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PtSVP, an *SVP* homolog from trifoliate orange (*Poncirus trifoliata* L. Raf.), shows seasonal periodicity of meristem determination and affects flower development in transgenic *Arabidopsis* and tobacco plants

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Abstract A MADS-box gene was isolated using the suppressive subtractive hybridization library between early-flowering mutant and wild-type trifoliate orange (*Poncirus trifoliata* L. Raf.). This gene is highly homologous with *Arabidopsis SHORT VEGETATIVE PHASE* (*SVP*). Based on real-time PCR and in situ hybridization during bud differentiation, *PtSVP* was expressed intensively in dormant tissue and vegetative meristems. *PtSVP* transcripts were detected in apical meristems before floral transition, then down-regulated during the transition. *PtSVP* expression was higher in differentiated (flower primordium) than in undifferentiated cells (apical meristems). The *PtSVP* expression pattern during apical meristem determination suggested that its function is not to depress flower initiation but to maintain meristem development. Transcription of *PtSVP* in *Arabidopsis svp-41* showed partially rescued *SVP* function. Ectopic overexpression of *PtSVP* in wild-type *Arabidopsis* induced late flowering similar to the phenotypes induced by other *SVP/StMADS-11-like* genes, but transformants produced additional trichomes and floral defects, such as flower-like structures instead of carpels. Ectopic expression of *PtSVP* in tobacco also caused additional florets. Overexpression of *PtSVP* in tobacco inhibited early transition of the coflorescence and prolonged coflorescence development, thus causing additional florets at the later stage. A yeast two-hybrid assay indicated that *PtSVP* significantly interacted with *PtAP1*, a homolog of *Arabidopsis APETALA1* (*AP1*). These findings suggest that citrus *SVP* homolog genes are involved in flowering time regulation and may influence inflorescence meristem identity in some conditions or genetic backgrounds. *SVP* homologs might have evolved among plant species, but the protein functions are conserved between *Arabidopsis* and citrus.

Keywords Floral transition · Meristem determination · *SVP/StMADS-11-like* gene · Trifoliate orange

Abbreviations

FT	*FLOWERING LOCUS T*
GFP	Green fluorescent protein
ORF	Open reading frame
SOC1	*SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1*
SP	Stamen primordium
SVP	*SHORT VEGETATIVE PHASE*
WUS	*WUSCHEL*

Zhi-Min Li and Jin-Zhi Zhang contributed equally to this work.

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Introduction

The reproductive transition in perennial woody plants does not occur until several years of repeated seasonal changes and alternative growth. Although many genes related to flowering regulation have been identified and a fine gene network has been described in *Arabidopsis*, the model herbaceous plant (Blázquez 2000, 2005), how this gene network works in woody plants remains a challenging subject in the field of plant molecular development. Recently, differences in rates of molecular evolution have been noted between woody perennials and annual herbaceous species (Dornelas et al. 2007; Wilkie et al. 2008; Yang et al. 2008). These differences have been presumed to reflect differences in generation times (Martín-Trillo and Martínez-Zapater 2002; Meilan 1997).

Extensive research on *Arabidopsis* has revealed that flowering can be triggered by light, temperature, gibberellins, and autonomous regulation pathways that initiate expression in several integrator genes that respond to both internal and environmental cues. These integrator genes include *FLOWERING LOCUS T* (*FT*), *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1* (*SOC1*), and *LEAFY* (Blázquez 2000; Fujiwara et al. 2005). The main theory is that the vegetative growth in *Arabidopsis* is maintained by repressing the function of flowering genes (Koornneef et al. 1998). *SHORT VEGETATIVE PHASE* (*SVP*), a MADS-box gene, is an important flowering repressor in *Arabidopsis* (Hartmann et al. 2000). Recent molecular cloning and functional analyses of *SVP* indicated that *SVP* may be another central regulator of the flowering regulatory network because the gene is controlled by the autonomous, thermosensory, and gibberellin pathways and directly represses *SOC1* transcription in the shoot apex and leaf (Li et al. 2008). Moreover, a moveable florigen *FT*, expressed in the leaf, is also modulated by *SVP* (Lee et al. 2007b). The SVP protein associates with the promoter regions of *SOC1* and *FT*, where the potent repressor *FLOWERING LOCUS C* (*FLC*) binds, and SVP interacts with FLC in vivo during vegetative growth (Li et al. 2008). Some *SVP/StMADS-11-like* genes from Chinese cabbage (Lee et al. 2007a) and barley (Trevaskis et al. 2007) also act as flowering repressors. Another *SVP* homolog, *PkMADS1*, from the woody plant *Paulownia kawakamii* promoted vegetative growth (Prakash and Kumar 2002), whereas the *SVP/StMADS-11-like* gene from *Eucalyptus* (*EgSVP*) seems to have a slightly different function. The ectopic expression of *EgSVP* in *Arabidopsis* caused a slight delay in flowering time and produced additional inflorescences (Brill and Watson 2004). Thus, in woody plants *SVP* might have evolved functional diversification not seen in annual herbaceous plants.

Precocious trifoliate orange was discovered as a spontaneous mutant from wild-type trifoliate orange (*Poncirus trifoliata* L. Raf.; Liang et al. 1999). A major characteristic of precocious trifoliate orange is that its juvenile phase is shortened to 1 to 2 years, whereas the wild-type plant has a juvenile period of 6 to 8 years. This precocious mutant is valuable for investigating the flowering mechanism in woody plants because of its short juvenile phase and special flowering behaviors.

In a previous study, we established a two-dimensional suppressive subtractive hybridization library to identify differential gene expression in shoot meristems between the precocious mutant and wild-type trifoliate orange. A total of 274 expressed sequence tags were discovered, including *FT*, *TFL1*, *BAM*, and other flowering-related genes (Zhang et al. 2009). In this study, *PtSVP*, a differentially expressed gene, was characterized. The temporal and spatial expression patterns of *PtSVP* were investigated using in situ hybridization and real-time PCR. Its function was further studied using ectopic transgenic analysis.

Materials and methods

Plant materials and sample collections

Plants of precocious and wild-type trifoliate orange were grown under natural environmental conditions in experimental fields of the National Citrus Breeding Center of Huazhong Agricultural University, Wuhan, China (30°35′ N, 114°17′ E). Plants in the first year after sowing were considered to be juvenile, and adult trees of the mutants were 3–5 years old and had flowered several times. All plant tissues were sampled according to the demands of each experiment, immediately frozen in liquid nitrogen, and stored at −80°C until used.

For morphological observation and floral development, 600 buds that displayed a similar growing condition were selected and tagged when they were sprouting. Ten shoots derived from these buds were sampled every 3 days in the first month and every 7 days thereafter. The shoots were separated into segments with one node, fixed, stored in FAA, and grouped according to their bud location. Paraffin section analysis followed the method of Yao et al. (2007).

To analyze the fluctuation of *PtSVP* expression over the annual cycle and its relationship to flower development, total RNA for RT-RCR and real-time PCR was isolated from bud samples from adult trees at the following developmental stages: dormant buds, sprouting buds, buds on the shoots in quick growth, and mature buds. Tissue samples from three randomly selected trees were collected at approximately 2-month intervals throughout the 2006 and 2007 growth cycles.

The expression patterns and relationship of *PtSVP* to *CiFT* and *CsWUS* were investigated during shoot development. The spring and summer shoots at three distinct phases (before, during, and after self-pruning) were collected from adult trees of both precocious mutant and wild-type trifoliate orange. Considering that *CiFT* might be regulated by light, all shoots were sampled at 10:00 AM to minimize the light effect. Shoot samples were collected from three groups of trees (each group containing three trees) used as biological repeats.

RNA extraction, first-strand cDNA synthesis, and *PtSVP* isolation

Total RNA was extracted according to the protocol of Zhang et al. (2008). Total RNA (3 mg) of each sample was treated with 3 U DNase (Promega, USA) and then further purified to remove the DNase. The total RNA yield and quality were determined spectrophotometrically at wavelengths of 230, 260, and 280 nm. Total RNA was adjusted to a final concentration of 1 μg/μl, and the first-strand cDNA synthesis was performed according to the manufacturer's instructions.

Full-length *PtSVP* was obtained by rapid amplification of cDNA ends, and the *PtSVP* DNA sequence was identified by a BD Universal Genome Walker kit (Clontech, USA) according to the manufacturer's instructions. The putative protein sequence was predicted using Blastx and MIT (http://genes.mit.edu/GENSCAN.html). The phylogenetic tree was constructed by the PHYLIP package using maximum likelihood.

Construction of PtSVP-GFP fusion protein and PtSVP localization

The open reading frame (ORF) without the terminator codon of *PtSVP* was constructed to the pCAMBIA1302 fusing to green fluorescent protein (GFP) using the restriction enzyme *Nco*I. The *PtSVP-GFP* expression vector was transformed into onion epidermis cells by particle bombardment, as described previously (Kinkema et al. 2000). After bombardment, the Petri dishes were sealed with Parafilm and placed in a 26°C incubator for about 24 h. GFP fluorescence was then detected using an 80i Nikon fluorescent microscope under a stimulating wavelength of 490 nm.

Analyzing *PtSVP* expression using real-time PCR

PtSVP expression level was measured by real-time PCR using SYBR green I chemistry (Qiagen, Germany). Primers for *PtSVP* were designed with the Primer Express software (PE Applied Biosystems, Foster City, CA, USA) and tested to ensure amplification of single discrete band with no primer-dimers. Product size was 137 bp. An amount of cDNA corresponding to 25 ng of input cDNA was used in each reaction. The real-time PCR was performed with 1 μl template of the real-time reaction mixture, 10 μl 2× SYBR Green Master Mix, 0.5 μl forward and reverse primers (10 μmol/μl), and water to a final volume of 20 μl. Gene expression levels were analyzed with ABI 7500 Sequence Detection System Software (PE Applied Biosystems) and normalized with the results of *β-actin*. Three biological repeats and four mechanical repeats were used for each reaction.

RNA in situ hybridization and detection

Materials for in situ hybridization were sampled and immediately fixed in RNAase-free FAA (4% formaldehyde, 10% acetic acid, 50% ethanol). Paraffin sections were made according to the method of Yao et al. (2007). A special 246-bp sequence in the middle of the *PtSVP* cDNA sequence was cloned into pGEM-T vector for probe synthesis (Supporting Information Table S1). Digoxigenin-labeled RNA probes were prepared using a DIG Northern Starter Kit (Roche, Germany). T7 and SP6 RNA polymerase were used to generate the sense and antisense RNA probes by in vitro transcription according to the manufacturer's instructions. The in situ hybridization experiment was performed as described in the Cold Spring Harbor *Arabidopsis* Molecular Genetics Course (www.Arabidopsis.org/cshl-course/5-in_situ.html).

Arabidopsis transformation

The *svp-41* mutant and wild-type (Col-0) *Arabidopsis* plants were used for transformation to confirm the function of *PtSVP*. Seeds of *svp-41* were provided by P. Huijser (Max-Planck-Institut für Züchtungsforschung, Molekulare Pflanzengenetik, Cologne, Germany). The floral dipping transformation method (Clough and Bent 1998) was used in this experiment. Seeds carrying *PtSVP* fused to the 35S promoter were selected on medium containing 75 mg/l kanamycin and grown under long-day conditions (16 h light/8 h dark) at 25°C. The transgenic plants T_1 and T_2 were also confirmed by PCR amplification. To investigate flowering time, days to flowering and the number of rosette leaves were counted when plants bore a 1-cm-long inflorescence.

Overexpression of *PtSVP* in tobacco and phenotype analysis of the transformants

PtSVP cDNA containing full-length ORF was introduced into the binary plant transformation vector pBI121, in which the transcription of *PtSVP* was driven by the CaMV 35S promoter. *PtSVP* transgenic tobacco plants were produced according to the method of Salehi et al. (2005). A total of 219 transgenic tobacco plants were screened from the kanamycin-selected culture medium. These 219 plants together with 54 nontransgenic controls were planted in an isolated greenhouse under the same conditions with uniform management. In the 219 screened plants, 37 (T_0) plants were confirmed to be true transformants by PCR detection using DNA templates with *PtSVP* RT–PCR primers (Supporting Information Table S1). Fifteen nontransgenic plants and 19 transformants were selected randomly for analysis of flowering time, flowering duration, number of flowers on an inflorescence, and average flower number per coflorescence. Flowering time was defined as days from transplanting in the greenhouse to blooming of the top flower. Flowering duration was the period from blooming of the top flower to formation of the last floret. Number of flowers on an inflorescence refers to the average number of flowers per inflorescence in the investigated group. Number of flowers per coflorescence was calculated as the mean of the averaged flower number on each coflorescence among the tested inflorescences. The data were analyzed using Excel (Microsoft, Redmond, WA, USA).

Yeast two-hybrid assays and library screens

Lateral buds, terminal buds, and shoot meristem at different developmental stages were sampled from precocious trifoliate orange. To prepare a representative sample of total RNA from the adult and juvenile tissues for cDNA library construction, different developmental stages of plant organs from roughly equal numbers of adult and juvenile plants were pooled. A cDNA library was constructed using the BD Matchmaker library construction and screening kits (Clontech) according to the manufacturer's instructions. Full-length *PtSVP* cDNA was cloned into pGBKT7 (Clontech). Yeast cells were transformed by the LiAc/DNA/PEG method according to the Yeast Protocols Handbook from Clontech (http://www.clontech.com). Constructs were tested for autoactivation as described by the manufacturer. Colonies growing on the SD/-Leu-Trp-His-Ade media with 10 mM of the competitive inhibitor 3-amino-triazole were transferred to selective medium containing X-Gal (80 mg/ml), and the blue colonies were characterized. Screening of interaction clones was carried out according to the manufacturer's instructions. Plasmids were isolated from colonies showing a positive (blue-colored) reaction and introduced into AH109 for confirmation of protein interaction. Inserts of the plasmids were sequenced by the Beijing Genomics Institute (Wuhan, China).

Results

Annual growth cycle and floral development course in trifoliate orange

Most citrus species have two or three periods of bud sprouting in a year to form different shoots (Davenport 1990). In wild-type trifoliate orange, only the shoots that sprout in late winter or early spring (March to April) are capable of forming a floral bud, whereas the summer (May to July) and autumn shoots (August to October) are vegetative and unable to form a floral bud (Fig. 1). In contrast, both the spring and summer shoots of the precocious mutant can generate flowers (Fig. S1). As a result, the precocious mutant has a lower ratio of leaf bud/flower bud than the wild type.

Self-pruning is a necessary but not sufficient condition for floral bud initiation. It is a typical in all kinds of shoots of citrus plants to cease vegetative growth temporarily by

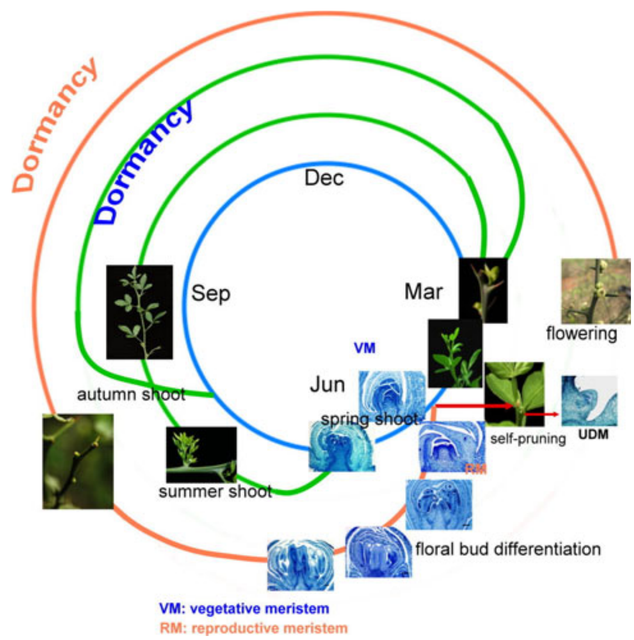

Fig. 1 The schematic developmental cycle of wild-type trifoliate orange. Spring shoot sprouted in early March and began vegetative growth which would last for about one month. In mid April, vegetative growth of the spring shoots was terminated by self-pruning and the lateral buds on spring shoots had to select their differential determination. At this time, the meristem was un-determinated (UDM). For the flowering-competent shoots (the spring shoots), the meristem at top several bud location had the advantage to begin floral differentiation (RM, reproductive meristem) and the meristem at bottom leaves' axilla was vegetative meristem (VM, vegetative meristem) which initiated leaf bud (vegetative) differentiation. For the flowering-incompetent shoot (the summer shoot and autumn shoot; green line) which sprouted in summer or autumn from the spring shoots or old shoots formed before the current year, all the lateral buds could only initiate leaf bud differentiation after the self-pruning

automatically removing the shoot tip (about 0.5–1 cm; Fig. 1). Cytological observation revealed that the floral buds in the wild type and the precocious mutant initiated their differentiation immediately after self-pruning on spring shoots. The shoot apical meristem was in an undetermined state (Fig. S2a) and floral primordia were not been observed until the late stage of self-pruning (mid-April in Wuhan, China). After self-pruning, differentiation occurred rapidly and produced the primordia of sepal, petal, stamen, and pistil sequentially (Fig. S2b, c, d, f, g, h). The whole integrated flower bud was formed in 1 month, then fell into dormancy until late February of the following year. In wild-type trifoliate orange, the summer shoots, which do not form floral buds, begin to produce vegetative buds after self-pruning. Thus, the self-pruning appears to be a demarcation point for the meristem to initiate leaf bud or floral bud development.

Cloning and annotation of the *PtSVP*

The *PtSVP* expressed sequence tag was identified from a previously constructed suppressive subtractive hybridization library; the 3’ and 5’ rapid amplification of cDNA ends was used to obtain the full-length cDNA, which was 1087 bp long with an ORF of 654 nucleotides (GenBank accession no. FJ373210). A 3834-bp sequence of *PtSVP* genomic DNA (GenBank accession no. FJ373211) was obtained by DNA genome walking. Structure analysis between the full-length cDNA and genomic DNA sequence revealed that *PtSVP* contained eight exons and seven introns. The DNA sequence between the first exon and the eighth exon was 3129 bp, the start codon ATG was in the second exon, and the stop codon was in the eighth exon (Fig. 2a). *PtSVP* cDNA encoded a MADS-box protein of 218 amino acids, containing a MEF2-like motif (MADS-box) in the N-terminus and a K-box motif in the middle.

Phylogenetic analysis using amino acid sequences showed that PtSVP belongs to the SVP group, which includes the SVP homologs (*StMADS11-like*) from *Arabidopsis* and other plant species. This group was distinct from other MADS-box proteins in *Arabidopsis*, such as AtAGL24 and AtSOC1 (Fig. 2b). To better understand their evolutionary relationships, the *SVP/StMADS-11-like* genes in other plant species, including *BrSVP* in Chinese cabbage (*Brassica*; Lee et al. 2007a), *EgSVP* in *Eucalyptus* (Brill and Watson 2004), *INCO* in *Antirrhinum* (Masiero et al. 2004), and *SVP/StMADS-11-like* genes in rice (Sentoku et al. 2005), were included in the phylogenetic analysis. They all were clustered in the SVP group. *PtSVP* was closest to *MEF2-LIKE*, a functionally unidentified MADS-box gene from apple. In *Arabidopsis*, *AtAGL24* is a MADS-box gene most similar to *SVP* (Gregis et al. 2006), but compared with *PtSVP*, it was even farther from *SVP*

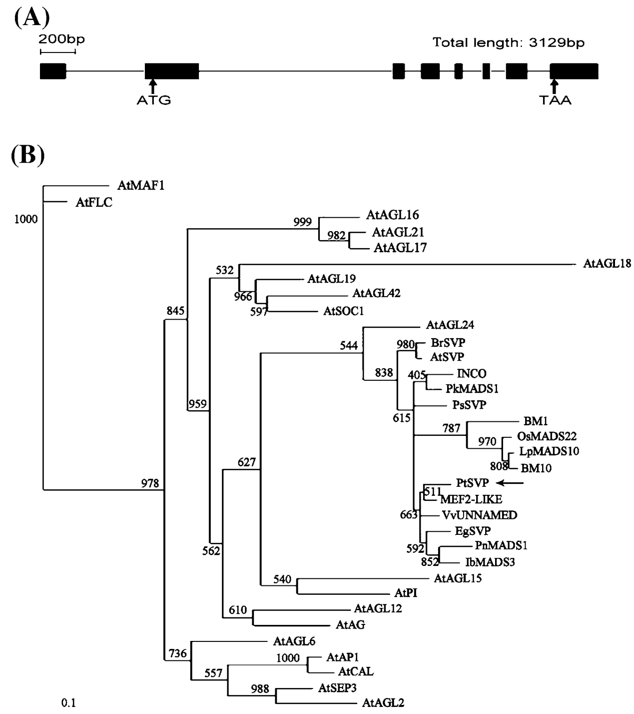

Fig. 2 Gene structures of *PtSVP* and phylogenetic relationship of the predicted amino acid sequences of PtSVP with SVP-like proteins from other plant species and other representative MADS-box proteins of *Arabidopsis*. **a** Structures of the genomic sequence of *PtSVP*. The closed boxes denote exons, and the lines between the closed boxes denote introns. **b** Maximum likelihood Bayesian phylogeny of *PtSVP*-related MADS-box protein in *Arabidopsis* and the SVP from other plant species. The phylogram tree shows that the PtSVP is a homolog to AtSVP. The scale bar indicates a divergence of 0.1 amino acid substitutions per site. The unrooted tree was generated using Phylip package sofyware by neighbor-joining method. Bootstrap values from 1,000 replicates are indicated at each node. The accession numbers of MADS proteins used in this analysis are: EgSVP (AAP40641), AtSVP (NP_179840), BrSVP (AAQ55452), MEF2-LIKE (ABD66219), PkMADS1 (AAF22455), VvUnnamed (CAO48343), OsMADS22 (NP_001048193), BM1 (CAB97350), BM10 (ABM21529), INCO (CAG27846), LpMADS10 (AAZ17549), IbMADS3 (AAK27150), PnMADS1 (BAF46766), PsSVP (AAX47170), AtAGL24 (NP_194185), AtFLC (NP_001078563), AtAGL6 (NP_182089), AtAGL19 (NP_194026), AtMAF1 (NP_850979), AtSOC1 (NP_182090), AtAGL15 (NP_196883), AtAGL18 (NP_191298), AtAGL42 (NP_568952), AtAG (NP_567569), AtAGL12 (NP_565022), AtSEP1 (NP_001119230), AtAGL17 (NP_179848), AtCAL (NP_564243), AtAP1 (NP_177074), AtAGL21 (NP_195507), AtAGL16 (NP_191282), AtSEP3 (NP_564214), and AtPI (NP_197524)

according to phylogenetic analysis. Thus, PtSVP might be an *SVP/StMADS-11-like* transcription factor.

The localization of PtSVP protein

To examine whether PtSVP was localized in the nucleus like other transcription factors, the PtSVP-GFP fusion protein was constructed to localize the PtSVP protein. The *PtSVP* ORF without the stop codon was fused to the 5’ end

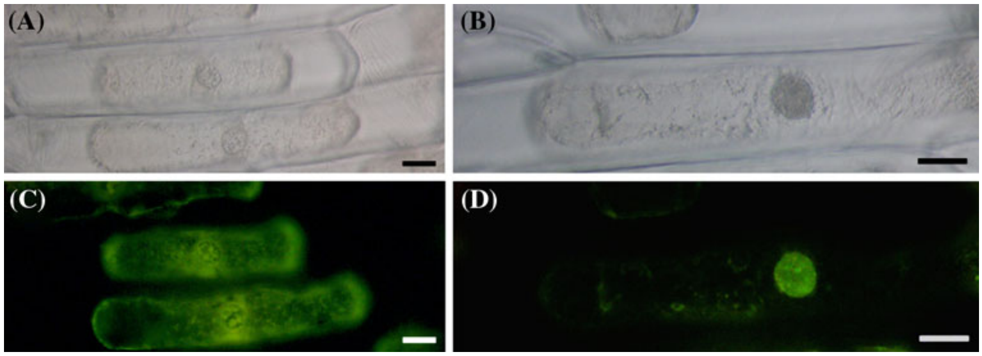

Fig. 3 Subcellular localization of PtSVP in cells. **a**, **c** Transient transformation with *35S::GFP*. **c** GFP alone was widespread in the whole cell. **b**, **d** Transient transformation with *35S::PtSVP-GFP* fusion construction. **d** PtSVP-GFP fusion protein was located in cell nucleus. *Bar* = 50 μm

of the GFP ORF in pCAMBIA1302. The pCAMBIA1302 with CaMV 35S promoter driving *GFP* alone was used as a negative control. The PtSVP-GFP signal was localized in the nucleus (Fig. 3d), whereas the fluorescence of the control was localized in both the nucleus and cytoplasm (Fig. 3c). The nuclear localization of PtSVP-GFP also suggests that PtSVP might be a transcription factor.

The annual fluctuation of *PtSVP* expression

To understand the relationship between floral bud initiation and the expression of *PtSVP* in trifoliate orange, the expression levels of *PtSVP* were measured in different stages of shoot, including newly formed young bud (July), mature bud (September), dormant bud (November to January), and sprouting bud (March to April) by real-time PCR (Fig. 4a).

The expression level of *PtSVP* fluctuated with season, shoot growth, and self-pruning, as revealed by quantitative PCR (Fig. 4a). *PtSVP* was expressed abundantly in the dormant vegetative bud (November). When the vegetative bud was sprouting in March, *PtSVP* expression level was down-regulated, and the expression level was even lower when the vegetative bud grew into a shoot in April (near the self-pruning stage). Similar results were obtained by in situ hybridization: *PtSVP* was expressed strongly in vegetative buds in November (Fig. 4b) and was down-regulated in sprouting shoots (Fig. 4c). In addition, the signal intensity of in situ hybridization indicated that *PtSVP* was expressed at high levels in spring shoots of juvenile wild-type (Fig. 4e) and summer shoots of adult wild-type trees (Fig. 4f), which are unable to form flowers. Based on these results, we conclude that a high expression level of *PtSVP* might help to maintain the tissue or meristem in a dormant state, whereas the down-regulation of *PtSVP* expression after winter might be closely related to the shift of cell activity or the change in flowering competence of buds on adult wild-type trifoliate orange.

Expression pattern of *PtSVP* during self-pruning

Self-pruning may be a key stage for meristem determination. To understand the role of *PtSVP* in the floral transition, the dynamic expression of *PtSVP* during self-pruning was monitored by real-time PCR. As shown in Fig. 5, *PtSVP* expression was markedly down-regulated during the floral transition (self-pruning) and then increased to higher levels after the lateral buds were differentiated (20 days after self-pruning) in the flowering-competent shoots (spring shoots of both trifoliate oranges and summer shoots of the precocious mutant). However, in the flowering-incompetent shoot (summer shoots of wild type), there was an increased trend of *PtSVP* expression during self-pruning compared with the prior stage. The dynamic expression of *PtSVP* in flowering-competent and -incompetent shoots suggests that *PtSVP* is involved in the floral transition.

Parallel analysis of the expression of two functionally confirmed genes, *CiFT* and *CsWUS* (Endo et al. 2005; Tan and Swain 2007), was carried out by real-time PCR to further investigate the role of *PtSVP* in floral bud initiation (Fig. 5). The expression of *CsWUS* was high before and after self-pruning, but it was down-regulated during the floral transition (self-pruning) in flowering-competent shoots. The expression of *CiFT* was low before floral transition and then up-regulated during the floral transition. Taken together, the expression patterns of these three genes suggest that the down-regulation of *PtSVP* is related to the floral transition. In other words, the high expression of *PtSVP* may maintain either vegetative or reproductive development but not directly cause development transition. It is the decrease of the expression level of *PtSVP* that leads to the development transition.

To precisely localize *PtSVP* expression in lateral buds during seasonal period of floral transition and flower differentiation, in situ hybridization was performed on sections of wild-type floral buds containing florets at various stages of development. Consistent with the results of real-

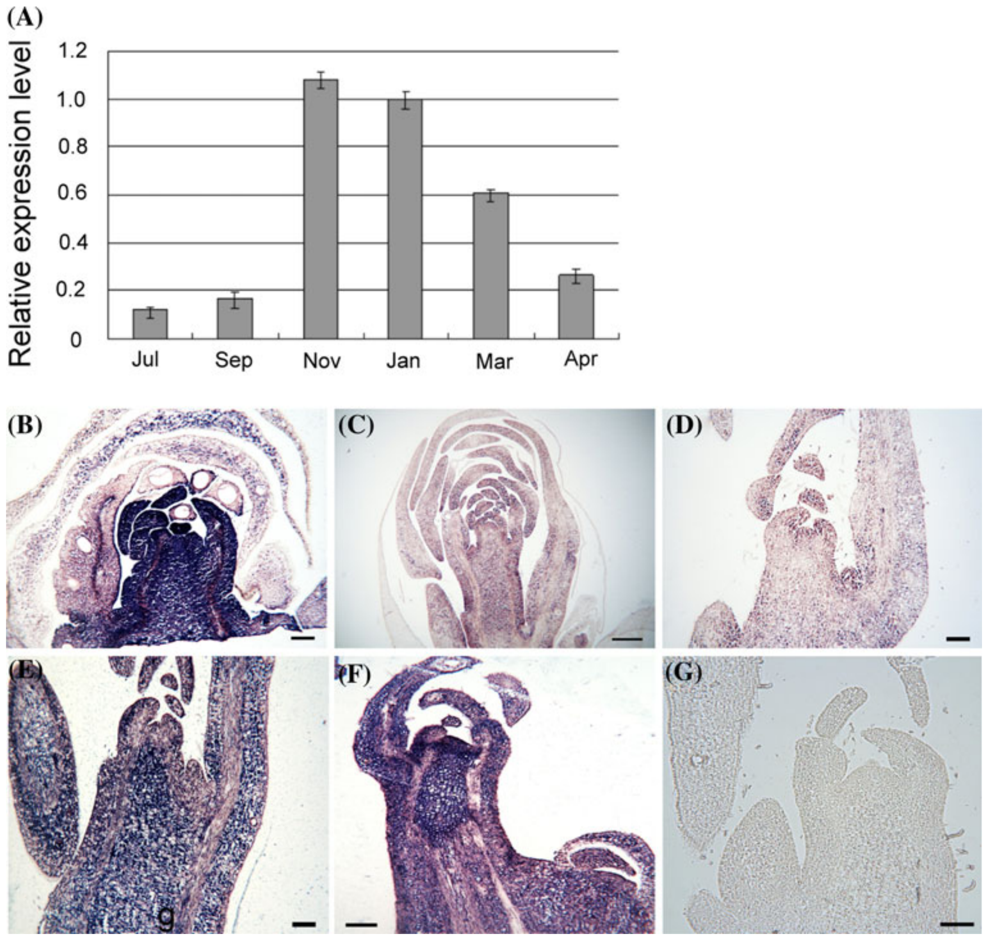

Fig. 4 The expression of *PtSVP* was investigated by real-time PCR and in situ hybridization. **a** Relative quantities of *PtSVP* in lateral vegetative buds on adult wild-type trifoliate orange. Data points represent the mean ± SE of at least four replications for the relative expression, which was normalized by the amount of the *Actin* control. The primers used for the analyses are shown in Supplement Table S1. **b**, **c**, **d** are corresponding to Nov, Mar, and Apr in panel, respectively. **b** The high expression of *PtSVP* in vegetative bud sampled in November. **c** The weak expression of *PtSVP* in the vegetative bud which was sprouting in March; **d** the weaker expression of *PtSVP* in the came-up spring shoot (in April, before self-pruning) which is flowering-competent. Contrary, *PtSVP* expressed strongly in the flowering-incompetent shoots, spring shoot of juvenile tree (**e**) and the summer shoot of adult tree (**f**) of the wild-type trifoliate orange. **g** The sense probe detection in the summer shoot tip is as a control. *Bar* = 50μm for panels **b**, **d**–**g**; 300 μm for panel **c**

time PCR, the signals of *PtSVP* transcripts were clearly detected in apical meristems before the floral transition (before self-pruning; Fig. 6a). But during the floral transition (at the late stage of self-pruning), the expression of *PtSVP* in apical meristems was down-regulated (Fig. 6b). In the course of flower differentiation, *PtSVP* expression was higher in differentiated cells (flower primordia) than in undifferentiated cells (apical meristems; Fig. 6c). When the flower bud fully developed and the flower apical meristem disappeared, *PtSVP* showed high levels in the whole flower bud except the bract (Fig. 6d). Therefore, the expression patterns of *PtSVP* in the lateral buds detected by in situ hybridization were consistent with the *PtSVP* expression pattern in whole shoots detected by real-time PCR. These results indicate that *PtSVP* is involved in maintaining cells in a state of ongoing activity, and its expression change is associated with floral bud initiation in trifoliate orange.

Functional analysis of *PtSVP* in transgenic *Arabidopsis*

To evaluate the function of *PtSVP* in the regulation of flowering, *PtSVP* was ectopically expressed in *svp-41* mutant and wild-type *Arabidopsis* driven by the 35S promoter. *Arabidopsis svp-41* mutant plants exhibit a strong early-flowering phenotype under long days. If *PtSVP* and *SVP* are functionally conserved, *PtSVP* should rescue the *SVP* defect in the mutants of *Arabidopsis* and result in normal flowering. Fourteen independent kanamycin-resistant plants were obtained in the T_1 generation. For expression analysis of *PtSVP* function, we randomly selected 13 T_2 transgenic plants by PCR detection using

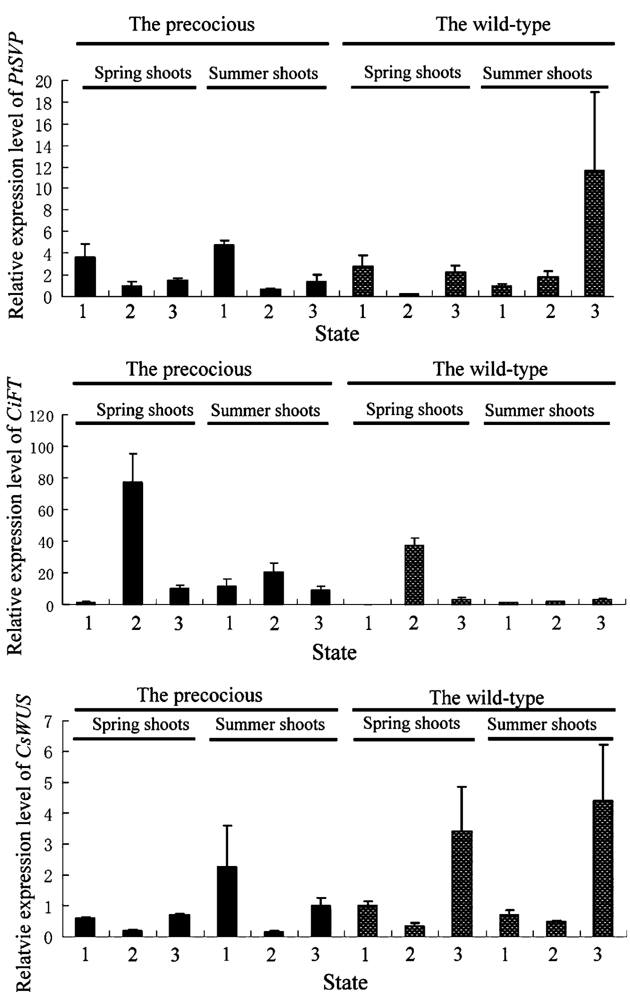

Fig. 5 The expression pattern of *PtSVP*, *CiFT* and *CsWUS* during self pruning. The relative quantities of *PtSVP*, *CiFT*, and *CsWUS* during floral transition in spring and summer shoots of both adult precocious and wild-type trifoliate orange were investigated by real-time PCR. *ACTIN* was used as a housekeeping control. Real-time PCR experiments were conducted using the primers displayed in Supplement Table S1. *Lane 1*, 15 days before self-pruning; *lane 2*, during self-pruning; *lane 3*, 20 days after self-pruning

DNA templates. Under long-day conditions, the flowering time of most of the transgenic lines was delayed, suggesting that *PtSVP* may function as a floral repressor and could partially recover the *svp* mutation (Fig. 7a; Table 1). Furthermore, ectopic expression of *PtSVP* in wild-type *Arabidopsis* also delayed the flowering time (Fig. 7b). These transgenic plants were grouped into three classes based on the total number of rosette leaves at flowering (Table 1). Class I plants flowered significantly later than the wild-type plants in terms of both days to flowering and the number of leaves, and the Class I plants also showed floral defects (Fig. 7d), including alterations in floral organ number, the appearance of excrescent trichomes on sepal, pale green sepals, and vestigial petals. These results suggest that the strong expression of *PtSVP* could disturb normal flower development. Although the class II and III plants showed fewer flower defects, they showed late flowering and had excrescent trichomes on sepal. More trichomes are usually considered to be a juvenile phenotype of *Arabidopsis* (Telfer et al. 1997). The overexpression of *PtSVP* delayed flowering time in transgenic lines and induced juvenile characteristics during the adult stage, demonstrating that the expression of *SVP* disturbs flower development, acts as a floral repressor similar to *SVP*, and is involved in organ determination. Concerning the length of the inflorescence and total number of flowers, however, the transgenic plants of *35S::PtSVP* displayed obvious prolonged inflorescence and many additional flowers (Fig. 7e).

Ectopic expression of *PtSVP* in tobacco

To further examine the function of *PtSVP*, we constitutively expressed *PtSVP* in tobacco under the control of the 35S promoter. The vegetative growth of 37 transformants (T_0; Supporting Information Fig. S3) was similar to that of the wild type, whereas the process of reproductive growth differed. Although the flowering time of transformants and nontransgenic tobacco plants were not significantly different with regard to anthesis of their top flower (Table 2), the coflorescence development of *35S::PtSVP* transgenic tobacco was delayed at the early stage (Fig. 8b), which was evidenced by the fact that the transformants had fewer flowers in the early stage (Supporting Information Fig. S4). The duration of coflorescence development in the *35S::PtSVP* transgenic tobacco plants was 1.4 times longer than in wild-type plants, and transgenic plants eventually generated significantly more florets than the wild type (Fig. 8d, e; Table 2). Although the number of coflorescences in transformants and nontransgenic plants showed no difference, the total numbers of flowers in each transgenic plant was greatly increased. The average flower number per coflorescence on *35S::PtSVP* transgenic tobacco plants was about 17, significantly more than that on the nontransgenic plants (about 11, Table 2). These results suggest that *PtSVP* not only stunted the coflorescence transition during the early stage but also maintained the reproductive growth after the floral transition. In addition, in transgenic plants *PtSVP* overexpression resulted in more abnormal flowers with late development of organs from the second whorl (petal) and finally sterility (Supporting Information Fig. S5).

Interacting partners of PtSVP in citrus

To find candidate citrus proteins interacting with PtSVP, a yeast two-hybrid system was used to screen citrus cDNA expression library, using *PtSVP* as a bait. Approximately

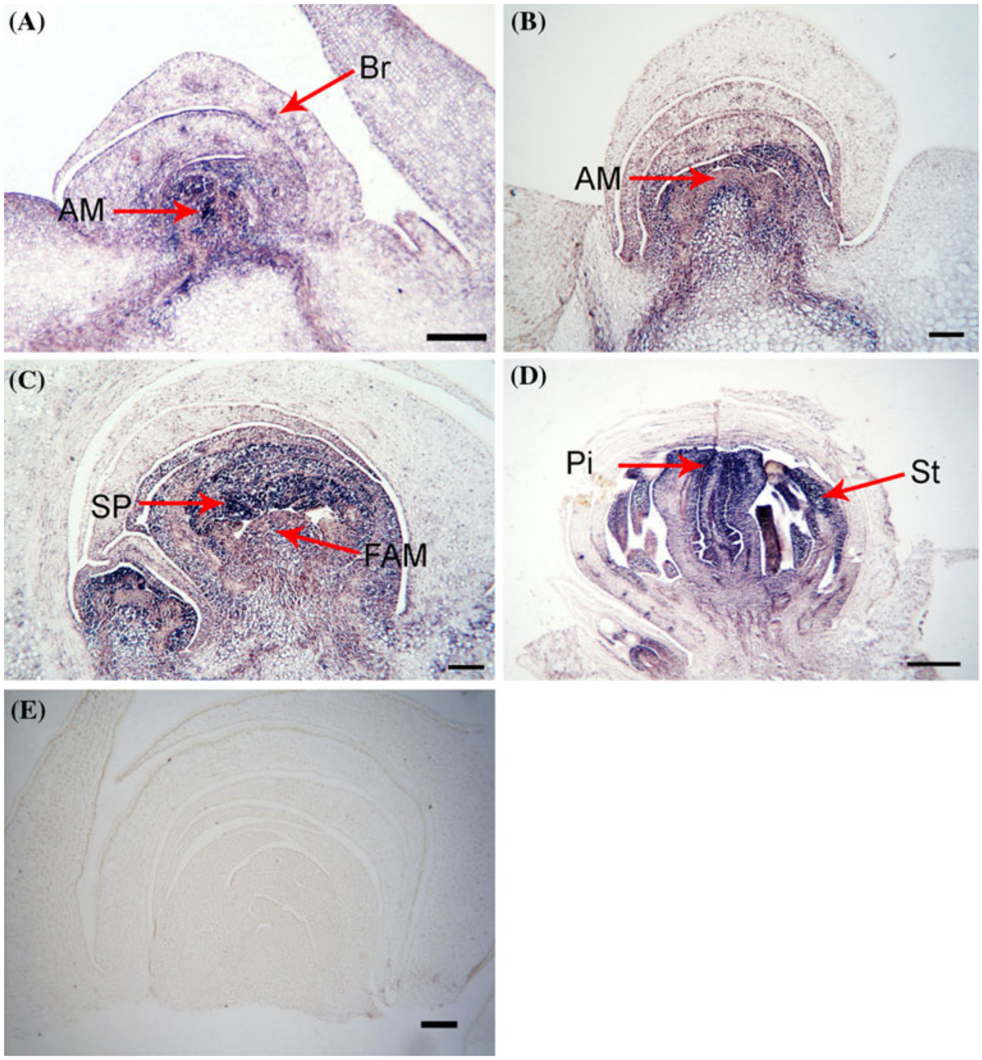

Fig. 6 The expressions of *PtSVP* in lateral buds at different developmental stages of trifoliate orange were shown by in situ hybridization; **a** is the lateral bud before self-pruning period; **b** floral transition phase; **c** flower bud differentiation phase; **d** whole mature flower bud; **e** hybridized with a sense *PtSVP* probe. *Bar* = 50 μm. *AM* apical meristem, *Br* bract, *FAM* flower apical meristem, *Pi* pistil, *SP* stamen primordium

1×10^6 yeast colonies were screened, and 17 positive clones were identified from the cDNA library. DNA sequencing showed that the 17 clones represented three genes (encoding an unknown protein, ubiquitin thiolesterase, and APETALA1-like protein). Of these selected clones, genes encoding APETALA1-like protein (PtAP1) were the most frequently identified proteins (8 clones). *PtAP1* shared a very high similarity (99% at the amino acid and nucleotide sequence level in the ORF; Supporting Information Fig. S7) with *CsAP1*, which was demonstrated to be a functionally active homolog of *AP1* (Cervera et al. 2009; Pillitteri et al. 2004). Thus, we conclude that *PtSVP* has the same mechanism of interacting with the *AP1* homolog as reported by Gregis et al. (2006). However, a positive interaction between PtSVP and the other two proteins needs to be investigated further.

Discussion

Gene structure and evolution of *PtSVP*

Analysis of the full-length cDNA and the genomic DNA sequence of *PtSVP* indicated that there were eight exons in *PtSVP* (Fig. 2a), which differs from the nine exons in *SVP* and *BrSVP* (Lee et al. 2007a). In addition, the initiation codon of *PtSVP* is located in the second exon, whereas those of *SVP* and *BrSVP* are in the first exon. Several predicted *cis*-acting regulatory elements, including the MYB protein (Schmitz et al. 2002) binding site, exist in the region between the first exon and the initiation codon. This region might be an important transcriptional regulatory position, because a similar regulation pattern has been reported in other genes (Jeon et al. 2000; Jeong et al. 2006;

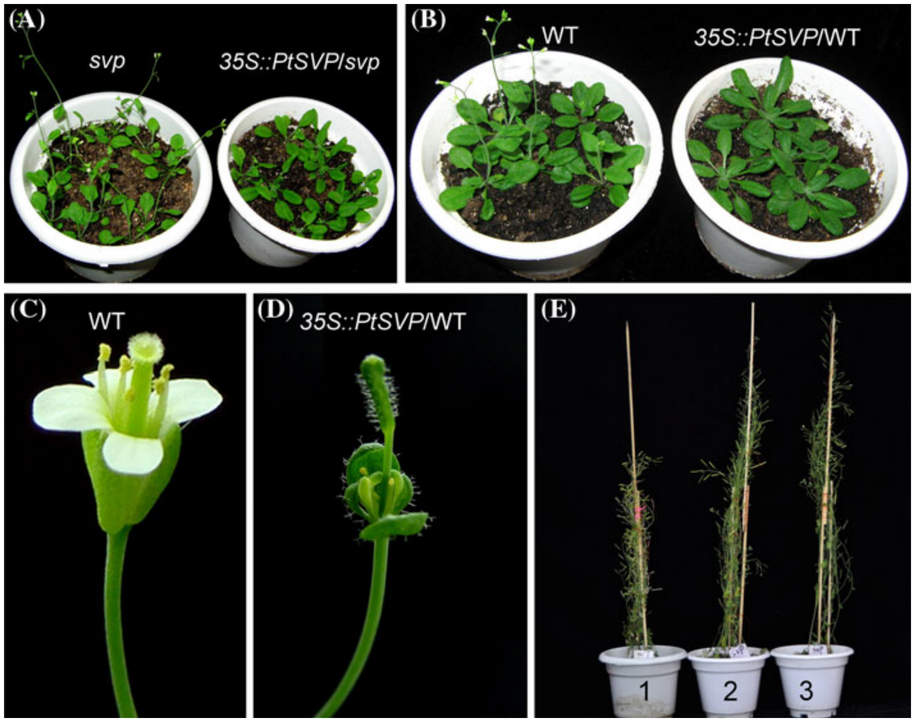

Fig. 7 Phenotypes of *PtSVP* transgenic *Arabidopsis*. **a** The expression of *PtSVP* rescued the early-flowering phenotype of *svp* mutant *Arabidopsis*. **b** Ectopic expression of *PtSVP* in wild-type *Arabidopsis* delayed the flowering time. **c** The normal flower of wild-type *Arabidopsis*. **d** In transgenic *35S::PtSVP*/WT, a flower-like structure instead of carpel and excrescent trichomes on flower. **e** Compared to wild-type *Arabidopsis* (*1*), *35S::PtSVP*/WT plants (*2*, *3*) grew higher and produced longer inflorescences. The longer inflorescence of *35S::PtSVP*/WT was caused by the unabated growth of inflorescence, with continuously emerging new flowers. *35S::PtSVP/svp: PtSVP* was expressed in the *svp* mutant driven by 35S promoter; WT: wild-type *Arabidopsis*; *35S::PtSVP*/WT: *PtSVP* was expressed in the wild-type driven by the 35S promoter

Table 1 Flowering time of transgenic Arabidopsis

Plant Genotype[a]	Number of Plants[b]	Days to Flowering[c]	Number of leaves[d]
svp-41	15	21.93±0.54	7.67±0.33
35S:PtSVP/svp-41 (T2)	13	28.08±0.62	9.46±0.35
Wt (Col)	15	33.07±0.39	10.13±0.19
*35S:PtSVP/*WT (T1)			
Class I	5	48.40±0.81	16.60±0.25
Class II	13	41.77±0.54	14.38±0.21
Class III	11	36.42±0.49	12.67±0.15

Twenty-nine *35S:PtSVP/*WTtransgenic plants of T1 generation were used for observation after kanamycin selection: [a] The groups are classified based on the total number of leaves at flowering: 12–13 (weak), 14–15 (intermediate), and 16–17 (strong); [b]Plants were grown on the potted soil at 22°C under the day length conditions at 16 h of light/8 h of darkness (long-day conditions); [c] Days from sowing to a 1-cm inflorescence (± SD). [d] Number of rosette leaves on plants with 1-cm inflorescence (±SD)

Kikuchi et al. 2000). Nevertheless, the predicted PtSVP protein has similar functional domains as those of SVP, with a MADS-box at the N-terminus and a K-box motif in the middle, and was most similar to MEF2-LIKE, a putative SVP/StMADS-11-like protein from apple. Phylogenetic analysis of PtSVP and other SVP/StMADS-11-like proteins also indicated that PtSVP was evolutionarily close to EgSVP from *Eucalyptus* (Brill and Watson 2004; Prakash and Kumar 2002), SVP from *Arabidopsis* (Hartmann et al. 2000), and BrSVP from Chinese cabbage (Lee et al. 2007a). All the genes clustered in the SVP group (Fig. 2b) have similar functions of suppressing flowering or promoting vegetative growth. Our findings suggest that this MADS-box protein might be a candidate *SVP* gene in citrus.

The expression of *PtSVP* was closely related to floral bud initiation in trifoliate orange. *SVP* is a transcription factor in *Arabidopsis* and suppresses the floral transition by directly binding to the promoter of *FT* and *SOC1* in the nucleus (Lee et al. 2007b; Li et al. 2008). PtSVP was localized in the nucleus (Fig. 3d), which is a feature of transcription factors. Similar to *SVP*, *PtSVP* was expressed intensively in vegetative meristems (Supporting Information Fig. S6). However, the expression pattern of *PtSVP* showed an annual fluctuation coincident with the flowering competence of shoots. The expression of *PtSVP* was down-regulated in the lateral vegetative bud after winter (Fig. 4a) and remained at a low level in the spring shoot (Fig. 4d). In the annual cycle of *PtSVP* expression, each down-regulation of the gene was followed by initiation of floral bud

Table 2 Phenotype analysis of non-transgenic and *35S::PtSVP* tobacco

Strain	No. of plants	Flowering time[a] (days ± SE)	Flowering duration (days ± SE)	No. of coflorescense	No. of flowers per coflorecence	No. of flowers in an inflorescence
Non-transgenic	15	81.01±15.03	51.33±3.51	5.20±0.41	10.93±2.02	56.73±12.71
35S::PtSVP	19	80.42±13.11	71.25±5.03*	5.25±0.44	17.19±1.77*	91.13±13.55*

* Indicates significant difference from control by Student *t* test ($P < 0.001$)

[a] Flowering time was defined as the days when the top flower blooming after transplanting in the greenhouse

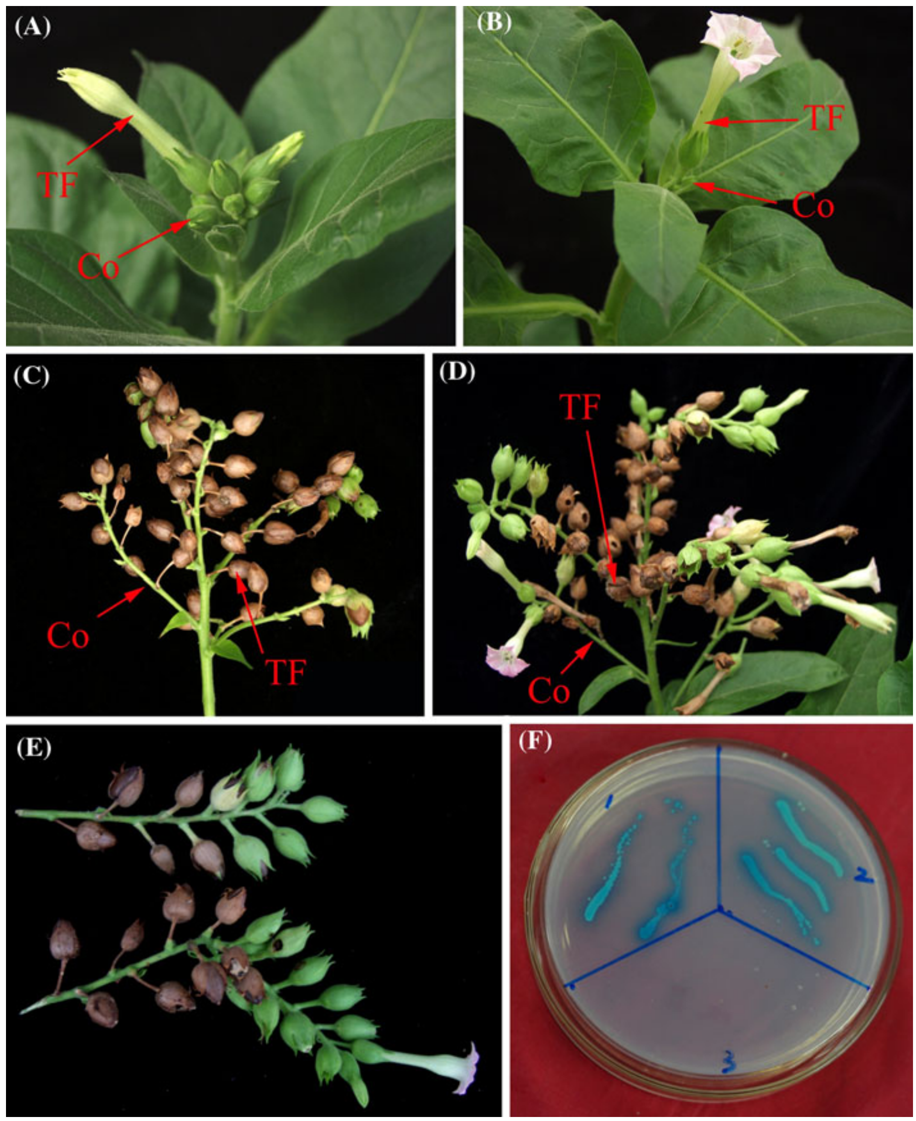

Fig. 8 Phenotype of the *35S::PtSVP* transgenic tobacco and the interaction experiment of *PtSVP*. **a**, **c** Inflorescences of the wild-type tobacco. **b**, **d** Inflorescences of *35S::PtSVP* transgenic tobacco. The coflorescense development of transgenic plant was postponed at the early state (**b**) but was prolonged at the later stage and resulted in more florets (**d**). **e** The comparison of coflorescences between the wild-type (*above*) and transgenic plants (*below*). **f** Yeast two hybrid system was used to detect the interaction between PtSVP and PtAP1. *Mark (1)* shows blue yeast colony indicating that PtSVP interacted with PtAP1; *Mark (2)* is a positive control (blue yeast colony); *Mark (3)* is a negative control (no colony). Co, coflorescense; TF, top flower

differentiation. In wild-type trifoliate orange, the spring shoot of the juvenile tree (Fig. 4e) and the summer shoot of the adult tree showed a strong expression level of *PtSVP* (Fig. 4f). These two kinds of shoots are incapable of floral bud formation. A similar expression pattern was reported in an *SVP* homolog from another perennial plant, raspberry (*Rubus idaeus* L.; Mazzitelli et al. 2007). This pattern was consistent with the yearly phase transition in woody plants, which does not occur in annual herbaceous plants.

Self-pruning is a self-regulation mechanism controlling the transition from vegetative to reproductive growth by removing the shoot apical meristem and top growth advantage. The earliest observable floral initiation occurs just after self-pruning. The results from real-time PCR

(Fig. 5) and in situ hybridization (Fig. 6) consistently showed that the expression of *PtSVP* sharply declined during self-pruning, the pivotal stage when the shoots shift from vegetative to reproductive growth. Taken together, these results suggest that *PtSVP* negatively regulates the floral transition in trifoliate orange. The phenotype of *35S::PtSVP* transgenic tobacco plants provided further evidence that the overexpression of *PtSVP* decelerated the floral transition of coflorescences (Fig. 8b). In *Arabidopsis*, SVP is associated with the *FT* promoter regions to regulate the expression of *FT* in the leaf (Lee et al. 2007b) and might be a flowering repressor. In our experiments, however, the expression patterns of *CiFT* and *PtSVP* did not shown a completely opposite trend. Therefore, whether *PtSVP* directly regulates *CiFT* in trifoliate orange must be further investigated. Our findings also suggest that the functions of *SVP* may be different between herbaceous and woody plants.

High expression of *PtSVP* prolongs reproductive growth

SVP is a transcription factor in *Arabidopsis* and suppresses the floral transition by directly binding to the promoter of *FT* and *SOC1* in the nucleus (Lee et al. 2007b; Li et al. 2008). Like transcription factors, PtSVP was localized in the nucleus (Fig. 3d). In *Arabidopsis*, *SVP* is expressed in functionally determined meristems (vegetative shoot apical meristem, flower primordium, and coflorescence primordium), but not in undetermined meristems (Hartmann et al. 2000). Likewise, extensive expression of *PtSVP* in trifoliate orange was detected in vegetative meristems (Supporting Information Fig. S6). Thus, it appears that the expression patterns of *SVP* are relatively similar between herbaceous and woody plants. In addition, our results indicated that *PtSVP* was down-regulated in apical meristems (Fig. 6b, c), which were functionally undetermined tissues. In contrast, *PtSVP* was expressed strongly in the functionally determined tissues, such as vegetative shoot tips (Fig. 4e, f) and flower organ primordia (Fig. 6b, c). The expression pattern of *PtSVP* was somewhat similar to that of *CsWUS* (Fig. 5). *CsWUS* is functionally similar to *WUS* in *Arabidopsis*, which maintain the identities of functionally determined meristems (Laux 1996; Tan and Swain 2007). However, real-time PCR also showed that *PtSVP* was highly expressed in the functionally determined shoots before and after the floral transition (Fig. 5). Based on the expression pattern of *PtSVP*, we propose that *PtSVP* actively maintains the duration of both vegetative and reproductive growth.

SVP is considered to be a flowering repressor in *Arabidopsis*, because it functionally delays flowering time. In this study, the flowering time of *PtSVP* transgenic *Arabidopsis* plants was delayed and the *35S::PtSVP*/WT showed a typical juvenile character (more trichomes). This result suggests that *PtSVP* regulates flowering time by maintaining the vegetative growth, which is functionally conserved to *SVP* in *Arabidopsis*. However, recent studies proved that *AGL24* and *SVP* are also floral meristem identity genes. Ectopic *AGL24* and *SVP* expression induces floral meristem indeterminacy by promoting the development of new ectopic floral meristems rather than causing floral reversions (Gregis et al. 2008). This finding suggested that expressions of *PtSVP* may control floral organ development. In this study, *PtSVP* induced a flower-like structure in place of the carpel (Fig. 7d). Moreover, ectopic overexpression of *PtSVP* in *Arabidopsis* and tobacco showed similar increases of flower numbers by prolonging the inflorescence length and flower formation time (Figs. 7e, 8d, e). This result coincides with the reports in transgenic of *EgSVP* to *Arabidopsis* (Brill and Watson 2004). An *SVP* homolog from *Antirrhinum*, *INCO*, functions not only as a negative regulator of phase transition, but also as a positive regulator of floral development (Masiero et al. 2004). Thus, we conclude that *PtSVP* might also play some role in maintaining reproductive growth.

In *Arabidopsis*, *AP1* is an identity gene to promote and maintain floral meristems. *AP1* binds to the promoter region of *SVP* to suppress its expression and then promote flower organ development after the floral transition, when the first-whorl organs are generated (Liu et al. 2007; Yu et al. 2004). The down-regulation of *SVP* at the proper time ensures normal flower development. This may explain why the constitutive expression of *PtSVP* (driven by the 35S promoter) disrupted the correlative gene expression order and thus caused abnormal flower development in *Arabidopsis* and tobacco. An alternative explanation of the floral defects is that the malapropos and excessive PtSVP protein may interact with AP1 or its homolog in the transgenic plants and disrupt flower development. During the floral transition in *Arabidopsis*, SVP interacts with other MADS-box proteins that determine floral organ identities during flower formation (DeFolter et al. 2005; Pelaz et al. 2001). This hypothesis is supported by findings that the SVP-AP1 heterodimer is involved in the recruitment of the corepressor complex for the regulation of *AGAMOUS* (*AG*) expression (Gregis et al. 2006). Thus, in our experiments the excess PtSVP may also function as SVP to effect the flower organ development of transgenic plants after the floral transition.

Regardless of the underlying mechanism, *PtSVP* effected different phenotypes in different genotypes (*Arabidopsis* and tobacco). Ectopic expression of *PtSVP* affected meristem determination during the reproductive transition, and thus resulted in disturbed flower development. The

SVP/StMADS11 group appears to have expanded in perennials in their functions as well as phylogenetically (Jimenez et al. 2009). Perennials might use *SVP/StMADS11* genes not only for functions that are required in annual plant models but also for the regulation of seasonal growth, and *PtSVP* might regulate the annual floral transition in trifoliate orange. These traits may include the formation of floral and vegetative bud structures, regulation of endodormancy cycling, and/or regulation of the juvenile–mature transition (Jimenez et al. 2009).

Conclusion

Our findings suggest that *PtSVP* plays an important role in floral bud initiation in trifoliate orange. We demonstrated that *PtSVP* acts as a transcription factor correlated with the floral transition and may be involved in meristem maintenance, retaining the states of both vegetative and reproductive growth. The down-regulation of *PtSVP* in annual spring shoots may be the cue triggering annual flowering in perennial trees. These findings provide a new understanding of the functions of the *SVP* clade. Further studies are required to understand how *PtSVP* is regulated by or regulates other genes to maintain the meristems and whether it directly regulates the flowering promoter *CiFT* to hamper the floral transition. Answers to these questions will greatly improve our understanding of the annual flowering mechanisms of citrus and other woody plants.

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