Molecular characterization and expression patterns of *PsSVP* genes reveal distinct roles in flower bud abortion and flowering in tree peony (*Paeonia suffruticosa*)

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Wang, S., Xue, J., Ahmadi, N., Holloway, P., Zhu, F., Ren, X. and Zhang, X. 2014. **Molecular characterization and expression patterns of *PsSVP* genes reveal distinct roles in flower bud abortion and flowering in tree peony (*Paeonia suffruticosa*)**. Can. J. Plant Sci. **94**: 1181–1193. Container culture and flower forcing are used for off-season production of tree peony for the Chinese Spring Festival. Storage of potted tree peony for 10 d at 12°C in a refrigerator before 4°C chilling treatment can help new root growth and promote leaf development. Development from bud swelling to anthesis was divided into nine stages. Some aborted flower buds usually emerge in Stage III. Removal of two to four leaflets in an alternating pattern and applying gibberellic acid 3 (GA_3) around the flower bud at Stage III can decrease the flower bud abortion rate and promote flower formation rate. Two MADS-box genes with homology to *Arabidopsis SVP*, designated *PsSVP1* and *PsSVP2*, which probably caused flower-bud abortion, were isolated by reverse transcription-PCR. Sequence comparison analysis showed that *PsSVP* was most similar to SVP-like gene in apple. Phylogenetic analysis indicates that *PsSVP* was evolutionarily close to *SVP*-like genes from *Malus domestica*, *SVP* genes from Cruciferae and *SVP*-like genes from *Vitis vinifera*. The qRT-PCR results suggested that expression of *PsSVP* was high in vegetative growth phase, especially in the leaves of tree peony, and its expression was regulated by GA_3. Further analysis showed that more *PsSVP* transcripted in the aborted flower bud, especially in the buds where leaflets grew well. It was deduced that *PsSVP* can promote vegetative growth and suppress flowering in tree peony. Thus, it is very important to further investigate *PsSVP* and decipher the mechanisms of flower-bud abortion to improve forcing culture of tree peony.

Key words: Tree peony, flower bud abortion, MADS-box gene, *PsSVP*, qRT-PCR

Wang, S., Xue, J., Ahmadi, N., Holloway, P., Zhu, F., Ren, X. et Zhang, X. 2014. **La caractérisation moléculaire et le mode d'expression des gènes *PsSVP* indiquent des rôles différents au niveau de l'avortement des bourgeons floraux et de la floraison chez la pivoine arbustive (*Paeonia suffruticosa*)**. Can. J. Plant Sci. **94**: 1181–1193. On recourt à la culture en conteneur et au forçage pour faire fleurir les pivoines arbustives en prévision du Festival du printemps, en Chine. La réfrigération des plantes en pot pendant 10 jours à 12 °C, puis leur refroidissement à 4 °C concourent à accélérer la croissance des racines et le développement des feuilles. Le passage du gonflement des bourgeons à l'anthèse a été divisé en neuf stades. Quelques bourgeons floraux apparaissent puis avortent habituellement au stade III. La suppression de 2 à 4 folioles en alternance et l'application d'acide gibbérellique 3 (GA_3) autour du bourgeon au stade III peut réduire le pourcentage d'avortements et promouvoir l'apparition des fleurs. Les chercheurs ont isolé deux gènes de la famille MADS-box homologues au *SVP* d'*Arabidopsis*, baptisés *PsSVP1* et *PsSVP2*, vraisemblablement à l'origine de l'avortement des bourgeons floraux, en recourant à la méthode RT-PCR. L'analyse comparative des séquences révèle que *PsSVP* ressemble le plus au gène similaire au *SVP* du pommier. L'analyse phylogénétique indique que, sur le plan de l'évolution, *PsSVP* est très proche des gènes similaires au *SVP* de *Malus domestica*, aux gènes *SVP* des Crucifères et aux gènes semblables au *SVP* de *Vitis vinifera*. Les résultats obtenus par qRT-PCR laissent supposer que le *PsSVP* s'exprime beaucoup durant la phase végétative, surtout dans les feuilles de la pivoine arbustive, et que le GA_3 en régule l'expression. Une analyse plus poussée montre que la transcription du *PsSVP* est plus grande dans les bourgeons floraux avortés, surtout ceux dont les folioles poussent bien. On en déduit que le *PsSVP* promeut la croissance végétative et entrave la floraison chez la pivoine arbustive. Par conséquent, il serait capital d'étudier davantage ce gène et de décrypter les mécanismes qui aboutissent à l'avortement des bourgeons floraux si l'on veut améliorer la culture des pivoines arbustives par forçage.

Mots clés: Pivoine arbustive, avortement des bourgeons floraux, gène MADS-box, *PsSVP*, qRT-PCR

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Abbreviations: **GA**, gibberellic acid; **SNP**, single nucleotide polymorphism

Tree peonies (Family Paeoniaceae, Section Moudan) include nine species, all originating in China: *Paeonia jishanensis*, *P. suffruticosa*, *P. cathayana*, *P. ostii*, *P. qiui*, *P. decomposita*, *P. rockii*, *P. delavayi* and *P. ludlowii*. They are distributed primarily in the Yunnan, Sichuan, Gansu, Shaanxi, Shanxi, Henan, Hubei, Anhui and Xizang (Tibet) provinces (Yuan et al. 2010, 2012). *Paeonia suffruticosa* Andrews is one of the earliest known horticultural and medicinal plants in the world, and is known as ''the king of flowers'' due to its large and colorful blooms. It is respected as a symbol of happiness, power and wealth in Chinese culture (Cheng et al. 2001; Yuan et al. 2011; Gai et al. 2013). There are more than 2000 cultivars of *Paeonia suffruticosa* worldwide, at least half of which are grown in China (Han et al. 2008; Yuan et al. 2011, 2012). Chinese tree peony can be geographically classified into four groups. They include Zhongyuan cultivars (distributed in the middle and downstream of the Yellow river, mainly in Heze, Luoyang and Beijing city), Xibei cultivars (West Shaanxi, South Ningxia and Gansu and East Qinghai Province), Jiangnan cultivars (the middle and downstream of the Yangtse River, mainly in Zhejiang, North Jiangxi, Shanghai, Jiangsu, South Anhui and Southeast of Hubei Province) and Xinan cultivars (Sichuan, Guizhou, Yunnan and South Tibet), respectively (Zhang et al. 2007).

The tree peony is the first candidate for China's national flower, loved by the Chinese people for centuries, and an integral part of Chinese art, music, literature, architecture, medicine and landscapes. It is a significant symbol during national holidays, especially, Chinese National Day (October 01), the New Year and the Spring Festival (January or February). However, the natural flowering period occurs in mid to late April (Central Plains Region) and lasts about 10 d. This short flowering period along with the fact that peony seedlings have a long juvenile phase, are major obstacles to the year-round flowering of tree peony for national ceremonies.

For the past three decades, studies on the physiological control of flowering, selection for spring-, autumn- and winter-blooming cultivars, shortening of the juvenility period and new cultivation practices have become important methods to achieve year-round flowering (Hosoki et al. 1983; Aoki and Yoshino 1989; Aoki 1992; Hosoki et al. 1992; Zhang 2004). For instance, Mornya et al. (2011a, b) found that various combinations of hormonal and sugar signals could regulate the flowering of in autumn- and non-autumn tree peonies. Despite advances in forcing technology, poor leaf development and flower-bud abortion are common during the flowering of tree peony especially in winter.

The genes involving in gibberellic acid (GA) biosynthesis, signaling and response may play a central role in the regulation of dormancy release in tree peony, a critical factor in flowering forcing (Huang et al. 2008; Gai et al. 2013). In *Arabidopsis*, gibberellins can affect the expression of gene *SHORT VEGETATIVE PHASE* (*SVP*), *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*) and *LEAFY* (*LFY*) to regulate flowering time (Wellmer and Riechmann 2010). However homologous *SVP*, *SOC1* and *LFY* in tree peony have not been isolated. In this paper, we tried to clone and analyze the potential function of homologous *SVP* in tree peony, regulated by GA.

The MADS box gene family derives its name from an acronym of the yeast protein MCM1 (Passmore et al. 1988), the plant proteins AGAMOUS (Yanofsky et al. 1990) and DEFICIENS (Schwarz-Sommer et al. 1990), and the mammalian protein SERUM RESPONSE FACTOR (Norman et al. 1988). The highly conserved MADS domain, encoding a length of about 60 amino acids, functions as a transcription factor involved in diverse aspects of plant development, due to its properties, including DNA binding, dimerization and nuclear localization (De Bodt et al. 2003). This large gene family is divided into two categories, type I and type II (Alvarez-Buylla et al. 2000). Members of type II in plants contain weakly conserved I (intervening) domain, the K (keratin-like) domain and variable region C domain. Plant type I MADS-domain proteins do not contain a well-defined K domain, and the C domain is still not well defined and is of variable length (De Bodt et al. 2003; Sentoku et al. 2005). *SVP*, the new *Arabidopsis* member of the type II subfamily was cloned through transposon tagging by Hartmann et al. (2000). It functions in the maintenance of vegetative shoot identity. Considerable effort has been made to identify and characterize *SVP*-like genes in many monocotyledons and dicotyledons (Carmona et al. 1998; Petersen et al. 2006), and annual (Lee et al. 2007a; Trevaskis et al. 2007; Fornara et al. 2008) and perennial species (Prakash and Kumar 2002; Diaz-Riquelme et al. 2009; Li et al. 2010; Wu et al. 2012). Furthermore, *SVP*-like genes function in the onset and/or release of endodormancy in raspberry (Mazzitelli et al. 2007) and leafy spurge (Horvath et al. 2010). Studies in kiwifruit showed that *SVP*-like genes have distinct roles in the regulation of flowering and bud dormancy (Wu et al. 2012). Tree peony (*Paeonia suffruticosa* Andr.) is a woody perennial plant, which goes through a period of bud dormancy in winter before flowering the next year. The dormancy of tree peony is endodormancy according to Lang and Martin (1987). Until now, no homologous *SVP* has been reported in tree peony and its function is unknown.

The objectives of this research were to advance the understanding of flower regulation and forcing of the tree peony by studying the effects of leaflet removal and GA_3 applications to flower buds, to isolate the homologous *SVP* genes regulated by GA in tree peony, and to elucidate the function of *SVP* genes on tree peony flowering.

MATERIALS AND METHODS

Plant Materials

In October and November, 144 four-year-old containerized (the size of the pot was $35 \times 28.4 \times 25$ cm) tree

peony plants (*P. suffruticosa* cv. Luoyang Hong) with 10 buds were randomly subdivided into two groups, viz. group I ($n=96$) and group II ($n=48$). Plants in groups I and II were stored in darkness at a constant 12 ± 1°C and 4 ± 0.5°C, respectively, for 10 d. Then containers were removed to a constant 4 ± 0.5°C in cool room in the dark for 30 d. After pre-chilling treatments, 48 randomly assigned plants in group I and II ($n=24$ each) were removed from their containers and the presence of new root growth was noted. After new growth root examined, the plants were discarded. The remained plants ($n=96$) were returned to the controlled greenhouse in the Chinese Academy of Agricultural Sciences, Beijing China from 2012 Dec. 20 to 2013 Feb. 01 under natural light. The range of greenhouse temperature was controlled at from 4 to 10°C in the first week. Then the greenhouse temperature was slowly increased day by day and finally controlled at 15–25°C in late January. Bud development was monitored daily and classified into one of nine stages according to Cheng et al. (2001) and Li (1999) (Supplementary Fig. 1).

Treatments and Material Collection

When the buds in group I reached stage II, they received one of three treatments (80 buds in eight pots per treatment) and each treatment was replicated three times: (1) buds remained intact with no further treatment (control, $n=24$); (2) buds were peeled using a thin blade to remove the bud scale and two to four leaflets in an alternating pattern around the bud ($n=24$); 3) buds were peeled as in treatment 2, then treated with 650 mg L^{-1} aqueous solution of GA_3 applied ($n=24$). Buds in group II were only manipulated by the third method ($n=24$). This treatment was also replicated three times.

Bud samples were harvested at stages II and III, approximately 1 d after treatment, for growth and genetic analysis (Supplementary Fig. 1 III a, b). In addition, roots, stems and leaves from plants at stage IX (2013 Feb. 01) were collected to study organ-specific gene expression. Eight different developmental types of buds (Fig. 1) (at least nine of each type of bud were collected) were sampled to evaluate *PsSVP* gene expression in normal and aborted flower buds. They were collected at stage III (Fig. 1A–E, 2012 Dec. 30), stages V and VI (Fig. 1F–H, 2013 Jan. 19), respectively. All the collected samples were immediately frozen in liquid nitrogen and stored at -80°C until analyzed.

Statistical Analysis

Analysis of variance was performed using SAS 9.2. software.

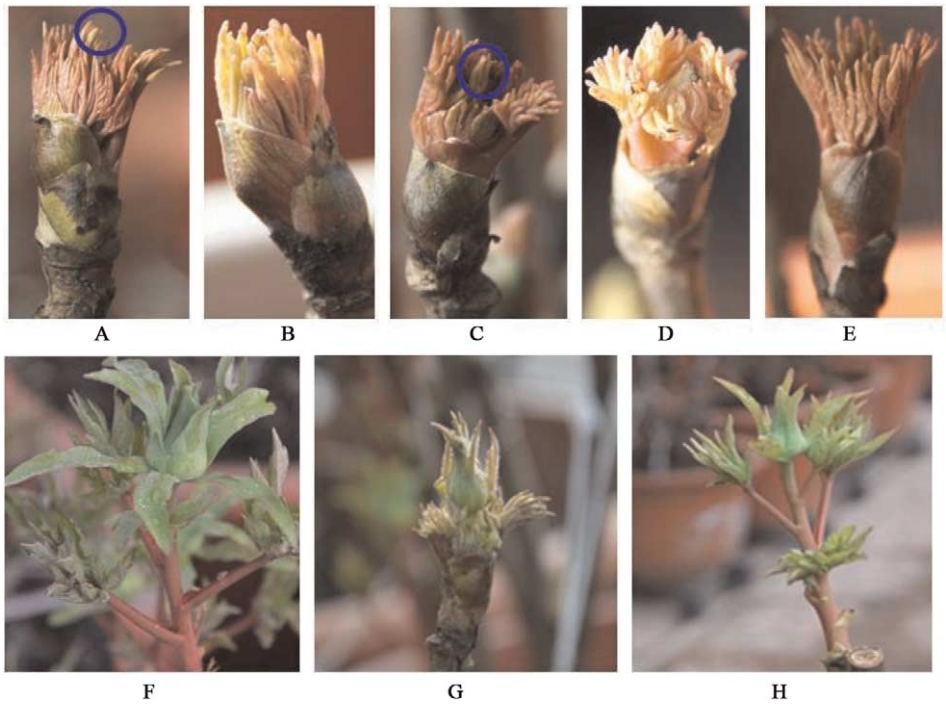

Fig. 1. The morphology of different developmental flower buds/flower-buds of tree peony. (A) normal flower bud, (B) aborted flower bud hidden completely within the leaves, (C) aborted flower bud with 1/3 partly covered by leaves, (D) aborted flower bud with 2/3 partly covered by leaves, (E) shoot-tip without flower bud, (F) big bell-like flower-bud stage with leaflets removed and GA_3 treatment in Stage II, (G) flower bud emerging phase (control) and (H) small bell-like flower bud stage with leaflet removal treatment. The tip of flower bud is marked by a blue circle.

mRNA Extraction, cDNA Synthesis, RT-PCR and qRT-PCR

The extraction of total mRNA from different developmental flower buds was performed according to Wang et al. (2013), using Trizol extraction kit (Invitrogen^{TM}). The detailed steps of mRNA purification and cDNA synthesis were outlined in Wang et al. (2012, 2013).

After cDNA synthesis, transcription-polymerase chain reaction (RT-PCR) and quantitative real time-pCR (qRT-PCR) were performed using gene specific primers designed by primer 5.0 (Table 1). Based on the conserved MADS domain of the 5′- terminal of homologous *SVP* genes retrieved from GenBank and partial C-terminal sequence of *PsSVP* obtained in our laboratory (by the result of RNA sequence), a pair of primers amplifying the *PsSVP* gene sequences were designed and used for PCR amplifications.

The actin gene from *P. suffruticosa* (GenBank accession number JN105298) was used as a control gene based on Zhang et al. (2011). The qRT-PCR reactions were performed according to Wang et al. (2012). An initial denaturation step of 4 min at 94°C was followed by 40 cycles of denaturation at 94°C for 15 s, hybridization at 57°C for 15 s, extension at 72°C for 20 s, and finally an extension step at 72°C for 10 min. Relative expression levels of *PsSVP* mRNA were calculated by normalizing to the level of actin mRNA according to CFX96 real-time system (Bio-Rad). Triplicates for each PCR reaction and at least three biological replicates were performed for each gene.

Sequence Comparison and Secondary Structure Prediction

Multiple alignments and analysis of complete *PsSVP* nucleotide sequences, and the putative amino acid sequences were deduced using Bio-Edit version 7.05 (Ibis Therapeutics, Carlsbad, CA). Variations in single nucleotide polymorphisms (SNPs) and insertions/deletions (InDels) among *PsSVP* genes were detected according to Wang et al. (2011).

Prediction of the secondary structure of deduced amino acid sequences of *PsSVP* genes was carried out by Protein Homology/analogy Recognition Engine V 2.0 (Phyre server[2]) based on Kelley and Sternberg (2009).

Table 1. List of primers for *PsSVP* gene cloning and expression

Primer name	Sequences of the primers
SVP1F	5′-ATGGCGAGAGAAAAGATTCA-3′
SVP1R	5′-TTATCAACCCGAATATGGCAG-3′
SVP2F:	5′-TTCAAGAAAGCCGAGGAGC-3′
SVP2R:	5′-GCCTCCTTGCTCAACATCG-3′
Actin1F	5′-GAGAGATTCCGTTGCCCAG-3′
Actin1R	5′-TCCTTGCTCATTCTGTCTGC-3′

Phylogenetic Study of *SVP*-like Genes in Tree Peony

Phylogenetic analysis was conducted using MEGA (Molecular Evolutionary Genetics Analysis) version 4.0 (Kumar et al. 2004; Wang et al. 2011). The bootstrap percentages were calculated based on 1000 replications. In addition, a phylogenetic tree was constructed based on clustering of amino acid sequences of *SVP* and *SVP*-like genes, using the neighbor-joining method. To get broad taxon spectra and to decrease the margin of error of phylogenetic trees, as many predicted amino acids sequences of SVP and SVP-like proteins from plant species as possible were chosen to construct the phylogenetic tree. Finally, the sequences that showed more than 50% similarity were used for the condensed tree. The sequences of *Arabidopsis FLC* (AY769356), *Arabidopsis SOC1* (NM_130128), *P. suffruticosa AP1* (HM143943) were used as an out-group.

RESULTS

Pre-chilling Conditions and New Root Growth and Bud Development

New root growth was observed in all old roots of the plants ($n = 24$) in pre-chilling group I (12°C for 10 d followed by 4°C for 30 d), while no new roots ($n = 24$) appeared in group II (Supplementary Fig. 2A, C) after 40 d pre-chilling treatment. Bud swelling stage occurred at the same time for each pre-chilling treatment and bud morphology at stage I (bud swelling) was also the same for plants regardless of pre-chilling treatment. At stages II and III (bud sprouting), plants receiving pre-chilling group II showed an average of twice as many abnormal leaflets the tips of which were higher than those of bud (indicated by a blue circle Fig. 1A) per bud as group I (Table 2). In order to guarantee tree peony flowering, leaflet removal and GA_3 treatment should be applied to the flower-bud at stage III.

Leaflet Removal and GA_3 Treatment on Bud Development

In pre-chilling group I, 98% of flower buds in the control treatment were aborted; only, leaflets emerged (Fig. 1E). The remaining 2% of buds stopped development in Stage IV, and they were not able to flower (Fig. 1G). In the second treatment, 85.31% of flower buds developed very slowly and finally aborted. The remaining flower buds were entirely or partially covered by leaflets, and these flower buds also aborted (Fig. 1B–E).

In the third treatment, in which bud scales and leaflets were removed, followed by GA_3 treatment, almost all the buds in group I developed normally and flowered, while an average of 2.58 ± 0.19 aborted flower-buds per plant were recorded in group II (Table 2). Leaves grew well in group I, compared with those in group II, and were on average 8 cm longer than those in group II (Table 2, Supplementary Fig. 2B, D). Average number of flowers per plant in groups I and II were 9.87 ± 0.06

Table 2. Average number of abnormal leaflets per bud, aborted flower bud, flowers and leaf length in two pre-chilling treatments of tree peony in per pot

	Pre-chilling treatment			
	Group I[z] (72 pots)		Group II[y] (24 pots)	
	Control	Removal leaflet	Removal leaflet with applying GA_3	Removal leaflet with applying GA_3
Abnormal leaflets per bud	2.04±0.07*b*	2.00±0.13*b*	1.96±0.07*b*	4.08±0.19*a*
Number of aborted flower buds per plant	10.00±0.00*a*	10.00±0.00*a*	0.13±0.06*c*	2.58±0.19*b*
Leaf length (cm)	22.80±0.85*b*	19.72±1.76*c*	25.63±1.21*a*	17.65±1.04*c*
Numbers of flowers	0.00±0.00*c*	0.00±0.00*c*	9.87±0.06*a*	7.53±0.25*b*

[z]Pre-chilling treatment: 10 d at 12°C, then 30 d at 4°C.
[y]Pre-chilling treatment: 40 d at 4°C.
a–c Means followed by different letters are significantly different at $P < 0.05$.

and 7.53±0.25, respectively (Table 2). Buds without treatment or only with leaflet removal treatment in group I were not able to flower (Table 2), while they developed relatively well in early stages.

Molecular Characterization of *PsSVP* Genes from *P. suffruticosa* cv. Luoyang Hong

Two new *PsSVP* genes were amplified and cloned from *P. suffruticosa* by using designed PCR primers. They were deposited in GenBank (accession numbers KF113362 and KF113363). The full open reading fragment (ORF) lengths of cloned *PsSVP* genes were 690 and 693 bp, respectively, encoding putative protein consisting of 228 and 229 amino acid residues. Sequence comparison of *Arabidopsis* SVP (JX863099) and AGL24 (NM_118587) and *Malus domestica* (DQ402055) SVP indicated that the deduced amino acid sequences of the two cloned coding sequences exhibited typical structural features of MADS genes, belonging to plant type II MADS-box genes. Both PsSVP proteins contained conserved MADS-box, composed of 58 amino acid residues, I-box, K-box and C-terminal domains. The high similarity with *Arabidopsis* SVP and AGL24 and *Malus domestica* SVP is most prominent in the MIK region, but similarity is also detected in the less conserved C-terminal end of predicted proteins (Fig. 2).

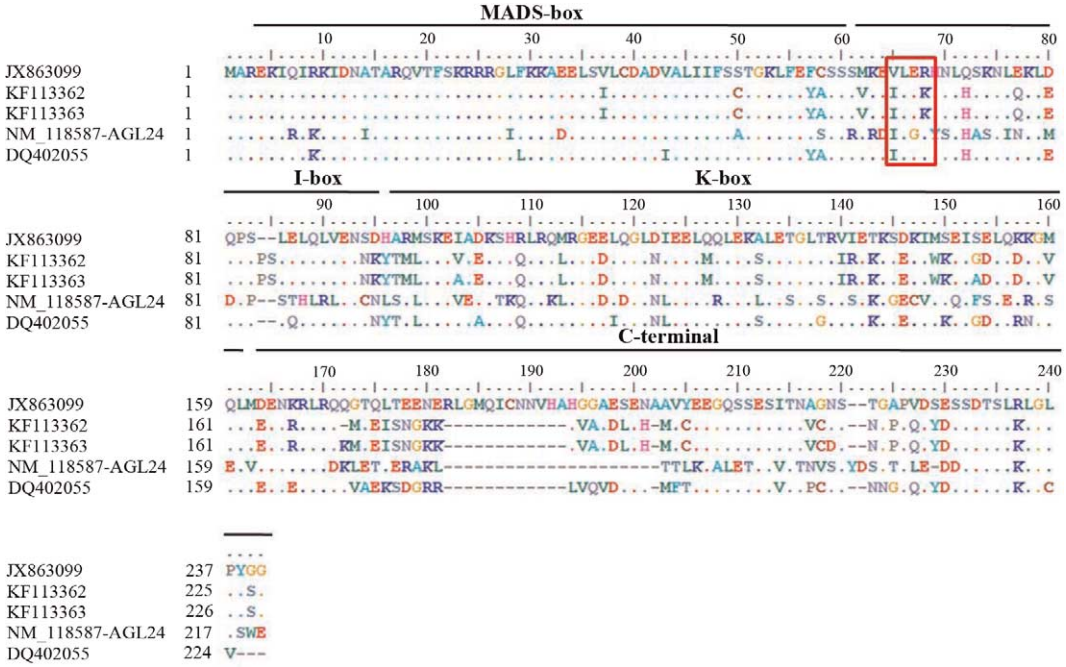

Fig. 2. Multiple alignment of the deduced amino acid sequences of five SVP and SVP-like genes. The GenBank accession number of five genes was JX863099 (*Arabidopsis thaliana*), KF113362 (*Paeonia suffruticosa*), KF113363 (*Paeonia suffruticosa*), NM_118587 (*Arabidopsis thaliana*) and DQ402055 (*Malus domestica*), respectively. The same amino acid residues and deletions with deduced protein sequence of JX863039 are indicated by dots and dashes, respectively, while variant amino acid residues are shown by abbreviation letters of the amino acid. A specific tetrapeptide was highlighted by red box in I-box domain. The gene function will be lost, if VLER →GHNL. MADS, I, K-boxes and C-terminal are marked in the figure.

In *Arabidopsis*, the SVP is a transcription repressor that inhibits floral transition in the autonomous flowering pathway. Together with AGL24 and AP1, it controls the identity of floral meristem and regulates expression of class B, C and E genes (Gregis et al. 2008). SVP has three variations and one mutagenesis in *Arabidopsis*. The mutagenesis was caused by 65th–68th amino acid residues change (VLER →GHNL), resulting of early flowering (NUCLEOTIDE SEQUENCE 1999). The four amino acid residues were marked in Fig. 2. In tree peony, the composition of amino acid was ILEK, while the first amino acid residue was not G. We speculate that the function of PsSVP is similar to *Arabidopsis* SVP.

KF113362 and KF113363 are closely related, sharing 98.25% sequence identity, and their similarity with *Arabidopsis* SVP and *Malus domestica* SVP were 69.42 and 68.60% and 77.29 and 74.42%, respectively. Based on sequence comparative analysis of KF113362 and KF113363, there were four single nucleotide polymorphisms (SNPs) and 1 InDel polymorphisms difference in KF113362 (Supplementary Fig. 3). The positions of SNPs and Indels were distributed at the 252th, 308th, 458th, 616th and 515th–517th nucleotide (causing Lys amino acid insertion). Of the four SNPs detected, the first was synonymous mutation, which was located in I-box domain, and the remaining three variations were nonsynonymous. These mutations were predicted to change Val, Gly and Asn in KF113362 to Ala, Ala and Asp in KF113363, respectively.

Secondary Structure Prediction and Phylogenetic Analysis of *P. suffruticosa* SVP-like Genes

The secondary structures of two SVP proteins were predicted by Phyre Server (http://www.sbg.bio.ic.ac.uk/phyre/) with high precision by combining in-house component algorithms under controlled conditions. Protein secondary structure prediction showed that both KF113362 and KF113363 have 56% alpha helix, 4% beta strand and 31% disordered (Supplementary Fig. 4), although there were few differences in 3D models. Further analysis showed that both SVP and two PsSVP all have 4% beta distributed at MADS-box domain, which is the main function domain (Supplementary Fig. 4). The two homologous *SVP* genes probably have similar functions as *SVP*.

Further evidence of possible phylogenetic evolutionary relationships was detected by considering the predicted SVP-like proteins from 26 other plants (Fig. 3). It is clear in Fig. 3 that the phylogenetic tree has separated into two branches. SVP proteins were clustered in one branch, and three genes as an outgroup were clustered together. Two sub-clades were apparently separated in the first branch. PsSVP-1 and PsSVP-2 were closely related to *Malus domestica* SVP, along with *Arabidopsis* SVP and SVP-like members from Cruciferae species (*Brassica juncea*, *Brassica napus* and *Brassica rapa*), woody perennial specie (*Vitis vinifera*) and Leguminosae sp. (*Pisum sativum* and *Medicago truncatula*). In further analysis of the phylogenetic tree, SVP proteins encoded by different species belonging to the same family were clustered together. Except for the above three families, SVP-like members from Poaceae, most of Solanaceae were tightly cluster together.

Transcriptional Expression Profiles of *PsSVP* in Different Flower Buds

As a way to discover transcriptional expression patterns of *PsSVP* genes in specific tissues–organs, reproductive and vegetative organs (bud, root, stem and leaf) were analyzed first by qRT-PCR (Fig. 4). This pair of primers (SVP-2F/2R) was designed on the basis of the sequences of two *PsSVP* genes to specifically amplify the desired sequences of *PsSVP* genes. The results showed that *PsSVP* genes were expressed in both reproductive and vegetative organs at different levels (Fig. 4). They were most strongly expressed in leaves and relatively highly expressed in stem. In bud and root, they had similar expression levels. However they were expressed higher in aborted flower bud (Fig. 4). *PsSVP* genes may play a role in vegetative production, and more highly expressed *PsSVP* genes may cause flower bud abortion in the reproductive growth phase. The result of qRT-PCR showed that the expression of *PsSVP* genes was decreased by GA_3 treatment (Fig. 4). The expression of *PsSVP* also decreases in the flower bud in the second treatment (Fig. 4). It was suggested that removing leaflets may decrease *PsSVP* expression in flower bud.

The transcriptional expression patterns of *PsSVP* genes in normal developmental and aborted flower buds were further investigated in this work (Fig. 5.1). As shown in Fig. 5.1, the expression of *PsSVP* genes was much lower in normal flower buds, and higher in aborted flower buds. Additionally, expression was higher in the aborted flower-buds with leaflets (Fig. 5.1 B, E), further support that higher expression of *PsSVP* genes may promote leaflet growth, which resulted in bud abortion.

The expression of *PsSVP* genes in flower buds with two treatments (removing leaflets and GA_3 treatment, removing leaflets treatment alone) and with no treatment were further investigated after 20 d (at stage V to VI) by qRT-PCR. It showed that they expressed higher in flower buds with no treatment than in those with the other two treatments (Fig. 5.2). It was suggested that GA_3 could boost flower bud development by suppressing *PsSVP* gene expression, and that removing the leaflet may partially replace the role of GA_3. Although the flower bud can emerge without any treatment, it develops slowly and finally aborts. The possible reason for the bud abortion is greater *PsSVP* genes expression.

DISCUSSION

How to Improve Flowering of Forced Tree Peony

In China, flower forcing of containerized tree peony has a long history, and it can be traced back to the Song dynasties (960–1279) (Li 1999). *Paeonia suffruticosa* cv.

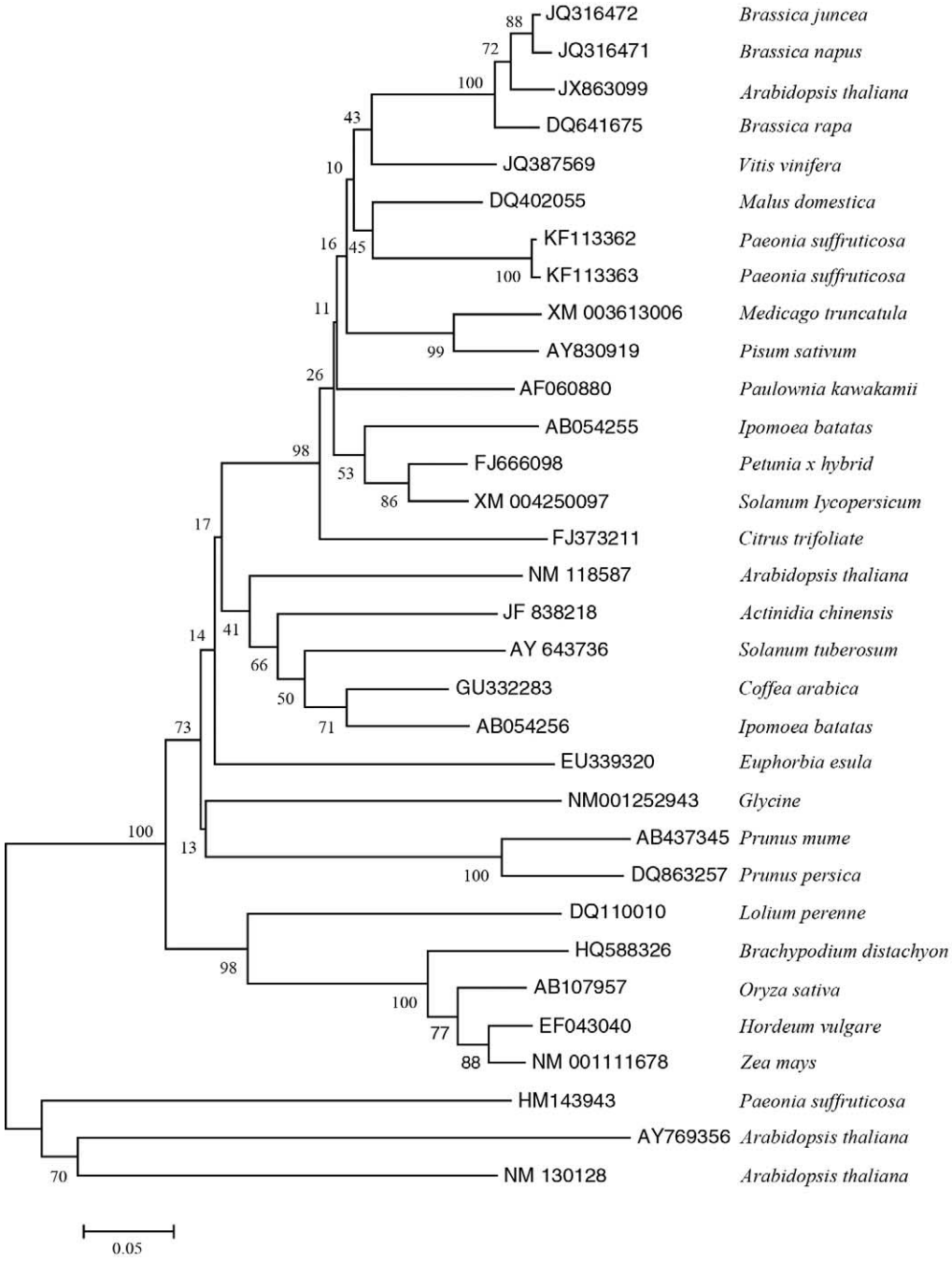

Fig. 3. The phylogenetic tree of 29 complete *SVP*, *SVP*-like genes constructed by MEGA4.1 software and calculated by the neighbor-joining method. Genes HM143943, AY769356 and NM_130128 were used as outgroup.

Luoyang Hong is a traditional cultivar and is commonly used in flower forcing. A storage period of 30–40 d at 4°C is considered sufficient to break bud dormancy (Gai et al. 2013). However, plants often exhibit poor leaf development and flower bud abortion. Our study showed that a 10-d treatment at 12°C followed by 30 d of chilling (4°C) enhanced leaf growth and flower bud development, in part because of new root growth during the 12°C exposure. New roots can absorb nutrition for leaf development and flowering.

Removing leaflets and applying GA_3 treatment in bud stage III also increased flower bud development and reduce flower bud abortion. We suggest that leaflet removal decreases *PsSVP* expression, which may, in turn, promote flower bud development. The expression of *SVP* is regulated by the autonomous and gibberellin

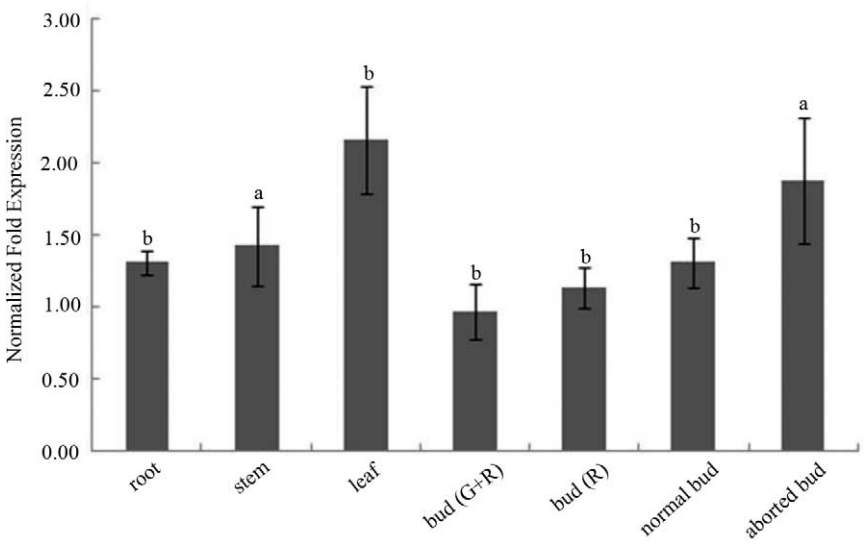

Fig. 4. Expression patterns of *PsSVP* genes in different organs of tree peony by qRT-PCR. Bud (G+R) and bud (R) represent *PsSVP* expression in flower-buds treated with GA_3 and removal of the leaflet, removal of the leaflet, respectively. Different lowercase letters above columns mean indicate significance at $P < 0.05$.

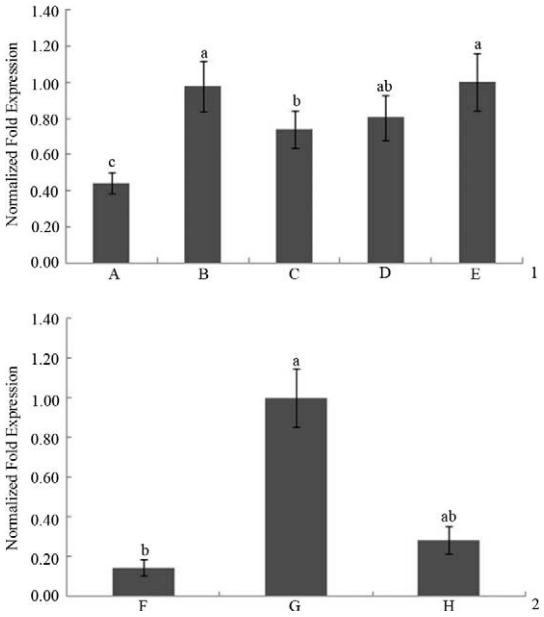

Fig. 5. Expression patterns of *PsSVP* genes in different developmental flower buds in tree peony by qRT-PCR. (1) *PsSVP* expression in normal development and aborted flower-bud. (2) *PsSVP* expression in a little big bell-like flower-bud, flower bud emerging phase, small bell-like flower bud stage. (A) normal flower bud, (B) aborted flower bud hidden completely within the leaves, (C) aborted flower bud with 1/3 partly covered by leaves, (D) aborted flower bud with 2/3 partly covered by leaves, (E) shoot-tip without flower bud, (F) a little big bell-like flower-bud stage with leaflets removed and GA_3 treatment in Stage II, (G) flower bud emerging phase (control) and (H) small bell-like flower bud stage with leaflet removal treatment. Different lowercase letters above columns indicate significance at $P < 0.05$.

pathways as well as by ambient temperature, thereby mediating delayed flowering in cool conditions (Lee et al. 2007b). Our results suggest that GA_3 application decreased *PsSVP* expression, and plants kept at 4°C for a long time were not active for tree peony new root growth and flowering. GA_3 may repress *PsSVP* expression and activate homologues *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*) expression (Wellmer and Riechmann 2010). Low expression of *PsSVP* could decrease the repression of *SOC1* expression. More *SOC1* expression could interact with its downstream gene *Leafy* (*LFY*) and the interaction of the two genes could promote tree peony flowering.

PsSVP Genes are Members of MADS-box Gene

Members of the *SVP* and SVP-like gene family have been identified in a wide range of species, and have been shown to perform diverse functions (Carmona et al. 1998; Hartmann et al. 2000; Sentoku et al. 2005; Li et al. 2010; Wu et al. 2012; Méndez-Vigo et al. 2013). In Chinese cabbage and barley they act as flowering repressors, while the *SVP* homolog, *PkMADS1*, plays a role in promoting vegetative growth in the woody plant *Paulownia kawakamii* (Lee et al. 2007a; Trevaskis et al. 2007; Prakash and Kumar 2002). The SVP-like gene from *Eucalyptus* (*EgSVP*) seems to have a slightly different function, which is active in producing additional inflorescences (Brill and Watson 2004). Thus, in perennial woody plants *SVP* might have evolved functional diversification not seen in annual herbaceous plants.

Two new MADS-box genes from tree peony were firstly isolated in trials. The predicted PsSVP proteins contained the typical domain structure, common to most plant MADS proteins, with the high conserved

MADS-box, relative conserved I, and K boxes as well as a C-terminal variable domain, and were most similar to DQ402055, a putative SVP/StMADS-11-like protein from apple. There were only three amino acid residue differences in MADS-box domain compared with that in *Malus domestica* or that in *Arabidopsis* (Fig. 2). Phylogenetic analysis suggested that *PsSVP* genes were most similar to SVP-like genes from *Malus domestica*, and they might be orthologues of Cruciferae SVP and *Vitis vinifera* SVP-like genes. Further studies suggest that all the genes clustered in the SVP group have a similar function of suppressing flowering or promoting vegetative growth. Wu et al. (2012) expected that kiwifruit *SVP1* had similar function of *SVP*, based on similarity to *SVP*. Constitutive overexpression verified kiwifruit *SVP1* caused a significant delay in flowering. It was suggested that kiwifruit *SVP1* was a functional equivalent of *SVP* and conservation of this gene was across eudicot genera. The secondary structure of PsSVP and SVP is also similar with the same beta strand composition (4%) distributed at MADS-box domain (Supplementary Fig. 4). These data indicate that *PsSVP* in tree peony may have a similar function (delay flowering) to that in *Arabidopsis*.

Expression and Function of *PsSVP* in Aborted Flower Buds

The onset of flower formation is a key regulatory event during the lifecycle of angiosperm plants, which marks the beginning of the reproductive phase of development. The transition from vegetative to reproductive growth is a key developmental step that is under tight genetic control. *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). Recent studies indicate that *SVP* may be another central regulator of the flowering regulatory network (Li et al. 2008). In the current work, *PsSVP* gene expression pattern in different tissues–organs and under different treatment were investigated by qRT-PCR. The expression result of *PsSVP* in tree peony, indicated that it had a new function (causing flower bud abortion), besides promoting vegetative growth.

Studies on flower buds that are completely covered by leaflets and shoot-tips (flower-bud not visible, with only leaflets seen) (Fig. 5.1 B, E) have shown that leaflet growth is faster than flower bud growth, and more leaves are formed in aborted buds. *PsSVP* expression was high in these types of buds (Fig. 5.1 B, E). Plants both heterozygous for *svp* mutants alleles and homozygous mutants formed fewer leaves than wt (Hartmann et al. 2000). It was suggested that greater *SVP* gene expression was beneficial for leaf growth. However, good leaf growth affects flowering in forcing culture of tree peony. Thus, high *PsSVP* expression in buds repressed flowering and finally caused flower bud formation to abort. This type of flower bud did not flower, indicating *PsSVP* is expressed in this type bud much more than in others (Fig. 1G). Removing two leaflets or applying GA_3 around flower bud, or using both methods, could decrease *PsSVP* expression. Lowered *PsSVP* expression could increase the speed of flower bud growth, and promote a transition from vegetative to reproduction growth.

Taken together, these results suggest that *PsSVP* negatively regulates flowering and positively regulate leaflet growth. GA_3 can improve floral bud development by suppressing *PsSVP* expression. Higher expression of *PsSVP* may be a cause for flower bud abortion in tree peony.

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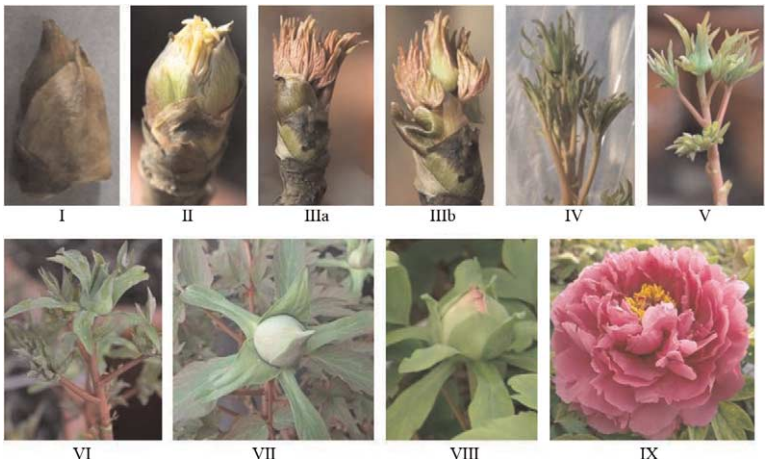
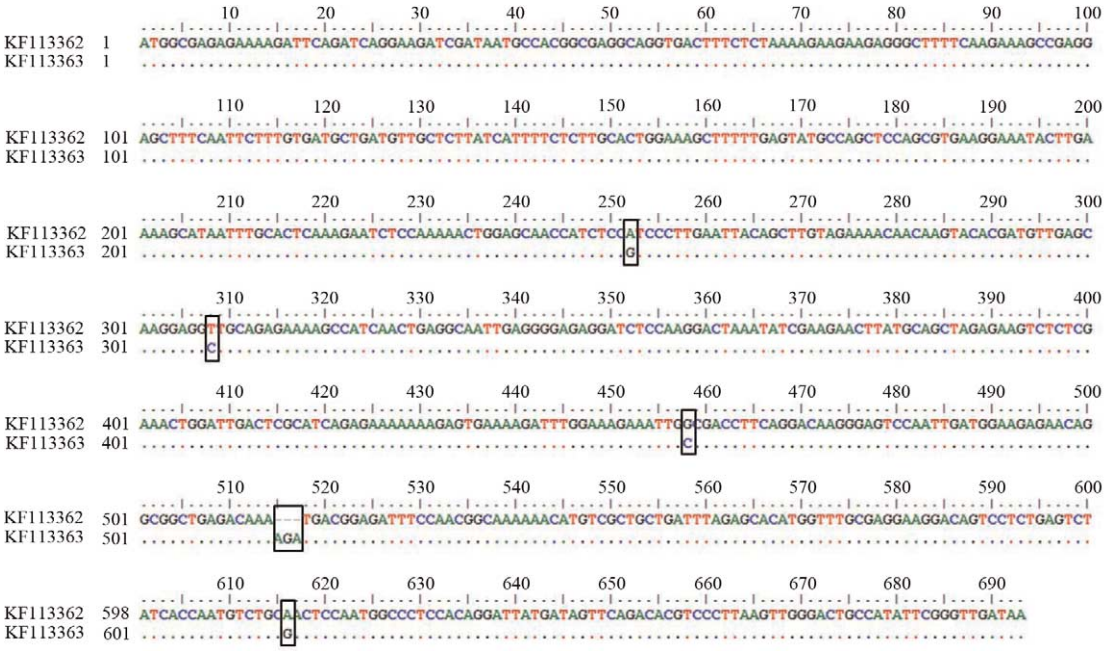
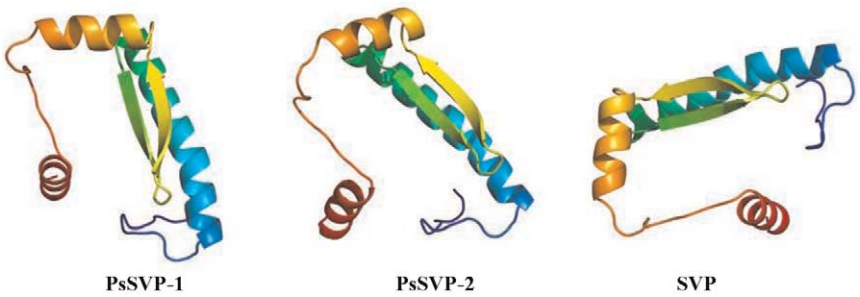


Supplementary Fig. 1. Morphological development of forced tree peony. The development from bud swelling to flowering of 'Luoyang Hong' was divided into nine stages. Stage I: Bud swelling. Stage II: Bud sprouting; flower buds tip exposed. Stage III: Leaflet emerging; leaflet kept incurved. IIIb represents two to four leaflets removed in stage III. Stage IV: Flower bud emergence; petiole extended and leaflet incurved. Stage V: Small bell-like flower bud visible; leaves unfolding and petiole expanded outward. Stage VI: Enlarged bell-like flower-bud; sepals flattened and leaves completely expanded. Stage VII: Bell-like flower-bud enlarged and elongated. Stage VIII: Petal color present, soft bud. Stage IX: Petal opening, anthesis, through petal abscission.

Supplementary Fig. 2. The root and flower of 'Luoyang Hong' observed on tree peony under 40 d pre-chilling treatment (2013 Dec. 20) and at stage IX (2013 Feb. 01), respectively. A and B show root and flowers with pre-chilling treatment at 4°C for 40 d. C and D show root and flowers with pre-chilling treatment at 12°C for 10 d, followed by 4°C for 30 d. New root marked with red arrow.



Sipplementary Fig. 3. Nucleotide sequence alignment of KF113362 and KF113363. The variant nucleotides are represented by a box.



Supplementary Fig. 4. 3D model of secondary structure prediction of *Paeonia suffruticosa* SVP-like protein and *Arabidopsis* SVP.