

The second intron of *AGAMOUS* drives carpel- and stamen-specific expression sufficient to induce complete sterility in *Arabidopsis*

Zongrang Liu · Zhongchi Liu

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Abstract Gene containment technologies that prevent transgene dispersal through pollen, fruit and seed are in immediate demand to address concerns of gene flow from transgenic crops into wild species or close relatives. In this study, we isolated the enhancer element of *Arabidopsis AGAMOUS* that drives gene expression specifically in stamens and carpels. By fusing this *AG* enhancer to a minimal 35S promoter fragment, two tissue-specific promoters, *fAGIP* and *rAGIP* in forward and reverse orientations, respectively, were created and fused to the GUS reporter. Transgenic *Arabidopsis* plants harboring either *fAGIP::GUS* or *rAGIP::GUS* displayed similar GUS expression specifically in carpel and stamen tissues and their primordial cells. To test their utility for engineering sterility, the promoters were fused to the *Diphtheria toxin A (DT-A)* gene coding for a ribosome inactivating protein as well as the *Barnase* gene coding for an extracellular ribonuclease, and tested for tissue-specific ablation. Over 89% of *AGIP::DT-A* and 68% of *AGIP::Barnase* transgenic plants displayed specific and precise ablation of stamens and carpels and are completely sterile. These transgenic plants showed normal vegetative development with prolonged vegetative growth. To evaluate the stability of the sterile phenotype, 16 *AGIP::DT-A* lines underwent two consecutive cutback generations and showed no

reversion of the floral phenotype. This study demonstrates a simple, precise and efficient approach to achieve absolute sterility through irreversible ablation of both male and female floral organs. This approach should have a practical application for transgene containment in ornamental, landscaping, and woody species, whose seeds and fruits are of no economic value.

Keywords *AGAMOUS* · Transgenic plants · Sterility · Transgene containment

Introduction

Despite the great potential of agrobiotechnology for crop improvement, growing concerns about pollen-, seed- and fruit-mediated transgene flow into closely related species have impacted the confidence of growers and consumers with regard to genetically modified crops and foods. Copious amounts of pollen from transgenic crops could pollinate and hybridize with their wild relatives, leading to introgression of superior engineered traits into wild relatives (Ellstrand 2001; Ellstrand et al. 1999; Mikkelsen et al. 1996; Snow and Palma 1997). Seeds or fruits released into the environment from transgenic crops could develop into unwanted 'weedy' plants that could compete with unmodified crops in subsequent years. These transgenic populations could naturalize and persist as feral weed populations, which could also pass the transgene into their wild relatives (Snow 2002). The consequence of transgene flow may create super or invasive weeds (Hall et al. 2000), adding management burdens to farmers and resulting in further invasion of natural habitats (Dale et al. 2002). Transgene flow may also have an unintended impact. For example, spreading of a transgene encoding a *Bt* toxin from

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Z. Liu (✉)
USDA-ARS, Appalachian Fruit Research Station,
2217 Wiltshire Road, Kearneysville, WV 25430, USA
e-mail: zongrang.liu@ars.usda.gov

Z. Liu
Department of Cell Biology and Molecular Genetics,
University of Maryland, College Park, MD 20742, USA

crop plants to wild populations could pressure the exposed insects to rapidly evolve resistance to *Bt*. In addition, transgenes created to produce pharmaceutical agents and industrial compounds could end up in unintended locations through transgene flow (Ellstrand 2003). Thus, containing and mitigating transgene flow is of paramount importance to the success of agrobiotechnology, preservation of ecological and environmental balance, and enhancement of economic development.

Diverse crop plants require an array of available technologies for gene containment. Many crops such as maize and soybean produce edible seeds; while others like apples and citrus produce fruits for human consumption. In contrast, forest and ornamental plants serve different purposes, and their fruit and seeds are of little economical value. Hence, the containment strategy should vary from crop to crop.

The generation of male and female sterility using a cytotoxic gene through tissue-specific ablation is an effective method to contain transgene flow for non-grain and non-fruit producing plants such as ornamental, landscape and forestry species. The *Barnase* gene under the control of a tapetum-specific promoter was used for engineering male sterility a decade ago (Mariani et al. 1990; Mariani et al. 1992). Since then, cytotoxic genes in combination with male or female tissue-specific promoters have been exploited for generating reproductive sterility in various species (Beals and Goldburg 1997; Block and Debrouwer 1993; Block et al. 1997; Burgess et al. 2002; Gleba et al. 2004). A similar approach has been applied to woody species such as *Populus* (Skinner et al. 2003; Wei et al. 2007) and birch trees (Lannenpaa et al. 2005; Lemmetyinen et al. 2004b; Lemmetyinen et al. 2001). In many cases, severe damage to vegetative growth due to basal or leaky promoter activity in non-targeted tissues was often observed in transgenic plants (Lannenpaa et al. 2005; Wei et al. 2007). This poses serious challenges in containing transgene flow without compromising vegetative growth. To address this problem, an additional gene repression system was introduced together with the gene ablation system so that *Barnase* activity could be repressed by *Barstar* expressed specifically in non-targeted tissue with a weak constitutive promoter (Kobayashi et al. 2006; Wei et al. 2007). This approach has been used successfully to engineer male and female sterility using gamete-specific or floral-specific expression of *Barnase* in tobacco or poplar, respectively (Kobayashi et al. 2006; Wei et al. 2007). Despite efficient attenuation of *Barnase* activity by *Barstar* in non-targeted tissues under laboratory and greenhouse conditions, vegetative growth of transgenic trees was, after one or two growing seasons in the field, often compromised (Wei et al. 2007), indicating that *Barstar*-mediated attenuation of *Barnase* in vegetative tissue is

reversible and conditioned at certain developmental stages or/and growth conditions. Hence, complete and precise containment of transgene flow requires highly specific promoters for reproductive tissues or organs.

Here, we tested tissue-specific ablation of male and female floral organs by expressing cytotoxic genes under a floral organ-specific promoter derived from an enhancer element in the second intron of the *Arabidopsis* *AGAMOUS* (*AG*) gene. This *AG* intron has been previously shown to direct β -glucuronidase (*GUS*) reporter gene expression specifically in stamen and carpel primordial cells at early stages of flower development (Busch et al. 1999; Deyholos and Sieburth 2000; Sieburth and Meyerowitz 1997). We demonstrate that the precise and efficient engineering of male and female sterility can be achieved using this *AG* enhancer-derived promoter.

Materials and methods

Plasmid construction and plant transformation

The approximately 3.0 kb *AGAMOUS* second intron (*AGI*) was isolated from *Arabidopsis* accession C10 genomic DNA by PCR amplification with primers AGIntU693 (gaaatctgggagaggaaagatcga) and AGIntL3962 (cattaattctgccagatatccggtgt). The amplified fragment was cloned into the pDRIVE plasmid (Qiagen, Valencia, CA), and verified by DNA sequencing. To create functional promoters, the minimal CaMV 35S promoter (-60 position) was fused to the *AGI* fragment in both forward and reverse orientations to generate *fAGIP* and *rAGIP*, respectively (Fig. 1). Fragments containing *fAGIP* and *rAGIP* promoters were isolated and inserted 5' to *GUS* or *DT-A* coding regions to create *fAGIP::GUS*, *fAGIP::DT-A*, *rAGIP::GUS* and *rAGIP::DT-A* fusions in the binary vector pBIN19 (Fig. 1). The control plasmid *CsVMV::GUS* was made by inserting an ~600 bp *HindIII*–*Bam*H I fragment from pAD3850 (a gift from The Scripps Research Institute, San Diego, CA) into the same sites of pR2059. *fAGIP::Barnase* and *rAGIP::Barnase* fusions were created by inserting *fAGIP* and *rAGIP* promoter fragments into the *Bam*HI site between *Barnase* and *Barstar* coding regions in pR2001, respectively. All plasmids were introduced into *Agrobacterium tumefaciens* strain GV3101 via electroporation. *Arabidopsis* transformations were performed using the floral dip method described by Clough and Bent (1998).

Tissue sectioning and histochemical GUS assays

Plant tissues were stained with X-gluc solution containing 10 mM EDTA, 100 mM NaH₂PO₄H₂O, 0.5 mM K₄Fe

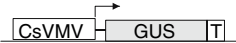
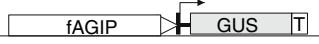

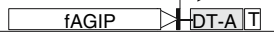
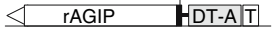
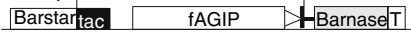
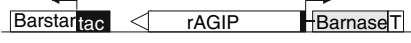
		Frequency of transgenic lines with the ablated stamens and carpels	
		%	n
CsVMV::GUS		0	15
fAGIP::GUS		0	12
rAGIP::GUS		0	20
fAGIP::DT-A		96	30
rAGIP::DT-A		89	55
fAGIP::Barnase		68	35
rAGIP::Barnase		80	36

Fig. 1 Diagram of various plasmid constructs and the efficiency of their tissue specific ablation. An approximately 3.0 kb *Arabidopsis AGAMOUS* second intron (*AGI*) was fused with the minimal CaMV 35S promoter sequence (illustrated as *filled bars*) to create a forward-oriented *AGI* promoter (*fAGIP*) and a reverse-oriented *AGI* promoter (*rAGIP*). The orientation of *AGI* is indicated by an *open arrow head* at one end. The resulting promoters were used to drive expression of *GUS*, *DT-A* and *Barnase*. All gene fragments illustrated above have

(CN)₆H₂O, 0.1% triton X-100 and 1 mM X-gluc. After overnight incubation at 37°C, tissues were bleached with 95% ethanol twice before photographing. Tissue section analysis, GUS staining, tissue fixation, sectioning, and visualization were based on a previously described protocol (Blazquez et al. 1997).

RT-PCR analysis

Total RNA was isolated from young flower buds using the RNeasy Plant Mini Kit (Qiagen), and was treated with DNase to remove contaminating genomic DNA using the DNA-free kit (Ambion, Austin, TX). RNA quality was examined by gel electrophoresis. RT-PCR analysis was performed using the Titan One Tube RT-PCR kit (Qiagen) according to the manufacture's instructions. Twenty ng of total RNA was used for RT-PCR amplification with primer pairs DTAU363 (cttcgtaccacgggactaaactggttatgt) with DTAL800 (aagtctacgcttaacgctttcgctgt), and EF1F (gcactgtcattgatg ctcc) with EF1R (gtcaagagcctcaaggagag). Amplification cycles included 45°C for 30 min for the reverse transcription step and 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.

been inserted between the right and left T-DNA transfer borders in the binary vector pBIN19. Transcriptional start sites and direction are indicated by *angled arrows*. *CsVMV* cassava vein mosaic virus promoter; *T* nos terminator. *tac* bacterial cell-expressing promoter. Floral morphology was examined in T₁ transgenic plants and percentages (%) of the total evaluated lines (*n*) that showed complete sterility are shown in the *right* column

Evaluation of vegetative stability of ablated carpels and stamens in transgenic plants

Approximately 400–700 flowers from the chosen eight lines for each construct were evaluated under a microscope for the presence of carpels and stamens in the T₁ generation. The same lines of plants evaluated in the T₁ generation were cut back and the re-grown generation (T₁C₁) was subjected to the same analysis. T₁C₁ plants were cut back one more time and re-growth (T₁C₂) was similarly evaluated. To ensure the robust growth of wild type plants in continuous cutback generations, all young siliques were removed from control *CsVMV::GUS* plants immediately after they were formed because we have found that this prevents plants from undergoing senescence, which enables plants to live as long as 4–5 months (data not shown). Usually, the T₁C₂ generation plants exhibited weak growth and could not be used for further cut back analysis.

Results

Developing stamen and carpel specific promoters using the second intron of *AG*

Arabidopsis AG encodes a key regulator of carpel and stamen organ identity in flowers (Yanofsky et al. 1990).

AG expression is initiated in the stage 3 floral meristem and is exclusively restricted to carpel and stamen primordia (Yanofsky et al. 1990; Drews et al. 1991). The second intron of *AG* (*AGI*) was previously identified to contain *cis* elements that direct carpel- and stamen-specific expression of *AG* (Busch et al. 1999; Deyholos and Sieburth 2000; Sieburth and Meyerowitz 1997). This intronic sequence contains conserved motifs and binding sites for several transcription factors with roles in floral development (Bao et al. 2004; Busch et al. 1999; Hong et al. 2003; Lohmann et al. 2001). These motifs are conserved across a wide range of species (Hong et al. 2003). Thus, *AGI* appears to qualify as an ideal regulatory element for directing stamen- and carpel-specific gene expression and ablation. To test this, we isolated the 3-kb *AGI* fragment from the *Arabidopsis* genome and placed it upstream of the minimal 35S promoter in both forward and reverse orientations to create *fAGIP* and *rAGIP* promoters, respectively. These promoters were then fused to the *GUS* coding region to create *fAGIP::GUS* and *rAGIP::GUS* fusions (Fig. 1). Control plants transformed with a *CsVMV::GUS* fusion displayed *GUS* expression in all tissues (Fig. 2a). In contrast, *AGIP::GUS* plants exhibited flower-specific expression. Eight of 12 *fAGIP::GUS* lines showed *GUS* expression only in stamens and carpels but not in sepals and petals nor in vegetative tissues such as leaves, roots and stems (Fig. 2b). Fifteen of 20 *rAGIP::GUS* lines displayed a similar stamen- and carpel- specific expression pattern (Fig. 2c), indicating that the forward- and reverse-oriented *AGIP* promoters confer similar specific promoter activity. Tissue section analysis of the representative *fAGIP::GUS* and *rAGIP::GUS* lines revealed that *GUS* staining is present in the primordia of carpels and stamens, but absent in the shoot apical meristem (SAM) or surrounding tissues

(Fig. 2d, e). These results are consistent with earlier findings (Busch et al. 1999; Deyholos and Sieburth 2000; Sieburth and Meyerowitz 1997).

Precise and specific ablation of carpels and stamens by *AGIP* promoters

To test if the *AGIP* promoters could be used for engineering male and female sterility, the *fAGIP* and *rAGIP* promoters were used to drive the *Diphtheria toxin A* (*DT-A*) coding region to create *fAGIP::DT-A* and *rAGIP::DT-A* (Fig. 1). Of 30 *fAGIP::DT-A* lines analyzed, 29 lines displayed normal sepals and petals but lacked stamens and carpels in all flowers (Fig. 3c, d). As a result, these lines produced no pollen, siliques or seeds, and were completely sterile (Fig. 4b, d, g) in comparison to wild type (Figs. 3a, b, 4a, f, i). These transgenic plants with sterile flowers continue to grow for up to 4 months, which is significantly longer than the two-month life span of wild type plants under the same conditions (data not shown). In addition, these plants grew taller and produced more biomass than wild type plants at week 6 (Fig. 4g, i). These results support the notion that reduced plant fertility diverts energy for vegetative growth and biomass production. This observation is of particular importance for species used for biomass production.

Similar phenotypes were also observed in 89% of 55 transgenic lines harboring the *rAGIP::DT-A* transgene (Figs. 3e, f, 4c, e, h). Interestingly, no intermediate or partially ablated floral phenotypes were observed. This is in contrast with earlier studies using various other promoters for flower-specific ablation, which usually displayed diverse phenotypes ranging from mild to severe retardation

Fig. 2 Tissue-specific *GUS* expression directed by the *AGIP* promoters. **a–c** are whole-mount histochemical staining by X-gluc; **d** and **e** are tissue sections of inflorescences. **a** *CsVMV::GUS*; **b** *fAGIP::GUS*; **c** *rAGIP::GUS*; **d** *fAGIP::GUS* expression in stamen and carpel primordia (stage 3–4 flower bud to the left and stage 7–8 flower bud on the right); **e** *rAGIP::GUS* expression in stamen and carpel primordia. *SAM* shoot apical meristem

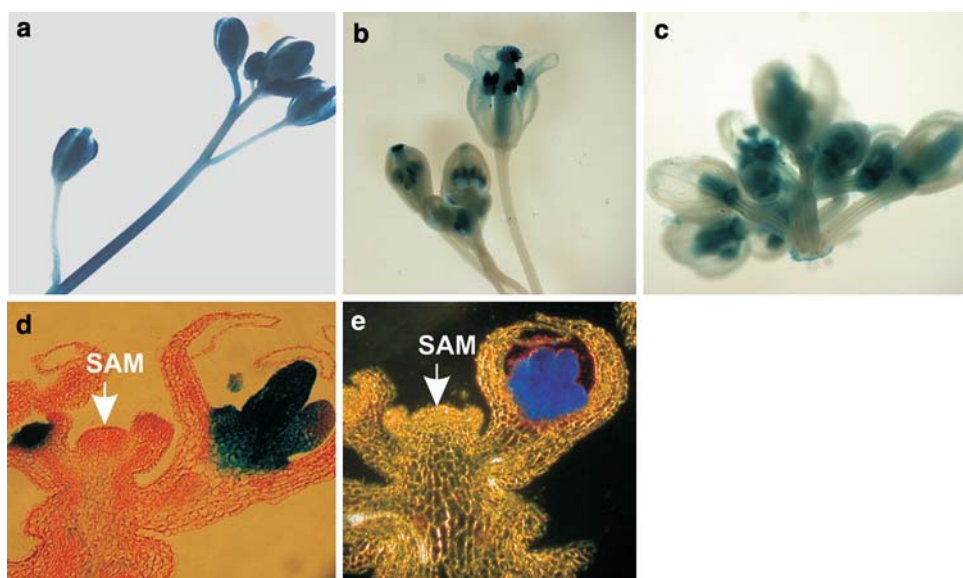
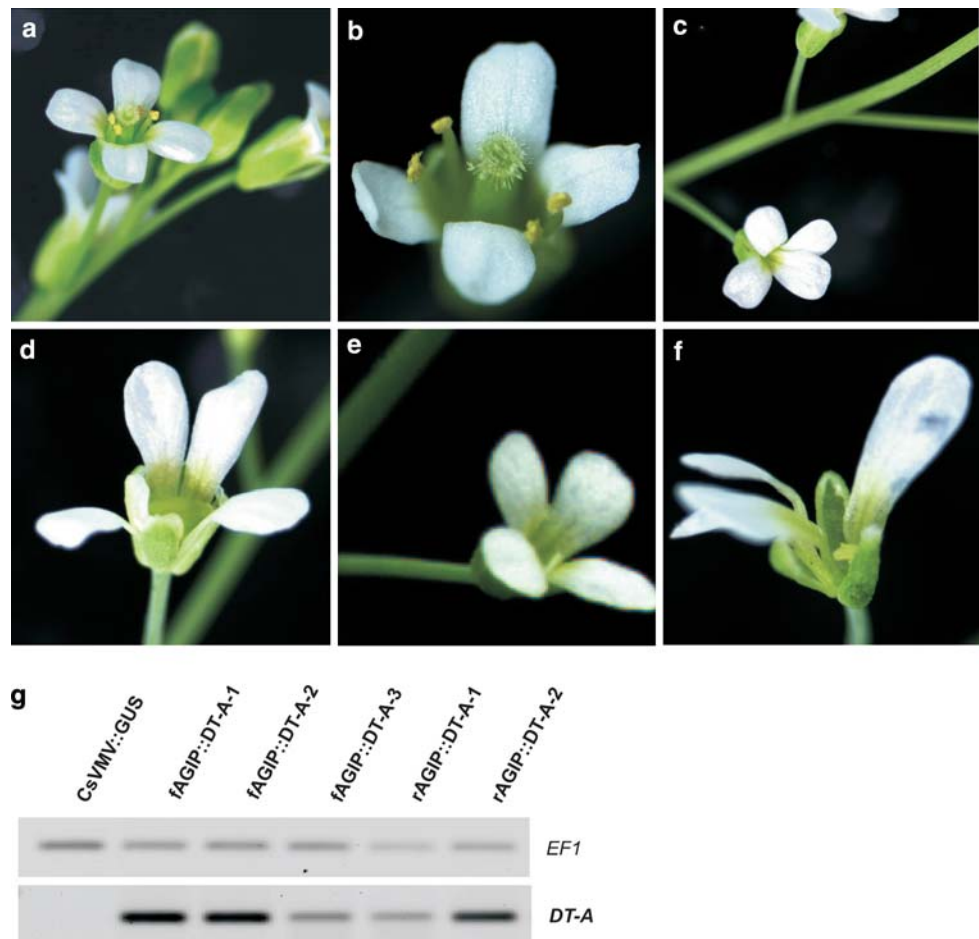


Fig. 3 Tissue-specific ablation of carpel and stamen organs in transgenic plants. (a, b) Wild type flowers containing both carpels and stamens; (c, d) *fAGIP::DT-A* transgenic flowers with no stamens or carpels; (e, f) *rAGIP::DT-A* transgenic plants with flowers missing stamens and carpels; (g) RT-PCR analysis of *DT-A* expression in floral tissues from the transgenic plants indicated. *CsVMV::GUS* transgenic plant serves as a control. *EF1a* serves as an internal amplification control



in vegetative growth (Lannenpaa et al. 2005; Nilsson et al. 1998; Skinner et al. 2003; Wei et al. 2007). *DT-A* expression was analyzed by RT-PCR and *DT-A* mRNAs were detected in flowers of 3 *fAGIP::DT-A* and 2 *rAGIP::DT-A* transgenic lines but were absent in the transgenic control line containing *CsVMV::GUS* (Fig. 3d). Therefore, *DT-A* expression in floral tissues of the transgenic lines correlated with the absence of stamens and carpels in these lines.

In a parallel experiment, the *fAGIP* and *rAGIP* promoters were fused to the *Barnase* coding region, and transgenic plants were tested for tissue-specific ablation. As illustrated in Fig. 1, an additional copy of the *Barstar* gene driven by the bacterial *tac* promoter was inserted into the vector backbone to neutralize the detrimental effect of *Barnase* in the bacterial host (Beals and Goldman 1997). The majority of *fAGIP::Barnase* or *rAGIP::Barnase* lines displayed complete ablation of stamens and carpels (data not shown). This phenotype is identical to that observed in *fAGIP::DT-A* and *rAGIP::DT-A* lines (Figs. 3, 4). However, both *AGIP::Barnase* fusions showed relatively low efficiencies in that only 68% *fAGIP::Barnase* and 80% *rAGIP::Barnase* displayed male and female sterility, in comparison to 89% and 96% sterility induced by

rAGIP::DT-A and *fAGIP::DT-A*, respectively (Fig. 1). Nevertheless, *AGIP*-directed expression of *DT-A* or *Barnase* is able to precisely and efficiently ablate both male and female organs without compromising vegetative growth.

Mitotic maintenance of male and female sterility in transgenic plants

The precise and efficient ablation of male and female reproductive organs by *AGIP* directed expression of *DT-A* and *Barnase* without affecting plant development and growth provides an ideal tool for containing gene flow. However, whether the engineered sterility is mitotically or meiotically stable is unknown. Due to their sterile nature, assessing meiotic stability in these lines was impossible. Accordingly, we tested only mitotic stability of the engineered sterility in transgenic *Arabidopsis* by continuously cutting back primary inflorescences of the same transgenic plants for two generations. Eight transgenic lines containing each construct (Table 1) as well as control transgenic lines were microscopically evaluated for floral structure in the T_1 generation. The same lines were cut back twice. The

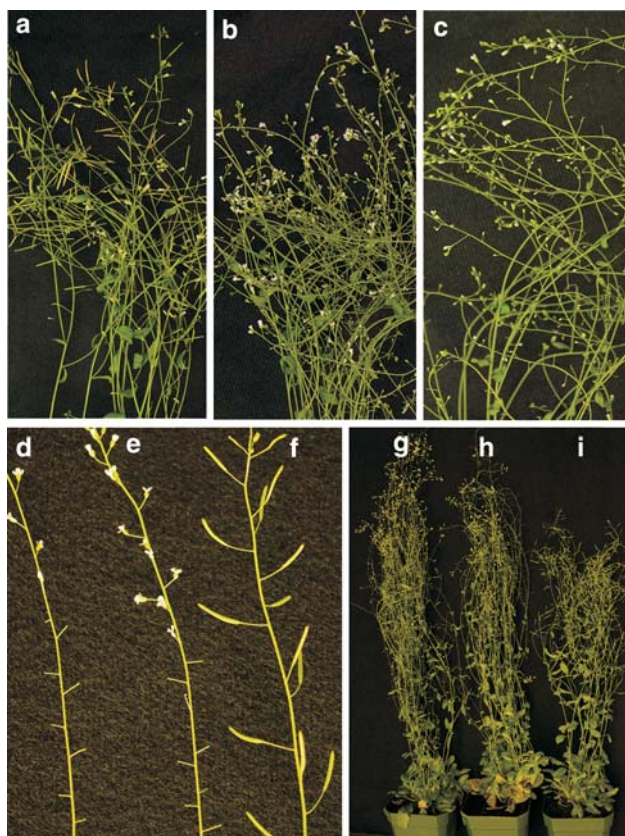


Fig. 4 Plant growth and sterile phenotype in transgenic plants. (a, f) *CsVMV::GUS* transgenic plants with normal silique formation; (b, d) ablated silique phenotype in *fAGIP::DT-A* plants; (c, e) ablated silique phenotype in *rAGIP::DT-A* plants; (g, h) vegetative growth of six-week-old *fAGIP::DT-A* and *rAGIP::DT-A* plants respectively; (i) vegetative growth of six-week-old *CsVMV::GUS* control plants

re-grown plants (designated T_1C_1 , and T_1C_2 respectively) were subjected to the same microscopic analysis. As summarized in Table 1, over 1,200 wild type flowers in *CsVMV::GUS* lines examined in T_1 , T_1C_1 and T_1C_2 displayed wild type floral morphology. Over 1,400 *fAGIP::DT-A* flowers and over 1,500 *rAGIP::DT-A* flowers examined in T_1 , T_1C_1 and T_1C_2 displayed the same ablated floral phenotype with normal sepals and petals but no carpels or stamens. Interestingly, no partial or complete reversion was observed in any of the *fAGIP::DT-A* and *rAGIP::DT-A* flowers examined. Evidently, *fAGIP::DT-A*

and *rAGIP::DT-A* mediated organ ablation can be stably maintained for at least two consecutive cutback generations.

Discussion

Male and female gametes are primary gene flow carriers and their complete elimination through tissue-specific ablation is ideal for the prevention of transgene flow. Key to generating precise, effective, and complete sterility is the use of highly specific and stringently regulated promoters. Previous work showed that *AG* intron-derived promoters can confer stamen and carpel specific *GUS* expression (Busch et al. 1999; Deyholos and Sieburth, 2000; Sieburth and Meyerowitz 1997). Our results shown here further confirms the highly specific nature of these *AG* intron-derived promoters as there appeared to be an absence of any adverse effect on vegetative tissues. Thus, *AG* intron-derived promoters developed for this study showed a highly tissue-specific and tightly regulated expression pattern, and *AGIP*-mediated expression of cytotoxic genes precisely eliminated both stamens and carpels without affecting plant vegetative growth, leading to absolute, bisexual sterility. This approach is simple, precise and efficient, and exhibits several promising improvements over existing approaches, which can be widely used for remedying gene flow in transgenic ornamental, landscaping, forestry, bioenergy and fruit root stock species, as well as improving their biomass production and environmental friendliness (e.g. reduction of excessive pollen and fruit).

Although several promoters and cytotoxic genes were previously used for engineering sterility, the most successful ones are limited to engineering male or female sterility (Beals and Goldberg 1997; Block and Debrouwer 1993; Block et al. 1997; Mariani et al. 1990; Mariani et al. 1992). However, engineering simultaneous male and female sterility without compromising non-targeted tissues remains challenging due to a lack of highly regulated promoters. Many promoters, including gamete-specific *Arabidopsis AtDMC* (Kobayashi et al. 2006), floral meristem-specific *LFY* (Nilsson et al. 1998) and *LFY* ortholog promoters (Wei et al. 2007), flower-specific poplar *PTD* promoter (Skinner

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

	T_1		T_1 Cut-back generation 1 (T_1C_1)		T_1 Cut-back generation 2 (T_1C_2)	
	No. flower	No. flower with carpel and stamen	No. flower	No. flower with carpel and stamen	No. flower	No. flower with carpel and stamen
<i>CsVMV::GUS</i> (8 lines)	488	488	356	356	298	298
<i>fAGIP::DT-A</i> (8 lines)	524	0	498	0	426	0
<i>rAGIP::DT-A</i> (8 lines)	602	0	478	0	433	0

et al. 2003), and birch *BpFULL* (Lannenpaa et al. 2005) and *BpMADS1* (Lemmettyinen et al. 2004b) promoters have been tested for engineering bisexual sterility in various species. Disappointingly, almost all of them showed, to various degrees, leaky expression in vegetative tissues as reflected by compromised vegetative growth or the requirement of an additional gene repression unit such as *Barstar*. In some cases, the engineered bisexual sterility was unstable and became reversible under certain circumstances such as outcrossing (Kandasamy et al. 1993; Thorsness et al. 1993). Hence, specifically targeting male and female organs and tight regulation in vegetative tissues makes the present system an ideal approach for engineering bisexual sterility for complete containment of transgene flow.

Previous reports indicated that flower-specific ablation using the various promoters mentioned above generated diverse phenotypes ranging from mild to severe retardation (Lannenpaa et al. 2005; Lemmettyinen et al. 2004b; Lemmettyinen et al. 2001; Nilsson et al. 1998; Skinner et al. 2003; Wei et al. 2007). Our observation of a uniform phenotype with a high ablation efficiency by *AGIP::DT-A* indicates that *AGIP*-mediated tissue ablation is extremely effective. Furthermore, in contrast to previous reports using different types of promoters, we did not observe partially developed stamens or carpels nor did we see partial or complete reversion of the phenotype after two cut back generations. This high tissue specificity and complete penetrance of *AGIP::DT-A* is reminiscent of the *AP3* promoter used to drive *DT-A* (Day et al. 1995). *AP3* is a promoter specific for petal and stamen primordia. When fused with *DT-A*, *AP3* was able to completely arrest petal and stamen primordia development and produced a uniform and complete ablation of petals and stamens (Day et al. 1995). Hence, the stage at which *DT-A* is expressed may be crucial to the effectiveness of tissue ablation. Since both *AG*- and *AP3*-derived promoters drive *DT-A* expression at the earliest stages of floral organ primordial initiation, they may contribute to the observed effectiveness and high efficiency.

Interestingly, the two *AGIP::DT-A* constructs, which displayed 89–96% ablation frequencies, appeared more efficient than *AGIP::Barnase* constructs, which yielded frequencies of only 68–80% (Fig. 1). This suggests that targeted stamen and carpel tissues or primordial cells might be more sensitive to *DT-A* than *Barnase* cytotoxicity. Alternatively, leaky expression of *tac::Barstar* in plant cells due to its proximity to *AG* enhancer elements present in the same vector within the T-DNA right and left borders (Fig. 1) might compromise the effectiveness of *Barnase* in the targeted cells or tissues. The fact that *Barnase*-mediated retardation in vegetative tissues can be readily repressed by weak promoter-mediated expression of *Barstar* (Wei et al. 2007) supports the alternative possibility.

The *AG* gene and its function are conserved across a wide range of species (Benedito et al. 2004; Kater et al. 1998; Kempin et al. 1993; Kramer et al. 2004; Lemmettyinen et al. 2004a; Pnueli et al. 1994; Zahn et al. 2006). Even *AG* orthologs from primitive gymnosperms share a conserved gene structure (intron position and number) and tissue-specific expression. Orthologous genes from other species were able to rescue *ag* loss-of-function mutants or confer a gain-of-function phenotype in *Arabidopsis* (Rutledge et al. 1998; Zhang et al. 2004). Strikingly, the size, position and even function of the second intron are highly conserved among many species (Hong et al. 2003). For example, the second intron of the cucumber *AG* ortholog can confer a stamen- and carpel-specific gene expression pattern in *Arabidopsis* (Hong et al. 2003). Phylogenetic footprinting reveals that binding motifs for several transcription factors, including *LFY* and other *MADS*-domain proteins, within the second intron are also highly conserved across a variety of species ranging from herbaceous to woody plants (Brunner et al. 2000; Hong et al. 2003). Hence, the second intron of *AG* orthologs in many plant species could be isolated and tested for their ability to drive cytotoxic gene expression. They can also be used to drive the expression of other types of genes such as the molecular scissor gene so as to excise transgenes in reproductive tissues to prevent pollen-, seed- and fruit-mediated gene flow (Luo et al. 2007).

Potential applications for *AGIP* promoter-mediated engineering of complete sterility go beyond transgene containment. Many ornamental and landscape species produce copious amounts of pollen, which contributes to abundant allergens, and generate excessive numbers of fruits and nuts that can be particularly problematic in urban settings. Plants with absolute sterility created using the method developed here can prevent the production of both pollen and fruit, minimizing the health and environmental hazards of ornamental and landscape species. Additionally, engineered sterility diverts energy to vegetative growth, enhancing wood production in forestry species and biomass production in bioenergy crops such as *Populus* tree. Engineered sterility will also help prevent the spread of invasive ornamental, landscape and forestry species, which has become a major threat to biological diversity, ecosystem function, and agricultural production world wide (Li et al. 2004).

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