Leaf-Like Sepals Induced by Ectopic Expression of a SHORT VEGETATIVE PHASE (SVP)-Like MADS-Box Gene from the Basal Eudicot *Epimedium sagittatum*

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**Epimedium** L. (Berberidaceae, Ranales), a perennial traditional Chinese medicinal herb, has become a new popular landscape plant for ground cover and pot culture in many countries based on its excellent ornamental characteristics and, distinctive and diverse floral morphology. However, little is known about the molecular genetics of flower development in *Epimedium sagittatum*. Here, we describe the characterization of *EsSVP* that encodes a protein sharing 68, 54, and 35% similarity with SVP, AGAMOUS-like 24 (AGL24) and SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 (SOC1) in *Arabidopsis*, respectively. Quantitative RT-PCR (qRT-PCR) indicated that *EsSVP* transcripts were principally found in petiole and leaf tissues, with little expression in roots and flowers and no in fruits. The highest *EsSVP* expression was observed in leaves. The flowering time of *35S::EsSVP* in most *Arabidopsis thaliana* and in all petunia plants was not affected in both photoperiod conditions, but *35S::EsSVP* Arabidopsis lines induced late and early flowering under long day (LD, 14 h light/10 h dark) and short day (SD, 10 h light/14 h dark) conditions, respectively. The *35S::EsSVP Arabidopsis* produced extra secondary inflorescence or floral meristems in the axils of the leaf-like sepals with excrescent trichomes, and leaf-like sepals not able to enclose the inner three whorls completely. Moreover, almost all transgenic *Arabidopsis* plants showed persistent sepals around the completely matured fruits. Upon ectopic expression of *35S::EsSVP* in *Petunia* W115, sepals were enlarged, sometimes to the size of leaves; corollas were greenish and did not fully open. These results suggest that *EsSVP* is involved in inflorescence meristem identity and flowering time regulation in some conditions. Although, the SVP homologs might have suffered functional diversification among diverse species between core and basal eudicots, the protein functions are conserved between *Arabidopsis/Petunia* and *Epimedium*.

**Keywords:** leaf-like sepal, floral reversion, floral transition, SVP, *Epimedium sagittatum*
INTRODUCTION

The ‘photoperiodic’, ‘autonomous’, ‘vernalization’, and ‘gibberellin’ pathways in Arabidopsis seem to focus on the transcriptional regulation of the floral integrator genes FLOWERING LOCUS T (FT) and SOC1, which accelerate expression of APETALA1 (API) and LEAFY (LFY), demanded to confer floral identity on developing floral primordia (Huala and Sussex, 1992; Weigel et al., 1992; Bowman et al., 1993; Mandel and Yanofsky, 1995; Ferrandiz et al., 2000; Corbesier and Coupland, 2005, 2006). FLOWERING LOCUS C (FLC) and SVP restrict the expression of the mobile floral inducer (florigen) FLOWERING LOCUS T (FT) and other genes that initiate floral transition, in a partly tissue-specific fashion (Searle et al., 2006; Li et al., 2008; Jang et al., 2009). During the early stages of flower development, API can interact with SVP and this complex may initially repress homeotic gene activity. During this stage, also SOC1 blocks the premature expression of floral homeotic B-, C- and E-class genes in inflorescence meristems (IMs) and early floral meristems (FMs) in a redundant manner with AGL24 and SVP, respectively (Gregis et al., 2009; Liu et al., 2009; Torti et al., 2012). Floral integrators (FT and SOCl), floral repressors (SVP and FLC), and a microRNA, targeting AP2-like factors (miR172a), were identified as possible AGL15 targets (Zheng et al., 2009). AGL15 and AGL18, along with SVP and AGL24, are necessary to repress initiation of floral programs in vegetative organs (Fernandez et al., 2014). Members of the SVP-like gene family have been ascertained in a great range of species and have been shown to perform diverse functions. In Arabidopsis, two paralogous genes, SVP and AGL24, perform opposite functions during the floral transition (Hartmann et al., 2000; Yu et al., 2002), and have redundant functions during early stages of flower development (Gregis et al., 2006, 2008).

Based on the position where flowers and branches are formed, inflorescences are classified into three broad types: racemes, cymes and panicles (Rickett, 1944; Weberling, 1989; Prusinkiewicz et al., 2007). Arabidopsis and Antirrhinum produce racemes, whereas petunia produces a cyme. Epipedium sagittatum, a member of the basal eudicots belongs to Ranunculaceae and produces panicles.

Epipedium plants are regarded as an excellent evolutionary model for their distinctive and diverse floral morphologies, displaying evolutionarily intermediate forms including petaloid sepals and petals with nectariferous (nectar secreting) tissue on their inner face (Stearn, 2002). Another member of basal eudicots Aquilegia (Ranunculaceae, Ranunculales) has been studied as a new model in plant development, ecology, and evolution (Kramer, 2009), but little is known about SVP genes in Aquilegia. In the past decade, the signaling pathways that promote flowering and the way floral architecture is determined has been described in Arabidopsis, a member of the core eudicots (Blázquez, 2000; Blázquez, 2005), but whether a similar gene network works in basal eudicots Epipedium L. remains a challenging subject in the field of plant molecular development. What is the difference between basal and core eudicots with regard to flower development leading to diverse inflorescences types? To understand the relationship between MADS-box genes and floral organ development in Epipedium, we previously isolated and characterized homologs of the API/SEPPALATA(SEP)/AGL6 superclade of MADS-box genes in E. sagittatum (Sun et al., 2014). Here, we isolated an SVP-like MADS-box gene from E. sagittatum. Transgenic approaches and transcriptional analysis were used to further investigate the potential role of EsSVP, pointing at an important role in inflorescence meristem development and flowering time regulation.

MATERIALS AND METHODS

Plant Material and Growth Condition

Plant material for gene cloning and qRT-PCR analysis were obtained from E. sagittatum. The samples were collected from individuals in the experimental field of Wuhan Botanical Garden, the Chinese Academy of Sciences that were originally introduced from Hunan province, China. Leaves, petioles, flower buds, flowers, and roots were sampled and immediately frozen in liquid nitrogen and kept at −70°C until required. Total RNA from flower buds and flowers was isolated for EsSVP cloning.

Overexpression of EsSVP was carried out in the Arabidopsis Columbia-0 (Col-0) ecotype. The svp-41 lines used for complementation were screened out by its phenotype combined with RT-PCR. The Col-0, svp-41 and transgenic Arabidopsis plants were grown at 22°C under LD and/or SD conditions. To investigate flowering time, the number of rosette and/or cauline leaves of transgenic plants T3 were counted when plants bore a 1-cm-long inflorescence. Before the first flower formation, the transgenic Arabidopsis seedlings were sampled for qRT-PCR.

Wild type Petunia W115 and extrapetals (exp*W2115) was grown at average 22°C under normal greenhouse conditions. The Petunia mutant exp*W2115, containing an insertion of a dTph1 transposon in an SVP-like gene, arose spontaneously among progeny of the line W138 (Castel, 2009). The exp*W2115 plants used for functional complementation were screened out by phenotype, which is characterized by an inflorescence with a single terminal flower compared with successive terminal flowers in the wild type (Supplementary Figures S1A,B). Because seasonal changes in day length and/or light intensity might influence plant development and flowering time, care was taken to grow different genotypes side by side under the same conditions for comparison. The transgenic progeny was scored by phenotype and analyzed by RT-PCR.

Cloning, Sequence Alignment, and Phylogenetic Analysis

The fragment of EsSVP cDNA of E. sagittatum was obtained by 454 GS-FLX pyrosequencing technology (Zeng et al., 2010). Cloning of the full-length EsSVP gene was accomplished by using a 3’/5’-RACE (rapid amplification of cDNA ends) strategy as previously reported (Li et al., 2012), using GSP5-3 and GSP3-5 to amplify the 3’ and 5’ terminal regions of SVP-orthologous gene, respectively. Universal 3’ and 5’ PCR primers were supplied by the SMART™ cDNA Library Construction Kit (Clontech, Palo Alto, CA, USA). For vector construction, the complete cDNA
sequence of EsSVP was obtained by primer pairs EsSVP-V-F and EsSVP-V-R (Supplementary Table S1; Supplementary Figure S2). The sequences of selected species were downloaded from the NCBI GenBank. Multiple alignment of predicted amino acid sequences were generated using Clustal W 1.83 (Thompson et al., 1997) with a gap open penalty of 10.00 and a gap extension penalty of 0.05. Neighbor-joining (NJ) bootstrap analysis (1,000 replications) with the maximum composite likelihood model for the DNAs and the Poisson correction for the amino-acids were performed by MEGA 4 (Tamura et al., 2007). During the analyses, Arabidopsis SOC1 was used as an out-group. Sequence data from this article can be found in the GenBank/EMBL databases under the following accession numbers: SVP (NM_127820), AGL24 (NM_118587) and SOC1 (NM_130128) in Arabidopsis, potato StMADS11 (AF008652), StMADS16 (AY643736), rice OsMADS22 (AB107957), OsMADS47 (AY345221), OsMADS55 (AY345223), barley HvBM1 (AJ249142), HvBM10 (EF043040), AfsVP1 (HQ173338) and AfsVP2 (HQ173339) in Aquilegia formosa, MpMADS1 (AB050643) in Magnolia praeccisissima and DiSVP (KM657947) in Dimocarpus longan.

Construction of EsSVP-Overexpression Lines

For 35S:EsSVP, the EsSVP cDNA including partial 5’ and 3’-untranslated regions (UTRs) was amplified using the primer pairs EsSVP-V-F and EsSVP-V-R (Supplementary Table S1; Supplementary Figure S2) with Pfu DNA Polymerase (Stratagene, La Jolla, CA, USA) and cloned in pMD18-T Vector. Sequence accuracy and insertion direction were confirmed by sequencing. After digestion with SalI and SacI restriction enzymes, the insert was ligated into the modified binary vector pBI121 containing the CaMV 35S promoter and the Nos terminator. The construct was transformed to Agrobacterium tumefaciens strain GV3101 or EHA105 and used to transform Arabidopsis by the floral dip method (Clough and Bent, 1998) or Petunia via leaf disk transformation (Horsch et al., 1985). Data shown are representative phenotypes based on the analysis of multiple independent transformants.

Analysis of Gene Expression

For expression analysis, total RNA was prepared using Trizol REAGENT (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. RQ1 RNase-Free DNase (Promega, USA) pre-treatment of the RNA samples was applied to eliminate all non-RT-dependent background. 3 µg of DNase pre-treated total RNA was reverse transcribed in a total volume of 20 µL with 0.5 µg oligo(dT)15, 0.5 mM dNTPs, 10 mM DTT, 40 U RNasin. TM Ribonuclease Inhibitor (Promega, USA) and 200 U SuperScript II RNase H- reverse transcriptase (Invitrogen, Carlsbad, CA, USA).

To determine the expression pattern of SVP and/or EsSVP in transgenic Arabidopsis and/or Petunia, RT and/or qRT-PCR was performed. The qRT-PCR assays of putative genes including CO, FT, FLC, SVP, SOC1, LFY, and API were performed as described below. The primers for RT and qRT-PCR are listed in Supplementary Table S1. Reactions were performed with the SYBR Premix Ex Taq (Bio-Rad, USA) and analyzed in Eco™ Real-Time PCR System (USA). Thermocycler conditions were 2 min at 50°C followed by 10 s at 95°C and 40 cycles of 15 s at 95°C, 20 s at 60°C, and 30 s at 72°C. Relative amounts of transcripts were calculated by the comparative CT (threshold cycle number at the cross-point between amplification plot and threshold) method, and values were normalized. The Actin homolog of E. sagittatum (Huang et al., 2015) and the Tubulin2 (ß-tubulin) gene of Arabidopsis (AY054693) were used as internal controls. The qRT-PCR products were amplified using 4 µl of the RT reaction mixture, 5 µl 2×SYBR Green Master Mix, 0.5 µl forward and reverse primer (10 µmol/µl) and water to a final volume of 10 µL. RT-PCR conditions were 94°C for 1 min, followed by 28 cycles at 94°C for 30 s, 58°C for 30 s, 72°C for 1 min, and a final extension of 72°C for 3 min. The qRT-PCR was performed in three replicates for each sample and data are shown as mean values ± SD (standard deviation).

Histological Section

Floral buds and open flowers were fixed in EAF buffer (50% Ethanol, 0.5% Acetic Acid, 3.7% Formaldehyde) by applying vacuum for 15 min, then incubate 1 h at 4°C in EAF. Vacuum and incubation steps were repeated for three times. For serial microtome sections, FMs were embedded in paraffine (Paraplast Plus) after dehydration and penetration of paraffine into the plant material. The 8-mm-thick sections were stained with toluidine blue for 10 min and rinsed by deionized water for 5 min, then eosin for 5 min and dehydration by 95 and 100% ethanol for three times 15 min, deparaffin the sections in xylene.

RESULTS

Sequence Alignment and Phylogenetic Analysis

The 1027 bp length EsSVP mRNA contains an open reading frame (ORF) of 678 bp with 5’ and 3’-UTRs of 87 and 241 bp respectively (GenBank accession no. KX250266, Supplementary Figure S2). The predicted EsSVP protein of 226 amino acid residues contains a MEF2-like motif in the N-terminus and a K-box motif in the middle (Figure 1A). The putative EsSVP protein shares high similarity with other SVP-related proteins, e.g., it shows 68, 54, and 35% identity with the products of the SVP, AGL24, and SOC1 in Arabidopsis, respectively (Figure 1A). Sequence comparison of the putative protein sequence with other published MADS-domain proteins indicates that EsSVP shows extensive similarity to StMADS11 and StMADS16-like proteins (around 52 and 55%, respectively).

To better understand the evolutionary relationships, phylogenetic analysis of EsSVP with the SVP/StMADS11-like genes from other plant species was performed. EsSVP clustered closely to AfsVP.1 and AfsVP.2 from Aquilegia formosa, DiSVP from Dimocarpus longan, SVP from Arabidopsis (Hartmann et al., 2000), OsMADS22, OsMADS47 and OsMADS55 from rice (Sentoku et al., 2005), HvBM1 and HvBM10 from barley (Trevaskis et al., 2007), and MpMADS1 from Magnolia.
FIGURE 1 | Comparison of predicted SVP-like protein EsSVP sequences. (A) Alignment of *Epimedium* sagittatum SVP-like amino acid sequences with *Arabidopsis* SVP, AGL24, SOC1, and potato StMADS11, StMADS16, and barley HvBM1, HvBM10, and rice OsMADS22, OsMADS47, OsMADS55. The amino acid residues in the alignment are colored according to the following scheme: white on black, identical or conservative residue; white on gray, similar residues; black on white, non-similar or weakly similar residues. Black boxes indicate fully conserved residues; shaded boxes indicate similar and partially conserved residues. A MEF2-like motif spanning amino acids 1–58 in the N-terminus and a K-box motif in the middle are overlined in blue and green. (B) Phylogenetic tree based on the amino acid datasets with MIKC domains alignment of EsSVP in *Epimedium* and SVP-like from other plant species. The tree was generated with MEGA4.1 software, using the Neighbor-Joining method and 1000 bootstrap replicates. The scale bar indicates a divergence of 0.05 amino acid substitutions per site. SVP (NM_127820), AGL24(NM_118587) and SOC1 (NM_130128) in *Arabidopsis*, potato StMADS11 (AF008652), StMADS16 (AY643738), rice OsMADS22 (AB107957), OsMADS47 (AY345221), OsMADS55 (AY345223), barley HvBM1 (AJ249142), HvBM10 (EF043040), AGL24 (HQ173338) and AGL24 (HQ173339) in Aquilegia formosa, MpMADS1 (AB0560643) in Magnolia praecocissima and DIBVP (KM657947) in Dimocarpus longan. SOC1 was used as an out-group.

They all cluster in the SVP/StMADS11-like group (Carmona et al., 1998; Figure 1B). This shows that EsSVP belongs to the SVP group, which includes the SVP homologs (StMADS11-like) from *Arabidopsis*. This group is distinct from *Arabidopsis* AGL24 and SOC1 proteins (Figure 1B). Thus, EsSVP appears to be an SVP/StMADS11-like transcription factor.

Expression Pattern of EsSVP in *E. sagittatum* The results of our qRT-PCR experiment indicated that EsSVP transcripts were principally found in leaf tissues and petioles, but hardly in flowers and roots, and no expression was found in fruits of *Epimedium*. The expression of EsSVP in flowers is approximately 2.7-fold higher than that in roots. The highest EsSVP expression was observed in leaves, reaching nearly 740 and 270-fold higher expression than in roots and flowers respectively, and about 1.6-fold of the petioles expression (Figure 2). Based on these results, we conclude that a high level of EsSVP expression occurs in leaves, especially in leaf lamina, which might contribute to maintaining vegetative growth.

Ectopic Expression of EsSVP in *Arabidopsis* Thirty independently transformed *Arabidopsis* plants containing the full-length sense EsSVP cDNA driven by the 35S promoter were generated (35S::EsSVP *Arabidopsis*). The presence of the transgene in these lines was confirmed by PCR on genomic DNA. Twelve of the transformed *Arabidopsis* T3 lines showed various degrees of phenotypic alterations in their reproductive organs, as compared to wild type plants. Ectopic expression of 35S::EsSVP caused floral reversion with the central primary flower forming extra secondary inflorescence meristems (2nd IM) and secondary floral meristems (2nd FM) in the axils of the leaf-like sepals (Figure 3D), and also excrescent trichomes on leaf-like sepals and fruits (Figures 3G, J, K, M). Based on the total number of cauline and/or rosette leaves, 35S::EsSVP 5# *Arabidopsis*/ Col-0 plants showed late flowering under LD
expression occurred in Arabidopsis lines induced late and early flowering under LD and SD conditions, respectively. In the 35S::EsSVP Arabidopsis seedlings, CO and FT transcripts were significantly downregulated, but FLC, SVP and SOC1 were upregulated in 35S::EsSVP #LD compared to Col-0, which showed late flowering under LD conditions (Figures 3 and 5A). On average, the total number of leaves at flowering was 31.7 compared to 13.2 in Col-0 (Table 1) showing late flowering, whereas floral organs developed normally (Figure 3). However, in the early flowering 35S::EsSVP 1#SD Arabidopsis line under SD conditions, CO and FT transcripts were significantly upregulated, but FLC, SVP and SOC1 were downregulated to different degrees (Figure 5B). The total number of leaves at flowering was 10.9 compared to 64 in Col-0 on average (Table 1).

Ectopic Expression of EsSVP in Petunia

Forty-two independent Petunia transformants containing a 35S::EsSVP transgene were generated, which were all confirmed by PCR of genomic DNA. Twelve of the transgenic Petunia lines showed various degrees of phenotypic alteration in the reproductive organs, as compared to wild type Petunia W115 plants. Expression of 35S::EsSVP in wild type Petunia W115 had little or no effect on flowering time and did not affect cymose branching, but clearly affected flower development. That is, the five separated sepals were enlarged, sometimes to the size of leaves. Furthermore, these leaf-like organs expanded during flower development and after anthesis (Figures 6D,H,I). The corollas were greenish and did not fully open (partial loss of petal identity; Figures 6A,B). The shortened style was not easily separated from the elongated and enlarged ovary (arrow shown, Figures 6E,N,P). A sunken surface on the top of the stigma appeared compared with wild type (red asterisk shown, Figures 6J–M). The base of the placenta elongated many fold (Figure 6F, paraffin section material just in Figure 6E, right) compared with WT (Figure 6O, paraffin section material just in Figure 6E, left). 35S::EsSVP fruits (Figure 6F), appears longer and narrower and with the base of the placenta elongated (Figure 6G, circled). EsSVP gene overexpression in Petunia resulted in a range of floral reversion phenotypes, but was not able to phenotypically complement a Petunia exp mutant, which is characterized by an inflorescence with a single terminal flower compared with wild type Petunia W115

SD and EsSVP expression levels in mature leaves of 35S::EsSVP transformants were investigated by qRT-PCR analysis (for T3 generation plants in 35S::EsSVP SD1# Arabidopsis, the plant just as Figure 3B). The relative expression of EsSVP reached over 1000-fold the background level (Figure 4B), while SVP was expressed at slightly lower levels, that is just 0.63-fold of the Col-0 (Figure 5B). EsSVP expression levels in seedlings of Arabidopsis transgenic plants varied among different lines. Relative EsSVP expression levels in 35S::EsSVP LD 2#, 4# were approximately 2.8 and 11-fold higher compared to LD 3#, respectively. The highest EsSVP expression occurred in 35S::EsSVP LD 1#, reaching nearly 14.8-fold the LD 3#. The relative EsSVP expression in 35S::EsSVP conditions (Figure 3A), while 35S::EsSVP 1# lines showed early flowering under SD conditions (Figure 3B; Table 1). 35S::EsSVP 1# Arabidopsis generated a central primary flower with an extra one, two or four 2nd FMs and one 2nd IM in the axils of leaf-like sepals. Often these extra flowers reiterated this pattern producing a highly branched floral structure (Figures 3D,H). Five sepaloids and petals produced in one of the primary FMs (Figure 3G). 35S::EsSVP 1#, 2#, and 4# generate leaf-like sepals that develop trichomes on their adaxial side and these leaf-like sepals are not able to enclose the inner developing organs completely (Figures 3D–J). 35S::EsSVP 2# Arabidopsis developed excrectent trichomes on fruits (Figures 3E,M). 35S::EsSVP 4# produced four or five leaf-like sepals (Figures 3J,K) and green sepaloid petals (Figures 3F,K,L), comparing with empty vector control (Figures 3C,I,L). In general, transgenic Arabidopsis showed persistent sepals around the completely matured fruit (Figure 3N). Apart from that, no morphological or microstructure alterations were detected in the third and fourth whorl organs. After kanamycin-resistance selection combined with RT-PCR screening, five 35S::EsSVP/svp-4I transformants were obtained, but none showed complementation of the mutant phenotype (data not shown).

SVP and EsSVP expression levels in mature leaves of 35S::EsSVP transformants were investigated by qRT-PCR analysis (for T3 generation plants in 35S::EsSVP SD1# Arabidopsis, the plant just as Figure 3B). The relative expression of EsSVP reached over 1000-fold the background level (Figure 4B), while SVP was expressed at slightly lower levels, that is just 0.63-fold of the Col-0 (Figure 5B). EsSVP expression levels in seedlings of Arabidopsis transgenic plants varied among different lines. Relative EsSVP expression levels in 35S::EsSVP LD 2#, 4# were approximately 2.8 and 11-fold higher compared to LD 3#, respectively. The highest EsSVP expression occurred in 35S::EsSVP LD 1#, reaching nearly 14.8-fold the LD 3#. The relative EsSVP expression in 35S::EsSVP
FIGURE 3 | Phenotypes of p3SS::EsSVP transgenic Arabidopsis. (A,B) Wild type (Columbia-0) (left) and ectopic expression of 3SS::EsSVP in wild type Arabidopsis (right). (A) 3SS::EsSVP 5# causes late flowering under LD conditions (right), (B) 3SS::EsSVP 1# causes early flowering under SD conditions (right). The arrow indicates the first flower. (C) Wild type Arabidopsis Col-0 inflorescence. (D) Inflorescence of 3SS::EsSVP 1# showing floral reversion. The arrow indicates a flower that is replaced by an indeterminate racemose shoot; The arrowhead points at the main stem, and the arrow indicates a 2nd IM, red snowflake shows four 2nd FM. (E) FM of 3SS::EsSVP 2# can cause excrescent trichomes on fruit with longer fruit stem (arrow). (F) FM of 3SS::EsSVP 4# leaf-like sepal, green sepaloid petals compared with empty vector control inflorescence (C). (G) Trichomes on the adaxial side of the leaf-like sepals. Sepals are not able to enclose the inner developing organs completely. Five sepals and petals produced in one of the primary FMs (arrow). (H) Four extra flowers develop next to carpel, with excrescent trichomes on leaf-like sepals. (I) Wild type Arabidopsis Col-0 flower. (J–L) Close inspection of 3SS::EsSVP 4# flowers and sepals. Four (J) and five (K) green sepaloid petals compared with empty vector control flower (I). Green sepaloid petals (L, right) compared to the normal white ones in wild type. (M) Close inspection of trichomes on fruit, longer fruit stem, leaf-like sepal (arrow) in 3SS::EsSVP 2# flower. (N) Persistent sepals (arrows) around the matured fruits.

detected in L6 and L37, respectively. Relative expression levels reached a peak in L25 in sepals (i.e., approximately 4.4, 5.4, and 12.3-fold higher expression levels than detected in petals, leaves, and pistils, respectively), reaching >6.7 and 13.7-fold higher expression level than detected in L6 and L37, respectively (Figure 7).

These results suggest that ectopic EsSVP expression in Petunia resulted in leaf-like sepals, not fully opened greenish corollas, and abnormal stigma and matured ovary.

DISCUSSION

Evolution of EsSVP and its Expression Profile

In early diverging eudicots, the basal eudicots (Buxaceae, Trochodendraceae, Proteales, Sabiaceae, and Ranunculales) form separate well-supported clades, which are clustered closely to monocots and Chloranthaceae (Li et al., 2012). *Epimedium*

belongs to Berberidaceae, Ranunculales, and is a member of the basal eudicots. Phylogenetic analysis showed that EsSVP was clustered closely to AfSVP.1 and AfSVP.2 in *Aquilegia formosa.*

| Light condition | Genotype             | Rosette leaf numbers | Cauline leaf numbers | Total numbers of leaves |
|-----------------|----------------------|----------------------|----------------------|-------------------------|
| LD              | 3SS::EsSVP LD5#      | 26.12 ± 3.20 **      | 5.59 ± 1.12**        | 31.71 ± 3.51**          |
| WT              | 11.00 ± 0.79         | 2.24 ± 0.44          | 13.24 ± 1.03         |
| SD              | 3SS::EsSVP SD1#      | 9.44 ± 0.89 **       | 1.44 ± 0.51 **       | 10.88 ± 1.31**          |
| WT              | 53.94 ± 1.18         | 10.06 ± 1.00         | 64.00 ± 1.03         |

**Significance at 1% level (P-value < 0.01) compared with wild type Arabidopsis.**

| a,b,c | Average numbers ± SD. Twenty-eight 3SS::EsSVP LD5# and 31 3SS::EsSVP SD1# transgenic plants were used for observation under LD and SD condition, respectively. Thirty WT Arabidopsis plants were used as a control under LD/SD condition. |
All these genes clustered in the SVP/ StMADS11-like group which have similar functions in promoting vegetative growth or suppressing flowering (Li et al., 2010). According to phylogenetic analysis, which included *AGL24*, a MADS-box gene most similar to *SVP* in *Arabidopsis* (Gregis et al., 2006), *EsSVP* was more closely related to *SVP*. These findings suggest that the *EsSVP* MADS-box protein in *Epimedium* might be orthologous to the *Arabidopsis SVP* gene.

In the past, members of the SVP-like gene family performing diverse functions have been identified in a range of species. In *Arabidopsis*, *SVP* is expressed during early stages of flower development together with *AGL24* (SVP until stage 3; Hartmann et al., 2000). Both *SVP* and petunia exp are expressed during vegetative growth throughout the shoot apical meristem, in leaf primordia and in the veins of leaves (Hartmann et al., 2000; Li et al., 2008; Castel, 2009). *SVP* is transiently expressed in lateral (floral) primordia, but absent from developing flowers and siliques (Hartmann et al., 2000; Castel, 2009). *INCOMPOSITA (INCO)* in *Antirrhinum* is transiently expressed in reproductive meristems (Masiero et al., 2004). Similar to the other SVP-like genes, *EsSVP* transcript was expressed intensively in the vegetative stage, as shown here. We found *EsSVP* expression in petiole and leaf tissues, with little in root and flower and no expression in fruit. The highest *EsSVP* expression was observed in leaves, reaching over 270-fold the flower level.

**High Expression of *EsSVP* Resulted in Leaf-Like Sepals and Abnormal Flowers**

Interestingly, late flowering, floral reversions and shoot-like flowers were induced by overexpression of *SVP* or *INCO* in transgenic *Arabidopsis*. This suggests that they could either directly repress the vegetative to floral transition in *Arabidopsis*, or could indirectly interfere with the function of proteins controlling flowering (Hartmann et al., 2000; Masiero et al., 2004). On the other hand, *INCO* inhibits the prophyll development as an important control gene, which has not been reported yet for MADS-box genes (Masiero et al., 2004). Ectopic
expression of SVP homologs from rice, barley, Chinese cabbage, Kiwifruit, trifoliate orange, and *Eucalyptus* in *Arabidopsis* causes similar phenotypes, including 2nd IM or 2nd FM in the axils of the leaf-like sepals with excrescent trichomes, leaf-like sepals not able to enclose the inner three whorls (Brill and Watson, 2004; Lee et al., 2007, 2012; Trevaskis et al., 2007; Fornara et al., 2008; Li et al., 2010; Wu et al., 2012).

Constitutive expression of *OsMADS22* and *OsMADS55* led to floral reversion phenotypes including leaf-like sepals, similar to that of the SVP floral overexpressor phenotypes, whereas only *OsMADS55* expression induced late flowering arising from the repression of *FT* and *SOC1*. The complementation experiments showed that *OsMADS55*, but not *OsMADS22*, was able to rescue the early flowering phenotype and ambient temperature-insensitive flowering of *svp-32* mutants (Lee et al., 2012). Some SVP/MADS11-like genes from herbaceous plants, Chinese cabbage (Lee et al., 2007) and barley (Trevaskis et al., 2007) also act as flowering repressors.

Over-expression and complementation of Kiwifruit SVP-like genes in *Arabidopsis* resulted in a range of abnormal floral morphologies via interactions with *Arabidopsis* MADS-box proteins (Wu et al., 2012). Ectopic overexpression of *PtSVP* in *Arabidopsis* resulted in late flowering, additional trichomes and floral defects, such as flower-like structures instead of carpels (Li et al., 2010). The ectopic expression of *EgSVP* in *Arabidopsis* caused a slight delay in flowering time and produced additional inflorescences (Brill and Watson, 2004). Transcription of kiwifruit SVP1/SVP3 or trifoliate orange *PtSVP* in *Arabidopsis*
**SVP in Basal Eudicot Epimedium**

SVP-41 was able to complement the svp mutant (Li et al., 2010; Wu et al., 2012).

We determined conservation of biological function of an Epimedium SVP-like gene by over expression and complementation tests performed in Arabidopsis and/or Petunia and compared the outcome to various experiments in which SVP-like genes from other species were ectopically expressed. *EsSVP* gene over-expression in Arabidopsis and Petunia resulted in a range of floral reversion phenotypes, but was not able to complement a mutant affected in an svp-like gene in Petunia. Either, slight differences in these proteins are accountable for this or expression from the 3S promoter does not generate the appropriate expression pattern regarding timing and place.

SVP is considered to be a flowering repressor in Arabidopsis as it functionally delays flowering time. In this study, flowering time of 35S::EsSVP transgenic Arabidopsis under SD conditions or Petunia plants was not delayed. Ectopic expression of 35S::EsSVP 5# and 35S::EsSVP 1# in Arabidopsis resulted in late flowering under LD conditions and early flowering under SD conditions, respectively (Figures 3A,B). The relative EsSVP expression level in 35S::EsSVP 5# was much lower than 35S::EsSVP 1#, 4#, 2# on LD (Figure 4A). Moderate expression of EsSVP in Arabidopsis indeed delays flowering in 35S::EsSVP 5# on LD, but 35S::EsSVP 1#, 4#, and 2# are not affected on flowering time in the same photoperiod conditions. It seems that there is no obvious correlation of the mutant phenotype (number of leaves in 35S::EsSVP on LD) and the expression levels of EsSVP. However, the higher expression levels of 35S::EsSVP transformants under LD conditions, such as 35S::EsSVP 1#, 4# and 2#, the more obvious phenotype was observed on floral organs variation, including abnormal IMs and FMts from axes of the leaf-like sepal with excrescent trichomes. The relative expression of SVP in 35S::EsSVP 1# line just 0.63-fold of the WT (Col-0; Figure 5B). 35S::EsSVP 1# showing early flowering under SD conditions might thus be a consequence from post-transcriptional gene silencing. Functional divergence of EsSVP might have occurred partly because *Epimedium* belongs to the Ranunculales, a member of the basal eudicots.

Recent studies demonstrated that SVP and AGL24 are also floral meristem identity genes. Ectopic SVP and AGL24 expression induces floral meristem indeterminacy by promoting the development of new ectopic floral meristems rather than causing floral reversions (Gregis et al., 2008). Ectopic expression of barley BM1 and BM10 (Treviskis et al., 2007), kiwifruit SVP-like (Wu et al., 2012), rice OsMADS55 (Fornara et al., 2008; Lee et al., 2012) in Arabidopsis, and petunia UNSHAVEN (UNS) and EXP overexpression in Petunia (Ferrario et al., 2004; Castel, 2009) can give rise to leaf-like sepalas. In this study, EsSVP induced one to four flower-like structures or an inflorescence in place of the carpel, with fully opened leaf-like sepalas and green sepaloid petals (Figures 3D,G,H). There are no leaf-like sepalas in E. sagittatum, but its sepalas are always bigger than petals. Whether EsSVP determines sepal size in Epimedium remains to be established. Outer four sepalas are purple spotted and apex blunt, outer pair narrowly ovate and inner pair oblong-ovate is ca. 3.5 × 1.5 and 4.5 × 2 mm, respectively; inner sepalas are white, ovate-deltoid, apex acute and ca. 4 × 2 mm in size. Petals are brownish yellow, saccate, blunt and 1.5-4 mm in size (Maximowicz, 2011).

More trichomes are usually considered to be a juvenile phenotype of Arabidopsis (Telfer et al., 1997). The 35S::EsSVP lines showed a typical juvenile character, excrescent trichomes on opened leaf-like sepalas and fruits. The overexpression of EsSVP induced floral reversions and 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.

Constitutive expression of 35S::EsSVP in petunia W115, caused little or no effect on flowering time and cymose branching, but clearly affected flower development, including not fully opened greenish corollas, not enclosed leaf-like sepalas, sometimes to the size of leaves. This result also coincides with the reports on overexpression of the SVP-like gene, p35S::EXP in wild type Petunia (Castel, 2009). EsSVP overexpression in Petunia shares some phenotypes with that of overexpression of Petunia UNS, a SOC homolog in Arabidopsis. 35S::UNS in petunia leads to not fully opened and greener corollas until full maturity and a stem-like structure at the base of the sepal in the elongated ovaries (Ferrario et al., 2004). The phenotype of 35S::EsSVP transgenic petunia plants also includes shortened style, a stem-like structure at the base of the sepal in a significantly longer ovaries, a sunken surface on the top of the stigma, persistent petals on the completely matured fruit (Figure 6C). When introduced in exp, 35S::EsSVP could not rescue the mutant phenotype. Like in Arabidopsis, differences in the proteins or the 35S promoter expression pattern might be accountable for the lack of complementation.

Recent study showed that SVP delays flowering by repressing integrator gene expression as well as the plant growth regulator gibberellin (GA) biosynthesis. The results link GA biosynthesis to the established regulatory cascade of flower development and illuminate one of the mechanisms by which levels of growth regulators are synchronized with floral transition (Andrés et al., 2014). Regardless of the underlying mechanism, EsSVP effected different phenotypes in different genotypes (Arabidopsis and petunia). Abnormal flower development was caused by ectopic expression of EsSVP, affecting flower and primordia development during the reproductive phase.

**AUTHOR CONTRIBUTIONS**

ZL designed the research, performed vector construction, plant transformation, histological section and wrote the manuscript. YL and SZ performed detection of mutations, gene isolation expression. ZL and ES contributed reagents and materials. ES performed critical editing of the manuscript. All authors reviewed the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2016.01461

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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