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RESEARCH ARTICLE

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Cloning of an *APETALA3* homologous gene (*PtAP3*) from *Populus tomentosa* and genetic transformation of its sense and anti-sense constructs in tobacco

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Abstract A pair of primers were designed according to published literature on *Populus trichocarpa* gene (PTD), and *PtAP3*, an *AP3* homologous gene from *Populus tomentosa* was isolated by PCR using genomic DNA of the male clone of *P. tomentosa* (L50) as a template. The result indicated that the sequence was 1,813 bp (*BamH* Ⅰ and *Sac* Ⅰ were introduced at the 5′ and 3′ end) including 7 extrons and 6 introns, coding 238 amino acids. It was found that there was 52%–82% homology to proteins from *Lilium regale* (AF503913), *Petunia hybrida* (AF230704), *Gerbera hybrida* (AJ009724), *Rosa rugosa* (AB055966), *Malus domestica* (AJ251116), and *P. trichocarpa* (AF057708) determined by blast analysis in the GenBank. There was a highly conserved MADS-box motif in the protein of *PtAP3*, so it was putatived to be a transcription factor. The result of Southern blot analysis indicated that there were double copies of *PtAP3* or two members which had a high homology to each other in *P. tomentosa* (L50, male) genomic DNA, and there was single copy *PtAP3* in *P. tomentosa* (5082, female) genomic DNA. Sense and antisense expression vectors of *PtAP3* were constructed by PCR and restriction enzymes digestion identification, and transformed into tobacco (*Nicotiana tabacum*) by *Agrobacterium* GV3101 and LBA4404. Some transgenic tobacco plantlets were obtained by PCR identification. The results mentioned above have provided important data to understand the molecular mechanism of male flower development of *P. tomentosa*, and has contributed to the study on controlling flowering of *P. tomentosa* using genetic engineering.

Keywords *Populus tomentosa*, *APETALA3* homologous gene, cloning, transformation

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1 Introduction

Extensive studies on floral development have revealed a general model for the control of floral organ identity based on three genetic functions A, B, and C (Coen and Meyerowitz, 1991, Chasan, 1991). Flowers of representative dicotyledons have a concentric arrangement of four types of organs: sepals in whorl 1, petals in whorl 2, stamens in whorl 3, and carpels in whorl 4. According to the model, the identity of these organs depends on the action in the combinations A, AB, BC, and C in whorls 1–4, respectively. A noticeable event is the antagonistic function of A and C, which indicates that when C is silenced, A expresses, and vice versa (Wang et al., 2003). Usually, genes *AP1* and *AP2* act as function A, gene *AG* as function C, whereas *AP3* and *PI* as function B.

The proteins contain a highly conserved DNA binding domain called the MADS domain that are encoded by the above-mentioned genes and may work as transcription factors or MADS-box genes (Yanofsky, 1990). Other two systems of MADS-box genes are: D-class genes that are confirmed to determine ovule development (Angenent, 1995, Colombo, 1995), and E-class genes that are proposed to be together with other genes required for the formation of protein compounds.

Populus tomentosa is a native tree species in China that has not been studied sufficiently compared with *Arabidopsis* and *Antirrhinum*; it is rather important to investigate its molecular mechanism of flowering and to control floral development. Floral development in poplars differs significantly from that of a typical hermaphroditic annual, the apices of the branches do not become inflorescences and the flowers are borne on axillary inflorescences or catkins, with male

and female flowers found on separate trees. Instead of four concentric whorls of organs, the *Populus* flower has only two whorls, a reduced perianth cup surrounding either stamens or carpels (Sheppard, 2000). The mechanism causing the difference between *Arabidopsis* and *Populus* is still unknown. In the early of 1980s, Dong (1982, 1984) observed embryological development of *P. tomentosa*, then Zhu (1990) studied the capacity of sexual reproduction, and Zhang et al. (1992, 2000) studied flowering, fruit bearing, and sexual reproduction of hybrid triploids. Recently, Sheppard et al., (2000) have isolated PTD, an *AP3* homologous gene, and studied its spatio-temporal expression. *LFY*, *AG,* and *AP1* etc from *Arabidopsis*, the homologue *PTLF*, *PTAG,* and *PTAP1* have been isolated one after the other (Rottmann, 2000, Meilan, 2001). Up to now, however the floral genes in *P. tomentosa* involved in flowering development has not been reported. To begin this study, *AP3* homologue from *P. tomentosa* was isolated and cloned. It is a good choice for revealing the molecular mechanism of flowering in *P. tomentosa* and plays as an important base for shortening the breeding cycle and suppressing contamination of pollen and catkins.

2 Materials and methods

2.1 Plant materials

In spring, DNA in young leaves of female (5082) and male (LM50) clones were extracted as template of cloning and Southern blot analysis from Beijing. DNA in young leaves of tobacco including transgenic and nontransgenic (control) plants was extracted as templates of PCR test.

2.2 Methods

2.2.1 Extraction of genomic DNA

Genomic DNA was extracted according to the methods of Wang et al. (2002).

2.2.2 PCR reaction

A pair of primers were designed according to published literature on *Populus trichocarpa* gene (PTD), the forward primer 5′-TTGGATCCATGGGTCGTGGAAAGA-3′ and the reverse primer 5′-AAGAGCTCTCAAGGAAGGCG-AAGTT-3′. Then *PtAP3*, an *AP3* homologous gene from *P. tomentosa* was isolated by PCR using genomic DNA of the male clone of *P. tomentosa* (L50) as template. The PCR reaction system of 50 μL was composed of a 10×PCR buffer (Tris 10 mmol/L, pH 8.3, KCl 50 mmol/L, $MgCl_2$ 2.5 mmol/L), genomic DNA 10–50 ng, 1 μL of 10 pmol/μL forward and reverse primers respectively, and 2.5 unit Taq DNA polymerase. Thermocycling was performed at 94°C for 5 min, then at 94°C for 40 s, at 60°C for 40 s, at 72°C for 2 min for 30 cycles, at 72°C for 10 min, and finally kept at 4°C.

2.2.3 Recombine to pGEM-T easy vector

PCR products were reclaimed and purified using the Promega Co. QIAquick™ Gel Extraction Kit, and were ligated with T_4-DNA ligase after which the products of ligation were transformed into competent cells of *Escherichia coli* TG1 and spread on a LB medium containing 50 μg/mL Amp, 20 μg/mL IPTG, and 20 ng/mL X-gal. The positive recombinant plasmids were identified by PCR tests and digested with restriction enzymes.

2.2.4 DNA sequencing and analysis

The sequencing method was based on the original "Sanger" methods of dideoxy chain termination and ABI-377 DNA autosequencing system (PE Co.,USA) was used as sequencing models to take full-length DNA sequences. The result of sequencing was analyzed by blast analysis in GenBank and some softwares were applied to sequence translation, alignment, and annotation.

2.2.5 DNA probe preparation and Southern blotting analysis

The purified PCR products were labeled as blotting probe by DIG-11-dUTP for 20 h in 37°C, 10 μg of genomic DNA from male and female *P. tomentosa* were digested with restriction enzymes, *BamH* I, *EcoR* I, and *Hind* III, respectively, and electrophoresed on 0.8% agarose gel The products were then blotted onto a nylon membrane positively charged by capillary transfer with 20×SSC, prehybridized for 30 min at 42°C, and hybridized with DIG-labeled DNA probe at 55°C for 14–16 h. They were then washed at a high stringency (2×SSC, 0.1% SDS, at 15–25°C for 2×5 min, then 0.5×SSC, 0.1% SDS at 65°C for 2×15 min). Immunological detection was carried out according to the protocol of DIG DNA Labeling and Detection Kit (Roche).

2.2.6 Construction and identification of sense and antisense expression vectors

The recombinant plasmid pGEM-T-*PtAP3* and pBI121 were digested with BamH I/Sac I and BamH I/Xba I respectively. The corresponding fragments were released, reclaimed, and purified by QIAquick™ Gel Extraction Kit (QIAGEN) and ligated in a sense and antisense-oriented manner with T_4-DNA ligase, then transferred into competent cells of *E. coli* TG1 and spread on a LB plate containing 100 μg/mL Kan respectively. The positive recombinant

plasmids were obtained by PCR tests (the forward primer 5′-GGATTGATGTGATATCTCCACTG-3′ and the reverse primer 5′-CCACAGTTTTCGCGATCCAGACT-3′ were used to identify the antisense construct, and the reaction system was the same as the above PCR), sequenced and digested with restriction enzymes.

2.2.7 Tobacco transformation and PCR tests of transgenic tobacco

pBI121-*PtAP3* and pBI121-*Ptap3* were introduced into *Agrobacterium* strains LBA4404 and GV3101. A single colony of *Agrobacterium* GV3101 containing target construction grew in LB medium with streptomycin (25 mg/L) and kanamycin (100 mg/L) at 28°C for 24 h. Leaves of sterile tobacco plant were cut into 1–2 cm^2 disks and immersed into a 10×dilution of the overnight culture for 5 min and the disks were dried by blotting onto the filter paper and placed on solid MS medium (callus induction medium MS containing 1 mg/L benzyladenine, 0.1 mg/L naphtalene acetic acid, 3% sucrose and 4.5 g/L phytoagar). Then they were cultured for two days in dark at room temperature whereafter they were transferred to a CIM containing 500 mg/L carbenicillin and 100 mg/L kanamycin for kan-resistant adventitious shoots to appear. Thereafter, they were transferred to fresh medium weekly until the transgenic shoots were big enough to root on 1/2 MS containing 1.5% sucrose and 100 mg/L kanamycin. Genomic DNA of young leaves of transgenic tobacco plants and control were extracted by cetyl-trimethylammonium bromide (CTAB)-based methods and used as a DNA template for PCR tests. The system and thermocycling conditions of PCR test were conducted according to the methods described previously.

3 Results and analysis

3.1 Isolation of *PtAP3*

A pair of primers were designed according to *P. trichocarpa AP3* gene (PTD), and *PtAP3*, an *AP3* homologous gene from *P. tomentosa* was isolated by PCR using genomic DNA of male clones of *P. tomentosa* (L50) as template. The corresponding fragment was obtained by PCR amplification whose length was about 1.8 kb. The results are shown in Fig. 1A. The purified PCR products was cloned into a pGEM®-T easy vector, then transferred into competent cells of *E. coli* TG1 and spread on a LB medium containing 100 μg/mL Kan. Two kinds of positive recombinant plasmids (PTAP3XA-1 and PTAP3XA-2) were obtained and were amplified using the recombined plasmids as DNA template by further PCR identification. The results indicated that they were absolutely coincident with the lengths of the previous PCR products (Fig. 1B). It revealed that the length of the recombined plasmids is obviously bigger than pBS (Fig. 1C). Meanwhile, the recombinant plasmids were digested with *BamH* I and *Sac* I; the length of corresponding fragments matched the length of the PCR products, too. It suggested that the PCR products had been cloned into the pGEM-T easy vector successfully.

3.2 Sequence analysis

The recombinant plasmids, which were verified by PCR, were sequenced by the Sangon Co. The results indicated that *PtAP3* from *P. tomentosa* was 1,813 bp long (including the introduced 5′ and 3′ end restriction sites). Combining with the rule of intron slice in eukaryotic genome DNA and the analysis of SPL & Seqaid II (search for potential splice sites) at http://dot.imgen.bcm.tmc.edu:9331/gene-finder/gf.htm, the results indicated that there were 7 extrons and 6 introns in this gene (1st extron 9–196, 1st intron 197–331; 2nd extron 332–398, 2nd intron 399–491; 3rd extron 492–553, 3rd intron 554–664; 4th extron 665–764 , 4th intron 765–1,323; 5th extron 1,324–1,365, 5th intron 1,366–1,446; 6th extron 1,447–1,491, 6th intron 1,492–1,592; 7th extron 1,593–1,805) and encoded 238 amino acids (Fig. 2). There was a highly conserved MADS-box motif in the 1^{st}extron (shown by underlined section in Fig. 2), so it was accepted to be a transcription factor.

In addition, the structure and map of the restriction site of homologous *AP3* from *P. tomentosa* were annotated by DNAMAN software (Fig. 3), the 1st, 2nd, 3rd, 4th extrons were distributed in the front half of the section, the 5th, 6th, 7th extrons in the back one-third section, and the 4th intron was the longest (559bp) in the sequence. Besides in the introduced *BamH* Ⅰ and *Sac* Ⅰ sites, there were *EcoR* Ⅴ, *Nde* Ⅰ, and *Xba* Ⅰ in 516 bp, 719 bp, and 862 bp respectively, which provided important information for further studies including preparing a probe of Southern and Northern blotting hybridization, genomic DNA function, and enzyme digestion.

It indicated that the deduced amino acid of *PtAP3* was up to 82% homologous to *P. trichocarpa* (AF057708); at the same time, 60%, 60%, 55%, 52% and 52% homologous to *Malus domestica*, (AJ251116, AB081093), *Rosa rugosa* (AB055966), *Lilium regale* (AF503913, AB071378), *Petunia hybrida* (AF230704), and *Gerbera hybrida* (AJ009724) respectively by blast analysis in GenBank. A highly conserved domain near the 5′ end of *AP3* homologues was found by aligning amino acid sequences of *AP3* homologous genes with different species (*P. tomentosa*, *P. trichocarpa*, *Malus domestica, Rosa rugosa, Lilium regale, Petunia hybrida, Gerbera hybrida,* and *Hemerocallis* hybrid cultivar (AF209729)) by Bioedit and Clustalx software (Fig. 4).

M: DL2000 marker; 1: PCR product of genomic DNA; 2 and 3: PCR products of PtAP3XA-1 and PtAP3XA-2; 4: The plasmid pBS; 5: The recombinant plasmid; 6v The recombinant plasmid digested with *BamH* Ⅰ and *Sac* Ⅰ; A: PCR product of genomic DNA; B: PCR tests of the recombinant plasmid; C: Test of length of the recombinant plasmid; D: The recombinant plasmid digested with restriction endonucleases.

Fig. 1 Cloning of *AP3* homologous gene from male clone of *P. tomentosa*

```
ttggatccATGGGTCGTGGAAAGATTGAAATCGAGAAGATCGAAAACCCCACAAACAGGCAAGTCACCTACTCGAAGAGAAGAAATGGTA   90
        M  G  R  G  K  I  E  I  E  K  I  E  N  P  T  N  R  Q  V  T  Y  S  K  R  R  N  G  I    28
TTTTCAAGAAAGCCCAGGAACTCACTGTTCTTTGTGATGCTAAGGTCTCTCTTATCATGTTCTCCAACACTAACAAACTCAATGAGTACA   180
 F  K  K  A  Q  E  L  T  V  L  C  D  A  K  V  S  L  I  M  F  S  N  T  N  K  L  N  E  Y  I    58
TTAGCCCCTCCACATCgtacgtatactcgtatcatgtttctgtctaagtatttcttccgtgctttctcttctttctttcttttcttgtct   270
 S  P  S  T  S                                                                                63
TTtatgttgcagttttatgaaaccttggtaatggaactgtagtttttattgttacgaccagGACGAAGAAGATCTACGATCAATATCAGA   360
                                                              T  K  K  I  Y  D  Q  Y  Q  N     73
ACACTTTAGGCATAGATCTGTGGGGCACTCAATACGAGgtgaacctttctttctgtctttctttcttctaatgtttgatctacagggcga   450
 T  L  G  I  D  L  W  G  T  Q  Y  E                                     EcoR V                 85
atatgagattcttcaaaggatttttctgtgaggtttgcagAAAATGCAAGAGCACTTGAGGAAGCTGAATGATATCAATCATAAGCTGA   540
                                          K  M  Q  E  H  L  R  K  L  N  D  I  N  H  K  L  R   102
GAAAAGAAATCAGgtgactttgaaaagaaataaccatcgcatatatgcatgtggttatggttttacgggaatatcgatctgtaaatttg   630
 K  E  I  R                                                                                   106
tggagctactaatcaaggtatttgtttttaacagGCAGACGAGAGGAGAGGGCCTGAATGATCTGAGCATTGATCATCTGCGCGGTCTTG   720
     Nde I                            Q  T  R  G  E  G  L  N  D  L  S  I  D  H  L  R  G  L  E   125
AGCAACATATGACTGAAGCCTTGAATGGTGTGCGTGGCCGGAAGgtcagttgttttcaagtgaacatctttatatgattatcaagttcta   810
 Q  H  M  T  E  A  L  N  G  V  R  G  R  K                   Xba I                            139
attcctaaaatttgagcttactagtgatttgagttcggtccggtgtatcaagcagttaatctagatgaacctaatttttttttcttaccaa   900
atcaaagtcattttgaggatttttttatatatataaaaaggctaaattttggataaacttgtataaattcatcaatctacaactcgaat   990
cttagacctaatcaaactctcaagttagatcttataaatatgatattaaccggtaggtgttttatgtgcatttttagttcaactatttta   1080
tcatttttttttaaatttgagttattttaatccttcaagtgacattccaatttttattattttgggattcttggaaaccctggttagaa   1170
agaaaatacacaccctttaacttgtgcttctttacctttggattatggatgttcatgaactggattttgggttaccgttacactcatctg   1260
tagaagggatatgccttgtaacgctttacacttacaatttcaacgttctttgattatttacagTTCCATGTGATCAAAACACAAAACGAA   1350
                                                                  P  H  V  I  K  T  Q  N  E    148
ACCTACAGGAAGAAGgttagtgataaaaaaaacattttacctcttcagtttcatgcatgtagcttttggaacaaattctctggcgattaa   1440
T  Y  R  K  K                                                                                  153
ttgcagGTGAAGAATCTAGAGGAGAGACATGGAAACCTCTTGATGGAATATgtaagaatctaaactttcatgtgcttgttttcgctaatt   1530
      V  K  N  L  E  E  R  J  G  M  L  L  M  E  Y                                              168
tcccaacttggaaaaacacatggattaaacctgagattttcttctgtgctttgggatttaagGAAGCAAAACTAGAGGATCTACAGGGTG   1620
                                                                 E  A  K  L  E  D  L  Q  G  G   178
GTTTAGTGGACAATGAAGCTGCTGTTGCACTTGTAGATGGGGCTTCGGACAATGAAGCTGCTGTTGCACTTGCAGATGGGGCTTCCAACC   1710
 L  V  D  N  E  A  A  V  A  L  V  D  G  A  S  D  N  E  A  A  V  A  L  A  D  G  A  S  N  L   208
TCTATGCAATCCGCCTGCATCACGGGCACAACCACCACCACCCTAATCTTCACCTTGAAGATGGATTTGGAGCCCATGAACTTCGCCTTC   1810
 Y  A  I  R  L  H  H  G  H  N  H  H  H  P  N  L  H  L  E  D  G  F  G  A  H  E  L  R  L  P   238
CTTGAgagctctt                                                                                 1813
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Fig. 2 The full nucleotide and deduced amino acid sequence of putative *AP3* homologous gene (*PtAP3*) from male clone of *P. tomentosa.* The small letters belong to noncoding sequence, and the capital letter belong to coding sequence

Fig. 3 Structural and restriction map of *AP3* homologous gene from male clone of *P. tomentosa* (*PtAP3*)

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                   10         20         30         40         50         60         70
PtAP3       MGRGKIEIEK IENPTNRQVT YSKRRNGIFK KAQELTVLCD AKVSLIMFSN TNKLNEYISP STSTKKIYDQ
AF05770     MGRGKIEIKK IENPTNRQVT YSKRRNGIFK KAQELTVLCD AKVSLIMFSN TNKLNEYISP STSTKKIYDQ
AJ251116.   MGRGKIEIKL IENQTNRQVT YSKRRNGIFK KAQELTVLCD AKVSLIMLSN TNKLNEYISP TTTTKSMYDD
AB081093.   MGRGKIEIKL IENQTNRQVT YSKRRNGIFK KAQELTVLCD AKVSLIMLSN TNKMHEYISP TTTTKSMYDD
AB055966.   MGRGKIEIKL IENQTNRQVT YSKRRNGIFK KAQELTVLCD AQVSLIMQSS TDKIHEYISP TTTTKKMFDL
AJ009724.   MGRGKIEIKK IENNTNRQVT YSKRRNGIFK KAHELTVLCD AKVSLIMFSN TDKFHEYISP STTTKKMYDQ
AF230704.   MGRGKIEIKK IENSTNRQVT YSKRRNGIFK KAKELTVLCD AKICLIMLSS TKKFHEYISP NTTTKKMIDL
AF503913.   MGRGKIEIKK IENSTNRQVT YSKRRTGIIK KAKELTVLCD AKVSLLMFSS TGKLSEFCSP STDTKKIFDR
AF209729.   MGRGKIEIKK IENSTNRQVT YSKRRSGIMK KAKELTVLCD ADVSIIMFSS TGKFSEVCSP GTDTKTVFFR
Clustal Co  ********;  *** ****** *****,*;;* ** ******* *,;,;;* *, * *; *; **  * **.; ;

                   80         90        100        110        120        130        140
PtAP3       YQNTLGIDLW GTQYEKMQEH LRKLNDINHK LRKEIRQT-R GEGLNDLSID HLRGLEQHMT EALNGVRGRK
AF05770     YQNALGIDLW GTQYEKMQEH LRKLNDINHK LRQEIRQR-R GEGLNDLSID HLRGLEQHMT EALNGVRGRK
AJ251116.   YQKTMGIDLW GTHEESMKDT LWKLKEINNK LRREIRQR-L GHDLNGLSFD ELASLDDEMQ SSLDAIRQRK
AB081093.   YQKTMGIDLW RTHYESMKDT LWKLKEINNK LRREIRQR-L GHDLNGLSYD DLRSLEDKMQ SSLDAIRERK
AB055966.   YQKNLQIDLW SSHYEAMKEN LWKLKEVNNK LRRDIRQR-L GHDLNGLSYA ELQDLEETMS QSVQIIRDRK
AJ009724.   YQSTVGFDLW SSHYERMKET MKKLKDTNNK LRREIRQRVL GEDFDGLDMN DLTSLEQHMQ DSLTLVRERK
AF230704.   YQRTLGVDIW NKHYEKMQEN LNRLKDINNK LRREIRQR-T GEDMSGLNLQ ELCHLQGNVS DSLAEIRERK
AF503913.   YQQLSGINLW SAQYEKMQNT LNHLSEINRN LRKEISQR-M GEELDGLDIK DLRGLEQNLD EALKLVRHRK
AF209729.   YQQATQTNLW STQYEKMQNT LNHLKEINHN LRKEIRQR-I GEELDGMDFK ELRGLEQNLD EALKSVRARK
Clustal Co  **      ;;*   ; * *;;  ;  ;*, ; *,; **;;* *    *,  ;,,;,   ,*  *;  ;  ,;;  ;* **

                  150        160        170        180        190        200        210
PtAP3       PHVIKTQNET YRKKVKNLEE RHGNLLMEY- --EAKLEDLQ GGLVDNEAAV ALVDGASDNE AAVALADGAS
AF05770     YHVIKTQNET YRKKVKNLEE RHGNLLMEY- --EAKLEDRQ YGLV------ -------DNE AAVALANGAS
AJ251116.   YHVIKTQTET TKKKVKNLEQ RRGNMLHGYF DQEAAGEDPQ YGYEDNEG-- -------DYE SALALSNGAN
AB081093.   YHVIKTQTET TKKKVKNLEE RRGNMLHGY- --EAASENPQ YCYVDNEG-- -------DYE SALVLANGAN
AB055966.   YHVLKTQAET TRKKVKNLEE RNSNLMHGYG --APGNEDPQ YGYVDNEG-- -------DYE SAVALANGAS
AJ009724.   YHVIKTQTDT CRKRVRNLEQ RNGNLRLDYE --TIHQLDKK YDTGENEG-- -------DYE DSLTLVRERK
AF230704.   YHVIKTQTDT CRKRVRNLEE QHGSLVHDL- --EAKSEDPT YGVVENEG-- -------HFN DSLAEIRERK
AF503913.   YHVINTQTET YKKKVKNSEE AHKNLLRDLV NREMKDENPV YGYVDEDP-- ------SNYD EALKLVRHRK
AF209729.   YHVITTQTDT YKKKVKNSQE AHKTLLHELD -------DAV YGYADEDP-- ------GNYD EALKSVRARK
Clustal Co   **;,** ;*   ;*;*;* ;;   , ,;              ;        ;                 ; ,  ;  ;,*

                   220        230        240        250        260        270
PtAP3       NLYAIR---- ---------- ---------- -----LHHGH NHHH---PNL HLEDGFGAHE LRLP-
AF05770     NLYAFR---- ---------- ---------- -----LHHGH NHHHHL-PNL HLGDGFGAHE LRLP-
AJ251116.   NLYTF----- ---------- --------HL H-------HR NHHHG--GSS LGSSITHLHD LRLA-
AB081093.   NLYTF----- ---------- --------QL HRNSDQLHHP NLHHHR-GSS LGSSITHLHD LRLA-
AB055966.   NLYFFNRVHN NHNLDHGHGG GSLVSSITHL G-NPNNHGNH NLENGHGGGS LISSITHLHD LRLA-
AJ009724.   NLYAF----- ---------- ---------- -----CVHPN NIPHG---AG YEL-----HD HQHTN
AF230704.   NLYAFR---- ---------- ---------L Q----TLHPN -LQN---GGG FGS-----RD LRLA-
AF503913.   NLYEFR---- ---------- ---------- ------VQPS Q-PNLH-GMG YGS-----HD LRLA-
AF209729.   NMYAYR---- ---------- ---------- ------VQPS Q-PNLH-GMS YGP-----HD LRLA-
Clustal Co  ;;*                                              ;                ;;    ; ,
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The amino acid sequences have been aligned by introducing gaps (---) to maximize homology (Clustal W software). Conserved MADS-box motif are underlined, and totally conserved and conservatively replaced amino acids are indicated by asterisks and dots, respectively. AF057708: *Populus trichocarpa*; AJ251116 and AB081093: *Malus domestica*; AB055966: *Rosa rugosa*; AJ009724, *Gerbera hybrida*; AF230704: *Petunia hybrida*; AF503913: *Lilium regale*; AF209729: Hemerocallis hybrid cultivar.

Fig. 4 Similarity of amino acid sequences coded by *AP3* homologous gene of several plants

3.3 Southern blot analysis

To verify whether the homologous *AP3* gene comes from male clones of *P. tomentosa*, *PtAP3* was labeled as probe and genomic DNA from male (LM50) and female (5082) clones was digested with *BamH* I, *Eco*RI, and *Hind* III, respectively, electrophoresed on 1.0% agarose gel and blotted onto the nylon membrane positively charged by capillary transfer with 20×SSC and washed at a high stringency. The result indicated that *PtAP3* actually comes from the genome of *P. tomentosa*, but there was remarkable difference between male and female clones—double copies or two high homologue members in male genomic DNA, whereas a single copy in female clones (Fig. 5).

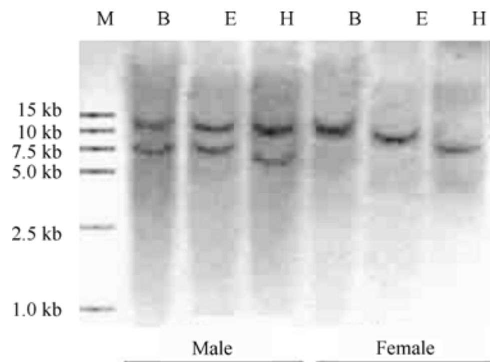
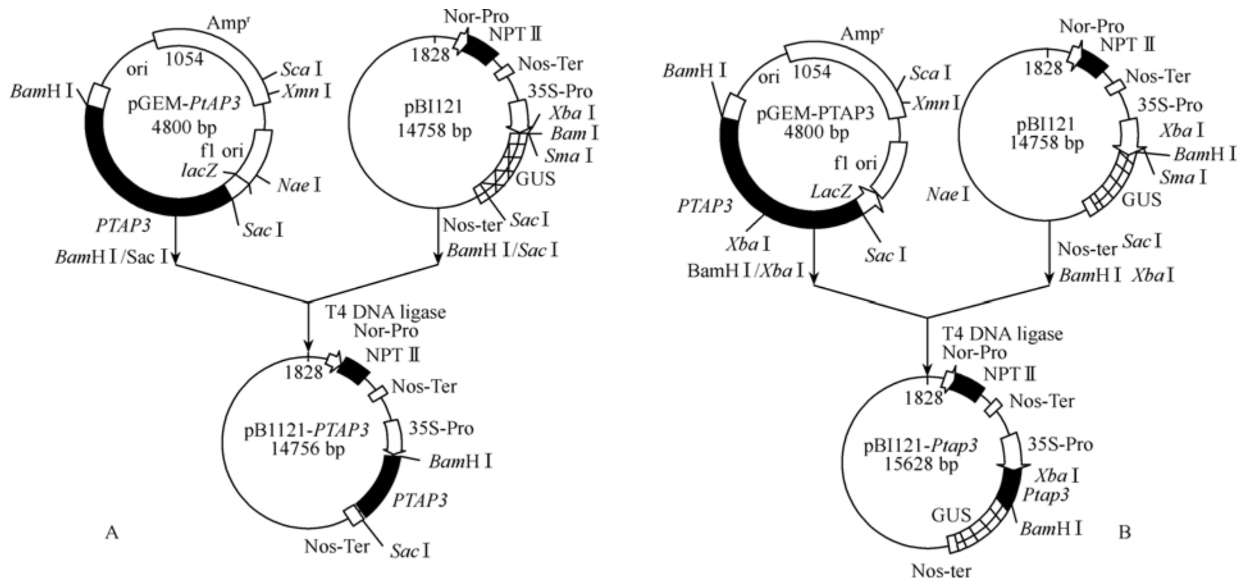


Fig. 5 Southern blot analysis of DNA from male and female clones of *P. tomentosa* using probe of *PtAP3*. M: DL 15,000 marker; B: *BamH* I; E: *EcoR* I; H: *Hind* III.

3.4 Construction of sense and antisense expression vectors

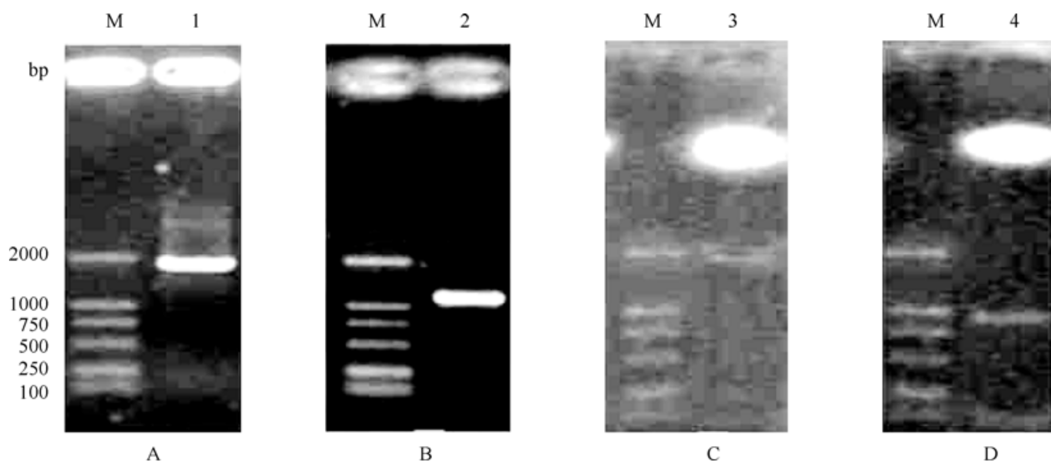
To study the function of *PtAP3* in stamen development, sense and antisense expression vectors of *PtAP3* were constructed. The recombinant plasmids pGEM-T-*PtAP3* and pBI121 were digested with *BamH* I and *Sac* I, the corresponding target fragments were recycled, purified, and ligased to produce a sense-oriented and anti-sense-oriented construction (Fig. 6).

The recombined plasmid pBI121-*PtAP3* and pBI121-*Ptap3* were used as DNA templates respectively, and the released fragments were also about 1.8 kb and 1.1 kb long (Fig. 7A, B) including partial sequences of the 35S promoter and GUS gene, which was about 230 bp. It showed that the results of the PCR test and digestion with *BamH* I/*Xba* I and BamH Ⅰ/*Xba* Ⅰ were coincident, that is, the sense and anti-sense constructs were also successful.



Construction of sense expression vector of *PtAP3*; B. Construction of anti-sense expression vector of *PtAP3*.

Fig. 6 Construction of sense and anti-sense expression vectors of *PtAP3*



A: Identification of PCR of pBI121-*PtAP3*; B: Identification of PCR of pBI121-*Ptap3*; C: Identification of digestion of pBI121-*PtAP3*; D: Identification of digestion of pBI121-*Ptap3*. M: Marker DL2000, 1: PCR product of pBI121 *PtAP3*, 2: PCR product of pBI121-*Ptap3*; 3: pBI121-*PtAP3* digested with *BamH* Ⅰ and *Sac* Ⅰ; 4: pBI121-*Ptap3* digested with *BamH* Ⅰ and *Xba* Ⅰ.

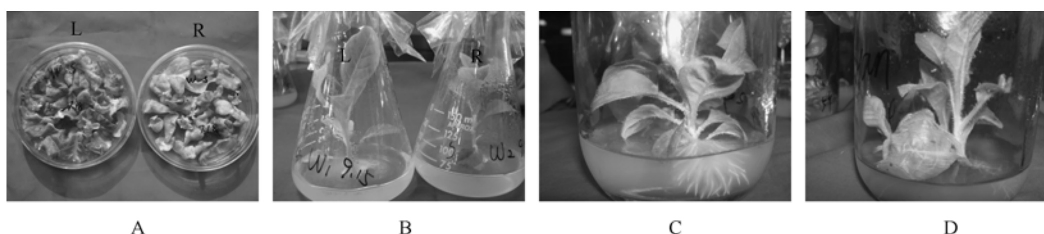
Fig. 7 Identification of PCR and digestion of both sense and anti-sense expression vectors of *PtAP3*

3.5 Tobacco transformation

Tobacco leaf disks without preculture were used as acceptor materials, cocultured with *Agrobacterium* for two days (Fig. 8A, L: sense and R: antisense), then transferred on screening MS media with carbenicillin 250 mg/L and kanamycin 100 mg/L. Some resistant buds from transformed disks were found in about seven days (Fig. 8B, L: sense and R: antisense). When resistant buds were long enough, they were cut and transformed on rooting MS media with carbenicillin 250 mg/L and kanamycin 200 mg/L to root (Fig. 8 C: sense and D: antisense). Some transformed plants were obtained, and DNA in leaves of transformed and nontransformed tobacco plantlets was used as the template of PCR reaction, and the recombined plasmid as positive control. The results of PCR showed that one sense transformant (Fig.9A) and three antisense transformants (Fig. 9B) had been introduced in these tobacco plants.

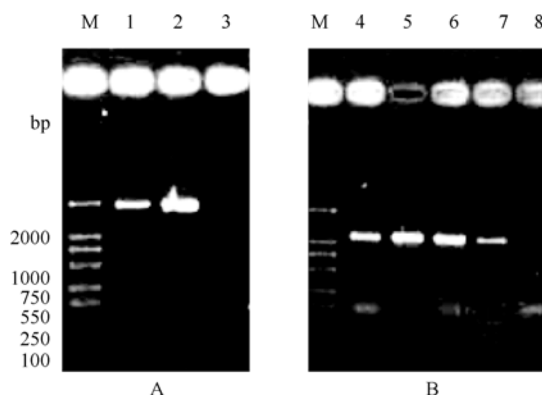
4 Discussion

So far *AP3* homologous gene has been well isolated in many herbs, such as, *Arabidopsis thaliana, Antrirrhinum majus, Petunie hybrida, Solanum tuberosum* etc., whereas only *Malus domestica* and *P. trichocarpa* for woody species. *PtAP3*, an *AP3* homologue, was isolated from male clones of *P. tomentosa* by PCR in this research, indicating that homologous *AP3* gene exist widely in higher plants. The prominent trait of the *AP3* homologue is with conservative MADS-box motif. It is believed that MADS-box might have functions of DNA integration and dimer formation while participating in transcriptional regulation, therefore the proteins that were coded by the genes belong to transcription factors. Conserved MIKC structure with the highly conserved DNA-binding MADS domain at the amino terminus exists widely in many plants (Alvarez-Buylla, 2000). The moderately conserved K domain in the downstream of MADS-box genes, the MADS, and K domains are linked to one another by a weakly conserved I domain, as well as a carboxyl-terminal (C) region. It indicates that the I region (which follows the MADS box) have been characterized as a key molecular determinant for the selective formation of DNA-binding dimers. The DNA-binding specificity is mediated to a large degree by AP3 protein located in I region and K region (Riechmann, 1996a, 1996b). The microarray analysis suggests that *AP3* and *PI* regulate a relatively small number of genes, implying that many genes used in petal and stamen development are not tissue specific and likely have roles in other processes as well (Moriyah, 2003). *PtAP3*



A: The tobacco leaf disks infected by *Agrobacterium*; B: Kan resistant shoots from tobacco leaf disks; C: Culture of regenerated shoots (sense transformation) on generation medium; D: Culture of regenerated shoots (anti-sense transformation) on generation medium.

Fig. 8 Acquirement of transgenic tobacco plantlets with sense and anti-sense construction



A: The sense transgenic plant verified by PCR, B: The anti-sense transgenic tobaccos verified by PCR. M. DL2000 Marker, 1: The sense transgenic plant; 2: The positive control (pBI121 *PtAP3*); 3: The nontransgenic tobacco; 4–6:The anti-sense transgenic tobaccos; 7: The positive control (pBI121 *Ptap3*); 8: The nontransgenic tobacco.

Fig. 9 Transgenic tobacco plantlets verified by PCR

is highly homologous to other plants, especially higher in conserved MADS-box region, so it is implicated to be a transcription factor. *APETALA3* (*AP3*) in *Arabidopsis* is one of the genes that confer a B-class function. In accordance with their roles in specifying petal and stamen identity, the expression of *AP3* or *PI* and *AG* are maintained throughout the development of petals and stamens (Honma, 2000, Pelaz, 2001). The floral homeotic genes *APETALA3* (*AP3*) and *PISTILLATA* (*PI*) have been proved crucial for understanding the molecular mechanisms that lead to petal and stamen formation in this research. *AP3* and *PI* expression is necessary and sufficient to regulate basic morphology of petals and stamens (Moriyah, 2003; Kramer, 2003). However, the result of the Southern blot analysis indicates that there are obvious differences; double copies of *PtAP3* or two members have high homology to each other in male clones (L50) in *P. tomentosa,* whereas this research indicated a single copy in *PtAP3* in *P. tomentosa* (5082, female) genomic DNA. Meilan et al. (2001) have also verified that *PTAP3* was expressed at high levels both in male flower primordia and female flower primordia.

The interrelated researches indicate that *P. tomentosa* is abundant in resource of male clones; however, abortive pollen are easier to happen (Zhang, 1992), and breeding cycle is rather long with a long juvenile phase of about 5–10 years, which mean that there are many difficulties in cross-breeding of *P. tomentosa*. To overcome the difficulties of abortive pollen and little flower and further study the function of *PtAP3*, sense and antisense expression vectors are constructed, and some transgenic tobacco transformants have been obtained by *Agrobacterium*; further molecular identification and morphologic observations are under way. At same time, the transgenic work of *P. tomentosa* is being carried out. In future the transformants from *P. tomentosa* may reduce abortive pollen thus promoting pollen living ability, which will also provide a new route for cross breeding of *P. tomentosa*.

Besides *P. tomentosa*, a native greenbelt species, plays a key role in beautifying the environment in north China, although pollen and catkin production contamination is another problem. Especially male clones with good reproductive ability release more pollen, and is one of the relatively serious sources of allergy, which increases the density of dust breathed in to bodies. In northern cities, the catkins from female clones in every spring have become an important factor affecting the environment and have attracted wide attention from society. As the part of anti-sense sequence (with intron sequence) of part *PtAP3* gene sequence is ligated into pBI121 and 35S promoters, *Ptap3* is constructed to transform *P. tomentosa* clones to suppress its expression. Introduced intron in anti-sense construct will help cosuppress expression of *PtAP3* gene, thus bringing about disturbing the floral development of *P. tomentosa* along with poplars and willows to improve the environment.

In this research, *PtAP3* homologue gene was isolated and anti-sense expression vector was constructed and transformed into *P. tomentosa* and tobacco. At present, *LEAFY* and *AGAMOUS* involved in flowering genes are being isolated, so that the rule of spatio-temporal expression will be analyzed as a whole. This will provide a molecular mechanism to explain flower development of *P. tomentosa*, and also contribute on long breeding cycle and pollen contamination. This study has provided transgenic safety of *P. tomentosa* by genetic engineering and affords us useful references for genetic improvement of other trees in the *Salix* section.

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