Cell PRESS

Keeping the genie in the bottle: transgene biocontainment by excision in pollen

Hong S. Moon[1], Yi Li[2] and C. Neal Stewart Jr[1]

[1] Department of Plant Sciences, University of Tennessee, Knoxville, TN 37996
[2] Department of Plant Science, University of Connecticut, Storrs, CT 06269

Gene flow from transgenic plants is an environmental and regulatory concern. While biocontainment might be achieved using male sterility or transgenic mitigation tools, we believe that perhaps the optimal solution might be simply to remove transgenes from pollen. Male sterility might not be ideal for many pollinators, and might not be implementable using standardized genes. Transgenic mitigation might not be useful to control conspecific gene flow (e.g. crop to crop), and relies on competition and not biocontainment *per se*. Site-specific recombination systems could allow highly efficient excision of transgenes in pollen to eliminate, or at least minimize, unwanted transgene movement via pollen dispersal. There are other potential biotechnologies, such as zinc finger nucleases, that could be also used for transgene excision.

Introduction

Transgenic plants have played important roles in solving current agricultural problems, and hold even greater prospects of alleviating poverty and malnutrition in developing countries. For example, Golden rice contains high levels of β-carotene and could be a great help for people with vitamin A deficiency [1]. Currently, over 3 billion people have micronutrient malnourishment [2]. Micronutrient deficiencies negatively affect human health and cause subsequent societal problems; nearly two-thirds of childhood deaths worldwide are caused directly by malnutrition [2]. Transgenic plants have demonstrated benefits including higher yields, enhanced nutrients, and easier pest control [3]. The Green Revolution has boosted crop yield in many parts of the world but Africa has not realized its benefits. In Africa, impediments such as insufficient water for irrigation and nutrient-depleted soils have resulted in low yields and often crop failure with conventional plant varieties [4]. Biotechnology has the potential to trigger drastically improved agriculture in Africa by adding traits, such as those conferring drought-, salt- or heat-tolerance.

Regardless of its potential benefit, biotechnology has not been exploited fully in very many crops, even in developed countries, because of regulatory and environmental concerns about gene flow. Following the example above, the large-scale deployment of Golden rice has been hampered mainly because of concerns about gene flow to neighboring farms that do not currently contain transgenic plants, and interference with existing vitamin A supplementation [5]. The concern that Golden rice could be grown in a country that lacks sufficient biosafety regulations and monitoring capabilities, coupled with potential gene flow from transgenic rice to weedy rice has stymied its cultivation [6]. Ingo Potrykus, a principal developer and advocate of Golden rice, considers this long delay in its cultivation to be a serious moral downfall [7]. In late 2008, the Rockefeller Foundation promised its financial support for the deregulation process of Golden rice cultivation in several developing countries [8]. It seems, however, that gene flow remains to be a significant regulatory hurdle.

In theory, gene flow could be prevented or rendered a negligible risk if strategies were realized that could contain transgenic traits within cultivated transgenic fields. Uncontrolled transgene escape to non-transgenic crop fields or sexually compatible wild relatives is a particularly important issue if transgene introgression is probable, or even possible, within a crop-wild system [9]. One especially problematic class of transgenes are those used for plant-made pharmaceuticals (PMPs) that are expressed in transgenic food crops or species that are prone to gene flow; that is, those that are outcrossers or have sexually compatible wild relatives [9]. Trace adventitious presence of PMP transgenes in food processed from other non-PMP transgenic crops is not acceptable by either regulators or food companies [10]. In 2002, highly stringent regulatory standard was applied to the biotechnology company ProdiGene for the presence of noncompliant PMP genes in their experimental field trials [11]. Therefore, PMPs probably will be the subject of even higher regulatory scrutiny with regards to gene flow, compared with non-PMP transgenic crops with input traits. These and other biotechnological applications beg for effective methods for biocontainment.

Removal of transgenes from pollen and/or seeds could minimize gene flow problems. Transgene movement from transgenic to non-transgenic plants typically occurs most frequently via pollen dispersal [12]. Therefore, for most plants, the first line of containment is pollen, the long-distance vector for hybridization and introgression. To prevent pollen dispersal, formation of sexual reproductive organisms can be suppressed simply under field conditions for some transgenic crops by harvesting leaves prior to flowering [13]. However, this is not a practically useful

Corresponding author: Stewart, C.N. Jr (nealstewart@utk.edu).

0167-7799/$ – see front matter doi:10.1016/j.tibtech.2009.09.008 Available online 24 October 2009

method for most crop species because the lack of seed production would be significantly disadvantageous. There are a number of biological transgene biocontainment tools that could eliminate, or at least minimize, unwanted transgene escape from transgenic to non-transgenic plant populations, including wild-relatives, or potential negative consequences of transgene flow [14]. These include male sterility and transgenic mitigation, whereas, perhaps the most effective method would be the removal of transgenes from pollen using site-specific DNA recombinases.

While pollen is considered to be the primary agronomy-based vehicle for long-range gene dispersal, it is not the only one. Transgenic seeds can be dispersed as volunteers in the next season, during harvest, transport, and sometimes also mediated by animals. Compared with pollen dispersal, seed dispersal is more predictable because it is most probably caused by human-mediated dissemination, which can be decreased with improved shipment and handling procedures [15]. However, transgene movement via pollen dispersal mediated by insects or wind is almost inevitable without an appropriate pollen biocontainment procedure in place.

Male sterility and chloroplast transformation as potential biocontainment tools

Male sterility is one of the most commonly used transgene biocontainment systems in commercial fields. Mariani *et al*. [16] have generated male-sterile tobacco and canola using a mechanism that prevents pollen formation through the expression of chimeric ribonuclease genes (Barnases). Male sterile plants are able to act as maternal parents and are fertilized with pollen from outside the field. Hybrid seeds from crossing between male-sterile plants and wild relatives can acquire fertility restoration in successive generations via the Barstar gene [17]. Male sterility, however, might have a negative effect on many pollinators that acquire food and nutrients from pollen. For example, the survival of pollen beetles to adulthood has been shown to be reduced in the absence of pollen compared to wild-type flowers [18]. Cytotoxicity of Barnase gene expression results in ablation of tapetal cells and embryos of plants [16,19]. Furthermore, because Barnase toxins have been shown to be cytotoxic in animal and human cell line models, their cell-specific expression to plants parts that are not consumed is required [20]. However, even low amounts of cytotoxic genes, such as Barnases in non-targeted plant parts, caused by leaky expression, might affect plant growth negatively [21]. The cytotoxicity and potentially unregulated expression of Barnase could result in cell death [22], therefore, male sterility might not be the best choice for transgene biocontainment.

Cytoplasmic male sterility (CMS) is another method to contain genes effectively though maternal inheritance, which has been demonstrated in proof-of-principle experiments in transgenic tobacco [23]. However, gene transfer from the cytoplasm to the nucleus occurs at high frequency [24,25]. A high transfer rate of integrated DNA from the cytoplasm to the nucleus, which results in termination of maternal inheritance, might not be an appropriate characteristic for reliable biocontainment systems [26]. Fertility of CMS in *Petunia* could be restored by nuclear gene expression [27]. Also, abnormal morphology of flower parts and poor nectar production in hybrid plants have been reported as unexpected consequences of some CMS systems [28]. CMS systems need to surmount these drawbacks to play effective roles as transgene biocontainment systems.

Plant plastids and their genomes are exclusively maternally inherited in many crop species, thus transplastomic approaches could be effective in biocontainment of male gametophyte-mediated transgene flow. However, maternal inheritance is not universal, which limits the use of plastid transformation for pollen-targeted biocontainment [29]. More than one-third of the species in angiosperm do not have a trait of strict maternal inheritance [30]. Also, efficient tissue culture and selection protocols that are required in order to obtain homoplastomic plants have not been established for most monocotyledonous species plants [13,30]. It has been suggested that additional methods should be paired with plastid transformation to achieve complete transgene containment [31,32].

Genetic engineering for transgene removal from pollen

With regards to pollen biocontainment, transgene removal is an alternative to male sterility, which as mentioned above, can be leaky, and to chloroplast transformation, for which maternal inheritance is typical (no transgenes in pollen), but nevertheless is rather difficult to accomplish in many plant species. One possibility for transgene removal is using site-specific recombination because this simply cuts the transgenes from pollen [33]. With this approach, the entire transgenic construct could be flanked with recognition sites for a site-specific recombinase gene, introduced under the control of a tightly regulated pollen-specific promoter (Figure 1). Upon expression of the recombinase in pollen, the entire transgenic cassette is excised, leaving only a short recognition site in the mature pollen (Figure 1). Similarly, zinc-finger nucleases (ZFNs), which can be designed specifically to bind and cleave target DNA sequences, could also be used to excise transgenes from pollen [34–36] (Figure 2).

In contrast to male sterility, a transgene removal strategy allows for normal production of pollen and fertilization, thus not adversely affecting the many flower-feeding herbivores. Concerns about reintegration of the excised transgene with reversible recombination systems including Cre-*lox* and FLP-*FRT* into pollen genome could be resolved with newly discovered, non-reversible recombination systems, such as ParA-*MRS* or CinH-*RS2* [37]. The potential of this approach has been demonstrated already with the successful removal of integrated transgenes in plants, in particular selectable marker genes, using site-specific recombination systems [38–40]. Furthermore, Luo *et al*. [41] have achieved dramatically increased efficiency of transgene removal in tobacco when they have combined *loxP-FRT* recognition sites with pollen-specific expression of either Cre or FLP recombinases, compared to non-fused recognition sites of Cre-*loxP* or the FLP-*FRT* recombination system. Based on screening of over 25 000 progeny per transgenic event, several transgenic tobacco events have shown complete transgene excision from their pollen [41]. Here, coexpression of both Cre

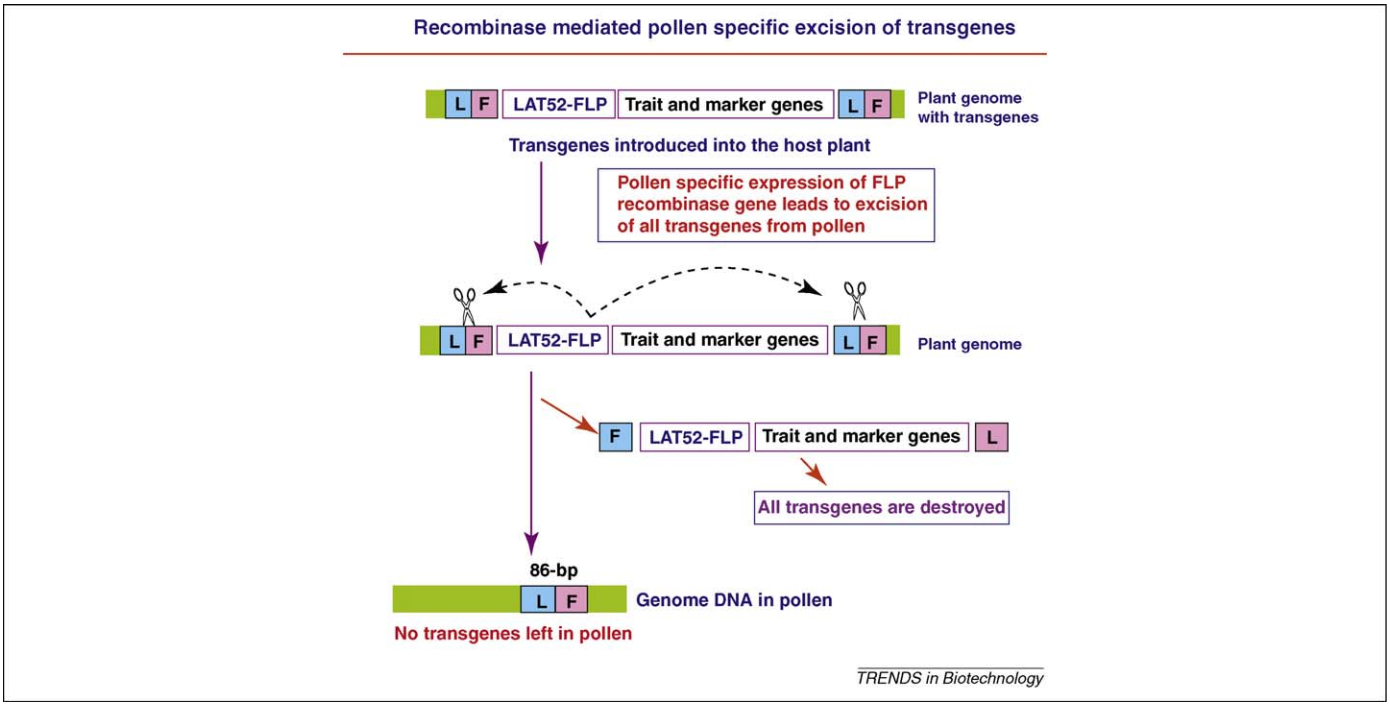

Figure 1. Principle of transgene excision from pollen. Here, L represents the *loxP* recognition sequence from the phage Cre/*loxP* system and F represents the *FRT* recognition sequence of the yeast FLP/*FRT* system. LAT52 is a pollen-specific gene promoter from tomato [42,53]. FLP is a DNA recombinase from the FLP/*FRT* system. Expression of FLP under the control of the LAT52 promoter leads to deletion of all transgenes between the two LF (*loxP-FRT* fusion) sites, including the recombinase gene in pollen specifically. The excised gene sequences will be destroyed by non-specific nucleases present in the cell. Reproduced from Ref. [52] with permission.

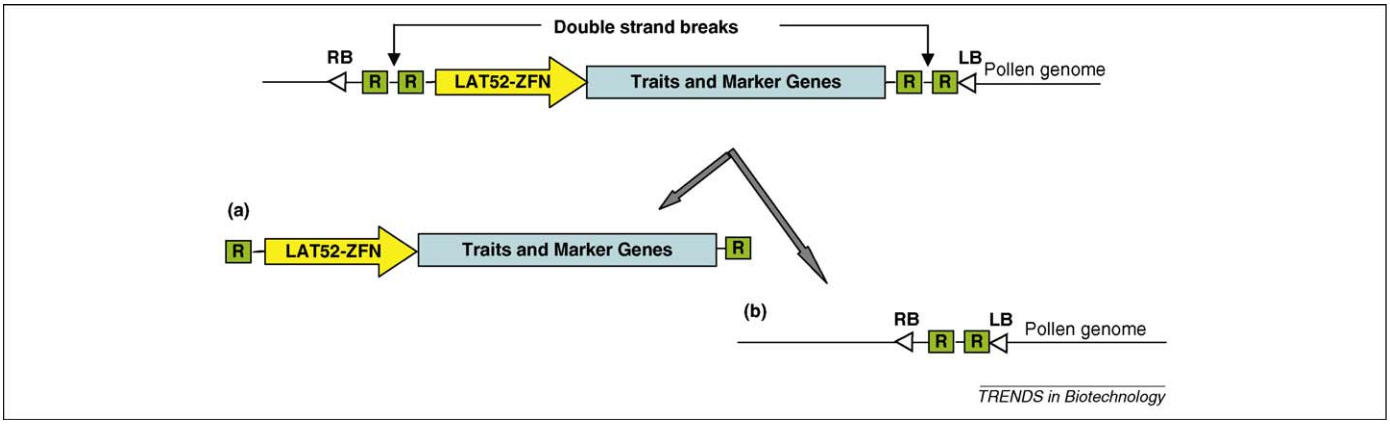

Figure 2. ZFN-mediated transgene excision from pollen. ZFN expression under the control of a pollen-specific promoter LAT52 creates a double-strand break in the spacer region between two adjacent ZFN recognition sites (R) that form one set of ZFN sites. This results in: **(a)** one DNA fragment that contains the functional transgenes, including trait and marker genes that have been excised from pollen genome, and which are destroyed in the cell; and **(b)** the pollen genome with only one set of two adjacent ZFN recognition sites, which by itself, is non-functional.

and FLP recombinases actually decreases the efficiency of transgene removal, which might originate from competition of the two recombinases to bind to adjacent recognition sites [41].

Transgene removal within a particular organ or tissue is made possible by judicious selection of tissue-specific promoters. Several pollen-specific promoters, such as LAT59 and LAT52 promoters from tomato [42,43], ZM13 promoter in maize [44], and DEFH125 promoter in *Antirrhinum* [45] have been characterized as being only activated in pollen cells, with non-detectable activity in other tissues or developmental stages. A site-specific recombinase or ZFN driven by a pollen- or microspore-specific promoter might also be useful for transgene removal from pollen [46]. Availability of several pollen-specific promoters from various sources might provide more chances to use the transgene removal system in many other crop species.

Regulatory and economic considerations

Recently, there has been a trend to decrease the amount of transgenic DNA in plants to the extent that is absolutely necessary to deliver a trait; a development that has been embraced by companies and regulators. The introduction of additional transgenes as means for biocontainment would thus run counter to this trend, except that biocontainment itself might be considered a valuable trait. From an economic perspective, sufficient benefits with regard to significant biosafety gains or sustainability would be

required to outweigh the additional costs for discovering and licensing of the promoters and genes required for transgene removal. Such a transgene removal system that requires initial investment probably would be deployed first in those crops that are the greatest risks with regard to introgression to weedy wild relatives, such as sorghum [9] and switchgrass [47]. However, once the system is established, transgene removal systems in other marketable crops would be significantly cost effective compared to the cost for extensive monitoring and clean-up of accidental transgene contaminants. From a regulatory perspective, it is uncertain at present what degree of decrease in transgene flow constitutes an acceptable risk. In addition, the components required for site-specific recombination would need to undergo a risk assessment analysis for various ecological and food safety parameters. In pollen, very little foreign DNA would remain after excision events because, for example, transgene removal using the Cre-*loxP* recombination system would leave just a single 34 bp *loxP* site in the pollen genome.

There are several choices of well- or partially characterized transgene removal systems. The well-characterized Cre-*loxP* that is derived from phage P1 and the yeast-derived FLP-*FRT* systems are both reversible, which potentially allows the transgene to reenter into the genome, although reintegration of the excised products has not been reported, probably because transgene excision is the preferable reaction in this system [48].

Non-reversible site-specific recombination systems are also available, such as ParA-*MRS* and CinH-*RS2*, which are both derived from the serine resolvase family of recombinases [37]. Transgene removal in plants by ParA recombinase, which was derived from bacterial plasmids RK2 and RP4, has been shown to be precisely site-specific for

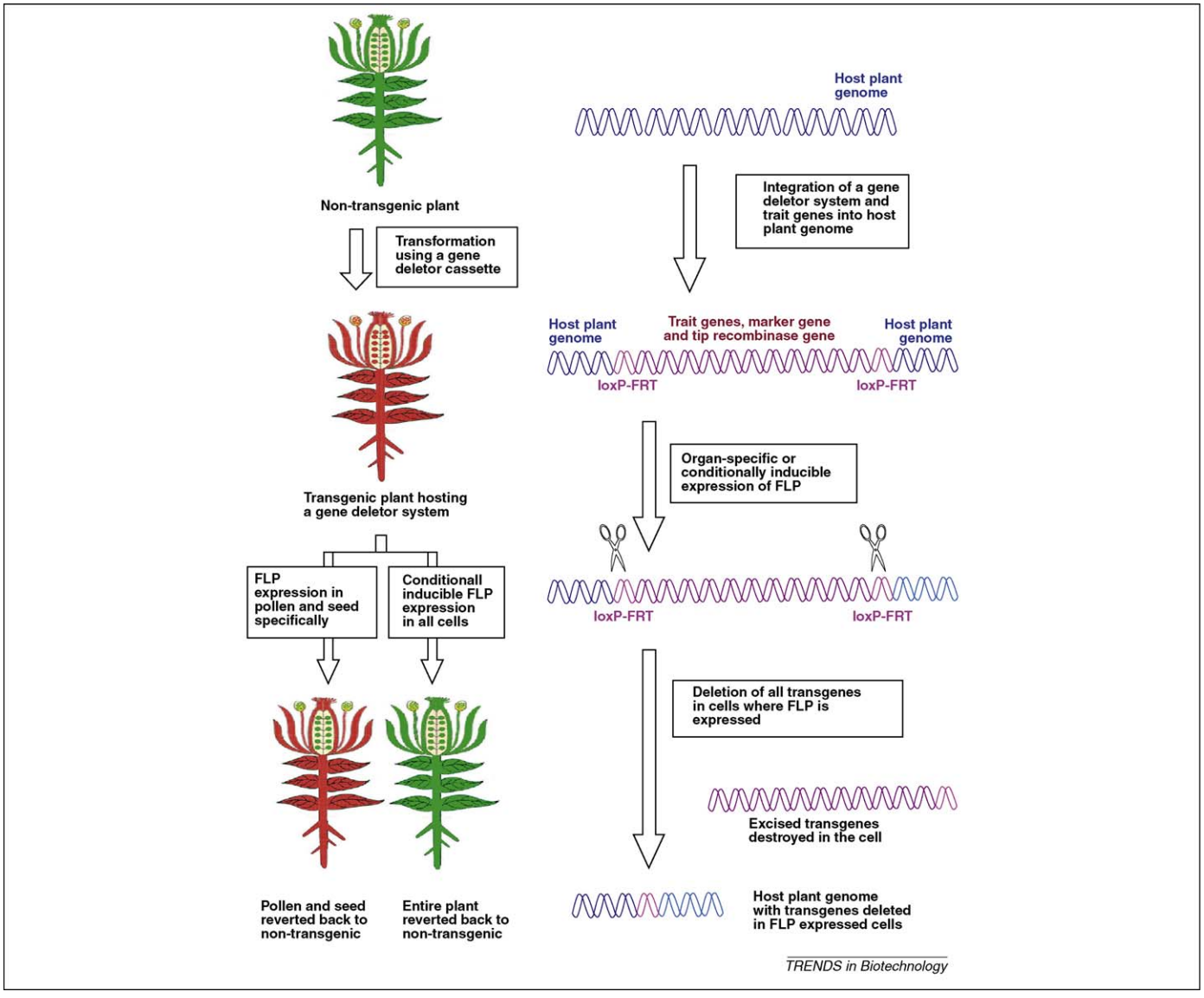

Figure 3. Schematic illustration of biocontainment using a gene deletor system. On the left, the use of a gene deletor system to produce non-transgenic pollen, seed or plants from a transgenic plant is demonstrated. The schematics shown on the right illustrate transgene removal. Any transgenes, such as trait genes, marker gene and FLP or Cre recombinase gene, that have been inserted into the two *loxP-FRT* sites (86 bp in length), will be deleted from any cell, in which the recombinase is expressed. When a pollen- and seed-specific gene promoter is used to control recombinase expression, all functional transgenes are deleted from these specific organs. On the other hand, using a conditionally inducible gene promoter, such as chemically or high-temperature inducible, to control recombinase expression results in the deletion of all functional transgenes throughout the plant upon induction. Adapted from Ref. [41] with permission.

excision of an embedded sequence between the recognition sites [49]. CinH recombinase derived from *Acetinetobacter* plasmids pKLH2, pKLH204 and pKLH205 has shown a site-specific gene excision function in yeast, but it has not yet been deployed in plants [50].

Perspectives

Transgene removal from pollen using site-specific recombination system could be an effective tool for transgene biocontainment; however, no system has so far been tested under agronomic conditions, or even in the field. Any transgene biocontainment system for commercial field application would probably be required to be not leaky and have no pleiotropic effects. Luo *et al*. [41] have been able to achieve complete transgene excision from pollen using fusion recognition sites of *loxP-FRT*, therefore, it appears feasible to employ site-specific recombination as a transgene biocontainment strategy. Further experiments, including those in field settings are needed to increase sample sizes and confidence limits, and also to test for reversal in the bidirectional recombination systems Cre-*loxP* and FLP-*FRT*. Non-reversible recombination systems, such as ParA-*MRS* and CinH-*RS2*, with their longer recognition site sequences, might provide more reliable transgene removal, while removing the possibility of potential transgene re-integration.

Homozygous transgenic seeds cannot be produced with transgene removal using a site-specific recombination system. This could be disadvantageous for seed-propagated plants for commercial purposes. If a transgene removal system were completely efficient, transgenic seed production would rely on the presence of transgenes in eggs; that is, the transgenic female parent (Figure 3). Seeds from transgenic plants that contain the transgene-removal trait by site-specific recombination in their pollen are hemizygous for the transgenic trait, or are non-transgenic. Practically speaking, half of the seeds that contain no transgenic traits could be eliminated for commercial purposes by soaking in a selection agent or by post-germination selection [51]. However, homozygous transgenic seeds could be produced if a conditionally expressed recombinase repression gene is incorporated into the transgene removal system [52]. In this case, expression of the recombinase gene is suppressed conditionally in pollen and seeds at generations in which transgenes need to be maintained, for example, in breeding stock (Figure 3) [52].

Our view on future perspectives on commercial use is cautiously optimistic. Transgenic tobacco plants with laboratory-effective, site-specific recombination systems that contain the fused *loxP*/*FRT* recognition sites [41] are being tested currently under agronomic conditions for the efficacy of transgene removal in pollen in the field. We are also testing multiple systems in *Brassica napus* (canola); again to be challenged under field conditions. If one or more systems perform as well in the field as they do under more controlled conditions, they could be good candidates in a commercially vectored system, and applied to transgenic crops that could otherwise be delayed by regulatory issues. Of special interest is the application in the foreseeable future to crops never before considered for transgenic release, such as outcrossing grasses for bioenergy production. Transgene removal from pollen using site-specific recombination might be the best choice as an environmentally friendly biocontainment strategy with high efficiency.

Acknowledgements

Research in the Stewart and Li Laboratories on transgene biocontainment is funded by USDA Biotechnology Risk Assessment Grants.

References

1 Al-Babili, S. and Beyer, P. (2005) Golden rice – five years on the road – five years to go? *Trends Plant Sci.* 12, 565–573
2 Welch, R.M. and Graham, R.D. (2004) Breeding for micronutrients in staple food crops from a human nutrition perspective. *J. Exp. Bot.* 55, 353–364
3 James, C. (2008) *Global Status of Commercialized Biotech/GM Crops* (ISAAA Briefs 39-2008), International Service for the Acquisition of Agri-biotech Applications
4 Frison, E. (2008) Biodiversity: indispensible resources. *Dev. Cooperation* 49, 190–193
5 Mayer, J. (2005) The golden rice controversy: useless science or unfounded criticism? *Bioscience* 55, 726–727
6 Lu, B.R. and Snow, A.A. (2005) Gene flow from genetically modified rice and its environmental consequences. *Bioscience* 55, 669–678
7 Korth, K.L. (2008) Genes and traits of interest for transgenic plant. In *Plant Biotechnology and Genetics: Principles, Techniques and Applications* (Stewart, C.N., Jr, ed), Wiley and Sons, Hoboken, New Jersey, pp. 193–216, 374 pp.
8 Miller, H.I. (2009) A golden opportunity, squandered. *Trends Biotechnol.* 27, 129–130
9 Stewart, C.N., Jr *et al.* (2003) Transgene introgression from genetically modified crops to their wild relatives. *Nat. Rev. Genet.* 4, 806–817
10 Stewart, C.N., Jr (2008) Pharming in crop communities. *Nat. Biotechnol.* 26, 1222–1223
11 Spok, A. *et al.* (2008) Evolution of a regulatory framework for pharmaceuticals derived from genetically modified plants. *Trends Biotechnol.* 25, 506–517
12 Conner, A.J. *et al.* (2003) The release of genetically modified crops into the environment. Part II. Overview of ecological risk assessment. *Plant J.* 33, 19–36
13 Verma, D. and Daniell, H. (2007) Chloroplast vector systems for biotechnology applications. *Plant Physiol.* 145, 1129–1143
14 Daniell, H. (2002) Molecular strategies for gene containment in transgenic crops. *Nat. Biotechnol.* 20, 581–586
15 Stewart, C.N., Jr (2004) *Genetically Modified Planet: Environmental Impacts of Genetically Engineered Plants*, Oxford University Press
16 Mariani, C. *et al.* (1990) Induction of male sterility in plants by a chimaeric ribonuclease gene. *Nature* 347, 737–741
17 Mariani, C. *et al.* (1992) A chimaeric ribonuclease-inhibitor gene restores fertility to male sterile plants. *Nature* 357, 384–387
18 Cook, S.M. *et al.* (2004) Do pollen beetles need pollen? The effect of pollen on oviposition, survival, and development of a flower-feeding herbivore. *Ecol. Entomol.* 29, 164–173
19 Kuvshinov, V. *et al.* (2001) Molecular control of transgene escape from genetically modified plants. *Plant Sci.* 160, 517–522
20 Prior, T.I. *et al.* (1996) Studies on the activity of Barnase toxins *in vitro* and *in vivo*. *Bioconjugate Chem.* 7, 23–29
21 Skinner, J.S. *et al.* (2000) Options for genetic engineering of floral sterility in forest trees. In *Molecular Biology of Woody Plants* (Jain, S.M. and Minocha, S.C., eds), pp. 135–153, Kluwer Academic Publishers
22 Ramos, H.J.O. (2005) A new system to control the *barnase* expression by a NifA-dependent promoter. *J. Biotechnol.* 118, 9–16
23 Ruiz, O.N. and Daniell, H. (2005) Engineering cytoplasmic male sterility via the chloroplast genome by expression of ß-Ketothiolase. *Plant Physiol.* 138, 1232–1246
24 Martin, W. (2003) Gene transfer from organelles to the nucleus: Frequenct and in big chunks. *Proc. Natl. Acad. Sci. U. S. A.* 100, 8612–8614
25 Stegemann, S. *et al.* (2003) High-frequency gene transfer from the chloroplast genome to the nucleus. *Proc. Natl. Acad. Sci. U. S. A.* 100, 8828–8833

26 Huang, C.Y. *et al.* (2003) Direct measurement of the transfer rate of chloroplast DNA into the nucleus. *Nature* 422, 72–76
27 Bentolila, S. *et al.* (2002) A pentatricopeptide repeat-containing gene restores fertility to cytoplasmid male-sterile plants. *Proc. Natl. Acad. Sci. U. S. A.* 99, 10887–10892
28 Jagannath, A. *et al.* (2002) Development of transgenic *barstar* lines and identification of a male sterile (*barnase*)/restorer (*barstar*) combination for heterosis breeding in Indian oilseed mustard (*Brassica juncea*). *Curr. Sci.* 82, 46–52
29 Hagemann, R. (2004) The sexual inheritance of plant organelles. In *Molecular Biology and Biotechnology of Plant Organelles* (Daniell, H. and Chase, C., eds), pp. 93–114, Springer
30 Maliga, P. (2004) Plastid transformation in higher plants. *Annu. Rev. Plant Biol.* 55, 289–313
31 Wang, T. *et al.* (2004) Low frequency transmission of a plastid-encoded trait in *Setaria italica*. *Theor. Appl. Genet.* 108, 315–320
32 Daniell, H. (2007) Transgene containment by maternal inheritance: Effective or elusive? *Proc. Natl. Acad. Sci. U. S. A.* 104, 6879–6880
33 Keenan, R.J. and Stemmer, W.P. (2002) Nontransgenic crops from transgenic plants. *Nat. Biotechnol.* 20, 215–216
34 Kim, Y-G. *et al.* (1996) Hybrid restriction enzymes: Zinc finger fusions to *Fok I* cleavage domain. *Proc. Natl. Acad. Sci. U. S. A.* 93, 1156–1160
35 Bibikova, M. *et al.* (2002) Targeted chromosomal cleavage and mutagenesis in *Drosophila* using zinc-finger nuclease. *Genetics* 161, 1169–1175
36 Tovkach, A. *et al.* (2009) A toolbox and procedural notes for characterizing novel zinc finger nucleases for genome editing in plant cells. *Plant J.* 57, 747–757
37 Thomson, J.G. and Ow, D.W. (2006) Site-specific recombination systems for the genetic manipulation of eukaryotic genomes. *Genesis* 44, 465–476
38 Dale, E.C. and Ow, D.W. (1991) Gene transfer with the subsequent removal of the selection gene from the host genome. *Proc. Natl. Acad. Sci. U. S. A.* 88, 558–562
39 Schaart, J.G. *et al.* (2004) Effective production of marker-free transgenic strawberry plants using inducible site-specific recombination and a bifunctional selectable marker gene. *Plant Biotechnol. J.* 2, 233–240
40 Gilbertson, L. (2003) Cre-*lox* recombination: Cre-active tools for plant biotechnology. *Trends Biotechnol.* 21, 550–555
41 Luo, K. *et al.* (2007) '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. *Plant Biotechnol. J.* 5, 263–274
42 Twell, D. *et al.* (1990) Pollen-specific gene expression in transgenic plants: coordinate regulation of two different tomato gene promoters during microsporogenesis. *Development* 109, 705–713
43 Twell, D. *et al.* (1991) Promoter analysis of genes that are coordinately expressed during pollen development reveals pollen-specific enhancer sequences and shared regulatory elements. *Genes Dev.* 5, 496–507
44 Hamilton, D.A. *et al.* (1998) A monocot pollen-specific promoter contains separable pollen-specific and quantitative elements. *Plant Mol. Biol.* 38, 663–669
45 Lauri, A. *et al.* (2006) The pollen-specific DEFH125 promoter from *Antirrhinum* is bound *in vivo* by the MADS-box proteins DEFICIENS and GLOBOSA. *Planta* 224, 61–71
46 Mlyunarova, L. *et al.* (2006) Directed micro-specific recombination of transgenic alleles to prevent pollen-mediated transmission of transgenes. *Plant Biotechnol. J.* 4, 445–452
47 Stewart, C.N., Jr (2007) Biofuels and biocontainment. *Nat. Biotechnol.* 25, 283–284
48 Hare, P.D. and Chua, N-H. (2002) Excision of selectable marker genes from transgenic plants. *Nat. Biotechnol.* 20, 575–580
49 Thomson, J.G. *et al.* (2009) ParA resolvase catalyzes site-specific excision of DNA from the *Arabidopsis* genome. *Transgenic Res.* 18, 237–248
50 Kholodii, G. (2001) The shuffling function of resolvases. *Gene* 269, 121–130
51 Conner, A.J. and Christey, M.C. (1997) A seed treatment for eliminating non-hybrid plants when using atrazine resistance as a genetic marker for hybrid seed production. *Annal. Bot.* 80, 561–564
52 Li, Y. *et al.* (2007): Gene-deletor: a new tool to address concerns over GE crops. *USDA Information Systems for Biotechnology News Report*. June 2007 (http://www.isb.vt.edu/articles/jun0701.htm)
53 Belostosky, D.A. and Meagher, R.B. (1996) Differential organ-specific expression of three poly(A) binding protein genes from *Arabidopsis*. *Proc. Natl. Acad. Sci. U. S. A.* 90, 6686–6690