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Full Length Research Paper

Cloning and expression analysis of an E-class MADS-box gene from *Populus deltoides*

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An E-class MADS-box gene, *PdMADS2*, was isolated from *Populus deltoides* Bartr. ex Marsh male floral buds by RT-PCR. As shown by relative–quantitative real-time polymerase chain reaction analysis, the expression of *PdMADS2* was high in apical and floral buds, intermediate in immature xylem and roots and low in mature leaves of adult male *P. deltoides*. In developing male floral buds, *PdMADS2* expression was high and remained constant from July to September, increased significantly on February 6 and quickly fell to a very low level with the maturation of the flowers. In male inflorescences, *PdMADS2* expression was abundant in the perianth cups and inflorescence peduncles; it was not detected in mature pollen. In female inflorescences, *PdMADS2* was highly expressed in both ovaries and inflorescence peduncles. Our results suggest that *PdMADS*2 plays an important role in the development of inflorescence meristems and flower organs in poplar.

Key words: Floral development, expression, *MADS-box*, E-class.

INTRODUCTION

The molecular mechanisms that regulate flower development are an important question in plant studies. The classic ABC model of flower development proposed in the early 1990s based on genetic studies in *Arabidopsis thaliana* and *Antirrhinum majus* has been broadly accepted and is applicable to a wide range of plant species (Schwartz-Sommer et al., 1990; Coen and Meyerowitz, 1991). This model has been revised with the discovery of D- and E-class genes in *Arabidopsis,* resulting in the ABC (DE) model (Pelaz et al., 2000; Theissen, 2001). All but one (*AP2*) of the genes in the ABC (DE) model of floral development encode putative transcriptional regulators and belong to the MADS-box gene family.

Studies in *Arabidopsis* and *Petunia* have indicated that proteins encoded by E-class genes participate in the formation of higher-order transcription factor complexes with other MADS-box proteins and play a central role in floral-meristem and organ identity (Pelaz et al., 2000; Vandenbussche et al., 2003; Ditta et al., 2004). In *Arabidopsis*, the E-class genes *SEP*1, *SEP*2 and *SEP*3 have been shown to interact with the products of the B and C organ identity genes to direct petal, stamen and carpel development (Bowman et al., 1991; Honma and Goto, 2001). *SEP*4 contributes to the development of sepals, petals, stamens and carpels and plays an important role in meristem identity (Ditta et al., 2004). In *Petunia*, it was shown that the B, C and D floral-organ identity functions require E-class genes (*FBP*2 and *FBP*5) (Vandenbussche et al., 2003). There have also been reports indicating that *SEP*-type genes play a role in fruit development and vegetative growth in some plant species, such as *MdMADS*1, 3 and 4 in apple trees and *NtMADS*4 in tobacco (Sung and An, 1997; Sung et al., 2000; Jang et al., 2002).

As in *Arabidopsis,* a family of E-class genes is involved in poplar reproductive viability (Cseke and Podila, 2004). *PTM*3 and its allele *PTM*4 from *Populus tremuloides* are related to the *SEP*1- and *SEP*2-type genes and another gene, *PTM*6, also from *P. tremuloides*, is related to *SEP*3, as demonstrated by sequence-based phylogenetic analyses. *PTM*3/4 and *PTM*6 are expressed at all stages of male and female aspen floral development. Transgenic experiments in both *P. tremuloides* and heterologous systems such as tobacco and *Arabidopsis* have indicated that the *PTM*3/4 genes participate in floral development

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(Cseke et al., 2005).

In this paper, we report the isolation of another E-class MADS-box gene from *Populus deltoids*, which is not homologous to the *SEP*1- , *SEP2* and *SEP*3-type genes reported in poplar and *Arabidopsis*. We present its expression pattern in male floral buds of different stages of development, in different parts of male and female inflorescences and in different tissues of *P. deltoides*. Possible regulatory roles of the E-class MADS-box genes in poplar flower development are discussed.

MATERIALS AND METHODS

Plant materials

Terminal buds, mature leaves, immature xylem and root tissues were collected on 3 September 2005 from adult male *P. deltoides* growing in Beijing, China (40°0'0"N, 116°14'24"E). Developing male floral buds were collected on 20 January, 6 February, 12 February, 8 July, 19 August and 7 September 2006. The buds gathered on 20 January, 6 February and 12 February were collected from branches that were excised on 30 December 2005 and maintained by water culture in a naturally lit greenhouse at temperatures between 20 and 28°C. Various floral parts were excised from male and female inflorescences collected from adult *P. deltoides* growing in Qinhuangdao, China (39°32'60"N, 119°20'60"E) just prior to flower opening, on 12 April 2007 and mature pollen was collected. Samples were immediately frozen in liquid nitrogen and stored at - 70°C until use.

Isolation and sequencing of the complete coding sequence

A complete coding sequence was amplified from cDNA of *P. deltiodes* male floral buds using *PfuUltra*™ high-fidelity DNA polymerase (Stratagene, USA), the primers P1 (5'-ATGGGGAGAGGTA GAGTGGAG-3') and P2 (5'-GTCCGATGGCAAATTCTTG-3'), which were designed according to the coding regions of high-scoring segment pairs (FL_GENBANK_97 and M129E09) in the PopulusDB (http://poppel.fysbot.umu.se/) and 3'-UTR from an 844-bp cDNA fragment isolated and sequenced from a male floral bud cDNA library of *P. deltoides* (Zhou et al., 2006). The polymerase chain reaction (PCR) conditions used were 94°C for 5 min; 36 cycles of 94°C for 20 s, 58°C for 20 s and 72°C for 50 s and a final extension of 7 min at 72°C. The amplified fragments were fused into the pGEM-T Easy vector (Promega, USA). Both strands of the full-length cDNA were sequenced using the dideoxynucleotide chain termination method (TaKaRa Bio, China). The cDNA sequence was analyzed with DNAMAN ver. 5.2.9 (Lynnon Biosoft). Comparison searches were performed using the NCBI BLASTN and tBLASTX programs.

Phylogenetic analysis

Major E-class MADS-box protein sequences from different plant species used in the phylogenetic analysis were downloaded from GenBank, including: from *A. thaliana*, *AP*3 (gi 543815), *SEP*1 (gi 26454603), *SEP*2 (gi 113514), *SEP*3 (gi 3912988) and *SEP*4 (gi 30678072); from *Eucalyptus grandis*, *EGM*1 (gi 3114584) and *EGM*3 (gi 3114588); from *Malus* × *domestica*, *MdMADS1* (gi 3290209), *MdMADS*3 (gi 5777904), *MdMADS*4 (gi 5777906), *MdMADS*6 (gi 3646322), *MdMADS*7 (gi 3646324), *MdMADS*8 (gi 3646334) and *MdMADS*9 (gi 3646336); from *P. tremuloides*, *PTM*3 (gi 28372802) and *PTM*6 (gi 30314024); from *Populus tomentosa*, *PtSEP*3 (gi 90903289); from *Petunia* x *hybrida*, *FBP*2 (gi 1345965); from *Pinus radiata*, *PrMADS*1 (gi 7446558) and from *Solanum lycopersicum*, *TM*5 (gi 3913002). Protein sequences encoded by the *P. trichocarpa* genes *PtMADS*6, *PtMADS*13, *PtMADS*31, *PtMADS*37, *PtMADS*40, *PtMADS*46 and *PtMADS*49 are from Leseberg et al. (2006). A multiple sequence alignment of MIK domains was performed using CLUSTALW. A phylogenetic tree was generated using the neighbor-joining method of MEGA ver. 3.1 (Kumar et al., 2004) based on the p-distance amino acid substitution model and the tree was tested using the bootstrap method with 1,000 replicates.

Relative–quantitative real-time PCR

Total RNA was extracted from poplar tissues and treated with RNase-free DNase as described (Zhang et al., 2008). cDNA was synthesized from 2.0 µg RNA using Superscript II RNase- Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions and checked for purity using specific genomic DNA primers. RQ real-time PCR reactions were carried out using an ABI Prism 7500 sequence detector (Applied Biosystems, USA). PCR reactions (final volume 20 µl) contained 1 µl first-strand cDNA (equal to 5ng total RNA), 200 nM primers and 1X SYBR PCR mixture (TaKaRa Bio). The amplification conditions were as follows: 10 s at 94°C, followed by 40 cycles of 5 s at 94°C and 34 s at 55°C. As a control, parallel amplification reactions were performed with primers specific for *ACT*11 as reference genes (Brunner et al., 2004). In all experiments, four replicates for each RNA sample were included. Relative quantification values and standard deviations were calculated using the standard-curve method according to the manufacturer's instructions (ABI Prism 7000 Sequence Detection System Users Guide).

The PCR primers used were, for *PdMADS2* 5'-AAAACGAGA GTGGACGTGCT-3' and 5'-GCTCTCCGACTTTCCATGAC-3', for *ACT*11, 5′-CACACTGGAGTGATGGTTGG-3′ and 5′-ATTGGCCTT GGGGTTAAGAG-3″.

Accession number

The GenBank accession number of PdMADS2 is EU121637. The sequence of "FL_GENBANK_97" and "M129E09" can be accessed at http://poppel.fysbot.umu.se/result.php?form_Keyword=121387 and http://poppel.fysbot.umu.se/result.php?form_Keyword=36337.

RESULTS

Sequence analysis of full-length cDNA

A total of 4,500 clones randomly selected from a *P. deltoides* male bud cDNA library were sequenced from their 5' ends (Zhou et al., 2006) and a 488-bp cDNA clone with a sequence similar to those of MADS-box genes was found through a GenBank BLASTX search. Sequencing of both strands of the cDNA clone indicated that it was 844 bp, comprised of a 630-bp incomplete open reading frame (ORF), a 206-bp 3' UTR and a 29-bp poly (A) tail. To isolate the full reading frame of cDNA, a fragment was amplified by PCR using primers designed from two high-scoring segment pairs found in PopulusDB and the 3' UTR of the 844-bp cDNA fragment. The fragment amplified is 765 bp and contains a complete ORF of 723 bp. The complete ORF encodes 241 amino acids, from the first ATG start codon at 1 - 3 bp to a TAG stop codon at 724 - 726 bp; the putative protein has a molecular weight of 27.59 kDa and a pI of 6.78. Figure 1 shows the

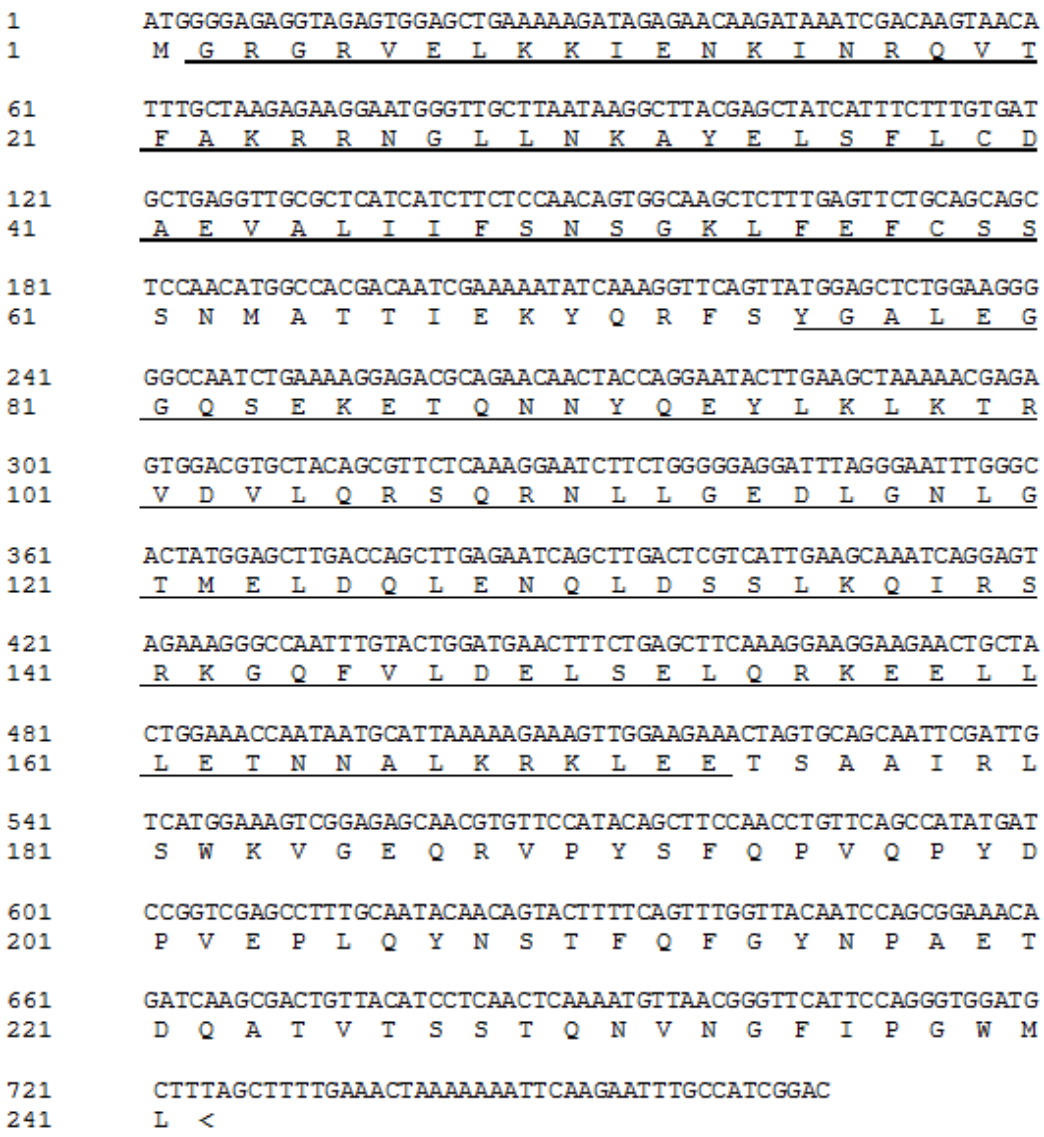

Figure 1. cDNA nucleotide and deduced amino acid sequences of *PdMADS*2 from *P. deltoides*. The double underlined region is the MADS-box domain and the single underlined region is the K-box domain.

sequence of the fragment amplified by PCR and its deduced amino acid sequences. We named it *PdMADS2* for research convenience.

A GenBank BLASTP search analysis of the *PdMADS2* deduced protein sequence showed a high similarity with predicted protein of *Populus trichocarpa* (named *PtMADS* 31 by Leseberg et al. (2006). A protein sequence alignment revealed 95.55% identity between *PdMADS2* and *PtMADS31* (Figure 2). Analysis using BLASTN from the DOE Joint Genome Institute web site (http://genome.jgi-psf.org/Poptr1/Poptr1. download.html) showed that *PdMADS2* is located on linkage group LG VIII. Therefore *PdMADS2* is homologous to *P. trichocarpa PtMADS*31and they correspond to the same locus in poplar genome. Unlike other MADS-box genes in poplar, such as *PdPI* in *P. deltoids* and *PTAG*1 in *P. trichocarpa* (Zhang et al., 2008; Brunner et al., 2000), GenBank BLASTP search analysis yielded low homology with MADS-box proteins of other plant species (62 - 68% identity and 76 - 81% similarity) (Table 1).

Phylogenetic analysis of PdMADS2

To better understand the relationship of *PdMADS*2 to the E-class of MADS-box genes in poplar and other plant species, phylogenetic analysis was performed using the deduced amino acid sequences of MIK domains of 26 previously reported E-class MADS-box proteins from *A. thaliana*, *S. lycopersicum*, *Petunia* x *hybrida* and several tree species. The E-class MADS-box proteins in plant can be roughly classified into several well-supported sub-

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PtMADS31.seq  MGRGRVELKRIENKINRQVTFAKRRNGLLKKAYELSVLCDAEVALIIFSNSGKLFEFCSS
PdMADS2 .seq  MGRGRVELKKIENKINRQVTFAKRRNGLLNKAYELSFLCDAEVALIIFSNSGKLFEFCSS
              *********.****************** ****** ************************

PtMADS31.seq  SNMATTIEKYQRFSYGALEGGQSEKETQLECSKQNNYQEYLKLKTRVDVLQRSQRNLLGE
PdMADS2 .seq  SNMATTIEKYQRFSYGALEGGQSEKET------QNNYQEYLKLKTRVDVLQRSQRNLLGE
              **************************      ****************************

PtMADS31.seq  DLGNLGTMELDQLENQLDSSLKQIRSRKGQFVLDELSELQRKEELLLETNNALKRKLEET
PdMADS2 .seq  DLGNLGTMELDQLENQLDSSLKQIRSRKGQFVLDELSELQRKEELLLETNNALKRKLEET
              ************************************************************

PtMADS31.seq  SAAIRLSWKVGEQRVPYSFQPVQPYDPIEPLQYNSTFQFGYNPAETDQATVTSSSQNVNG
PdMADS2.seq   SAAIRLSWKVGEQRVPYSFQPVQPYDPVEPLQYNSTFQFGYNPAETDQATVTSSTQNVNG
              *************************.************************.*****

PtMADS31.seq  FIPGWML
PdMADS2 .seq  FIPGWML
              *******
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Figure 2. Alignment of the amino acid sequences of *PdMADS*2 of *P. deltoides* and its homolog *PtMADS*31 of *P. trichocarpa*.

Table 1. Comparison of the deduced full-length amino acid sequence of *PdMADS2* with full-length sequences of MADS-box genes from *Populus trichocarpa* and three plant species.

Plant species	Accession no.	Sequence identity	Sequence similarity
Populus trichocarpa	EEE88719	236/242 (97%)	239/242 (98%)
Vitis vinifera	CAO41836	167/243 (68%)	198/243 (81%)
Vitis vinifera	CAN84110	165/243 (67%)	197/243 (81%)
Vitis vinifera	CAO49209	157/243 (64%)	194/243 (79%)
Petunia x *hybrida*	AAK21254	160/245 (65%)	193/245 (78%)
Vitis vinifera	AAM21342	158/244 (64%)	194/244 (79%)
Capsicum annuum	AAF22138	157/250 (62%)	192/250 (76%)

classes: *SEP1*/*2*-, *SEP3*- and three unnamed subclasses. *PdMADS2* falls within one of the unnamed subclass and was grouped together with *PtMADS*31 of *P. trichocarpa*, with a 100% bootstrap value (Figure 3). That is why GenBank BLASTP search analysis got low homology with MADS-box proteins of other plant species.

Expression patterns of *PdMADS2*

To determine the expression profile of *PdMADS2* in different tissues of the adult male *P. deltoides* tree, RQ real-time PCR was performed to detect transcripts in reproductive and vegetative tissues. *PdMADS2* was found to be expressed in flower buds, roots, immature xylem, leaves and terminal buds, but the relative expression levels varied. *PdMADS2* showed the highest transcript abundance in terminal buds, moderate levels in flower buds, low levels in immature xylem and roots and the lowest levels in mature leaves (Figure 4).

Studies in *Arabidopsis* and *Petunia* have shown that the E-class genes *SEP*1, *SEP*2, *SEP*3 and *SEP*4 play a central role in flower meristem and organ identity (Pelaz et al., 2000; Vandenbussche et al., 2003; Ditta et al., 2004). RQ real-time PCR was also performed to detect the expression of *PdMADS2* during various developmental phases in male *P. deltoides* floral buds. Figure 5 shows that *PdMADS*2 RNA could be detected in all of the developing male floral buds collected. The levels of relative expression were highest from 8 July to 7 September. During this period, the inflorescence meristem developed and floral organs formed and grew larger as observed using microscopy (Zhang et al., 2008). From 20 January to 6 February, the floral organs became larger and the pollen mother cells underwent meiotic division. At this point, *PdMADS*2 expression quickly increased again to the levels observed on 8 July. By 12 February, tetrahedral microspores had developed in the pollen sacs and *PdMADS2* expression had decreased to its lowest level.

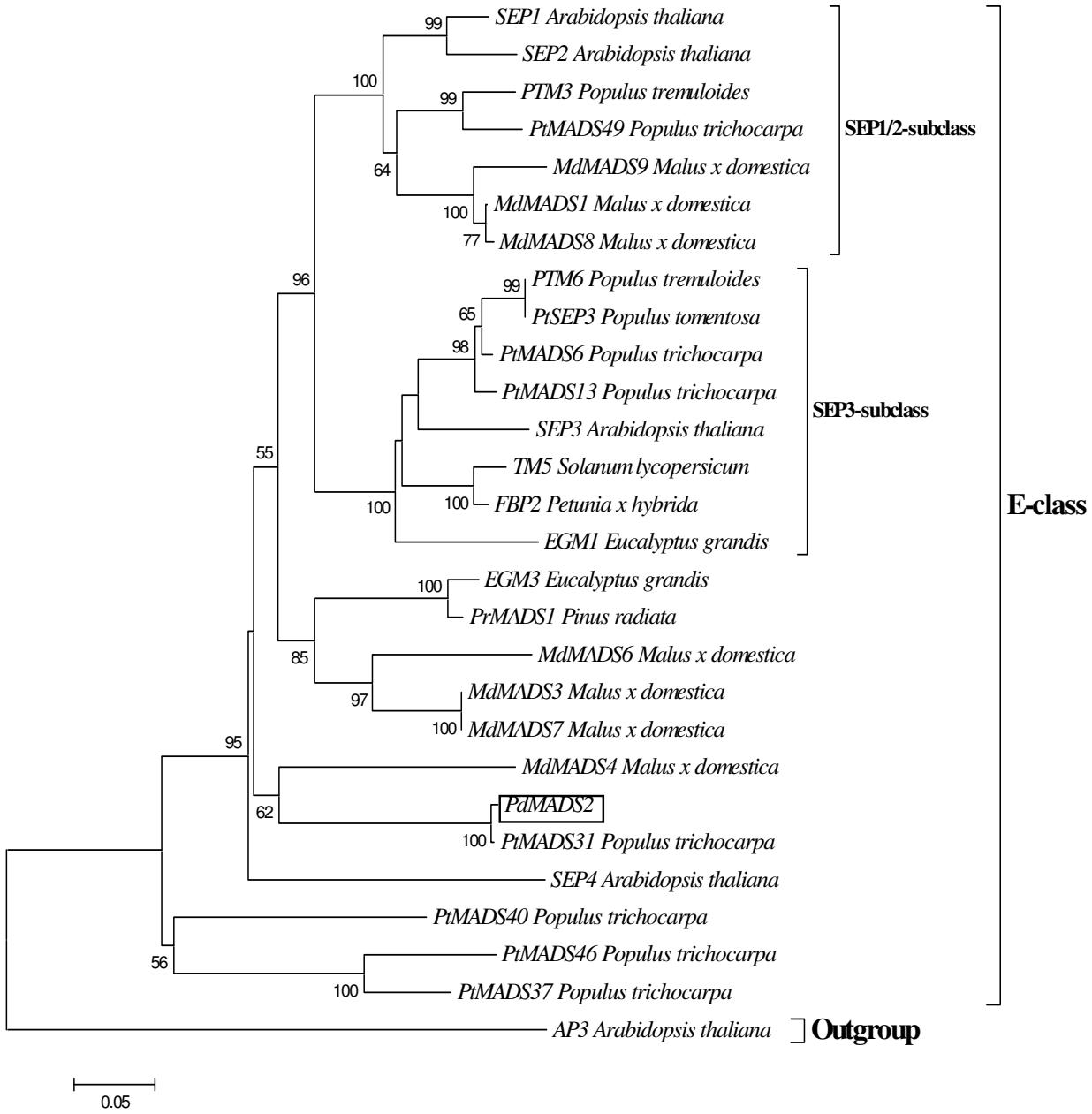

Figure 3. Phylogenetic tree of *PdMADS2* from *P. deltoides* and E-class MADS-box genes from other plant species. Branches with less than 50% bootstrap support are collapsed. The numbers are the bootstrap value for each node. The *PdMADS2* gene is boxed.

The functions of the floral MADS-box genes are very well correlated with their expression patterns, particularly when expression levels are high (Kim et al., 2005). These results suggest that the *PdMADS*2 gene might play an important role in the development of inflorescence meristems and flower organs in male *P. deltoides*.

We performed real-time PCR analysis of *PdMADS2* in male and female *P. deltoides* inflorescences that had been collected just before anthesis and were large enough to provide sufficient material for RNA extraction from various floral parts. The male inflorescences were carefully dissected into several parts, including the inflorescence peduncle, perianth cup and anther. The female inflorescences were carefully dissected into the inflorescence peduncle and ovary (together with the perianth cup). As shown in Figure 6, *PdMADS2* transcripts were detected in both male and female inflorescences. In male inflorescences, *PdMADS2* expression was abundant in the perianth cup and inflorescence peduncle, weak in the anthers and could not be detected in mature pollen. In female inflorescences, expression was high in both the ovary and the inflorescence peduncle.

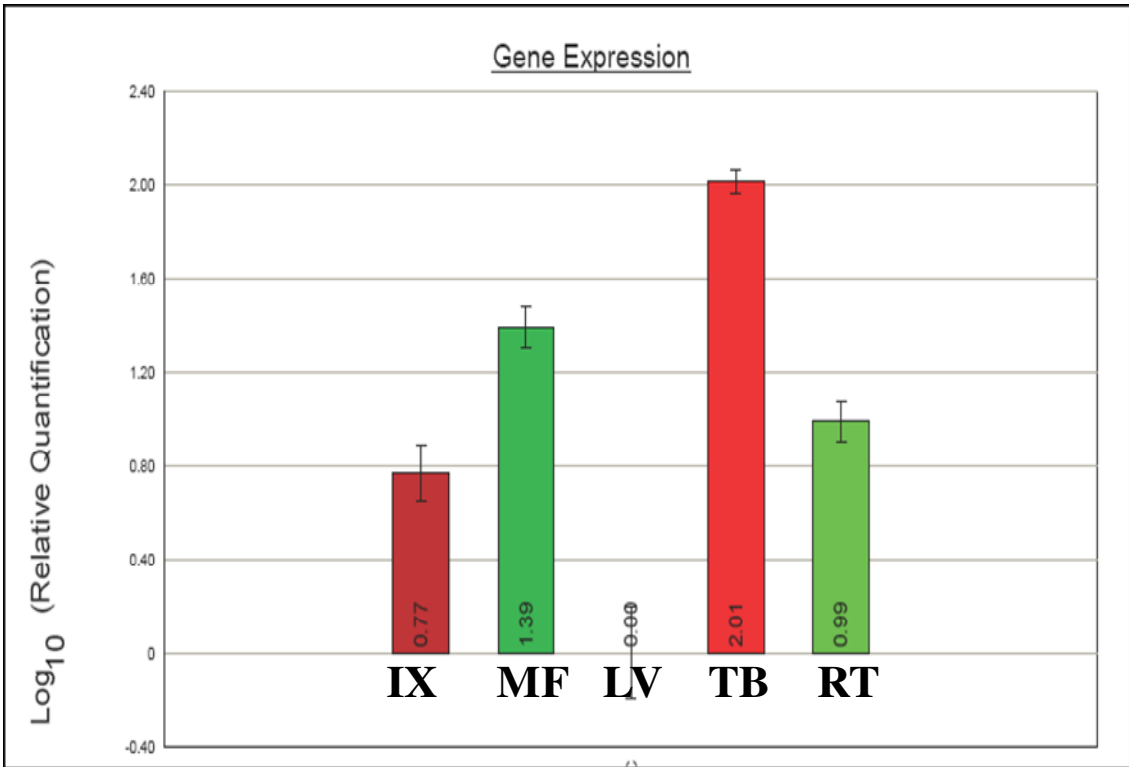

Figure 4. Expression of *PdMADS2* in different tissues collected at the same time. From left to right: immature xylem (IX), male flower buds (MF), leaves (LV), terminal buds (TB), roots (RT). The expression of *PdMADS2* in leaves was used as a calibration standard.

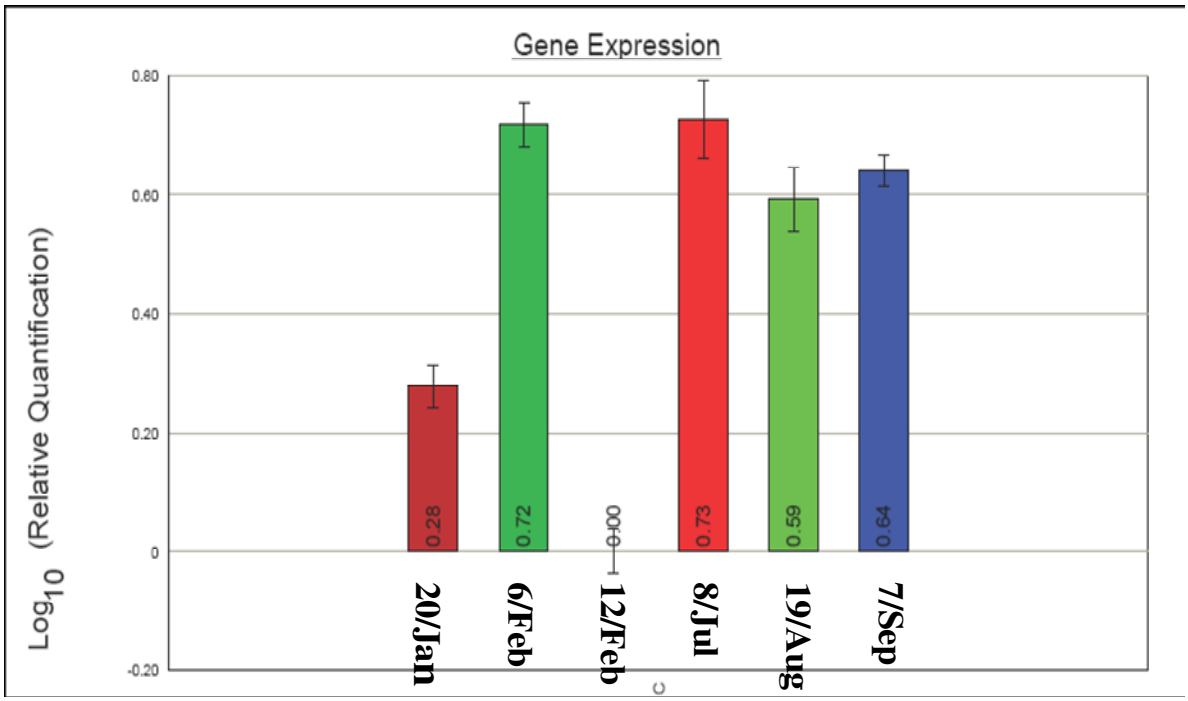

Figure 5. Expression of *PdMADS2* in *P. deltoides* male flower buds at different stages of development. From left to right: expression in buds collected on 20 January, 6 February, 12 February, 8 July,19 August and 7 September. The expression of *PdMADS2* on February 12 was used as a calibration standard.

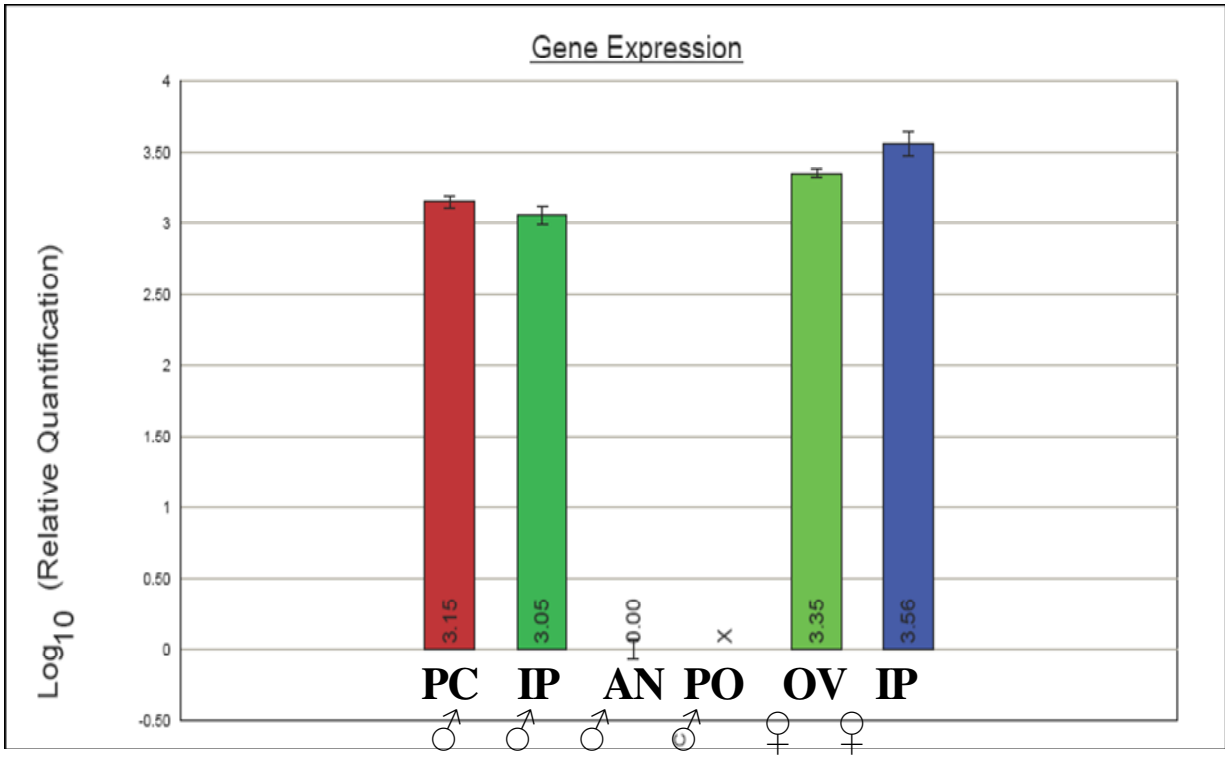

Figure 6. Expression of *PdMADS2* in male and female inflorescences of *P. deltoides*. From left to right: male perianth cup (♂PC), male inflorescence peduncle (♂IP), anther (♂AN), pollen (♂PO), ovary (♀OV) and female inflorescence peduncle (♀IP). The expression of *PdMADS2* in anther was used as a calibration standard.

DISCUSSION

We isolated a MADS-box gene (*PdMADS2*) from cDNA of *P. deltoides* male flower buds. Phylogenetic analysis showed that the gene is homologous to *P. trichocarpa PtMADS31* and encodes an E-class MADS-box protein. We also present the first report of the expression pattern of *PdMADS2*.

Previous studies have shown that plant MADS-box genes sort into the same gene family in phylogenetic analysis, have similar expression patterns and share highly related functions in flower development (Purugganan et al., 1995; Theissen and Saedler, 1995). We observed that *PdMADS2* expression remained high during floral organ differentiation and growth, but decreased to its lowest levels as the flower neared the maturation stage when the tetrahedral microspores had developed in the pollen sac. The expression pattern of *PdMADS2* during male flower development in *P. deltoides* is similar to those of some E-class genes from other plant species, such as *SEP1* in *A. thaliana* and *MdMADS1* in *Malus × domestica*. Both of these genes show high expression levels during the early stages of flowering (Flanagan and Ma, 1994; Sung and An, 1997). Other poplar E-class genes, such as *PTM3/4* and *PTM6* of *P. tremuloides* (Cseke et al., 2005), are expressed during all stages of flower development, but their expression patterns differ from that of *PdMADS2*. The expression of *PTM3/4* and *PTM6* is higher at the start of the spring growing season when the catkins have mature floral organs, whereas *PdMADS2* shows high expression during the early stages of floral development, decreasing to the lowest levels as the flowers near maturation. Therefore, *PdMADS2* in *P. deltoides* might function in poplar flower development similarly to the E-class genes in other plants, but also have functions different from those of the E-class genes in *P. tremuloides*.

In general, poplar trees are dioecious and each flower has only two whorls (a highly reduced perianth cup and stamens or a pistil). It is unknown whether the perianth cup is derived from the equivalent of sepals and/or petals. It is assumed that if the poplar perianth cup is homologous to petals and develops through a mechanism similar to four-whorled species, then both B- and E-function genes would be expected to be expressed in that tissue (Cseke and Podila, 2004; Kaufmann et al., 2005; Cseke et al., 2005). Both *PdMADS2* in this study and *PdPI*, a B-class *PI* homolog (Zhang et al., 2008) showed high expression levels in the male flower perianth cup of *P. deltoides*. It is likely that the perianth cup of poplar trees is homologous to perianth organs. Since our results are from dissected tissues, further studies, including *in situ* hybridization, are needed.

Our results and previous reports have shown that the E-class genes in poplars have a broader expression pat-

tern than in other plant species. In our study, *PdMADS2* of *P. deltoides* was expressed not only in the floral buds, but also in terminal buds, roots, immature xylem and leaves. In *P. tremuloides*, the E-class genes *PTM3/4* are expressed in developing flowers but also in terminal buds, young stems and young leaves (Cseke et al., 2005). The E-class genes *SEP1/2* of *Arabidopsis* are expressed specifically in floral organs, but *PrMADS* RNA of *P. radiata* was not detected in vegetative organs and *MdMADS1/6/7/8/9* are also not expressed in leaves (Flanagan and Ma, 1994; Sung and An, 1997; Yao et al., 1999; Cseke et al., 2005). Therefore, we speculate that E-class genes in poplar have additional functions in vegetative tissues. It has been suggested that the broader and stronger expression patterns of floral MADS-box genes in basal angiosperms, as compared to their counterparts in the eudicots and monocots, represent the ancestral pattern for all angiosperms (Kim et al., 2005). Because no conclusions can be made about the ancestral patterns from the present studies, this issue will be addressed in a future study.

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