Plant Mol Biol (2011) 75:621–631
DOI 10.1007/s11103-011-9756-2

Transgene excision in pollen using a codon optimized serine resolvase CinH-*RS2* site-specific recombination system

Hong S. Moon · Laura L. Abercrombie · Shigetoshi Eda · Robert Blanvillain · James G. Thomson · David W. Ow · C. N. Stewart Jr.

Received: 28 October 2010 / Accepted: 11 February 2011 / Published online: 26 February 2011

Abstract Transgene escape, a major environmental and regulatory concern in transgenic crop cultivation, could be alleviated by removing transgenes from pollen, the most frequent vector for transgene flow. A transgene excision vector containing a codon optimized serine resolvase CinH recombinase (CinH) and its recognition sites *RS2* were constructed and transformed into tobacco (*Nicotiana tabacum* cv. Xanthi). CinH recombinase recognized 119 bp of nucleic acid sequences, *RS2,* in pollen and excised the transgene flanked by the *RS2* sites. In this system, the pollen-specific LAT52 promoter from tomato was employed to control the expression of CinH recombinase. Loss of expression of a green fluorescent protein (GFP) gene under the control of the LAT59 promoter from tomato was used as an indicator of transgene excision. Efficiency of transgene excision from pollen was determined by flow cytometry (FCM)-based pollen screening. While a transgenic event in the absence of CinH recombinase contained about 70% of GFP-synthesizing pollen, three single-copy transgene events contained less than 1% of GFP-synthesizing pollen based on 30,000 pollen grains analyzed per event. This suggests that CinH-*RS2* recombination system could be effectively utilized for transgene biocontainment.

Keywords CinH-*RS2* · Pollen · Transgene excision · Site-specific recombination · Biocontainment · Green fluorescent protein (GFP) · Flow cytometry

H. S. Moon · L. L. Abercrombie · C. N. Stewart Jr. (✉)
Department of Plant Sciences, University of Tennessee, Knoxville, TN 37996, USA
e-mail: nealstewart@utk.edu

S. Eda
Center for Wildlife Health, Department of Forestry, Wildlife and Fisheries, University of Tennessee, Knoxville, TN 37996, USA

R. Blanvillain · D. W. Ow
Plant Gene Expression Center, 800 Buchanan Street, Albany, CA 94710, USA

J. G. Thomson
Crop Improvement and Utilization Research Unit, Western Regional Research Center, USDA-ARS, 800 Buchanan Street, Albany, CA 94710, USA

Present Address:
R. Blanvillain
Laboratoire de Biochimie et Physiologie Moléculaire des Plantes, CNRS UMR 5004, 2 Place Viala, 34060 Montpellier Cedex 1, France

Present Address:
D. W. Ow
Gene Engineering Center, South China Botanical Garden, 723 Xingke Road, 510650 Tianhe, Guangzhou, China

Introduction

The transfer of transgenes conferring traits including herbicide and insect resistance from transgenic plants to sexually compatible non-transgenic plants has occurred via several vectors such as pollen, seeds, and propagules. Uncontrolled transgene escape that has been an important issue to environmentalists, regulators, scientists, and farmers could result in undesirable consequences such as creation of more invasive or competitive weeds, and admixture in crops that are intended to be non-transgenic. Transgene transfer through pollination via mediators including wind, insects, and birds is possible between transgenic and non-transgenic plants, including many commercially cultivated crop species (Stewart et al. 2003). Transgene escape has occurred between two different types

of transgenic *Brassica napus* (canola) by outcrossing with each other under field conditions (Beckie et al. 2003; Aono et al. 2006) and canola to its wild relative *Brassica rapa* (Chevre et al. 2000; Halfhill et al. 2004). Transgene presence in native Mexican landrace populations of maize (*Zea mays* L.) has been documented with molecular evidence (Piñeyro-Nelson et al. 2009).

Transgene containment strategies have been proposed to suppress or eliminate unintentional transgene escape from transgenic plant populations. Two categories of transgene contaminant strategies exist: physical and biological. Physical transgene containment strategies include trap crops, spatial isolation, fences, the removal of flowers, or even cultivation underground (Ingram 2000; Morris et al. 1994; Whittington 2006. http://www.associatedcontent.com/article/27102/underground_farming.html?cat=15). Most physical transgene containment strategies are not considered to be very effective. Underground cultivation, suggested for pharmaceutical production, is limited by the number of available abandoned mines (Whittington 2006).

Several biological transgene containment strategies—biocontainment—such as male sterility, maternal inheritance, transgenic mitigation, and transgene excision, have been the subject of research for many years. Male sterility was successfully achieved using chimeric ribonuclease genes (Barnase) in tobacco and canola (Mariani et al. 1990). The drawbacks of this approach, including fertilization with non-transgenic plants as a maternal parent and potential negative effects on pollen-feeding pollinators including pollen beetles make this strategy inappropriate for widespread use in commercial fields (Daniell 2002; Cook et al. 2004). Transgenes targeted to the maternally inherited chloroplast genome has been used as a containment strategy in some plant species (Daniell et al. 1998; Iamtham and Day 2000; Ruf et al. 2001). Despite maternal inheritance of the chloroplast transgenes in many species, low levels of transgenes can be transmitted via pollen in some species (Haygood et al. 2005; Svab and Maliga 2007). Also, commercial utilization requires maternally inherited chloroplast genes be engineered to homoplasmy, which appears to be very difficult in most of crop species. Chloroplasts in one-third of angiosperm species are not maternally inherited, thus this strategy could not be used in these species (Maliga 2004). A mitigating gene linked to a transgene can be used to reduce the competitiveness of transgene that could be introgressed weeds (Al-Ahmad et al. 2004, 2006). However, this mitigation strategy might not be effective for transgene escape within the same plant species (Moon et al. 2010).

Since most transgene dispersal occurs by pollen movement, biocontainment targeting pollen has attracted considerable attention (Gray and Raybould 1998). A transgene excision strategy, where transgenes are excised from the pollen genome, appears to be one of the most attractive transgene biocontainment strategies (Luo et al. 2007; Moon et al. 2010). Unlike male sterility, the pollen reproductive process in plants would be uninterrupted using the transgene excision strategy. Therefore, seeds would be produced normally and pollen-feeding insects would not suffer from a lack of food. Several site-specific recombination systems have shown to effectively remove a transgene from transgenic plant, including pollen and seed genomes (Mlynárová et al. 2006; Luo et al. 2007; Kobertekh et al. 2010). Recently, zinc-finger nuclease (ZFN)-mediated transgene excision has been demonstrated in transgenic tobacco containing a reporter gene flanked by ZFN cleavage sites by crossing with a tobacco plant carrying a ZFN gene (Petolino et al. 2010). Most site-specific recombinase-mediated transgene excision occurs by activating a transgenically-encoded recombinase with an inducible or tissue-specific promoter (Fig. 1). When expressed, the recombinase recognizes its recombination sites that flank all functional transgene cassettes and excises the sandwiched transgene between two recombination sites in the target cells, in this case, pollen. Once excised from genomic DNA, all transgenes are degraded and no longer functional (Fig. 1). Some small components of the integrated transgene such as the left and right border and one recombination site would remain in the pollen genome after transgene excision (Fig. 1). Site-specific recombination is involved in many natural biological functions including DNA transposition, integration of viral DNA into a host chromosome, excision or inversion of DNA segment, and regulation of gene expression (Grindley et al. 2006). Several fully reversible site-specific recombination systems including Cre-*lox*, FLP-*FRT*, Gin-*gix* and R-*RS* have shown transgene excision in transformed plants (Dale and Ow 1990; Lyznik et al. 1993; Maeser and Kahmann 1991; Onouchi et al. 1991). Cre-*lox* mediated-transgene excision by chemical induction has been demonstrated in *Arabidopsis* (Zuo et al. 2001). Recombination by the yeast FLP recombinase at two *FRT* sites resulted in the deletion of the embedded sequence between the sites (Lloyd and Davis 1994; Rao et al. 2010). A mutant Gin recombinase mediated deletion of the DNA sequences between the specific recombination sites called *gix*; however, wild-type Gin recombinase failed to rearrange the DNA sequences (Klippell et al. 1993). The DNA segment between the recombination sites has shown to be excised by activation of the R recombinase in *Arabidopsis* and rice (Onouchi et al. 1995; Nakagawa et al. 2001). Luo et al. (2007) reported that highly improved efficiencies of transgene excision using Cre or FLP recombinase have been achieved by the fusion of *loxP* and *FRT* recognition sites. Transgenes in pollen and/or seeds were excised by Cre or FLP recombinase with the fused *loxP-FRT* recognition sites in several transgenic tobacco events, whereby a 100% excision efficiency was achieved in some events

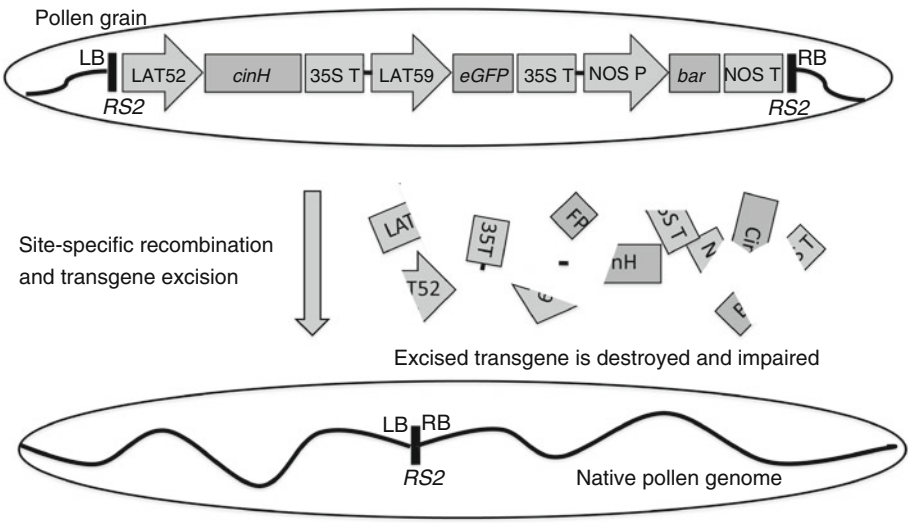

Fig. 1 Schematic illustration of CinH recombinase-mediated transgene excision in pollen. Pollen specific promoter LAT52 drives CinH recombinase in pollen. CinH recombinase recognizes 119 bp sequence sites, *RS2*, all functional transgenes flanked by the *RS2* sites excised leaving only one *RS2* site and T-DNA borders in the pollen genome. *LAT52* pollen-specific LAT52 promoter, *cinH* codon optimized CinH recombinase gene, *35S T* 35S terminator, *LAT59* pollen-specific LAT59 promoter, *eGFP* enhanced GFP gene, *NOS P* nopaline synthase promoter, *bar* herbicide resistant bar gene, *NOS T* nopaline synthase terminator, *RS2* CinH recombinase recognition site

based on the analysis of 25,000 progeny seedlings (Luo et al. 2007). However, the progeny seeds were produced by crossing without emasculation of maternal non-transgenic tobacco plants. Therefore, a large portion of the seeds might come from self-fertilization of the maternal plants.

The serine resolvase family site-specific recombinases, including ParA and CinH recombinases, have shown efficient excision of plasmid DNA in transformed fission yeast (*Schizosaccharomyces pombe*) (Thomson and Ow 2006). ParA recombinase was effective in catalyzing DNA excision in transgenic *Arabidopsis thaliana* plants too (Thomson et al. 2009). An intervening transgene was excised by *Streptomyces*-derived phiC31-mediated recombination in transgenic *Arabidopsis* and wheat (Thomson et al. 2010; Kempe et al. 2010). CinH recombinase from *Acetinetobacter* plasmids pKLH2, pKLH204, and pKLH205 has shown activity in site-specific recombination in yeast, but this system has yet to be characterized in plants (Kholodii 2001; Thomson and Ow 2006). Here, we demonstrated transgene excision in pollen by the plant codon usage optimized CinH recombinase for use in transgene biocontainment. This is the first report of the CinH-*RS2* system-mediated recombination in transgenic plants.

Materials and methods

Vector constructs

CinH and CinH_Drec vectors (Fig. 2a, b) were constructed for plant transformation. Plasmids containing the CinH recombinase optimized for codon usage in plants and the CinH recombination site (*RS2*), 5′-CGTTACTTTGGGGTATACCCTAAAGTTACAATATAAAAGTTCTTAAAACTATGTAACATTTAAATGATTTTTAACCATATATAACATGTAACTTTGATATTTAAAGTTTATAATTTACG-3′, were constructed. The pLAT59-12 plasmid containing the LAT59 promoter (Genbank accession X56488) and tomato genomic DNA were gifts from the McCormick Lab (Plant Gene Expression Center, Albany, CA, USA). pPK100 containing the *tevL-eGFP* cassette was a gift of Patrick Gallois (University of Manchester, Manchester, UK). The two flanking CinH recognition sites, *RS2*, one designed for the right border (BamHI-BglII–*RS2*-SpeI) and the other designed for the left border (EcoRI-ApaI-*RS2*-PmeI-AatII-SacI) were PCR amplified and cloned in pGemT Easy to yield pBioC#461 and pBioC#464 respectively. Primers used were (attRB1: 5′-ggatccccagatctCGTTACTTTGGGGTATAC-3′, attRB2: 5′-gcgttaacactagtCGTAAATTATAAACTTTAAATATC-3′ and attLB1: 5′-gaattcgggcccCGTTACTTTGGGGTATAC-3′, attLB2: 5′-gagctcgacgtcgtttaaacCGTtAATTATAAACTTTAAATATC-3′). Enzyme sites underlined.

The LAT52 promoter (Twell et al. 1990) was PCR amplified from *Solanum lycopersicum* gDNA using primers (Lat52(F): 5′-gtttaaacgacgtcCCTATACCCCTTGGATAAG-3′, Lat52(R) 5′-ggcgcgccTTTAAATTGGAATTTTTTTTTTTGGTGTGTGTAC-3′) cloned in pGemT Easy to yield pBioC#499. The PmeI to AscI promoter fragment was cloned in place of the CaMV35S promoter of pC35S CinH to yield pBioC535 (pLAT52::CinH). The NOS terminator of pCambia 0390 (http://www.cambia.org) was removed by

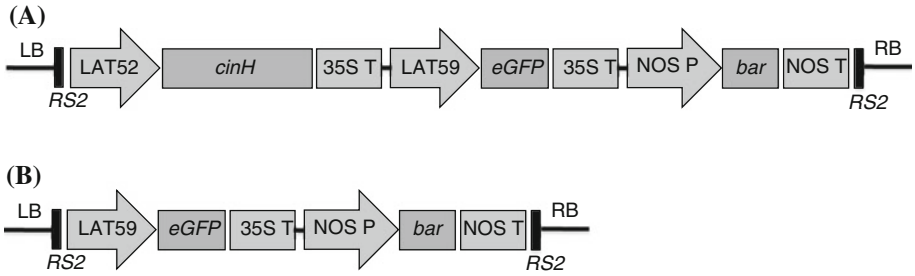

Fig. 2 CinH and CinH_Drec vector constructs **a** CinH recombinase is under the control of pollen-specific LAT52 promoter. Enhanced GFP gene is driven by pollen-specific LAT59 promoter. Bar gene confers resistance to herbicide glufosinate ammonium. **b** CinH_Drec vector was constructed from the CinH vector by removing CinH recombinase cassette. *LAT52* pollen-specific LAT52 promoter, *cinH* codon optimized CinH recombinase gene, *35S T* 35S terminator, *LAT59* pollen-specific LAT59 promoter, *eGFP* enhanced GFP gene, *NOS P* nopaline synthase promoter, *bar* herbicide resistant bar gene, *NOS T* nopaline synthase terminator, *RS2* CinH recombinase recognition site, *LB* left border, *RB* right border

inserting a SpeI site using PCR (primers—0390F: 5′-aact actagtgtttgacaggatatattggc-3′; 0390R: 5′-aacgtcagaagccg actgcac-3′); the amplified fragment was cloned in pGemT Easy, digested with SpeI and SphI and inserted in place of the original fragment. The resulting plasmid was then digested with PstI and BamHI for ligation of the PstI to BamHI *Bar*-selection cassette of pGreen0229 (http://www.pgreen.ac.uk) to yield pBioC#538. Then the BglII to SpeI fragment of pBioC#461 containing *RS2* designed for the right border was cloned in pBioC#538 to yield pBioC#542. As a preparative step, pPK100 was digested with NotI and BamHI, filled with dNTPs using T4 polymerase and religated to yield pRC10. Then pRC10 was digested XhoI and EcoRI and filled with primers (CPe1: 5′-TCGAcatcgatcacgtgc-3′, CPe2: 5′-aattgcacgtgatcgatg-3′) to yield pRC28 with a PmlI site. The HindIII to PmlI fragment of pRC28 containing the CaMV35S promoter was replaced with the HindIII to SmaI LAT59 promoter fragment of pLAT59-12 to yield pBioC#401.

The *RS2* EcoRI to SacI fragment of pBioC#464 designed for the left border was cloned into pBioC#401 digested with EcoRI and SacI to yield pBioC#472 (*RS2*, linker, LAT59::*eGFP*). The fragment from PmeI to SacI of pBioC#535 (pLAT52::CinH) was then cloned in pBioC#472 to yield pBioC#545 (*RS2*, LAT52::CinH, LAT59::*eGFP*).

Finally, the ApaI to HindIII fragment of pBioC#472 or pBioC#545 was cloned into pBioC#542 to yield CinH_Drec (Fig. 2b) (Moon et al. 2011) and CinH vectors (Fig. 2a).

Plant transformation

Each CinH or CinH_Drec vector was transformed into *Agrobacterium tumefaciens* strain EHA105. *Agrobacterium*-mediated tobacco (*Nicotiana tabacum* L.) transformation was performed using a standard protocol (Horsh et al. 1985). All cultures were maintained at 24 ± 2°C under a 16/8 h light/dark photoperiod. Rooted shoots were transplanted to soil and acclimated for 2 weeks. T_0 transgenic events from tissue culture were confirmed by polymerase chain reaction (PCR) and grown in the greenhouse. Pollen grains collected from the T_0 events were screened under epifluorescence microscopy as described in the next section.

Microscopic visual assay

Pollen grains from two plants of each CinH_Drec T_1 event were collected to confirm transgenicity and to estimate GFP synthesis level. CinH T_1 event pollen grains were screened to estimate the frequency of GFP synthesizing pollen. Pollen was collected by tapping flowers so that pollen fell into a 1.5 ml microfuge tube and was then suspended in sterile water. The microfuge tubes were immediately shaken in a mixer (Eppendorf 5432 mixer) for 10 min to minimize clumping of pollen. The pollen suspension was taken from the tube and placed on a slide glass and covered with a glass cover slip. Pollen screening was performed under an epifluorescence (FITC filtered) microscopy (Olympus BX51 model) with blue light excitation at 200× magnification. QCapture software (Qimaging, Surrey, BC, Canada) was used to acquire pollen images.

Progeny analysis

T_1 seeds were produced by self-pollination of T_0 events. Progeny analysis with the harvested T_1 seeds was performed on MSO medium containing glufosinate ammonium at 5 mg/l. After 10–12 days of seed placement on the media, the total number of germinated seeds and number of negative and positive seedlings for glufosinate ammonium selection were recorded. Chi-square goodness of fit test was used to analyze fitness of the data.

Southern blot analysis

Positive T_1 seedlings for glufosinate ammonium selection were grown in the greenhouse. PCR and pollen screening

under epifluorescence microscopy were performed to confirm transgenicity. Genomic DNA was extracted from 2 g of macerated snap-frozen leaf tissue using a modified CTAB extraction method (Stewart and Via 1993). Resulting genomic DNA was purified by repeated phenol chloroform extractions. Ten micrograms of genomic DNA was digested to completion with HindIII. Control plasmid DNA from the binary vector CinH was also digested with HindIII. Resulting fragments were separated on a 1.2% agarose gel. Fragments were transferred to Zeta-Probe GT membrane (Bio-Rad, Hercules, CA, USA) by high salt Southern transfer (Brown 2001). A PCR product containing the full-length open reading frame of *cinH* was radioactively labeled with α-^{32}P dCTP using Prime-It II Random Primers Labeling Kit (Stratagene, La Jolla, CA, USA). The 0.5 kb *cinH* ^{32}P-labeled probe was produced by GoTag® Green master mix (Promega, Madison, WI, USA) using primers 5′-CTACGTTCGTGTTT-CATCGG-3′ and 5′-CTGGATATGCCGAACGCTTT-3′. Labeled probe was purified using mini Quick Spin DNA columns (Roche Diagnostics, Indianapolis, IN, USA). Southern blots were hybridized with labeled probe in ULTRAhyb hybridization buffer (Ambion, Austin, TX, USA) and washed according to the manufacturer's protocol. Blots were visualized by exposure to phosphor-imaging screens (Storage Phosphor Screen GP, Eastman Kodak, Rochester, NY, USA) and scanned using Personal FX (Bio-Rad, Hercules, CA, USA). Image analysis was undertaken using Quantity One software (Bio-Rad, Hercules, CA, USA).

Pollen collection

All plants including non-transgenic tobacco, CinH_Drec-1 T_1 event, and CinH T_1 events were grown in the greenhouse. Four plants per each type were grown with 2 m distance from other plant types to prevent potential cross-contamination. Pollen collections were conducted for 10 days during flowering period by tapping the mature flowers each into a 1.5 ml microfuge tube and immediately freezing at −80°C. At the time of assay, 1 ml of sterile water was added into each tube containing pollen grains and the tubes were shaken in a mixer (Eppendorf 5432 mixer) for 10 min. Pollen suspensions were filtered with a 132 μm pore nylon mesh (Sefar Nitex 03-132/43, Sefar filtration Inc., Depew, NY, USA) to remove non-pollen debris such as anthers and clumps of pollen that may clog the fluidic system of the flow cytometer. Filtered pollen suspension was transferred into 5 ml polystyrene round bottom tubes (BD falcon, San Jose, CA, USA) for FCM analysis.

Flow cytometry (FCM) analysis

FCM-based transgenic pollen analysis was performed using methods described in Moon et al. (2011). Briefly, a LSR II flow cytometer (BD Biosciences, San Jose, CA, USA) was used to detect GFP fluorescence in pollen grains with the voltage settings of 200 eV for forward scatter channel, 190 eV for side scatter channel, and 468 eV for FL1 channel. Data were obtained by counting 30,000 pollen grains for each sample and analyzed using DiVa software (BD Biosciences, San Jose, CA, USA) and CyflogicTM software (CyFlo Ltd, Finland).

Results

Regeneration of transgenic tobacco events

Twenty independent transgenic events were generated containing the CinH vector (Fig. 2a). Of the nine CinH_-Drec events, one high GFP-synthesizing event was selected by visual screening of pollen grains under a blue-light microscopy. T_1 seeds from PCR-confirmed T_0 events were selected on selective media containing glufosinate ammonium 5 mg/l. Selected T_1 seeds were germinated and plants grown in the greenhouse. Visual screening of pollen from the selected CinH_Drec T_1 event allowed differentiation between different zygosity status including homozygous, hemizygous, and nullizygous for the transgene (data not shown).

Progeny analysis

Progeny analysis was performed with all twenty CinH events. Non-transgenic tobacco seeds did not survive on glufosinate ammonium selection media (Fig. 3a), while all non-transgenic seeds survived on non-selection media (Fig. 3b). T_1 seeds from CinH transgenic events were segregated for glufosinate ammonium resistance (Fig. 3c, d). Surviving seeds were a mixture of hemizygous and homozygous seeds for the transgene. For CinH events, should the transgene excision system completely excise all DNA between the *RS2* sites in pollen, transgenic DNA would exist only in maternal reproductive cells. Pollen that failed to excise transgene by CinH-*RS2* recombination system retain *eGFP* expression cassette, and exhibit GFP fluorescence. The expected progeny segregation ratio for completely effective pollen transgene excision for a single locus would be 1:1 for transgenic: non-transgenic T_1 individuals. However, all 20 CinH events significantly differed from the expected 1:1 ratio (Table 1). This could be due to incomplete excision in pollen and/or the presence of multiple transgenic loci segregating in the female gametes. Eight events with relatively small deviations from the expected ratio, CinH-2, 3, 4, 6, 9, 10, 20, and 21, were selected for further analysis. In addition to these 8 events, 2 more CinH events, CinH-19 and 22, that had very small

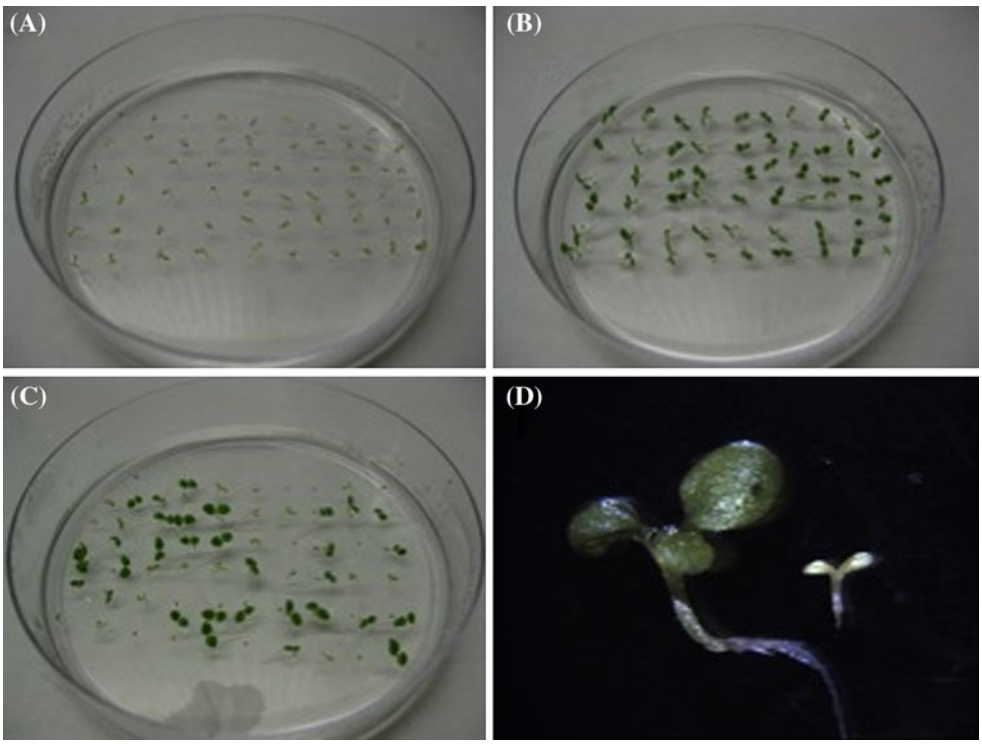

Fig. 3 T_1 progeny selection on selection media containing glufosinate ammonium. **a** Non-transgenic tobacco (Xanthi) seeds did not survived on selection media containing 5 mg/l of glufosinate ammonium. **b** Non-transgenic tobacco seeds on non-selection media. **c** CinH event seeds were selected on the herbicide selection media. **d** Representation of living and dead CinH seedlings

numbers of non-transgenic seedlings were also selected for further analysis.

Microscopic visual assay of pollen

Four T_1 plants per each event were grown in the greenhouse and had confirmed transgenicity by PCR (data not shown). Phenotypic differences were not found between transgenic tobacco events and non-transgenic tobacco. Pollen grains from CinH or CinH_Drec T_1 transgenic events were not phenotypically different from non-transgenic tobacco pollen under white light. However, the CinH_Drec event had large numbers of GFP positive pollen grains under blue light, while non-transgenic tobacco had no green-fluorescent pollen grains. Compared to the CinH_Drec event, CinH T_1 events had significantly less or lacked GFP positive pollen in the microscopic images (Fig. 4). Elimination of GFP expression would be expected from transgene excision of the *eGFP* marker occurring in pollen via site-specific recombination (Fig. 1). Therefore, the loss of green florescence serves as an indicator of transgene excision in pollen.

Southern blot analysis

Ten CinH events were subject to Southern blot analysis to confirm transgene integration and copy number. As expected, genomic DNA from non-transgenic tobacco and the CinH_Drec event did not reveal hybridizing bands with the *cinH*-specific probe. Five CinH events, CinH-2, 4, 9, 10, and 21, exhibited a single hybridization band which indicated a single copy of transgene integration (Fig. 5). The remainder of the events appeared to contain multiple transgene copies in their genomes (Fig. 5). Southern blot analysis also confirmed that the 10 selected CinH events were independently transformed events.

Flow cytometry (FCM) analysis

FCM analysis was performed to determine efficiency of CinH-*RS2* recombination-mediated transgene excision in large numbers (ca., 30,000) of pollen. FCM has been shown to be fast and effective in discriminating between GFP-synthesizing transgenic pollen and non-expressing pollen (Moon et al. 2011). In this study, FCM was used to develop a system for GFP expression in pollen. Here, GFP positive particles were detected in non-transgenic pollen sample at a very low rate (0.02%), even after extensive flushing of FCM fluidic system between measurements (Fig. 6). GFP positive pollen ratio ranged from 0.46 to 17.48% for single transgene copy-inserted events (Fig. 6). Three events, CinH-4, 10, and 21, had 0.93, 0.47, and 0.46% of GFP positive pollen, respectively, out of 30,000

Table 1 Segregation analysis of T_1 progeny

	Total germinated	Total transgenic (T)	Total non-transgenic (N)	Observed ratio (T:N)	X^2 value under the 1:1 expected ratio
CinH-2	370	250	120	2.1:1	45.6*
CinH-3	224	150	74	2.0:1	25.7*
CinH-4	387	250	137	1.8:1	32.9*
CinH-5	258	222	36	6.2:1	134.0*
CinH-6	190	151	39	3.9:1	66.0*
CinH-7	295	280	15	18.7:1	238.0*
CinH-9	412	289	123	2.3:1	66.8*
CinH-10	294	213	81	2.6:1	59.2*
CinH-11	338	333	5	66.6:1	318.2*
CinH-12	311	290	21	13.8:1	232.6*
CinH-13	312	229	83	2.8:1	68.3*
CinH-14	317	236	81	2.9:1	75.7*
CinH-15	337	315	22	14.3:1	254.7*
CinH-16	325	317	8	39.6:1	293.7*
CinH-17	329	248	81	3.1:1	84.7*
CinH-18	282	204	78	2.6:1	56.2*
CinH-19	729	716	13	55.1:1	677.9*
CinH-20	226	139	87	1.6:1	11.9*
CinH-21	472	288	184	1.6:1	22.9*
CinH-22	250	243	7	34.7:1	222.7*

*$Pr > \chi^2 < 0.0001$

pollen grains analyzed (Fig. 6). The CinH_Drec-1 event had more than 50% GFP positive pollen, which is consistent with the expectations of collecting pollen from a mixture of hemizygous and homozygous T_1 plants.

Discussion

Transgene excision via site-specific recombination could be an effective transgene biocontainment strategy when gene flow from pollen is the main concern for transgenes leaving the field of interest (Luo et al. 2007; Moon et al. 2010). A codon optimized serine resolvase recombinase CinH recognizes specific 119 base sequence recombination sites and excised embedded functional transgenes including the *eGFP* gene from the pollen genome. An absence of GFP expression served as an indicator of transgene excision. Three independent CinH tobacco T_1 events exhibited less than 1% GFP positive pollen, based on FCM analysis of 30,000 pollen grains. As compared to a control CinH_Drec event, which had GFP expression in 70% of pollen, CinH events had a significant decrease in percentage of GFP positive pollen. This indicated that transgenic events containing CinH recombinase efficiently excised transgenes from the pollen genome. Unlike the tyrosine family of recombinases including Cre and FLP recombinases, CinH is an irreversible recombinase that prevents possible re-insertion of excised transgenes into the genome. CinH recombinase has superior specificity by recognizing a 119 nucleic acid base sequence known as *RS2* (NCBI ref# AF213017). As compared to Cre and FLP recombinases, which have 34 bp recombination sites, CinH has recombination sites of 119 bp that should provide high specificity to the site-specific recombination, this negating the risk of illicit recombination within plant genomes. One *RS2* recombination site would remain in the pollen genome after transgene excision occurs. However, this single recombination site in the absence of a recombinase and another *RS2* sites should not lead to recombination in pollen genome.

None of CinH transgenic events had the expected 1:1 segregation ratio. Since none of the CinH events demonstrated complete transgene excision in pollen, deviation from the expected 1:1 T_1 segregation ratio of transgenic to non-transgenic would most likely be due to incomplete excision in T_1 pollen. Sample size of progeny analysis might need to be larger to make a confident conclusion. In addition, to acquire more reliable progeny analysis data with this transgene excision system, transgenic plants should be crossed with emasculated or male-sterile non-

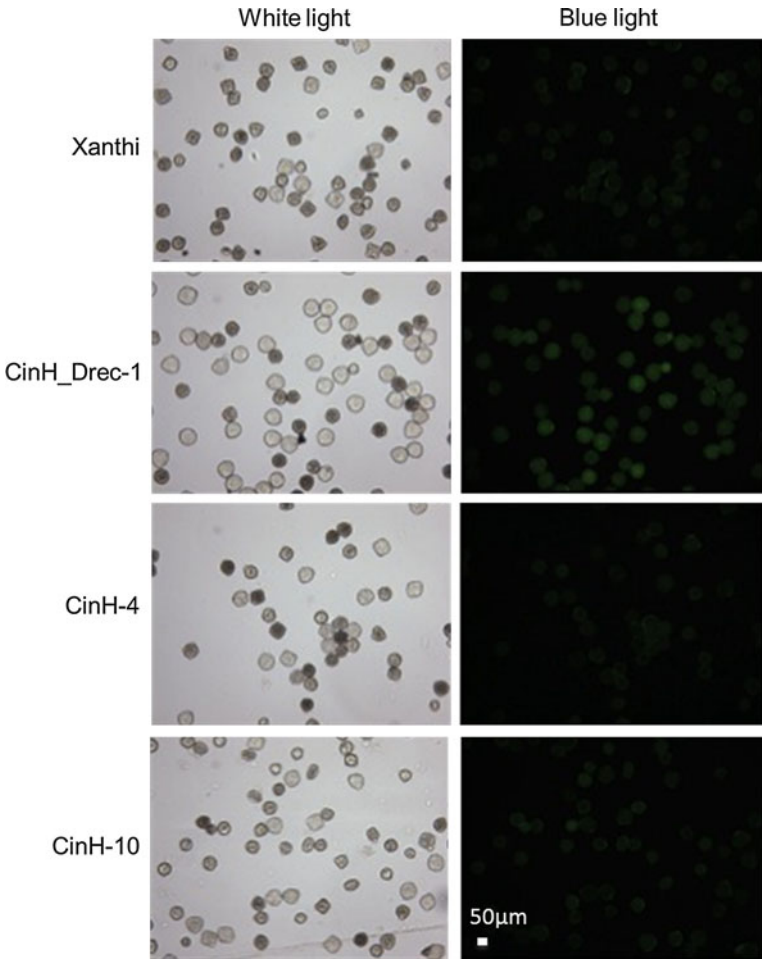

Fig. 4 Microscopic images of pollen grains. Pollen grains from non-transgenic tobacco (Xanthi), CinH_Drec event, and 2 CinH events were collected and screened under the FITC filtered epifluorescent microscopy. *Left panel* images were taken under white fluorescent light with 1.67 ms exposure time. *Right panel* images were taken under blue light with 3 s exposure time. All images were taken at ×200 magnification

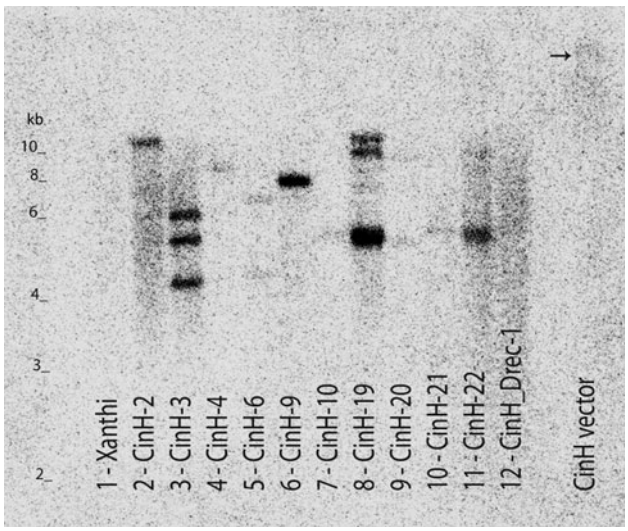

Fig. 5 Southern blot analysis of T_1 CinH transgenic events. Genomic DNA from non-transgenic tobacco (Xanthi), 10 CinH events (CinH-2, 3, 4, 6, 9, 10, 19, 20, 21, 22), and CinH_Drec event (CinH_Drec-1) were digested with HindIII and hybridized to *cinH* probe. HindIII digested binary CinH vector used for tobacco transformation is shown in CinH vector lane. Relative migration distances of DNA ladder fragments are shown in the *left lane*

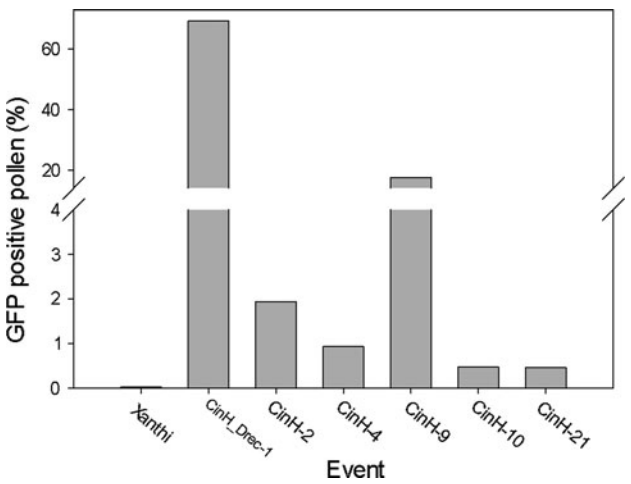

Fig. 6 Percentage of GFP positive pollen in single transgene copy integrated CinH transgenic events. Loss of GFP expression served as an effective indicator for transgene excision. Each event including non-trasngenic tobacco contained GFP positive pollen as follows. Non-transgenic tobacco (Xanthi)—0.02%, CinH_Drec—69.36%, CinH-2—1.94%, CinH-4—0.93%, CinH-9—17.48%, CinH-10—0.47%, CinH-21—0.46%. Percentage of GFP positive pollen was based on 30,000 pollen grains analyzed

transgenic maternal plants. Transgene excision system with a fused recombination sites—*loxP*/*FRT*—has shown high efficient transgene excision in pollen (Luo et al. 2007). However, the efficiency of transgene excision was estimated by reciprocal cross with non-transgenic tobacco plants without emasculation. The efficiency might not reflect the fact that large portion of progeny seeds could come from self-fertilization of non-transgenic tobacco. Field experiments with larger sample sizes should be useful to better define real-world utility.

Various efficiencies of recombination systems could be optimized by choice of promoter (Kopertekh et al. 2010). The tomato-derived LAT52 pollen-specific promoter has shown high specificity to pollen (Luo et al. 2007), and indeed, transgenes were efficiently excised, although the efficiency of this promoter in tobacco could possibly be overestimated due to non-emasculated maternal plants. Reverse transcriptase (RT)-PCR has revealed high specificity of the LAT52 promoter to pollen, when other plant parts including root, stem and leaf were subject to RT-PCR (Luo et al. 2007). This indicates that CinH-*RS2* recombination system coupled with the LAT52 pollen-specific promoter could be a strong candidate to excise transgenic DNA for the transgene biocontainment strategy, but perhaps higher expression is needed for complete excision.

FCM analysis of pollen detected fluorescent signal even in non-transgenic pollen sample at a very low level (0.02%). There are several possible explanations of GFP positive particles in non-transgenic pollen sample. Carry-over of GFP positive pollen from previous measurements, bright autofluorescent non-pollen particles in similar size range, or contamination of transgenic pollen during pollen collection process could contribute to fluorescent signal detection (Moon et al. 2011).

Different levels of GFP fluorescence in pollen among the independently transformed events might be expected. GFP fluorescence levels could not be determined due to a small number of GFP positive pollen grains in the CinH events. It could be possible to underestimate the frequency of GFP positive pollen with the FCM-based method for some low GFP fluorescence events. However, a significantly low GFP fluorescence event was detected with the accuracy of 75% using the FCM-based method (Moon et al. 2011). Therefore, maximum inaccuracy of possible low GFP expressing events would not be more than 25% of measured percentage. Analysis of large number of pollen grains would provide comparable data to other small number sampled studies, even though there is a possible underestimation with the minimum accuracy of 75%.

FCM analysis provided fast and efficient detection of GFP synthesizing transgenic pollen. Most transgene excision studies have reported their results based on small numbers of samples analyzed. This FCM-based transgenic pollen screening method allowed analysis of large numbers of pollen samples in short time. FCM is technically capable of analyzing thousands of particles per second based on the sample concentrations. Data was acquired at a speed of 10,755 pollen grains per minute on one sample that had optical density 0.336 at 600 nm. FCM analysis could eliminate laborious progeny analysis and microscopic screening and the inherent errors in manual counting to analyze efficiency of transgene excision in pollen (Moon et al. 2011).

GFP-synthesizing pollen was an efficient marker for transgene excision. Transgene excised pollen was visualized under microscope and easily indentified in the FCM by the absence of GFP expression in pollen. Most transgene excision studies in plants have confirmed transgene excision using molecular biological techniques including PCR (Woo et al. 2009; Rao et al. 2010; Thomson et al. 2010), However, each pollen grain is considered as a single event of the transgene excision system in pollen. PCR seems to be unfeasible for transgene excision system in pollen due to the technical challenge of DNA isolation from a single pollen grain. In this study, loss of GFP expression in pollen served as an effective indicator of transgene excision in pollen. Non-GFP synthesizing pollen grains are the mixture of non-transgenic segregant pollen and transgene excised pollen grains. It is impossible to distinguish the transgene excised pollen from non-transgenic segregant pollen. However, the efficiency of the transgene excision system is determined based on the number or percentage of GFP synthesizing pollen grains. Unlike β-glucuronidase (GUS), GFP gene does not require chemical treatments for visualization of transgene excision. Therefore, GFP synthesizing pollen or transgene-excised pollen can be sorted and utilized for further research.

Efficiency of CinH-*RS2* recombination system-mediated transgene excision is comparable to other recombination systems including Cre-*loxP* and FLP-*FRT* systems (Luo et al. 2007; Verweire et al. 2007). The FLP-*FRT* system using the pollen-specific LAT52 promoter has shown efficiency of transgene excision from pollen ranging from 0 to 99% with a 32% average among events (Luo et al. 2007).

The Cre-*loxP* recombination system under the control of a chemical-induced promoter has shown a wide range of transgene excision efficiency in different plant species including *Arabidopsis*, rice, and tomato (Zuo et al. 2001; Sreekala et al. 2005; Zhang et al. 2006). This suggested that transgene excision efficiency could be influenced by different genomes. Therefore, this CinH-*RS2* recombination system should be tested in various plant species to acquire species-specific recombination efficiency before the practical employment of the system for crops.

Variable transgene excision efficiencies among transgenic events in this system were most likely the result of position effects of transgene insertion in the genome. It is feasible that an insertion site that enabled higher gene expression with stability across generations could be located to improve efficiency. If so, it is also technically possible to use ZFN-mediated targeting to place a transgene at the target locus (Reviewed in Weinthal et al. 2010). Based on high efficiency of transgene excision in pollen, this CinH-*RS2* recombination system would be a good candidate for highly efficient transgene excision system by itself or coupling with other site-specific recombinases or zinc-finger nucleases.

Acknowledgments The authors would like to thank Murali R. Rao for helping on statistical analysis and Spencer Wei for his help with vector construction. We thank Patrick Gallois for the gift of pPK100 that was included in our vectors. This research was funded by USDA Biotechnology Risk Assessment Grants to C. N. Stewart, Jr. and D. W. Ow. We especially wish to express our gratitude to collaborator Yi Li, also a co-PI on the above grant, for the extensive help in this line of research and for the many conversations and innovations he and his lab has accomplished.

References

Al-Ahmad H, Galili S, Gressel J (2004) Tandem constructs to mitigate transgene persistence: tobacco as a model. Mol Ecol 13:697–710

Al-Ahmad H, Dwyer J, Moloney M, Gressel J (2006) Mitigation of establishment of *Brassica napus* transgenes in volunteers using a tandem construct containing a selectively unfit gene. Plant Biotechnol J 4:7–21

Aono M, Wakiyama S, Nagatsu M, Nakajima N, Tamaoki M, Kubo A, Saji H (2006) Detection of feral transgenic oilseed rape with multiple-herbicide resistance in Japan. Environ Biosafety Res 5:77–87

Beckie HJ, Warwick SI, Nair H, Séguin-Swartz G (2003) Gene flow in commercial fields of herbicide-resistant canola (*Brassica napus*). Ecol Appl 13:1276–1294

Brown T (2001) Southern blotting. Curr Protocols Mol Biol 2.9.1–2.9.20

Chevre AM, Eber F, Darmency H, Fleury A, Picault H, Letan-neur JC, Renard M (2000) Assessment of interspecific hybridization between transgenic oilseed rape and wild radish under agronomic conditions. Theor Appl Genet 100:1233–1239

Dale EC, Ow DW (1990) Intra- and intermolecular site-specific recombination in plant cells mediated by bacteriophage P1 recombinase. Gene 91:79–85

Daniell H (2002) Molecular strategies for gene containment in transgenic crops. Nature Biotechnol 20:581–586

Daniell H, Datta R, Varma S, Gray S, Lee SB (1998) Containment of herbicide resistance through genetic engineering of the chloroplast genome. Nature Biotechnol 16:345–348

Gray AJ, Raybould AF (1998) Crop genetics—reducing transgene escape routes. Nature 392:653–654

Grindley NDF, Whiteson KL, Rice PA (2006) Mechanisms of site-specific recombination. Annu Rev Biochem 75:567–605

Halfhill MD, Zhu B, Warwick SI, Raymer PL, Millwood RJ, Weissinger AK, Stewart CN Jr (2004) Hybridization and backcrossing between transgenic oilseed rape and two related weed species under field conditions. Environ Biosafety Res 3:73–81

Haygood R, Ives AR, Andow DA (2005) Population genetics of transgene containment. Ecol Lett 7:213–220

Horsh RB, Fry JE, Hoffmann NL, Eichholtz D, Rogers SG, Fraley RT (1985) A simple and general method for transferring genes into plants. Science 227:1229–1231

Iamtham S, Day A (2000) Removal of antibiotic resistance genes from transgenic tobacco plastids. Nat Biotechnol 18:1172–1176

Ingram J (2000) The separation distance required to ensure cross-pollination is below specified limits in non-seed crops of sugar beet, maize and oilseed rape. Plant Var and Seeds 13:181–199

Kempe K, Rubtsova M, Berger C, Kumlehn I, Schollmeier C, Gils M (2010) Transgene excision from wheat chromosomes by phage phiC31 integrase. Plant Mol Biol 72:673–687

Kholodii G (2001) The shuffling function of resolvases. Gene 269:121–130

Klippell A, Kanaar R, Kahmann R, Cozzarelli NR (1993) Analysis of strand exchange and DNA binding of enhancer-independent Gin recombinase mutants. EMBO J 12:1047–1057

Lloyd AM, Davis RW (1994) Functional expression of the yeast FLP/*FRT* site-specific recombination system in *Nicotina tabacum*. Mol Gen Genet 242:653–657

Luo K, Duan H, Zhao D, Zheng X, Deng W, Chen Y, Stewart CN Jr, McAvoy R, Jiang X, Wu Y, He A, Pei Y, Li Y (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

Lyznik LA, Mitchell JC, Hirayama L, Hodges TK (1993) Activity of yeast FLP recombinase in maize and rice protoplasts. Nucleic Acids Res 21:969–975

Maeser S, Kahmann R (1991) The Gin recombinase of phage Mu can catalyze site-specific recombination in plant protoplasts. Mol Gen Genet 230:170–176

Maliga P (2004) Plastid transformation in higher plants. Annu Rev Plant Biol 55:289–300

Mariani C, DeBeuckeleer M, Trueltner J, Leemans J, Goldberg RB (1990) Induction of male sterility in plants by a chimeric ribonuclease gene. Nature 347:737–741

Moon HS, Li Y, Stewart CN Jr (2010) Keeping the genie in the bottle: transgene biocontainment by excision in pollen. Trends Biotechnol 28:3–8

Moon HS, Eda S, Saxton AM, Ow D, Stewart CN Jr (2011) An efficient and rapid transgenic pollen screening and detection method using a flow cytometry. Biotechnol J 6:118–123

Morris WF, Kareiva PM, Raymer PL (1994) Do barren zones and pollen traps reduce gene escape from transgenic crops? Ecol Appl 4:157–165

Nakagawa Y, Machida C, Machida Y, Toriyama K (2001) A system to induce the deletion of genomic sequences using R/*RS* site-specific recombination and the *Ac* transposon in transgenic rice plants. Theor Appl Genet 102:1136–1141

Onouchi H, Yokoi K, Machida C, Matsuzaki H, Oshima Y, Matsuoka K, Nakamura K, Machida Y (1991) Operation of an efficient site-specific recombination system of *Zygosaccharomyces rouxii* in tobacco cells. Nucleic Acids Res 19:6373–6378

Onouchi H, Nishihama R, Kudo M, Machida Y, Machida C (1995) Visualization of site-specific recombination catalyzed by a recombinase from *Zygosaccharomyces rouxii* in *Arabidopsis thaliana*. Mol Gen Genet 247:653–660

Petolino JF, Worden A, Curlee K, Connell J, Strange Moynahan TL, Larsen C, Russel S (2010) Zinc finger nuclease-mediated transgene deletion. Plant Mol Biol 73:617–628

Piñeyro-Nelson A, Van Heerwaarden J, Perales HR, Serratos-Hernandez JA, Rangel A, Hufford MB, Gepts P, Garay-Arroyo

A, Rivera-Bustamante R, Alvarez-Buylla ER (2009) Transgenes in Mexican maize: molecular evidence and methodological considerations for GMO detection in landrace populations. Mol Ecol 18:750–761

Rao MR, Moon HS, Schenk TMH, Becker D, Mazarei M, Stewart CN Jr (2010) FLP/*FRT* Recombination from yeast: application of a two gene cassette scheme as an inducible system in plants. Sensors 10:8526–8535

Ruf S, Hermann M, Berger IF, Carrer H, Bock R (2001) Stable genetic transformation of tomato plastids- high-level foreign protein expression in fruits. Nature Biotechnol 19:870–875

Sreekala C, Wu L, Gu K, Wang D, Tian D, Yin Z (2005) Excision of a selectable marker in transgenic rice (*Oryza sativa* L.) using a chemically regulated Cre/*loxP* system. Plant Cell Rep 24:86–94

Stewart CN Jr, Via LE (1993) A rapid CTAB DNA isolation technique useful for RAPD fingerprinting and other PCR applications. BioTechniques 14:748–751

Stewart CN Jr, Halfhill MD, Warwick SI (2003) Transgene introgression from genetically modified crops to their wild relatives. Nature Rev Genet 4:806–817

Svab Z, Maliga P (2007) Exceptional transmission of plastids and mitochondria from the transplastomic pollen parent and its impact on transgene containment. Proc Natl Acad Sci USA 104:7003–7008

Thomson JG, Ow DW (2006) Site-specific recombination systems for the genetic manipulation of eukaryotic genomes. Genesis 44:465–476

Thomson JG, Yau Y-Y, Blanvillain R, Chinquy D, Thilmony R, Ow DW (2009) ParA resolvase catalyzes site-specific excision of DNA from the *Arabidopsis* genome. Transgenic Res 18:237–248

Thomson JG, Chan R, Thilmony R, Yau Y-Y, Ow DW (2010) PhiC31 recombination system demonstrates heritable germinal transmission of site-specific excision from the *Arabidopsis* genome. BMC Biotechnol 10:17

Twell D, Yamaguchi J, McCormick S (1990) Pollen-specific gene expression in transgenic plants: coordinate regulation of two different tomato gene promoters during microsporogenesis. Development 109:705–713

Verweire D, Verleyen K, De Buck S, Claeys M, Angenon G (2007) Marker-free transgenic plants through genetically programmed auto-excision. Plant Physiol 145:1220–1231

Weinthal D, Tovkach A, Zeevi V, Tzfira T (2010) Genome editing in plant cells by zinc finger nucleases. Trends Plant Sci 15:308–321

Whittington M (2006) Underground farming. http://www.associatedcontent.com/article/27102/underground_farming.html?cat=15. Accessed 24 Oct 2010

Woo H-J, Cho H-S, Lim S-H, Shin K-S, Lee S-M, Lee K-J, Kim D-H, Cho Y-G (2009) Auto-excision of selectable marker genes from transgenic tobacco via a stress inducible FLP/*FRT* site-specific recombination system. Transgenic Res 18:455–465

Zhang Y, Li H, Quyang B, Lu Y, Ye Z (2006) Chemical-induced autoexcision of selectable markers in elite tomato plants transformed with a gene conferring resistance to lepidopteran insects. Biotechnol Lett 28:1247–1253

Zuo J, Niu Q-W, Møller SG, Chua N-H (2001) Chemical-regulated, site-specific DNA excision in transgenic plants. Nature Biotechnol 19:157–161