

Functional analysis of the promoters of B-class MADS-box genes in London plane tree and their application in genetic engineering of sterility

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Received: 11 September 2016 / Accepted: 20 April 2017 / Published online: 4 May 2017
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Abstract Breeding flowerless and/or fruitless varieties are highly desirable for London plane tree because it can prevent pollen- and fruit-mediated environmental contamination. Floral tissue-specific cell ablation is an efficient method to create such sterile plants. Here we isolated and characterized *APETALA3* (*AP3*)-like and *PISTILLATA* (*PI*)-like genes and the promoters of *PaAP3* and *PaPI*, in London plane tree respectively. The promoter fragments were fused to *GUS* (β -glucuronidase) and *BARNASE* gene, respectively, and transformed into tobacco plants. In *pPaAP3::GUS* transgenic plants, the GUS activity could be detected in various organs, including leaves, stems and all floral organs. Furthermore, most tobacco plants transformed with *pPaAP3::BARNASE* were dead and the survivals showed abortion of inflorescence. In contrast, heterologous expression of *pPaPI::GUS* in tobacco plants led to specific GUS activity in the inner three whorls of flowers. Accordingly, tobacco plants transformed with *pPaPI::BARNASE* lack petal, stamen and pistil, with only sepal left. The results suggest that sterile lines of *P. acerifolia* may be obtained by genetic engineering with *pPaPI::BARNASE* construct, which might solve the problems of shedding fruit hairs and disseminative pollens, reducing air pollution and reducing the allergens that harmful to human health.

Keywords *Platanus acerifolia* · *AP3* · *PI* · Promoter · *BARNASE* · Ablation

Introduction

The London plane tree (*Platanus acerifolia*) is widely planted as a roadside and parkland tree in cities, and it is considered as an interspecific hybrid of the oriental plane (*Platanus orientalis*) and American sycamore (*Platanus occidentalis*) (Zhang et al. 2011a, b; Lu et al. 2012). It has a lot of desirable characters, such as rapid growth, huge crown for shading, attractive colorful leaves in fall season, and good adaptability to urban environment (Li et al. 2007). However, this species also has a few unfavorable traits, and the greatest negative impact on human life is the pollen shedding of male flowers in spring and fruit hair releasing in the late spring. It results in the pollution of environment, leading to some serious allergic symptoms (such as rhinitis and bronchial asthma, etc.) (Han et al. 2016). In order to solve the problems, breeding non-flowering or fruitless varieties is imperative.

The development of genetic engineering and molecular biology provides an effective new way to generate genetically modified (GM) plants including sterile plants (Mariani et al. 1990). And the utilization of tissue-specific promoters has important practical application value by controlling the timing and precision of foreign gene expression and reducing the impact on plant growth, development, and metabolic pathways (Lv and Zhang 2016). Among these promoters, flower-specific promoters were proved to be applicable to generate complete sterility (Kobayashi et al. 2006; Liu and Liu 2008; Yang et al. 2010). A number of flower-specific promoters have been isolated and identified to engineer sterility. The anther tapetum-specific tobacco

Communicated by WenWu Guo.

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promoter (*TA29*) was fused to *BARNASE* or *RNase T1* and transformed into tobacco, and the results showed that transgenic plants with *BARNASE* specifically expressed in anther and tapetum cells were dead (Mariani et al. 1990). Expression of *BpFULL1: BARNASE* in tobacco and *Arabidopsis* resulted in ablation of inflorescences (Lan-nenpaa et al. 2005). In addition, expression of *BARNASE* in tobacco reproductive tissues driven by the *FBP6* promoter (*pFBP6*) from petunia also resulted in the ablation of flower buds (Liao et al. 2012). Above evidences suggested that flower-specific promoters can be used for engineering sterility in these species mentioned above.

According to the ABC model of flower development, B-class MADS-box transcription factors, *APETALA3* (*AP3*) and *PISTILLATA* (*PI*), are key regulators of petal and stamen development (Jack et al. 1992; Goto and Meyerowitz 1994). In eudicots and monocots, loss of function of B-class genes exhibited similar phenotypes, i.e. the development of second and third whorl floral organs are disrupted (Jack et al. 1992; Schwarz-Sommer et al. 1992; Trobner et al. 1992; Goto and Meyerowitz 1994; Ambrose et al. 2000; Nagasawa et al. 2003; Vandenbussche et al. 2004; Drea et al. 2007; Kramer et al. 2007; Roque et al. 2013; Bartlett et al. 2015; Otani et al. 2016), suggesting that the function of B-class genes is largely conserved during evolution. Expression of B-class genes in core eudicots and gramineous monocots is relatively conserved, predominantly found in developing petals or lodicules (organs likely to be homologous to petals) and stamens (Jack et al. 1992; Ambrose et al. 2000; Nagasawa et al. 2003; Vandenbussche et al. 2004). In species outside the core eudicots, however, the B-class genes showed a higher degree of expression divergence (Kim et al. 2005; Kramer and Irish 1999; Zhao et al. 2010; Zhang et al. 2011a, b). Previously, we have isolated *AP3* and *PI* homologues (*PaAP3* and *PaPI*) from London plane tree. *PaAP3* showed high expression in male inflorescences, low expression in female inflorescences, and weak expression in stems of 1-year-old seedlings and adult plants, but no expression was detected in roots, leaves and vegetative buds (Li et al. 2012). There are at least five *PI* homologous genes (*PaPI1/2/3/4/5*) that show different expression profiles and transcript abundance in London plane tree, among which *PaPI2* may be the major functional *PI* homolog and expressed predominantly in male and female inflorescences (Zhang et al. 2011a, b).

In this study, the *PaAP3* and *PaPI2* (thereafter named *PaPI* for convenience) promoter were isolated from London plane tree, and their expression features were characterized by fusing to *GUS* report gene followed by introduction into tobacco plants. Histochemical staining and gene expression analysis showed that *GUS* activity of *pPaAP3::GUS* transgenic plants could be detected evidently in various floral organs while in *pPaPI::GUS* transgenic plants *GUS*

activity predominantly existed in the inner three whorls of flowers. In addition, the expression of *BARNASE* driven by *PaAP3* and *PaPI* promoter was also investigated.

Materials and methods

Plant materials

London plane tree grown at Huazhong Agricultural University was used for promoter cloning. *Nicotiana tabacum* ‘Xanthi’ was used for genetic transformation experiment. Transformed plants were grown in a containment greenhouse with a 14 h photoperiod and a 25 °C/17 °C day/night temperature regime.

Isolation of *PaAP3* and *PaPI* promoters

Total DNA was extracted with CTAB method (Li et al. 2008). Thermal asymmetric interlaced-PCR (TAIL-PCR) was used to obtain the promoter region of the *PaAP3* and *PaPI* (Liu et al. 1995). The eight universal primers used for TAIL-PCR were listed in Table 1. We designed three gene-specific reverse primers (*AP3-GSP1/2/3*) according

Table 1 Primer sequences used for TAIL-PCR

Primer	Sequence
AP3-GSP1	5'-CTTCTTGGTGATTCCGCCTCTT-3'
AP3-GSP2	5'-AGTGACCTGCCTGTTTGTAGTATTC-3'
AP3-GSP3	5'-GAAATGGAAGCAACGATGTCAGA-3'
PI-GSP1-1	5'-TACTCAGACATCTTCCCAGAACTTG-3'
PI-GSP1-2	5'-TCTTTGAGTATGTCACTTGCCTGTT-3'
PI-GSP1-3	5'-CTCAACTTCTCAACTCTGGCTTCT-3'
PI-GSP2-1	5'-TTCTGTTCTACATCAGTCTCCCCC-3'
PI-GSP2-2	5'-GTTGAAAGTAGGGGAAATAGCCATA-3'
PI-GSP2-3	5'-CCTTTCGCAGGTAGACCATCAC-3'
AD1	5'-NTCGASTWTSWGWT-3'
AD2	5'-NGTCGASWGANAWGAA-3'
AD3	5'-WGTGNAGWANCANAGA-3'
AD4	5'-AGWGNAGWANCAWAGG-3'
AD5	5'-NGTAWAASGTNTSCAA-3'
AD6	5'-NGACGASWGANAWGAC-3'
AD7	5'-GTNCGASWCANAWGTT-3'
AD8	5'-NCAGTWSCTNTSCTT-3'

The gene-specific primers (*AP3-GSP1*, *AP3-GSP2*, and *AP3-GSP3*) are complementary to the first exon and 5'UTR region of *PaAP3*. The primers (*PI-GSP1-1*, *PI-GSP1-2*, and *PI-GSP1-3*) are complementary to the *PaPI* as well. In addition, the primers (*PI-GSP2-1*, *PI-GSP2-2*, and *PI-GSP2-3*) are seated on the the sequence which were confirmed by the previous TAIL-PCR. Eight arbitrary degenerate (AD) primers used in the PCR analysis are also shown above N-A/T/C/G, S-C/G, W-A/T

to the base sequence of exon 1 and 5'UTR of *PaAP3* (*PaAP3*: GenBank accession No. EF488452). In the meantime, we designed three gene-specific reverse primers of *PaPI* (*PI-GSP1-1/2/3*) following the same principle of *PaAP3* (*PaPI*: GenBank accession no. EF488452). After the *PaPI* promoter sequences were isolated and analyzed, another three gene-specific reverse primers for TAIL-PCR (*PI-GSP2-1/2/3*) were designed to isolate longer promoter sequence. All the gene-specific primers are listed in Table 1 while the steps of TAIL-PCR program are listed in Table 2.

Analysis of *PaAP3* and *PaPI* promoter sequences

Fundamental functions of the promoters were confirmed by the Promoter Scan program (<http://www.bimas.cit.nih.gov/molbio/proscan/>). PlantCARE (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>) and PLACE (<http://www.dna.affrc.go.jp/PLACE/index.html>) were used to analyze potential *cis*-acting regulatory elements of *PaPI* and *PaAP3* promoters.

Vector construction

Primers *pPaAP3-F* and *pPaAP3-R* were used to amplify a 2.9 kb *PaAP3* promoter (*pPaAP3*) (Table 3); meanwhile, primers *pPaPI-F* and *pPaPI-R* were used to amplify a 2.6 kb *PaPI* promoter (*pPaPI*) (Table 3). Both of the reverse primers, *pPaAP3-R* and *pPaPI-R*, were exactly next to the initiation codon of gene coding region. Both of the amplified fragments were inserted into pMD-18T (Takara Biomedical Technology, Beijing, China) and sequenced (Sangon Biotech, Shanghai, China). Then, they were double digested by *Bam*HI and *Pst*I, and both were introduced

Table 2 The modified protocol of TAIL-PCR used in study

Reaction no.	Cycle no.	Thermal settings
Primary	1	94 °C, 3 min
	5	94 °C, 30 s; 66 °C, 1 min; 72 °C, 2.5 min
	1	94 °C, 30 s; 25 °C, 3 min, ramping to 72 °C over 3 min
		72 °C, 2.5 min
	15	94 °C, 30 s; 66 °C, 1 min; 72 °C, 2.5 min 94 °C, 30 s; 66 °C, 1 min; 72 °C, 2.5 min 94 °C, 30 s; 44 °C, 1 min; 72 °C, 2.5 min
	1	72 °C, 10 min
Secondary	12	94 °C, 30 s; 62 °C, 1 min; 72 °C, 2.5 min 94 °C, 30 s; 62 °C, 1 min; 72 °C, 2.5 min 94 °C, 30 s; 44 °C, 1 min; 72 °C, 2.5 min
	1	72 °C, 10 min
	Tertiary	20

Table 3 Primers used for vector construction

Primer	Sequence
pPaAP3-F	5'-CTCTCCTCTGCAACTCCCCCTC-3'
pPaAP3-R	5'-CTCTCCTCTGCAACTCCCCCTC-3'
pPaPI-F1	5'-AAGACTTTGTGGAAGCGGTTTAGC-3'
pPaPI-R	5'-GCTCAACTTCTCAACTCTGGCTTCTT-3'
GUS-F	5'-CACACCGATACCATCAGAGATC-3'
GUS-R	5'-TCACCGAAGGGCATGCCAGTCC-3'
BARNASE-F	5'-TGACGGGGTTGCGGATTAT-3'
BARNASE-R	5'-GGTGGAGGTCGCTGATACTTCT-3'
EF1-F	5'-TGTTGTGACTTTTGGTCCCA-3'
EF1-R	5'-ACAAACCCACGCTTGAGATCC-3'
GUS-vF	5'-CACACCGATACCATCAGAGATC-3'
GUS-vR	5'-TCACCGAAGGGCATGCCAGTCC-3'
BARNASE-vF	5'-TGACGGGGTTGCGGATTAT-3'
BARNASE-vR	5'-GGTGGAGGTCGCTGATACTTCT-3'

into pCAMBIA1391Z vector (Fig. 1b, c). The empty pCAMBIA1391Z vector was used as a negative control construct (Fig. 1a), and the CaMV 35S promoter was introduced into pCAMBIA1391Z as a positive control (Fig. 1d). In addition, the *pPaAP3* and *pPaPI* were introduced into *BpFULL1::BARNASE* binary construct (Lannenpaa et al. 2005), separately with *Sal*I and *Bam*HI digestion to produce the *pPaAP3::BARNASE* and *pPaPI::BARNASE* plasmids (Fig. 1e–g).

Tobacco transformation

All plasmids were transferred into the *Agrobacterium tumefaciens* EHA105. The transformation method for tobacco was according to Ning et al. (2012). Transgenic tobacco lines were verified by PCR with the primer pairs *GUS-F/GUS-R* and *BARNASE-F/BARNASE-R* (Table 3).

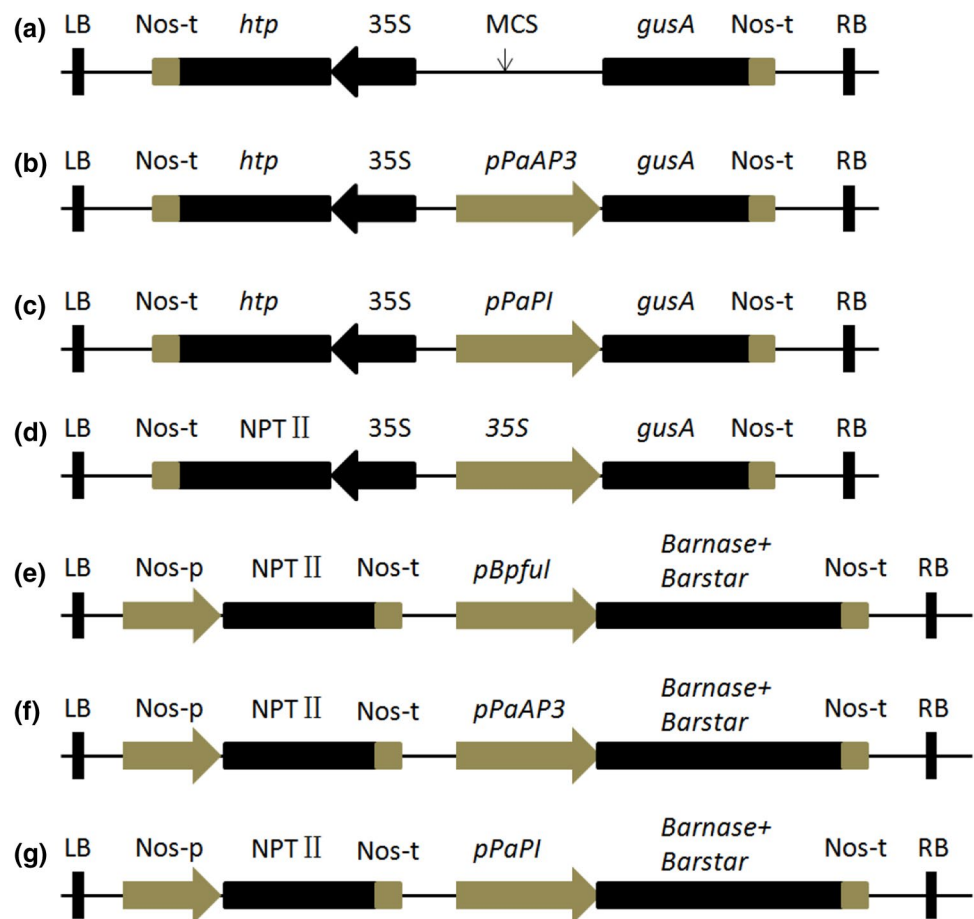
Histochemical assays

The method of histochemical staining was described by Liao et al. (2012). various tissues of each transgenic line and control were immersed in a 1 mg/ml solution of X-Gluc, prepared with 50 mM sodium phosphate (pH 7.0), 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 10 mM Na₂-EDTA and 0.1% w/v Triton X-100, for 12–18 h under 37 °C.

RT-PCR analysis

Total RNA was extracted from leaves, stems and flowers with the RNA extraction kit (Trizol Reagent, Invitrogen). The *NtEF1α* was used as internal control. The primers used for RT-PCR are listed in Table 3. The PCR program

Fig. 1 Schematic diagram of constructs utilized in this study. **a** The original vector of pCAMBIA1391Z containing a promoterless *GUS* reporter gene. **b** The *pPaAP3::GUS* construct, containing the 2.9 kb upstream promoter region of *PaAP3* placed in front of the *GUS* reporter gene. **c** The *pPaPI::GUS* construct, containing the 2.6 kb upstream promoter region of *PaPI*. **d** The positive control vector pCAMBIA1391Z containing a 35S promoter placed in front of the *GUS*. **e** The original *pBpFULL1::BARNASE* construct, containing the promoter region of *BpFULL1* placed in front of *BARNASE* and *BARSTAR* gene (from Dr. M. Länneppää of University of Joensuu, Finland). **f** The *pPaAP3::BARNASE* construct, with *pBpFULL1* replaced with *pPaAP3*. **g** The *pPaPI::BARNASE* construct, with *pBpFULL1* replaced with *pPaPI*



was 3 min at 94 °C for initial denaturation, followed by 30 cycles of 94 °C for 30 s, 59 °C for 30 s and 72 °C for 50 s.

Results

Sequence information of *pPaAP3* and *pPaPI*

In order to isolate the promoter sequence of *PaAP3* and *PaPI*, TAIL-PCR strategy was adopted. After first three rounds of amplification, a 3.1 kb fragment upstream of *PaAP3* gene and a 2.2 kb fragment upstream of *PaPI* gene were isolated and sequenced, using the AD primer and *AP3*-GSP1/2/3 or *PI*-GSP1-1/2/3 (Table 1). Based on the obtained sequence information, another three specific reverse primers *PI*-GSP2-1/2/3 were designed to isolate longer *PaPI* promoter, which resulted in a 700 bp extension to the promoter fragment, making its length up to 2.9 kb. Sequence accuracy was identified by repeatedly sequencing.

Bioinformatics analysis of the *pPaAP3* and *pPaPI*

The sequence of *pPaAP3* was submitted to the PlantCARE and PLACE, and confirmed as the “Highly likely prediction”. The result indicated that *pPaAP3* fragment contains several basic motifs, including six putative TATA box (i.e., TATTAAT, TATATAA, TTATTT), ten CAAT box (CAAT), one MADS-box protein binding CArG box (CWWWWWWWWG) (Table 4) and a target sequence named LEAFYTAG (CCAATGT). When the sequence of *pPaPI* was analyzed, similar result was displayed, in which the detected motifs include one putative TATA box (TTATTT) and ten CAAT box (CAAT) (Table 5). In addition, a *cis*-acting regulatory element AGL3ATCONSENSUS, which was the target sequence of *AGL3*, was detected in both promoter fragments. Nevertheless, some different motifs were also observed between the two promoter fragments. For example, AGL2ATCONSENSUS was found in *pPaAP3* but not in *pPaPI* (Tables 4, 5).

Table 4 Analysis of putative *cis*-acting regulatory elements in the *pPaAP3* promoter

Motif	Sequence	Loction	Function
TATABOX3/4/5	TATTAAT, TATATAA, TTATTT	–2919, –3027, –1010, –974, –519, –115	Core promoter element for transcription start
CAATBOX1	CAAT	–192, –396, –747, –792, –883, 1040, –1330, –1592, –1620, 1728...	Common <i>cis</i> -acting element in promoter and nhancer regions
LEAFYATAG	CCAATGT	–193	Target sequence of LEAFY
DPBFCOREDCDC3	ACACNNG	–2622, –1716	Motif found in embryo-specific gene promoter
DOFCOREZM	AAAG	–281, –343, –486, –524, –635, –652, –823, –1186, –1397, –2133, –2267, –2320, –2335, –2591, –2651, –2969, –3089	Core site required for binding of Dof proteins, involving PBF and zinc finger
AGL2ATCONSENSUS	NNWNCCAWWWTRGWAN	–232	Binding consensus sequence of Arabidopsis (A.t.) AGL2
AGL3ATCONSENSUS	TTWCYAWWWWTRGWAA	–231	Binding consensus sequence of Arabidopsis (A.t.) AGL3
CARGCW8GAT	CWWWWWWWG	–1011, –1550, –2341, –2821	<i>Cis</i> -acting element in promoter of MADS-box genes. Binding site for AGL15
EECCRCAH1	GANTTNC	–16, –1772	Consensus motif of the two enhancer elements
CACGTGMOTIF	CACGTG	–1459	Binding site of Arabidopsis GBF4, acting as transcriptional repressors
WUSATAg	TTAATGG	–2917	Target sequence of WUS in the intron of AGAMOUS gene in Arabidopsis

Table 5 Analysis of putative *cis*-acting regulatory elements in the *pPaPI* promoter

Motif	Sequence	Loction	Function
TATABOX5	TTATTT	–922	Core promoter element for transcription start
CAATBOX1	CAAT	–134, –161, –194, –479, –497, –589, –673, –792, –934, –991...	Common <i>cis</i> -acting element in promoter and nhancer regions
LTRE1HVBLT49	CCGAAA	–157	Low-temperature-responsive element
DPBFCOREDCDC3	ACACNNG	–375, –1878, –1922, –2586	Motif found in embryo-specific gene promoter
DOFCOREZM	AAAG	–39, –130, –154, –214, –285, –615, –954, –1220, –1907, –2189...	Core site required for binding of Dof proteins, involving PBF and zinc finger
CARGATCONSENSUS	CCWWWWWWGG	–135, –1565	Binding site of FLC in SOC1 promoter blocking transcriptional activation
AGL3ATCONSENSUS	TTWCYAWWWWTRGWAA	–1587	Binding consensus sequence of Arabidopsis (A.t.) AGL3
CARGCW8GAT	CWWWWWWWG	–1516, –1584, –1706, –1724	<i>Cis</i> -acting element in promoter of MADS-box genes. Binding site for AGL15
EECCRCAH1	GANTTNC	–1302, –1310, –1830, –1885, –1929	Consensus motif of the two enhancer elements
CACGTGMOTIF	CACGTG	–374	Binding site of Arabidopsis GBF4, acting as transcriptional repressors
WUSATAg	TTAATGG	–2398	Target sequence of WUS in the intron of AGAMOUS gene in Arabidopsis

Spatial expression patterns of *pPaAP3* and *pPaPI*

To investigate the tissue-specific expression patterns directed by the *pPaAP3* and *pPaPI*, expression vectors

containing a 2.9 kb length fragment of *pPaAP3* and a 2.6 kb length fragment of *pPaPI* fused with *GUS* were constructed, separately, and used to transform tobacco plants (Fig. 2). 11 *pPaAP3::GUS* transgenic lines and

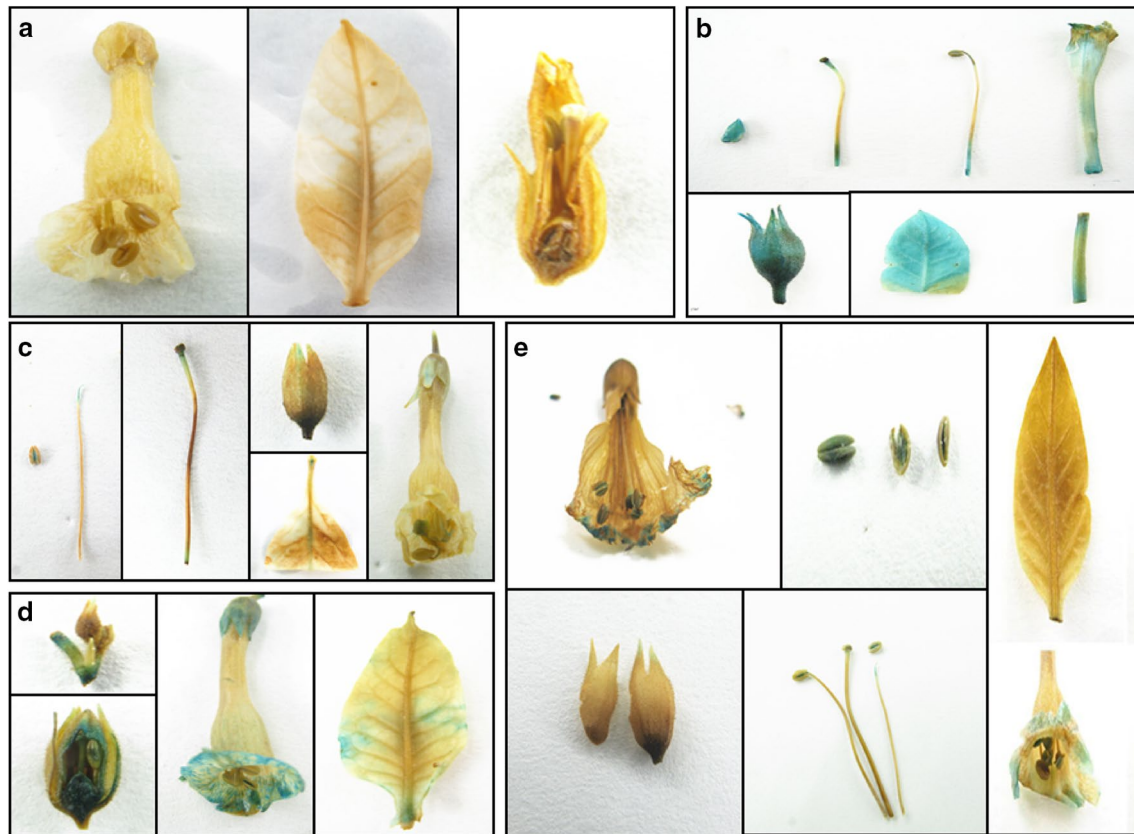


Fig. 2 Histochemical GUS staining of *pPaAP3::GUS* and *pPaPI::GUS* transgenic tobacco plants. **a** Histochemical GUS staining of non-transgenic tobacco plants. **b** Histochemical GUS staining of *35S::GUS* transgenic tobacco plants. **c, d** Sepal, petal, stamen,

anther, pistil, ovary, leaves and stem from *pPaAP3::GUS* transgenic tobacco plants. **e** Sepal, petal, stamen, anther, pistil and leaves from *pPaPI::GUS* transgenic tobacco plants

13 *pPaAP3::GUS* transgenic lines were obtained, and all of them were used for histochemical staining analysis. The results demonstrated that the transgenic lines of *pPaAP3::GUS* not only showed GUS activity in sepal, petal, stamen and pistil, but a certain level of activity was also detected in vegetative organs (Fig. 2c, d). In contrast, only petal, stamen and pistil were stained in the transgenic lines of *pPaPI::GUS* (Fig. 2e). As positive control, transgenic lines of *35S::GUS* demonstrated GUS activity in all the tissues and organs (Fig. 2b). No GUS activity was detected in wild-type (WT) and transgenic tobacco plants with empty control construct (Fig. 2a).

Heterologous expression of *BARNASE* driven by *pPaAP3* or *pPaPI* in tobacco

Considering temporal and spatial expression patterns of *pPaAP3* and *pPaPI*, it is potential to create flower organ-specific ablation by using the promoter fragments fused

with *BARNASE* gene. Thus, we transformed tobacco plants with *pPaAP3::BARNASE* and *pPaPI::BARNASE* construct, aiming to observe the effect of heterologous expression before applying them to *P. acerforlia*.

Eighteen transgenic lines of *pPaPI::BARNASE* were obtained, and it demonstrated that 76% plants formed depauperate flower buds—which abscised from pedicels in an early stage of floral development (Fig. 3b). Based on the morphology anatomy, we found only sepals were persistent, and all the petals, stamens, stigma and ovary were completely ablated (Fig. 3d) compared to the flowers of wild-type and transgenic tobacco plants with empty construct (Fig. 3e). Vegetative growth was not affected, except lateral branches increased (Fig. 3a). In contrast, transgenic plants of *pPaAP3::BARNASE* showed the inhibition of vegetative growth. More than half of transgenic lines (ten lines) were either small or weak, and the flower buds were completely withered and browning in the early stage of development, resulting in abnormal structures of the flower buds (Fig. 3c).

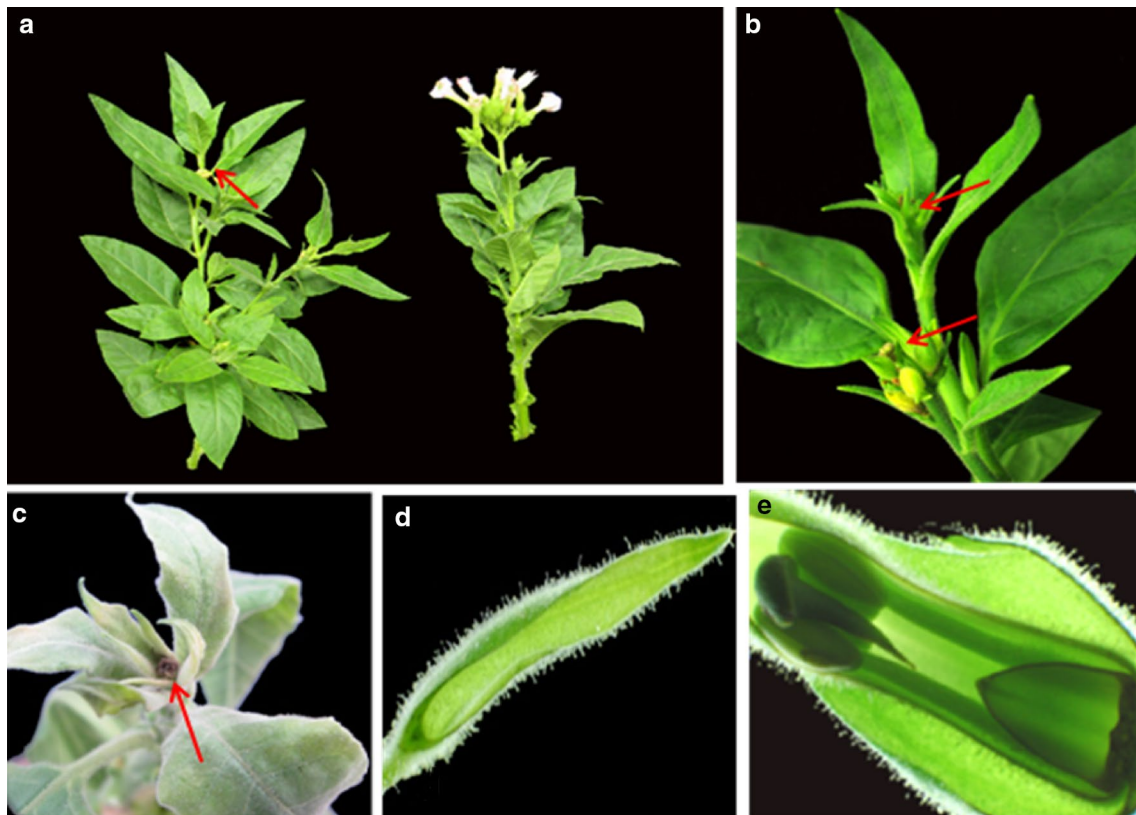


Fig. 3 The phenotype of tobaccos transformed with *pPaAP3::BARNASE* and *pPaPI::BARNASE*. **a** Compared to the wild-type tobacco (*right*), plant transformed with *pPaPPI::BARNASE* (*left*) showed complete bud abscission and lateral branches increased. **b** Tobacco transformed with *pPaPI::BARNASE* showed floral abscis-

sion. **c** Plants transformed with *pPaAP3::BARNASE* showed browning and withering of top meristem. **d** Longitudinal section of flower buds from the *pPaPI::BARNASE* transgenic plants revealed ablation of inner three whorls of floral organs. **e** Longitudinal section of flower buds from WT tobacco

Semi-quantitative expression in transgenic tobacco

Using the RT-PCR analysis, the floral organ-specific ablation and the influence of vegetative growth were clearly proved to be related with the expression patterns directed by the promoter fragments. Total RNA was extracted from leaf, stem, and the floral organs in the transgenic lines of *GUS* constructs, and from leaf, stem and the residual floral organs (petal) in the transgenic lines of *BARNASE* constructs, which then were reversely transcribed into cDNA. Besides, a whole plant of 1-month old wild-type tobacco was also applied for the RT-PCR analysis as negative control. The concentration of all the cDNA samples used for detection of target gene was balanced by the internal control gene *NtEF1 α* . The RT-PCR results demonstrated that *GUS* activity was only detected in the inner three whorls of floral organs in transgenic plants with *pPaPI::GUS* constructs (Fig. 4a), but in all the whorls of floral organs in transgenic lines of *pPaAP3::GUS*, including sepals (Fig. 4b). In line with this, the expression of *BARNASE* in transgenic lines of *pPaPI::BARNASE* was not detected in the sepals (floral organ left only), and it was detected a

certain expression in the stem and leaf (Fig. 4c); but in the transgenic lines of *pPaAP3::BARNASE*, *BARNASE* could be detected obviously in stem and weakly in leaf (Fig. 4d). In wild-type tobacco plants, neither *GUS* nor *BARNASE* transcripts were detected in all the plant tissues.

Discussion

In this study, we mainly investigated the possibility of breeding sterile London plane trees by using the genetic engineering with the flower tissue-specific promoters of the B-class MADS-box genes. Compared to means of artificial microRNA or RNA interference of floral related genes, flower tissue-specific promoters fused with cytotoxic genes such as *BARNASE* may be more efficient approaches to create sterile plants (Lannenpaa et al. 2005), because the flowering and floral development progress is regulated by a large number of genes, which usually show overlapping or redundant functions leading to inefficient results by silencing a single gene.

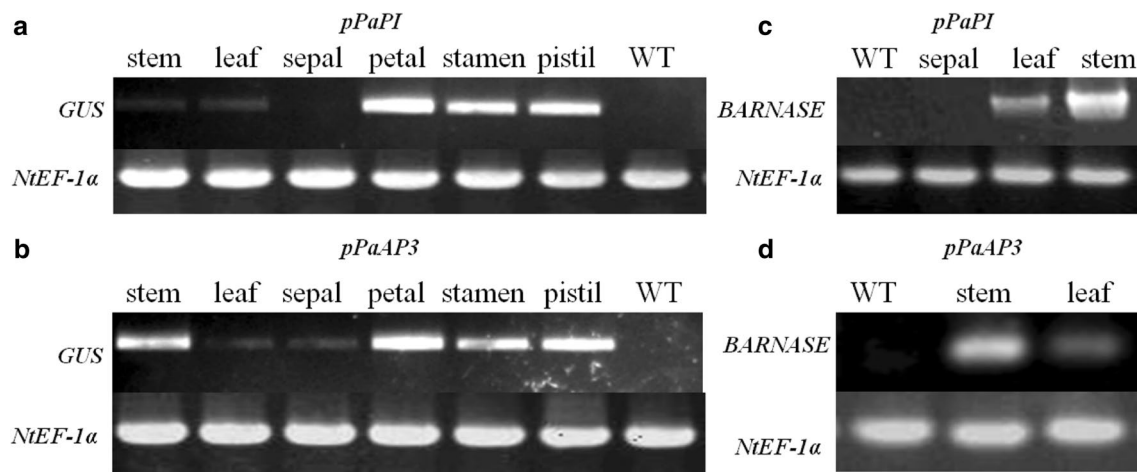


Fig. 4 RT-PCR analysis of *GUS* and *BARNASE* gene expression in transgenic tobacco. **a** Semi-quantitative RT-PCR analysis of *GUS* expression in WT and *pPaPI::GUS* transgenic tobacco. **b** Semi-quantitative RT-PCR analysis of *GUS* expression in WT and *pPaAP3::GUS* transgenic tobacco. **c** Semi-quantitative RT-PCR

analysis of *BARNASE* expression in WT and *pPaPI::BARNASE* transgenic tobacco. **d** Semi-quantitative RT-PCR analysis of *BARNASE* expression in WT and *pPaAP3::BARNASE* transgenic tobacco. The *NtEF1α* gene of tobacco was used as an internal control

At first, we isolated the promoter fragments upstream of the 5'UTR of *PaAP3* and *PaPI* from *P. acerifolia*. Bioinformatics analysis confirmed that both fragments contain the basic promoter domains and regulation elements. Then, both of them were fused with *GUS* reporter gene and cytotoxic gene *BARNASE*, and transformed into tobacco, respectively. The expression of *GUS* and *BARNASE* demonstrated that the two promoter fragments have different expression specificity, with the *PaPI* promoter driving gene expression more particularly in floral organs, which can lead to flower organ-specific cell ablation and sterile plants as expected.

As we known, B-class MADS-box genes regulate the development of the second and third whorl floral organs, and their expression is primarily limited in petals and stamens, hardly detected in vegetative organs in the majority of plant species (Jack et al. 1992; Trobner et al. 1992; Vandebussche et al. 2004). In our study, when *pPaAP3::GUS* was transformed into tobacco, the activity of *GUS* was detected in all four whorls of flower. RT-PCR analysis further confirmed the expression of *GUS* at various levels not only in all floral organs, but also in vegetative organs including stems and leaves, although lower than that in floral organs (Fig. 4b). In contrast, the *pPaPI::GUS* transgenic lines exhibited a high level activity of *GUS* in petal, stamen and pistil, with no or low expression, if any, in sepal and vegetative tissues (Fig. 4a). Our previous qRT-PCR analysis indicated *PaAP3* was expressed mainly in the male inflorescences of London plane tree, but that its expression was also detected in female inflorescences and even weakly in the stems of juvenile and adult plants (Li et al. 2012); *PaPI* was likewise expressed in both male and female

inflorescences, as well as in mature embryos, but almost no expression could be detected in vegetative tissues (Zhang et al. 2011a, b). The expression patterns of *PaAP3* and *PaPI* in London plane suggest that their promoters (*pPaAP3* and *pPaPI*) may contain special and distinct *cis*-regulatory elements, which may explain the different activities and the broader expression region of *pPaAP3* and *pPaPI* in London plane than that in most core eudicots. Alternatively, the expression pattern of heterologously introduced promoters may be regulated by the upstream transcription factors in receptor plants (Kusters et al. 2015), such as tobacco used here. In tobacco, differences were observed in the expression patterns of *NTDEF* and *NTGLO* (*AP3* and *PI* ortholog, respectively). Expression of *NTGLO* was restricted to the regions of petals and stamens, but no expression was detected in stem, root, leaf, sepal and gynoecium. Whereas the expression of *NTDEF* was more widespread, which was easily detected in the fourth whorl and faintly detected in the first whorl, with relatively high expression in the second and third whorls (Davies et al. 1996; Hansen et al. 1993).

When *pPaAP3::BARNASE* construct was transformed into tobacco, the apical meristem and flower buds were browned to death in the early stage of development. The transcripts of *BARNASE*, in few survivals, were detected in stem and leaf, and the expression level was higher than the parallel group containing the *pPaPI::BARNASE*. The expression of *BARNASE* could be detected in all lines transformed with either of the two constructs, although at different expression levels. The reason of survivals may due to a small 35S core fragment fused with *BARSTAR* gene following the *BARNASE* gene in the constructs (Lan-nenpaa et al. 2005). Instead of inhibiting the transcription

of *BARNASE* gene, *BARSTAR* gene encodes a kind of enzymatic inhibitor of RNase which is encoded by *BARNASE* gene on protein level. Therefore, the transgenic plants kept normal growth even though the transcript of *BARNASE* was detected in the vegetative organs. In this study, when *BARSTAR* was expressed at a low but ubiquitous level in the transgenic lines with *pPaPI::BARNASE*, the leakage of *BARNASE* gene at a certain level did not cause damage to vegetative organs and sepals. However, in the transgenic plants of *pPaAP3::BARNASE*, the expression of *BARSTAR* was not high enough to inhibit the toxicity of *BARNASE*. This result also confirmed the good specificity of *pPaPI* as well. Although the leakage of *BARNASE* was detected in the transgenic plants, the expression in leaf, stem and sepal was much lower than that in the inner three whorls of floral organs. In contrast, the poor specificity of *pPaAP3* leading to the leakage of *BARNASE* in the transgenic plants resulted in the death of most transformants.

In summary, our study characterized the expression specificity and function of the promoters of *PaAP3* and *PaPI* in transgenic tobacco plants. The results revealed that *pPaPI* has stronger specificity than *pPaAP3*, which can specifically control gene expression within the inner three whorls of floral organs, and so may be used efficiently in the gene engineering of sterility. Although the transgenic lines of *pPaAP3* fused with *BARNASE* gene also resulted in floral abortion, the poor specificity limited its potential for application. Based on the present results and the expression pattern of *PaPI* in London plane, the *pPaPI::BARNASE* construct may have the potential to be applied in breeding programs for sterile lines of London plane tree. This will be benefit for the environment and human health by reducing the fruit hairs and pollens. In addition, this approach can also be used for obtaining sterile transgenic plants, thereby preventing transgene flow through pollens, fruits, or seeds.

Acknowledgements This research was funded by the “Twelfth Five-Year Plan” of the National Science and Technology Research (Grant No. 2012BAD01B04), the National Natural Science Foundation of China (Grant Nos. 31672187, 31272206), the Special Fund for Forest Scientific Research in the Public Welfare (201304103) and the National Key Technology R&D Program (2012BAD01B04). We thank Dr. M. Länneppää of University of Joensuu, Finland for providing the *BpFULL1::BARNASE* plasmid and all colleagues in our laboratory for discussions and technical assistance.

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