotes flowering by regulating *FT* and *FLC* in *35S::PvMADS56* plants, however, whether the regulation on *FLC* and *FT* is indirect or direct needs further experimental evidences.

The identity genes of floral organs such as *AP1* (A class), *AP3*/*PI* (B class), and *AG* (C class) function to produce floral organs (sepals, petals, stamens and carpels) individually or in combination (Coen and Meyerowitz 1991). *SOC1*, *AGL24*, and *SVP* are required for the timely activation of B and C floral genes (Liu et al. 2009). *AP1* contributes to floral patterning through regulation of the repression of *SEP3* by *SVP*, *SOC1* and *AGL24* (Liu et al. 2009). Moreover, these proteins such as AP1, AP3, PI, AG and SEP3 could form heterodimers (AP1-SEP3 and AP3-PI) or trimmers (AP3-PI-AP1 and AP3-PI-AP3) to specify the floral organs (Honma and Goto 2001; Pelaz et al. 2001). In our study, the *35S::PvMADS56* transgenic plants showed abnormal floral organs and significant reduced expression levels for *AP1*, *AP3*, *PI*, and *AG*. *AP3*/*PI* and *AG* expressions were not detectable in line 4 from Group IV with the most severe phenotype. Considering the observation that the abnormal severity of floral organs correlated with the expression levels of the aforesaid genes, we proposed that *PvMADS56* might involve in the development of floral organs in a manner dependent either on *AP1*, *AP3*, *PI*, *AG* individually or on their complex in *Arabidopsis*.

PvMADS56 was widely expressed in the tested tissues in both flowering and non-flowering *P. violascens*. In non-flowering plants, its expression level was different from the other *SOC1*-like genes (Ferrario et al. 2004; Lei et al. 2013; Zhong et al. 2012), higher in culms and young leaves than in other tissues. In both flowering and non-flowering plants, the level of *PvMADS56* mRNA gradually decreases in leaves along with leaf development, also was different from *SOC1* and *OsSOC1*, whose transcripts gradually increased with leaf development (Lee et al. 2000, 2004). *35S::PvMADS56* transgenic *Arabidopsis* plants exhibited phenotypes of dwarfism and abnormal leaves (rosette and cauline leaves), suggesting that *PvMADS56* involves in the development of culms and leaves.

GA-responsive elements exist in *SOC1* promoter region in *Arabidopsis* and GA could activate *SOC1* expression under short days (8 h light/16 h dark) (Moon et al. 2003). To further explore if the expression pattern is associated with the regulation of *PvMADS56* promoter, we analyzed the *cis*-regulatory elements within the promoter. It turned

out that the promoter contains no GA-responsive element but ABRE and CGTCA-motif (*cis*-acting elements), which respond to ABA and MeJA, respectively. To testify if ABA and MeJA affect the activity of *PvMADS56* promoter, exogenous phytohormonal treatments in *Arabidopsis* were performed using *Agrobacterium*-mediated transient expression. Histochemical staining showed that GUS activity driven by *PvMADS56* promoter was significantly increased after ABA and MeJA treatments. Consistent with the role of endogenous ABA which activates flowering in several species including the key floral gene *FT* in *Arabidopsis* (Conti et al. 2014), early study has shown that higher level of ABA promoted flower bud differentiation in *P. violascens* (Lu et al. 2012). MeJA also affected flowering time and floral structure in some species (Diallo et al. 2014). Thus, ABA and MeJA might affect bamboo flowering by regulating *PvMADS56* expression. In addition, ABA was gradually increased with leaf senescence in bamboo (Xie 2004). In our study, the leaves of transgenic *Arabidopsis* overexpressing *PvMADS56* were apt to enter senescence. Therefore, we concluded that *PvMADS56* might involve in flowering time, development of floral organs and leaves in bamboo by responding to ABA and MeJA.

SOC1-like proteins are located in cytoplasm, but SOC1 can move into nucleus from cytoplasm when it combines with AGL24 (Lee et al. 2008, 2013; Zhong et al. 2012). The particle bombardment assays showed PvMADS56 locates only in the nucleus, suggesting that the protein might be different in function. *SOC1* expression is regulated in emerging floral meristem by AP1 which binds to *SOC1* promoter (Liu et al. 2007, 2009). SOC1 can interact with SEP3 as demonstrated by yeast two-hybrid assay (de Folter et al. 2005). In this aspect, PvMADS56 was similar to SOC1 and interacted with PvSEP3 in the yeast two-hybrid assay. Interestingly, PvMADS56 also interacted with bamboo PvAP1, different from SOC1 whose promoter does interact with AP1. These data implied that PvMADS56 might differ from *Arabidopsis* SOC1 in signal transduction pathways. Overexpression of *AP1* or *SEP3* both causes early flowering besides abnormal floral organs (Mandel et al. 1992; Wuest et al. 2012). In deed, *PvAP1* and *DlMADS8* (an ortholog of *PvSEP3*) exhibit the phenotype of early flowering in *Arabidopsis* when they are overexpressed (Lin et al. 2009; Tian et al. 2006). Thus, *PvMADS56* might be more similar to *SOC1* in the aspect of functioning on flowering time than in the aspect of regulating flower development by regulating *PvAP1* and *PvSEP3* in bamboo.

In conclusion, our data suggested that *PvMADS56* in association with *PvAP1* and *PvSEP3* may involve in regulation of flowering time and development of floral organs and leaves in bamboo through responding to ABA and MeJA. *PvMADS56* shared high sequence similarity with while functions differently from many other *SOC1* homologs. Our results promote a better understanding of the underlying mechanism of bamboo flowering.

Author contribution statement Shinan Liu conducted RT-qPCR assays, promoter cloning, activity measurement, *Arabidopsis* cultivation, phenotype observation, subcellular location, yeast two-hybrid assay, analyzed data and wrote the manuscript. Tiantian Qi and Jingjing Ma performed the cultivation of *Arabidopsis*. Tengfei Ma carried out gene cloning. Xinchu Lin and Luyi Ma designed the experiments and revised the manuscript.

Acknowledgments We are grateful to Professor Erh-min Lai (Institute of Plant and Microbial Biology, Academia Sinica, Taipei, Taiwan) for supplying the *efr-1 Arabidopsis* seeds and *Agrobacterium tumefaciens* strain *C58C1*. This study was funded by the National Natural Science Foundation of China [Grant Nos. 31000295 and 31270715] and the China Scholarship Council to X Lin.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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