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Petunia *AGAMOUS* Enhancer-Derived Chimeric Promoters Specify a Carpel-, Stamen-, and Petal-Specific Expression Pattern Sufficient for Engineering Male and Female Sterility in Tobacco

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Abstract Previous studies have shown that the *AtAGIP* promoter derived from the *Arabidopsis AGAMOUS* (*AG*) second intron/enhancer specifies a carpel- and stamen-specific expression in its native host species, but not in heterologous species such as tobacco, which restricts its application in the engineering of male and female sterility. These findings also imply that the *AG* regulatory mechanism that has evolved in *Arabidopsis* may, to some extent, have diverged from that of tobacco. To test whether a similar chimeric promoter created using the *AG* second intron/enhancer can overcome this barrier of evolutionary divergence in closely related species, we generated forward- and reverse-oriented chimeric promoters, *fPtAGIP* and *rPtAGIP*, from the petunia *AG* second intron/enhancer (*PtAGI*) fragment and tested them in tobacco, which, like petunia, belongs to the *Solanaceae* family. Our results demonstrate that both *fPtAGIP* and *rPtAGIP* confer similar carpel- and stamen-specific expression without any leaky activity in vegetative tissues in tobacco as revealed by tissue-specific gene expression and tissue ablation. This pattern resembles that driven by the *AtAGIP* in *Arabidopsis* and indicates that the *AG* regulatory mechanism is more conserved between tobacco and petunia than between tobacco and *Arabidopsis*. The petunia-derived promoters also exhibited petal-specific activity, and their activities in floral organs were substantially influenced by the orientation of the *PtAGI* enhancer, with reverse-oriented enhancers displaying approximately double the effectiveness of forward-oriented enhancers. These two properties are novel and have not been observed previously with *AtAGIP* promoters. Furthermore, we found that *PtAGIP* promoter-driven tissue ablation is effective for engineering complete sterility in plants, and the resulting sterile trait is stable for at least three mitotic generations at various temperature regimes, which is important for the complete containment of seed-, pollen-, and fruit-mediated gene flow in field conditions.

Keywords Petunia · *AGAMOUS/pMADS3* · Enhancer · Transgenic plants · Sterility · Gene containment

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Introduction

The tissue-specific ablation of floral organs or tissues provides an effective means for generating complete sterility, which is useful for containing the flow of transgenes into wild or closely related species, potentially leading to the creation of super weeds or highly adaptable pests. Successful engineering of stable sterility in plants requires the identification and utilization of promoters that can drive the expression of chosen genes specifically in targeted tissues or organs. Earlier work has shown that male organ-specific promoters can, when fused to the *Barnase*

gene coding for an extracellular ribonuclease, specifically ablate tapetal cells and generate plant sterility (Mariani et al. 1990, 1992). This approach has been widely used for engineering male sterility in numerous species, including *Arabidopsis* (Konagaya et al. 2008), *Brassica* (Block and Debrouwer 1993; Roque et al. 2007), cabbage (Lee et al. 2003), creeping bentgrass and rice (Luo et al. 2006), tobacco (Hofig et al. 2006; Twell 1995), tomato (Gomez et al. 2004; Roque et al. 2007), and wheat (Block et al. 1997). As promoters specific for floral meristems or reproductive tissues become available, it is more feasible to generate both male and female sterility in transgenic plants. For example, the *SLG* promoter from *Brasicca*, which drives expression primarily in the stigmatic tissues of gynoecia and to a lesser extent in tapetal cells of anthers, is one of the pioneer promoters that has been used for engineering complete sterility. *SLG* is able to, when fused to the *DT-A* gene, which encodes a ribosomal inactivation factor, arrest the development of targeted tissues in tobacco and *Arabidopsis*, making plants completely sterile (Thorsness et al. 1991, 1993). Similar sterility was also produced with the use of the *Arabidopsis LFY* promoter, which is specific for floral meristem cells, and when fused to *DT-A* completely ablated sepals, petals, stamens, and carpels (Nilsson et al. 1998). Meiosis-specific promoters, such as the *AtDMC* promoter, further improve the accuracy and specificity of tissue ablation. As such, when this promoter is fused to *Barnase*, it enables the precise ablation of both male and female gametes without sacrificing flower appearance and architecture (Kobayashi et al. 2006), which is of aesthetic value for economically important ornamental cultivars. Furthermore, with an array of flower-specific promoters that have now been characterized in woody species, engineering male and female sterility in tree species is also becoming feasible. *PTLF* and *PTD* promoters from poplar, as well as *BpFULL* and *BpMADS1* from birch, are able to direct either *Barnase* or *DT-A* expression specifically in floral tissues, rendering transgenic trees completely sterile (Lannenpaa et al. 2005; Lemmetyinen et al. 2004; Skinner et al. 2003; Wei et al. 2007).

Despite its great effectiveness, current tissue- or cell-specific ablation systems suffer several undesirable drawbacks. The most prevailing of these is the presence of leaky promoter activity in non-targeted tissues, which often causes adverse effects on vegetative growth (Kobayashi et al. 2006; Lannenpaa et al. 2005; Lemmetyinen et al. 2004; Nilsson et al. 1998; Skinner et al. 2003; Wei et al. 2007). Although co-expression of *Barnase* together with its inhibitor, *Barstar*, could lessen such vegetative damage, this attenuation appears to be restricted to certain developmental stages and/or growth conditions (Wei et al. 2007). Whether this approach is effective and practically applicable under field conditions remains unknown. Another drawback of these ablation systems is the reversibility of the engineered sterility. For example, transgenic *Arabidopsis* plants harboring an *SLG::DT-A* cassette display perfect self-sterility, but become partially fertile when out-crossed with either wt male or female gametes (Thorsness et al. 1993). Since engineering sterile traits for transgene containment will eventually need to be deployed in the field, the influence of environmental or genetic factors could significantly impact the stringency of containment. Hence, engineering stable sterility without impairing non-targeted tissue is highly desirable, but is still a challenge and requires a continuous effort in the search and characterization of highly specific and tightly regulated promoters.

Recently, we have taken advantage of the tissue specificity of the enhancer within the *AGAMOUS* (*AG*) second intron for engineering complete sterility. *AG* encodes a floral homeotic MADS-box factor and acts to specify stamen and carpel identity and floral meristem determinacy in the inner two whorls of flowers, which has been very well characterized in *Arabidopsis* (Bowman et al. 1989, 1991). *AG* mRNA expression is highly specific for carpel and stamen primordial cells and tissues, but is absent in any other tissues (Drews et al. 1991; Yanofsky et al. 1990). An enhancer located within the *AG*'s second intron was found to primarily regulate this tissue-specific expression (Busch et al. 1999; Deyholos and Sieburth 2000; Sieburth and Meyerowitz 1997). Intriguingly, the *AG* promoter alone has been found to drive *GUS* expression ubiquitously (Sieburth and Meyerowitz 1997), while the isolated enhancer can, when fused with the minimal 35S promoter, confer a carpel- and stamen-specific expression pattern in transgenic plants (Busch et al. 1999; Deyholos and Sieburth 2000), a pattern that is identical to that of endogenous *AG* transcript accumulation in plants (Yanofsky et al. 1990). When the same enhancer fusion promoter was used for directing tissue-specific expression of *DT-A*, over 90% of transgenic *Arabidopsis* lines displayed precise ablation of both carpels and stamens without compromising vegetative growth (Liu and Liu 2008). The ensuing sterility was stable and persisted for several mitotic generations (Liu and Liu 2008). This *AG* enhancer-mediated tissue-specific ablation appears to be more efficient and precise than any other system reported to date. However, a similar construct, when introduced into tobacco, failed completely to ablate carpels and stamens in flowers, and instead, a mildly retarded floral phenotype was observed in only two of 25 lines analyzed (Wang et al. 2008). The failure of the *Arabidopsis AG* enhancer-derived promoter to instigate floral organ-specific ablation in tobacco suggests that the *AG* regulatory apparatus that has evolved in tobacco might, to a certain degree, have diverged from that in *Arabidopsis*, and the *AG* second intron may only interact or function effectively in more closely related species (e.g., same family). Since the tobacco *AG* ortholog(s) and its gene

organization, as well as its second intron/enhancer, has not been characterized as of yet, we set out to analyze and evaluate the tissue specificity of the second intron/enhancer from the *AG* ortholog of petunia, *pMADS3*, and assess its potential application for engineering complete sterility in tobacco plants to determine whether an *AG* enhancer can function in closely related species.

Materials and Methods

Isolation and Cloning of Petunia *AG* Second-Intron Fragments

Genomic DNA was extracted from the leaves of *Petunia hybrida* cultivar V26 grown in a greenhouse using the D-TAB method (Gustincich et al. 1991). Three hundred nanograms of genomic DNA was used as template for amplifying the approximately 4-kb second intron of *pMADS3* (*PtAGI*), one of two *AG* paralogs in petunia, using primer pair PMADS3U3151 (CTGTGCTCTGTGATGCT GAAGTTGCTTTGATT) and PMADS3L7275 (CTGAAC AAGCTTTCTTGTACCTCTCAATTGTTGCT). These primers were designed to immediately flank the splice junctions of the *PtAGI* in *pMADS3* (Kapoor et al. 2002; GenBank accession no. AB076051). Amplified fragments were cloned into the pGEM-T easy vector (Promega, Madison, WI) and verified by DNA sequencing. All PCR amplifications were performed using the following conditions: 2 min at 95°C, followed by 30 cycles of 40 s at 95°C, 1 min at 60°C, and 5 min at 72°C with a final extension of 10 min at 72°C.

Plasmid Construction and Plant Transformation

The *PtAGI* fragment was fused with the 60-bp minimal 35S promoter at its 5′ and 3′ ends to create functional *fPtAGIP* and *rPtAGIP* promoters, respectively. These chimeric promoters were isolated as *Fse*1-*Sbf*I fragments and inserted 5′ of the *GUS* and *DT-A* coding regions in a pBIN19 background to generate *fPtAGIP::GUS*, *rPtAGIP::GUS*, *fPtAGIP::DT-A*, and *rPtAGIP::DT-A* fusions, respectively, as illustrated in Fig. 1a. Plasmid DNA was isolated and introduced into *Agrobacterium tumeficiens* strain GV3101, which was subsequently used for the transformation of tobacco SR1 cultivar leaves in vitro (Horsch et al. 1985). Transformed cells and shoots were selected on standard MS medium containing 1 mg/L BA, 500 mg/L carbenicillin, and 200 mg/L kanamycin and were subsequently rooted in the same media lacking BA.

Histochemical GUS Assays

Tobacco plantlets grown in vitro, as well as hand-sectioned flower buds from 2-month-old plants grown in a greenhouse, were histochemically analyzed according to a previously described protocol (Jefferson et al. 1987). Briefly, all tissues were incubated in X-gluc solution (10 mM EDTA, 100 mM $NaH_2PO_4H_2O$, 0.5 mM $K_4Fe(CN)_63H_20$, 0.1% triton X-100, and 1 mM X-gluc) at 37°C overnight and were subsequently depigmented with 95% ethanol three to five times before photographing.

RT-PCR Analysis of Gene Expression in Transgenic Plants

Flower buds at the 0.2- to 0.4-cm stages were harvested for isolation of total RNA using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA). Genomic DNA in the RNA samples was removed by DNase digestion with the DNA-free kit (Ambion, Austin, TX) before RT-PCR analysis with 20 ng of treated RNA as template and the Titan One Tube RT-PCR kit (Qiagen). Primer pairs DTAU363 (CTTCGTACCACGGG ACTAAACTGGTTATGT) and DTAL800 (AAGTTCTA CGCTTAACGCTTTCGCCTGT) were used for analysis of *DT-A* transcripts, while NTACT66U1 (GTTATCTGAT TTGGCATAGCCT) and NTACT66L1 (TGGAATTGTAA GTTGTTTCGTG) were used to amplify the internal control, *Actin 66*. Amplification cycles included 45°C for 30 min for reverse transcription, 95°C for 15 min for the activation of Hotstart Taq DNA polymerase, followed by 27 cycles of 94°C for 1 min, 48–50°C for 1 min, and 72°C for 2 min. All amplified DNA fragments were resolved on a 1.2% agarose gel.

Evaluating the Stability of Engineered Sterility in Various Mitotic Generations

Three *CsVMV::GUS*, two *fPtAGIP::DT-A*, and three *rPtAGIP::DT-A* T_0 lines were chosen for stability analysis and were visually and microscopically evaluated for floral phenotypes. After flower development had terminated in the T_0 generation, the plants were cut back to approximately the fourth or fifth node from the base of the plant, and subsequent re-growth (termed T_0C_1) was subjected to similar phenotypic analyses. T_0C_1 plants were cut back one more time and T_0C_2 plants were reevaluated. The entire evaluation lasted approximately 8 to 9 months in a greenhouse. All data are summarized in Table 1.

Results

The *PtAGIP* Promoter Confers Distinct Floral Organ-Specific Expression

The petunia genome contains two *AG* homologs, *FBP6* and *pMADS3*, both of which are specifically expressed in carpel and stamen primordia and assume similar functions as

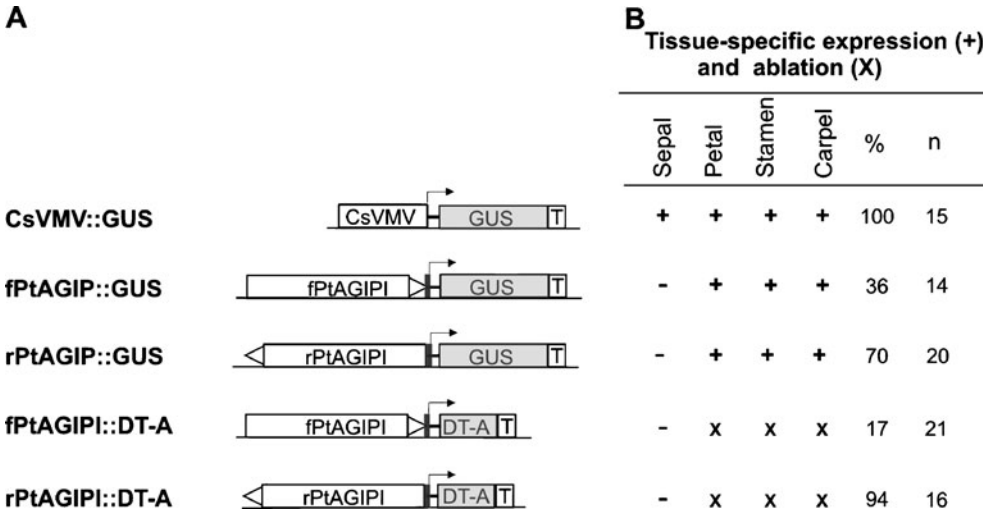

Fig. 1 Plasmid construction and tissue-specific analysis of chimeric *PtAGIP* promoters. **a** Gene constructs. Petunia *pMADS3*'s second intron (*PtAGI*) was fused with the minimal 35S promoter (illustrated as *filled bars*) at its 5′ and 3′ ends, respectively, to create forward-oriented *fPtAGIP* and reverse-oriented *rPtAGIP* promoters. The orientation of *PtAGI* is indicated by an *open arrowhead* at one end. The resulting promoters were placed in front of *GUS* and *DT-A* coding regions, respectively. Transcriptional start sites and direction of transcription are indicated by *angled arrows* above the minimal 35S promoter (*filled bars*). All gene fragments were inserted between the right and left T-DNA transfer borders in the binary vector pBIN19. *CaMV* cauliflower mosaic virus, *CsVMV* cassava vein mosaic virus promoter, *T* nos terminator. **b** Summary of tissue-specific expression and ablation examined in T_0 transgenic plants. Percentages (%) of the total evaluated lines (*n*) are indicated in each column. Floral organ tissues that express *GUS* are denoted by (+), while those that are completely ablated by *DT-A* expression are denoted by (X) and partially ablated or retarded by (X/−) symbols. The tissues that do not express detectable *GUS* or are not ablated by *DT-A* expression are denoted by (−)

demonstrated by ectopic expression studies (Kater et al. 1998). Further analyses showed that the *pMADS3* genomic region contains nine exons and eight introns with sizes and locations similar to those characterized in the *Arabidopsis AG* gene (Busch et al. 1999; Deyholos and Sieburth 2000; Kapoor et al. 2002). The second intron in *pMADS3* is about 1 kb longer than the *Arabidopsis AG*'s second intron, but they both share similar transcription factor binding sites (Hong et al. 2003; Kapoor et al. 2002), which strongly suggests that the second intron of *pMADS3* assumes a similar regulatory role as its *Arabidopsis* counterpart. Based on this information, we cloned and verified the second intron fragment from *pMADS3*. For convenience and consistency with earlier work, we termed this fragment *PtAGI*. To create functional promoters, we fused a minimal 35S promoter sequence to the *PtAGI* fragment at its 5′ and 3′ ends, respectively, generating forward- and reverse-oriented chimeric promoters, *fPtAGIP* and *rPtAGIP*, which were used to drive *GUS* expression in tobacco plants (Fig. 1a). At least 30 in vitro grown plantlets harboring each gene fusion, *fPtAGIP::GUS*, *rPtAGIP::GUS*, *CsVMN::GUS*, and empty vector, were initially screened with an X-gluc staining assay. Like empty vector plants (Fig. 2b), no visible GUS activity was detected in root, leaf, or stem tissues in either *fPtAGIP::GUS* or *rPtAGIP::GUS* lines (Fig. 2c, d), which is in contrast to *CsVMV::GUS* lines (Fig. 2a). Similarly, greenhouse-grown *fPtAGIP::GUS* and *rPtAGIP::GUS* plants also showed no detectable GUS activity in vegetative tissues (data not shown). However, strong *GUS* expression was detected in flower tissue specifically in the carpels and stamens, and these plants also exhibited weak but detectable GUS activity in petal tissues (Fig. 2k, l), which has never been observed for the *Arabidopsis AtAGIP* promoter (Liu and Liu 2008). GUS

Table 1 Mitotic stability of male and female sterility in transgenic plants

	T_0		T_0C_1		T_0C_2	
	No. of flowers	No. of flowers with carpels, petals, and stamens	No. of flowers	No. of flowers with carpels, petals, and stamens	No. of flowers	No. of flowers with carpels, petals, and stamens
CsVMV::GUS (3 lines)	283	283	265	265	228	228
fPtAGIP::DT-A (2 lines)	346	0	226	0	256	0
rPtAGIP::DT-A (3 lines)	574	0	321	0	435	0

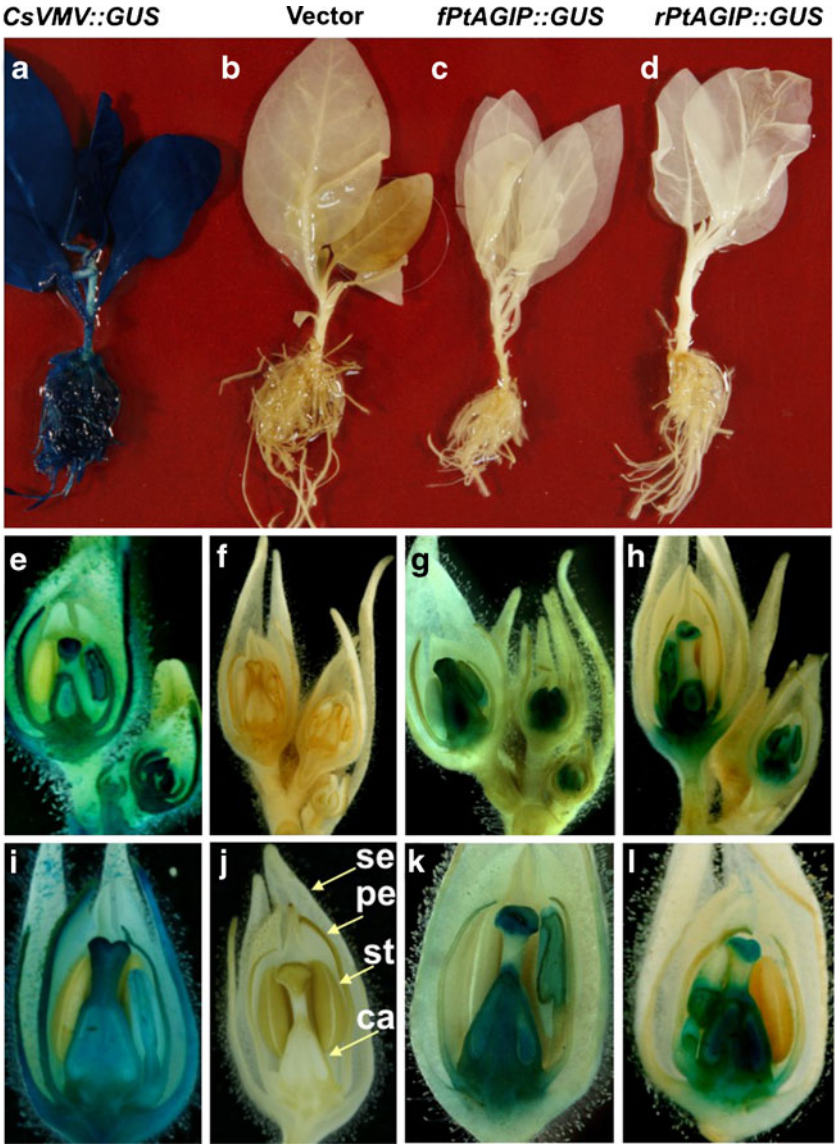

Fig. 2 Floral organ-specific *GUS* expression. Four- to five-week-old *in vitro* grown plantlets (**a**, **b**, **c**, **d**), longitudinally hand-sectioned flower buds at stages 9 – 12 (**i**, **j**, **k**, **l**) and floral inflorescences with stage 4 – 10 flower buds (**e**, **f**, **g**, **h**), which contain *CsVMV::GUS* (**a**, **e**, **i**), control vector pBIN19 (**b**, **f**, **j**), *fPtAGIP::GUS* (**c**, **g**, **k**) or *rPtAGIP::GUS* (**d**, **h**, **l**) transgenes, respectively, were histochemically analyzed with X-gluc staining. Flower developmental stages were defined based on earlier descriptions (Mandel et al. 1992). Note that only tissues that were cut or subjected to section were accessible to X-gluc staining and displayed blue coloration. se – sepal; pe – petal; st – stamen; ca – carpel

activity could be detected in flower buds as early as stages 4–5 and persisted through the remaining flower development stages (Fig. 2g, h). Interestingly, we noticed that *fPtAGIP::GUS* and *rPtAGIP::GUS* plants differed in *GUS* expression efficiency. While approximately 70% of 20 *rPtAGIP::GUS* lines exhibited *GUS* expression in flowers, only 36% of 14 *fPtAGIP::GUS* lines displayed a *GUS* expression phenotype (Fig. 1b). Evidently, the activity of the chimeric promoters is influenced by the orientation of the incorporated *PtAGI* fragment.

Floral Organ-Specific Ablation by *PtAGIP* Fused to a Cytotxic Gene

Arabidopsis AtAGIP promoters displaying carpel- and stamen-specific expression can direct a cytotoxic gene for specific ablation of both carpels and stamens, generating complete sterility (Liu and Liu 2008). To ascertain whether the *PtAGIP* promoters have a similar function, we fused them to the *DT-A* coding region, which encodes a ribosome inactivating protein (Palmiter et al. 1987), to create *fPtAGIP::DT-A* and *rPtAGIP::DT-A* fusions, respectively (Fig. 1a), and tested them in tobacco plants. As expected, both *fPtAGIP::DT-A* and *rPtAGIP::DT-A* lines showed normal vegetative growth, with similar numbers of leaves produced during development, and no growth damage was observed until the reproductive process began (Fig. 3b, c). Perturbed flower buds became apparent at very early stages of inflorescence development in *fPtAGIP::DT-A* and *rPtAGIP::DT-A* lines (Fig. 3b, c). The abnormal flower buds were small and lacked visible petals (Fig. 3e, f, g) compared to wt flowers with large, visible petals in *CsVMV::GUS* plants (Fig. 3d). Longitudinal tissue sections revealed that all carpel and stamen tissues were completely ablated and the

◀ **Fig. 3** Tissue-specific ablation and generation of complete sterility. Two-month-old *CsVMV::GUS* plants grown in a greenhouse displayed normal flower buds (**d**, **h**, **i**), inflorescence development (**a**, **h**) and reproductive processes (**a**), but both *fPtAGIP::DT-A* (**b**, **e**, **f**, **j**, **k**) and *rPtAGIP::DT-A* (**c**, **g**, **l**, **m**) plants manifested a very similar abnormal flower phenotype and reproductive processes with severely perturbed inflorescences (**j**, **l**), flower buds (**e**, **f**, **g**), as well as completely ablated carpels and stamens (**k**, **m**). Wt flower buds from the *CsVMV::GUS* plants at stages of 0.7, 1.0, and 1.5 cm (**i**) and perturbed flower buds of from *fPtAGIP::DT-A* (**k**) and *rPtAGIP::DT-A* (**m**) plants at corresponding developmental stages were longitudinally hand-sectioned

residual tissues observed within perturbed flower buds in the early stages of floral development were primarily retarded petal tissue that eventually died (Fig. 3k, m), again confirming weak activity of the chimeric promoters in petal tissues. Consistent with the influence of the orientation of the *PtAGI* on *GUS* expression, the *rPtAGIP::DT-A* fusion was much more efficient than *fPtAGIP::DT-A* in directing the tissue-specific ablation of floral organs in transgenic plants. Figure 1b shows that as many as 94% of 16 *rPtAGIP::DT-A* plants exhibited floral organ ablation in comparison to only 17% of 21 *fPtAGIP::DT-A* plants with ablated floral organs.

The relationship between *DT-A* expression and floral organ-specific ablation was examined using RT-PCR analysis. Figure 4 shows that *DT-A* mRNA was detected in perturbed flowers from all six lines analyzed harboring each *PtAGIP::DT-A* fusion, but not in two *CsVMV::GUS* lines. Conversely, tobacco *Actin* 66 gene expression was detected in both *CsVMV::GUS* and *PtAGIP::DT-A* lines, indicating that the abnormal flower phenotype corresponded to *DT-A* expression.

Analysis of the Mitotic Stability of Engineered Sterility

The effective containment of transgene flow in ornamental, forestry, landscape, and bioenergy crops requires stable and irreversible sterility. Because of their sterile nature, it was impossible to analyze the meiotic stability of engineered sterility in either *fPTAGIP::DT-A* or *rPtAGIP::DT-A* plants. Instead, we analyzed their mitotic stability by continuously cutting back the plants into successive generations (Liu and Liu 2008). Two *fPtAGIP::DT-A* and three *rPtAGIP::DT-A* lines bearing the same flower phenotype, as well as three *CsVMV::DT-A* control lines, were chosen for this analysis. All T_0 plants were subjected to two consecutive cutbacks with the mitotic generations designated T_0C1 and T_0C2, respectively. Table 1 shows that the more than 700 flowers examined in the *CsVMV::GUS* plants from T_0, T_0C1, and T_0C2 generations exhibited the same flower phenotype with no altered flower or floral organ observed. Similarly, over 2,000 flowers evaluated in the *fPtAGIP::DT-A* and *rPtAGIP::DT-A* lines over three mitotic generations, respectively, displayed identical ablated flower phenotypes with no revertants observed. The entire evaluation process took more than 8 months in a greenhouse and experienced a wide range of temperature fluctuations ranging from as low as 10°C in the winter and as high as 40°C in the summer. The fact that no flower revertants were observed during our analyses strongly suggests that the perturbed phenotype induced by either *rPtAGIP::DT-A* or *fPtAGIP::DT-A* in tobacco is thermally stable.

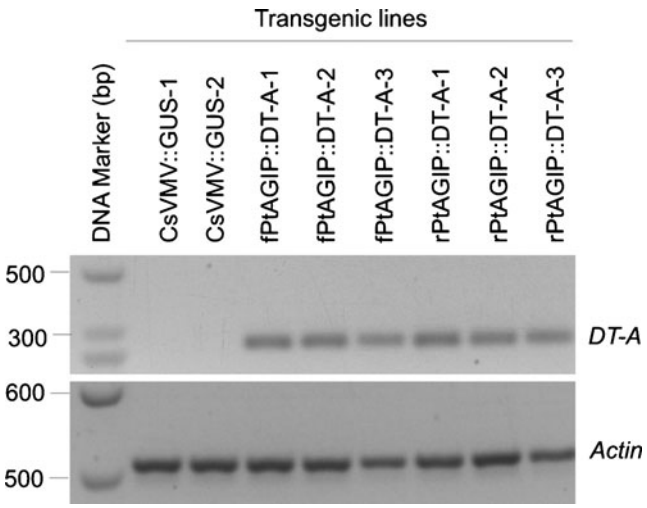

Fig. 4 Verification of *DT-A* expression in perturbed flowers by RT-PCR. Total RNA samples were isolated from young flower buds at the 0.2- to 0.4-cm stages and treated with DNase prior to RT-PCR amplification. Tobacco *Actin* 66 served as an internal control

Discussion

In this study, we analyzed chimeric promoters derived from the *AG* enhancer of petunia, which is taxonomically closely related to tobacco, and showed that this promoter can, unlike its *Arabidopsis* counterpart, confer floral organ-specific expression and tissue ablation in tobacco (Figs. 2 and 3). The distinct behaviors of the petunia and *Arabidopsis AGIP* promoters in the same heterologous tobacco host likely reflects the degree of evolutionary conservation and divergence of the *AG* regulatory mechanism in tobacco, *Arabidopsis*, and petunia.

AG plays a critical role in specifying both male and female floral organ identity in plants (Bowman et al. 1989, 1991; Yanofsky et al. 1990) and is specifically expressed in carpel and stamen primordial cells and tissue (Drews et al. 1991; Yanofsky et al. 1990), which is dictated by a enhancer element that resides within the second intron rather than its promoter (Busch et al. 1999; Deyholos and Sieburth 2000; Sieburth and Meyerowitz 1997). The *AG* enhancer bears a multitude of protein factor binding sites where both positive and negative regulators such as LEAFY, WUSCHEL, APETALA2, LEUNIG, SEUSS, BELLRINGER, and SEP3 bind to help define *AG* enhancer-governed tissue specificity in response to developmental cues (Bao et al. 2004; Bomblies et al. 1999; Busch et al. 1999; Deyholos and Sieburth 2000; Lohmann et al. 2001; Mayer et al. 1998; Parcy et al. 1998; Weigel et al. 1992). Such a complex interaction probably demands a high degree of host specificity of the *AG*'s enhancer function because any significant divergence in either enhancer or regulatory factors could impair their intimate interactions. Hence, the inability of the *Arabidopsis AG* second intron-derived *AtAGIP* promoters to properly function in tobacco (Wang et al. 2008) could be attributable to the physical and functional divergence of both the introduced *Arabidopsis AG* enhancer and native transcription factors in tobacco. If this notion holds true, the *AG* second intron/enhancer from closely related species (e.g., tomato or petunia) should faithfully function in tobacco, assuming that all *AG* enhancers in their native hosts play similar regulatory roles. In agreement with this prediction, the isolated petunia *AG* enhancer and its derived chimeric *PtAGIP* promoters were able to drive carpel- and stamen-specific *GUS* expression and *DT-A*-mediated tissue ablation in tobacco (Figs. 1b, 2, and 3), which resembles that observed with the *AtAGIP* promoter in *Arabidopsis*, indicating that the role of the *AG* enhancer in the regulation of their residential gene expression is broadly conserved in plants but its regulatory mechanism and apparatus is relatively conserved in evolutionarily related species (e.g., tobacco and petunia).

The observation of additional petal-specific activity manifested by *GUS* expression and a partially retarded petal phenotype in *PtAGIP::DT-A* lines suggests that the petunia *AG* enhancer has gained additional tissue specificity that has not been found in its counterpart in *Arabidopsis*. However, previous studies showed that *pMADS3* is specifically expressed in stamens and carpels in petunia and its transcript is undetectable in petal tissue (Kapoor et al. 2002; Kater et al. 1998), but this research was based on RNA blot analysis, which may not be as sensitive as the transgenic expression and tissue ablation used in our study to detect weak activity. Hence, whether the observed petal-specific activity is a result of its inherent activity or stochastically gained in tobacco awaits further characterization of the function of the *AG* enhancers in their native petunia and tobacco hosts.

We previously demonstrated that *Arabidopsis AtAGIP* promoters with either forward- or reverse-oriented *AG* enhancer fragments yielded similar *GUS* expression activity and tissue ablation efficiencies (Liu and Liu 2008). However, the activity of the petunia *PtAGIP* promoters is substantially influenced by the orientation of the *AG* second intron/enhancer (Fig. 1b), which raises interesting questions about the underlying mechanisms. In *Arabidopsis*, the *AG* enhancer is located within the 3-kb second intron, but the 1.7-kb region located at its 3′ end is critical for its function (Busch et al. 1999; Deyholos and Sieburth 2000). This critical region has also been found to house eight of the nine binding sites for regulatory factors discussed above (Bao et al. 2004; Bomblies et al. 1999; Busch et al. 1999; Lohmann et al. 2001; Mayer et al. 1998; Parcy et al. 1998). These transcription factor binding sites were also found to be conserved in the enhancer of *pMADS3* from petunia, but their locations are quite different (Hong et al. 2003).

Instead, eight of the nine binding sites were found to reside in the first 2-kb region of the 4-kb enhancer fragment. Conceivably, the reverse orientation of the petunia *AG* enhancer positions the key regulatory region in closer proximity to the minimal 35S promoter, allowing more efficient or intimate physical interactions between the *AG* enhancer and minimal 35S promoter, which could contribute to the observed orientation-dependent promoter activity.

Our work revealed that the petunia *PtAGIP* promoters can effectively function in evolutionarily closely related tobacco species, while the *Arabidopsis AtAGIP* promoter fails in this same species. *PtAGIP*-conferred tissue specificity is analogous to that conferred by the *AtAGIP* promoter in general (Liu and Liu 2008), indicating that *AG* enhancer-mediated regulation of its residential gene is widely conserved among plants. Importantly, the chimeric promoters created here can direct the tissue-specific ablation of carpels and stamens without compromising vegetative growth, and the *rPtAGIP* promoter in particular can achieve up to 94% ablation efficiency in evolutionarily closely related tobacco plants (Fig. 1b), which is comparable to the efficiency obtained with the *AtAGIP::DT-A* fusion in its native host (Liu and Liu 2008). This signifies that *AG* enhancer-derived promoters are ideal for engineering male and female sterility in their native hosts as well as in closely related species. Given that *AG* orthologs and their tissue-specificity are widely conserved in many species (Kramer et al. 2004; Zahn et al. 2006), engineering complete sterility for containing pollen-, fruit-, and seed-mediated gene flow could be achieved across a wide range of species using a similar approach. The demonstrated stability of the engineered sterile trait in various mitotic generations in a greenhouse with significant temperature fluctuations further validates our earlier report on the mitotic stability of sterility with the *AtAGIP* promoters in *Arabidopsis* (Liu and Liu 2008) and is of significance for the application of this engineered sterility as a practical tool for gene containment in field conditions.

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