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ORIGINAL RESEARCH PAPER

Ectopic expression of a poplar *APETALA3*-like gene in tobacco causes early flowering and fast growth

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Abstract A MADS-box gene, designated *PtAP3*, was isolated from a floral bud cDNA library derived from *Populus tomentosa.* Analysis by multiple alignments of both nucleotide and amino acid sequences, together with phylogenetic analysis, revealed that *PtAP3* is an ortholog of *Arabidopsis AP3*. Analysis of RNA extracts from vegetative and reproductive tissues of *P. tomentosa* by RT-PCR indicated that *PtAP3* is expressed in roots, stems, leaves and vegetative and floral buds. Notably, the expression of *PtAP3* fluctuated during floral bud development between September and February with differences between male and female buds. In the former, a gradual down-regulation during this period, interrupted by a slight up-regulation in December, was followed by a sharper up-regulation on February. In developing female floral buds, expression was stable from September to November, sharply up-regulated in December, and then gradually down-regulated until February. The functional role of *PtAP3* was investigated in transgenic tobacco plants. Of 25 transformants, nine displayed an earlier flowering phenotype compared with the wild type plants. Furthermore, transgenic tobacco had faster growth and more leaves than untransformed controls. The traits proved to be heritable between the T0 and T1 generations. Our results demonstrate a regulatory role of the *PtAP3* gene during plant flowering and growth and suggest that the gene may be an interesting target for genetic modification to induce early flowering in plants.

Keywords *APETALA3* · *Populus tomentosa* · Early flowering · Fast growth · Tobacco

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Introduction

Current knowledge concerning the ABCDE gene model comes largely from extensive studies on the model plant *Arabidopsis*. Within the B-class genes, *APETALA3* (*AP3*) and *PISTILLATA* (*PI*) are of interest because they act as identity genes required for petal and stamen morphogenesis (Jack et al. 1992). Both *AP3* and *PI* gene products belong to the MADS-box super family of proteins, which are

known as the MIKC-domains (Theißen et al. 2000). The MADS-box domain contains approx. 60 aa and is highly conserved across the entire super family. It is essential for binding at conserved DNA sites containing the sequence CC $(A/T)_6GG$, known as CArG elements, and is considered to play a role in protein dimerization (Riechmann et al. 1996). The gene products of *AP3* form heterodimers that recognize and bind to these sites (Hill et al. 1998).

The particular importance of understanding the flowering mechanism in poplar is due to the economic and ecological importance of the species for forest production, reclamation and biomass (Polle and Douglas 2010). *Populus* differs from *Arabidopsis* in many aspects. It is a woody perennial tree with a long juvenile phase and a long life span (Hsu et al. 2006). It flowers annually or seasonally during the reproductive developmental phase (Yuceer et al. 2003) and each seasonal flowering period is interrupted by a vegetative period. Furthermore, the structures of poplar flowers are distinct from those of *Arabidopsis* (Rottmann et al. 2000). At present, the functional role of *AP3* and/or its orthologs in the development of woody plant flowers is unclear. In this study, we report the isolation of *PtAP3*, an *AP3*-like gene in *Populus tomentosa*, and the analysis of its function both in situ and in heterogeneous transformed tobacco plants. Our data provides new insights into the molecular mechanism underlying the development of floral buds in *P. tomentosa* and, additionally, one possible approach to accelerating early flowering in plants via genetic modification.

Materials and methods

Plant materials

Vegetative and floral buds of *P. tomentosa* were rapidly frozen in liquid N_2 after collection from the adult trees and stored at −80°C until use. Additionally, root, stem and leaf samples were taken from 30-day-old tissue-cultured plantlets of *P. tomentosa*, followed by immediate RNA extraction. Sterilized tobacco (*Nicotiana tabacum*) leaf discs were used as transgenic acceptor materials. The young leaves of transgenic tobacco plants were used for extraction of DNA and RNA.

Cloning and characterization of *PtAP3*

The primers P1F and P1R (Table 1) used for the screening of *PtAP3* were designed according to *PTD* (accession AF057708), an *AP3* homolog from *Populus trichocarpa*. *PtAP3* was obtained by PCR screening of the *P. tomentosa* male floral bud cDNA library. SM buffer containing recombinant bacteriophages was used as DNA template. Thermal cycling was performed at 94°C for 5 min, 94°C for 30 s, 60°C for 20 s, 72°C for 30 s, for 35 cycles, then at 72°C for 5 min. The positive recombinant bacteriophage clones were isolated by PCR.

The amino acid sequences of *AP3* homolog genes were retrieved from GenBank (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi) and aligned with CLUSTALX1.81 and Bioedit software. Conserved domain sequences and functional domains were analyzed using the Expert Protein Analysis System (ExPASy) proteomics server of the Swiss Institute of Bioinformatics (SIB) (http://cn.expasy.org/). The tertiary structure of the PtAP3 protein was built using software 3D-JIGSAW 2.0 (http://bmm.cancerresearchuk.org/~3djigsaw/).

Phylogenetic relationship analysis

Genetic relationships were analyzed using a number of B-class MADS-box protein sequences of plant species retrieved from GenBank. Multiple sequence alignment was performed with ClustalW, genetic distance matrices were obtained from the alignments and a neighbor-joining tree was constructed with bootstrap sampling of 1,000 replications using MEGA 4.1(Tamura et al. 2007).

RT-PCR analysis

Total RNAs were extracted from vegetative and reproductive tissues of *P. tomentosa* according to the method of Chang et al. (1993). Total RNA was pretreated with RQ1 DNase I (Promega) to remove contaminating genomic DNA. The first-strand cDNA was synthesized using 1 μg treated total RNA, Superscript III (Invitrogen) and oligo $d(T)_{20}$, subsequently diluted 1:10 with water as template. P2F and P2R primers (Table 1) were used for PCR amplification. Conditions of thermal cycling were identical to those described above. No-template controls

Table 1 Primers used for screening the cDNA library, labelling DNA probes, construction of the expression vector, PCR amplification, DNA gel blot analysis, RT-PCR analysis of poplar tissues and qRT-PCR analysis of transformants

Primers	Oligonucleotide*	Features
P1F forward	5′-TTGGATCCATGGGTCGTGGAAAGA-3′(*Bam*HI)	For screening cDNA library
P1R reverse	5′-AAGAGCTCTCAAG GAAGGCGAAGTT-3′(*Sac*I)	
P2F forward	5′-GAAGCAAAACTAGAGGATCTACAGG-3′	For RT-PCR analysis
P2R reverse	5′-TCAAGGAAGGCGAAGTTCAT-3′	
P3F forward	5′-CTCCATCATGAAATGCGATG-3′ (*P. tomentosa ACTIN*)	
P3R reverse	5′-TTGGGGCTAGTGCTGAGATT-3′ (*P. tomentosa ACTIN*)	
P4F reverse	5′- TTGGATCCATGGGTCGTGGAAAGA-3′(*Bam*HI)	For PCR, DNA blot analysis
P4R reverse	5′-TAAGCTTGATGTGGAGGGGCTAAT-3′	
P5F forward	5′- CTGCTGGAATTCACGAAACA-3′ (Tobacco *ACTIN*)	For qRT-PCR analysis
P5R reverse	5′-GCCACCACCTTGATCTTCAT-3′ (Tobacco *ACTIN*)	

*The added restriction sites are *underlined*

for each primer pair were included in each run. *P. tomentosa* actin was used as an internal reference to normalize small differences in template amounts with P3F and P3R primers (Table 1).

Construction of expression vectors and transformation of tobacco

Both the plasmid DNA containing *PtAP3* and the binary vector pBI121 DNA were digested with both *Bam*HI and *Sac*I. The corresponding fragments were recycled and purified using QIAquick Gel Extraction Kit (Qiagen) and ligated with T4-DNA ligase (Promega) at 16°C overnight. The ligation product was then transferred into competent cells of *E. coli* TG1. The pBI121-*PtAP3* was verified by PCR (using P4F and P4R primers) (Table 1) and digestion. Tobacco leaf disc transformation was performed using the *Agrobacterium*-mediated method.

Molecular identification of transgenic tobacco plants

PCR and DNA gel blot analysis

Genomic DNA was extracted from young leaves of transformed plants and used as the template for PCR identification. The PCR reaction system and conditions of thermal cycling were identical to those described in the previous sections. PCR products were separated by electrophoresis on a 0.8% agarose gel and blotted onto positively charged nylon membrane by capillary transfer in the presence of 20 × SSC buffer. A 203 bp fragment used as DNA probe for gel blot analysis was amplified from genomic DNA of *P. tomentosa* using P4F and P4R primers (Table 1). The blots were hybridized with a DIG-labeled DNA probe at 58°C, and washed at high stringency. Immunological detection was performed with the DIG DNA Labeling and Detection Kit (Roche) according to the manufacturer's protocol.

Quantitative RT-PCR analysis

Total RNA was extracted from transgenic tobacco leaves using the SV Total RNA Isolation System (Promega). Reverse transcription was performed as described in previous sections. As template, first-strand cDNA was diluted 1:10 with water. P2F and P2R were used for qPCR analysis. The qPCR reaction was performed using PowerSYBR Green PCR Master Mix (Invitrogen) on a DNA Engine Opticon 2 system (MJ Research). The program included a preliminary step of 2 min at 50°C, predenaturation at 94°C for 2 min, followed by 35 cycles of 94°C for 15 s, 60°C for 30 s and 72°C for 30 s, with a final extension of 7 min at 72°C. No-template controls for each primer pair were included in each run. Tobacco *actin* was used as an internal reference to normalize small differences in template amounts with P5F and P5R primers (Table 1). Three replicates were employed for the qPCR analysis of each sample.

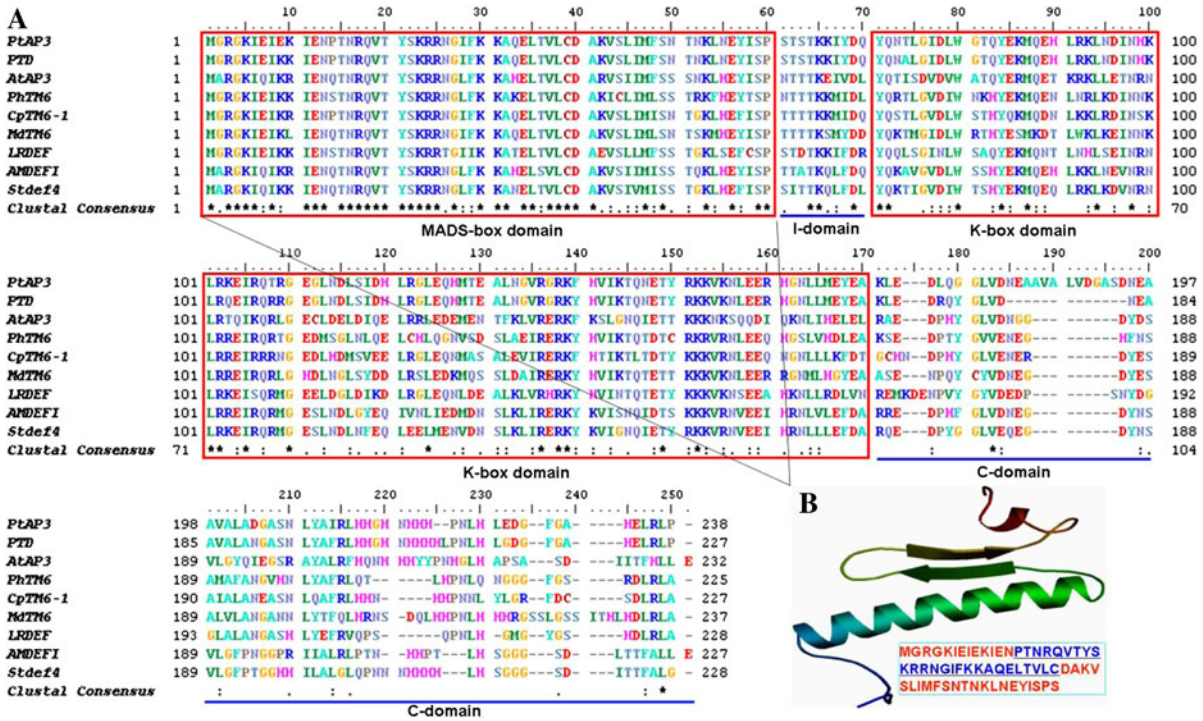

Fig. 1 Sequence alignment and predicted tertiary structure of PtAP3 protein. **a** Sequence alignment of MADS-box domain proteins from several plant species. The *M*- and *K*- and the *I*- and *C*-domains are highlighted using *red bold frames* and *blue bold lines* respectively. **b** The tertiary structures of *M*-domain in *PtAP3*. The helix was formed by "PTNRQVTYSKRR NGIFKKAQELTVLC"

Phenotypic analysis of transgenic tobacco plants

Twenty-five transgenic plantlets were transplanted into individual pots with artificial soil mix as growing support. The plants were moved outside the greenhouse when they had produced four to five leaves (June 15). Plant height and number of leaves of each T_0 transgenic line were recorded on August 11 and 21, September 1, 11 and 21, and October 2. The date when flower primordia were first visible was also recorded.

Results and discussion

A positive phage plaque was isolated from the *P. tomentosa* male floral bud cDNA library by PCR. Sequencing revealed the length of *PtAP3* cDNA to be 717 bp, encoding 238 amino acids. The sequence alignments suggest that *PtAP3* belongs to the MADS-box gene family. It contains M-, I-, K- and C-domains, which are typical characteristics of MIKC-type genes (Fig. 1a). The predicted tertiary structure of the MADS-box domain of PtAP3 (Fig. 1b) consists of one helix and two sheet structures. MADS-box genes play crucial roles in plant growth and development (Causier et al. 2002). The M-domain itself is the most highly conserved of the four major MADS-box protein domains and has been widely studied across the taxonomic kingdoms (Leseberg et al. 2006). Proteins incorporating the M-domain are involved in DNA binding and dimerization with other MADS-box proteins (Diaz-Riquelme et al. 2009). The protein PtAP3 reported here shares 82% amino acid similarity with PTD, an AP3 homolog from *P. trichocarpa*. The data described in the present report will add to existing knowledge in this field (Sheppard et al. 2000).

To understand the relationships between *PtAP3* and the B-class MADS-box genes of other species, a neighbor-joining tree was constructed from *PtAP3* together with 23 previously reported B-class MADS-box genes from *Arabidopsis*, gymnosperm, eudicot and monocot species. Similarly to the previous

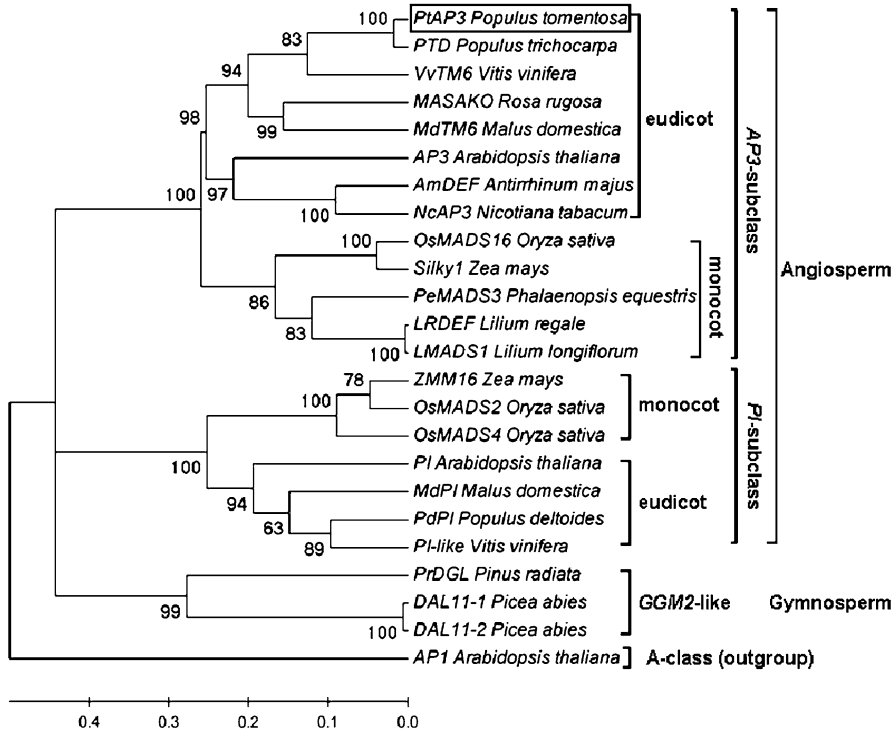

Fig. 2 Neighbor-joining tree representing relationships of *PtAP3* with gymnosperm and other angiosperm MADS-box gene homologs. Bootstrap support values (%) from 1,000 replications are indicated when over 50%. GenBank accession number for each sequence: *PtAP3* (AY210488), *AP1* (BT00 4951), *AP3* (AY142590), *PI* (NM_122031), *VvTM6* (BQ97 9341), *PI-like*(DQ059750), *MASAKO* (AB055966), *MdTM6* (AB081093), *MdPI* (AJ291491), *Silky1* (AF181479), *ZMM16* (NM_001111666), *OsMADS2* (L37526), *OsMADS4* (L37527), *OsMADS16* (AF077760), *PeMADS3* (AY378150), *LMADS1*(AF503913), *LRDEF* (AB071378), *PTD* (AF057708), *PdPI* (EU029172), *DAL11-1*(AF158539), *DAL11-2*(AF158540), *PrDGL* (AF120097), *AmDEF* (AB516402), *NcAP3* (X96428)

studies (Winter et al. 2002), the B-class MADS-box genes were classified into *AP3*, *PI* and *GGM2* subclasses (the *AP3* and *PI* subclasses associated with the angiosperm group and the *GGM2* subclass with the gymnosperm group). The analysis shows that *PtAP3* falls into the eudicot and monocot *AP3* subclass of B-class MADS-box proteins (Fig. 2). The result suggests that *PtAP3* shares similar functions with those of other genes in the same clade. In other words, *PtAP3* is likely to fulfil a B-function role during the development of floral organs in *P. tomentosa*.

The expression profiles of *PtAP3*, analyzed by RT-PCR, indicate that the gene is expressed in various tissues of *P. tomentosa* including roots, tender stems and leaves, vegetative buds and male and female floral buds. In floral buds, its relative expression levels fluctuated and differences were apparent between the two sexual forms (Fig. 3a, b, c). In developing male floral buds, a gradual down-regulation between September 13 and January 25 was interrupted by a slight up-regulation, demonstrated by the data point in December and expression was then sharply up-regulated on February 25 (Fig. 3b). In contrast, transcript levels in developing female floral buds were stable from September 13 to November 24, sharply up-regulated in December and then gradually down-regulated until February 25th (Fig. 3c). These results imply that *PtAP3* is associated with sexual differentiation of *P. tomentosa* floral organs.

Sixty-three positive transgenic tobacco plants were identified by PCR. The results of both PCR and DNA gel blot analysis of partial transgenic tobacco plants showed that the constructed plasmid 35S::*PtAP3* (Fig. 4A) had been integrated into the tobacco genome (Fig. 4B). Relative differences in *PtAP3* expression in the transgenic tobacco was also indicated by qRT-PCR (Fig. 4C). Statistical analysis of

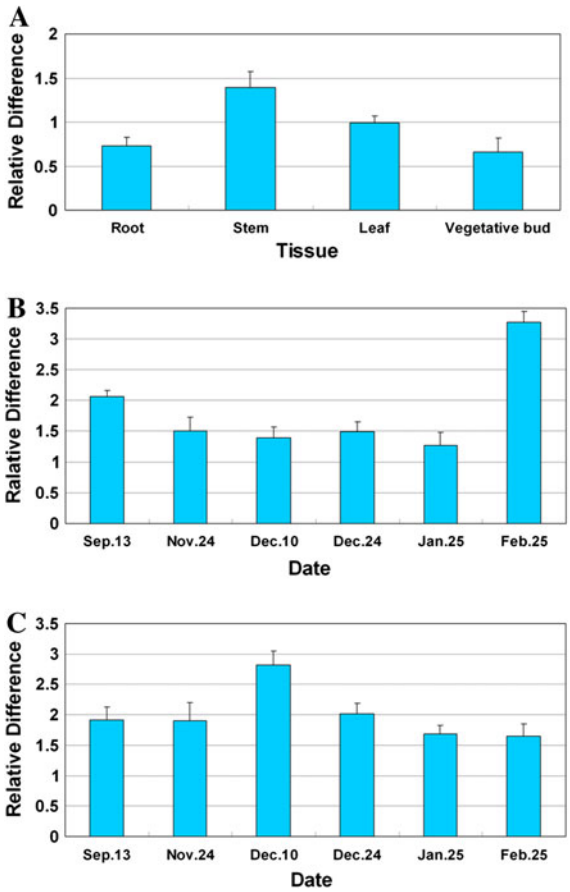

Fig. 3 Expression patterns of *PtAP3* in *P. tomentosa*. **a** Expression in different tissues. Roots, stems and leaves were collected from one-month-old tissue-cultured plantlets. Vegetative buds were collected from adult trees. **b** and **c** The expression patterns of *PtAP3* during floral bud development derived from real time RT-PCR analysis in male floral buds (**b**) and female floral buds (**c**)

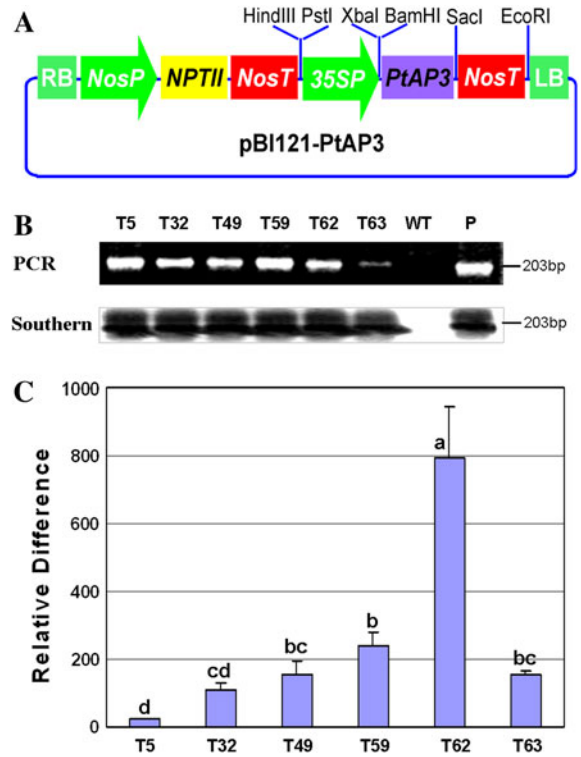

Fig. 4 Molecular identification of transgenic tobacco plants carrying the 35S::*PtAP3* construct. **A** Schematic representation of pBI121-PtAP3 for over-expression of *PtAP3* in transgenic tobacco. RB (T-DNA *right border*), LB (T-DNA *left border*), *Nosp* (Nos promoter), *nptII* (Neomycin phosphotransferase II), *Nost* (Nos terminator), *35S* (CaMV 35S promoter), *gus* (b-glucuronidase gene), *PtAP3* (an *AP3*-like gene from *P. tomentosa*). **B** Transgenic tobacco plants identified by PCR amplification and DNA gel blot analysis. *T5*, *T32*, *T49*, *T59*, *T62* and *T63* are six transgenic lines, *WT* is the wild type, *P* is positive control (the recombinant plasmid pBI121-PtAP3). **C** The over-expression of *PtAP3* in transgenic tobacco plants identified by quantitative RT-PCR (using tobacco *ACTIN* as reference control). *T5*, *T32*, *T49*, *T59*, *T62* and *T63* are six transgenic lines. Significance was assessed using the ANOVA Fisher's LSD test ($*P < 0.05$)

these results shows that expression was significantly higher in line T62 than in other lines. Significant differences in expression of *PtAP3* were also demonstrated between partial lines (T63, T59 and T49) and T5. The difference between T5 and T62 was approx. 400-fold. Investigation of the phenotypic traits of the transgenic tobacco indicated correlations between these and the expression levels of *PtAP3*. Line T62, with the highest expression level, flowered 25 days earlier than wild type plants, while T63, T59, T49 and T32 flowered 15, 12, 10 and 8 days earlier than wild type, respectively. Faster growth than wild type was observed in lines T62, T63, T59, T49 and T32 while T5 also grew faster but did not flower earlier.

Twenty-five independent T0 generation transgenic tobacco plants and ten wild type were transferred to conditions exterior to the greenhouse for further study. The average height of all these transgenic plants was statistically higher than that of wild type during development (Fig. 5A) and the transgenic plants also had more leaves than the control plants (Fig. 5B). Nine flowered earlier than the wild type plants by between 4 and 25 days (Table 2; Fig. 5C). No obvious phenotypic variation of floral organs was observed in the transgenic tobacco compared with the

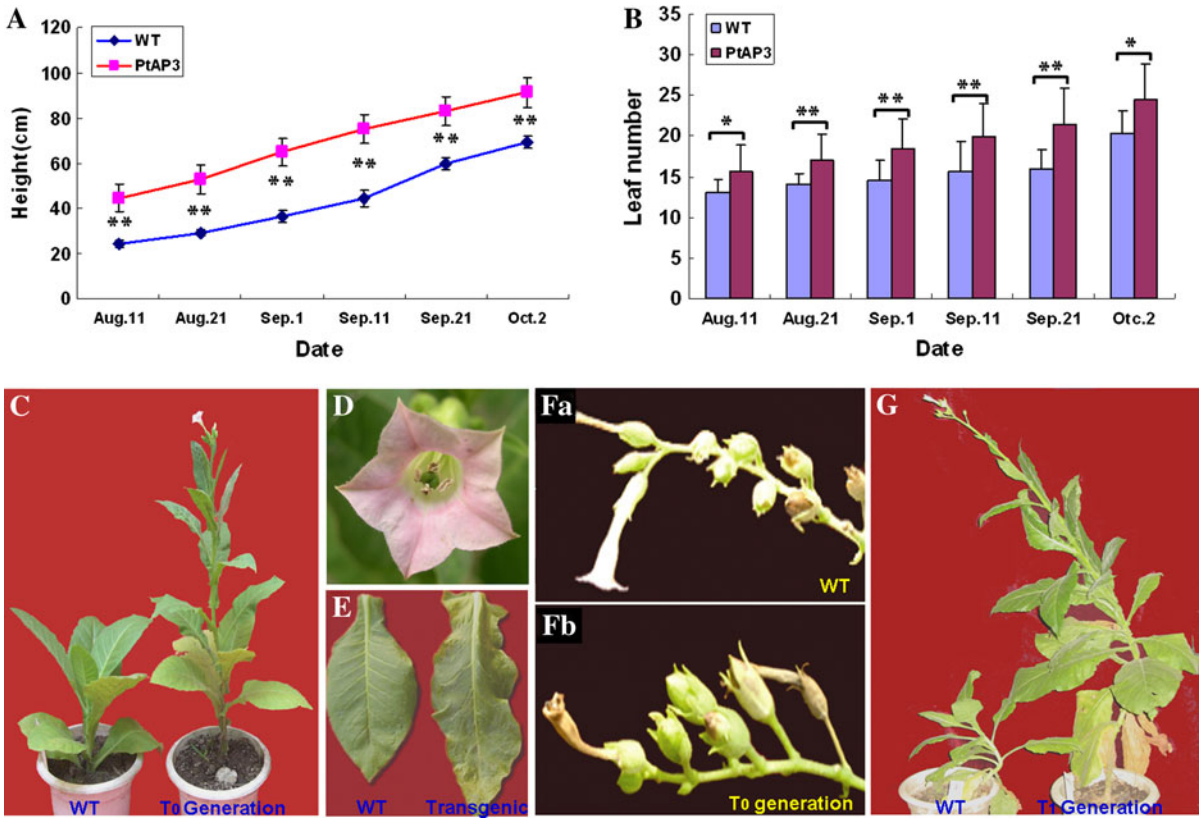

Fig. 5 The phenotypic characteristics and growth traits of tobacco plants carrying pBI121-PtAP3 grown in natural conditions. **A** The height of transgenic and wild type tobacco plants from August 11th to October 2nd. Significance was assessed using the ANOVA Fisher's LSD test ($^{**}P < 0.01$) **B** Change in leaf number during the development of transgenic tobacco plants and wild type. Significance was assessed using the ANOVA Fisher's LSD test ($^{*}P < 0.05$, $^{**}P < 0.01$). **C** Early flowering phenotype produced in T_0 generation of transgenic tobacco. **D** A flower of transgenic tobacco (T_0 generation). **E** The altered leaf shape of a T_0 generation transgenic tobacco plant. **Fa** and **Fb** were fruits from wild type and transgenic tobacco plants (T_0 generation), respectively. **G** The early flowering trait was inherited in the T_1 generation of transgenic tobacco

wild type (Fig. 5D). Leaves, however, appeared to be narrower, longer and uneven (Fig. 5E). No difference was observed in fruit shape (Fig. 5Fa, Fb). The T_1 generation transgenic plants derived from T_0 seeds flowered earlier than wild type (Fig. 5G), indicating that the early flowering trait had been inherited in the T_1 generation.

Despite varying levels of expression of *PtAP3*, all transgenic tobacco plants grew faster and produced more leaves, while 9 of the 25 plants flowered earlier. We speculate that the essential genetic factor for such phenotypic traits is the conserved MADS-box domain. The MADS-box domain of the PtAP3 protein bears very close homology to those of the *Arabidopsis* proteins AtAP3 and AtAP1 (Fig. S1) and it is expected that its function during flower development is similar to that of the *Arabidopsis* proteins. The effects of over-expression of *PtAP3* in transgenic tobacco plants suggest that one of the protein's major functions concerns the regulation of flowering time. It is possible that faster growth and more leaves also contribute to the transition to flowering.

Our study should contribute to the greater understanding of the genetic factors controlling flower development in poplar. The *PtAP3* gene represents a valuable element to further this knowledge not only in poplar but possibly other woody species. Based on these findings, our work will be extended to the production of transgenic poplar expressing altered levels of the endogenous poplar gene to further

Table 2 Comparison of flowering traits in T0 transgenic tobacco lines and wild type tobacco plants grown in natural conditions

Transgenic line	Date of flowering	Number of leaves at time of flowering	Final height (cm)	Note
1	Aug. 3	20.0	110.0	Early flowering
2	Aug. 1	16.0	105.0	Early flowering
3	Aug. 6	19.0	145.0	Early flowering
4	Aug. 10	19.0	126.0	Early flowering
5	Sep. 1	20.0	82.0	No early flowering
6	Sep. 3	15.0	82.0	No early flowering
7	Aug. 14	20.0	112.0	Early flowering
8	Aug. 16	23.0	124.0	Early flowering
9	Aug. 29	17.0	86.0	No early flowering
10	Sep. 12	16.0	77.0	No early flowering
11	Aug. 26	18.0	85.0	No early flowering
12	Sep. 3	16.0	80.0	No early flowering
13	Sep. 12	15.0	72.0	No early flowering
14	Aug. 29	19.0	84.0	No early flowering
15	Sep. 15	15.0	68.0	No early flowering
16	Sep. 16	15.0	72.0	No early flowering
17	Aug. 31	16.0	82.0	No early flowering
18	Sep. 16	17.0	80.0	No early flowering
19	Sep. 13	18.0	82.0	No early flowering
20	Aug. 30	16.0	84.0	No early flowering
21	Aug. 22	15.0	92.0	Early flowering
22	Sep. 11	18.0	78.0	No early flowering
23	Sep. 10	19.0	72.0	No early flowering
24	Aug. 18	18.0	100.0	Early flowering
25	Aug. 20	16.0	100.0	Early flowering
WT	Aug. 26	14.5 (average)	69.2 (average)	Control

investigate its involvement in flowering in this species. Control of flowering time is also an important factor in other domains of plant culture. For instance, speed to flowering has an important effect on production costs in floriculture, as it determines the productivity of a defined area of greenhouse space (Chandler and Brugliera 2010). Although some control of flowering can be achieved by manipulation of photoperiod and/or use of plant growth regulators, ectopic expression of *PtAP3* might be one approach using genetic modification to achieve early flowering induction in these plants.

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