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'GM-gene-deletor': fused *loxP-FRT* recognition sequences dramatically improve the efficiency of FLP or CRE recombinase on transgene excision from pollen and seed of tobacco plants

Keming Luo[1,2,†], Hui Duan[1,†,‡], Degang Zhao[1,†,§], Xuelian Zheng[1], Wei Deng[1], Yongqin Chen[1,¶], C. Neal Stewart Jr[3], Richard McAvoy[1], Xiangning Jiang[1,**], Yanhong Wu[1], Aigong He[1,§], Yan Pei[2,*] and Yi Li[1,*]

[1]*Department of Plant Science, University of Connecticut, Storrs, CT 06269, USA*
[2]*Biotechnology Center, Southwest University, Chongqing 400716, China*
[3]*Department of Plant Sciences, University of Tennessee, Knoxville, TN 37996-4561, USA*

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**Correspondence* (fax +1 860 486 6777; e-mail yi.li@uconn.edu) or (fax +1 86 23 63250515; e-mail peiyan@swu.edu.cn)
†These authors contributed equally to this work
‡*Present address*: Department of Cell and Structural Biology, University of Illinois, Urbana, IL 61801, USA
§Visiting scientists (postdoctorates) from Guizhou University, Guizhou, China
¶Visiting scientist from Hubei University, Hubei, China
**Visiting scientist from Beijing Forestry University, Beijing, China
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Summary

Pollen- and seed-mediated transgene flow is a concern in plant biotechnology. We report here a highly efficient 'genetically modified (GM)-gene-deletor' system to remove all functional transgenes from pollen, seed or both. With the three pollen- and/or seed-specific gene promoters tested, the phage CRE/*loxP* or yeast FLP/*FRT* system alone was inefficient in excising transgenes from tobacco pollen and/or seed, with no transgenic event having 100% efficiency. When *loxP-FRT* fusion sequences were used as recognition sites, simultaneous expression of both FLP and CRE reduced the average excision efficiency, but the expression of FLP or CRE alone increased the average excision efficiency, with many transgenic events being 100% efficient based on more than 25 000 T_1 progeny examined per event. The 'GM-gene-deletor' reported here may be used to produce 'non-transgenic' pollen and/or seed from transgenic plants and to provide a bioconfinement tool for transgenic crops and perennials, with special applicability towards vegetatively propagated plants and trees.

Introduction

In the greater public discussion of plant biotechnology, transgene flow is a serious concern, with transgene containment being the ultimate goal (Dale *et al*., 2002; Konig, 2003). However, gene transfer through pollination routinely occurs in many commercially important plants, and between these crops and their wild relatives (Conner *et al*., 2003; Stewart *et al*., 2003). It has been reported that cross-pollination between commercial canola fields can occur over considerable distances (Rieger *et al*., 2002; Conner *et al*., 2003), and pollen-mediated gene flow from creeping bentgrass can take place as far away as 21 km (Watrud *et al*., 2004). Seed dispersal is another important vehicle in the spread of transgenes and, in some cases, seeds can be dispersed via spillage during handling and transportation (Stewart, 2004). The lack of effective biological containment technologies to prevent pollen- and seed-mediated gene flow has hindered the commercialization of many economically important, long-lived, genetically modified (GM) perennials, such as trees, that freely outcross in nature (Bradshaw and Strauss, 2001).

There are several candidate technologies for transgene containment with pollen or seed as targets. Inserting transgenes into the chloroplast genome provides an excellent method to reduce pollen-mediated gene flow (Daniell *et al*., 2005a,b), but the technology is not effective in addressing the seed-mediated gene flow problem, and some species have biparental chloroplast inheritance. Although 'terminator gene' technology (Oliver *et al*., 1998) may reduce the seed-mediated spread of GM genes, its potentially negative perceived impact on farmers in developing countries has created obstacles for its commercialization (Giovannetti, 2003).

Male and female sterility technologies can be used to reduce the gene flow problem from vegetatively propagated trees (Khan, 2005; Ruiz and Daniell, 2005), but the anticipated adverse ecological effects of large-scale plantations of sterile plants, such as sterile trees, are of concern, because fauna that feed on pollen, seed or fruit may be negatively impacted, probably resulting in a loss of biodiversity in these ecosystems.

Site-specific recombination systems (e.g. bacterial phage CRE/*loxP* and *Saccharomyces cerevisiae* FLP/*FRT*) function through interactions of a recombinase with its specific recognition sites (van Duyne, 2001; Ow, 2002; Chen and Rice, 2003; Lyznik *et al*., 2003). Recombinase-mediated excision between directly orientated recognition sites results in the removal of the intervening DNA, leaving one recognition site intact (Odell *et al*., 1990; Dale and Ow, 1991; Bayley *et al*., 1992; Russell *et al*., 1992; Gilbertson, 2003). Previously, strategies for removing marker genes or short spacer sequences from host plants using site-specific recombinases have been developed (Russell *et al*., 1992; Lyznik *et al*., 1996; Gleave *et al*., 1999; Iamthan and Day, 2000; Luo *et al*., 2000; Sugita *et al*., 2000; Corneille *et al*., 2001; Hoff *et al*., 2001; Ow, 2001, 2002; Zuo *et al*., 2001; Hare and Chua, 2002; Hoa *et al*., 2002; Zhang *et al*., 2003; Klaus *et al*., 2004). Keenan and Stemmer (2002) proposed the use of a site-specific recombinase to remove all transgenes from target organs to address the gene flow problem, but a major technical challenge may be that the efficiencies of the site-specific recombinase systems used in higher plants may not be sufficiently high for commercial scale crop production applications. More recently, Mlynarova *et al*. (2006) used a CRE/*loxP* system to excise transgenes from pollen; their lowest leakage rate was 0.024% based on 16 800 seeds examined. A calculation by Haygood *et al*. (2004), however, suggests that a leakage rate of much lower than 10^{-3} may be needed for large-scale plantations in order to alleviate concerns over GM gene escape through pollen, seed or both.

To develop a highly efficient method for the targeting of GM gene removal from pollen and/or seed of GM crops, we designed several novel gene cassettes using components from both FLP/*FRT* and CRE/*loxP* recombination systems. With the loxP-*FRT* hybrid sequences as the recognition sites to flank all transgenes, we demonstrated that the expression of either the *FLP* or *Cre* gene alone could lead to 100% efficiency in deleting all functional transgenes from pollen, or from both pollen and seed, of transgenic tobacco plants based on the examination of more than 25 000 progeny seedlings per transgenic event.

Results

Fusion gene construction

We constructed a series of fusion gene cassettes using components from both FLP/*FRT* and CRE/*loxP* systems and inserted them into pBIN19 Ti-plasmid vector (Bevan, 1984). Our hope was that a combined use of both FLP/*FRT* and CRE/*loxP* systems might enhance the efficiency of deletion of transgenes from target organs. Because the DNA sequences within the two T-DNA borders of pBIN19 Ti-plasmid were non-essential for *Agrobacterium* infection of plant tissues (Xiang *et al*., 1999), we deleted most of these sequences before introducing the fusion gene cassettes into the T-DNA region of pBIN19.

The gene cassette pLF (LF is an abbreviation for the *loxP-FRT* fusion sequence) (see Figure 1a), which served as a control, contained the GUS::NPTII fusion gene (Fabijanski *et al*., 2001) and two sets of 86-bp *loxP-FRT* sequences to flank all transgenes. As a recombinase gene was not included in this cassette, excision was not expected to occur in pLF plants. Cassettes pF_polB-FLP and pF_polseed-FLP contained the 48-bp *FRT* recognition sites, and pLF_polB-FLP, pLF_polL-Cre, pLF_pol-Cre + FLP and pLF_polseed-FLP contained the 86-bp *loxP-FRT* (34 bp for *loxP*, 48 bp for *FRT* and 4 bp for the spacer between *loxP* and *FRT*) recognition sites. We used pollen-specific *BGP1* (Xu *et al*., 1993) and *LAT52* (Twell *et al*., 1990) and pollen- and seed-specific *PAB5* (Belostotsky and Meagher, 1996) gene promoters to control the expression of the *FLP* gene. The *BGP1* gene promoter was used to drive FLP expression (BGP1-FLP-nos) in pF_polB-FLP and pLF_polB-FLP. The pollen- and seed-specific PAB5 gene promoter was chosen to control FLP expression (PAB5-FLP-nos) in pF_polseed-FLP and pLF_polseed-FLP. Gene cassettes pF_polB-FLP vs. pLF_polB-FLP and pF_polseed-FLP vs. pLF_polseed-FLP were designed to determine the effect of the *loxP-FRT* fusion sequences on the FLP-mediated excision efficiency. The cassettes pF_polB-FLP and pF_polseed-FLP contained the native FLP/*FRT* system, whereas pLF_polB-FLP and pLF_polseed-FLP contained both *loxP* from the CRE/*loxP* system and *FRT* from the FLP/*FRT* system as recognition sequences. The cassettes pLF-polB-FLP, pLF_polL-Cre and pLF_pol-Cre + FLP were used to study the effect of the simultaneous expression of both FLP and Cre genes on the excision efficiency.

Figure 1b shows a schematic diagram of transgene deletion from pollen and seed using gene cassette pLF_polseed-FLP as an example. Transgenic plants hosting pLF_polseed-FLP would be expected to have transgenes present in all organs during the plant life cycle, except in seed and pollen. Because of pollen- and seed-specific FLP expression, all functional transgenes

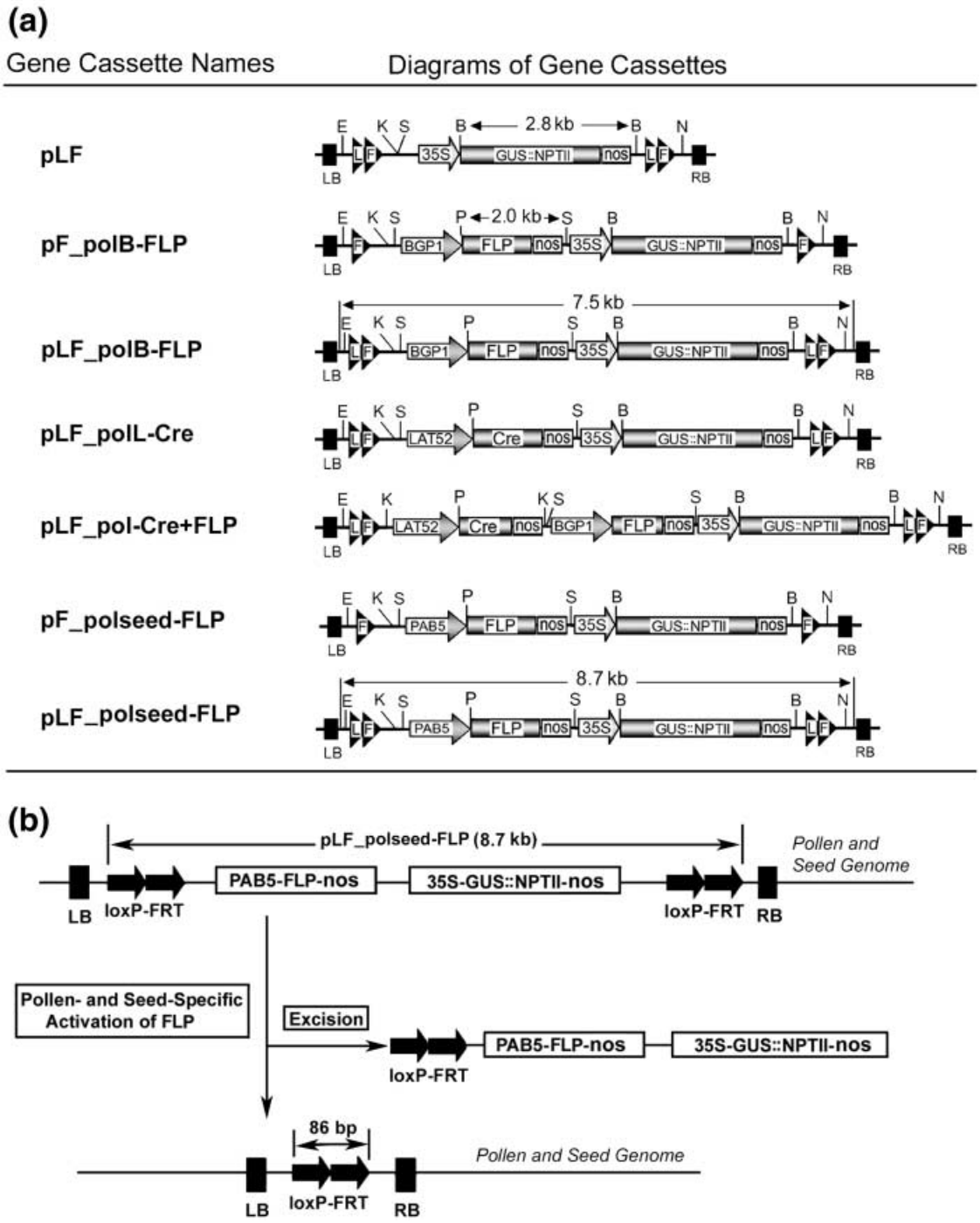

Figure 1 Schematic illustration of gene cassettes and the principles of 'genetically modified (GM)-gene-deletor' technology. (a) Gene cassettes used in this study. The FLP or *Cre* gene was driven by pollen- and/or seed-specific gene promoters. The GUS::NPTII fusion gene was driven by the 35S cauliflower mosaic virus (CaMV) gene promoter. All functional transgenes are flanked by *FRT* or *loxP-FRT* sequences. B, *Bam*HI; E, *Eco*RI; K, *Kpn*I; N, *Nhe*I; P, *Pst*I; S, *Sal*I (restriction enzyme sites); 35S, 35S CaMV gene promoter; BGP1, pollen-specific BGP1 gene promoter; LAT52, pollen-specific LAT52 gene promoter; PAB5, pollen- and seed-specific *PAB5* gene promoter; nos, 3′-untranslated sequence from the *Agrobacterium* NOS gene; F, *FRT* recognition sequence from the FLP/*FRT* system; LF, fused *loxP* and *FRT* (*loxP-FRT*) recognition sequence; *Cre* and *FLP*, *Cre* and *FLP* gene coding sequences from CRE/*loxP* and FLP/*FRT* systems, respectively; LB and RB, left border and right border (25 bp each) of T-DNA, respectively. (b) Schematic illustration of the FLP-mediated excision of all transgenes from pollen and seed. Between LB and RB sequences of T-DNA, two sets of 86-bp *loxP-FRT* fusion sequences were inserted in direct orientation to flank all trait genes including the *FLP* or *Cre* gene. The 35S-GUS::NPTII-nos gene represents a surrogate trait gene. FLP expression should lead to the deletion of all functional transgenes, including *FLP*, specifically from pollen and seed. The deleted 8.7-kb DNA sequence should be destroyed by nonspecific nucleases in the cell. All other gene cassettes shown in (a), except pLF, should work in the same fashion.

including the *FLP* gene, except an 86-bp non-protein encoding the *loxP-FRT* fusion sequence, should be excised from pollen and seed. The 8.7-kb excised DNA sequence, consisting of one *loxP-FRT* site, the PAB5-FLP-nos gene and the 35S-GUS::NPTII-nos gene, should be destroyed by nonspecific nucleases in the cell (Wilson, 1975; Srivastava and Ow, 2003). All other gene cassettes shown in Figure 1a, except cassette pLF, should work in the same fashion as pLF_polseed-FLP, although their excision efficiencies may be different.

Production and verification of transgenic plants

We produced more than 35 independent transgenic tobacco events for each gene cassette, except pLF, using an *Agrobacterium*-mediated plant transformation method (Li *et al*., 1992). Putative transgenic plants were initially screened for kanamycin resistance and β-glucuronidase (GUS) activity, and then confirmed with Southern blot hybridization (data not shown). Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of representative transgenic events showed that FLP or Cre genes, under the control of *BGP1*, *LAT52* and *PAB5* gene promoters, were transcribed in pollen and/or seed, but not in leaves or other non-reproductive organs (Figure 2).

Histochemical characterization of GUS gene expression in transgenic plants

Detailed histochemical analysis for GUS activity of T_0 plants and T_1 progeny of representative transgenic events from each group was performed. Because the GUS gene was under the control of the constitutive 35S cauliflower mosaic virus (CaMV) gene promoter (Odell *et al*., 1985), GUS expression served as an excellent marker for the presence of transgenes in the cell. We examined GUS activity in vegetative organs and young flowers of transgenic plants hosting gene cassettes pLF_polB-FLP, pLF_polL-Cre and pLF_polseed-FLP, and observed GUS activity in all tissues of leaves, stems, roots and young flowers (Figure 3a–d). These results revealed that neither the FLP nor *Cre* gene under the control of the *LAT52*, *BGP1* or *PAB5* gene promoters was active in vegetative organs or in young flowers. However, based on whole-tissue GUS staining patterns, we cannot exclude the possibility that excision may have occurred in a small number of cells at a low frequency in these organs.

To examine the FLP- or CRE-mediated excision of the transgenes in pollen, we conducted histochemical staining of GUS activity in pollen at different stages of flower development. As shown in Figure 3e,f, GUS activity was observed in both immature and mature pollen grains of plants hosting cassette pLF (containing the 35S-GUS::NPTII-nos gene but lacking an *FLP* or *Cre* gene). For transgenic events that harboured cassettes pLF_polB-FLP, pLF_polL-Cre and pLF_polseed-FLP, GUS activity was detected in immature pollen (Figure 3g), but disappeared in mature pollen (Figure 3h), suggesting that the transgenes were deleted from pollen. In transgenic plants hosting cassette pLF_polseed-FLP (containing the pollen- and seed-specific *PAB5-FLP* gene), no GUS activity was observed in the progeny plants derived from self-pollination (Figure 3l) or reciprocal cross-pollination with wild-type plants (Figure 3m,n), suggesting that the transgenes were excised from both pollen and seed. In contrast, GUS activity was detected in progeny seedlings of pLF plants when selfed (Figure 3i) and crossed with wild-type plants (Figure 3j,k), indicating that no excision occurred when neither *FLP* nor *Cre* was included.

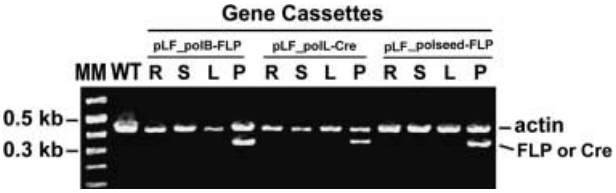

Figure 2 Reverse transcriptase-polymerase chain reaction (RT-PCR) analyses of FLP transcript levels in vegetative organs and young pollen of transgenic plants. For RT-PCRs, 2 μg of total RNA isolated from the pollen grains of stage 9 flowers was used to synthesize cDNAs with a cDNA synthesis kit (Invitrogen, Life Technologies, Carlsbad, CA). One twentieth of the volume of the first-strand cDNA reaction was used for PCRs to amplify the FLP and *actin* cDNAs. The PCR conditions were as follows: 95 °C for 1 min, 62 °C for 1 min and 72 °C for 1 min, 20 cycles in total. The primers 5′-GTTCGAATCGGAAGAAGC-3′ and 5′-GCTTGTCTTTGTCTCTGTCAC-3′ were used to amplify the FLP cDNA. The primers 5′-GAGGTTCGCAAGAACCTGATG-3′ and 5′-TCAGTGCGTTCGAACGCTAG-3′ were used to amplify Cre cDNA. The primers 5′-ATGCCCTCCCCACATGCTATTt-3′ and 5′-AACATGGTAGAGCCACTG-3′ were used to amplify *actin* cDNA. MM, DNA size marker; WT, mRNA from leaf tissue of a wild-type plant; R, S, L and P, mRNA from root, stem, leaf and pollen of pF_polB-FLP (event 12), pLF_polL-Cre (event 4) and pLF_polseed-FLP (event 7) plants.

Determination of efficiency of transgene excision from pollen and seed

To characterize the excision efficiency of each gene cassette, we vegetatively propagated all transgenic events and used these T_0 plants to conduct self- or cross-pollination (between transgenic and wild-type plants) for each transgenic event obtained. We analysed the GUS gene expression in T_1 progeny seedlings and used the number of GUS-positive progeny seedlings from each self- or cross-pollination to calculate the excision efficiency. Table 1 shows the total number of transgenic events analysed for each gene cassette, the average number of progeny seedlings per event examined for GUS activity, the ranges and means of the excision efficiencies observed, the

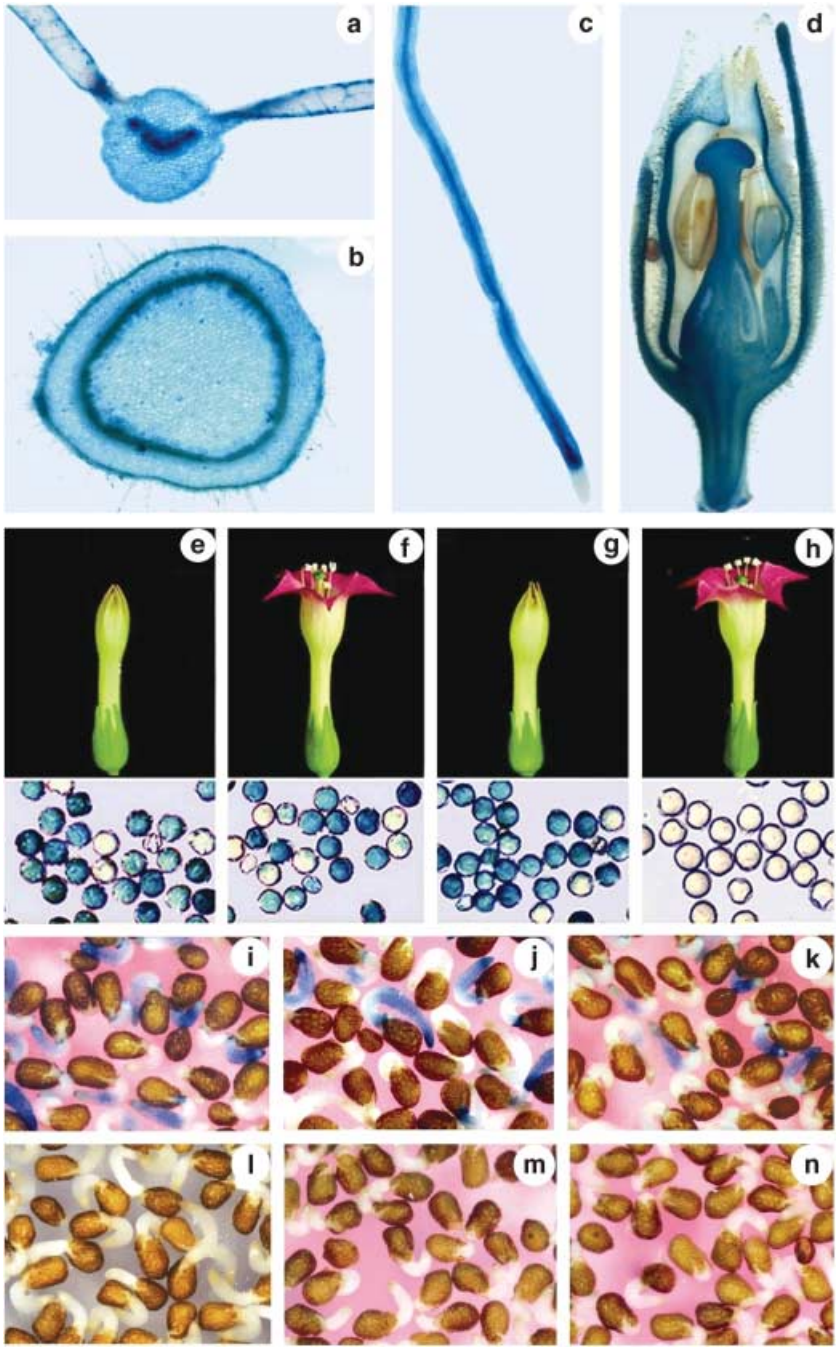

Figure 3 Histochemical visualization of β-glucuronidase (GUS) activity in transgenic tobacco plants. (a–d) GUS activity detected in leaf, stem, root and flower of a T_0 pLF_polseed-FLP plant (event 7). (e, f) GUS activity detected in immature and mature pollen of a pLF plant (event 5). (g, h) GUS activity observed in immature (g) but not mature (h) pollen from a pLF_polseed-FLP plant (event 7). Below each flower in (e–h) are the stained pollen grains from the corresponding flowers. (i–k) GUS activity detected in T_1 seedlings of a pLF event (event 5) from self- (i) or cross-pollination with wild-type plants [female (j) or male (k) plants]. (l–n) No GUS activity observed in T_1 seedlings of a self- (l) or cross-pollinated pLF_polseed-FLP plant (event 7) with wild-type plants [female (m) or male (n) plants].

number of transgenic events with 100% excision efficiency, and the average number of progeny seedlings analysed for those exhibiting 100% excision efficiency. None of the pF_polB-FLP or pF_polseed-FLP transgenic events (containing the *FRT* sequences as recognition sites) achieved 100% excision efficiency for transgenes from pollen and/or seeds. By contrast, a significant number of transgenic plants hosting cassettes pLF_polB-FLP or pLF_polseed-FLP (containing the *loxP-FRT* fusion sequences as recognition sites) were 100% efficient in deleting all functional transgenes from pollen and/or seed. For the transgenic events that exhibited 100% excision efficiency, vegetatively propagated T_0 plants were used to conduct additional self- or cross-pollination with wild-type plants to produce more than 25 000 T_1 progeny seeds for each event. As shown in Table 1, 13 of 42 pLF_polB-FLP plant events and eight of 45 pLF_polseed-FLP transgenic events were 100% efficient in deleting all transgenes from pollen and/or seed. Because cassettes pLF_polB-FLP and pLF_polseed-FLP

Table 1 Transgene excision efficiencies in pollen and seed as determined by the analysis of β-glucuronidase (GUS) activity in T_1 seedlings

Gene cassette names*	No. of independent transgenic events analysed	Average no. of progeny analysed per event	Excision efficiency (%) of all transgenic events		No. of events with 100% excision efficiency (% of all events)	Average no. of progeny analysed for each event that exhibited 100% efficiency
			Range	Mean		
pLF	14	3801	0	0	0 (0%)	
pF_polB-FLP	40	4115	0–99	32	0 (0%)	
pLF_polB-FLP	42	10154	0–100	79	13 (31%)	25106
pLF_polL-Cre	38	8432	0–100	33	6 (13%)	26665
pLF_pol-Cre + FLP	39	6300	0–45	11	0 (0%)	
pF_polseed-FLP	36	7216	0–89	26	0 (0%)	
pLF_polseed-FLP	45	11489	0–100	55	8 (18%)	30642

Cre, *Cre* recombinase gene; F, *FRT* recognition sequences; FLP, FLP recombinase gene; LF, fused loxP and *FRT* recognition sequences; p, plasmid; polB, pollen-specific *BGP1* gene promoter; polL, pollen-specific *LAT52* gene promoter; polseed, pollen- and seed-specific *PAB5* gene promoter.
*Two sets of *loxP-FRT* (LF) or *FRT* (F) in direct orientation were used to flank all functional transgenes delivered into host plants.

contained *loxP-FRT* fusion sequences as recognition sites, whereas cassettes pF_polB-FLP and pF_polseed-FLP contained only FRT sequences as recognition sites, it is clear that the combined use of the *loxP* and *FRT* sequences (loxP-FRT fusion) as the recognition sites dramatically enhances the excision efficiency.

Table 1 also shows that transgenic events hosting pLF_pol-Cre + FLP (with the *loxP-FRT* fusion sequences as recognition sites and both FLP and CRE recombinase genes expressed simultaneously) had an average excision efficiency of 11% from a total of 39 independent transgenic events, whereas transgenic plants hosting pLF_polB-FLP (containing the *loxP-FRT* fusion sequence with BGP1-FLP-nos) and pLF_polL-Cre (containing the *loxP-FRT* fusion sequence with *LAT52-Cre-nos*) had average excision efficiencies of 79% based on a total of 42 independent transgenic events and 33% from 38 independent events, respectively. Furthermore, none of the pLF_pol-Cre + FLP transgenic events showed 100% efficiency in deleting transgenes from pollen. In contrast, 31% of pLF_polB-FLP events and 13% of pLF_polL-Cre events were 100% efficient based on more than 25 000 T_1 progeny seedlings examined per event. These results demonstrate that simultaneous expression of FLP and CRE leads to a marked decrease in excision efficiency. In addition, the data in Table 1 reveal that, of the three gene promoters used, BGP1 appeared to be more effective than LAT52 or PAB5, because pLF_polB-FLP plants had an average excision efficiency of 79% based on a total of 42 independent events, whereas pLF_polL-Cre and pLF_polseed-FLP plants had efficiencies of 33% from a total of 38 independent events and 55% based on 45 independent events, respectively.

To confirm the high excision efficiencies observed, we vegetatively propagated representative pLF_polB-FLP, pLF_polL-Cre and pLF_polseed-FLP plants. Although we observed 100% excision efficiencies from pollen and/or seed in a number of transgenic plants hosting two copies of pLF_polB-FLP, pLF_polL-Cre or pLF_polseed-FLP, the plants used for propagation were those that contained a single copy of the transgenes. Using sexually and/or vegetatively propagated plants, we conducted additional self-pollination and cross-pollination with wild-type plants. As shown in Table 2, for plants hosting pLF (no recombinase gene included), we observed a positive GUS stain in about 75% of the progeny when self-pollinated, consistent with the expected 3 : 1 simple Mendelian ratio for genetic segregation. When pollen grains from pLF plants were used to cross with wild-type plants or wild-type plant pollen grains were used to pollinate pLF plants, approximately 50% of the progeny were GUS positive. These results show that, as expected, no excision of transgenes occurred in plants hosting pLF.

For plants hosting one copy of pLF_polB-FLP (containing the pollen-specific BGP1-FLP-nos gene), 50% of progeny seedlings were GUS positive if the plants were pollinated with wild-type pollen (Table 2). If the pollen of pF_polB-FLP plants was used to pollinate wild-type plants, the resulting T_1 progeny seedlings were 0% GUS positive, demonstrating that transgenes were deleted from pollen. Similar results were also obtained for the plants that hosted one copy of pLF_polL-Cre (the pollen-specific *LAT52-Cre-nos* gene). For pLF_polseed-FLP plants (containing the pollen- and seed-specific *PAB5* gene promoter used to drive FLP expression), none of the T_1 progeny seedlings from either self-pollinated or reciprocal wild-type crosses were GUS positive. The results demonstrate that pLF_polB-FLP, pLF_polL-Cre and pLF_polseed-FLP are 100% efficient for the deletion of all transgenes from pollen and/or seed based on more than 25 000 T_1 seedlings examined per event.

Table 2 Analysis of transgene excision efficiencies of representative plants*

Gene cassette names†	Transgenic event	GUS-negative : GUS-positive (GUS^- : GUS^+) ratio						Anticipated expression/ observed effect
		Self-pollinated		WT as pollen recipient‡		WT as pollen donor§		
		Expected¶	Observed**	Expected	Observed	Expected	Observed	
pLF	5	1 : 3	776 : 2127	1 : 1	987 : 1028	1 : 1	1009 : 985	No FLP gene/no excision in either pollen or seed
pLF_polB-FLP	12	1 : 3	12693 : 12287	1 : 1	25883 : 0	1 : 1	11944 : 11718	Pollen-specific FLP expression/100% excision in pollen but no excision in seed
pLF_polB-FLP	19	1 : 3	11892 : 12001	1 : 1	27954 : 0	1 : 1	19765 : 19041	Pollen-specific FLP expression/100% excision in pollen but no excision in seed
pLF_polL-Cre	4	1 : 3	12376 : 12964	1 : 1	26556 : 0	1 : 1	12356 : 12583	Pollen-specific CRE expression/100% excision in pollen but no excision in seed
pLF_polL-Cre	10	1 : 3	11468 : 11292	1 : 1	24398 : 0	1 : 1	16790 : 17657	Pollen-specific CRE expression/100% excision in pollen but no excision in seed
pLF_polseed-FLP	7	1 : 3	24103 : 0	1 : 1	25223 : 0	1 : 1	31790 : 0	Pollen- & seed-specific FLP expression/100% excision in both pollen & seed
pLF_polseed-FLP	26	1 : 3	32990 : 0	1 : 1	26343 : 0	1 : 1	32120 : 0	Pollen- & seed-specific FLP expression/100% excision in both pollen & seed

GUS, β-glucuronidase; WT, wild-type.

*Transgenic events hosting a single copy of the transgenes were analysed.

†A detailed explanation of the gene cassettes used is given in Figure 1.

‡Transgenic plants were pollen donors and wild-type plants were seed parents for these crosses.

§Transgenic plants were seed parents and wild-type plants were pollen donors for these crosses.

¶'Expected' ratio for GUS^- : GUS^+ was derived according to Mendel's law of genetic segregation assuming that there was no excision of transgenes.

**'Observed' number of GUS^- : GUS^+ progeny seedlings was obtained from each self-pollination or cross-pollination experimentally. If the number of GUS^+ progeny seedlings was zero, the excision efficiency in the pollen and/or seed of the relevant parental plant(s) was 100%.

Molecular characterization of transgene excision from pollen and seed

The deletion of all transgenes from pollen and/or seed was verified using Southern hybridization and highly sensitive PCR techniques. Southern blot analysis confirmed the presence of the GUS and FLP genes in vegetative organs of T_0 pLF_polB-FLP, pLF_polL-Cre or pLF_polseed-FLP plants, but these genes were absent in progeny from self-pollinated pLF_polseed-FLP plants or cross-pollinated pLF_polB-FLP or pLF_polL-Cre plants with wild-type plants being pollen recipients (Figure 4a,b). In addition, using the genomic DNA isolated from T_0 pLF_polB-FLP and pLF_polseed-FLP plants as templates and a set of oligos annealing the DNA sequences outside the two *loxP-FRT* sites for PCRs, we amplified 7.5- and 8.7-kb DNA products, respectively (Figure 4c).

DNA sequencing analysis confirmed that the 7.5- and 8.7-kb products contained pLF_polB-FLP and pLF_polseed-FLP (Figure 1a). Using the same oligos and the genomic DNA from T_1 seedlings from crosses of pLF_polB-FLP (male) × wild-type (female) plants and from self-pollinated pLF_polseed-FLP plants, we amplified a 0.2-kb fragment instead of 7.5- or 8.7-kb fragments (Figure 4c). This 0.2-kb fragment was not found in wild-type plants or in vegetative organs of T_0 pLF_polB-FLP or

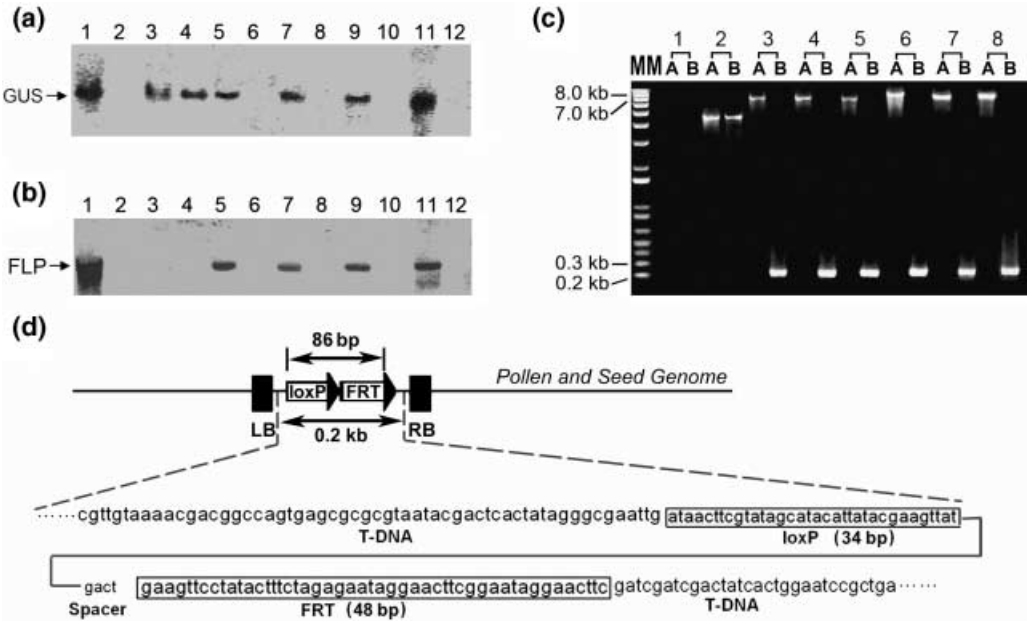

Figure 4 Molecular characterization of pollen- and/or seed-specific excision of all transgenes in tobacco plants. (a, b) Southern blot analysis of the β-glucuronidase (GUS) and FLP genes in T_0 and T_1 transgenic plants. As shown in Figure 1a, genomic DNA of T_0 or T_1 plants was digested with *Bam*HI and hybridized with a GUS DNA fragment (a) or digested with *Sal*I and hybridized with an FLP DNA fragment (b). Lane 1, pLF_polseed-FLP plasmid DNA; lane 2, T_0 wild-type plant; lane 3, T_0 pLF plant (event 5); lane 4, T_1 seedlings from self-pollinated pLF plant (event 5); lane 5, T_0 pLF_polB-FLP plant (event 12); lane 6, T_1 seedlings from cross between pLF_polB-FLP (event 12) and wild-type (female) plants; lane 7, T_0 pLF_polB-FLP plant (event 19); lane 8, T_1 seedlings from cross between pLF_polB-FLP (event 19) and wild-type (female) plants; lane 9, T_0 pLF_polseed-FLP plant (event 7); lane 10, T_1 seedlings from self-pollinated pLF_polseed-FLP plant (event 7); lane 11, T_0 pLF_polseed-FLP plant (event 26); lane 12, T_1 seedlings from self-pollinated pLF_polseed-FLP plant (event 26). (c) Polymerase chain reaction (PCR) detection of transgenes in T_0 and T_1 plants. MM, DNA size marker; lane 1A, T_0 wild-type plant; lane 1B, T_1 seedlings from self-pollinated wild-type plant; lane 2A, T_0 pLF plant (event 5); lane 2B, T_1 seedlings from self-pollinated pLF plant (event 5); lane 3A, T_0 pLF_polB-FLP plant (event 12); lane 3B, T_1 seedlings from cross of pLF_polB-FLP (event 12) × wild-type (female) plants; lane 4A, T_0 pLF_polB-FLP plant (event 19); lane 4B, T_1 seedlings from cross of pLF_polB-FLP (event 19) × wild-type (female) plants; lane 5A, T_0 pLF_polB-FLP plant (event 23); lane 5B, T_1 seedlings from cross of pLF_polB-FLP (event 23) × wild-type (female) plants; lane 6A, T_0 pLF_polseed-FLP plant (event 7); lane 6B, T_1 seedlings from self-pollinated pLF_polseed-FLP plant (event 7); lane 7A, T_0 pLF_polseed-FLP plant (event 26); lane 7B, T_1 seedlings from self-pollinated pLF_polseed-FLP plant (event 26); lane 8A, T_0 pLF_polseed-FLP plant (event 31); lane 8B, T_1 seedlings from self-pollinated pLF_polseed-FLP plant (event 31). (d) DNA sequence of the 0.2-kb fragment cloned with PCR using the genomic DNA of T_1 seedlings from the self-pollinated pLF_polseed-FLP plant (event 26) as template.

T_0 pLF_polseed-FLP plants. Furthermore, DNA sequencing analysis also revealed that the 0.2-kb signal consisted of a single *loxP-FRT* site and the deletion junction located within the two *loxP-FRT* direct repeats (Figure 4d). Disappearance of the 7.5- or 8.7-kb PCR products and appearance of the 0.2-kb post-excision PCR product in progeny further confirmed that all transgenes were deleted from the pollen and/or seed.

Discussion

As shown here, with the combined use of *loxP* and *FRT* sequences (86 bp in length) as the flanking sites for FLP or CRE recombinase, we have developed an exceptionally efficient system for deleting all functional transgenes from pollen and/or seed of transgenic plants (Figure 5). Based on the analysis of more than 25 000 progeny for each representative transgenic event, efficiencies for the automatic deletion of all functional transgenes from pollen and/or seed are as high as 100% under glasshouse conditions. In addition, we have demonstrated that the high excision efficiency trait remains stable in vegetatively propagated progeny plants. Our results suggest that the 'GM-gene-deletor' system reported here should be readily applicable for the production of 'non-transgenic' pollen and seed from vegetatively propagated GM plants that are important to forest, ornamental and paper industries.

In our study, the deletion of all functional transgenes from pollen and seed was confirmed using three different techniques: histochemical assay for GUS activity, Southern blot hybridization and PCR. Histochemical assay of GUS activity offers a simple, easy and highly sensitive method for both the initial screen of transgene activity in pollen and seed, and for the determination of the excision efficiency in large numbers of progeny seedlings from self- or cross-pollinated events. PCR amplification provides a highly sensitive method to verify the deletion of all functional transgenes from pollen and seed, and Southern blot hybridization offers additional

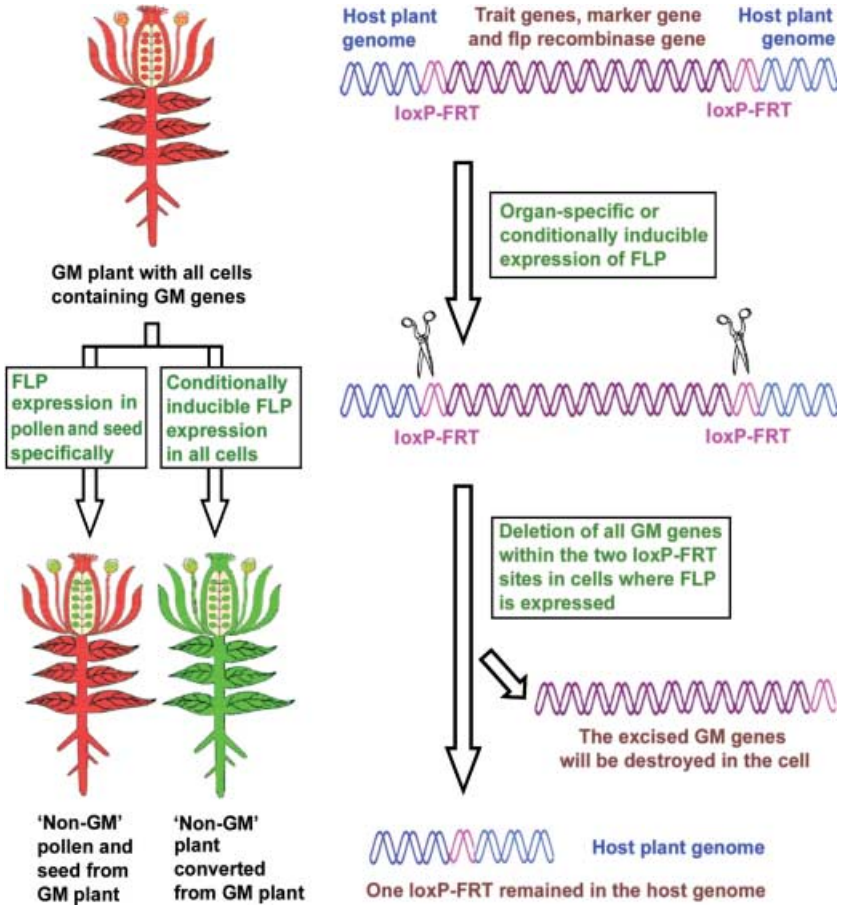

Figure 5 Schematic illustration of the production of non-genetically modified (non-GM) pollen and seed or a non-GM plant from a GM plant using the 'GM-gene-deletor' system. The left panel shows the use of the 'GM-gene-deletor' system to produce non-GM pollen, seed or plant from a GM plant. The right panel shows that, if all GM genes, such as trait genes, marker gene and FLP or *Cre* recombinase gene, are inserted into the two *loxP-FRT* sites (86 bp in length), these DNA sequences should be deleted from cells in which the recombinase is expressed. When a pollen- and seed-specific gene promoter is used to control recombinase expression, all functional GM genes should be deleted from these organs. When a conditionally inducible gene promoter, such as a chemically inducible or high-temperature-inducible gene promoter, is used to control recombinase expression, all functional GM genes should be deleted throughout the plant on application of the inducer. Thus, the 'GM-gene-deletor' system should provide a useful tool to reduce pollen- and seed-mediated GM gene flow problems of vegetatively propagated plants, and may also have many other applications, as described in the 'Discussion' section.

confirmation. It should be noted that, although all functional transgenes were deleted, a short, non-native, non-protein encoding an 86-bp *loxP-FRT* sequence remained behind in pollen and seed. However, the potential for adverse environmental and health effects from this non-expressed DNA sequence should be minimal or relatively easy to determine. Moreover, removal of all foreign DNA sequences, including the *loxP-FRT* recognition sequence, may be possible with the use of a recombinase that either recognizes related native plant sequences or cuts outside of their recognition sequences (Keenan and Stemmer, 2002).

Because the efficiencies of systems with *FRT*, *loxP* or the *loxP-FRT* fusion as flanking sites were compared side by side, our results clearly demonstrate that the *loxP-FRT* fusion sequences as recognition sites dramatically enhance the FLP- or CRE-mediated excision efficiency. We speculate that the fused *loxP-FRT* sequences may enhance the alignment of the recognition sequences, DNA bending or cleavage, or the formation of a Holliday junction or DNA–recombinase complex (van Duyne, 2001; Chen and Rice, 2003), thus leading to improved efficiency. In addition, the presence of loxP on one side and FRT on the other side of the flanking DNA sequence may enhance the excision efficiency, as the use of *loxP* on one side and FRT on the other results in higher rates of FLP- or CRE-mediated recombination (Lauth *et al*., 2002). However, when FLP and CRE were expressed simultaneously with *loxP-FRT* fusion sequences as the recognition sites (pLF_pol-Cre + FLP), the excision efficiency was decreased relative to pLF_polL-Cre and pLF_polB-FLP events (Table 1). Because we originally thought that the combined use of the CRE/*loxP* and FLP/*FRT* systems would enhance the excision efficiency, this result was surprising and unanticipated. It is possible that both FLP and CRE may bind to the same *loxP-FRT* molecule and such binding may affect the formation or stability of the DNA–recombinase complex (van Duyne, 2001; Chen and Rice, 2003), thus reducing the excision efficiency. However, additional experiments, preferably using a bacterial system, are needed to test this possibility.

Transgene containment technologies with little 'leakage' are highly desirable to avoid potentially ecologically important rare hybridization and introgression events (Stewart *et al*., 2003). Although the system reported by Mlynarova *et al*. (2006), using a CRE/*loxP* system to delete all functional transgenes from pollen to produce 'non-transgenic' pollen,

was high, as suggested by Haygood *et al*. (2004), a higher excision efficiency may be needed under large-scale field conditions. Our data demonstrate that the 'GM-gene-deletor' system, with a unique use of *loxP* and *FRT* sequences, is 100% efficient based on more than 25 000 progeny examined, a significantly higher efficiency than that observed by Mlynarova *et al*. (2006). Furthermore, the 'GM-gene-deletor', when the *PAB5* gene promoter is used to control FLP or CRE expression, can delete all functional transgenes from both pollen and seed, whereas the Mlynarova system deletes transgenes from pollen only. Thus, the 'GM-gene-deletor' system reported here should offer a better technique than the Mlynarova system to address the GM gene flow problem.

A modified version of the 'GM-gene-deletor' technology could also be useful in preventing gene flow from both pollen and seed of sexually propagated crops, such as sunflower, canola and sorghum, which outcross to wild relatives (Conner *et al*., 2003; Stewart *et al*., 2003). If a non-phytotoxic, chemically inducible RNAi-FLP gene is introduced into the pLF_polseed-FLP cassette, FLP expression in pollen and seed could be conditionally repressed (via a spray application of an induction agent), which should prevent the deletion of transgenes from pollen and seed, and allow the production of certified or registered seed stocks. Without application of the induction agent, sexually produced plants would produce transgene-free pollen and/or seed in the subsequent generation. This system may be especially applicable for genetically encoded and potentially controversial traits, such as industrial or pharmaceutical proteins.

There may be additional applications for 'GM-gene-deletor' technology. First, non-transgenic organs or plants from transgenic plants could be produced, mitigating consumer concerns over transgene presence in food (Figure 5). For example, with a fruit-specific gene promoter to drive FLP gene expression, all transgenes in fruit could be deleted. Second, the 'GM-gene-deletor' could provide farmers with the ability to replant viable, but non-transgenic, seed harvested from transgenic plants. This benefit stands in contrast with the controversial 'terminator seed' technology (Shand, 2002; Giovannetti, 2003) that produces non-viable seed. The 'GM-gene-deletor' would have both technology restriction and biosafety utilities without seed sterility. Third, 'GM-gene-deletor' technology, when it can be used in grain crops, would eliminate the need for labelling and physical separation of transgenic from non-transgenic grains after harvest. Fourth, the combined use of *loxP* and *FRT* as the recognition sequences for FLP or CRE recombinases, the key element of the 'GM-gene-deletor' system, may also be able to enhance the efficiencies of CRE or FLP in some non-plant systems.

Experimental procedures

Construction of gene cassettes

To construct the fusion gene cassettes used in this study, we synthesized the *loxP-FRT* fusion sequences with a spacer and a multiple cloning site in the middle: 5′-GGGAATTC*ATAA-CTTCGTATAGCATACATTATACGAAGTTAT*GACT***GAAGTTCC-TATACTTTCTAGAGAATAGGAACTTCGGAATAGGAACT-TC**GGTACCTATGTCGACGAACTCGAGTAGAGCTCAAGGA-TCCTT*ATAACTTCGTATAGCATACATTATACGAAGTTAT*GAC-T**GAAGTTCCTATACTTTCTAGAGAATAGGAACTTCG-AATAGGAACTTC**GCTAGCGG-3′. We synthesized the FRT recognition sequences with a spacer and a multiple cloning site in the middle: 5′-GGGAATTC**GAAGTTCCTATACTTTC-TAGAGAATA GGAACTTCGGAATAGGACTTC**GGTACCTA-TGTCGACGAACTCGAGTAGAGCTCAAGGATCCTT**GAA-GTTCCTATACTTTCTAGAGAATAGGAACTTCGGAATAG-GAACTTC**GCTAGCGG-3′. The italic letters represent the loxP site (34 bp) and the bold letters represent the FRT sequence (48 bp). The four base pairs, GACT, between the loxP and FRT sites denote the spacer sequence. The DNA sequence flanked by two *loxP-FRT* sites or two FRT sites, GGTACCTATGTCGAC-GAACTCGAGTAGAGCTCAAGGATCCTT, is a multiple cloning site containing *Kpn*I, *Sal*I, *Xho*I, *Sac*I and *Bam*HI restriction enzyme sites. The 8-bp DNA sequence at the 5′-end contains an *Eco*RI site (GAATTC), and the 8-bp DNA sequence at the 3′-end contains an *Nhe*I site (GCTAGC).

We deleted most of these sequences within two T-DNA borders of the pBIN19 Ti-plasmid before introducing the fusion gene cassettes. The synthetic loxP-FRT fusion and FRT sequences were inserted into the pBIN19 vector separately within *Eco*RI and *Nhe*I, respectively. A GUS and NPTII fusion gene (Fabijanski *et al*., 2001), under the control of the globally active 35S CaMV gene promoter (Odell *et al*., 1985) (35S-GUS::NPTII-nos), was then cloned into two FRT or loxP-FRT sites. The NPTII gene (the kanamycin resistance gene) served as a marker gene for the selection of transgenic plants during transformation, and the GUS reporter gene provided a convenient tool to monitor the presence or absence of the transgenes in specific organs of transgenic plants. The 35S-GUS::NPTII-nos fusion gene was excised from pBluescript KS II with *Sal*I plus *Xho*I, and cloned into pBIN19-LF, which was digested with *Sal*I to produce the pBIN19-LF-GN construct (pLF).

The resulting pBIN19 derivative vectors were used to host the *BGP1-FLP-nos*, *LAT52-Cre* and *PAB5-FLP* genes. To amplify a 660-bp fragment of the BGP1 5′-upstream region, we used genomic DNA of *Brassica campestris* as the template, 5′-GCGGTACCTATCATTCCTTTAATTTCAAGG-3′

as the 5′-end primer and 5′-GTCTGCAGTTGGAGAGGAGAT-GGGGTTG-3′ as the 3′-end primer for a 40-cycle PCR: denaturation of the DNA template at 94 °C for 1 min, followed by primer annealing at 58 °C for 1 min and extension at 72 °C for 1 min. The *Kpn*I- and *Pst*I-digested 660-bp BGP1 5′-upstream fragment was fused to the 5′-end of the FLP-nos sequence in pBluescript KS II (kindly provided by Dr G. M. Wahl, Salk Institute, San Diego, CA, USA). The BGP1-FLP-nos fusion gene was excised from pBluescript KS II with *Kpn*I and *Sac*I digestion and ligated into the *Kpn*I and *Sac*I sites of the pBIN19-LF-GN vector, resulting in the pLF_polB-FLP construct. The LAT52-Cre-nos fusion gene was constructed using the same cloning strategy as for the BGP1-FLP-nos gene. The coding sequence of the Cre gene was kindly provided by Dr David Ow, USDA Plant Gene Expression Center, Albany, CA, USA. The LAT52 gene promoter and PAB5 gene promoter were cloned from tomato and *Arabidopsis* plants using PCR-based technology with the following two pairs of specific DNA primers: 5′-GGC**GGATCC**TATACCCCTTGGATAAG-3′ (*Kpn*I site in bold), 5′-CGG**CTGCAG**AGCACAATAGCCTTTGCC-3′ (*Pst*I site in bold) and 5′-GGC**GGATCC**GGCAAGACTCTTCGTCTTTG-3′ (*Kpn*I site in bold), 5′-CGG**CTGCAG**GGGCAATTCCAGAT-GCAAC-3′ (*Pst*I site in bold), respectively. The same strategy was used to construct all other gene cassettes shown in Figure 1a with the help of the restriction enzyme sites indicated. Most of these restriction enzyme sites were introduced during the cloning processes.

Plant transformation, growth conditions and histochemical staining

Agrobacterium tumefaciens LBA 4404 manipulation, *Nicotiana tabacum* leaf disc transformation and histochemical staining for GUS activity were performed as described previously (Li *et al*., 1992). Plants used for this study were grown in the glasshouse. All plants used for the experiments were T_0 generation plants that had been vegetatively propagated.

Southern blot, PCR and DNA sequencing analysis

Southern blot hybridization was performed using 10 μg of *Pst*I-, *Bam*HI- or *Sal*I-digested tobacco genomic DNA with α-^{32}P-labelled FLP or GUS DNA fragments as hybridization probes (RadPrime Labelling Kit, GibcoBRL, Grand Island, NY). The hybridization signals were visualized using a Packard Cyclone Storage Phosphor System (Packard Instruments, Meriden, CT). Genomic DNA isolated from immature pollen (stage 5 flowers) (Koltnow *et al*., 1990) of T_0 plants was used to establish the pre-excision signal, and from T_1 seedlings from self- or cross-pollination with wild-type plants to establish the post-excision signal. Two oligos, 5′-GAACGTGGCGA-GAAAGGAAGG-3′ and 5′-ACTGACAGAACCGCAACGTTG-3′, specific to the T-DNA sequences outside the two loxP-FRT fusions were used as primers for PCRs at 94 °C for 1 min, 60 °C for 1 min and 72 °C for 8 min for 40 cycles. DNA fragments were amplified by Ex Taq polymerase (TaKaRa, Madison, WI, USA) and sequenced using an Applied Biosystems 377 DNA sequencer (Perkin-Elmer, Wellesley, MA).

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