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REGULAR PAPER

# Phylogenetic and evolutionary analysis of A-, B-, C- and E-class MADS-box genes in the basal eudicot *Platanus acerifolia*

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**Abstract** London plane tree (*Platanus acerifolia* Willd.) is an important member of the Platanaceae family, being popular as an urban landscaping tree. Here, we report the isolation of five MADS-box genes from the basal angiosperm, *Platanus acerifolia*. Sequence and phylogenetic analyses identified *FRUITFUL*-like, *APETELA3*-like, *AGAMOUS*-like, *SEPALLATA1*-like and *SEPALLATA3*-like sequences and, hence, we term the respective *Platanus acerifolia* genes as *PlacFUL*, *PlacAP3*, *PlacAG*, *PlacSEP1* and *PlacSEP3*. From these identities we infer that they represent candidate A-, B-, C-class and two E-class genes, respectively. The conserved MIK or MIKC domains from the nucleotide and protein sequences of *PlacFUL*, *PlacAP3*, *PlacAG*, *PlacSEP1* and *PlacSEP3* were analyzed using the maximum-likelihood, MrBayes and neighbor-joining methods. The results confirmed *P. acerifolia* as a basal eudicot. Expression pattern was determined by reverse transcriptase PCR, which showed all paralogous genes have distinct expression patterns, suggesting that they had undergone functional divergence.

**Keywords** *Platanus acerifolia* · MADS-box gene · Phylogenetic evolution

The gene sequences have been deposited in GenBank and will appear under the accession numbers *PlacFUL* (GU296505), *PlacAP3* (EF488452), *PlacAG* (GU296506), *PlacSEP1* (EF686225) and *PlacSEP3* (GU296507).

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## Introduction

MADS-box genes encode a large family of transcriptional regulators with diverse developmental functions. However, the floral organ identity genes are, by far, the most intensively researched. These encode a family of transcription factors that have key roles in specifying the identity of each whorl of angiosperm flowers. Recent progress in understanding the floral developmental program has predominantly come from the study of two model systems, *Arabidopsis thaliana* and *Antirrhinum majus*. Genetic studies with these two species have shown that floral organ identities are determined by a combination of five classes of genes (A, B, C, D and E). This work has produced an expanded ABCDE model of floral development and also the "floral quartet" model that predicts how the different floral homeotic genes interact at the molecular level to specify the various floral organ identities (Coen and Meyerowitz 1991; Theissen and Saedler 2001). In the ABCDE model, A- and E-class genes specify sepal structures, A + B + E classes determine petal formation, B + C + E direct the formation of stamens, and C + E class factors interact to specify the identity of carpels; D- and E- class genes are involved in ovule development. In the model plant *Arabidopsis*, class A genes are represented by *APETALA1* (*AP1*) and *APETALA2* (*AP2*; Mandel et al.

1992; Jofuku et al. 1994), class B genes by *APETALA3* (*AP3*) and *PISTILLATA* (*PI*; Goto and Meyerowitz 1994; Yang et al. 2003), class C genes by *AGAMOUS* (*AG*; Yanofsky et al. 1990), class D genes by *SEEDSTICK* (*STK*; formerly *AGL11*) (Pinyopich et al. 2003; Kaufmann et al. 2005) and class E genes are represented by *SEPALLATA1*, *2*, *3* and *4* (*SEP1*, *2*, *3* and *4*; previously known as *AGL2*, *4*, *9* and *3*, respectively; Pelaz et al. 2000; Honma and Goto 2001; Ditta et al. 2004). With the exception of *AP2*, each of these ABCDE floral homeotic genes represents a MIKC$^C$-type MADS-box gene, this term referring to their conserved structure comprising an M (MADS), I (intervening), K (keratin-like) and C (C-terminal) domain (Sommer et al. 1990; Bowman et al. 1991, 1993; Becker and Theissen 2003).

*Platanus acerifolia* is a monoecious tree species that is frequently used in city landscaping schemes due to its desirable vegetative growth characteristics. However, undesirable traits of *P. acerifolia* include the abundant release of pollen and seed hairs that pollute the environment and are known to be problematic as allergens. Thus, floral organ identity MADS-box genes are of interest to us as potential targets to engineer male- and/or female-sterile lines (Li et al. 2008).

By studying the MADS-box genes in *P. acerifolia*, we hope to further clarify the phylogenetic position of the Platanaceae family. The isolation of these full-length MADS-box gene clones offers the potential for genetic transformation techniques (Li et al. 2007) to be applied to develop male and/or female sterile lines in *P. acerifolia* by gene silencing (Wesley et al. 2001; Helliwell et al. 2002).

## Materials and methods

### Biological material, RNA isolation and sequencing

All plant material used in this study was taken from trees of *Platanus acerifolia* Willd. growing within the campus grounds of Huazhong Agricultural University, Wuhan, China. The trees were of mixed ages, with 0.5-year-old seedlings or 4-year-old seedlings having been generated by the germination of seeds taken from 40-year-old adult trees. During the period April–December 2007, various tissue samples were collected from the juvenile and adult plants. Juvenile samples comprised: leaves from 0.5-year-old seedlings (JL); stems from 0.5-year-old seedlings (JS); roots of 0.5-year-old seedlings (JR); subpetiolar buds of 4-year-old saplings (JSB). Samples from the 40-year-old adult trees included: mature embryos (ME); subpetiolar buds (ASB, Fig.S1-b, c); leaves (AL); stem tissue (AS). Inflorescences were also collected from the adult trees: male inflorescences designated 7MF, 8MF, 10MF, 12MF, 3MF and 4MF were collected during the months July (stamen and pistil differentiation phase, Fig.S1-h; Fig.S2-b), August (completion of stamen and pistil differentiation, Fig.S1-i; Fig.S2-c), September (there is no obvious difference in differentiation on spherical head but size, Fig.S1-i; Fig.S2-d), December (stop of floral growth and development, there is no obvious difference comparing with September but size), March (microspore mother cell in meiosis) and April (pollen mature, stigma to be red), respectively; female inflorescences designated 12FF and 4FF were collected during December and April. Male inflorescences at specific ontogenetic stages were collected on July 15, August 15, October 16, December 15, March 15 and April 16 of 2007. Female inflorescences at various ontogenetic stages were collected on April 16 and December 15, 2007. All samples were immediately frozen in liquid nitrogen but, in the case of the flower samples, bud scales were removed before freezing. Total RNA was prepared from the individual tissues using CTAB (Li et al. 2008).The construction of double-stranded cDNA, to be used for rapid amplification of cDNA end (RACE), was performed using the SMART™ cDNA Library Construction Kit (Li et al. 2008). For amplification of the *P. acerifolia* MADS-box sequences, 3′-RACE experiments were conducted using a 3′ PCR primer and a degenerate primer (PMADSF, 5′-GTKCTHTGYGAYGCYGARRTTGC-3′) (additional data are given in Online Resource Supplementary Table S1) that corresponded to the conserved MADS-box amino acid sequence, VLCDAEV (Van der Linden et al. 2002). The PCR reagents comprised 5 μL 10× DNA polymerase buffer, 3 μL $MgCl_2$ (25 mM each), 1 μL 10 mM dNTP (2.5 mM each), 1 μL of each GSP (gene specific primer) (additional data are given in Online Resource Supplementary Table S1) (10 mM each), 1 μL template, 0.5 μL DNA polymerase (MBI Fermentas) and adjusted with water to a final volume of 50 μL. Touchdown-PCR reactions were initially heated to 94°C for 2 min, followed by 5 cycles of 94°C for 20 s, 64°C for 50 s, 72°C for 90 s; the annealing temperature was reduced in each subsequent set of 5-cycles by 3°C until it reached 58°C, after which there followed 25 cycles of: 94°C for 20 s, 55°C for 50 s, 72°C for 90 s; a final extension step was performed at 72°C for 10 min. 5′-RACE was performed with a 5′ PCR primer and gene-specific primer (GSP) (additional data are given in Online Resource Supplementary Table S1). The reaction was initially heated at 94°C for 2 min, followed by 35 cycles of 94°C for 30 s, 60°C for 45 s and 72°C for 90 s, and followed by a final extension step at 72°C for 10 min. The PCR products were purified with a gel PCR purification kit (Omega, USA) and cloned into the pMD-18T Vector system (Takara, Japan). Ligation products were transformed into *Escherichia coli* DH5a-competent cells (Takara, Japan), following the manufacturer's instructions. Sequencing of cDNA from three

independent clones was performed using bigdye terminator V3.1 cycle sequencing kit with an ABI 3700 sequencer.

## Sequence alignment and phylogenetic analysis

The sequences of selected species were downloaded from the NCBI GenBank (http://www.ncbi.nlm.nih.gov). The various taxa included in the analysis (additional data are given in Online Resource Supplementary Table S2–5) were chosen to represent angiosperm diversity at the order level. All homologues or paralogues from each selected taxon were included in the analysis.

The DNA and amino acid sequences of A-, B-, C- and E-class MADS-box genes were aligned by ClustalX 1.83 respectively (Thompson et al. 1997). The conserved MIK or MIKC domains were included for phylogenetic analysis. We first used Model Test 3.7 to select the best-fit distance models and parameters for the DNA datasets with MIKC domains (Posada and Crandall 1998), and Tree-Puzzle 5.2 for the amino-acid datasets with MIK domains (Schmidt et al. 2002). The individual maximum-likelihood (ML) trees for each of the A-, B-, C- or E-class MADS-box genes were constructed by Phyml 2.4.4 with GTR + I + Γ model. The combined ML tree for all four classes of MADS-box genes was built with JTT + I + Γ model (Guindon and Gascuel 2003). The bootstrap support values (500 replications) were also computed. Neighbor-joining (NJ) bootstrap analysis (2,000 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). The 50% majority rule consensus trees were inferred using MrBayes v3.12 with the GTR + I + Γ model for DNAs and WAG + I + Γ model for amino-acids (Ronquist and Huelsenbeck 2003). Metropolis-coupled MCMC (MCMCMC) method from a random starting tree was initiated in the Bayesian inference and run for 2,000,000 generations with trees sampled every 100th generation. The first 5,000 trees were discarded as ''burn-in'' and the subsequent 15,000 were used to calculate the consensus tree. During the analyses, *AGL6*, *GGM2*, *GGM3*, *AcMADS600* and *AGL15* were used as out-groups for the individual A-, B-, C-, E-classes and the combined four-class MADS-box genes, respectively.

## Expression analysis by RT-PCR

Samples of total RNA were treated with RQ1 RNase-Free DNase (Promega, USA) in order to remove DNA contamination. Single-stranded cDNA was synthesized with M-MLV Reverse Transcriptase and Oligo $(dT)_{15}$ primer (Promega, USA). In order to amplify cDNA from the different tissues we used a reverse primer located in the *PlacAG* 3′ UTR in conjunction with a specific forward primer, or specific forward and reverse primers in the 5′ and 3′ UTR of *PlacFUL*, *PlacAP3*, *PlacSEP1* and *PlacSEP3* genes (additional data are given in Online Resource Supplementary Table S6). The *Histone* gene of *P. acerifolia* was identified and primers designed to act as an internal control for the RT-PCR (Li et al. 2008).

# Results

## Sequence identification of MADS-box genes and conserved C-terminal motifs analysis in *P. acerifolia*

A total of 39 cDNA clones were isolated from *P. acerifolia* flowers by 3′ RACE, and 22 of these were identified as

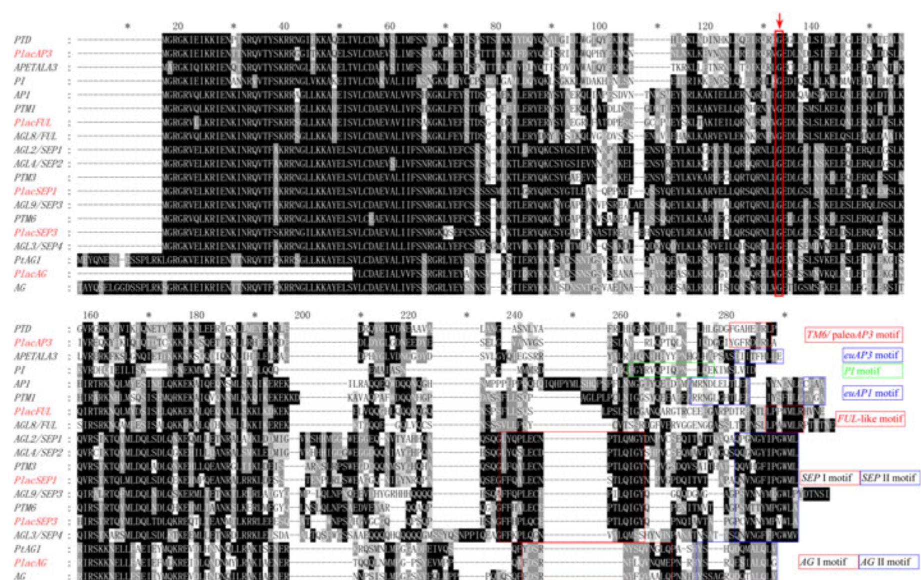



**Fig. 1** Representative predicted amino acid sequences alignment of ABCE genes from *P. acerifolia* and model plant (*Arabidopsis* and poplar). Conserved motifs are *boxed*, as defined by previous studies for the *FUL/SEP* motif (Litt and Irish 2003), the *PI* and *TM6*/paleo-*AP3* motifs (Kramer et al. 1998), and the *AG* motif (Kramer et al. 2004). Gly-110 is indicated by the *downward arrowhead* and *solid box outline*

homologues of floral organ identity genes. These comprised *FUL*, *AP3*, *AG*, *SEP1* and *SEP3* homologues, each homologue type being represented by 2, 1, 1, 7 and 11 of the clones, respectively. GenBank blast and phylogenetic analysis showed these to be homologous to floral A-, B-, C- and E-class genes (Fig. S4).

Full-length *P. acerifolia* sequences for four of the genes (namely, *PlacFUL*, *PlacAP3*, *PlacSEP1* and *PlacSEP3*) were obtained by RACE and a partial clone of *PlacAG* was also obtained. *PlacFUL* is 996 bp in length and has an ORF of 735 bp which encodes a predicted polypeptide of 245 aa with 5′/3′-UTR of 128 and 103 bp. *PlacAP3* is 930 bp in length and has an ORF of 678 bp which encodes a predicted polypeptide of 226 aa with 5′/3′-UTR of 83 and 139 bp. *PlacSEP1* is 1,120 bp in length and has an ORF of 735 bp which encodes a predicted polypeptide of 245 aa with 5′/3′-UTR of 123 and 251 bp. *PlacSEP3* is 993 bp in length and has an ORF of 720 bp which encodes a predicted polypeptide of 226 aa with 5′/3′-UTR of 64 and 179 bp. Clone *PlacAG* contains 838 bp from the gene's 3′-end and this contains a partial ORF of 570 bp, which encodes a predicted polypeptide of 190 aa and a 3′-UTR of 243 bp.

The predicted amino acid sequences of the genes included in this analysis have the typical "MIKC" structure of plant type II MADS domain-containing proteins (Alvarez-Buylla et al. 2000). A-, B-, C- and E-class genes each typically have a characteristic C-terminal motif. Thus, most known *AP1/FUL* sequences encode C-terminal regions that are rich in glutamine, although regions rich in proline, serine or glycine are also common. The C-terminal ends of all known *FUL*-like and eu*FUL* codons contain a highly conserved hydrophobic six-amino-acid sequence (known as the *FUL*-like motif: L/MPPWML) and this is generally followed by either two basic residues or one polar and one basic residue (Fig. 1). The eu*AP1* sequences, instead, encode a distinct C terminus with two short conserved motifs namely, the eu*AP1* motif (RRNaLaLT/NLa, where "a" is an acidic residue) and the farnesylation motif (CFAT/A) which terminates the protein (Fig. 1). The *PI*, eu*AP3*, *TM6* and paleo*AP3* sequences are each characterised by a highly conserved amino acid sequence (i.e. *PI* motif: F??RVQPMQPNLQE; eu*AP3* motif: D??TF?LLE; *TM6*/paleo*AP3* motif: F/YG??DRLR) (Fig. 1). Most published *AG* or *SEP* sequences display two C-terminal motifs. Thus, C-class genes contain the *AG* I and *AG* II motifs, and E-class genes contain the *SEP* I and *SEP* II motif (including WML or WL) (Fig. 1). The *P. acerifolia* genes isolated in this study contained the respective C-terminal motifs, according to the A-, B-, C- or E-class of each gene, which the *FUL*-like motif of *PlacFUL* was LPPWML, the paleo*AP3* motif of *PlacAP3*: YGFRDRLR, the AG I and AG II motif of *PlacAG*: FDSRNFLQVNQME and YSRQESIALQLG, the *SEP* I and *SEP* II motif of *PlacSEP1*: FFQALECNSTLQIGY and AQNVNGFIPGWML, the *SEP* I and *SEP* II motif of *PlacSEP3*: FFHPLE CEPTLQIGY and PCVNNYMPVWLA (Fig. 1).

**Fig. 2** Phylogenetic tree of A-class genes based on maximum-likelihood (ML) analysis. *AGL6* is shown as an out-group. *Numbers on the branches* are bootstrap values obtained from ML analysis followed by posterior probabilities from Bayesian inference (BI) and bootstrap from neighbor-joining (NJ). Only values over 50% in the bootstrap analyses and Bayesian posterior probability are indicated. The two *stars* indicate the *AP1/FUL* and eu*FUL*/core eudicot *FUL*-like gene duplication, respectively, prior to the diversification of core eudicots, which might have happened very closely in time. In monocots, the two major duplication events are indicated with *black circles*, also the other mall-scale

## Phylogenetic analysis

Sequence information for 599 MADS-box genes was collated by using published MADS-box gene sequences, database mining and sequences isolated by degenerate PCR and RACE analyses. These 599 sequences originated from phylogenetically diverse species and represented each of the MADS-box lineages, with 162 belonging to class A, 186 to class B, 111 to class C/D and 140 to class E (additional data are given in Online Resource Supplementary Table S2–5). The sequences of the five *P. acerifolia* floral organ identity gene homologues were aligned with the respective A-, B-, C- or E-class genes of the multiple angiosperm taxa for phylogenetic analysis.

The *PlacFUL* sequence was found to form a clade with none-core eudicot *FUL*-like homologues from other none-core basal eudicots, i.e. *TraFUL1* and *TraFUL2* of *Trochodendron aralioides* (Trochodendrales) (Wu et al. 2007), *NEnuFL1* of *Nelumbo nucifera* (Nelumbonaceae, Proteales), *PapsFL1* of *Papaver somniferum*, *RbFL3* of *Ranunculus bulbosus*, *PapsFL1* of *Papaver somniferum* (Ranunculales), *BUseFL1*, *BUseFL2* and *BUseFL3* of *Buxus sempervirens* and *PatFL1* or *PAteFL1* of *Pachysandra terminalis* (Buxales) (Fig. 2).

The *AP3* homologue of *P. acerifolia*, *PlacAP3*, formed a sister group to *PlaocAP3-1* and *PlaocAP3-2* genes of *P. occidentalis* and formed a clade with other paleo*AP3* homologues from basal eudicots, namely, *AktAP3* of *Akebia trifoliata* and *DeAP3 of Dicentra eximia* (Ranunculales), *MdAP3* of *Meliosma dilleniifolia* (Sabiaceae), *NnAP3* of *Nelumbo nucifera* (Nelumbonaceae, Proteales), *TraAP3* of *Trochodendron aralioides* (Trochodendrales), *PpAP3-1* of *Pachysandra procumbens*, *PtAP3-1* of *Pachysandra terminalis*, *MdAP3-1* of *Meliosma dilleniifolia* (Buxales) (Fig. 3).

The *AG* homologue from *P. acerifolia*, *PlacAG*, also aligned near the base of the core eudicots, i.e. before the

euFUL

core eudicot FUL-like

euAP1

Trochodenrales

Proteales

Ranunculales

Buxales

none-core eudicot FUL-like

Chloranthales

monocot

Nymphaeaceae

Magnoliid

AGL6

0.1

separation of the two major eu*AG* clades, *SHP/PLE* and *AG/FAR*. *PlacAG* formed a clade with other none-core eudicot *AG* homologues of the basal eudicots, namely, *MdAG1* of *Meliosma dilleniifolia* (Sabiaceae, Buxales), *TraAG1* and *TraAG2* of *Trochodendron aralioides* (Trochodendrales), *EScaAG1* of *Eschscholzia californica*, *BgAG* of *Berberis gilgiana*, *AkqAG1* of *Akebia trifoliate*, *RfAG2* of *Ranunculus ficaria* (Ranunculales) and *CsAG1* of *Chloranthus spicatus* (Chloranthales) (Fig. 4).

The two *SEP* homologues from *P. acerifolia* separated into two basal eudicot groups, namely, *PlacSEP1* in the *SEP1* clade and *PlacSEP3* in the *SEP3* clade. *PlacSEP1* was placed close to *EScaAGL2* of *Eschscholzia californica* (Ranunculales). *PlacSEP3* formed a sister group to the cluster containing *PEamAGL9.1* of *Persea americana* (Laurales), *Ma.gr.AGL9* of *Magnolia grandiflora* (Magnoliales), *EScaAGL9* of *Eschscholzia californica* (Ranunculales) and *AktSEp3-1* of *Akebia trifoliata* (Ranunculales) (Fig. 5).

Phylogenetic evolution analysis of sequence databases of A-, B-, C- and E-class MADS-box genes with the *PlacFUL*, *PlacAP3*, *PlacAG, PlacSEP1* and *PlacSEP3* sequences indicates that *P. acerifolia* is one of the basal eudicots and is close to Trochodendrales, Buxales, Ranunculales and Chloranthales (Fig. 6).

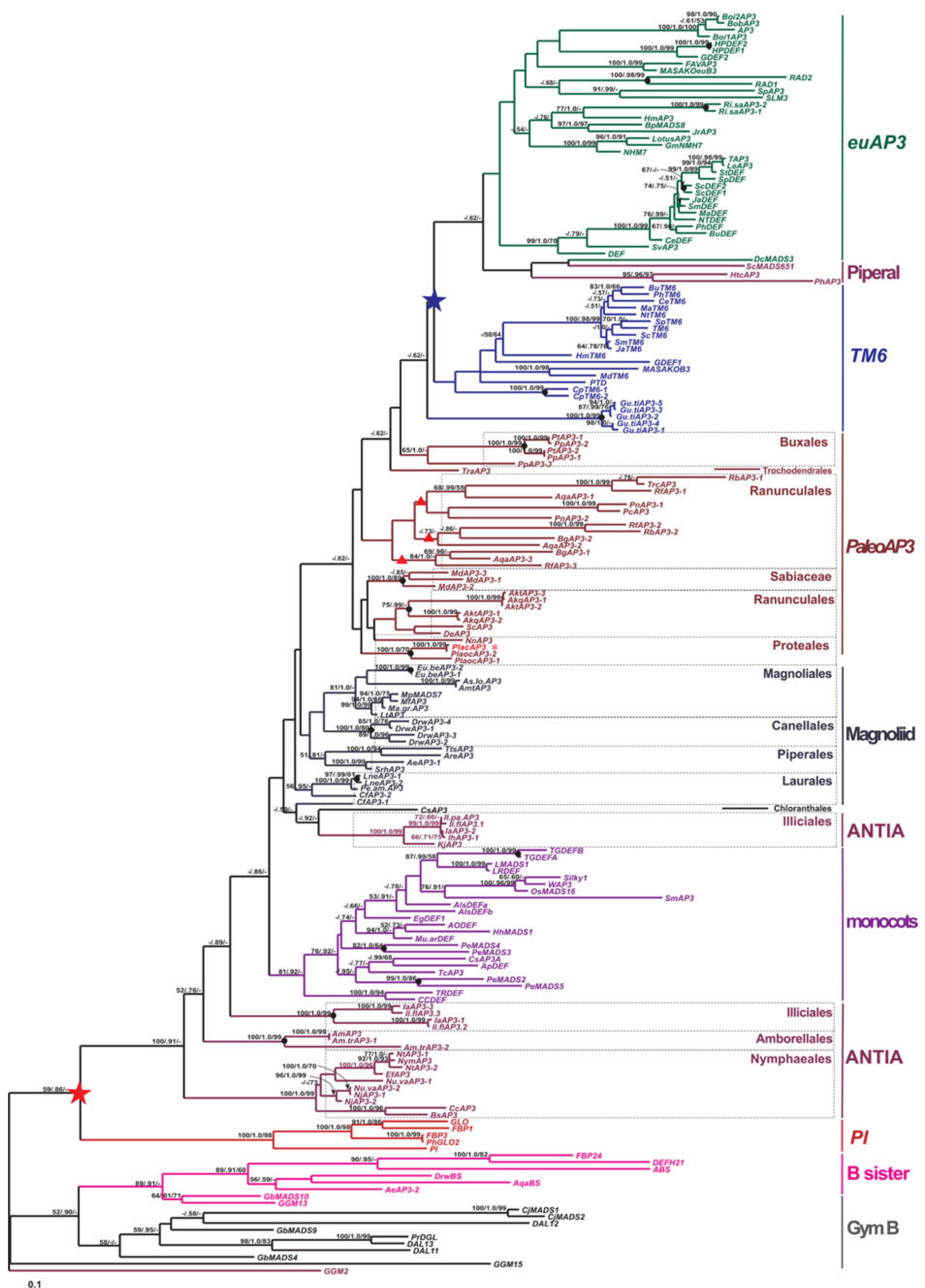
Expression analysis

RT-PCR was used to detect the expression of the *P. acerifolia* floral identity gene homologues within various tissues, including the vegetative organs of juvenile and adult plants and male and female inflorescences taken at a range of developmental stages. The results obtained following 35 cycles of PCR are shown in Fig. 7. Transcripts of the genes (excluding *PlacAG*) were detected, albeit weakly, within some vegetative organs. Thus, *PlacSEP3* transcripts were detected in embryos (ME) and seedlings roots (JR), but not in leaves (JL, AL) or stems (JS, AS). Transcripts of *PlacSEP3*, *PlacSEP1* and *PlacFUL* (but not *PlacAG* or *PlacAP3*) were detected in the subpetiolar buds of adult trees (ASB). *PlacSEP1* appeared to be expressed in AS but not in AL, whereas, the other four genes were not detected to any significant level in either AL or AS. All five *Platanus* MADS-box genes were expressed to significant levels within the reproductive structures, although the abundance of the respective transcripts varied according to the inflorescence type. *PlacFUL* was found to be expressed in the male inflorescence at stages 10MF and 12MF (and weakly in 3MF) and female inflorescences at stage12FF (and weakly in 4FF). *PlacAP3* was expressed in all eight samples of the male and female inflorescences, with particularly strong signals detected in 12MF and 3MF and the weakest levels found in 4MF. Expression of *PlacAG* was detected in 8MF, 10MF and 12MF, and also 12FF and 4FF samples. *PlacSEP1* and *PlacSEP3* transcripts were both found throughout the male and female inflorescence samples, although levels varied with sampling stage.

**Fig. 3** Phylogenetic tree of B-class genes based on maximum-likelihood (ML) analysis. *GGM15* is used as outgroup. *Numbers on the branches* are bootstrap values obtained from ML analysis followed by posterior probabilities from Bayesian inference (BI) and bootstrap from neighbor-joining (NJ). Only values over 50% in the bootstrap analyses and Bayesian posterior probability are indicated. The two *stars* indicate the paleo-*AP3/PI*, and eu*AP3/TM6* gene duplication, respectively; the *red triangle*, a gene duplication in the paleo-*AP3*; *black circles*, the other mall-scale gene duplications ▸

## Discussion

We obtained five MADS-box homologues from *Platanus acerifolia*, namely, *PlacFUL*, *PlacAP3*, *PlacAG*, *PlacSEP1* and *PlacSEP3*. Phylogenetic analyses grouped these *Platanus* genes according to the respective A-, B-, C- or E-class of MADS-box floral organ identity genes, typical of angiosperm species. In addition, the C-terminal regions of the respective *P. acerifolia* genes contained conserved signature motifs, as typical of A-, B-, C- or E-class genes (Vandenbussche et al. 2003) (Fig. 1). These conserved polypeptide motifs support the possibility that the *P. acerifolia* genes fulfill similar functions to their homologues in other flowering plants. Recent studies have indicated that a major duplication event may have occurred for floral ABC-class genes, at the base of the core eudicot lineages (Kramer et al. 1998, 2004; Litt and Irish 2003). Studies of *AP3* gene phylogeny have provided evidence that such a duplication event occurred after the branching of *P. acerifolia* (Kramer et al. 2006). Immediately following gene duplication, a frame shift mutation occurred in one of the *AP3* copies giving rise to the eu*AP3* lineage (Kramer et al. 2006). The results of our study with *Platanus acerifolia* indicate that A- and C-class gene phylogenies are also consistent with this scenario. We found that each class of the *P. acerifolia* homologues includes a preduplication form of the floral identity genes, as characterized by typical paleotypes of gene product motifs (Irish 2003; Vandenbussche et al. 2003) (Fig. 1). By contrast, the three *PlacFUL* homologues found in *P. acerifolia* form sister groups in A-class phylogenies (Fig. 2) and this could reflect an ancient duplication of a

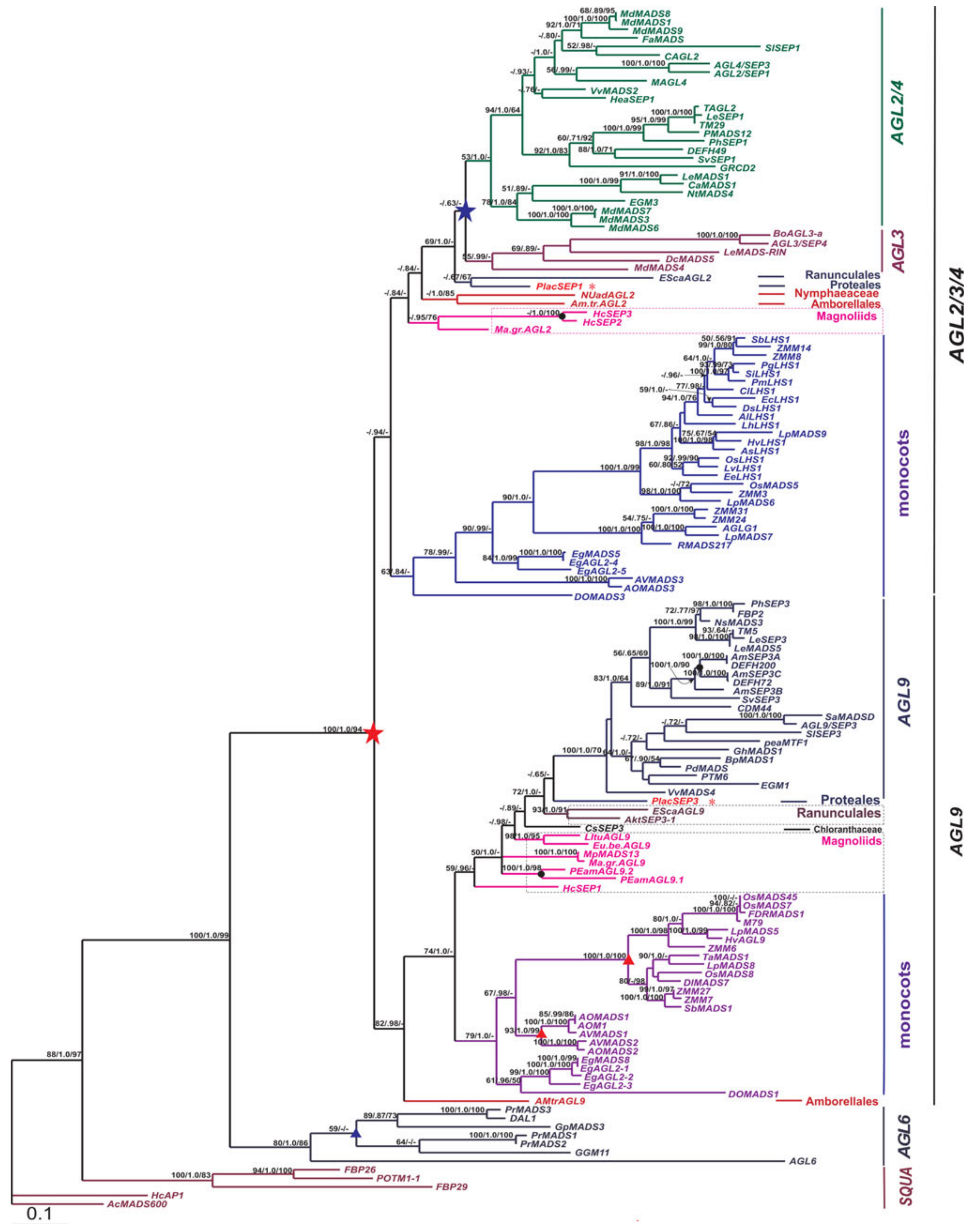

euAP3
Piperal
TM6
Buxales
Trochodendrales
Ranunculales
PaleoAP3
Sabiaceae
Ranunculales
Proteales
Magnoliales
Canellales
Piperales
Laurales
Magnoliid
Chloranthales
Illiciales
ANTIA
monocots
Illiciales
Amborellales
Nymphaeales
ANTIA
PI
B sister
Gym B
0.1

partial or whole chromosome within the *Platanus* lineage. The ABCDE model of floral development was largely derived from studies of two key model systems, *Arabidopsis* and *Antirrhinum majus* (Coen and Meyerowitz 1991; Theissen and Saedler 2001). Evidence of true A-class function has been limited to the *Arabidopsis* (*AP1* and *AP2*) species (Keck et al. 2003; Ferrario et al. 2004), with *AP1/FUL* homologues in other model plants, such as rice and petunia, failing to demonstrate A-class function (Ferrario et al. 2004). This restricts the inference of A-class function for homologues in other plant species. Thus, although we successfully identified the *AP1* homologue *PlacFUL*, we do not necessarily infer class-A function in *P. acerifolia.* Another A-function candidate, *AP2*, was not included in this study but is worthy of further investigation. By contrast, B and C functions appear highly conserved amongst the various B- and C-class gene homologues found in different angiosperm species (Kramer and Irish 2000; Ferrario et al. 2004). Nonetheless, direct evidence is required for functionality of the ABCE genes in *Platanus* and, since genetic experiments are very difficult to conduct in woody perennials, this is likely to come indirectly from complementation or over-expression experiments in *Arabidopsis* mutant backgrounds. In a similar evolutionary path to the B-class genes (Kramer et al. 2006), it has been proposed that the *AP1* lineage resulted from a major duplication event that occurred near the base of the core eudicots, giving rise to the eu*AP1* and eu*FUL* lineages of the core eudicots (Litt and Irish 2003; Shan et al. 2007). Although eu*AP1* is consistently expressed in sepals and petals (Irish, 2003), expression of eu*FUL* homologues is usually restricted to the carpels and bracts (Mandel and Yanofsky 1995; Gu et al. 1998; Müller et al. 2001). Previous studies have indicated that the B-class genes of core eudicots are stably expressed in petals and stamens. However, this is not always true of the B-class genes of basal eudicots and basal angiosperms (Zahn et al. 2005b). For instance, *AP3* and *PI* homologues from Ranunculaceae are expressed throughout the petaloid perianth and the stamens, but some homologues are also expressed in the carpels (Kramer et al. 2003). In *Eupomatia*, a member of the Eupomatiaceae (Magnoliales), the *AP3* homologues are also weakly expressed in the calyptras and leaves, while in *Magnolia*, the *AP3* homologue is expressed in spathaceous bracts (Kim et al. 2005). The *AG*-like homologues are usually expressed exclusively in stamens and carpels and play specific roles relating to C function (De Bodt et al. 2003; Irish 2003). Based on the reconstructed phylogeny of E-class genes, conducted in this study, *PlacSEP3* is assigned to belong to the *SEP3* clade (Malcomber and Kellogg 2005) or the *AGL9* clade (Zahn et al. 2005a). *PlacSEP1* appears to belong to the *LOFSEP* clade (Malcomber and Kellogg 2005) or, perhaps, the *AGL2/3/4* clade (Zahn et al. 2005a). Expression of *PlacSEP1* and *PlacSEP3* was found to be strongest in female inflorescences, compared to the male flowers, and this is consistent with the expression patterns of homologous genes in other species. Our results in *Platanus* are consistent with the general finding that E-class homologues are usually present as multiple copies in flowering plants and may have redundant or very diverse functions (this has been discussed more fully in reviews; Malcomber and Kellogg 2005; Zahn et al. 2005a). In *Arabidopsis*, *LFY* and *FUL* floral meristem identity genes have been shown to be the dominant B- and C-class genes, respectively, and show the appropriate distinct expression patterns (Busch et al. 1999; Lamb et al. 2002). In *P. acerifolia*, however, RT-PCR and qRT-PCR results indicated that *PaLFY* is expressed in a similar manner to *PlacFUL* (data not shown here). Since the four whorls of the *P. acerifolia* flower are not readily separated for RT-PCR analysis, in situ hybridization studies will need to be performed to determine the detailed expression patterns.

**Fig. 4** Phylogenetic tree of C-class genes based on maximum-likelihood (ML) analysis. *GGM3* is used as outgroup. *Numbers on the branches* are bootstrap values obtained from ML analysis followed by posterior probabilities from Bayesian inference (BI) and bootstrap from neighbor-joining (NJ). Only values over 50% in the bootstrap analyses and Bayesian posterior probability are indicated. The *red triangle* and *diamond* indicate the C/D and eu*AG/PLE* gene duplication, respectively; the *red triangle*, a gene duplication in the *SHP1/SHP2* lineage; the *black circles*, gene duplications in the grass C and D lineages; the *black triangle*, a gene duplication in the Ranunculales C lineage

Phylogenetic evolution analysis of A-, B-, C- and E-class MADS-box genes, *PlacFUL*, *PlacAP3*, *PlacAG,* *PlacSEP1* and *PlacSEP3*, shows that *P. acerifolia* is one of the basal eudicots and is close to Trochodendrales, Buxales, Ranunculales and Chloranthales. Thus, together with Proteaceae and Nelumbonaceae, Platanaceae belongs to the well-established order Proteales. These three families are morphologically very disparate and the fact that they assemble together exemplifies the diversity of floral structure and organization that is present among basal eudicot lineages. At this level of angiosperm evolution, floral structure is characterized by a wide range of variations relating to the number and arrangement of the reproductive organs and there is often weak differentiation, only, of the tepals and a variable phyllotaxis (Endress 1987a, b, 1994; Drinnan et al. 1994; von Balthazar and Endress 2002; Ronse DeCraene et al. 2003; Soltis et al. 2003, 2005).

RAG
MASAKOC1
STAG
TrAG
MdMADS15
CaMADS1
BpMADS6
JrAG
CAG3
CUM1
CAG2
AGAMOUS
TcAG
GhMADS3
PTAG2
PTAG1
GAGA2
GAGA1
HaAG
CDM37
GAG2
DcMADS4
PhaAG1
SLM1
RAG1
NAG1
TAG1
pMADS3
FAR
SxcAG1
euAG lineage
MASAKOD1
TrSHP
MdMADS14
VvMADS1
LAG
NTPLE36
TAGL1
FBP6
PLENA
BnSHP1
SHP1/AGL1
SHP2/AGL5
PLE lineage
HoAG1a
HoAG1b
CliAG1
RfAG1
AqaAG1
ThdAG1
AktAG1
AkqAG
BgAG
RfAG2
CliAG2
AqaAG2
ThdAG2
ScAG
EScaAG1
EScaAG2
Ranunculales
Trochodendrales
TraAG2
TraAG1
MdAG1
PlacAG
CsAG1
MpMADS2
SrhAG
HtcAG
Sabiaceae
Proteales
Basal eudicots
Chloranthace
Magnoliid
ZMM23
ZMM2
OsMADS3
HvAG1
WAG
HvAG2
ZAG1
AVAG1
HAG1
EgMADS12
PeMADS1
DthyrAG1
monocots
NymAG2
NymAG1
Nymphaeaceae
AG lineage
CAG1
CUM10
McAG
STK/AGL11
MdMADS10
GhMADS2
PhaAG2
SxcAG2
TAG11
FBP7
FBP11
VvMADS5
Core eudicots
ZMM1
ZAG2
OsMADS13
ZMM25
monocots
ApMADS2
HoMADS1
DthyrAG2
AktAG2
EScaAG11
MdAG2
CsAG2
MpMADS11
NymAG3
Ranunculales
Basal eudicots
Sabiaceae
Chloranthaceae
Magnoliales
Nymphaeaceae
AGL11 lineage
DAL2
SAG1
PrMADS
CyAG
GBM5
GGM3
Gymnosperms
0.1


MdMADS8
MdMADS1
MdMADS9
FaMADS
SlSEP1
CAGL2
AGL4/SEP3
AGL2/SEP1
MAGL4
VvMADS2
HeaSEP1
TAGL2
LeSEP1
TM29
PMADS12
PhSEP1
DEFH49
SvSEP1
GRCD2
LeMADS1
CaMADS1
NtMADS4
EGM3
MdMADS7
MdMADS3
MdMADS6
BoAGL3-a
AGL3/SEP4
LeMADS-RIN
DcMADS5
MdMADS4
EScaAGL2
PlacSEP1 *
NUadAGL2
Am.tr.AGL2
HcSEP3
HcSEP2
Ma.gr.AGL2
SbLHS1
ZMM14
ZMM8
PgLHS1
SiLHS1
PmLHS1
ClLHS1
EcLHS1
DsLHS1
AlLHS1
LhLHS1
LpMADS9
HvLHS1
AsLHS1
OsLHS1
LvLHS1
EeLHS1
OsMADS5
ZMM3
LpMADS6
ZMM31
ZMM24
AGLG1
LpMADS7
RMADS217
EgMADS5
EgAGL2-4
EgAGL2-5
AVMADS3
AOMADS3
DOMADS3
PhSEP3
FBP2
NsMADS3
TM5
LeSEP3
LeMADS5
AmSEP3A
DEFH200
AmSEP3C
DEFH72
AmSEP3B
SvSEP3
CDM44
SaMADSD
AGL9/SEP3
SlSEP3
peaMTF1
GhMADS1
BpMADS1
PdMADS
PTM6
EGM1
VvMADS4
PlacSEP3 *
EScaAGL9
AktSEP3-1
CsSEP3
LltuAGL9
Eu.be.AGL9
MpMADS13
Ma.gr.AGL9
PEamAGL9.2
PEamAGL9.1
HcSEP1
OsMADS45
OsMADS7
FDRMADS1
M79
LpMADS5
HvAGL9
ZMM6
TaMADS1
LpMADS8
OsMADS8
DlMADS7
ZMM27
ZMM7
SbMADS1
AOMADS1
AOM1
AVMADS1
AVMADS2
AOMADS2
EgMADS8
EgAGL2-1
EgAGL2-2
EgAGL2-3
DOMADS1
AMtrAGL9
PrMADS3
DAL1
GpMADS3
PrMADS1
PrMADS2
GGM11
AGL6
FBP26
POTM1-1
FBP29
HcAP1
AcMADS600
0.1
Ranunculales
Proteales
Nymphaeaceae
Amborellales
Magnoliids
Chloranthaceae
AGL2/4
AGL3
AGL2/3/4
monocots
AGL9
AGL6
SQUA

◀ **Fig. 5** Phylogenetic tree of E-class genes based on maximum-likelihood (ML) analysis. *AcMADS600* is used as outgroup. *Numbers on the branches* are bootstrap values obtained from ML analysis followed by posterior probabilities from Bayesian inference (BI) and bootstrap from neighbor-joining (NJ). Only values over 50% in the bootstrap analyses and Bayesian posterior probability are indicated. The *red* and *blue star* indicate *AGL2/3/4*-like/*AGL9* and *AGL2/4/AGL3* gene duplication, respectively; the *triangles*, a gene duplication in the gymnosperm *AGL6* and the monocots *AG9* lineage; *black circles*, the other mall-scale gene duplications

The A-, B-, C-, E-class genes from the basalmost angiosperms (Amborellaceae, Nymphaeaceae, and Austrobaileyaceae, Schisandraceae and Illiciaceae), always hold the basalmost position, followed by genes from magnoliids (Magnoliales, Laurales, Piperales, Canellales), monocots, Chloranthaceae, early-diverging eudicots, and core eudicots (Fig. 6). In early-diverging eudicots, genes from each of the Buxaceae, Trochodendrales, Proteales,

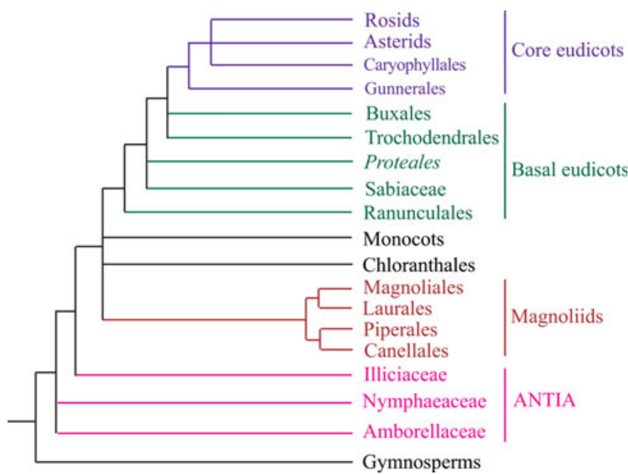



**Fig. 6** Outline of angiosperm phylogeny. Species used in this study are indicated according to taxonomic group

Sabiaceae and Ranunculales form separate well-supported clades, although the relationships among these clades are still uncertain. Within core eudicots, three distinct gene lineages are evident, each of which contains genes from rosids, asterids, Caryophyllales, and Saxifragales, suggesting that the two gene duplication events giving rise to these three clades predated the diversification of core eudicots.

Thus, in summary, we obtained five MADS-box homologues from *P. acerifolia*, identified as *PlacFUL*, *PlacAP3*, *PlacAG*, *PlacSEP1* and *PlacSEP3*, which group with none-core eudicot *FUL*-like, Paleo-*AP3*, none-core eudicot *AG,* none-core eudicot *AGL2* and *AGL9*. Phylogenetic analyses grouped these *P. acerifolia* genes according to the respective A-, B-, C- or E-class of MADS-box floral organ identity genes, typical of angiosperm species. The C-terminal regions of the *P. acerifolia* genes possess the conserved signature motifs of the respective A-, B-, C- or E-class genes (Vandenbussche et al. 2003) (Fig. 1), and therefore it seems possible that these *P. acerifolia* genes may fulfill similar functions to their homologues in other flowering plants. RT-PCR analysis detected expression of the genes within the male and female inflorescences. Unfortunately, however, the anatomy of the *P. acerifolia* flowers prevented us from separating the four whorls for RT-PCR analysis of the individual floral structures. Therefore, more detailed, whorl-specific expression analysis requires future investigation using in situ hybridization techniques.

In conclusion, the various floral homeotic gene phylogenies consistently placed *P. acerifolia* very close to the basal eudicots. This putative position of *P. acerifolia* as a basal eudicot makes it a particularly interesting candidate species for further studies regarding the timing and role of MADS-box gene duplications.

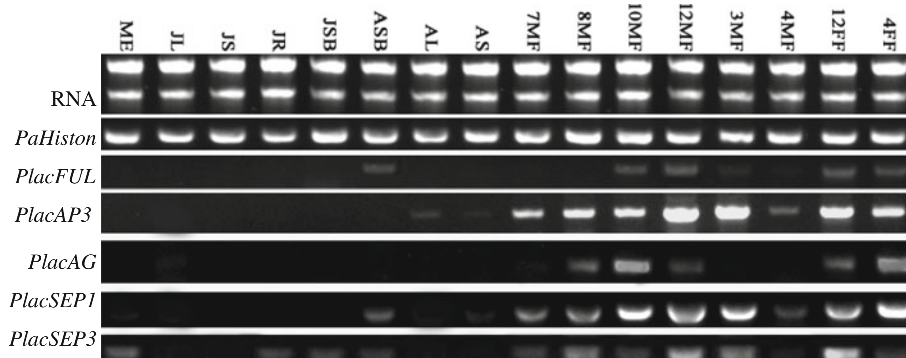



**Fig. 7** RT-PCR analysis of expression of *Platanus acerifolia* A-, B-, C- and E-class MADS-box gene homologues (*PlacFUL*, *PlacAP3*, *PlacAG*, *PlacSEP1* and *PlacSEP3*) in vegetative organs (*ME* mature embryo, *JL* juvenile leaf, *JS* juvenile stem, *JSB* juvenile subpetiolar bud, *ASB* adult subpetiolar bud, *AL* adult leaf, *AS* adult stem) and inflorescences (7MF, 8MF, 10MF, 12MF, 3MF, 4MF = various stages of male flower—see "Materials and methods" for details; 12 FF, 4FF = various stages of female flower). Results following 35-cycle reactions are shown. The *PaHiston* gene, from *P. acerifolia*, was included as an internal control for the reactions

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