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Directed microspore-specific recombination of transgenic alleles to prevent pollen-mediated transmission of transgenes

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Summary

A major challenge for future genetically modified (GM) crops is to prevent undesired gene flow of transgenes to plant material intended for another use. Recombinase-mediated auto excision of transgenes directed by a tightly controlled microspore-specific promoter allows efficient removal of either the selectable marker gene or of all introduced transgenes during microsporogenesis. This way, transgene removal becomes an integral part of the biology of pollen maturation, not requiring any external stimulus such as chemical induction by spraying. We here show the feasibility of engineering transgenic plants to produce pollen devoid of any transgene. Highly efficient excision of transgenes from tobacco pollen was achieved with a potential failure rate of at most two out of 16 800 seeds (0.024%). No evidence for either premature activation or absence of activation of the recombinase system was observed under stress conditions in the laboratory. This approach can prevent adventitious presence of transgenes in non-GM crops or related wild species by gene flow. Such biological containment may help the deployment and management of coexistence practices to support consumer choice and will promote clean molecular farming for the production of high-value compounds in plants.

Keywords: gene flow, marker-free transgenic plants, cre-*lox* recombination, pollen, biosafety, molecular farming.

Introduction

The total area of genetically modified (GM) crops continues to increase and public concerns over the adoption of GM crops in agriculture are slowly diminishing (James, 2004). For future generations of GM crops, major challenges will be (i) to prevent GM material from entering the food chain, when this material is either unfit, unintended or undesired for consumption (Dale *et al*., 2002); (ii) to prevent mixing GM material with plant material that is aimed at other uses, such as in molecular farming aimed at producing a variety of pharmaceuticals and other high-value compounds in plants (Fischer *et al*., 2004; Teli and Timko, 2004) and (iii) to prevent the presence of GM material in wild relatives and nonagricultural ecosystems (Ellstrand, 2003). A major route for undesired mixing of GM and non-GM or other crops is gene flow by pollen (Rieger *et al*., 2002; Conner *et al*., 2003). In addition to agronomic practices such as buffer zones or the use of nonfood crops, various molecular strategies have been developed that aim at the removal of undesired transgenes, notably selectable marker genes (Dale and Ow, 1991; Zubko *et al*., 2000; Schaart *et al*., 2004). This is summarized in various reviews on this matter (Hohn *et al*., 2001; Hare and Chua, 2002; Mascia and Flavell, 2004). Methods such as cotransformation (McKnight *et al*., 1987) or transposon-mediated transgene repositioning (Goldsbrough *et al*., 1993) can be used for the removal of marker genes via segregation, but do not prevent gene flow and inadvertent mixing of production lines.

Molecular containment strategies based on recombination, which were initially targeted towards marker gene removal (Daniell, 2002), also offer strategies to generate nontransgenic crops from transgenic plants (Keenan and Stemmer, 2002). One of the more promising containment technologies is chloroplast transformation (Daniell *et al*., 2005). Cytoplasmic sterility engineered via the chloroplast genome offers a new tool for transgene containment (Khan, 2005; Ruiz and Daniell, 2005). Advanced recombination-based methods (Keenan and Stemmer, 2002; Gilbertson, 2003) for molecular containment are based on auto excision (Konig, 2003). The introduced recombinase removes itself and the genes to be removed after being induced by either heat shock or spraying with chemicals (Hoff *et al*., 2001; Zuo *et al*., 2001; Zhang *et al*., 2003). The need for external induction, plus problems with efficacy, chimerism, and possibly cost, has hampered widespread field applications of such advanced approaches. Ideally, auto excision should be driven by an endogenous trigger that is an intrinsic part of plant development.

The tobacco NTM19 promoter drives a gene of unknown function, the expression of which is highly specific for the microspore. The promoter is inactive in all vegetative tissues as well as during female gametogenesis, embryo or seedling development (Oldenhof *et al*., 1996). The first detectable promoter activity is in early uninucleate microspores (Oldenhof *et al*., 1996; Custers *et al*., 1997), and when fused to *gus*, GUS activity declines rapidly to moderate levels after the first pollen mitosis (Custers *et al*., 1997). To investigate the feasibility of generating marker- and transgene-free pollen with this promoter, two T-DNA-containing plasmids were constructed. The creation of *lox*P-embedded cassettes with a plant intron-containing recombinase gene driven by the NTM19 microspore-specific promoter linked to a selectable marker gene and a gene of interest allows recombinase-mediated auto excision of either the marker gene or of all transgenes during microsporogenesis. This results in a simple approach to obtain marker-free GM plants or GM plants that produce only pollen without transgene. We present here the feasibility of this approach for *Arabidopsis thaliana* and tobacco (*Nicotiana tabacum* L.).

Results and discussion

Efficient excision of transgenes during microsporogenesis

To determine the application of the NTM19 promoter in excision of transgenes in pollen, two T-DNA-containing plasmids were constructed: pEX-NGC and pEX-CN (Figure 1A). In both, the NTM19 promoter drives an intron-containing *cre* gene (*cre*^{int}) (Mlynarova and Nap, 2003). Plasmid pEX-NGC contains *lox*P sites at the T-DNA borders. Upon activation of the NTM19 promoter, active CRE should remove all transgenes from the plant DNA. Plasmid pEX-CN contains essentially the same configuration as pEX-NGC, but by relocating one *lox*P site, the *gus* transgene is no longer between the *lox*P sites. With active CRE, the NPT gene (*npt*) as selectable marker will be removed and the GUS gene (*gus*) will be retained (Figure 1A). This allows for easy monitoring and quantitative analyses of transformants and offspring. With either plasmid, independent kanamycin-resistant transformants were obtained for both tobacco and arabidopsis via *Agrobacterium tumefaciens*-mediated plant transformation. Transformation efficiencies were normal, indicating no premature activity of NTM19-*cre*^{int} in somatic cells during transformation, regeneration or rooting. Most transformants had easily detectable GUS activity (data not shown). Transformants were analysed in considerable detail to identify single-locus and single-copy lines.

Upon selfing, all single-copy transformants with T-DNA from either pEX-NGC or pEX-CN segregated 1 : 1 for kanamycin-resistant (Km^R) vs. kanamycin-sensitive (Km^S) seed, consistent with *nptII* only being transmitted through egg formation (Figure 1B). When crossed to the wild type, no Km^R offspring were generated in small scale assays (n = about 100 progeny) of both tobacco and arabidopsis seeds (Table 1). As predicted on theoretical grounds (Figure 1C), no *gus* was detected in the Km^S offspring of EX-NGC-backcrossed plants, whereas half of the Km^S offspring of EX-CN-backcrossed plants still carried *gus* and showed GUS activity. Deletion of the transgenic DNA from the plant genome was investigated by detailed PCR for pEX-NGC-derived plants, as well as by DNA

Table 1 Efficiency of transgene removal by NTM19-*cre*^{int}

Tobacco line*	No. of seeds analysed†	Km^R	Failure (%)‡
All 18§	~1800	0	< 0.12
All 5¶	~15000	2	0.027
Total	~16800	2	0.024

*All single-locus lines used as a pollen parent in backcrosses.

†Numbers are estimates.

‡Calculated as [(no. Km^R)/(no. total/2)] * 100; where no. Km^R seeds = 0, no. Km^R seeds is taken as 1, that is, the lowest possible number. A correction factor of 2 is used in calculating the failure rate. Since the transgenic lines analysed are hemizygous, half of the seedlings are the result of fertilization with wild-type pollen.

§Small-scale germination assays, using about 100 seeds per line; figures are the pooled results of the assays of all 18 lines.

¶Large-scale assays, using ~3000 seeds per line; figures are the pooled results of the assays of all five lines. The two putative Km^R seedlings came from the same transgenic line.

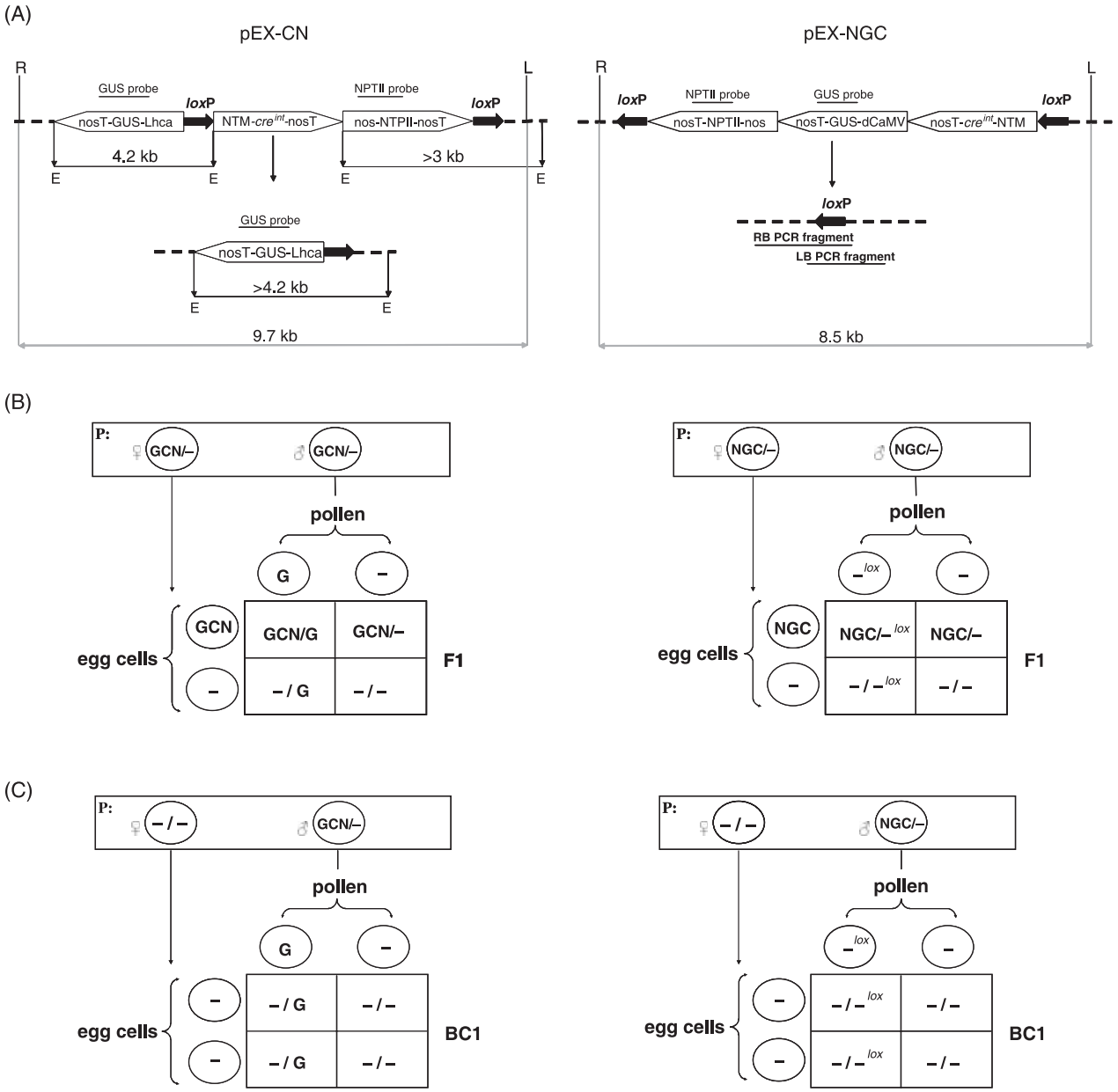

Figure 1 Configuration and behaviour of T-DNA alleles in crosses. (A) T-DNA configurations used for plant transformation. Black arrows indicate the presence and orientation of the *lox*P sites. The configuration generated after excision of *lox*P embedded DNA is given below. The right and left borders of T-DNA are indicated with R and L. The positions of the enzyme used for DNA blot analyses (pEX-CN) and expected PCR fragments (pEX-NGC) following excision are indicated. The size of the complete T-DNA is indicated in the lower part of the panel. E, *Eco*RI. (B) Predicted genetic segregation upon self-pollination of the transgenic plants. T-DNA configurations are given by the sequences present: *nptII* gene: N; *cre* gene: C; *gus* gene: G; sequence remaining after removal of NGC: -^{lox}; wild-type allele: –. (C) As (B) describing segregation upon crossing the transgenic plants as a pollen parent to the wild type.

blot analyses with *nptII* and *gus* probes for pEX-CN-derived plants (Figure 1A). Results (Figure 2) indicated that the offspring obtained through selfing (F1; Figure 1B) or backcrossing (BC1; Figure 1B) behave as predicted. Transgene-free offspring segregated in Mendelian ratios, indicating that the microspore-specific recombination does not interfere with subsequent pollen development. As an example, single-copy tobacco line EX-CN-6 generated as offspring plant f12 after selfing and plant bc17 after backcrossing to the wild type. Both offspring plants have no *nptII* and still carry *gus* (Figure 2A). Tobacco line EX-CN-6 (f10) is offspring obtained after selfing. This line carries one complete T-DNA allele including *nptII* (a 4.2-kb DNA fragment) and a second recombined T-DNA allele without *nptII* (a DNA fragment >4.2 kb). Line

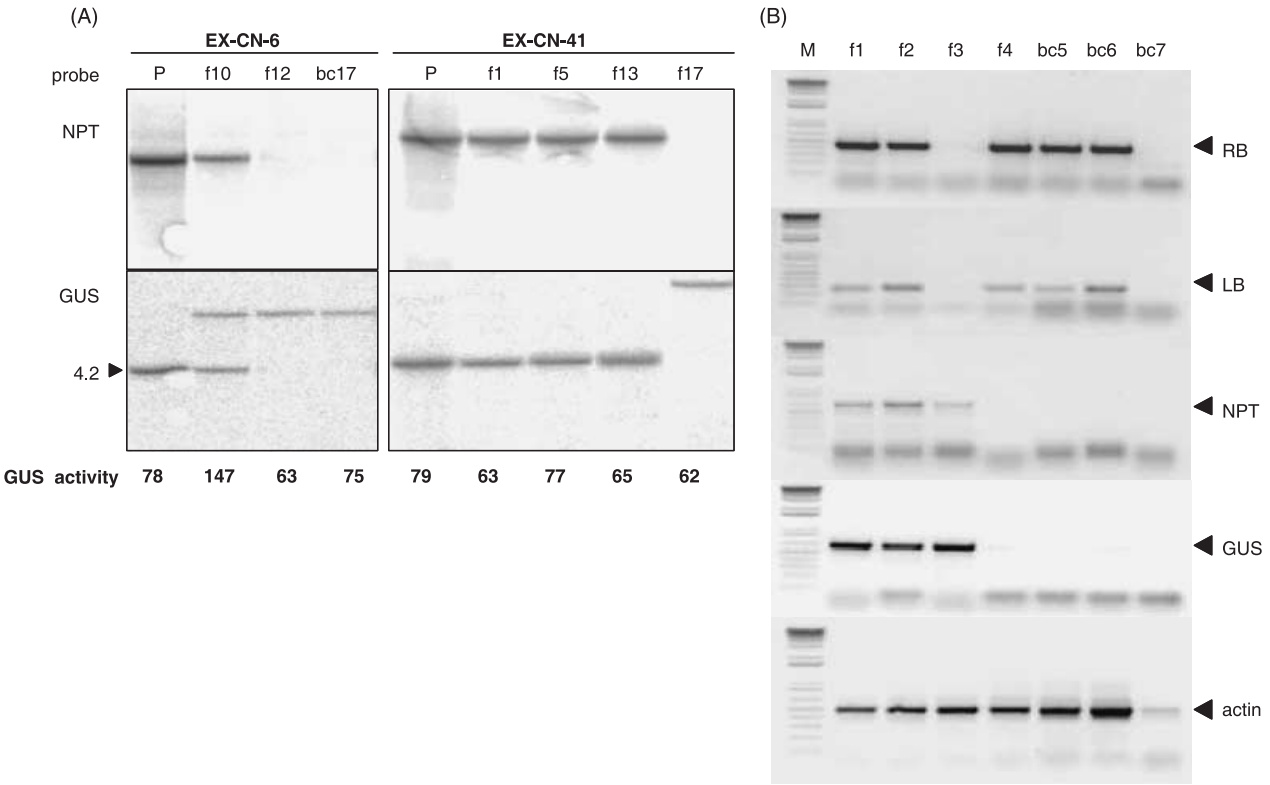

Figure 2 Generation of transgene-free pollen from transgenic plants. (A) Phosphor images of DNA gel blots of agarose gel-separated, *Eco*RI-digested genomic DNA from leaves of parental plants (lanes labelled P) and offspring plants of single-copy EX-CN-6 and EX-CN-41 tobacco transformants. The lanes labelled f# represent selfed offspring; the lane labelled bc17 was obtained after backcrossing. The blots were hybridized with *nptII* (top panel) and *gus* (lower panel) probes. The expected 4.2-kb *gus* fragment is indicated with an arrow. Under the lower panel, the GUS activity in leaves of plants is given in pmol methylumbelliferon per minute per microgram soluble protein. (B) Photograph of an ethidiumbromide-stained agarose gel of PCR fragments obtained from leaf DNA of selfed (f#) or backcrossed (bc#) progeny of transformed arabidopsis line EX-NGC-18. Positions of expected PCR fragments detected at right (RB; 450 bp) and left (LB; 250 bp) T-DNA borders after *lox*P excision are illustrated in Figure 1A. M contains the 1-kb DNA ladder (Life Technologies) as size marker.

EX-CN-6 (f10) is homozygous for the *gus* portion of the transgenic insert and has twice the activity of the hemizygous plants (Figure 2A). In line EX-CN-41, selfed progeny f17 carries only *gus*. GUS activity was stable over three generations in the greenhouse, irrespective of the presence or absence of *nptII* or *cre*.

The presence of some vector DNA sequences on either site of the remaining *lox*P site (Figure 1A) allowed analysing the offspring of EX-NGC transformants by PCR. The vector sequences present in the T-DNA vector used in this study are not essential and can be omitted in improved versions of the approach. All four genotypes generated by selfing (Figure 1B) or backcrossing (Figure 1C) were identified by PCR (Figure 2B). This is shown for two different single-copy arabidopsis transformants. For two more arabidopsis and a tobacco transformant, essentially identical results were obtained. Lanes f1 and f2 show offspring from self-pollinations with border fragments that contain the single remaining *lox*P site (Figure 1A) in addition to the transgene allele obtained via the mother (NGC/–^{lox}). Lane f3 shows a plant where only *gus* and *nptII* are present (genotype NGC/–; identical to the primary transformant), lanes f4 to bc6 show plants that no longer contain transgenes (–/–^{lox}) after selfing (lane f4) or backcrossing (lanes bc5 and bc6). Here, transgenic arabidopsis plants have produced transgene-free pollen. Lane bc7 shows an example of the wild type (–/–) after backcrossing. In this design, any transgenic line receiving pollen from the wild-type generates Km^R Km^S segregation ratios of 1 : 1, equivalent to selfing. This way, auto excision lines can be maintained for breeding purposes.

Robustness of transgene removal from pollen

The robustness of transgene removal was assessed by backcrossing to wild-type plants, thereby mimicking gene flow in nature. Each pollen grain in which NTM19-*cre*^{int} failed to remove *nptII* will be able to generate a Km^R seedling. Therefore, pooled seeds rather than individual seed capsules were

analysed. All progeny of tobacco backcrosses in small-scale germination analysis (~100 seeds per individual line) were kanamycin-sensitive. Among the progeny of about 300 backcrossed arabidopsis seeds, also no kanamycin-resistant offspring was observed. Because of the difficulties associated with obtaining backcrossed seed in arabidopsis, large-scale assessments were only performed in tobacco. In large-scale analyses of ~3000 seeds obtained from separate backcrosses of independent single-copy tobacco transformants, only two putative Km^R seedlings were observed (Table 1). A correction of a factor 2 is used in calculating the failure rate, because the transgenic lines used for analysis are hemizygous. As a consequence, half of all seedlings are the result of fertilization with wild-type pollen (Figure 1C,D). Two Km^R seedlings among about 16 800 Km^S seedlings indicated a failure rate of transgene removal of at most 0.024% (Table 1). Large-scale analyses of individual transformation events could, however, reveal a variability between events. After selfing or in crosses with wild-type plants as a pollen donor, no significant deviations of the expected 1 : 1 segregation were observed, indicating that the transgene is efficiently transmitted through maternal inheritance. Analysis of GUS activities in leaves shows that the transgene expression is stable in other somatic tissue. NTM19-driven *cre* therefore appears highly reliable for eliminating transgenes in pollen under the controlled greenhouse conditions used.

The approach as presented requires very high tissue specificity and reliability of the promoter driving the *cre* gene to prevent premature activation in somatic tissue or absence of activation in pollen. Growing plants or germinating seeds at elevated temperatures (30 °C), well above our normal temperature conditions of 24 °C, also showed no evidence for either premature activation (transgene loss from somatic cells, a production risk) or absence of activation (transgene presence in pollen, a containment issue) in either tobacco or arabidopsis. This indicates that the microsporogenesis-induced transgene excision is robust under stress conditions. It will be interesting to see how the NTM19-driven recombinase system behaves under (a)biotic stress conditions in the field. As microsporogenesis is an integral part of plant development, there may be room for cautious optimism. The tissue specificity and reliability of the tobacco promoter apparently extends to arabidopsis. It should be noted that the NTM19 promoter has no obvious counterpart in the arabidopsis genome (TIGR5 annotation) or to any other sequence represented in current databases. In tobacco, the activity of the NTM19 promoter has a striking limitation to early pollen development (Custers *et al*., 1997). In hybridization studies, similar sequences were identified in other Solanaceae (Oldenhof *et al*., 1996), but the sequence is apparently absent from the arabidopsis genome. In arabidopsis, genome-wide expression analysis has shown that gene expression in (mature) pollen is strikingly different from other plant tissues (da Costa-Nunes and Grossniklaus, 2003; McCormick, 2004) and relatively large numbers of genes may have pollen specific expression. These studies will help to identify the precise timing of genes and promoters during pollen development in arabidopsis, but such data will have to be confirmed with other tests (McCormick, 2004). The results demonstrated here indicate that the tobacco NTM19 promoter works remarkably well in arabidopsis.

Pollen transmission of CRE protein

Another key issue to consider is pollen transmission of transgene-encoded CRE protein (Custers *et al*., 1997) via tube cell and/or sperm cell cytoplasm carry over. Although only a minor fraction of tube cell cytoplasm may come into contact with the female genome, it could carry sufficient CRE protein to catalyse additional and undesired recombination. Lines EX-CN-6 and EX-CN-41 were used as pollen source in crosses with homozygous AGCN-61 plants (Figure 3). If active CRE is carried over via pollen, it could trigger excision of the *lox*511-embedded DNA in AGCN-61. In none of the cases analysed by DNA blot analysis, the anticipated results of CRE carry over were observed (Figure 3). Apparently there is no, or not sufficient, functional CRE available to result in excision.

Potential benefits and drawbacks of generating transgene-free pollen from GM plants

The results obtained show the feasibility of generating transgene-free pollen from transgenic plants, as proposed theoretically earlier (Keenan and Stemmer, 2002). In addition to a simple and efficient method for obtaining selectable marker-free GM plants, this presents a new and conceptually simple approach to prevent or dramatically reduce gene flow because of transgene-free pollen. Transgene removal as accomplished this way is an intrinsically iterative process: each plant in which the transgenes are maintained via the female lineage will produce transgene-free pollen upon microsporogenesis. This prevents fixation in wild populations and reduces the potential area of uncontrolled spread in ecosystems.

GM plants generated this way will, however, never be homozygous and should be maintained for breeding and production as hemizygous lines. For vegetatively propagated crops, such as potatoes and fruit trees, transgenic plants are maintained as clones in the hemizygous state from the initial

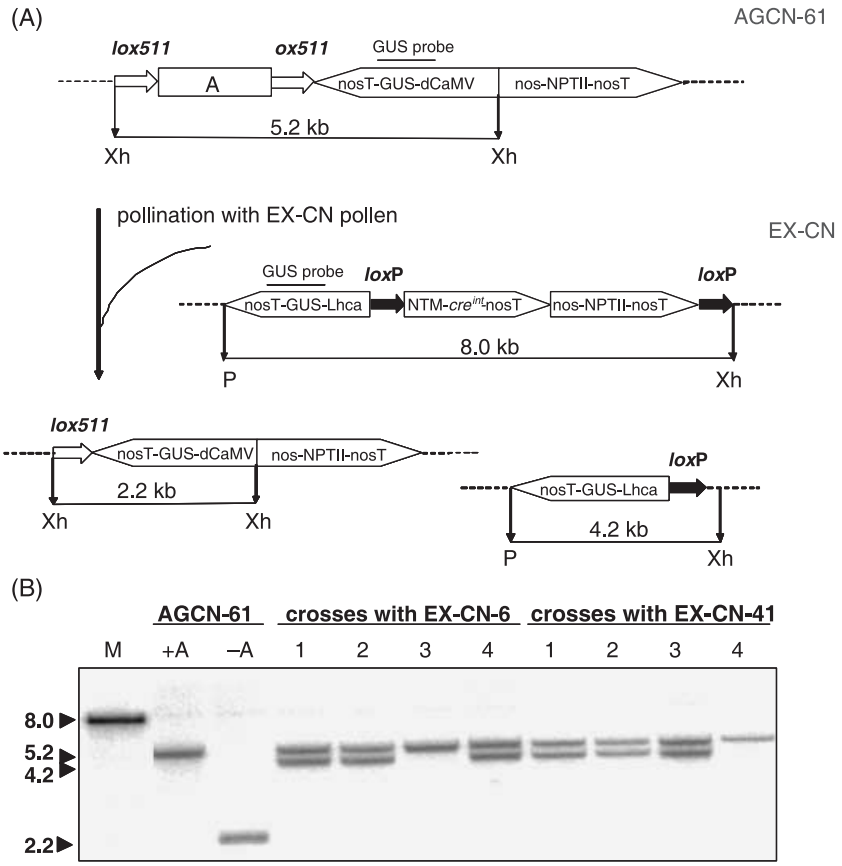

Figure 3 NTM19-*cre*^{int} -produced CRE protein is not transmitted in pollen. (A) T-DNA configuration used for investigation CRE carry over. Symbols are as in Figure 1A. Plant AGCN-61 carries additional DNA in-between heterospecific *lox*511 sites. T-DNA configuration after CRE action is given in the lower part. The enzymes used and the predicted length of the hybridizing fragments are indicated. Xh, *Xho*I; P, *Pac*I. (B) Phosphor image of a blot of agarose gel-separated genomic leaf DNA hybridized with a *GUS* probe. DNA is from AGCN-61 with (+A) and without (–A) DNA between *lox*511 sites and from four individual offspring plants. The upper 5.2-kb band represents the *GUS*-hybridizing fragment from AGCN-61; the lower 4.2-kb band, if present, originates from the T-DNA allele of the pollen donor, which recombined before pollen maturation. M is a size marker isolated as restriction fragment from pEX-CN.

plant regeneration event through to commercial release (Conner and Christey, 1994). In such crops, where sexual transmission of the transgenes is not necessary, generating transgene-free pollen has immediate application. This will be especially important to reduce gene flow to wild relatives of clonal cultivars, especially near the centres of genetic diversity in such crops.

The necessary maintenance of hemizygosity may be a disadvantage in seed propagated crops engineered for protein accumulation, but many other factors may play a role in the yield of protein. For example, different T-DNA insertions on different chromosomes would be feasible. In seed production, the hemizygosity will cause that half of the seed will not have the engineered trait. Standard herbicide tolerance (e.g. BASTA) could replace the kanamycin resistance gene (*nptII*) as the selectable marker and allow the subsequent elimination of such plants. This could be achieved by either a seed-priming step with herbicide solutions prior to sowing (Conner and Christey, 1997) or at an early stage in crop development (Conner and Field, 1995). The sowing of mixtures with herbicide-resistant and herbicide-sensitive seed at a higher density followed by chemical thinning of the herbicide-sensitive seeds has also been suggested as a management tool to facilitate crop establishment (Conner and Field, 1995). Such applications will depend on the economics of production and the value of the new GM trait. As always in risk – benefit balancing, the potential disadvantages of the use of hemizygous plants will have to be weighed with the perceived gain of safety in the field. When the value of compounds produced in plants, and/or the environmental burden of undesired gene flow, is high, then carrying non-GM seed through seed production for subsequent elimination will be more acceptable in agricultural systems. It should be investigated whether induction of distorted segregation towards 'true breeding' herbicide-resistant plants by the application of herbicide treatments to the hemizygous plants is feasible. For herbicides with high translocation in plants, such treatments have been reported to effectively prevent the full development of herbicide-sensitive pollen, ovule and seeds on the otherwise herbicide-resistant plants (Conner, 2000).

In the system as proposed, the biosafety discussions regarding gene flow through pollen can now be restricted to the presence of a 34-bp *lox*P site in the genome of a crop. Generating transgene-free pollen offers a new possibility for biosafe engineering of GM crops. Ideally, this may develop into an aid towards the design of GM crop management and

coexistence protocols that conform to consumer desire, offer the choice demanded and, at the same time, allow to generate added value for agriculture, for example, by improved biological containment in molecular farming approaches.

Experimental procedures

Plasmid construction and DNA analysis

All plasmids used are pBinPlus derivatives (Mlynarova *et al*., 2002), the components of which have been described before (Mlynarova and Nap, 2003). Binary vector DNA was prepared and introduced into *Agrobacterium tumefaciens* LBA4404 (pAL4404) (for tobacco transformation) or C58C1 (pMP90) (for arabidopsis transformation) by standard procedures (Sambrook *et al*., 1989). In plasmid pEX-NGC (Figure 1A), the *gus* gene (GUS) is driven by the doubled cauliflower mosaic virus (dCaMV) 35S promoter (Mlynarova *et al*., 1995). In plasmid pEX-CN (Figure 1A), *gus* is driven by a light-regulated potato *Lhca3.St.1* promoter (Lhca) (Nap *et al*., 1993), because that made an easier cloning scheme. The NTM19 promoter sequence (GenBank accession number X88847, nucleotides 1–954) was obtained from plasmid pEMBL_NTM19-GUS (Custers *et al*., 1997), subcloned and used to exchange with the CaMV 35S promoter as *Xba*I-*Nco*I fragment to become promoter of the plant intron-containing variant of *cre* (*cre*^{int}) as described previously (Mlynarova and Nap, 2003). The selectable marker gene encoding neomycin phosphotransferase II (NPTII) is driven by the nopaline synthase (nos) promoter. All genes also contain the nos polyadenylation region (nosT). For DNA blot analysis, 10 μg of genomic DNA was isolated as described (Mlynarova and Nap, 2003), digested with the appropriate restriction enzyme(s), separated on a 1% agarose gel and vacuum blotted on Hybond-N$^+$ (Amersham Biosciences NL) membranes. Gene-specific probes were isolated as restriction fragment from an agarose gel and labelled with ^{32}P-dATP using the MEGAPRIME^{TM} DNA labelling system (Amersham). Hybridization was performed overnight at 65 °C in 10% Dextran sulphate, 1% SDS, 1 M NaCl and 0.1 mg/mL sonicated salmon sperm DNA. After hybridization, the membrane was washed first in 2 × SSC, 1% SDS, followed by washing in 1 × SSC, 1% SDS at 65 °C. Hybridizing signals were visualized with a Fuji Bas 2000 PhosphoImager and Bas-Reader software. PCR analyses were performed as described previously (Mlynarova and Nap, 2003). Primers used to detect the remaining part of right border (RB) plasmid DNA after *lox*P excision were 5′-GGAAACAGCTATGACCATG-3′ (M13rev) and 5′-CACTGATAGTTTGTGAACCATC-3′; primers detecting the remaining left border (LB) plasmid sequence were 5′-GTAAAACGACGGCCAGT-3′ (M13for) and 5′-GCAACGCAATTAATGTGAGTTAGC-3′; for *nptII*, 5′-TGGGCACAACAGACAATCGGCTGC-3′ and 5′-TGCGAATCGGGAGCGGCGATACCG-3′; for *gus*, 5′-GCAGGAGAAACTGCATCAG-3′ and 5′-CGATAATTTATCCTAGTTTGC-3′, and for an actin gene 5′-GTGTTGGACTCTGGAGATGGTGTG-3′ and 5′-GCCAAAGCAGTGATCTCTTTGCTC-3′.

Transgenic plants and crossing schemes

Tobacco (*Nicotiana tabacum* cv. Petit Havana SR1) was transformed as described, with an *in vitro* selection of 50 μg/mL kanamycin (Mlynarova *et al*., 1994). Pollination, seed germination assays as well as quantitative GUS assays were performed as described (Mlynarova *et al*., 1994; Conner *et al*., 1998). Transgenic tobacco line AGCN-61 was described previously (Mlynarova and Nap, 2003). *Arabidopsis thaliana* (ecotype Wassilewskija) was transformed using the floral-dip method (Clough and Bent, 1998). Transformants were selected on medium with 50 μg/mL kanamycin and after rooting transferred to soil. All plants were grown and maintained in a climatized greenhouse. For the seed germination assays, hemizygous transgenic plants were used as pollen donor to the wild type. Seed capsules from individual lines were pooled.

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References

Clough, S.J. and Bent, A.F. (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J.* **16**, 735–743.

Conner, A.J. (2000) True breeding transgenics from plants heterozygous for transgene insertions. US patent 6057496 (2 May 2000).

Conner, A.J. and Christey, M.C. (1994) Plant breeding and seed marketing options for the introduction of transgenic insect-resistant crops. *Biocontrol Sci. Technol.* **4**, 463–473.

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. *Ann. Bot.* **80**, 561–564.

Conner, A.J. and Field, R.J. (1995) Herbicide-resistant crops: a new approach to an old problem or a radical new tool? In

Herbicide-Resistant Crops and Pastures in Australian Farming Systems (McLean, G.D. and Evans, G., eds), pp. 53–71. Canberra: Bureau of Resource Sciences.

Conner, A.J., Mlynarova, L., Stiekema, W.J. and Nap, J.P. (1998) Meiotic stability of transgene expression is unaffected by flanking matrix-associated regions. *Mol. Breed*. **4**, 47–58.

Conner, A.J., Glare, T.R. and Nap, J.P. (2003) The release of genetically modified crops into the environment. Part II. Overview of ecological risk assessment. *Plant J*. **33**, 19–46.

da Costa-Nunes, J.A. and Grossniklaus, U. (2003) Unveiling the gene-expression profile of pollen. *Genome Biol*. **5**, 205.

Custers, J.B., Oldenhof, M.T., Schrauwen, J.A., Cordewener, J.H., Wullems, G.J. and van Lookeren Campagne, M.M. (1997) Analysis of microspore-specific promoters in transgenic tobacco. *Plant Mol. Biol*. **35**, 689–699.

Dale, E.C. and Ow, D.W. (1991) Gene transfer with subsequent removal of the selection gene from the host genome. *Proc. Natl Acad. Sci. USA*, **88**, 10 558–10 562.

Dale, P.J., Clarke, B. and Fontes, E.M. (2002) Potential for the environmental impact of transgenic crops. *Nat. Biotechnol*. **20**, 567–574.

Daniell, H. (2002) Molecular strategies for gene containment in transgenic crops. *Nat. Biotechnol*. **20**, 581–586.

Daniell, H., Kumar, S. and Dufourmantel, N. (2005) Breakthrough in chloroplast genetic engineering of agronomically important crops. *Trends Biotechnol*. **23**, 238–245.

Ellstrand, N.C. (2003) Going to 'great lengths' to prevent the escape of genes that produce specialty chemicals. *Plant Physiol*. **132**, 1770–1774.

Fischer, R., Stoger, E., Schillberg, S., Christou, P. and Twyman, R.M. (2004) Plant-based production of biopharmaceuticals. *Curr. Opin. Plant Biol*. **7**, 152–158.

Gilbertson, L. (2003) Cre–*lox* recombination: cre-ative tools for plant biotechnology. *Trends Biotechnol*. **21**, 550–555.

Goldsbrough, A.P., Lastrella, C.N. and Yoder, J.I. (1993) Transposition mediated re-positioning and subsequent elimination of marker genes from transgenic tomato. *Biotechnology*, **11**, 1286–1292.

Hare, P.D. and Chua, N.H. (2002) Excision of selectable marker genes from transgenic plants. *Nat. Biotechnol*. **20**, 575–580.

Hoff, T., Schnorr, K.M. and Mundy, J. (2001) A recombinase-mediated transcriptional induction system in transgenic plants. *Plant Mol. Biol*. **45**, 41–49.

Hohn, B., Levy, A.A. and Puchta, H. (2001) Elimination of selection markers from transgenic plants. *Curr. Opin. Biotechnol*. **12**, 139–143.

James, C. (2004) Global status of commercialized biotech/GM crops. *ISAAA Briefs*, **232**.

Keenan, R.J. and Stemmer, W.P. (2002) Nontransgenic crops from transgenic plants. *Nat. Biotechnol*. **20**, 215–216.

Khan, M.S. (2005) Plant biology: engineered male sterility. *Nature*, **436**, 783–785.

Konig, A. (2003) A framework for designing transgenic crops – science, safety and citizen's concerns. *Nat. Biotechnol*. **21**, 1274–1279.

Mascia, P.N. and Flavell, R.B. (2004) Safe and acceptable strategies for producing foreign molecules in plants. *Curr. Opin. Plant Biol*. **7**, 189–195.

McCormick, S. (2004) Control of male gametophyte development. *Plant Cell*, **16** (Suppl.), S142–S153.

McKnight, T.D., Lillis, M.T. and Simpson, R.B. (1987) Segregation of genes transferred to one plant cell from two separate *Agrobacterium* strains. *Plant Mol. Biol*. **8**, 439–445.

Mlynarova, L. and Nap, J.P. (2003) A self-excising Cre recombinase allows efficient recombination of multiple ectopic heterospecific *lox* sites in transgenic tobacco. *Transgenic Res*. **12**, 45–57.

Mlynarova, L., Loonen, A., Heldens, J., Jansen, R.C., Keizer, P., Stiekema, W.J. and Nap, J.P. (1994) Reduced position effect in mature transgenic plants conferred by the chicken lysozyme matrix-associated region. *Plant Cell*, **6**, 417–426.

Mlynarova, L., Jansen, R.C., Conner, A.J., Stiekema, W.J. and Nap, J.P. (1995) The MAR-mediated reduction in position effect can be uncoupled from copy number-dependent expression in transgenic plants. *Plant Cell*, **7**, 599–609.

Mlynarova, L., Libantova, J., Vrba, L. and Nap, J.P. (2002) The promiscuity of heterospecific *lox* sites increases dramatically in the presence of palindromic DNA. *Gene*, **296**, 129–137.

Nap, J.P., van Spanje, M., Dirkse, W.G., Baarda, G., Mlynarova, L., Loonen, A., Grondhuis, P. and Stiekema, W.J. (1993) Activity of the promoter of the *Lhca3.St.1* gene, encoding the potato apoprotein 2 of the light-harvesting complex of Photosystem I, in transgenic potato and tobacco plants. *Plant Mol. Biol*. **23**, 605–612.

Oldenhof, M.T., de Groot, P.F., Visser, J.H., Schrauwen, J.A. and Wullems, G.J. (1996) Isolation and characterization of a microspore-specific gene from tobacco. *Plant Mol. Biol*. **31**, 213–225.

Rieger, M.A., Lamond, M., Preston, C., Powles, S.B. and Roush, R.T. (2002) Pollen-mediated movement of herbicide resistance between commercial canola fields. *Science*, **296**, 2386–2388.

Ruiz, O.N. and Daniell, H. (2005) Engineering cytoplasmic male sterility via the chloroplast genome by expression of beta-ketothiolate. *Plant Physiol*. **138**, 1232–1246.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Spring Harbor: Cold Spring Laboratory Press.

Schaart, J.G., Krens, F.A., Pelgrom, K.T.B., Mendes, O. and Rouwendal, G.J.A. (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.

Teli, N.P. and Timko, M.P. (2004) Recent developments in the use of transgenic plants for the production of human therapeutics and biopharmaceuticals. *Plant Cell Tiss. Org. Cult*. **79**, 125–145.

Zhang, W., Subbarao, S., Addae, P., Shen, A., Armstrong, C., Peschke, V. and Gilbertson, L. (2003) Cre/*lox*-mediated marker gene excision in transgenic maize (*Zea mays* L.) plants. *Theor. Appl. Genet*. **107**, 1157–1168.

Zubko, E., Scutt, C. and Meyer, P. (2000) Intrachromosomal recombination between *att*P regions as a tool to remove selectable marker genes from tobacco transgenes. *Nat. Biotechnol*. **18**, 442–445.

Zuo, J., Niu, Q.W., Moller, S.G. and Chua, N.H. (2001) Chemical-regulated, site-specific DNA excision in transgenic plants. *Nat. Biotechnol*. **19**, 157–161.