Creation and analysis of a novel chimeric promoter for the complete containment of pollen- and seed-mediated gene flow

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Abstract Effective containment of gene flow in transgenic plants requires a promoter that is highly specific for male and female gametes or tissues. Here, we report the creation of a novel pollen-, stigma- and carpel-specific (*PSC*) promoter through the fusion of the pollen-specific *LAT52* and carpel-specific *AGL5* enhancers to a stigma-specific *SLG* promoter. Gene expression analysis showed that fusion of the *LAT52* enhancer to the *SLG* promoter enables the latter to gain pollen-specific activity while the acquirement of carpel-specific activity requires the correct orientation of the inserted *AGL5* enhancer in the *PSC* promoter, and only a forward- but not a reverse-oriented one is functional. The resulting *fPSC* promoter, when fused to *DT-A*, generated at least three aberrant gynoecium phenotypes. Type I plants exhibited shortened stigmatic tissues, resembling plants containing the *DT-A* gene controlled by the *SLG* promoter. However, type II and III plants displayed partial or complete ablation of gynoecia, and were unable to support the reproductive process. Type II and III plants also produced severely perturbed anthers and pollen in comparison to type I or *SLG::DT-A* plants, and transgenic pollen

grains were unable, when out-crossed with control plants, to pass the transgene to the next generation in all plants examined, indicating that they are selectively eliminated. This tissue-specific ablation or perturbation is highly specific, and does not compromise vegetative growth. Evidently, the *fPSC* promoter faithfully acquires tissue specificity from the incorporated enhancers and promoter, and should have a practical application for transgene containment in non-fruit and -grain producing plant crops.

Keywords Tissue-specific enhancer · Chimeric promoter · Tissue ablation · Gene flow and containment

Introduction

Concerns regarding pollen- and seed-mediated gene flow and potential food safety of transgenic crops are growing and spurring intense debates, which has a significant impact on grower and consumer confidence, acceptance and adoption of food derived from transgenic crops. A primary concern is that copious amounts of pollen derived from genetically modified (GM) crops could pollinate and hybridize with their wild relatives. It is also a concern that seeds released into the environment from transgenic crops could cause introgression of the transgene into the genome of a wild relative in subsequent generations (Snow 2002). Introgression of these superior traits into wild type plants or their close wild relatives through gene flow (Mikkelsen et al. 1996; Snow and Palma 1997; Ellstrand et al. 1999; Ellstrand 2001) could boost the fitness of their progenies, which could facilitate the evolution of more aggressive and invasive weeds or species (Snow 2002; Hall et al. 2000). This could potentially pose a major threat to biological

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diversity, ecosystem function and agricultural productivity (Li et al. 2004). Hence, properly containing and mitigating gene flow is of ecological, economical and environmental significance.

Diverse plant crops and species are of distinct economic value and each requires a specific containment strategy. For example, many crops produce edible seeds (e.g. maize, soybean, etc.) or fruits (e.g. apple, citrus) that serve as sources of food for human consumption, while others, such as forestry and ornamental species, serve different purposes and their fruit and seeds are of little economical value. The same gene containment method, such as reproductive sterility, would likely compromise the productivity of food-producing crops but would surely benefit that of GM forestry or other non-food producing plants by conserving nutrients which are otherwise exhausted by growing fruit and seeds. Thus, distinct gene containment strategies are required to meet the special needs of each individual crop.

A few potential containment strategies for grain- and fruit-producing crops have been under extensive study. One of the strategies is to exploit the unique maternal inheritance of chloroplasts for the restriction of pollen-mediated gene flow (Daniell 2002). Chloroplasts are known to pass their genome on to the next generation through female but not male gametes, which provides a natural containment for gene flow. Thus, a transgene integrated into the chloroplast genome of GM crops is passed on to the next generation only through female gametes, while male gametes remain free from the introduced transgene. As chloroplast transformation systems become available in cotton (Kumar et al. 2004b), carrots (Kumar et al. 2004a), tomato (Ruf et al. 2001), tobacco (Avni and Edelman 1991; Daniell et al. 1998) and rice (Khan and Maliga 1999), chloroplast-mediated transgene containment should be achievable in many agriculturally important crops.

Transgene mitigation offers an alternative approach that is based on the ecological principle that by reducing the fitness of transgene recipients in hybrids, the eventual extinction of all hybrids will result after a few generations (Gressel 1999). Accordingly, any gene that is positive or neutral to the crop but deleterious to hybrids and their progeny (such as dwarfing) would serve as a mitigating tool to weaken the fitness of the hybrids, leading eventually to the elimination of the hybrids from the wild population. An exquisite example of this is the observation that transgenic plants with an engineered dwarf phenotype are unable to compete for light with wild-type plants. As a consequence, most of them died or formed no flowers when grown in proximity to wild-type plants (Al-Ahmad et al. 2004).

Genetic use restriction technology (GURT) is based on repressible two-component systems (e.g. *Barnase–Barstar*)

in which seeds produced by the plants are non-viable unless the plants are exposed to a specific activator molecule or stimulus. One example is the recoverable block of function (RBF) system, which relies on an integrated transgene that carries seed-specific *Barnase*, and heat-inducible *Barstar* expression units (Kuvshinov et al. 2001). Escaped pollen from transgenic plants carrying RBF pollinates wild-type plants and produces seeds. These hybrid seeds, however, cannot germinate unless they are treated with a heat shock.

The tissue-specific gene excision approach proposed by Keenan and Stemmer (2002) is a comprehensive strategy to address the concerns of both gene flow and food safety of transgenic plants. The transformation vector can be designed such that all transgene components necessary for selection and crop improvement can be excised by “molecular scissors”, in the pollen, seed and fruit tissues or in the early stage of floral initiation so that the derived pollen, fruit and seed are expected to carry no transgene except for a small residual DNA footprint. With the creation of a novel chimeric LoxP-FRT site, Luo et al. (2007) have demonstrated that a transgene excision efficiency of up to 100% can be achieved in pollen or seeds when either *Flp* or *Cre* is specifically expressed. This study provides the first evidence that highly efficient tissue-specific transgene excision is achievable in plants.

Tissue-specific ablation of flowers or male and female gametes by a cytotoxic gene provides a direct and effective means to contain transgene flow for non-grain and -fruit producing plants such as ornamental, landscape and forestry species. The *DT-A* gene coding for a ribosome inactivating protein (Palmiter et al. 1987), and *Barnase* gene coding for an extracellular ribonuclease that is inhibited by Barstar (Hartley 1988) have been used as effective genetic agents for engineering reproductive sterility in many different species (Beals and Goldberg 1997; Block and Debrouwer 1993; Block et al. 1997; Burgess et al. 2002; Gleba et al. 2004; Mariani et al. 1990, 1992). A similar approach has also been applied to gene containment in woody species such as *Populus* (Skinner et al. 2003; Wei et al. 2007) and birch trees (Lännenpää et al. 2005a, b; Lemmetyinen et al. 2001, 2004). However, a lack of tightly regulated floral organ- or gamete-specific promoters often leads to severe damage to non-targeted vegetative tissues in transgenic plants (Lännenpää et al. 2005a, b; Skinner et al. 2003; Wei et al. 2007), and an additional gene repression system is required to specifically repress the detrimental effects of the expressed cytotoxic gene product on vegetative tissues (Kobayashi et al. 2006; Wei et al. 2007). Hence, highly specific and tightly controlled, floral organ- or gamete-specific promoters are important for the

successful containment of transgene flow without compromising vegetative growth. In this study, we report the creation of a novel chimeric promoter through an enhancer–promoter fusion approach and the characterization of its tissue specificity as well as its potential utility for gene containment in transgenic plants.

Materials and methods

Plasmid construction

A 410-bp *SLG* stigma-specific promoter (Thorsness et al. 1993) isolated from plasmid pMKT11 was fused to *GUS*, *Barnase* and *DT-A* coding regions, respectively, to create *SLG::GUS*, *SLG::DT-A* and *SLG::Barnase* constructs. The *SLG::Barnase* construct contained a copy of *Tac-Barstar* in order to neutralize the *Barnase*'s detrimental effect on bacterial cells. A 440-bp *LAT52* pollen-specific enhancer (Bate and Twell 1998) was amplified by PCR from tomato genomic DNA with a primer pair of LAT52U (5'-gtctctgtctgaagctttcgacatact-3') and LAT52L (5'-gagatatcaatagtggtgagctattattgtgt-3'), and fused to a minimal 35S promoter (−60) to create the *LATp* promoter. *LATp* was placed in front of the *GUS* gene to generate the *LATp::GUS* construct. To create a chimeric pollen- and stigma-*(PS)* specific promoter, the 440-bp *LAT52* enhancer was inserted upstream to the *SLG* promoter in *SLG::GUS* to generate *PS::GUS*. A 1,972 bp *AGL5* carpel-specific promoter (Savidge et al. 1995) isolated from the *Arabidopsis* genome with a primer pair of AGL5U (5'-ctgtgacgttggtatatacacaaaagcttgaa-3') and AGL5L2082 (5'-tccgttcattggcctaagatcctattct-3') was inserted in front of *GUS* and *DT-A* coding regions, respectively, to create *AGL5::GUS* and *AGL5::DT-A* constructs. The 1,885-bp *AGL5* enhancer was amplified using a primer pair of AGL5U and AGL5L1996 (5'-tttcttttagaggaaagcttcttgaagt-3') and inserted in forward and reverse orientations upstream of the *LAT52* enhancer in *PS::GUS* to create *fPSF::GUS* and *rPSF::GUS* fusions, respectively. The *dPSF::GUS* construct was created by restriction digestion-mediated deletion of a 200-bp region located at the 3' end of the *AGL5* enhancer in *fPSC::GUS*. *fPFS::DT-A* was made by replacing the *GUS* coding region in *fPSC::GUS* with the *DT-A* gene fragment isolated from pR2283. All gene fusions were based on the binary vector *pBin19* (Bevan 1984) and are illustrated in Fig. 1.

Plant material and transformation

Arabidopsis thaliana C10 (ecotype Columbia) was grown in a growth chamber (16 h of light and 8 h of darkness at 22°C). *Arabidopsis* transformation was carried out as

described previously (Clough and Bent 1998). Primary transformants (T_0) were selected on medium containing half-strength Murashige and Skoog (MS) salts (Sigma, St Louis, MO, USA) supplemented with 1% sucrose, pH 5.7, 0.8% (w/v) agar and 50 mg/l of kanamycin.

Histochemical GUS assay

GUS assays were performed according to the described protocol (Jefferson 1988). *Arabidopsis* tissue was incubated in a 0.5 mg/ml solution of 5-bromo-4-chloro-indolyl β -D-glucuronide (X-Gluc) in 100 mM sodium phosphate buffer, pH 7.0 at 37°C overnight, followed by washing with 70% ethanol to remove the chlorophyll.

Pollen survival assay

Survival of pollen grains was indicated by intracellular retention of fluorescein produced by enzymatic hydrolysis of fluoroscein diacetate (Sigma, St Louis, MO, USA). Fluorescein diacetate was prepared in acetone (2 mg/ml) and diluted in sterile distilled water at a concentration of 0.2 mg/ml. Pollen grains were dispersed in a drop of fluorescein diacetate solution on a microscope slide and incubated for about 10 min. The image was captured using an Olympus florescent microscope.

Plant pollination

Arabidopsis flower buds with emerging petals (white tip) were opened with the tip of a pair of forceps between the petals and sepals, and immature anthers were removed. After 2–3 days, the stigmas in the emasculated flowers were pollinated with pollen from open, mature flowers two- to three times.

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, USA). Twenty nanograms of total RNA was used for RT-PCR amplification using the RT-PCR kit (Qiagen) with primer pairs DTAU363 (cttcgtaccacgggactaaactgggtatgt) and DTAL800 (aagttctacgcttaac gcttctcgctgt) for *DT-A*, and EF1F (gcactgtcattgatg ctcc) and EF1R (gtcaagagcctcaag gagag) for *EF1a*. 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.

Fig. 1 Construction and testing of a series of tissue-specific promoters. Promoter construction and *GUS* fusion (**a**). *Brassica* stigma-specific promoter *SLG*, pollen-specific promoter *LATp* containing the tomato *LAT52* enhancer fused to the 60-bp minimal CaMV 35S promoter (marked as a filled triangle), pollen- and stigma-specific *PS* promoter, and carpel-specific promoter *AGL5* were placed in front of the *GUS* coding region, respectively. Promoters *fPSC* and *rPSC* were identical except for the orientation of the *AGL5* enhancer. Promoter *dPSC* was derived from *fPSC* by the deletion of a 200-bp fragment at the 3' end of the *AGL5* enhancer as indicated by dashed dots. Promoter-cytotoxic gene fusion (**b**). The promoters *SLG*, *AGL5* and

fPSC were fused to *DT-A* or *Barnase*, or both genes. The direction of transcription is indicated by arrows above the genes. The orientation, start and end positions of the *AGL5* enhancer as well as its derivative in the *PSC* promoter are marked by arrows or numbers relative to the ATG start codon. Results of tissue-specific gene expression and perturbation/ablation are summarized in the right panel. *GUS*: *Escherichia coli* β -glucuronidase; *DT-A*: Diphtheria toxin A chain; *Barnase*: *Bacillus amyloliquefaciens* extracellular ribonuclease; Tn: the terminator of nopaline synthase gene. *pBin19* served as backbone vector for all constructs (Bevan 1984)

Results

Verification of tissue-specific promoters in transgenic *Arabidopsis*

Engineering complete sterility for containing transgenes requires a promoter specific for male and female tissues or gametes. However, such a promoter is rarely available in nature. Since promoters specific for pollen, stigmas or carpels have been reported in many plant species, combining these promoter elements together through an enhancer–promoter fusion would generate an ideal promoter to meet the needs of transgene containment. The *Brassica* *SLG* promoter is specific for stigmatic and tapetum tissues and *SLG*-driven expression of *DT-A* generated male and female

sterility but it is reversible (Thorsness et al. 1993), indicating that it lacks adequate strength for irreversible tissue retardation. The pollen-specific *LAT52* enhancer from tomato (Bate and Twell 1998) and carpel-specific *AGL5* enhancer from *Arabidopsis* (Flanagan et al. 1996; Roeder et al. 2003; Savidge et al. 1995) have been very well characterized and could serve as potential elements for reinforcing the *SLG* promoter's specificity in pollen and carpel tissues. To test this possibility, we first tested and verified the reported tissue specificity of these promoters in plants through *GUS* fusions (Fig. 1a). Histochemical staining revealed that 19 of 26 *SLG::GUS* lines were positive for *GUS* in stigmatic tissue (Fig. 2a). Earlier studies also showed that the *SLG* promoter has additional weak activity in tapetum tissue and the *SLG* promoter-mediated expression of *DT-A* specifically

perturbed tapetum tissue development, which led to male sterility (Kandasamy et al. 1993; Thorsness et al. 1993). Similarly, *AGL5* promoter activity in carpel tissues was confirmed in 17 of 20 *AGL5::GUS* lines (Fig. 2e), which is in agreement with earlier findings that *AGL5* is broadly expressed in the gynoecium in stage 10 flowers and is specifically limited to the valve margins and developing ovules through stage 17 (Flanagan et al. 1996; Roeder et al. 2003). To confirm tissue specificity of the *LAT52* enhancer, we first created a functional *LATp* promoter by fusing the *LAT52* enhancer to a 60-bp minimal 35S promoter (Fig. 1a). Of 32 *LATp-GUS* lines, 30 showed positive GUS staining in anthers (Fig. 2b) and pollen (Fig. 2c). Taken together, our results confirmed the tissue specificity of the promoters and enhancers we cloned.

Creation and testing of the pollen-, stigma- and carpel-specific promoter

To create the pollen-, stigma- and carpel (*PSC*) promoter, we performed a stepwise fusion of the *LAT52* and *AGL5* enhancers to the *SLG* promoter. The *LAT52* enhancer was first inserted upstream of the *SLG* promoter to create a pollen- and stigma-specific (*PS*) promoter (Fig. 1a). Thirteen of eighteen *PS::GUS* lines were positive for GUS in stigmatic tissues, anthers and pollen but not vegetative tissues (Fig. 2d), indicating that the *PS* promoter acquires additional pollen specificity from the *LAT52* enhancer. To enable the *PS* promoter to gain additional carpel tissue specificity, an approximately 1.9-kb *AGL5* enhancer fragment was inserted upstream of the *PS* promoter in forward

Fig. 2 Analyses of promoter activity in transgenic *Arabidopsis*. Histochemical GUS staining analysis was performed in stigmas (a) of *SLG::GUS*, anthers (b) and pollen grains (c) of *LATp::GUS*, stigmas and anthers (d) of *PS::GUS*, carpels (e) of *AGL5::GUS*, anthers, stigmas and carpels (f) of *fPSC::GUS*, anthers and stigmas (g) of *rPSC::GUS*, and anthers and stigmas (h) of *dPSC::GUS* plants. GUS staining of flowers from empty vector *pBin*-transformed plants is shown as a control (i)

and reverse orientations to create *fPSC* and *rPSC* promoters, respectively (Fig. 1a). Of 26 *fPSC::GUS* lines, 12 showed *GUS* expression in anthers and stigmas, as well as carpels, particularly in stage 10 onward flowers (Fig. 2f). Surprisingly, 20 of 23 *rPSC::GUS* lines displayed positive *GUS* expression only in anthers and stigmas but not in carpels (Fig. 2g). These results indicate that only the forward-oriented *AGL5* enhancer inserted in the *PSC* promoter was able to penetrate and express its activity in transgenic plants. These results also suggest that a core enhancer region could be located near the 3' end of the *AGL5* enhancer fragment, and a reverse orientation of the *AGL5* enhancer fragment in the *rPSC* promoter likely positions the core enhancer region distal to the *SLG* promoter's transcription initiation sites, which might attenuate or prevent the *AGL5* enhancer activity in the promoter. To test this possibility, we deleted an approximately 200 bp region located at the 3' end of the *AGL5* fragment in *fPSC* to create a *dPSC* promoter (Fig. 1a). All 20 *dPSC::GUS* lines lost *GUS* expression in carpels but not in stigmas and anthers (Fig. 2h), confirming that this 200 bp region is essential to the *AGL5* enhancer.

Tissue-specific ablation by *fPSC*-directed expression of *DT-A*

Barnase and *DT-A* have been widely used for cell and tissue ablation and engineering of reproductive sterility in plants (Mariani et al. 1990; Thorsness et al. 1993). To compare their efficiency, we made *SLG::DT-A* and *SLG::Barnase* fusions (Fig. 1b) and analyzed their effects on transgenic plants. Over 60% of *Arabidopsis* lines transformed with either of the gene fusions showed very similar phenotypes: delayed anther dehiscence, reduced pollen production and perturbed anther morphology (Fig. 3f) and self sterility (Table 1). The most obvious feature was the stigmatic tissues with shortened papillae cells (Fig. 3d, e) compared to those in vector-transformed wild-type flowers (Fig. 3b). However, plant growth and development, as well as flower structure, were not affected at all. These phenotypes are consistent with those reported in a previous study (Thorsness et al. 1993). The *DT-A* and *Barnase* genes appear to be functionally indistinguishable in tissue retardation and ablation. For the sake of convenient cloning, we chose *DT-A* for the remaining analyses because the presence of the *Barnase* gene poses a difficulty for cloning in bacterial cells (Beals and Goldberg 1997).

The *fPSC::DT-A* and *AGL5::DT-A* fusions were constructed and tested for tissue-specific perturbation and ablation (Fig. 1b). The *AGL5::DT-A* plants gave rise to two ablated gynoecium phenotypes. The first phenotype exhibited underdeveloped and bent gynoecia without style tissue, which was observed in 5 of 17 lines (Fig. 3g). The

Fig. 3 Tissue-specific perturbation and ablation in transgenic plants. Wild-type flower (a), stigmas (b) and anthers (c) observed in a *pBin*-transformed wild-type plant; mildly perturbed flower (d), stigmatic tissue (e) and anther (f) in *SLG::DT-A* plants; perturbed gynoecium with ablation of style (g) and absence of gynoecium (h, i) in *AGL5::DT-A* plants; gynoecium with ablated style and unfused apex (j), severely perturbed anther (k) and absence of gynoecium (l) in *fPSC::DT-A* plants. Pollen viability was assayed by fluorescent staining of anthers from *pBin* (m), type I *SLG-DT-A* (n) and type II *fPSC::DT-A* (o) plants

second phenotype showed an absence of gynoecia in 8 of 17 lines (Fig. 3h). In these plants, gynoecia were replaced by two leaf-like structures (Fig. 3i) that appear to represent two perturbed carpel residue tissues because the gynoecium in *Arabidopsis* is formed by the congenital fusion of two carpels. However, these plants displayed normal vegetative growth and development (data not shown). The ablated phenotypes are consistent with the demonstrated *AGL5* tissue specificity (Fig. 2e; Flanagan et al. 1996;

Table 1 Pollination analysis of transgene inheritance

	Pollination and cross		
	Fertile	<i>n</i>	Kan ^R
Self-cross			
Wild type (Wt)	98	100	0
<i>SLG::DT-A</i>	0	100	0
<i>fPSC::DT-A</i>	0	100	0
Outcross			
[Wt]♀ × [<i>SLG::DT-A</i>]♂	29	50	29
[Wt]♀ × [<i>fPSC::DT-A</i>]♂	4	54	0

In self-crosses, 100 flowers each from three wild type, *SLG::DT-A*- (type I phenotype), and *fPSC::DT-A*- (type II phenotype) transformed lines, respectively, were chosen for analyses. Successful pollination and fertilization was judged by the production of viable siliques and seeds. Outcrosses were performed by pollinating flowers of the wild type plants with pollen from the chosen three lines of *SLG::DT-A* type I and *fPSC::DT-A* type II plants, respectively. The F₁ seeds from viable siliques were harvested and germinated on selection media containing 50 mg/l kanamycin. Any seedlings from each cross that showed kanamycin resistance in the F₁ population were scored as a resistant cross

Roeder et al. 2003). As expected, *fPSC::DT-A* plants displayed perturbed anthers and ablated gynoeceia with a range of severities. Collectively, at least three types of perturbed gynoeceia were observed. The type I phenotype displaying shortened stigmatic tissues was observed in 16 of 32 lines, and this phenotype resembled that of *SLG::DT-A* plants (Fig. 3d, e). The type II phenotype with complete ablation of style tissue and an unfused apex in the gynoeceium (Fig. 3j) was observed in 5 of 32 lines. The type III phenotype with no visible gynoeceia, which had been replaced by two leaf-like tissues, was observed in 4 of 32 lines (Fig. 3l), and represented the most severe ablated phenotype. Type II and III phenotypes were very much like those observed in *AGL5::DT-A* plants (Fig. 3g–i), and were unable to support pollination or fertilization processes. Type II and III plants also exhibited similarly perturbed anthers (Fig. 3k), which are morphologically more severely

malformed than those present in either type I or *SLG::DT-A* plants (Fig. 3f). Type II and III anthers produced and released very few viable pollen grains (Fig. 3o) in comparison to the abundant ones in anthers of *pBin*-transformed wild type plants (Fig. 3m) and countable ones in *SLG::DT-A* or type I plants (Fig. 3n). The transgenic plants with all three types of retarded or ablated gynoeceia were self sterile and did not produce viable siliques and seeds (Fig. 4b–d; Table 1) in comparison to the control transformed plants (Fig. 4a), but displayed a robust and prolonged vegetative growth that continued growing for up to 4 months (Fig. 4e, f), while the control transformed wild-type plants lasted approximately 2 months (Fig. 4g). RT-PCR analysis showed that a 450-bp *DT-A* transcript was consistently detected in flower tissues from all three types of *fPSC::DT-A* as well as *SLG::DT-A* plants, respectively, but not in control transformed plants (Fig. 5).

Sterility generated by *fPSC::DT-A* prevented transgene flow events

Complete containment of seed- and pollen-mediated gene flow cannot be achieved unless male and female gametes or organs are irreversibly perturbed or ablated. Type II and III *fPSC::DT-A* plants, due to the ablated style or gynoeceium, were unable to support any pollination events and could not mediate gene flow through female gametes. Despite the fact that pollen viability was almost compromised in type II and III *fPSC::DT-A* plants as judged by fluorescent staining (Fig. 3o), whether transgenic pollen was still able to function as a gene flow carrier remains unknown. To address these questions, we chose three *fPSC::DT-A* lines with a type II phenotype for performing self-crossing and out-crossing analyses. As a comparison, we also chose three *SLG::DT-A* lines with a type I phenotype and three wild-type plants for the same crossing analyses. Table 1 shows that both the chosen *SLG::DT-A* and *fPSC::DT-A* lines were self sterile with the production of no viable siliques in 100 self-crossed flowers examined, while 98%

Fig. 4 *fPSC::DT-A* plant reproduction and growth phenotypes. Six-week-old *pBin*-transformed control plants displayed regular fertilized siliques (a) and growth termination (g) while the same aged *fPSC::DT-A* plants showed a self sterile phenotype with unfertilized silique residues in type I (b), type II (c) and type III (d) as well as continuing flowering and vegetative growth in type II (e) and type III (f) lines

Fig. 5 RT-PCR analysis of gene expression in transgenic plants. RT-PCR was performed for the analysis of *DT-A* expression using RNA samples isolated from floral tissues of one *pBin*-transformed (Wt), two *SLG::DT-A*, and six *fPSC::DT-A* transgenic lines with distinct retarded flower phenotypes. Expression of the gene coding for elongation factor (*EF1a*) served as an internal control

of self-crossed wild-type flowers produced normal siliques and seeds. In out-crossing analyses, 29 of 50 outcrosses in which the wild-type plants were pollinated with pollen from three *SLG::DT-A* plants produced siliques and seeds. About 15–40% of F₁ seedlings from each cross could root and grow normally in media containing kanamycin, indicating that the kanamycin resistance gene, *NPT-II*, was successfully transmitted into the control wild-type plants via pollen from the *SLG-DT-A* lines. This is also consistent with an earlier report (Thorsness et al. 1993). Although 4 of 54 outcrosses in which the wild-type plants were pollinated with pollen from the three type II *fPSC::DT-A* lines produced siliques and seeds, none of the derived F₁ seedlings showed resistance to kanamycin, as judged by the observation of seedlings with albino cotyledons and stunted roots in kanamycin-containing media. This indicates that transgenic pollen produced in the type II *fPSC::DT-A* plants failed to pollinate and transmit the transgene into the wild-type plants.

Discussion

Complete containment of transgene flow in transgenic plants is largely dependent on the elimination of gene flow carriers such as pollen, fruit and seeds by irreversible ablation or perturbation of male and female organs or gametes. Although several approaches have been developed to address this, most of them are limited to the specific eradication of either male gametes or tissues (Beals and Goldberg 1997; Block and Debrouwer 1993; Block et al. 1997; Mariani et al. 1990; Mariani et al. 1992), but specific elimination of both male and female gametes or tissues without compromising vegetative tissues remains challenging in large part because of the lack of desired promoters. The present study presents a new approach for creating novel chimeric promoters through enhancer–

promoter fusion. The resulting promoter is specific for pollen, stigmatic and carpel tissues and its mediated expression of a cytotoxic gene can specifically perturb or ablate the targeted tissues without compromising vegetative growth.

The *fPSC* promoter acquires the desired tissue specificity from the incorporated enhancer elements. This is supported by two lines of evidence. First, as revealed by *GUS* expression analysis, the *fPSC* promoter gains additional tissue specificity in pollen and carpels (Fig. 2f). Second, as revealed by tissue ablation analysis, *fPSC* promoter-mediated *DT-A* expression can completely or partially ablate gynoecium tissue (Fig. 3j, l), severely perturb anthers (Fig. 3k) and compromise pollen viability (Fig. 3o) compared to *SLG::DT-A*-mediated expression (Fig. 3d–f, n). The lack of *GUS* expression in non-targeted tissues, and the observed integrity of vegetative growth in *fPSC::DT-A* plants further confirms that the *fPSC* promoter activity is specifically restricted to targeted floral tissues, and the fusion of multiple flower-specific enhancers together does not alter, or interfere with the individual element's specificity.

Previous reports have shown that flower-specific ablation using various promoters often generated diverse phenotypes ranging from mild to severe retardation (Lännenpää et al. 2005a, b; Lemmetyinen et al. 2001, 2004; Nilsson et al. 1998; Skinner et al. 2003; Wei et al. 2007). *fPSC* promoter-mediated flower-specific ablation also produced at least three different gynoecium phenotypes. The type I phenotype with shortened papillae cells in stigmatic tissue resembles that commonly observed in *SLG::DT-A* plants (Fig. 3d, e, k). Consistent with earlier studies (Thorsness et al. 1993), this phenotype is self sterile but outcross fertile (Table 1). Therefore, the type I phenotype observed in *fPSC::DT-A* lines primarily results from the stigma-specific tissue specificity of the base *SLG* enhancer/promoter in the *fPSC*. Type II and III plants displayed perturbed gynoecia with different severities (Fig. 3j, l), and are absolutely female sterile because of the lack of stylar tissue. Type III represents the strongest phenotype with no visible gynoecium except for two leaf-like tissues (Fig. 3l) that presumably represent two residual carpels retarded at an early stage of development. The type II and III phenotypes resemble two phenotypes observed in *AGL5::DT-A* lines (Fig. 3g–i). Since the *AGL5* promoter is specific in carpel and carpel margin tissue (Flanagan et al. 1996; Savidge et al. 1995; Roeder et al. 2003) that gives rise to placenta, ovules, septa, abaxial repla, and the majority of stylar and stigmatic tissue (Liu et al. 2000), the activity of the *AGL5* enhancer in the *fPSC* promoter likely contributes to both type II and III phenotypes observed in *fPSC::DT-A* plants.

The fact that the *PSC* promoter with a forward-oriented but not a reverse-oriented *AGL5* enhancer fragment gains carpel-specific expression (Fig. 2f, g) indicates that *AGL5*

enhancer function is orientation-dependent, which could be better explained by the distance of the inserted enhancer to the transcriptional start sites (TSS) provided by the *SLG* base promoter (Fig. 1a). The inserted *AGL5* fragment, which is approximately 1.9 kb in size, may bear a core enhancer element near its 3' end, and the reverse orientation of the fragment accordingly positions the core enhancer element distal to the promoter's TSS region, which likely poses a physical distance barrier to block or attenuate penetration of *AGL5* enhancer activity. Consistent with this speculation, the deletion of a 200-bp region at the 3' end of the *AGL5* fragment in the *fPSC* promoter completely abolishes carpel-specific *GUS* expression in *dPSC::GUS* plants (Fig. 2h), indicating that the *AGL5* 3' region is critical. Despite the conventional wisdom that enhancer activity is independent of distance and orientation, the evidence to support a distance- and orientation-dependent enhancer has also been reported for the immunoglobulin heavy chain (IgH) enhancer in mammalian cells (Collins et al. 2006). Similarly, the fact that gene activation by the CaMV35S enhancer, one of the strongest ones characterized in plants is observed only within a 3.8-kb region but not beyond (Weigel et al. 2000), also supports the distance-dependent action of enhancers in plant cells.

The pollen-specific *LAT52* enhancer incorporated into the *fPSC* promoter likely contributes to permanently perturbing pollen viability and pollination ability in *fPSC::DT-A* plants. Two lines of evidence support this conclusion. First, anthers and pollen in type II and III *fPSC::DT-A* plants displayed severe retardation as judged by anther morphology (Fig. 3k) and pollen viability (Fig. 3o) compared to type I or *SLG::DT-A* plants (Fig. 3f, n) and control-transformed wild type plants (Fig. 3c, m). Second, genetic crosses showed that no transgenic pollen from the selected type II plants was, when out-crossed, able to pollinate and transmit the transgene into wild-type plants, indicating that transgenic pollen containing the *fPSC::DT-A* transgene has completely lost its viability. Our results are consistent with an earlier work in which *LAT52*-directed *DT-A* expression was able to specifically kill transgenic pollen in most cases, as well as to indiscriminately eliminate all pollen production in some cases (Twell 1995).

Taken together, we present a new strategy for creating a *fPSC* promoter with novel tissue specificity which is rarely available in nature and *fPSC*-directed expression of *DT-A* can completely prevent gene flow through selective elimination of male and female tissues or gametes that carry a transgene. Since a suite of genes involved in self incompatibility, pollination events and fruit development are found to be highly conserved for their function and tissue specificity across all plant taxa (Dwyer et al. 1994; Lee et al. 1994; Nasrallah and Nasrallah 1993; Gasser et al. 1989; Nasrallah et al. 1988), isolating these regulatory elements

and fusing them together for the creation of various promoters with similar tissue specificity will be of importance for precisely and specifically containing transgene flow in a wide range of species, including ornamental, landscaping, forestry and bioenergy crops whose fruits and seeds are of little economic value. Importantly, this strategy can be used for creating any promoter with desired tissue specificity to meet various research and industrial needs.

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