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Isolation and characterization of the promoter of *SEPALLATA1*-like gene from *Platanus acerifolia*

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Abstract

London plane (*Platanus acerifolia* Wild.) is a famous landscape plant because of its numerous desirable traits except the abundant pollens and seed hairs, which not only pollute the environment but also affect human health. To resolve these problems, we herein isolated and functionally analyzed the promoter of *PlacSEP1.1*, an orthologous gene of *Arabidopsis SEPALLATA1*, and investigated the potential usability for cell ablation strategies to engineer reproductive sterility in plants. A 2130 bp 5' upstream region of *PlacSEP1.1* was isolated and termed *pPlacSEP1.1*. Putative motif detections show that there were several types of motifs in *pPlacSEP1.1* including core promoter elements, tissue-specific expression regulatory elements, and some negative regulatory elements. β -Glucuronidase histochemical and quantitative assay showed that *pPlacSEP1.1* of all deletions was active in all detected tissues except the shortest deletion D5 in roots. In order to test whether *pPlacSEP1.1* could be used for London plane sterility breeding with a cytotoxic gene *Barnase*, the *pPlacSEP1.1::Barnase* and *pPlacSEP1.1::Barnase-mic35S-Barstar* vectors were constructed and transformed into tobacco. The *pPlacSEP1.1::Barnase* transgenic tobacco showed serious defects with respect to vegetative development and died within a couple of weeks after transplantation. On the other hand, most *pPlacSEP1.1::Barnase-mic35S-Barstar* transgenic tobacco showed normal vegetative growth and inflorescence, and flower development prevented phenotype.

Additional key words: *Agrobacterium* mediated transformation, *Arabidopsis thaliana*, cytotoxic gene *Barnase*, London plane, *Nicotiana tabacum*, pollution, sterility.

Introduction

London plane (*Platanus acerifolia* Wild.) is a famous landscape plant and known as 'the king of street trees' because of its numerous desirable traits, such as rapid growth, excellent shading capability, strong stress resistance, wide adaptation, etc. It is widely used as a roadside and parkland tree in cities throughout the temperate and subtropical regions. But it has a few unfavourable characteristics in urban use, most notably is the abundant pollens shed by mature male flowers in spring which cause pollen allergy and resulted in pollinosis and asthma (Asturias *et al.* 2002, Enrique *et al.* 2002). Moreover, the dispersing achenes with numerous stiff hairs released from spring to early summer not only pollute the environment but also cause serious damage to human health. In order to resolve these problems, breeding non-flowering or fruitless cultivars by engineered sterility

is imperative.

The genetic engineering research showed that there are a variety of techniques being utilized to genetically engineer reproductive sterility in plants. Techniques currently under development include gene suppression, dominant negative mutants (DNMs), and cell ablation (Skinner *et al.* 2000). Gene suppression strategies include antisense suppression, co-suppression, and RNA interference. Suppression necessitates the use of gene sequences displaying high similarity to the target gene, and frequently limiting the use of constructs to closely related species. The DNM strategies work at the protein level and theoretically function across more divergent genera, but production of successful DNM transgenes is complex and currently relies on trial and error mutation of conserved amino acids or domains. Cell ablation strategies direct to ablate specific tissues by the controlled expression of a cytotoxic gene. This strategy does not target specific genes and applicable

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Abbreviations: DNM - dominant negative mutant; GUS - β -glucuronidase; SEP - *SEPALLATA*.

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across the broadest range of genera; however, it requires highly specific floral regulatory elements (*i.e.*, promoters) to direct expression of deleterious gene.

Barnase is a cytotoxic gene encoding an RNase originally from *Bacillus amyloliquefaciens* (Hartley 1988). Since Mariani *et al.* (1990, 1992) first developed a male sterility system with the *barnase* gene regulated by the tapetum-specific promoter *TA29*, cytotoxic genes in combination with male or female tissue-specific promoters have been exploited for generating reproductive sterility in various species, such as tobacco (Goldman *et al.* 1994, Beals and Goldberg 1997, Roque *et al.* 2007), wheat (Block *et al.* 1997), *Brassica napus* (Denis *et al.* 1993, Roque *et al.* 2007), *Kalanchoe blossfeldiana* (Garcia-Sogo *et al.* 2010), *Pelargonium* (Garcia-Sogo *et al.* 2012), cabbage (Lee *et al.* 2003), *Solanum lycopersicon* and *Arabidopsis thaliana* (Roque *et al.* 2007). Moreover, besides herbaceous plant studied, there have been several reports of *barnase* gene employed for sterility breeding in trees. For example, the *BpFULL1::BARNASE* and *BpMADS1::BARNASE* constructs showed prevented inflorescence development and flowering in birch (Lemmetynen *et al.* 2004, Länneppää *et al.* 2005). In *Populus*, *TA29::BARNASE* transgenic poplar showed stable male sterility but slow field growth (Elorriaga *et al.* 2014). The *PrMC2-barnaseH102E* construct was found to efficiently ablate pollen in pine and *Eucalyptus* spp., and the transgenic plant grew similarly to control trees in all observed attributes except the less pollen (Zhang *et al.* 2012).

All the studies showed that reproductive tissue-specific promoter is crucial for cell ablation strategies engineering reproductive sterility in plants. Previous studies showed that floral organ development of flowering plants is determined mainly by a group of MADS-box genes. The ABCDE model has been proposed from developmental and molecular studies of *Arabidopsis*, *Antirrhinum*, and *Petunia* (Theissen 2001). According to ABCDE model, AP1-AP1-SEP-SEP, AP1-AP3-PI-SEP, AP3-PI-AG-SEP, and AG-AG-SEP-SEP are required for the formation of sepal, petal, stamen, and carpel, respectively, highlighting the importance of the SEP protein in specifying all four whorls of the flower (Theissen 2001, Krizek and Fletcher 2005). In *Arabidopsis*, E function genes consist of four *SEP* genes, *i.e.*, *SEP1* (*AGL2*), *SEP2* (*AGL4*), *SEP3* (*AGL9*), and *SEP4* (*AGL3*) (Ma *et al.* 1991, Huang *et al.* 1995, Mandel and Yanofsky 1998, Theissen 2001, Ditta *et al.* 2004). The four *SEP* genes are expressed before the onset of B and C class gene expression, and *SEP1*, *SEP2*, *SEP4* are expressed in all floral whorls, while the expression of *SEP3* starts slightly later and is restricted to the inner three whorls, and all *SEPs* are expressed in the developing ovules (Ma *et al.* 1991, Flanagan and Ma 1994, Savidge *et al.* 1995, Mandel and Yanofsky 1998, Pelaz *et al.* 2000, Ditta *et al.* 2004). Beside *Arabidopsis*, the expression of *SEP*-like genes showed floral-specific in several angiosperm species, including tomato (Uimari *et al.* 2004), petunia (Ferrario *et al.* 2003, Van den Bussche *et al.* 2003, Matsubara *et al.* 2008), peach (Xu *et al.* 2008), tobacco (Jang *et al.* 2002), rice (Cui *et al.* 2010), oil palm

(Adam *et al.* 2006), and orchids (Chang *et al.* 2009). In addition to floral-specific expression, members of *SEP* gene family are expressed in vegetative tissues, seeds, and fruits (Tzeng *et al.* 2003, Matsunaga *et al.* 2004, Xu *et al.* 2008, Tani *et al.* 2009, Ireland *et al.* 2013, Pan *et al.* 2014, Sun *et al.* 2014, Cheng *et al.* 2016).

In London plane, the sequence and expression patterns of *SEP*-like genes have been described previously (Zhang *et al.* 2017). There are five *SEP* genes in London plane, and three of the five *SEPs* (designated *PlacSEP1.1*, *PlacSEP1.2*, and *PlacSEP1.3*) belong to the *SEP1/2/4* clade, while the other two (designated *PlacSEP3.1* and *PlacSEP3.2*) are grouped into the *SEP3* clade. All *PlacSEPs* except for *PlacSEP1.1* and *PlacSEP1.2* were expressed during the male and female inflorescence initiation stages and throughout the flower and fruit development process, *PlacSEP1.1* is just express during subsequent floral and fruit development stages, while *PlacSEP1.2* was only detected clearly in female inflorescence at April. *PlacSEP1.3* and *PlacSEP3.1* were also expressed in vegetative tissues of adult plants, including the shoot apical buds, vegetative subpetiolar buds, and vegetative tissues in mixed flower buds (Zhang *et al.* 2017). These results showed that the *SEPs* from London plane may be appropriate candidate for cell ablation strategies engineering reproductive sterility in plants, especially *PlacSEP1.1*. So, in this study, we isolated and characterized the *PlacSEP1.1*, and analyzed the potential usability for cell ablation strategies engineering reproductive sterility in plants.

Materials and methods

Plant materials: Young leaves for DNA extraction were collected from field-grown 50-year-old London plane (*Platanus acerifolia* Wild.) trees in campus of Huazhong Agricultural University. Tobacco (*Nicotiana tabacum* L.) cv. Xanthi used for transformation were cultured in the Murashige and Skoog (1962) solid medium at a temperature of 25 °C, a 14-h photoperiod, and an irradiance of 80 μmol m⁻² s⁻¹.

Isolation and sequence analysis of the *PlacSEP1* promoter: London plane genomic DNA was extracted from young leaves, following the cetyltrimethyl ammonium bromide (CTAB) isolation procedure (Li *et al.* 2007). The 5' upstream region of *PlacSEP1.1* was isolated by thermal asymmetric interlaced PCR (Liu *et al.* 1995, Liu and Huang 1998). According to the cDNA sequence of *PlacSEP1.1*, three specific primers SP1, SP2 and SP3 were designed (Table 1 Suppl.), and eight arbitrary degenerate (AD) primers, AD1-AD8 were employed (Lu *et al.* 2012). The *cis*-acting regulatory elements in the 5' upstream region of *PlacSEP1.1* were predicted by *PLACE* (Higo *et al.* 1999) and *PlantCARE* (Lescot *et al.* 2002).

Vector construction: Based on the motifs location, the full length region and five 5'-deletion fragments were selected and amplified for expression vectors construction. Regulatory regions of full length, 1 686, 1 328, 1 083,

862, and 564 bp upstream from the translation initiation site of *PlacSEP1.1* sequences (termed FL, D1, D2, D3, D4, and D5, respectively), were amplified by PCR using forward primers, pPlacSEP1.1FL, pPlacSEP1.1D1, pPlacSEP1.1D2, pPlacSEP1.1D3, pPlacSEP1.1D4, pPlacSEP1.1D5 (Table 1 Suppl.), combined with the same reverse primer pPlacSEP1VR (Table 1 Suppl.), respectively. The 5' end of the forward primers was extended 8 bp to generate a *Sph*I digest site and *Bgl*II in reverse primer, respectively. The amplified products were digested with *Sph*I and *Bgl*II, and then ligated into the likewise digested plasmid *pCAMBIA1303*. The constructed vectors were verified by digestion with *Sph*I and *Bgl*II, and by PCR amplification using primers pPlacSEP1FL, pPlacSEP1D1, pPlacSEP1D2, pPlacSEP1D3, pPlacSEP1D4, pPlacSEP1D5, and 1303R (Table 1 Suppl.), respectively.

In order to test whether *pPlacSEP1.1* could be used for London plane sterility breeding with cytotoxic gene *Barnase*, the *pPlacSEP1.1::Barnase-Barstar* and *pPlacSEP1.1::Barnase-mic35S-Barstar* vectors were constructed. *Barnase-Barstar* was amplified from *BpFUL::Barnase* plasmid (Lännenpää *et al.* 2005) by primers BarnaseVF and BarstarVR (Table 1 Suppl.) which contained *Bgl*II and *Bst*PI digest site. The *Barnase-mic35S-Barstar* construct was built based on the *pMD18-Barnase-Barstar* as follows: The 110 bp length mic35S and *Barstar* were amplified by 35SVF (contained *Xba*I digest site), 35SVR and BarstarVF, BarstarVR (Table 1 Suppl.), and then built into pMD18-T, respectively. The mic35S was digested with *Xba*I and *Kpn*I, and then cloned into the *pMD18-Barstar* plasmid in front of the *Barstar* gene. Based on the constructed *pMD18-Barnase-Barstar*, the *Barstar* was removed (*Pst*I/*Sph*I) and replaced by *mic35S-Barstar*, giving the *Barnase-mic35S-Barstar* construct. And then, based on the constructed FL plasmid, β -glucuronidase (*GUS*) gene was removed (*Bgl*II/*Bst*PI) and replaced with *Barnase-Barstar* and *Barnase-mic35S-Barstar*, respectively, giving *pPlacSEP1.1::Barnase-Barstar* and *pPlacSEP1.1::Barnase-mic35S-Barstar* construct. Subsequently, all the constructed vectors were introduced into the *Agrobacterium tumefaciens* strain EHA105 by electroporation method (Mahmood *et al.* 2008).

Plant transformation: *Agrobacterium tumefaciens* strain EHA105 containing the constructed plasmids were grown at 28 °C in a Luria-Bertani liquid medium supplemented with 100 mg dm⁻³ kanamycin. Tobacco leaf disc transformation was performed according to Horsch *et al.* (1985). The transformants were selected by 25 mg dm⁻³ hygromycin, then transplanted into soil and grown in greenhouse at normal conditions. The transgenic plants were confirmed by PCR analysis with primers pPlacSEP1ZF, GUSVR, and BarnaseVR (Table 1 Suppl.), respectively.

Histochemical GUS assays: Histochemical localization of GUS activity in FL, D1, D2, D3, D4, and D5 transgenic plants was carried out as described previously (Jefferson *et al.* 1987). Various tissues of the transgenic and control

plants, including young flower, full expanded flower (divided into sepal, petal, stamen, and stigma), young fruit and semi-mature fruit, apical bud, young stem, old stem, young leaf, old leaf, and root, were incubated in X-gluc buffer [50 mM sodium phosphate pH 7.0, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 10 mM Na₂-EDTA, 0.1 % (m/v), *Triton X-100*, and 1 mg cm⁻³ *X-Gluc*] at 37 °C for 4 - 7 h. After staining, the samples were immersed in 70 % (v/v) ethanol and incubated at room temperature to remove the chlorophyll, and then photographed.

Quantification of GUS activity: For quantitative measurement of GUS activity in transgenic tobacco, we homogenized plant tissues in GUS extraction buffer [50 mM NaH₂PO₄, pH 7.0, 10 mM EDTA, 0.1 % (v/v) *Triton X-100*, 0.1 % (m/v), sodium lauryl sarcosine, and 10 mM β -mercaptoethanol]. The homogenate was centrifuged at 16 100 g and 4 °C for 10 min, and the GUS activity of the supernatant was assessed according to the method described by Jefferson *et al.* (1987). Protein content was assessed by the Bradford (1976) method, using bovine serum albumin as a standard. Activity of GUS was normalized to the protein content in each of the crude extracts and was calculated as picomoles of 4-methylumbelliferone (4-MU) produced per minute, per microgramme of soluble protein.

Results

Through TAIL-PCR, a 2 130-bp 5' upstream region of *PlacSEP1.1* was obtained, termed *pPlacSEP1.1*. It was submitted to *PlantCARE* and *PLACE* to detect putative motifs. The result showed that there were several types of motifs in *pPlacSEP1.1* (Table 2 Suppl.), including thirteen core promoter elements for transcription start TATA-box, fifteen CAAT-box, one *cis*-acting regulatory element A-box, several light response elements, hormone response elements, and stress response elements (Table 2 Suppl.). More important, there were several tissue-specific expression regulatory elements in *pPlacSEP1.1*, such as GATABOX, MADS-box protein binding motif CARG-box, meristem-specific activation element CCGTCC-box, pollen-specific expression motifs, endosperm-specific expression motifs, seed development related element, xylem development regulatory elements, trichome-specific regulatory elements, *etc.*, and some negative regulatory elements such as WBOXATNPR1, WBOXNTERF3, WRKY71OS, *etc.* (Table 2 Suppl.).

In order to analyze the regulatory effect of different regions of the *pPlacSEP1.1*, six constructs with full length (FL) or deletions (D1, D2, D3, D4, and D5) of the *pPlacSEP1.1* fused to *GUS* were generated (Fig. 1) and transformed into tobacco. After selected by hygromycin and confirmed by PCR analysis, 25, 18, 30, 16, 20 and 30 independent tobacco transformants of FL, D1, D2, D3, D4, and D5 were obtained. After a histochemical GUS assay (Fig. 2), we found that, in FL, D1, D2, and D3 transgenic tobacco, GUS staining was observed in apical bud, young

Fig. 1. A schematic representation of expression vectors of the *PlacSEPI.1* gene promoter and β -glucuronidase (*GUS*) gene constructs.

stem, old stem, young fruit, old fruit, young flower, and sepals of mature flower, and weak staining in young leaf, old leaf, root, and sepals, anther, and stigma of mature flower (Fig. 2). In D4 transgenic tobacco, GUS staining was observed in apical bud, young stem, old stem, young fruit, young flower, and sepals of mature flower, and weak staining in old fruit, young leaf, old leaf, root, and sepal, anther, and stigma of mature flower (Fig. 2). In D5, GUS staining was observed in apical bud, young stem, old stem, and weak staining in the young leaf, old leaf, young fruit, old fruit, young flower and four whorls of mature flower, and no GUS staining was observed in root (Fig. 2).

Quantitative GUS assays were performed to determine the expression strength of different deletions of *pPlacSEPI.1* in different tissues of transgenic tobacco by measuring the GUS activity (Fig. 3). The result showed that the FL, D1, D2, D3, and D4 transgenic tobacco had the highest GUS activity in fruit, and followed by apical bud, young stem, old stem, flower, root, old leaf, and young leaf. In flower, sepal had highest GUS activity, followed by petal, carpel, and stamen. Different to FL, D1, D2, D3, and D4 transgenic tobacco, the D5 transgenic tobacco had the highest GUS activity in apical bud and young stem, followed by old stem, sepal, carpel, fruit, petal, stamen, old leaf, and young leaf, and root had a similar GUS staining pattern as wild type.

To test whether *pPlacSEPI.1::Barnase-barstar* and *pPlacSEPI.1::Barnase-mic35S-Barstar* can provide a general tool to create flowerless plants, we transformed them into tobacco and generated *pPlacSEPI.1::Barnase-barstar* and *pPlacSEPI.1::Barnase-mic35S-Barstar* transgenic tobacco. After selected by hygromycin and confirmed by PCR analysis, one line of *pPlacSEPI.1::Barnase-barstar* and eight lines of *pPlacSEPI.1::Barnase-mic35S-Barstar* transgenic tobacco were obtained, respectively. The one line carrying *pPlacSEPI.1::Barnase-barstar* showed serious defects with respect to vegetative development, for example narrow and pale leaves and strong lateral bud development (Fig. 4A,B), and this line died within a couple of weeks after transplantation. One line carrying *pPlacSEPI.1::Barnase-mic35S-Barstar* also showed serious defects in vegetative development, for example yellowing and wrinkled leaves (Fig. 4C), and this line also died within a couple weeks after transplantation.

The other seven lines carrying *pPlacSEPI.1::Barnase-mic35S-Barstar* showed normal vegetative development (Fig. 4D,E), however, when the plants began reproductive phase transition, the browning spots appeared on young leaves and expanded gradually (Fig. 4F), and then leaves turned yellow and died from tip to base (Fig. 4G), but the inflorescence and flower development was inhibited (Fig. 4G,H).

Discussion

London plane is one of most important ornamental plant widely used as roadside and parkland tree throughout the temperate and subtropical regions of the world. However, its abundant pollens shed by mature male flowers and the dispersing achenes with numerous stiff hairs released from broken seed balls not only pollute the environment but also cause serious damage to human health. Our major goal was to identify a promoter with high specific activity in reproductive tissues to fuse to cytotoxic gene to targeted ablated reproductive tissues. For this purpose, in this study, based on the previous expression analysis, we isolated the promoter of *PlacSEPI.1* from London plane, fused to the *GUS* gene and transformed in tobacco to analyze its tissues specific activity, and then fused to cytotoxic gene *barnase* and a modified vector *barnase-mic35S-barstar* to verify if it could prevent flowering in transgenic tobacco.

In this study, a 2130 bp 5' upstream region of *PlacSEPI.1* was isolated, and the putative *cis*-element analysis showed that it contains a core promoter element (TATA-box and CAAT-box) and several elements related to flower-, pollen-, and embryo/endosperm-specific development. For example, the CArG-box is the binding site of MADS-box transcription factors (Riechmann *et al.* 1996, Riechmann and Meyerowitz 1997) and could be the binding site of SOC1-like protein, as observed in *Arabidopsis* (Lee *et al.* 2008), the pollen-specific expression motifs GTGANTG10 and POLLEN1LELAT52 are involved in pollen-specific development in tobacco and tomato (Bate and Twell 1998, Rogers *et al.* 2001), and the motifs Skn-1-motif, DOFCOREZM, PROLAMINBOXOSGLUB1, and SEF1MOTIF are related to endosperm/embryo development in rice, wheat, and soybean (Mena *et al.* 1998,

Fig. 2. Histochemical β -glucuronidase staining in transgenic tobacco lines harboring FL, D1, D2, D3, D4, and D5 constructs: *a* - young flower, *b* - sepal, *c* - petal and stamen, *d* - stigma, *e* - young fruit, *f* - semi-mature fruit, *g* - apical stem, *h* - young stem, *i* - old stem, *j* - young leaf, *k* - old leaf, and *l* - root.

Washida *et al.* 1999, Wu *et al.* 2000, Yoshino *et al.* 2006). Some light responsive elements, hormone regulation motifs, and negative regulatory motifs were also observed.

Although London plane is transformable (Li *et al.* 2007), it takes a long time to flower, so we use tobacco as a model system. The histological expression analysis and

quantification of *GUS* activity showed that the transgenic tobacco with full length and all deletions of *pPlacSEP1.1* showed high *GUS* activity in vegetative tissues as well as in reproductive organs. However, previous study showed that *PlacSEP1.1* is just expressed during floral and fruit development stages and not in vegetative tissues (Zhang

Fig. 3. Fluorometric analysis of β -glucuronidase (GUS) activity in different tissues of non-transgenic tobacco and transgenic tobacco lines harboring FL, D1, D2, D3, D4, and D5 constructs. S - sepal, P - petal, ST - stamen, C - carpel, F - fruit, AB - apical bud, YS - young stem, OS - old stem, YL - young leaf, OL - old leaf, and R - root.

Fig. 4. The phenotype of *pPlacSEP1.1::Barnase* transgenic tobacco and the prevention of flower development in *pPlacSEP1.1::Barnase-mic35S-Barstar* transgenic tobacco. One line of *pPlacSEP1.1::Barnase* transgenic tobacco showed serious defects with respect to vegetative development such as narrow and pale leaves (A). Non-flowering line showed dramatic defects in vegetative growth three weeks after transplantation: narrow, pale, and wrinkled leaves and strong lateral bud development (B). One line carrying *pPlacSEP1.1::Barnase-mic35S-Barstar* also showed serious defects with respect to vegetative development, for example yellowing and wrinkled leaves (C). Seven lines carrying *pPlacSEP1.1::Barnase-mic35S-Barstar* showed normal vegetative development (D,E), these transgenic tobacco lines showed dramatic disorders at the flower formation stage: browning spots which expanded gradually (F), and then turned yellow and the leaf died from tip to base (G), and the inflorescence and flower development were inhibited (G,H).

et al. 2017). The broader expression pattern conferred by the *pPlacSEP1.1* fragment in tobacco model system might be due in part to evolutionary distance or an incomplete promoter region missing key upstream regulatory element. Alternatively, it is possible that post-transcriptional regulation mechanism acts on the native *PlacSEP1.1* transcript, but tobacco has no post-transcriptional regulation mechanism and then it contribute to the expression pattern of *PlacSEP1.1* in London plane and the GUS in tobacco. It is in accord with the opinion of Hernandez-Garcia and Finer (2014) that the absence of transcription factors require to interact with *cis*-elements to modify the expression patterns in heterologous systems.

The purpose of this research is to determine whether *pPlacSEP1.1* could be used for London plane sterility breeding by driving a cytotoxic gene *barnase*. However, even though the stable *Agrobacterium*-mediated transformation of London plane was constructed, we used the tobacco for the detection, because normal London plane tree start flowering after about 6 - 10 years of growth. In this study, we obtained one line of transgenic tobacco which contained the construct *pPlaceSEP1.1::Barnase* and showed serious defect in vegetative development. It indicted that *pPlaceSEP1.1* could drive *Barnase* slightly expressed in vegetative tissues of young transgenic tobacco. In order to eliminate the cytotoxicity in vegetative tissues, the *Barnase-mic35S-Barstar* construct was adopted in this research. The phenotype of *pPlacSEP1.1::Barnase-mic35S-Barstar* transgenic tobacco showed that the cytotoxicity in vegetative tissues could be eliminated, and flower formation could be prevented. This may explain by the fact that the cytotoxicity in vegetative tissues which was produced by slight expression of *Barnase* was completely eliminated by *Barstar* promoted by *mic35S*.

The prevention of flower formation and maintenance of normal vegetative growth was the aim of this research. Seven transgenic tobacco lines were non-flowering and showed no phenotypic defects in vegetative growth at the vegetative development stage. However, in the flowering transition stage, the transgenic tobacco showed defects in young leaves. These defects might be caused by the low activation of *pPlacSEP1.1* at the vegetative development stage, while at the flowering transition stage, *pPlacSEP1.1* activation increased and the *Barstar* was unable to completely eliminate the cytotoxicity in vegetative tissues. However, whether *pPlacSEP1.1::Barnase-mic35S-Barstar* transgenic woody plants generate this phenotype is still unknown, and additional studies are needed to clarify this.

Taken together, the phenotype of transgenic tobacco containing constructs *pPlacSEP1.1::Barnase* and *pPlacSEP1.1::Barnase-mic35S-Barstar* indicated that *pPlacSEP1.1* had slight activity in vegetative tissues of transgenic tobacco. It was different to the GUS expression in transgenic tobacco. It may be due to that the GUS expression was affected by post-transcriptional regulation mechanism in tobacco, and the expression of *Barnase* and *Barnase-mic35S-Barstar* did not. In conclusion, the *pPlacSEP1.1::Barnase-mic35S-Barstar* transgenic tobacco showed that *pPlacSEP1.1::Barnase-mic35S-*

Barstar could prevent tobacco flowering, but more studies are needed to confirm whether it could be used in sterility breeding programs for woody plants.

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