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BRIEF COMMUNICATION

Cloning and characterization of paleo*AP3*-like MADS-box gene in London plane tree

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Abstract

We isolated *PlacAP3,* a homolog of the class B MADS-box transcription factor gene *APETALA3* (*AP3*), from the monoecious plant London plane tree (*Platanus acerifolia* Willd.). *PlacAP3* encodes a protein that shares good levels of identity with class B genes from *Arabidopsis thaliana* (35 and 51 % identity with *PISTILLATA* (*PI*) and *AP3,* respectively), and also with class B genes of other woody species (59 % identity with *PTD* from *Populus trichocarpa* and 66 % with *TraAP3* from *Trochodendron aralioides*). Reverse transcription polymerase chain reaction showed that *PlacAP3* was expressed in both the female and male flowers of *P. acerifolia*, but almost no signal was detected in the vegetative tissues or mature embryos. The *PlacAP3* expression in male flowers showed a relationship with developmental stage. There was a small transient increase during differentiation of the flower primordia in June, but maximal levels occurred during December when flower development appeared arrested. Increased *PlacAP3* expression was also detected in March of the following year, corresponding to meiotic divisions of the microspore mother cells, but this was lost by April when the pollen was mature.

Additional key words: *APETALA3,* female and male flowers, *Platanus acerifolia,* RACE, RT-PCR, transcription factors.

In higher eudicotyledonous angiosperms the floral organs are typically arranged in four different whorls, containing sepals, petals, stamens and carpels, respectively. Genetic studies with *Arabidopsis thaliana* and *Antirrhinum majus* 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 2001). B class genes act in combination with A or C class genes to specify petals or stamens, respectively.

APETALA3 (*AP3*) genes, or homologues, have been identified as critical regulators of floral organ identity in a wide range of species, including *Arabidopsis* (Jack *et al*. 1992), *Antirrhinum* (*DEFICIENS*; Sommer *et al*. 1990), *Petunia* (*pMADS1*; Van der Krol *et al*. 1993) and *Populus* (*PTD*; Sheppard *et al*. 2000). In *Arabidopsis*, *AP3* and *PISTILLATA* (*PI*; Goto and Meyerowitz 1994) are MADS-box genes belonging to sister clades and are necessary to specify B-function. In snapdragon the corresponding orthologs are *DEFICIENS* (*DEF*) and *GLOBOSA* (*GLO*). *Arabidopsis* plants carrying a

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Abbreviations: *AP3* - *APETALA3*; CTAB - cetyl-trimethyl-ammonium bromide; *DEF* - *DEFICIENS*; *GLO* - *GLOBOSA*; GSP - gene-specific primer; ORF - open reading frame; *PI* - *PISTILLATA*; PCR - polymerase chain reaction; RACE - rapid amplification of cDNA ends; RT-PCR - reverse transcription polymerase chain reaction; SOE - splicing overlap extension; TPI - triosephosphate isomerase.

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defective *AP3* gene produce flowers in which the second and third whorls, normally occupied by petals and stamens, are substituted by sepals and carpeloid structures, respectively (Jack *et al*. 1992).

We wish to characterize the genetic factors involved in floral development of *P. acerifolia*. This is partly with a view to facilitating the genetic engineering of reproductively sterile trees for the purposes of transgene containment. Towards this end, we have isolated and characterized one MADS-box gene, *PlacAP3*, from *Platanus acerifolia* Willd., a monoecious and diclinous hybrid of *P. orientalis* × *P. occidentalis*. This is a popular street tree belonging to the family *Platanaceae* (Besnard *et al*. 2002).

Total RNA was isolated from individual tissue types using CTAB method (Li *et al*. 2008) and synthesis of the double-stranded cDNA used for rapid amplification of cDNA ends (RACE) were performed using the *SMART*™ cDNA library construction kit (*Clontech*, Mountain View, CA, USA). For amplification of the *Platanus* MADS-box sequences, 3'-RACE experiments were conducted using a 3'PCR primer and a degenerate primer (PMADSF, 5'-GTKCTHTGYGAYGCYGARRTTGC-3') that corresponded to the conserved MADS-box amino acid sequence, VLCDAEV (Van der Linden *et al*. 2002). Consequently, a PCR fragment of approximately 739 bp was isolated and sequenced. The 5'/3'-RACE reactions were performed with a 5'/3'PCR primer and a gene-specific primer (GSP) (PlacAP3R: 5'-TTCTCTGTG CGTTTCCTGGGAGTTCTTT-3', PlacAP3F: 5'-AGC ATAGAGGAACTGCGTGGTCTTGAGC-3'). Splicing overlap extension (SOE) was performed using PCR reagents that comprised: 1 mm^3 of a 10-fold dilution of each of the 5' and 3'-RACE products (572 and 499 bp, respectively), 5 mm^3 dNTPs (2 mM), 5 mm^3 10× *Pyrobest* DNA polymerase buffer, 0.25 mm^3 *Pyrobest* DNA polymerase (*Takara*, Dalian, China), and 36 mm^3 ddH_2O. The mix was initially denatured at 94 °C for 2 min, and SOE was then carried out at 75 °C for 20 min. Subsequently, 1 mm^3 of each of the 5' and 3'PCR primers was added and 20 cycles of 94 °C for 30 s, 68 °C for 30 s, 72 °C for 90 s, were performed; a final extension step was performed at 72 °C for 10 min. The SOE product (930 bp) was identified by nested PCR using the PlacAP3F1 (5'-GCTTCTTCCCATCGCCTTCTACTT-3') and PlacAP3R1 (5'-AATGGAATGTCTGAAAAGGTT ACG-3') primer pair (predicted product size 755 bp). The full-length cDNA was sequenced and registered in *GeneBank* (accession number EF488452), named *PlacAP3*.

For analysis of *PlacAP3* expression patterns, plant materials were obtained either from 1-, 4- or 40-year-old, London plane trees that were field-grown at Huazhong Agricultural University, Wuhan, China. Juvenile tree samples were collected from seedlings that had been grown from seeds taken from the adult trees; RNA was extracted from fully expanded leaves, stems and roots of 1-year-old plants, and from subpetiolar buds of 4-year-old plants. For adult tree samples, RNA was extracted from fully expanded leaves, stems, subpetiolar leaf buds, mature embryos, and male and female inflorescences. Male inflorescences were sampled at various ontogenetic stages, including reproductive buds collected on 25 May (inflorescence primordium differentiation), 16 June (floral primordia differentiation), 15 July (stamen and pistil differentiation phase), 15 August (completion of stamen and pistil differentiation), 16 October (developmentally similar to August with larger head diameter) and 15 December (apparent arrest of floral growth and development) during the first year (2007), and on 15 March (microspore mother cells undergoing meiosis) and 16 April (mature pollen, stigma surface turns red) during the second year (2008). Female inflorescences at various ontogenetic stages were obtained by collecting them on 16 June and 15 December 2007, and 16 April 2008. Subpetiolar leaf buds from adult and juvenile trees were collected on 15 December 2007.

Total RNA samples from each of the tissue types were prepared and treated with RQ1 RNase-Free DNase (*Promega*, Madison, WI, USA) to remove DNA contamination. Single-stranded cDNA was synthesized with M-MLV Reverse Transcriptase and Oligo $(dT)_{15}$ primer (*Promega*). Gene-specific primers PlacAP3F1 (5'-CTC GCTTCTTCCCATCGCCTTCTACTT-3') and PlacAP3R1 (5'-CTTGAGCTTTCTTGG TGATTCCGCCTC-3') (Tm 60 °C) were designed using *Primer3* software and were predicted to amplify a 152 bp fragment. Real-time PCR products were amplified using 0.5 mm^3 of template from the RT reaction mixture, with 5 mm^3 2× *SYBR Green Master Mix* (*PE Applied Biosystems*, Foster City, CA, USA) and 0.5 μmol forward and reverse primers in a final volume of 10 mm^3. Gene expression was quantified using the *ABI 7500* sequence detection system software (*PE Applied Biosystems*). *PlacAP3* expression levels were normalized against the expression of the *P. acerifolia* house-keeping gene *TPI* (triose-phosphate isomerase), for which a 168 bp fragment was amplifed using the primers PaTPIF (5'-GCCACAACAAGGATTATTTATGGAG-3') and PaTPIR (5'-TGA TTCCCAAGTTTAAGCACTC TTC-3'). Data shown represent the means ± SD values from four replicates of the real-time quantitative RT-PCR reactions performed for each RNA sample. Thermocycler conditions were 2 min at 50 °C followed by 10 s at 95 °C, and then 40 cycles of: 15 s at 95 °C, 20 s at 60 °C, 30 s at 72 °C.

Phylogenetic analysis was carried out by aligning the MIKC domain of *PlacAP3* with the same region of homologous genes from 16 other species, and this was conducted using *ClustalX* (Thompson *et al*. 1997). Neighbor-joining (NJ) bootstrap analysis (1 000 replications) with the Poisson correction for the amino acids was performed using *MEGA 4* software (Tamura *et al*. 2007).

Here, we report that the *PlacAP3* gene is 930 bp in

length, has an open reading frame (ORF) of 678 bp encoding a predicted polypeptide of 226 amino acids, and contains 5' and 3'-UTRs of 83 and 139 bp, respectively (Fig. 1*A*). *PlacAP3* encodes a protein that shares 35 and 51 % identity with the products of the *Arabidopsis* class B genes *PISTILLATA* (*PI*) and *AP3*, respectively. *PlacAP3* also shares 59 % identity with *PTD* from (*Populus trichocarpa*), 66 % with *TraAP3* from *Trochodendron aralioides*, and 98 % with *PloAP3-2* from *Platanus occidentalis*. Sequence alignment showed that *PlacAP3* contains a highly conserved MADS domain, K domain, and also the conserved *PI* motif and *TM6*/paleo*AP3* motif (Fig. 1*A*) (Aoki *et al*. 2004). The *PI*, eu*AP3*, *TM6* and paleo*AP3* sequences are characterized by a highly conserved amino acid sequences (*i.e*. *PI* motif: FxxRVQPMQPNLQE; eu*AP3* motif: DxxTFxLLE; *TM6*/ paleo*AP3* motif: F/YGxxDRLR) (Fig. 1*A*). Phylogenetic analysis showed that *PlacAP3* was clustered closely to *Platanus occidentalis AP3* homologs *PloAP3-2* and *PloAP3-1* (Kramer *et al*. 2003), *Trochodendron aralioides TraAP3* gene (Wu *et al*. 2007), and petunia *pMADS1* (Fig. 1*B*); each of these genes has been identified as sharing similar functions with *Arabidopsis AP3*.

RT-PCR indicated that *PlacAP3* transcripts were principally found in inflorescence tissues, with little or no expression in vegetative parts or mature embryos (Table 1). Thus, expression of *PlacAP3* is similar to that of the *Platanus PISTILLATA* (*PaPI*) (Zhang *et al*. 2011) and *Populus trichocarpa DEFICIENS* (*PTD*) (Sheppard *et al*. 2000) genes that also show floral-specific patterns. Furthermore, *PlacAP3* expression levels in the flowers varied according to ontogenetic stage and showed a complex pattern with respect to time-of-year (Table 1). During the first year of bud development, relative *PlacAP3* expression in male inflorescences increased between May and June by *ca*. 3-fold, and this increase corresponded to progress from the initiation of inflorescence primordia to the development of individual flower primordia. Expression decreased again by July when floral development entered the phase of stamen and pistil differentiation. The highest *PlacAP3* expression was observed in male flowers during December, reaching >13-fold the May level, and this corresponded to the arrest of floral growth and development according to visual inspections. In the second year of flower development, significant *PlacAP3* expression was detected during March and this correlated with meiotic divisions of the microspore mother cells. By April, expression decreased to basal levels and this ontogenetic stage corresponded to maturation of the pollen grains. Expression in the female flowers tended to be lower than in the male flowers. Thus, during June 2007 and April 2008 *PlacAP3* expression was similar to the basal levels seen in male flowers (Table 1). The highest expression in female flowers occurred in December, but this was *ca*. 6-fold lower than that seen in corresponding male samples (Table 1). The higher expression in the male flowers is consistent with the pattern predicted for B-function genes by the ABC model.

Table 1. Relative quantification (RQ) of real-time RT-PCR expression analysis of *PlacAP3*. Analysis was carried out for various tissues: ME - mature embryos; 1-YL, 1-YS and 1-YR - leaves, stems and roots of 1-year-old seedlings; 4-YSB - subpetiolar buds of 4-year-old seedlings; ASB - subpetiolar buds of the adult tree; ANL and ANS - new leaves and stems of the adult tree; 5MF, 6MF, 7MF, 8MF, 10MF, 12MF, 3MF, 4MF - male inflorescences collected in May, June, July, August, October, December (2007), and March, April (2008), respectively; 6FF, 12FF, 4FF - female inflorescences collected in June, December (2007) and April (2008). *TPI* transcript abundance was used as a control in quantitative RT-PCR.

Samples	*PlacAP3* expression	RQ Min	RQ Max
ME	0	0	0
1-YL	0	0	0
1-YS	0.048	0	0
1-YR	0	0	0
4-YSB	0.005	0	0
ASB	0	0	0
ANL	0	0	0
ANS	0.07	0.043	0.114
5MF	1	0.257	0.296
6MF	3.041	0.188	0.179
7MF	0.27	0.193	0.379
8MF	1	0.422	0.308
10MF	1.393	0.392	0.308
12MF	13.232	0.555	0.536
3MF	2.489	0.277	0.379
4MF	0.83	0.207	0.219
6FF	0.829	0.346	0.326
12FF	2.254	0.277	0.379
4FF	0.832	0.177	0.179

Although both *AP3* and *DEF* share similar expression pattern, subtle differences in gene expression are detected in unexpected tissue. For instance, *DEF* transiently express in the carpel primordia and *AP3* expressed in a small adaxial patch of sepal cells (Jack *et al*. 1992, Schwarz-Sommer *et al*. 1992). Additionally, *AP3* expression in seed coat is high, but in ovary and silique low (https://www.genevestigator.com/ gv/index.jsp). The transcript of poplar *AP3* ortholog *PTD* is restricted to stamen primordia as reproductive primordia began to form, but not in carpel primordia and vegetative tissues (Sheppard *et al*. 2000). However, *PTD* expression in female catkin is higher than in male catkin, young and mature leaves according to the *Populus tremuloides* standard *61K affymetrix* dataset (http://www. plexdb. org/modules/tools/plexdb_blast.php). Due to the difficulty in separating flower whorls from inflorescences, *Platanus* inflorescences was selected for detecting *PlacAP3* spatio-expression in this study. The quantitative expression of *PlacAP3* in the flowers varied similarly in male and female inflorescences according to ontogenetic stages. The amount of *PlacAP3* transcripts in male

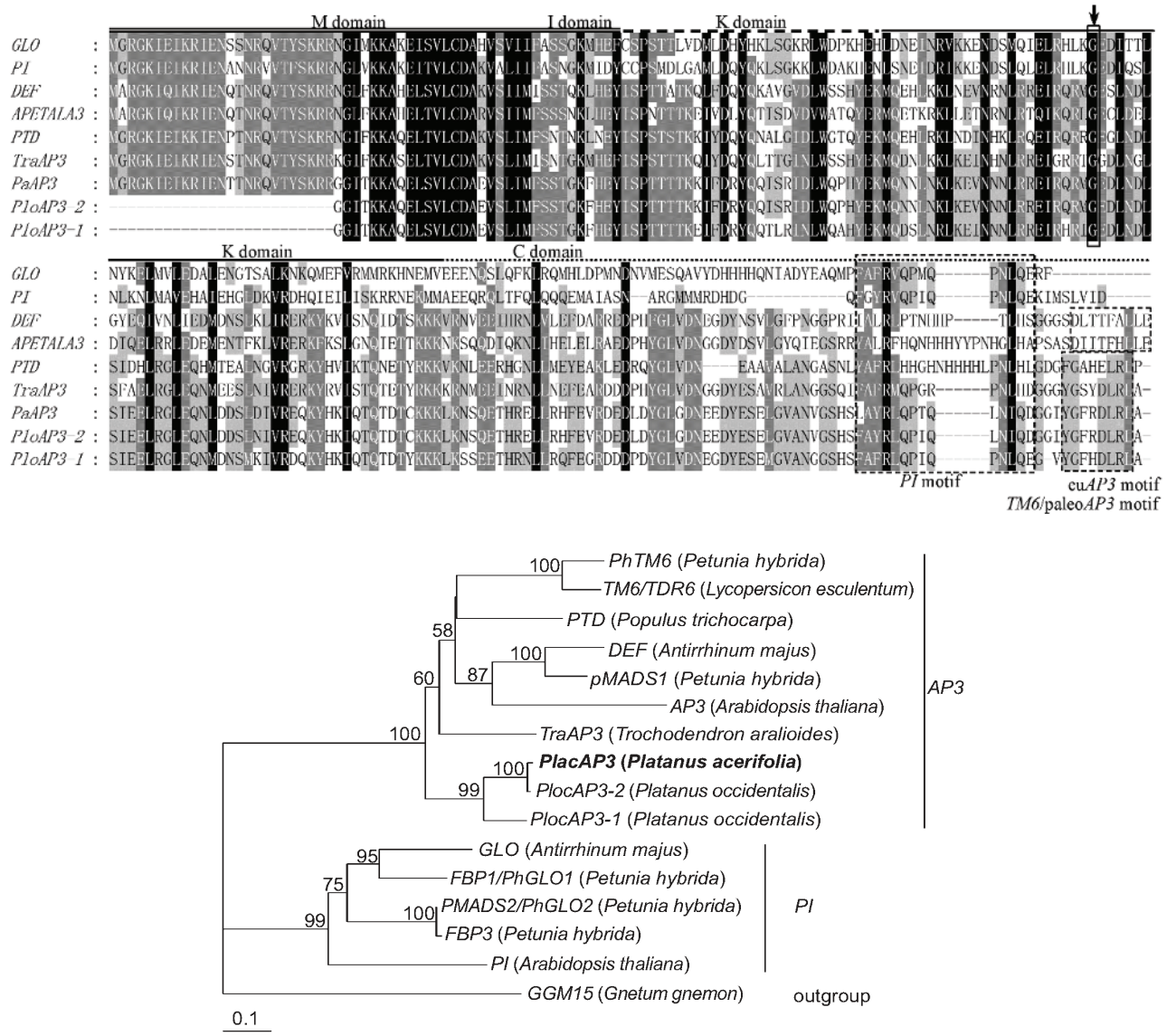

Fig. 1. Sequence alignment and phylogenetic analysis of *PlacAP3*. Alignment analysis of *PlacAP3*. MADS, I, K and C domains are underlined and defined by *double*, *single*, *dashed* and *dotted lines*, respectively (Aoki *et al*. 2004). Gly-110 is indicated by the *downward arrowhead* and *solid box outline*. PI motif, eu*AP3* motif (up) and *TM6*/paleo*AP3* motif (down) are annotated and highlighted with *dashed box outlines*. The genes and their accession numbers are as follows: *PhTM6* (AF230704), TM6/TDR6 (X60759/AY098734), *PTD* (AF057708), *DEF* (X52023), *pMADS1* (X69946), *AP3* (AF115814), *TraAP3* (DQ453774), *PlacAP3* (EF488452), *PloAP3-1* (AY162881), *PloAP3-2* (AY162882), *GLO* (X68831), *FBP1/PhGLO1* (M91190), *PMADS2/PhGLO2* (X69947), *FBP3* (X71417), *PI* (D30807), *GGM15* (AJ251555). The phylogeny of *PlacAP3* and *AP3* genes from other plants. The phylogenetic trees were constructed using *MEGA 4.0* software. Bootstrap values (1000 replications) are shown near the branch points, and sequence distances are shown at the bottom of the trees. *GGM15* is included as an out-group.

inflorescences is higher than in female inflorescences, which resemble *PTD* expression pattern (Sheppard *et al*. 2000), but opposite to the *Populus tremuloides* standard 61K affymetrix dataset, partially because *PlacAP3* was confined to stamen primordia but not in carpel primordia. In addition, no significant changes in *PlacAP3* transcripts are discovered in April, which might be as the result of pollen grains maturation and pollen emission.

In summary, we have isolated the MADS-box gene *PlacAP3* from *P. acerifolia* and shown it to be homologous to class B genes involved in floral organ identity. *PlacAP3* was found to be expressed in female and male inflorescences, but almost no signal was detected in vegetative parts or mature embryos. In many plant species, more than one *AP3* and/or *PI* family

member has been isolated, and most of these duplicated genes display differential functions (Zhou *et al*. 2010). Recently, we isolated five *PI*-like genes from *P. acerifolia* (Zhang *et al*. 2011). Therefore, it is possible that a sister *PlacAP3* gene remains to be identified in *P. acerifolia*.

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