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ORIGINAL ARTICLE

Functional conservation and divergence of five *SEPALLATA*-like genes from a basal eudicot tree, *Platanus acerifolia*

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Abstract

Main conclusion **Five *SEP*-like genes were cloned and identified from *Platanus acerifolia* through the analysis of expression profiles, protein–protein interaction patterns, and transgenic phenotypes, which suggested that they play conservative and diverse functions in floral initiation and development, fruit development, bud growth, and dormancy.**

SEPALLATA (*SEP*) genes have been well characterized in core eudicots and some monocots, and they play important and diverse roles in plant development, including flower meristem initiation, floral organ identity, and fruit development and ripening. However, the knowledge on the function and evolution of *SEP*-like genes in basal eudicot species is very limited. Here, we cloned and identified five *SEP*-like genes from London plane (*Platanus acerifolia*), a basal eudicot tree that is widely used for landscaping in cities. Sequence alignment and phylogenetic analysis indicated that three genes (*PlacSEP1.1*, *PlacSEP1.2,* and *PlacSEP1.3*) belong to the *SEP1/2/4* clade, while the other two genes (*PlacSEP3.1* and *PlacSEP3.2*) are grouped into the *SEP3* clade. Quantitative real-time PCR (qRT-PCR) analysis showed that all *PlacSEPs*, except *PlacSEP1.1* and *PlacSEP1.2*, were expressed during the male and female inflorescence initiation, and throughout the flower and fruit development process. *PlacSEP1.2* gene expression was only detected clearly in female inflorescence at April. *PlacSEP1.3* and *PlacSEP3.1* were also expressed, although relatively weak, in vegetative buds of adult trees. No evident *PlacSEPs* transcripts were detected in various organs of juvenile trees. Overexpression of *PlacSEPs* in *Arabidopsis* and tobacco plants resulted in different phenotypic alterations. *35S:PlacSEP1.1*, *35S:PlacSEP1.3,* and *35S:PlacSEP3.2* transgenic *Arabidopsis* plants showed evident early flowering, with less rosette leaves but more cauline leaves, while *35S:PlacSEP1.2* and *PlacSEP3.1* transgenic plants showed no visible phenotypic changes. *35S:PlacSEP1.1* and *35S:PlacSEP3.2* transgenic *Arabidopsis* plants also produced smaller and curled leaves. Overexpression of *PlacSEP1.1* and *PlacSEP3.1* in tobacco resulted in the early flowering and producing more lateral branches. Yeast two-hybrid analysis indicated that PlacSEPs proteins can form homo- or hetero-dimers with the *Platanus* APETALA1 (AP1)/FRUITFULL (FUL), B-, C-, and D-class MADS-box proteins in different interacting patterns and intensities. Our results suggest that the five *PlacSEP* genes may play important and divergent roles during floral initiation and development, as well as fruit development, by collaborating with FUL, B-, C-, and D-class MADS-box genes in London plane; *PlacSEP1.3* and *PlacSEP3.1* genes might also involve in vegetative bud growth and dormancy. The results provide valuable data for us to understand the functional evolution of *SEP*-like genes in basal eudicot species.

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Keywords Floral development · Landscape plant · London plane · MADS-box · Protein–protein interaction · *SEP*

Abbreviations

AG	AGAMOUS
AGL	AGAMOUS LIKE
AP1(3)	APETALA1(3)
ARF	Auxin response factor
FUL	FRUITFULL
GRF	Growth-regulating factor
PPI	Protein–protein interaction
SEP	SEPALLATA
STK	SEEDSTICK

Introduction

Angiosperm flowers are quite diverse in their morphology. Orthologous genes from different species can display divergent functions, which may provide the genetic basis for the floral diversification of flowering plants (Irish and Litt 2005; Theissen and Melzer 2007). In the early 1990s, the ABC model that elucidates how three functionally defined groups of genes (A, B and C) specify the four organ types of a typical eudicot flower was established based on molecular and genetic studies of homeotic mutants in *Arabidopsis thaliana* and *Antirrhinum majus* (Coen and Meyerowitz 1991; Bowman et al. 1991). According to this model, the petals are specified by the combination of A and B function genes, the stamens are specified by the combination of B and C function genes, while the sepals and carpels are specified alone by the A and C function genes, respectively; in addition, the A and C genes are mutually antagonistic. It is interesting that all genes involved in the ABC model, except *APETALA2* (*AP2*), encode putative transcriptional regulators and belong to the MADS-box gene family (Weigel and Meyerowitz 1994).

The ABC model has broadly been used as a framework for understanding the flower development of a wide range of angiosperm species (Causier et al. 2010), and later, it was extended into the ABCE and quartet model (Pelaz et al. 2000; Theissen 2001), with the addition of D- and E-class MADS-box genes. The D-class genes were first identified in *Petunia hybrida* (Angenent et al. 1995), where they specified the ovule identity. In *Arabidopsis*, the D-function gene, *SEEDSTICK* (*STK*), is also required for specifying the identity of ovules, together with *AGAMOUS* (*AG*), *SHATTERPROOF1* (*SHP1*), and *SHP2*, in addition, it plays a role in seed abscission and funiculus development (Pinyopich et al. 2003). The E-function genes are proposed based on a reverse genetic approach showed that four *SEPALLATA* (*SEP*) genes (*SEP1/2/3/4*; formerly *AGAMOUS LIKE* (*AGL)2/4/9/3*, respectively) are required for development of sepals, petals, stamens, and carpels in *Arabidopsis* (Pelaz et al. 2000; Theissen 2001; Ditta et al. 2004). In *Arabidopsis*, single *sep* mutants have only subtle phenotype, while *sep1 sep2 sep3* triple mutants produce flowers in which all organs develop as sepals (Pelaz et al. 2000), and the *sep1 sep2 sep3 sep4* quadruple mutants show an even stronger phenotype than the triple mutants, in which all floral organs are replaced by leaf-like organs (Ditta et al. 2004). Therefore, the different SEP proteins have largely, but not completely redundant functions in determining floral organ identity (Pelaz et al. 2001); they may bind to largely overlapping, but not identical sets of target genes that differ in the arrangement and spacing of the CArG-boxes in their cis-regulatory regions (Jetha et al. 2014).

Besides *Arabidopsis*, the E-class genes have been isolated from various angiosperms, including eudicots, monocots, and several basal angiosperm species (Li et al. 2005, 2015; Malcomber and Kellogg 2005; Zahn et al. 2005). Phylogenetic analysis of the available *SEP* genes shows multiple duplications within this subfamily, the first occurring before the origin of extant angiosperms producing the *SEP3* and *LOFSEP* clades (Zahn et al. 2005). Within the *SEP3* clade, an early diverging cluster of Asteraceae genes (*ASTERACEAE SEP3*) hints a second duplication early in angiosperm evolution, and additional duplications occurred at the base of grasses and more recently in some other families. Within the *LOFSEP* clade, duplications at or near the base of core eudicots produced the *SEP1/2*, *FBP9/23,* and *SEP4* subclades, and the monocot genes show duplications near the base of grasses, producing the *LHS1*, *OsMADS5,* and *OsMADS34* subclades (Zahn et al. 2005).

Functional data showed that *SEP* genes play a crucial role in floral meristem and organ identity. In *Arabidopsis*, the SEP proteins have been shown to participate in the formation of multimeric complexes with other MADS-box proteins, including the products of A, B, and C genes, to direct flower organ and meristem identity (Honma and Goto 2001; Ditta et al. 2004; Immink et al. 2009). In *petunia*, the B, C, and D functions require E-class genes (*FBP2* and *FBP5*) to specify petal, stamen, carpel, and ovule development (Vandenbussche et al. 2003; Matsubara et al. 2008). The *SEP* homologs in tomato, gerbera, birch, poplar, rice, and phalaenopsis have also been shown to be necessary for floral meristem and organ identity, even for inflorescence development (Pnueli et al. 1994; Kotilainen et al. 2000; Lemmetyinen et al. 2004; Uimari et al. 2004; Cseke et al. 2005; Cui et al. 2010; Gao et al. 2010; Pan et al. 2014). In addition, *SEP* genes have been increasingly proved to play a role in fruit development and ripening, such as *LeMADS-RIN* and *TM29* in tomato (Ampomah-Dwamena et al. 2002; Vrebalov et al. 2002), *FaMADS9* in

strawberry (Seymour et al. 2011), *MdMADS8/9* in apple (Ireland et al. 2013), *CaMADS-RIN* in pepper (Dong et al. 2014), *MgAGL2/9* and *MgSEP2* in lotus magnolia (Lovisetto et al. 2015), and *MaMADS1/2* in banana (Elitzur et al. 2016). Expression of *SEP*-like genes in vegetative tissues of some plant species suggests that they may also function in vegetative growth (Ferrario et al. 2003; Tzeng et al. 2003; Cseke et al. 2005; Elitzur et al. 2010; Liu et al. 2010; Li et al. 2014), which remains to be characterized. Recently, the tomato SEP protein SlMBP21 was shown to form protein complexes with JOINTLESS and MACROCALYX as a transcription activator for the development of the flower abscission zone (Liu et al. 2014), suggested that the *SEP*-like genes may play more diverse roles than what we recognized in plant development via subfunctionalization or even neofunctionalization. So far, *SEP* genes have been well characterized in some core eudicots and monocots (i.e. *Arabidopsis*, tomato, and rice); however, we still have very limited knowledge on the evolution and function of *SEP* genes from basal eudicot and basal angiosperm species (Li et al. 2005, 2015; Liu et al. 2010; Lovisetto et al. 2015).

London plane (*Platanus acerifolia*) is a basal eudicot tree belonging to the order Proteales that was widely planted in roadside and courtyard for shading. Flowering in trees is often associated with bud dormancy, a physiological state typical for most perennials, where growth is repressed within the buds, usually over the winter. *Platanus* species are monoecious with unisexual flowers aggregated into compact, spherical inflorescence heads (capitula), and the flowers are unusual in their structure, where the male flowers consist of one whorl of perianth organs followed by an alternating whorl of three-ridged organs and a whorl of stamens, while the female flowers are composed of a whorl of perianth organs followed by a whorl of small club-shaped organs, a whorl of staminodes, and two whorls of carpels (von Balthazar and Schonenberger 2009). Flowering of London plane is spread over two growing seasons. During the first season, lateral buds are formed under the petiole base (namely subpetiolar buds) on developing shoots (April–May), followed by two developmental fates. Most subpetiolar buds of adult trees (frequently located at the middle and upper part of the shoots) differentiate inflorescence and secondary shoot meristems individually in the same bud, hereinafter referred to as mixed flower buds, where floral meristem and floral organ differentiation occur at the early summer (June–July). While some subpetiolar buds located at the bottom part of the shoots or lower shoots of the tree can only differentiate shoot meristems without inflorescence meristems, hereinafter referred to as vegetative subpetiolar buds. By autumn, these lateral buds have ceased growth and become dormant. Flower development continues as the buds resume growth during the spring of the second growing season (Li et al. 2012b). This floral initiation and development process can be divided into inflorescence primordium differentiation phase (late May–early June), flower meristem initiation phase (mid-June), floral organ differentiation phase (late June–late July), first floral organ development phase (August–Oct), bud dormancy stage (Nov–Jan), and second floral organ development phase (Feb–April of next year) followed by anthesis and pollination at April (Li et al. 2012b). The fruits develop from the late April and mature till October.

As an excellent landscaping plant, London plane has an unfavorable characteristic that the abundant pollens shed by mature flowers and the dispersing achenes with numerous stiff hairs released from broken seed balls not only pollute the environment but also may result in serious pollinosis and breathing difficulties if breathed in by people (Lu et al. 2012). To remove this disadvantage, understanding the molecular regulatory mechanisms of flowering and flower development and then breeding non-flowering and fruitless varieties are meaningful and imperative. In this paper, we cloned and characterized five E-class MADS-box genes from *P. acerifolia*, homologous to the *SEP1/2/4* and *SEP3* genes in *Arabidopsis*, respectively. The results indicated that the five genes have divergent expression patterns, and may also possess distinct functions for flower development and bud dormancy, even for fruit and seed development, like the orthologous genes in other plant species.

Materials and methods

Plant materials

Plant samples used in this study were collected from the juvenile (2-year-old) or adult (over 30-year-old) London plane (*Platanus acerifolia*) trees in the campus of Huazhong Agricultural University on April 2013–April 2014. As described above, *Platanus* undergo a long period to complete its flower and fruit development process, which span two growing seasons. To catch the comprehensive gene expression profiles during the whole flower and fruit development process, we sampled monthly for different developmental stages according to the previous observation (Li et al. 2012b). Various samples from adult trees include the stems (S), young leaves (YL), mature leaves (ML), shoot apical buds (AB), and lateral subpetiolar buds (SB) in mid-April and mid-May, respectively; vegetative subpetiolar buds (VB), mixed flower buds (MB) containing differentiated inflorescences and vegetative tissues, vegetative tissues in mixed flower buds (MB-V), inflorescences in mixed flower buds (MB-F), and developing fruits

(F) during June to September when the male and female inflorescences and flowers begin to differentiate but cannot be distinguished clearly (Li et al. 2012b); different ontogenetic stages of male inflorescences (MF), female inflorescences (FF), and fruits (F) from October to April of second year. The male inflorescences at mid-March and April were sampled by separating into flowers mainly consisting of anthers (MF-A) and the fleshy inflorescence peduncles (MF-P). In addition, roots (JR), stems (JS), young leaves (JYL), mature leaves (JML) and subpetiolar buds (JSB) were sampled from juvenile individuals at June. All samples were collected from three individual trees, respectively, and immediately frozen in liquid nitrogen.

Cloning of *Platanus acerifolia SEP*-like genes

Total RNA was extracted from London plane tissues using the CTAB method as described by Li et al. (2008). Two micrograms of total RNA were reverse-transcribed using PrimeScript™ RT reagent Kit with gDNA Eraser (Takara, Otsu, Japan) according to the manufacturer's instructions. RNA and cDNA were stored at −80 °C for long-term use. Primers for PCR of the *P. acerifolia SEP*-like genes were designed according to the transcriptome sequencing data of London plane (unpublished), and were listed in Table S1. The amplified products were cloned into a pMD18-T vector (Takara), and 4–5 positive clones were randomly selected for sequencing.

Sequence alignment and phylogenetic analysis

Multiple sequence alignment for motif identification was performed using Vector NTI version 11.5 (Invitrogen) Clustal W program with default settings. A total of 75 *SEP*-like genes (cf. Fig 2, Table S2) were downloaded from the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov) for phylogenetic analysis. The *AGL6* and *AGL13* genes in *AGL6* subfamily of *A. thaliana* were used as outgroup. Full-length amino-acid sequences were first aligned using the default settings in MUSCLE implemented in MEGA version 6.0 (Kumar et al. 2004), and then adjusted manually with the reference alignment provided by Zahn et al. (2005). Phylogenetic tree was constructed using MEGA v6.0 by the neighbor-joining (NJ) method with 1000 bootstrap replicates.

Gene expression analysis by real-time quantitative RT-PCR

Expression of the *PlacSEP1* and *PlacSEP3* genes in different tissues and development phases of London plane was investigated by real-time quantitative RT-PCR (qRT-PCR) analysis. Gene-specific primers for qRT-PCR were designed within the non-conservative C-terminal region and 3′ UTR (untranslated region) using the Primer 5.0 software to amplify products between 90 and 300 bp in size (Table S1). The expected size of PCR products was confirmed by agarose gel electrophoresis. Amplification and quantification were carried out using the SYBR Premix Ex Taq (Takara) and the ABI Prism 7500 Sequence Detection System (Applied Biosystems). qRT-PCR products were amplified using 1.0 µl of template from the RT reaction mixture, 5 µl 2 × SYBR Green Master Mix, 0.2 µl forward and reverse primer (10 µmol/µl), and water to a final volume of 10 µl. PCR amplification parameters were as described previously by Zhang et al. (2013). Each PCR was performed in triplicate and data are shown as mean values ± SE (standard error). PCR efficiency for each primer pair was determined by a standard curve generated with serially diluted cDNA. Output data generated by the instrument onboard software Sequence Detector Version 1.3.1 (PE Applied Biosystems) were transferred to a custom-designed Microsoft Excel macro for analysis. Relative expression levels of the target genes were determined using the Relative Expression Software Tool (Multiple Condition Solver REST-MCS v2), normalized to the references genes *TPI* (*triose phosphate isomerase*) of *P. acerifolia* (Zhang et al. 2011, 2013; Lu et al. 2012).

Vector construction

The pMD18-T vectors containing full-length coding sequences (CDS) of *PlacSEP1* or *PlacSEP3* genes were digested by *Sal*I and *Kpn*I or *Sal*I and *Sac*I restriction enzymes, and the target fragments were ligated into the corresponding sites of vector pMV, modified from the binary vector pBI121 (Zhang et al. 2011, 2013; Lu et al. 2012) containing the CaMV 35S promoter and the Nos 3′ transcriptional terminator, resulting in *35S:PlacSEP1.1*, *35S:PlacSEP1.2*, *35S:PlacSEP1.3*, *35S:PlacSEP3.1,* and *35S:PlacSEP3.2* constructions, respectively. All the constructed plasmids were confirmed by PCR and restriction digestions. The resulting plasmids were then transformed into the *Agrobacterium tumefaciens* strain EHA105 or GV3101 by the electroporation method.

Plant transformation and phenotype analysis

Overexpression of *PlacSEP* genes in *Arabidopsis* was carried out in wild-type Columbia (Col-0). *Agrobacterium tumefaciens*-mediated *Arabidopsis* transformation was performed by the floral-dipping method (Clough and Bent 1998). The transformed seeds were selected on Murashige and Skoog (MS) agar with 50 µg ml^{-1} kanamycin and 50 µg ml^{-1} cefotaxime. Following the segregation tests, 16

kanamycin-resistant transgenic plants of the T_2 generation lines which fitted a segregation ratio of 3:1 were chosen to record flowering time and floral phenotype. Seedlings were grown in a growth incubator at 22 °C under a long day (LD) conditions (16/8 h, light/dark).

Agrobacterium tumefaciens EHA105 containing the *35S:PlacSEP1.1* or *35S:PlacSEP3.1* construction was also used to transform tobacco (*Nicotiana tabacum*) cultivar 'Xanthi'. Tobacco plants used for transformation were cultured in the plant growth regulator-free MS solid medium at 25 °C under a 16 h photoperiod. Transformation of tobacco plants was performed as described by Horsch et al. (1985). Transgenic lines of T_0 and T_1 generations were verified by genomic amplification of the transgene using a primer to the 35S promoter (*35SF*) and a *PlacSEP*-specific primer (*PlacSEP1.1R* or *PlacSEP3.1R*) (Table S1). Phenotype changes of transgenic tobacco plants were investigated in both T_0 and T_1 generations.

Transgene and downstream target gene expression analysis

To confirm the relationship between *PlacSEP* transgenes and phenotypic changes of transgenic plants, RT-PCR was performed to analysis the expression of the transgene in *Arabidopsis* and tobacco, which were carried out on 14-day-old seedlings for *Arabidopsis* and young leaves and flower buds for tobacco. Total RNA was isolated from wild-type and T_1 transgenic lines using the Trizol reagent (Takara), and then reverse-transcribed with the same reagent kit described above. Arabidopsis *EF1α* (*AtEF1α*, AT5G60390) and tobacco *EF1α* (*NtEF1α*, GenBank accession: D63396.1) were used as the endogenous reference genes to normalize small differences in template amounts. Primers used for the detection of *PlacSEPs* expression levels in the transgenic plants were listed in Table S1.

To understand the functional conservation of *Platanus* SEP-like proteins and the underlying mechanism of differential phenotypic changes of *35S::PlacSEPs* transgenic *Arabidopsis* plants, the expression of several flowering time and leaf development-related genes that are regulated directly by *SEP3* in *Arabidopsis*, including *FT*, *SOC1*, *LFY*, *AP1*, *SEP3*, *AG*, *GRF1*, *GRF2*, *GRF5*, *TCP3*, *TCP18*, *TCP20,* and *ARF2* (Kaufmann et al. 2009; Pajoro et al. 2014), were investigated using semi-quantitative and quantitative RT-PCR analysis. Total RNA was isolated from *35S::PlacSEPs* transgenic (T_2) or wild-type *Arabidopsis* seedlings at 10 days after sowing. Conditions for reverse transcription and RT-PCR are the same as above. Primers used for PCR were listed in Table S1.

The yeast two-hybrid assay

The full-length open reading frame of London plane *SEP*-like genes, *PlacFUL*, *PlacAP3*, *PlacPI2a* (previously named *PaPI2a*) (Zhang et al. 2011; Li et al. 2012a), *PlacAG,* and *PlacSTK,* were amplified by PCR with primers as listed in Table S1. The PCR products were simultaneously introduced into the pGBKT7 and pGADT7 vector, fused in-frame to the GAL4 binding domain (pBDGAL4, bait) and the GAL4 activation domain (pADGAL4, prey), respectively. The pBDGAL4-PlacPI2a, pADGAL4-PlacAP3, pADGAL4-PlacAG, and pBDGAL4-PlacAG constructs were previously described (Zhang et al. 2011, 2013; Lu et al. 2012). All constructed were confirmed by sequencing analyses. Both of the bait and prey plasmids were transformed into yeast strain AH109 using the Frozen-EZ Yeast Transformation II Kit (Zymo Research Corp, Irvine, CA, USA). The colonies were selected on SD plates lacking Leu and Trp. Interactions between the tested proteins were determined by spotting assay on selective SD media lacking Leu, Trp, His, and Ade, supplemented with X-α-gal.

Results

Identification and phylogenetic analysis of London plane *SEPALLATA*-like genes

Five London plane *SEP*-like genes were cloned and identified according to the transcriptome sequencing data of *P. acerifolia* (GenBank accession nos KT380052–KT380056), amongst them two members show high-sequence identity, over 99% identity at nucleotide sequence and 98.8% identity at amino-acid sequence, respectively, with the *PaSEP1* and *PaSEP3* isolated previously (Li et al. 2012b), suggesting that they may be the alleles from the same gene locus. Therefore, only the newly isolated genes were investigated further in this study. Sequence alignment and phylogenetic analysis suggested that three of the five *P. acerifolia SEPs* (designated *PlacSEP1.1*, *PlacSEP1.2,* and *PlacSEP1.3*) belong to the *SEP1/2/4* clade, while the other two (designated *PlacSEP3.1* and *PlacSEP3.2*) were grouped into the *SEP3* clade (Figs. 1, 2). The full-length CDS of these *SEP*-like genes encode 244-, 243-, 243-, 240-, and 240 amino-acid proteins, respectively. All predicted amino-acid sequences of PlacSEP proteins possess the conserved MIK domain and a divergent C-terminal domain with the conserved SEP I and SEP II motifs (Fig. 1; Zahn et al. 2005). The three *PlacSEP1* sequences share 79.9% nucleotide identity and 74.6% amino-acid identity in their coding regions, and all contain an characteristic SEP1 terminal motif

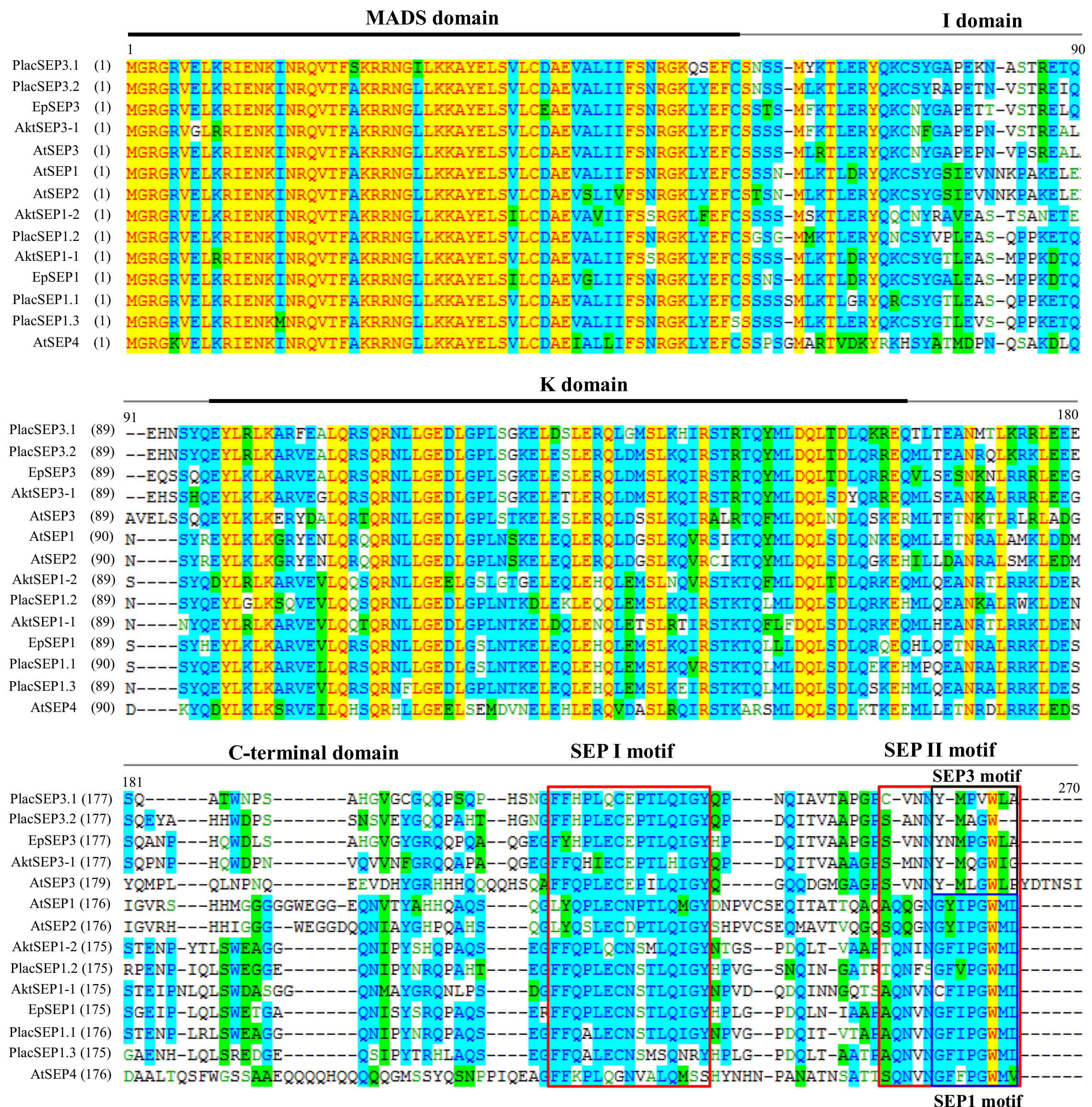

Fig. 1 Alignment of *Plactanus* SEP-like amino-acid sequences with *Arabidopsis*, *Akebia,* and *Euptelea*. The MADS and K domains are marked with *black bold lines*. The I domain and C-terminal domain are marked with *gray lines*. SEP I motif and SEP II motif are highlighted by *red box*, and SEP1 motif and SEP3 motif are marked in *dark and blue boxes*, respectively

(Malcomber and Kellogg 2005), but *PlacSEP1.1* and *PlacSEP1.3* are more closely related to each other than either is to *PlacSEP1.2* (Fig. 2). The two *PlacSEP3* CDS regions share 86.7% nucleotide identity and 83.1% amino-acid identity, and contain an SEP3 motif that is different from the SEP1 terminal motif (Malcomber and Kellogg 2005), whereas the PlacSEP3.2 protein has an incomplete motif with two amino-acid deletion, and the PlacSEP3.1 protein has an amino-acid substitution of G to V in the motif (Fig. 1).

Expression of *PlacSEP1* and *PlacSEP3* genes in London plane

To investigate the spatio-temporal expression pattern of *PlacSEP1* and *PlacSEP3* genes in London plane, qRT-PCR

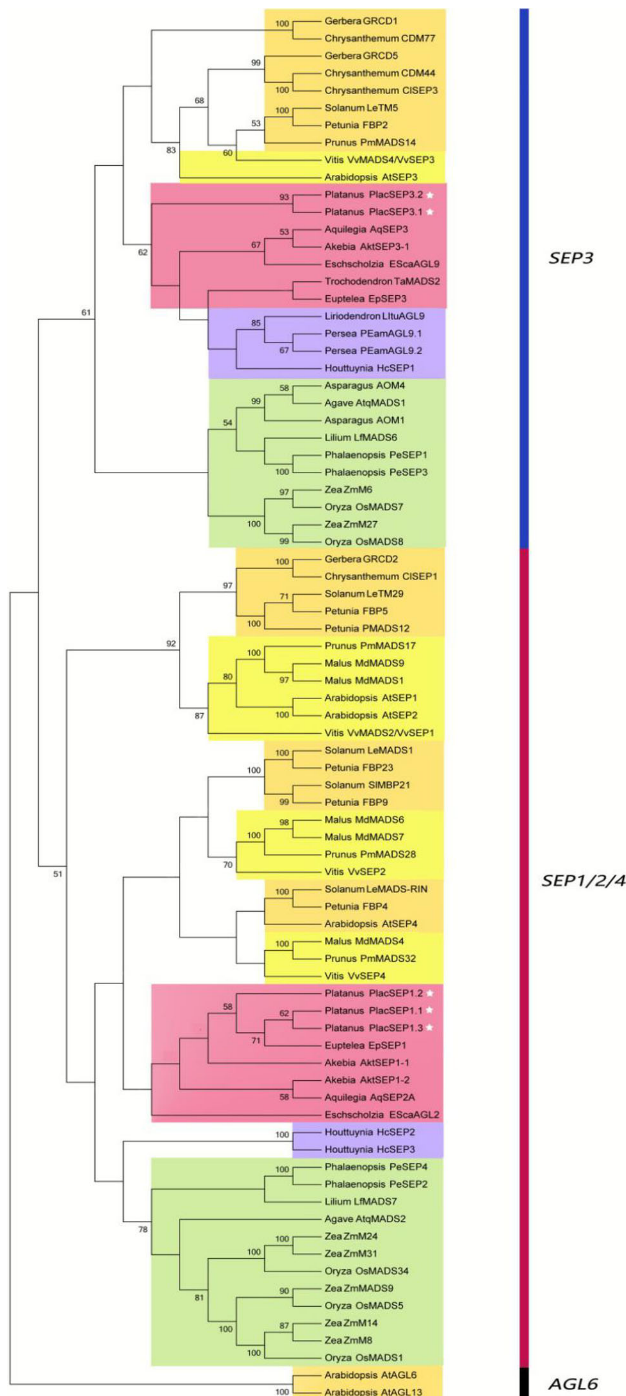

Fig. 2 Phylogenetic tree based on the amino-acid alignment of predicted PlacSEP proteins and SEP-like proteins from other plant species. The tree was generated with the MEGA v6.0 software, using the neighbor-joining (NJ) method and 1000 bootstrap replicates, including monocots (*green*), magnoliids (*purple*), basal eudicots (*pink*), rosids (*yellow*), and asterids (*orange*). Bootstrap values above 50% are indicated, and five *Platanus SEPs* are marked with *stars*

was performed for various plant tissues and ontogenetic stages. The results showed that neither *PlacSEP1* gene nor *PlacSEP3* gene was expressed obviously in juvenile tissues of 2-year-old plants (Fig. 3a). All *PlacSEPs* except for *PlacSEP1.1* and *PlacSEP1.2* were expressed during the male and female inflorescence initiation stages and throughout the flower and fruit development process (Fig. 3a). *PlacSEP1.1* was not expressed evidently in the mixed flower buds of June (6MB), inflorescences of July to September (7MB-F, 8MB-F and 9MB-F), and the fruits of June–August (6F and 8F), but expressed during subsequent floral and fruit development stages, while *PlacSEP1.2* was only detected clearly in female inflorescence at April, absent, or expressed very weakly from all the other tissues during flower initiation and development. *PlacSEP1.3* and *PlacSEP3.1* were also expressed in vegetative tissues of adult plants, including the shoot apical buds, vegetative subpetiolar buds, and vegetative tissues in mixed flower buds, although the expression levels were lower than that in the reproductive tissues. *PlacSEP1.1* and *PlacSEP3.2* transcripts were not detectable or only very weakly expressed in the adult vegetative tissues investigated. In addition, two *PlacSEP3* genes expressed much higher than *PlacSEP1* genes in most tissues and development stages, but all *PlacSEPs* showed relatively lower expression levels when compared with the housekeeping gene of London plane (Fig. 3a).

During the different ontogenetic stages, *PlacSEP3.2* started to express in mixed buds at June when the inflorescence and flower meristem began to differentiate (Li et al. 2012b), and then maintained a relative high expression level in both male and female inflorescences till the anthesis stage during March to April in the next year. During the whole annual development cycle, *PlacSEP3.2* showed three expression peaks during inflorescence and flower development, at July, October (male) or December (female), and February (male) or March (female), respectively. In the developing fruits, the highest expression level of *PlacSEP3.2* was detected at October. *PlacSEP3.1* showed a similar expression patterns as that of *PlacSEP3.2* during the inflorescence and fruit development processes, but had a relative lower expression levels, especially in the male inflorescences (Fig. 3b; Fig. S1). *PlacSEP1.1* increases its expression significantly in the male and female inflorescences at October, later than *PlacSEP3.1*, and then maintained a relative low expression level till April in the next year, up to the highest expression level in the mature male and female inflorescences, especially in the fleshy inflorescence peduncle. *PlacSEP1.3* showed a similar expression tendency as that of *PlacSEP1.1* during the male and female flower development processes, but possessed a broader expression patterns and started its expression earlier than *PlacSEP1.1* (Fig. 3b; Fig. S1).

Overexpression of *PlacSEP* genes in *Arabidopsis* resulted in different phenotypic alterations

To investigate the potential functions of *PlacSEP*1 and *PlacSEP3* genes in flowering transition, floral determination,

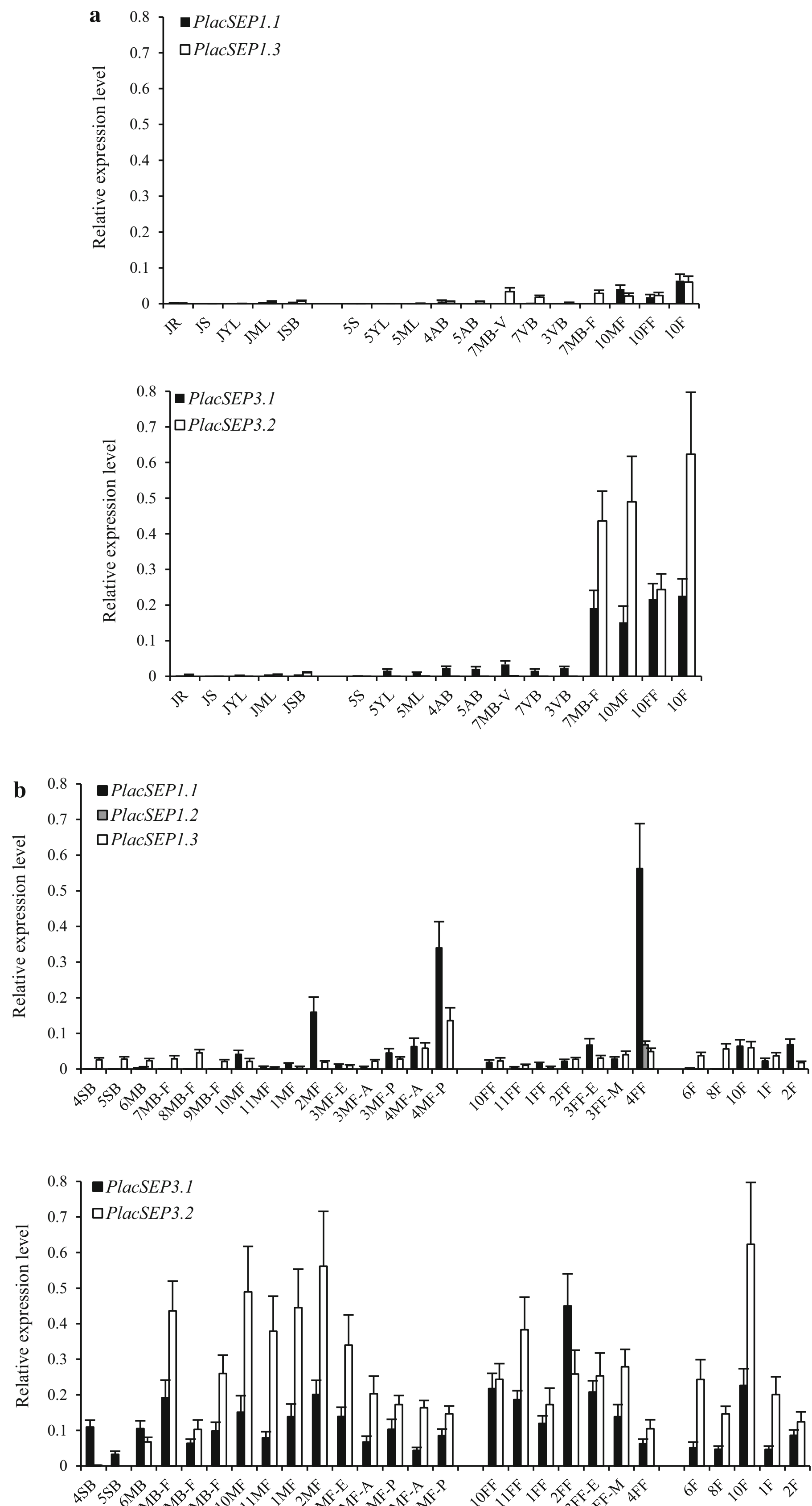

Fig. 3 Expression profiling of *PlacSEP1* and *PlacSEP3* in London plane. **a** Relative expression of *PlacSEP1* and *PlacSEP3* genes in vegetative tissues of juvenile and adult plants (July mixed flower buds and October inflorescences and fruits were placed here for comparison). PlacSEP1.2 didn't show here as no expression was detected in all of these tissues. **b** Relative expression of *PlacSEP1* and *PlacSEP3* genes in different development stages of inflorescences and fruits. *JR* roots of juvenile; *JS* stems of juvenile; *JYL* young leaves of juvenile; *JML* mature leaves of juvenile; *JSB* subpetiolar buds of juvenile; *S* stems; *YL* young leaves; *ML* mature leaves; *AB* shoot apical buds; *SB* subpetiolar buds; *VB* vegetative subpetiolar buds; *MB* mixed flower buds; *MB-V* vegetative tissues in mixed flower buds; *MB-F* inflorescences in mixed flower buds; *MF* male inflorescences; *FF* female inflorescences; *MF-A* male flowers mainly consisting of anthers; *MF-P* fleshy peduncles of male inflorescences; *FF-E* female inflorescences at the first day of the month; *FF-M* female inflorescences at the fifteenth day of the month; *F* fruits. The numbers indicate the sampling month of the tissues. The level of expression was normalized to London plane *TPI* (triose phosphate isomerase) gene. Error bars represent SE for three replicates

and organ identity, cauliflower mosaic virus 35S promoter (CaMV*35S*) was used to ectopically express *PlacSEPs* in *Arabidopsis.* Forty-one, eighty-nine, forty-four, twenty-five, and thirty independent T_1 transgenic lines were achieved for *35S:PlacSEP1.1*, *35S:PlacSEP1.2*, *35S:PlacSEP1.3*, *35S:PlacSEP3.1,* and *35S:PlacSEP3.2*, respectively. According to the phenotypic alterations, two to six T_1 transgenic lines whose progenies showed a 3:1 segregation ratio for kanamycin resistance, which may indicate a single-copy insertion of transgenes, were chosen for further experiment. Sixteen T_2 transgenic plants for each line were used to investigate the flowering time and floral phenotypes.

Compared with the wild-type plants, overexpression of the *PlacSEP1* and *PlacSEP3* genes in *Arabidopsis* resulted in different phenotypic alterations. *35S:PlacSEP1.1* and *35S:PlacSEP3.2* transgenic plants showed obvious early flowering with small plant size and curled leaves (the rosette leaves as well as cauline leaves curl inwards and upwards along the axis of the major leaf vein) (Fig. 4a, b, f). Some transgenic lines that exhibit extremely severe phenotypes bolted and flowered after only producing two small rosette leaves, or even immediately after only forming two cotyledons (Fig. 4h, i). In this case, the secondary inflorescence shoots were usually converted into solitary flowers, while the primary inflorescence terminated into an enlarged flower frequently derived from the fusion of several defective flowers, and no progenies can be obtained from these plants (Fig. 4j, k). Most transgenic lines showed moderate phenotypes, which bolted and flowered after production of four to eight rosette leaves and three to six cauline leaves, while the wild-type *Arabidopsis* plants frequently produced ten to thirteen (11.06 ± 0.97) rosette leaves and two to three (2.13 ± 0.48) cauline leaves before bolting and flowering under the same conditions (Fig. 4a, b; Table S3). Transgenic lines with weak phenotypes, such as *PlacSEP3.2*-27, showed only smaller curled rosette leaves and more cauline leaves compared with the wild-type plants, without the early flowering (Fig. 4e; Table S3). In addition, it is interesting that two different plant architectures were frequently observed within the same *35S:PlacSEP1.1* or *35S:PlacSEP3.2* transgenic lines, i.e., some individuals produced condensed inflorescences that contained several flowers subtended by sepal-shaped cauline leaves directly within the rosette leaves without evident bolting and peduncle elongation (Fig. 4h, i), while the other individuals bolted extremely early with few rosette leaves and produced elongated inflorescences with more cauline leaves (Fig. 4j, k). The former plants were largely sterile with short empty pods. Most *35S:PlacSEP1.3* transgenic lines also exhibited early flowering with less rosette leaves and more cauline leaves than the wild-type *Arabidopsis*, but no curled leaves was observed, which was different from that of the *35S:PlacSEP1.1* and *35S:PlacSEP3.2* transgenic plants (Fig. 4c, d). *35S:PlacSEP1.2* and *35S:PlacSEP3.1* transgenic plants did not show severe phenotypic changes with only few lines that expressed high levels of the transgenes (*PlacSEP1.2*-47, 62 and *PlacSEP3.1*-17, 20) displaying slightly the early flowering or a little more cauline leaves (Table S3).

RT-PCR analysis indicated that the phenotypic variations of *35S:PlacSEP1.3* and *35S:PlacSEP3.2* transgenic plants are related in a certain extent to the expression levels of the transgenes, namely higher expression levels tend to result in more severe phenotypic changes, such as earlier bolting and flowering time or more cauline leaves, but this correlation seems to be not exist in the *35S:PlacSEP1.1* transgenic lines, such as *PlacSEP1.1,* which is strongly expressed in lines 3, 17, and 38, moderately expressed in line 40 and weakly expressed in lines 10 and 20; however, the number of rosette leaves in line 38 is even higher than that in lines 10 and 20 (Fig. 4m, l; Table S3). Expression analysis of the *SEP3* downstream flowering time and leaf development-related genes in *35S:PlacSEPs* transgenic *Arabidopsis* seedlings indicated that *FT*, *SEP3*, *AP1,* and *AG* are significantly activated in 10-day-old seedlings of *35S:PlacSEP1.1,* *35S:PlacSEP1.3,* and *35S:PlacSEP3.2* transgenic lines that display the early flowering when compared with their expression levels in wild-type seedlings (Fig. 5). *SEP3* and *AP1* genes show the strongest activation in *35S:PlacSEP1.1* and *35S:PlacSEP3.2* transgenic lines with the early flowering and evident leaf curling, followed by *AG* and *FT* activation. These genes also showed evidently higher expression levels in transgenic lines displaying both the early flowering and leaf curling (such as *PlacSEP1.1*-3 and *PlacSEP3.2*-8) than those in transgenic plants displaying only the early flowering without leaf curling (*PlacSEP1.3*-9). Expression levels of *SOC1* and *LFY* in these transgenic lines are also significantly higher than that in wild-type seedlings, but they are not activated as strongly as *FT*, *SEP3*, *AP1,* and *AG* genes. No significantly higher expression levels of *FT*, *SEP3*, *AP1,* and *AG* were detected in *35S:PlacSEP1.2* and *35S:PlacSEP3.1* transgenic plants relative to wild-type seedlings (Fig. 5). In *PlacSEP3.2*-27 transgenic line that displays visible leaf curling but no early flowering, only *SEP3* and *AP1* were upregulated significantly. As for the leaf development-related genes investigated in this study, including *GRF1*, *GRF2*, *GRF5*, *TCP3*, *TCP18*, *TCP20,* and *ARF2*, only *ARF2* showed significantly higher levels of expression in *35S:PlacSEP1.1*, *35S:PlacSEP1.2,* and *35S:PlacSEP3.1* transgenic lines than in wild-type seedlings (Fig. 5; Fig. S2).

Ectopic expression of the *PlacSEP1.1* and *PlacSEP3.1* genes in tobacco plants caused early flowering but different axillary shoot branches

The functional roles of the *PlacSEP1.1* and *PlacSEP3.1*, representative of the two clades of *SEP* gene subfamily in

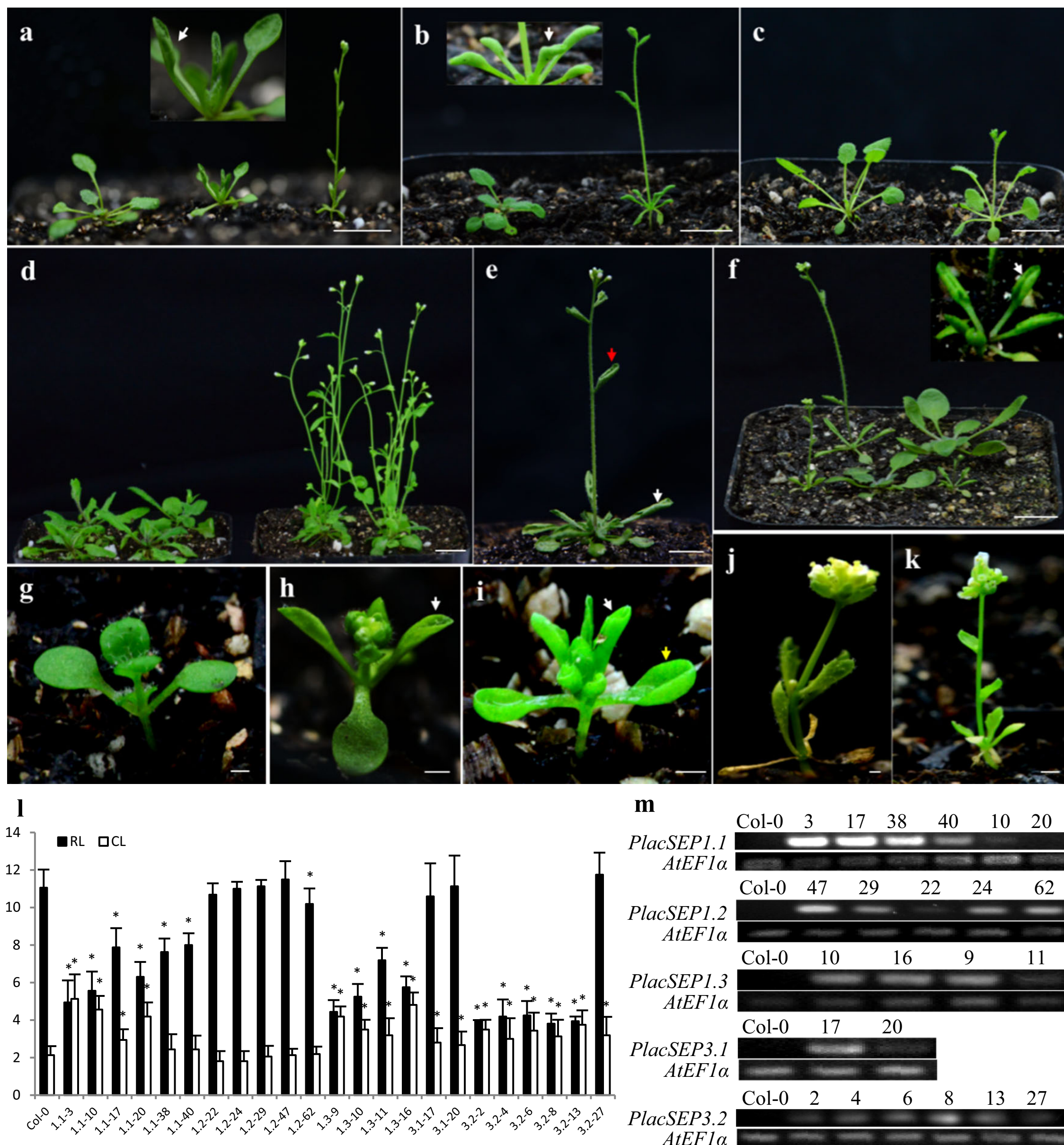

Fig. 4 Phenotype analysis of transgenic *Arabidopsis* plants ectopically expressing *PlacSEP1* and *PlacSEP3* genes. **a** Wild-type (*left*) and *35S:PlacSEP1.1* transgenic plants (*middle* and *right*) showing early flowering with curled leaves. **b** Wild-type (*left*) and *35S:PlacSEP3.2* transgenic plant (*right*) with early flowering and curled leaves. **c**, **d** Wild-type (*left*) and *35S:PlacSEP1.3* transgenic plants (*right*) displaying early flowering without curled leaves. **e** *35S:PlacSEP3.2* transgenic plant that showed curled leaves and more cauline leaves without early flowering. **f** Phenotypic segregation of T2 generation in *35S:PlacSEP3.2* transgenic line. **g** Wild-type Col-0 seedling. *35S:PlacSEP1.1* transgenic plants with early flowering, curled leaves (**h**) and large fused terminal flowers (**j**). *35S:PlacSEP3.2* transgenic plants with early flowering, curled leaves (**i**), and fused terminal flower (**k**). **l** The numbers of rosette leaves (RL) and cauline leaves (CL) in wild-type and *35S:PlacSEPs* lines; *asterisks* indicate statistically significant differences ($P < 0.05$) from the wild-type plants. **m** RT-PCR analysis of transgenes in wild-type *Arabidopsis* (Col-0) and transgenic lines. *Yellow*, *white,* and *red arrows* indicate curled cotyledon, rosette leaves, and cauline leaf, respectively. *Bars* 10 mm (**a**–**f**), 1 mm (**g**–**k**)

London plane, respectively, were also investigated by ectopic expression in tobacco plants. After selected by kanamycin and confirmed by PCR, thirty-six and thirty-two independent T_0 transgenic lines of *35S:PlacSEP1.1* and *35S:PlacSEP3.1* were obtained, respectively. Eighteen of the transgenic lines carrying the *35S:PlacSEP1.1* flowered obviously earlier than the control plants, and exhibited a dwarf phenotype with some plants only approximately 10 cm in height at the time of flowering. RT-PCR analysis was conducted to investigate the expression levels of the transgene in plants with severe (line 33), moderate (line 35), and weak (line 25) phenotypes base on the plant

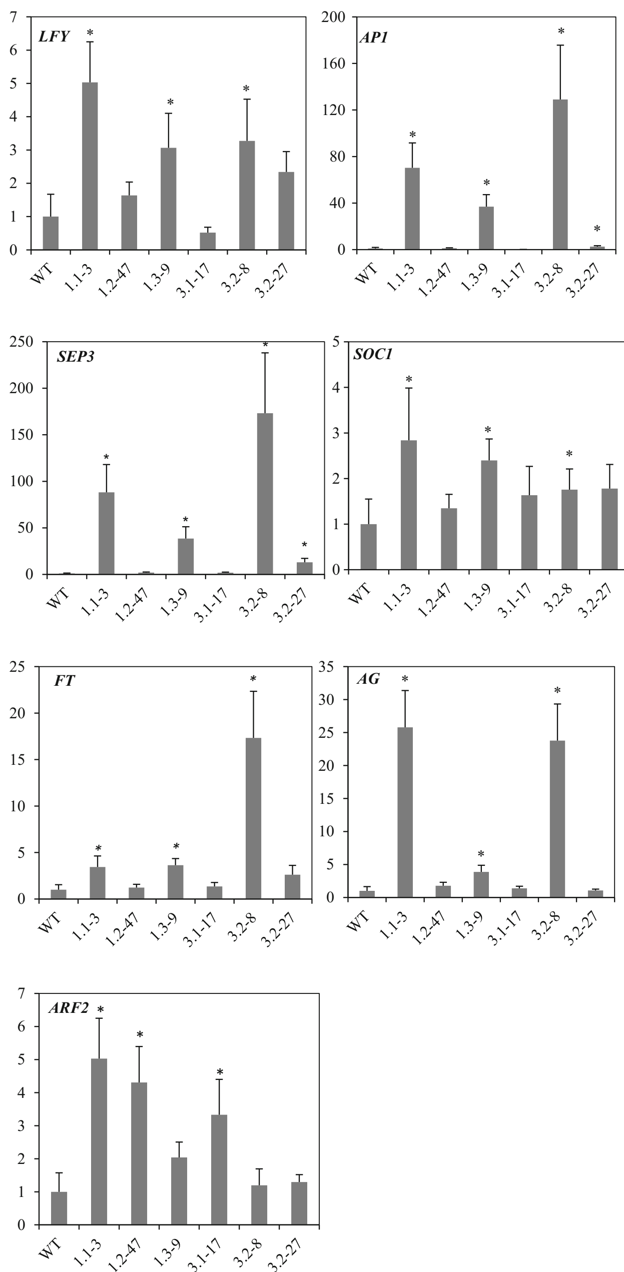

Fig. 5 qRT-PCR analysis of endogenous flowering and leaf development-related genes in 10-day-old seedlings of *Arabidopsis* wild-type and *35S::PlacSEP* transgenic lines. 1.1–3, 1.2–47, 1.3–9, 3.1–17, 3.2–8, and 3.2–27 indicate transgenic lines of the five *35S::PlacSEP* genes, respectively. Data represent the mean ± SE from three biological replicates, and *AtEF1α* was used as internal control. *WT* wild-type seedlings. The *asterisks* indicate significant differences compared with the WT plants ($P < 0.01$)

height, internode length, and leaf size, etc. (Table S4). The results revealed that plants showing severe phenotype expressed higher levels of the transgene compared with the transgenic plants exhibiting moderate or weak phenotype (Fig. 6h, i; Table S4), indicating that the transgene is responsible for the phenotypic alterations. Transgenic lines *PlacSEP1.1*-25, 33, and 35 were selected to examine the heritability of the phenotypes. The results showed that the dwarf and early flowering phenotypes were co-inherited with the *35S:PlacSEP1.1* transgene to the next generation. The T_1 transgenic plants displayed early flowering, lower plant height, shorter internode, less leaf number, smaller and greener crumpled leaves, and more lateral branches even before flowering, which was significantly different from those of the wild-type plants (Fig. 6b, e; Table S4).

In the case of *35S:PlacSEP3.1* transgenic tobacco, 16 out of 32 T_0 transgenic plants showed similar phenotypes to that of *35S:PlacSEP1.1* transgenic plants, including early flowering, dwarf plant stature, less leaf number, small and green leaves, and more lateral branches (Fig. 6c, d; Table S4). The inflorescence architecture appears to be simpler in the transgenic plants than in the wild-type plants, with less secondary inflorescences and less flowers for each inflorescence (Fig. 6f, g). It was interesting that the transgenic plants carrying the *35S:PlacSEP3.1* showed more reduced apical dominance and produced more lateral branches which generate secondary branches more easily than the *35S:PlacSEP1.1* transgenic lines, but there was no significantly different internode length were observed between these plants and the wild-type plants (Fig. 6a, d; Table S4). These phenotypic alterations were due to the overexpression of *PlacSEP3.1*, and the phenotype intensity was related to the expression level of the transgene (Fig. 6h, i). Three transgenic lines, *PlacSEP3.1*-9, 25 and 7 with severe, moderate, and weak phenotypes, respectively, were selected for further phenotype identification in T_1 generation. Unexpectedly, only the *PlacSEP3.1*-9 T_1 plants showed evident early flowering and shorter plant height, while the progenies of *PlacSEP3.1*-25 and 7 showed no significant difference from the wild-type plants, except for producing more lateral branches (Table S4). No visible changes were observed in flower organs or fruits of both *35S:PlacSEP1.1* and *35S:PlacSEP3.1* transgenic plants. In addition to the overexpression of *PlacSEP1.1* and *PlacSEP3.1*, we also produced transgenic lines constitutively expressing one or both of the antisense transcripts of the two genes, but no plant showed any phenotypic alterations (Table S4).

Interactions between PlacSEPs and other MADS-box proteins from London plane

As overexpression of the three *PlacSEP1* and two *PlacSEP3* genes in *Arabidopsis* and tobacco plants resulted in different phenotypes, a yeast two-hybrid analysis was performed to evaluate if it could be caused by their differential ability of protein interactions. The coding sequences of *FUL*-, *APETALA3* (*AP3*)-, *PISTILLATA* (*PI*)-, *AGAMOUS* (*AG*)-, *SEEDSTICK* (*STK*)-, and *SEP*-like

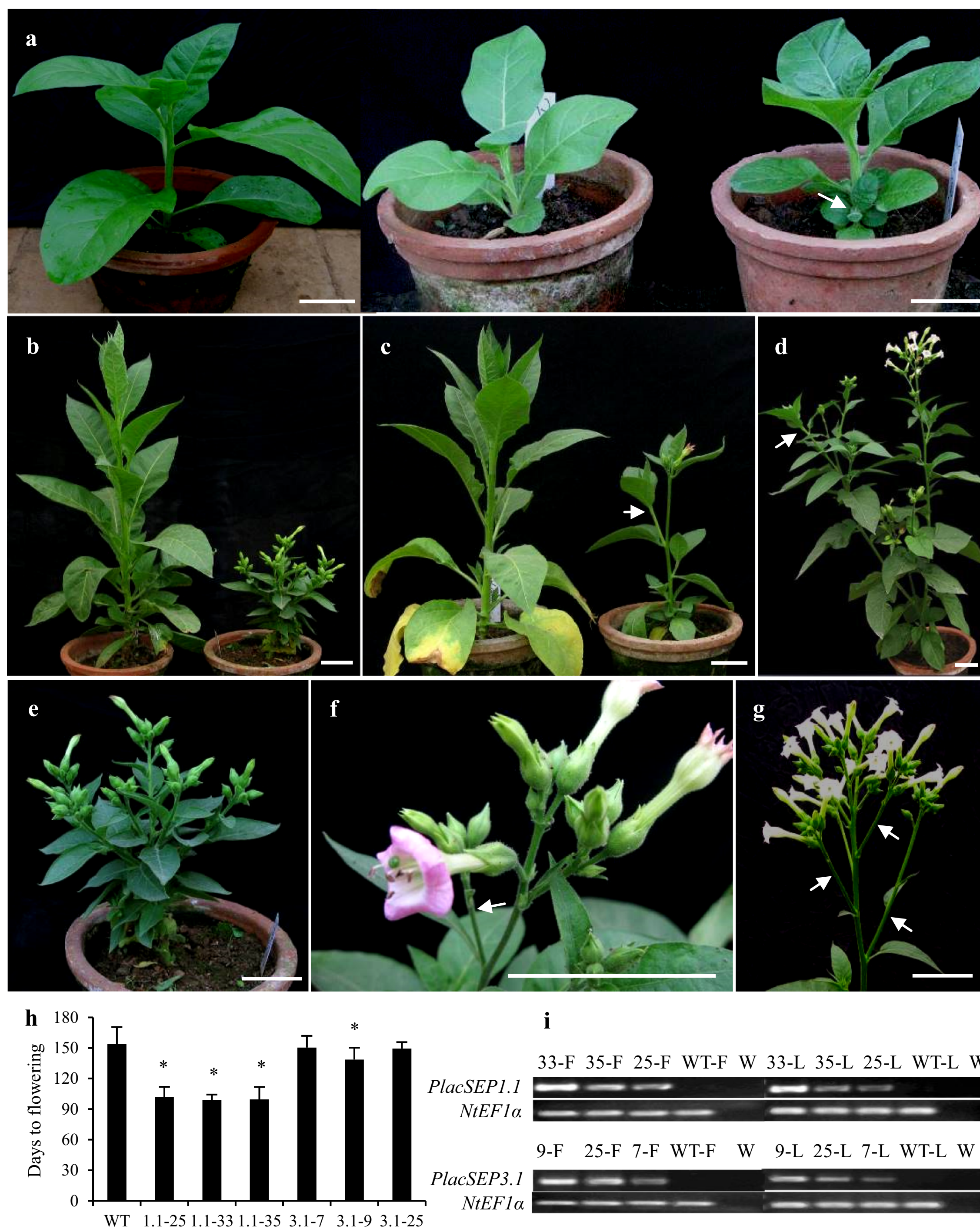

Fig. 6 Phenotype analysis of transgenic tobacco plants constitutively expressing *PlacSEP1.1* and *PlacSEP3.1* genes. **a** Wild-type (*middle*) and *35S:PlacSEP1.1* (*left*) and *35S:PlacSEP3.1* (*right*) transgenic tobacco seedlings; *white arrow* indicates the lateral shoot sprouting from axillary bud. **b** Wild-type (*left*) and dwarf *35S:PlacSEP1.1* transgenic plant showing early flowering (*right*). **c** Wild-type (*left*) and *35S:PlacSEP3.1* transgenic plant (*right*) with early flowering and more lateral branches (*white arrow*). **d** *35S:PlacSEP3.1* transgenic plant showing more primary and secondary lateral branches (*white arrow*) during later development stage. **e** Close up of the *35S:PlacSEP1.1* transgenic plant in (**b**). **f**, **g** Inflorescence architecture of *35S:PlacSEP3.1* transgenic plant and wild-type plant, respectively; *white arrows* indicate secondary inflorescences. **h** Days to flowering of wild-type and *35S:PlacSEPs* lines; *asterisks* indicate statistically significant differences ($P < 0.05$) from the wild-type plants. **i** Transgene expression in flowers (*left*, *line* number followed by F) and leaves (*right*, *line* number followed by L) of T1 transgenic lines with strong, moderate, and weak phenotypes, respectively. *WT* wild type, *W* deionized water. *Bars* 5 cm

genes were fused to the AD and BD domains, respectively, and used for comparison. The results indicated that all PlacSEPs interacted with PlacFUL, PlacAP3, PlacAG, and two PlacSTK proteins in different intensity, among which the interactions of PlacSEP3 with PlacAG as well as PlacSEP3.1 with all tested proteins, were weaker than that of the other PlacSEP interactions (Table 1; Fig. S3). All PlacSEPs were also capable of forming homodimers, except for PlacSEP3.1 in which the interaction was weak. Three PlacSEP1 proteins were failed to interact with PlacPI2a, but two PlacSEP3 had a capacity to interact with PlacPI2a, although the interaction between PlacSEP3.1 and PlacPI2a was weak and only in one direction (Table 1; Fig. S3).

Discussion

Evolutionary and functional conservation and diversity of *Platanus SEP*-like genes

Members of the *SEP* gene subfamily have been identified and characterized in a wide range of species and have been shown to take on roles in floral meristem identity, floral organ specification, fruit ripening, and plant architecture, and play a major role in the floral evolution of diverse plants (Malcomber and Kellogg 2005). In this study, five *SEP*-like genes were identified from *P. acerifolia*, a basal eudicot tree belonging to the family Platanaceae. A phylogenetic tree constructed using amino-acid sequences showed that *PlacSEP1.1*, *PlacSEP1.2,* and *PlacSEP1.3* belong to the *SEP1/2/4* clade, while *PlacSEP3.1* and *PlacSEP3.2* were grouped into the *SEP3* clade (Fig. 2), which were further verified by C-terminal sequence alignment (Fig. 1). Platanaceae together with Proteaceae and Nelumbonaceae form the well-supported order Proteales, which plus Sabiales, Trochodendrales, and Buxales compose the basal eudicot lineages between the first branching eudicot lineage Ranunculales and the core eudicots (Bremer et al. 2009). In accordance with this, the *SEP*-like genes from *P. acerifolia* show the highest degree of nucleotide and amino-acid sequence identity with their homologous genes from the species of Nelumbonaceae, Trochodendrales, or Ranunculales, such as *Nelumbo nucifera*, *Trochodendron aralioides,* and *Euptelea pleiosperma* (Table S5). *PlacSEP1.1* and *PlacSEP3.2* also show high-sequence identity with *SEP*-like genes of *Vitis vinifera*. Sequences that have similar structures and features usually have a relatively closer evolutionary relationship, especially if the features appear in a non-conserved region. Several conserved motifs in highly variable C-terminal regions of the *SEP* gene lineage were found in five *P. acerifolia SEP*-like genes (Fig. 1), such as the SEP I and SEP II motifs (Zahn et al. 2005); moreover, three *PlacSEP1* and two *PlacSEP3* genes share the clade-specific *SEP1* motif and *SEP3* motif in their C-terminal, respectively, suggesting that they are recent duplicated orthologs of *SEP1/2/4* and *SEP3*, respectively.

Based on the expression patterns, protein–protein interactions (PPIs), and phenotypic changes of transgenic *Arabidopsis* and tobacco plants, we showed that the *SEP*-like genes of *Platanus* could have not only conservative but also diverse functions. The previous studies indicated that *SEP*-like genes from core eudicots and even monocots have relatively conservative expression patterns and/or functions. For instance, almost all *SEP3*-clade members are expressed in inflorescences and in the inner three floral whorls and fruits; within the *LOFSEP* clade, all *SEP1/2* genes are also expressed in inflorescences, the inner three floral whorls and fruits, most homologs are additionally expressed in sepals and some even in vegetative tissues (Malcomber and Kellogg 2005). In our study, the five

Table 1 *Platanus acerifolia* SEPALLATA-like protein interactions with other MADS-box proteins detected by yeast two-hybrid assays. +++; very strong interaction, ++; strong interaction, +; weak interaction, − no interaction

	PlacSEP1.1		PlacSEP1.2		PlacSEP1.3		PlacSEP3.1		PlacSEP3.2		Empty	
	AD	BD	AD	BD	AD	BD	AD	BD	AD	BD	AD	BD
PlacFUL	++	+++	+++	+	+++	+	−	++	++	++	−	−
PlacAP3	++	++	+++	++	+++	++	+	++	+++	+++	−	−
PlacPI2a	−	−	−	−	−	−	−	+	++	++	−	−
PlacAG	++	−	+++	+	+++	+	+	−	+	+	−	−
PlacSTK1	+++	−	+++	−	+++	/	++	−	+++	−	−	−
PlacSTK2	+++	++	+++	+++	+++	/	+	+	+++	+++	−	−
PlacSEP1.1	++		/	/	/	/	/	/	/	/	−	−
PlacSEP1.2	/	/	+++		/	/	/	/	/	/	−	−
PlacSEP1.3	/	/	/	/	++		/	/	/	/	+	−
PlacSEP3.1	/	/	/	/	/	/	+		/	/	−	−
PlacSEP3.2	/	/	/	/	/	/	/	/	+++		−	−

Platanus SEP-like genes showed significantly different spatio-temporal expression patterns (Fig. 3). *PlacSEP3.2* gene is expressed exclusively in reproductive organs and tissues, including male and female inflorescences, flowers, and fruits, which is similar to that of most *SEP* homologs in other species (Malcomber and Kellogg 2005). *PlacSEP1.1* is also expressed mainly in reproductive tissues. However, the expression profile of the two genes is not identical in both the temporal and quantitative levels. First, the *PlacSEP3.2* gene starts its expression at June when the inflorescence began to initiate, then followed by three peaks of expression in male and female inflorescences at the stamen and pistil differentiation stage (July), the first floral organ development stage (October to December), and the second floral organ development stage (February to March), respectively (Fig. 3); while *PlacSEP1.1* is activated significantly much later till October when all the floral organ primordia already completed the differentiation, followed by two peaks of expression in male and female inflorescences at the second floral organ development stage (February to March) and the anthesis stage (April), respectively (Fig. 3). In addition, *PlacSEP3.2* has a significantly higher expression level than *PlacSEP1.1* almost in all the flower and fruit development stages. Another difference between the two genes is that *PlacSEP1.1* expression increases gradually during the second floral development period in the spring, while the expression of *PlacSEP3.2* declines at the same duration, resulting in the only higher expression level of *PlacSEP1.1* than that of *PlacSEP3.2* at April (Fig. 3b; Fig S1). In contrast, *PlacSEP1.3* and *PlacSEP3.1* genes are expressed not only in the reproductive organs like *PlacSEP1.1* and *PlacSEP3.2* but also in vegetative tissues of adult plants, including the shoot apical buds, vegetative subpetiolar buds, and the vegetative tissues in mixed flower buds (Fig. 3a, b). Likewise, there are evident differences between the expression of *PlacSEP1.3* and *PlacSEP3.1*; for example, *PlacSEP3.1* has an evident higher expression level than *PlacSEP1.3*. Besides, it is notable that the variation tendency of *PlacSEP1.3* and *PlacSEP3.1* expression is very similar to that of *PlacSEP1.1* and *PlacSEP3.2*, respectively, during the flower and fruit development processes, although they have a relatively lower expression levels, suggesting that *PlacSEP1.3* and *PlacSEP3.1* may acquire the new expression location in vegetative tissues from *PlacSEP1.1* and *PlacSEP3.2*, respectively, or vice versa, through the *cis*-regulatory element changes of the genes after their duplication. The most intriguing gene is *PlacSEP1.2*, which is only expressed at low level in female inflorescence at the anthesis stage and cannot result in visible phenotypic changes when overexpressing in *Arabidopsis*, indicating that it might have lost the functions, but it still maintains the same interacting capability with other floral MADS-box proteins as the PlacSEP1.1 and PlacSEP1.3 (see below). The differential expression patterns of the *SEP*-like genes in London plane suggest that they may not only play conservative roles in *Platanus* flower and fruit development, some members may also play divergent roles in other aspects, such as bud growth and dormancy, after their duplication and subfunctionalization events.

As the E-class MADS-box genes, SEP proteins are involved in the formation of multimeric complexes that contain other floral organ identity proteins. In *Arabidopsis*, SEP3 was proved to be a ‘glue’ protein to mediate the multimeric complex formation in various development processes (Immink et al. 2009). In other angiosperm species, SEP-like proteins have a conservative tendency of protein–protein interacting behavior with AP1/FUL, B-, C-, and D-function proteins (Malcomber and Kellogg 2005; Ruokolainen et al. 2010; Hu et al. 2015). However, a slightly different situation was also found in various species, especially those that are distantly different from the studied models (Liu et al. 2010; Li et al. 2015). In the previous studies, six PPIs between SEP proteins and other floral MADS-box proteins (A-, B-, C-, and D-class), i.e., AGL2-AP1, AGL9-AP1, AGL2-AG, AGL9-AG, AGL2-STK, and AGL9-STK, was shown to be conservative in angiosperm species, while B-class proteins (AP3 and PI) have gradually lost their interaction capability with SEP proteins, resulting in AGL2-AP3, AGL2-PI, AGL9-AP,3 and AGL9-PI remaining variable, and if any, usually weaker PPIs (Liu et al. 2010; Melzer et al. 2014; Li et al. 2015). Likewise, all five SEP-like proteins in *Platanus* showed conservative PPIs with AP1/FUL-like (PlacFUL), AG-like (PlacAG), and STK-like (PlacSTK1 and PlacSTK2) proteins in yeast, although in differential intensity and some PPIs merely positive in one-direction assays, confirming the conservative PPIs during angiosperm evolution (Table 1; Fig. S3). However, different from the situation in most angiosperm species, including basal eudicots, where the direct AGL2-AP3 and SEP-PI interactions have lost (Liu et al. 2010), PlacSEP1 and PlacSEP3 (especially PlacSEP3.2) proteins retained their interactions with AP3-like (PlacAP3) and PI-like (PlacPI2a) proteins, respectively, which is similar to the situation in basalmost angiosperm species, *Amborella trichopoda* (Melzer et al. 2014), suggestive of a slow evolution of PPIs between SEP and B-class proteins in *Platanus*. Like the situation in most other angiosperms, PlacSEP1 proteins did lost their interacting capability with the PI-like proteins, supporting the quick loss of AGL2-PI interaction during angiosperm evolution (Melzer et al. 2014). Within the SEP subfamily, AGL2-AGL9 has conservative interactions, while AGL2 and AGL9 show variable capability of homodimerization (Liu et al. 2010). In *Platanus*, all SEP-like proteins can

form homodimers varying from weak to strong interaction (Table 1; Fig. S3), which is similar to that in *Amborella trichopoda* (Melzer et al. 2014), while different from that in the basal eudicot species, *Euptelea pleiospermum*, where only SEP3-like protein (EUplSEP3) rather than SEP1-like protein (EUplSEP1) can form homodimer (Liu et al. 2010), indicating again the low evolution rate of SEP-related PPIs in London plane. The conservative and differential protein–protein interaction patterns of PlacSEPs with AP1/FUL-like, B-, C-, and D-class MADS-box proteins suggested that they play conservative and divergent functions in *Platanus*.

The evolutionary conservation and divergence of *Platanus SEP*-like genes were further suggested by transgenic studies in *Arabidopsis* and tobacco. Overexpressing three *PlacSEP1* and two *PlacSEP3* genes in *Arabidopsis* resulted in different degrees of phenotypic alterations. *35S:PlacSEP1.1* and *35S:PlacSEP3.2* transgenic plants showed most evident phenotypic changes, including the early flowering, reduced plant size, small curled leaves, and usually determinant primary and secondary inflorescences terminating or transforming into solitary flowers (Fig. 4), which is similar to the phenotypic results of ectopic expression of *Arabidopsis SEP3* genes or its homologs from some other species, such as petunia *FBP2* (Pelaz et al. 2001; Ferrario et al. 2003). *35S:PlacSEP1.3* transgenic plants also bolted and flowered early with small plant and leaf size related to the wild type, but without leaf curling. In contrast, *35S:PlacSEP1.2* and *35S:PlacSEP3.1* transgenic plants did not show any severe phenotypic changes, except few lines with high level of transgene expression flowered slightly early or seemingly produced a little more cauline leaves (Fig. 4l; Table S3). The severity of phenotypes in *SEP*-like gene overexpressing *Arabidopsis* plants is frequently reported to be correlated to the levels of transgenes (Zhao et al. 2006; Chang et al. 2009). This relationship was also observed in our experiments for the *35S:PlacSEP1.3* and *35S:PlacSEP3.2* transgenic *Arabidopsis* plants but not in the *35S:PlacSEP1.1* transgenic lines (Fig. 4m, l; Table S3), which implied that *PlacSEP1.1* may have more strong and complicated activity. In addition, differential phenotypes were observed in *35S:PlacSEP1.1* and *35S:PlacSEP3.1* transgenic tobacco plants, with the latter showing easier lateral shoot sprouting (Fig. 6), which may be resulted from their distinct coding sequence and PPI potential (Fig. 1; Table 1). Alternatively, those phenotypic differences just relate to differences in transgene expression could not be excluded. The discrepant phenotypes resulting from the overexpression of *PlacSEPs* suggested their divergent functions and probably differential capability of activating downstream target genes in *Arabidopsis*. Genome-wide approaches, such as ChIP-Seq, have identified many growth and development regulators as direct targets of SEP3 protein, including flowering time genes, floral meristem and organ identity genes, and growth regulatory genes, such as *FLOWERING LOCUS T* (*FT*), *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*), *AP1*, *FUL*, *AP3*, *PI*, *AG*, *TCPs*, *ARFs*, *GRFs*, and *SEP3* itself (Kaufmann et al. 2009; Pajoro et al. 2014). Moreover, it has been proved that ectopic expression of *Arabidopsis SEP3* is sufficient to ectopically activate *AP3* and *AG* in the curled leaves (Castillejo et al. 2005). To understand the molecular basis of differential transgenic phenotypes, the expression levels of a dozen candidate target genes of *SEP3* in different transgenic lines were investigated. In accordance with the phenotypic changes, *FT*, *SEP3*, *AP1*, *AG*, *SOC1,* and *LFY* are significantly activated in 10-day-old seedlings of *35S:PlacSEP1.1, 35S:PlacSEP1.3* and *35S:PlacSEP3.2* transgenic lines that display strong early flowering and/or leaf curling, while they are not significantly different in *35S:PlacSEP1.2* and *35S:PlacSEP3.1* transgenic plants showing no visible phenotypic effects related to the expression levels in wild-type seedlings (Fig. 5). The results indicate that *PlacSEP1.1*, *PlacSEP1.3,* and *PlacSEP3.2* have conservative roles like *Arabidopsis SEP3* in activating flowering time and floral development genes, while *PlacSEP1.2* and *PlacSEP3.1* might lose this capability by subfunctionalization and neofunctionalization. It is worth noting that the expression levels of direct downstream *ARF2* gene of *SEP3* are significantly higher (although only 3–5 fold) in *35S:PlacSEP1.1, 35S:PlacSEP1.2,* and *35S:PlacSEP3.1* transgenic plants, but not in *35S:PlacSEP1.3* and *35S:PlacSEP3.2*, than that in the wild-type plants (Fig. 5), which further indicates the divergent functions of *PlacSEPs*.

The phenotype of *35S:PlacSEP1.1* and *35S:PlacSEP3.2* transgenic plants is also similar to that of *35S:FT* transgenic plants (Teper-Bamnolker and Samach 2005) and the *curly leaf* (*clf*) or *agl15 agl18 agl24 svp* mutants in *Arabidopsis* (Goodrich et al. 1997; Fernandez et al. 2014), including the early flowering and leaf curling. In *clf* mutants, leaf curling was supposed to be caused by misexpression of the floral homeotic gene *AG* in leaves (Goodrich et al. 1997); however, *AG* mRNA and protein were expressed as strongly in *clf fpa* double mutants as in *clf* mutants, despite *clf fpa* mutants lack leaf curling (Lopez-Vernaza et al. 2012), suggesting that *AG* may be not the only or direct regulator of *clf* phenotype. Several other genes, including *AP3*, *SEP3*, *FT,* and *FLC,* have also been found to be misexpressed in *clf* mutants, among which mutual activation of *SEP3* and *FT* activity are required for the *clf* phenotype, and *SEP3* transcription requires *AG* activity, while *FLC* antagonizes the effects of *FT/SEP3/AG* on flowering and leaf phenotype in *clf* mutants (Lopez-Vernaza et al. 2012). In *agl15 agl18 agl24 svp* mutants, the

change in leaf morphology is associated with the elevated levels of *FT* and ectopic expression of *SEP3*, leading to ectopic expression of floral genes, such as *AP1* and *AP3* (Fernandez et al. 2014). In *35S:PlacSEPs* transgenic *Arabidopsis* plants, leaf curling was seemingly related to the activated expression levels of *SEP3*, *AG* and *FT*. For example, the expression levels of *SEP3*, *AG* and/or *FT* genes in transgenic lines with leaf curling (*35S:PlacSEP1.1*-3 and *35S:PlacSEP3.2*-8) are evident higher than that in transgenic plants without leaf curling (Fig. 5), suggesting that there may be a threshold of *SEP3* activity required for leaf curling and early flowering. Consistent with this, *35S:SEP3* transgenes give variable effects on leaf curling, presumably relating to expression levels (Honma and Goto 2001). However, it is noteworthy that *35S:PlacSEP3.2*-27 showed evident leaf curling but with a lower level of *SEP3* expression than *35S:PlacSEP1.3*-9, indicating that there may be some other regulatory factors involved in the leaf curling phenotype. In addition, the expression of *SEP3*, *AG,* and *FT* is evident higher in lines showing the higher expression of the transgene, such as *PlacSEP3.2*-8 vs. *PlacSEP3.2*-27, indicating that the expression levels of *SEP3*, *AG,* and *FT* genes are related to and resulted from the transgene expression (Figs. 4, 5).

Potential roles of London plane *SEP*-like genes

To exactly understand the functions of a gene usually need to obtain its loss-of-function mutants by gene knockdown or knockout technology, such as transposon or T-DNA insertion, gene inhibition (cosuppression, RNAi, or amiRNA), and targeted genome editing, etc. In woody plants, especially those recalcitrant species for genetic transformation; however, it is difficult and time consuming to obtain the natural or transgenic loss-of-function mutants. In this case, gene expression pattern analysis and ectopically expressing in model species can provide important cues to understand the gene function. Therefore, we investigated the functions of five London plane *SEP*-like genes in this study by analyzing their expression patterns during growth and flower development processes, the protein–protein interaction manners with other floral MADS-box genes, and transgenic phenotypes in *Arabidopsis* and tobacco plants.

As discussed above, the five *PlacSEP* genes showed diverse spatio-temporal expression patterns, with *PlacSEP1.1* and *PlacSEP3.2* expressed exclusively in reproductive development process, while *PlacSEP1.3* and *PlacSEP3.1* also transcribed in some vegetative tissues of adult plants (Fig. 3). In favor of the qRT-PCR results, high levels of *PlacSEP3.2* transcripts were detected in samples of June other than in samples of the other months by sequencing transcriptomes of London plane subpetiolar buds of April, May, and June, respectively, while *PlacSEP1.3* and *PlacSEP3.1* transcripts were detected in samples of all the 3 months, although the expression in samples of June is higher (unpublished data). The gene expression patterns indicate that *PlacSEP1.1* and *PlacSEP3.2* should play crucial but not necessarily identical roles in flower initiation and development as well as fruit development and maturation in *Platanus*, like most *SEP* homologs in other species. Consistent with this, transgenic *Arabidopsis* and tobacco plants ectopically expressing *PlacSEP1.1* or *PlacSEP3.2* gene showed strong early flowering phenotypes (Figs. 4, 6). *PlacSEP1.3* and *PlacSEP3.1* genes may not only play roles in flower and fruit development but also have functions in vegetative growth and development, such as bud growth and dormancy. In favor of this, *35S:PlacSEP1.3* transgenic *Arabidopsis* plants also showed early flowering, but no leaf curling phenotype was observed; *35S:PlacSEP3.1* transgene in *Arabidopsis* leads to no visible phenotype, but it does result in the early flowering in tobacco plants, and that more lateral branches are produced in transgenic plants (Fig. 6). As for *PlacSEP1.2*, it is unexpected that no visible early flowering is observed in transgenic *Arabidopsis* plants, because it maintains similar protein–protein interaction pattern and strength as PlacSEP1.1 and PlacSEP3.2 with other floral MADS-box proteins (Table 1; Fig. S3). Based on its very weak expression levels in all tested tissues and extremely limited expression position during the flower and fruit development processes, *PlacSEP1.2* may have lost or changed dramatically its functions, which is supported further by the phylogenetic analysis results that display *PlacSEP1.2* is more distantly related to *PlacSEP1.1* and *PlacSEP1.3*.

The *SEP* genes were first proposed to be the E-function genes that determine all the four whorls of floral organs in the ABCE flower development model (Pelaz et al. 2001; Ditta et al. 2004). Recently, more and more studies showed that *SEP*-like genes may play complex roles beyond floral organ identity (Seymour et al. 2011; Ireland et al. 2013; Dong et al. 2014; Liu et al. 2014; Elitzur et al. 2016). As in the previous works, our study shows the functions of *SEP*-like genes are not necessary to correlate with the flower phenotype.

In summary, five *SEP*-like genes were isolated and characterized from a basal eudicot tree, *P. acerifolia*. According to the expression profiles, protein–protein interaction patterns, and transgenic phenotype analysis in model species, we suggest that two *Platanus SEP*-like genes, *PlacSEP1.1* and *PlacSEP3.2*, play crucial and conservative functions in floral initiation and development, as well as in the fruit development; two *Platanus SEP*-like genes, *PlacSEP1.3* and *PlacSEP3.1*, may also play important roles in vegetative development, such as bud growth and dormancy, besides their functions in flower and fruit development. The results are valuable and informative

for us to understand the functional evolution of SEP-class genes in angiosperms and the molecular basis of flower and fruit development in *Platanus*. However, further studies including in situ hybridization analysis, promoter isolation, and characterization, etc., are needed to uncover the accurate expression location and the underlying mechanism of the divergent expression pattern and functions of these genes.

Author contribution statement GFL, MZB, and SSZ designed the research. SSZ performed most experiments. SJL, SSY, and LL performed tobacco transformation. HJH performed transcriptome sequencing. JQZ constructed PlacAP3, PlacPI2a, and PlacAG yeast vectors. SSZ, SJL, and GFL analyzed the data and wrote the manuscript. All of the authors approved the final manuscript.

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