Theor Appl Genet (2003) 107:1157–1168
DOI 10.1007/s00122-003-1368-z

W. Zhang · S. Subbarao · P. Addae · A. Shen · C. Armstrong · V. Peschke · L. Gilbertson

Cre/*lox*-mediated marker gene excision in transgenic maize (*Zea mays* L.) plants

Received: 19 November 2002 / Accepted: 17 April 2003 / Published online: 25 September 2003

Abstract After the initial transformation and tissue culture process is complete, selectable marker genes, which are used in virtually all transformation approaches, are not required for the expression of the gene of interest in the transgenic plants. There are several advantages to removing the selectable marker gene after it is no longer needed, such as enabling the reuse of selectable markers and simplifying transgene arrays. We have tested the Cre/*lox* system from bacteriophage P1 for its ability to precisely excise stably integrated marker genes from chromosomes in transgenic maize plants. Two strategies, crossing and autoexcision, have been tested and demonstrated. In the crossing strategy, plants expressing the Cre recombinase are crossed with plants bearing a transgene construct in which the selectable marker gene is flanked by directly repeated *lox* sites. Unlike previous reports in which incomplete somatic and germline excision were common, in our experiments complete somatic and germline marker gene excision occurred in the F_1 plants from most crosses with multiple independent Cre and *lox* lines. In the autoexcision strategy, the *cre* gene, under the control of a heat shock-inducible promoter, is excised along with the *nptII* marker gene. Our results show that a transient heat shock treatment of primary transgenic callus is sufficient for inducing *cre* and excising the *cre* and *nptII* genes. Genetic segregation and molecular analysis confirmed that marker gene removal is precise, complete and stable. The autoexcision strategy provides a way of removing the selectable marker gene from callus or other tissues such as embryos and kernels.

Communicated by D. Hoisington

W. Zhang · S. Subbarao · P. Addae · A. Shen · C. Armstrong · V. Peschke · L. Gilbertson (✉)
Monsanto Company, 700 Chesterfield Parkway North, St. Louis, MO 63017-1732,
e-mail: Larry.A.Gilbertson@Monsanto.Com
Fax: +1-636-7375454

Introduction

Over the last decade, transgenic plants have moved from being solely laboratory vehicles for basic research work to providing new varieties grown on large areas throughout the world. A number of transformation methods have been developed to transfer agronomically important genes into plant cells. Selectable marker genes are needed in virtually all plant transformation approaches, but generally the marker gene is not required for the expression of the trait gene in the transgenic plants. There are advantages to removing the selectable marker gene after transgenic plants have been generated, such as the "recycling" of selectable markers to facilitate gene stacking and simplifying transgene constructs by removing an entire cassette. Although most selectable marker genes, including those conferring resistance to non-clinically important antibiotics, such as the neomycin phosphotransferase gene (*nptII*), raise no significant safety concerns, their removal should help eliminate public concerns over the safety of transgenic plants.

A number of methods for marker gene removal from transgenic plants have been reported in the literature, including cotransformation of T-DNAs followed by segregation of the marker gene from the trait gene (Depicker et al. 1985; de Frammond et al. 1986; McKnight et al. 1987; De Block and Debrouwer 1991; Komari et al. 1996; De Neve et al. 1997; Daley et al. 1998; Xing et al. 2000; Lu et al. 2001; McCormac et al. 2001), homologous recombination between direct repeats (Lichtenstein et al. 1994; Zubko et al. 2000) and site-specific recombination. A number of site-specific recombinases of prokaryotic or yeast origin have been shown to function in transgenic plants for marker removal, including Cre/*lox* from bacteriophage P1 (Dale and Ow 1990, 1991; Odell et al. 1990; Russell et al. 1992; Srivastava et al. 1999; Zuo et al. 2001; Hoa et al. 2002), Flp/*frt* from *Saccharomyces cerevisiae* (Lyznik et al. 1993; Lloyd and Davis 1994; Kilby et al. 1995; Sonti et al. 1995; Luo et al. 2000), R/*RS* from *Zygosaccharomyces rouxii* (Onouchi et al. 1991; Sugita et al. 2000) and Gin/*gix* from bacterio-

phage Mu (Maeser and Kahmann 1991). These simple site-specific recombination systems all consist of two basic components: a recombination enzyme and small DNA recognition sites. These two components are sufficient to perform precisely defined recombination reactions in heterologous systems, thereby enabling a variety of applications such as site-specific integration, copy number reduction and marker gene removal. We chose the Cre/*lox* system to develop the marker excision technologies for transgenic maize plants.

A number of strategies can be used with Cre/*lox* to remove marker genes from transgenic plants. In all strategies the marker gene is flanked by directly repeated *lox* sites, and excision occurs when Cre activity is present. The strategies differ in how Cre function is delivered. In one strategy, transgenic plants that carry a marker gene flanked by *lox* sites can be retransformed with a Cre-expressing plasmid. The *cre* gene can then be segregated away in the next generation (Odell et al. 1990; Dale and Ow 1991; Russell et al. 1992). Alternatively, the *lox* plants can be crossed with plants that express Cre activity (herein referred to as the "crossing strategy"), in which case marker excision occurs in the F_1 progeny, followed by loss of the *Cre* gene by genetic segregation in the F_2 generation (Odell et al. 1990; Bayley et al. 1992; Russell et al. 1992; Hoa et al. 2002). Finally, the *cre* gene can be included on the DNA segment that is flanked by *lox* sites. In this strategy, herein referred to as the "autoexcision strategy", it is critical that the *cre* gene be regulated such that expression is activated (induced) after the marker gene is no longer needed. Autoexcision has recently been demonstrated in *Arabidopsis thaliana* using a chemically inducible promoter (Zuo et al. 2001) and using a heat shock-inducible promoter (Hoff et al. 2001).

We have tested the crossing and autoexcision strategies to remove an *nptII* marker gene from transgenic maize and found that both can efficiently and completely remove a marker gene.

Materials and methods

Plasmids

To test the efficiency of marker excision, we constructed two constructs and transferred these into maize plants. pMON36133 (Fig. 1A) has an e35S promoter (Kay et al. 1987) with an HSP70 intron in the 5′ untranslated region (Brown and Santino 1999), an *nptII* gene (Bevan et al. 1983) flanked by *lox511* sites (Hoess et al. 1986), followed by a promoter-less *gfp* green fluoresent protein gene (Pang et al. 1996). Upon Cre-mediated excision of the *nptII* gene, the g*fp* gene becomes fused to the e35S/HSP70 promoter/intron and is expressed constitutively. Cre expression construct pMON36136 (Fig. 1B) contains *cre* driven by the rice *Act1* gene promoter and the first intron of the *Act1* gene (McElroy et al. 1990; Zhang et al. 1991; Wang et al. 1992), and the *nptII* gene driven by e35S/HSP70. The *cre* gene is interrupted by the second intron (IV2) of the potato ST-LS1 gene (Vancanneyt et al. 1990) placed between nucleotides 431 and 432 in the *cre* open reading frame. Constructs were made in T-DNA binary vectors, and transferred into the ABI (*Agrobacterium* binary) *Agrobacterium tumefaciens* strain derived from C58 transconjugent carrying pMP90RK (Koncz and Schell 1986) for maize transformation.

pMON36159 (Fig. 1C) was made to test the autoexcision strategy. In this construct, *cre* is under the control of the HSP17.5E promoter from soybean (Ainley and Key 1990). The *cre* open reading frame was inserted between the *Hin*dIII and *Sal*I sites of pMA406 (Ainley and Key 1990), and the resulting *Bgl*II fragment was inserted into pMON36133 between the *lox* sites. pMON36159 was then transferred into the ABI *A. tumefaciens* strain.

Plant transformation

The transformation methods used were basically as described by Ishida et al. (1996), Cheng et al. (1997) and Armstrong and Rout (2001), with modifications. *Agrobacterium tumefaciens* (ABI strain) was cultured in Luria-Bertani (LB) liquid medium (1% tryptone, 0.5% yeast extract, and 1% NaCl, pH 7.0; 50 ml medium

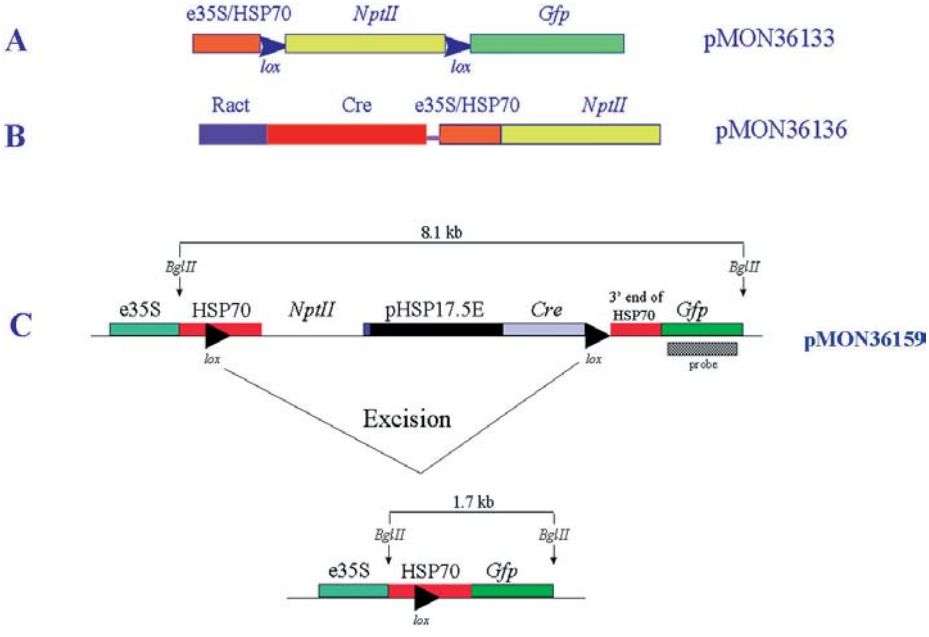

Fig. 1A–C Marker excision constructs. **A** pMON36133 is a *lox* construct with the *NPTII* marker gene, **B** pMON36136 is a *Cre* construct with *NPTII* as a selectable marker gene, **C** pMON36159 is an autoexcision construct with a *Cre* gene and *NPTII* flanked by two direct repeat *loxP* sites

per 250-ml flask) containing 100 mg/l kanamycin, 50 mg/l spectinomycin and 25 mg/l chloramphenicol for about 24 h at 27 °C, on a rotary shaker at 150–160 rpm. The *Agrobacterium* cells were spun down at 3,400 rpm and re-suspended into AB liquid medium (0.1 *M* MES, 0.5 m*M* NaH_2PO_4, 2% glucose, 1 g/l NH_4Cl, 300 mg/l $MgSO_4 \cdot 7H_2O$, 150 mg/l KCl, 10 mg/l $CaCl_2$, 2.5 mg/l $FeSO_4 \cdot 7H_2O$, pH 5.4; the OD was adjusted to 0.2–0.5 at 660 nm) containing one-half the level of spectinomycin and kanamycin used for LB, plus 200 μM acetosyringone (AS; used for the induction of virulence genes), in a 250 ml flask. After culturing for 15–16 h under the same conditions as for LB culture, the *Agrobacterium* cells were harvested and washed in 1/2-strength MS (Murashige and Skoog 1962) VI medium (half-strength MS salts, half-strength MS vitamins, plus 1 m*M* proline, 1% glucose, 2% sucrose and 200 μM As) and centrifuged again before re-suspending in 1/2-strength MS PL medium (half-strength MS salts, half-strength MS vitamins plus 1 m*M* proline, 3.6% glucose, 6.85% sucrose and 200 μM As). The final concentration of *Agrobacterium* was about 1×10^9 cfu/ml (OD = 1.0 at 660 nm).

A three-way cross of maize inbreds (Pa91 × H99) × A188 was employed for stable transformation. Immature embryos 1.0–2.0-mm-long were aseptically isolated and immersed into 1/2-strength MS PL liquid medium containing *Agrobacterium* with 200 μM AS for 30 min. The immature embryos were placed briefly on a piece of sterile Whatman filter paper (grade no.1) to remove excess liquid before plating onto 1/2-strength MS co-culture medium containing 3.0 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 200 μM AS, 2% sucrose, 1% glucose, 12 m*M* proline and 20 μM silver nitrate, and cultured at 23 °C, in the dark for 2–3 days. Embryos were then transferred to a modified Medium D (Duncan et al. 1985) consisting of Medium D macro- and micro-nutrients, 1 mg/l 2,4-D, 12 m*M* proline and 500 mg/l carbenicillin and cultured at 27 °C for 5 days. The embryos were then transferred to the first selection Medium D containing 1.0 mg/l 2,4-D, 12 m*M* proline, 750 mg/l carbenicillin and 50 mg/l paromomycin and cultured for 2 weeks at 27 °C. Then the embryos were transferred to the same basal medium but containing 500 mg/l carbenicillin and 100 mg/l paromomycin. After an additional 2 weeks, these embryos were transferred to Medium D containing 200 mg/l paromomycin for one or two more rounds of selection. Selected calli were transferred to MS 6BA regeneration medium containing 3.5 mg/l of 6-benzylaminopurine for about 1 week before moving them to MS medium without plant growth regulators in phytatrays and incubated at 28 °C under light. Plantlets with vigorous root and shoot development were selected after 2–3 weeks, and placed into soil and hardened off for 7 days before placement in the greenhouse.

Plant assays

The NPTII whole plant bioassay (Howe and Feng 2003) was used to assay transgenic plants for the presence/absence of the *nptII* gene. A 10-μl application of 1 g/l kanamycin and 1 g/l paromomycin with 0.06% silwet is applied to the whorl of V1–V2 maize seedlings using a pipettor. The assay is done in the greenhouse. The plants are scored for *nptII* expression 4–5 days after application of the above solution. Necrotic patches and bleaching of the younger leaves indicate absence of the *nptII* gene and is comparable to the symptoms seen on a negative control. Positive plants show no necrosis or bleaching and are confirmed positive by NPTII ELISA.

NPTII ELISA assays were performed on leaf tissue using the NPTII ELISA kit from Agdia according to the manufacturer's directions. Genomic DNA for Southern blots was extracted from leaf tissue using a modified CTAB DNA extraction protocol. Two to three grams fresh weight of tissue were ground in liquid nitrogen using a mortar and pestle. Approximately 100 mg of ground tissue was resuspended in 700 μl CTAB extraction buffer, followed by incubation at 65 °C for 30–45 min. This was followed by two extractions with equal volumes of chloroform/isoamyl alcohol (24:1, v/v), with the first extraction including a 10 min period of gentle mixing of the tube. After the second extraction, two volumes of 100% EtOH were added, at which point DNA precipitated out in a form that was easy to "hook out" with a pipette tip and transferred to a tube with 1 ml 70% EtOH. This tube was spun in a microcentrifuge to pellet DNA. After drying briefly, the DNA was resuspended in 50 μl of TE. Fifteen micrograms of DNA was digested for 4–5 h with the appropriate enzymes, followed by gel electrophoresis on a 0.7% agarose gel in TBE buffer. Subsequent steps for gel treatment, transfer to nylon membranes, DNA crosslinking and hybridization with radioactive probes were all done by standard methods described by Sambrook et al. (1989). Details about specific enzymes used for digests and DNA fragments used as probes are given in figure legends or text.

Transgenic plants were either self-pollinated, or crosses were made between *lox* and Cre plants. R_1 immature embryos were dissected and investigated under a GFP Plus fluorescence microscope (Leica M28) with two GFP filters (no. 446148: excitation at 425 ± 30 nm; diaphragm, 505 nm Long Pass; barrier, 475 nm; no. 44643: excitation at 480 ± 20 nm; diaphragm, 505 nm Long Pass; barrier 510 nm Long Pass) for *gfp* expression (described in the figures as under UV light). Segregation of GFP in R_1 embryos was analyzed by the Chi-square test.

Plant nomenclature

Independent transformed calli are called "events" and given a unique event name, which is generally recorded as a six digit number; for example 2265-1-2, where 2265 is the experiment number, 1 is the treatment number, and 2 is the unique transformant from that treatment. Since each event was derived from a unique immature embryo used in transformation experiments, all events are independent transformants. Frequently a single event produces multiple plants, i.e. multiple shoots regenerated from a single transgene callus. Unless otherwise noted, multiple plants from a single event are assumed to be clones and collectively represent a single transgenic event or line. Conversely, plants originating from different events are assumed to be genetically distinct.

The plants derived from tissue culture are called R_0 plants, and each plant is given a unique plant number, starting with the prefix "ZM", e.g. ZM_1004. In our tables we list both the number of the plant actually used in the cross and the event from which it was derived. When one plant is used to cross to another, the progeny of the cross are called F_1 plants. If F_1 plants are selfed, the subsequent generations are called F_2, F_3, etc. If an R_0 plant is selfed, the progeny are called R_1 plants. If R_1 plants are selfed, subsequent generations are called R_2, R_3, etc.

Results

Marker excision from transgenic maize plants by the crossing strategy

The constructs pMON36133 and pMON36136 (Fig. 1) were made and transferred into maize plants [(Pa91 × H99] × A188) using the *Agrobacterium*-mediated approach (Ishida et al. 1996; Cheng et al. 1997; Armstrong and Rout 2001) to test marker excision by crossing and autoexcision strategies. pMON36133 is a *lox* "reporter" construct that allows visual assays for the excision of the marker gene. It has *lox511* sites flanking the *nptII* gene; one *lox* site is placed between the *nptII* open reading frame and the e35S promoter, while the other is downstream of the transcriptional termination site. This is followed by a promoter-less *gfp* gene. Cre/*lox*-mediated excision of the *nptII* gene results in the *gfp* gene being placed under the control of the e35S promoter, thus providing a convenient visual assay for marker gene

Table 1 Plants transformed with the *lox* and *cre* gene constructs

Constructs	Number of experiments	Number of embryos	Number of independent transformants	Transformation frequency (%)
pMON36133 (*lox*/*nptII*/*lox*/*gfp*)	3	632	120	18.9
pMON36136 (*cre*/*nptII*)	2	366	26	7.1
pMON36159 (*lox*/*nptII*/*cre*/*lox*/*gfp*)	6	1,511	16	1.0

Table 2 GFP segregation in corn F_1[a] embryos (parents contain a single copy of the transgene) of pMON36133 × pMON36136 (or reciprocal crosses): R_0 (*lox*) × R_0 (*cre*)

lox transformants		Cre transformants		Observation segregation				
Zm no.[b]	Event no.	Zm no.	Event no.	GFP+	GFP–	Segregation ratio	Chi-square	*P* value
R_0 (female)		R_0 (male)						
2118	2265-1-21	2132	2265-2-09	59	198	1:3	0.47	0.5–0.9
2098	2265-1-07	2133	2265-2-10	80	65	1:3	68.8	<0.005
2121	2265-1-23	2124	2265-2-02	61	148	1:3	1.74	0.1–0.5
2089	2265-1-02	2145	2265-2-17	49	161	1:3	0.23	0.5–0.9
2116	2265-1-20	2142	2265-2-16	8	22	1:3	0.0	>0.995

[a] F_1 was defined as progeny from the cross of an R_0 plant (the original, regenerated trangenic plant) with another plant; R_1 was defined as the progeny of a self-pollinated R_0 plant
[b] Zm numbers here are the number assigned to each transgenic plant

Table 3 GFP segregation in corn F_1 embryos (parents contain a single copy of the transgene) of pMON36133 × pMON36136 (or reciprocal crosses): R_1 (*lox*) × R_0 (*cre*)

lox transformants		Cre transformants		Observed segregation		Expected segregation ration	Chi-square	*P* value
Zm no.	Event no.	Zm no.	Event no.	GFP+	GFP–			
R_1 (female)		R_0 (male)						
1005-01[a]	2339-1-37	5080-01-01[b]	2344-2-01	81	84	1:1	0.02	>09
1005-02	2339-1-37	5080-01-01	2344-2-01	30	50	1:1	4.51	0.025–0.05
1005-03	2339-1-37	5080-01-01	2344-2-01	47	48	1:1	2.47	>0.995
1009-01	2339-1-39	5080-01-01	2344-2-01	38	53	1:1	2.47	0.05–0.1
995-01	2339-1-32	5080-01-07	2344-2-07	67	222	1:3	0.34	>0.9
997-01	2339-1-33	5080-01-04	2344-2-04	27	113	1:3	2.14	0.1–0.5
R_1 (male)		R_0 (female)						
995-01	2339-1-32	5080-01-07	2344-2-07	55	171	1:3	0.02	>0.975
997-01	2339-1-33	5080-01-04	2344-2-04	28	126	1:3	3.46	0.05–0.1

[a] Zm no. 1005-01 is an R_1 plant derived from R_0 plant Zm no. 1005
[b] 5080-01-01 was an R_0 plant in the cross. 5080 is an experiment number, the -01 following is a treatment number and 01 is an event number

excision. pMON36136 is a *cre* construct that is needed for marker gene excision in the crossing strategy.

As shown in Table 1, the transformation frequency was 18.9% with pMON36133 and 7.1% with pMON36136. Over 120 independent events with pMON36133 and 26 events with pMON36136 were generated using (Pa91 × H99) × A188 as the genetic background.

To test the crossing strategy, three types of crosses were made: $R_0 \times R_0$, $R_0 \times R_1$ and $R_1 \times R_1$ (see Materials and Methods for nomenclature definitions). All parental plants (lines) used in crosses were first assayed for NPTII activity. Only the kanamycin-resistant plants were selected. These plants were then analyzed by Southern blot, probing for the *nptII* gene, to assess transgene copy number. Only plants with low molecular complexity (one to two copies of the transgene) were selected for crossing. For $R_0 \times R_0$ crosses (Table 2), R_0 plants from five different *lox* events and five different Cre events were crossed. R_0 plants are generally assumed to be hemizygous, so *gfp* expression should segregate 1+:3– if excision is complete. This was the case for four of the five crosses made. In the progeny from one cross, 2098 × 2133, GFP segregated close to 1:1.

Results of crosses between R_1 *lox* plants and R_0 Cre plants, representing 4 different *lox* events and 3 different Cre events, are shown in Table 3. For $R_1 \times R_0$ crosses, it is assumed that the R_0 plants are hemizygous, while the R_1 plants could be hemizygous or homozygous. Assuming Mendelian segregation, segregation of GFP activity should fit either a 1+:3– or 1+:1– ratio if excision is complete. Most of the segregation data are close to one of these two ratios, suggesting that excision occurred efficiently in these crosses.

Table 4 GFP segregation in corn F_1 embryos (parents contain a single copy of the transgene) of pMON36133 × pMON36136 (or reciprocal crosses): R_1 (*lox*) × R_1 (*cre*)

lox transformants		Cre transformants		Observed segregation		Expected segregation ration	Chi-square	*P* value
Zm no.	Event #	Zm no.	Event no.	GFP+	GFP–			
R_1 (female)		R_1 (male)						
937-04	2339-1-01	1094-02	2344-2-03	76	220	1:3	0.07	0.5–0.9
1004-02	2339-1-37	1103-01	2344-2-07	21	88	1:3	1.91	0.1–0.5
936-01	2339-1-01	1089-01	2344-2-01	10	74	1:3	7.00	0.005–0.01
943-01	2339-1-05	1089-02	2344-2-01	25	46	1:3	3.42	0.05–0.1
1004-01	2339-1-37	1089-03	2344-2-01	264	17	1:3	708.8	<0.005
937-01	2339-1-01	1089-04	2344-2-01	25	39	1:3	6.02	0.01–0.025
943-02	2339-1-05	1103-02	2344-2-07	0	206	1:3	67.34	<0.005
937-02	2339-1-01	1103-03	2344-2-07	0	226	1:3	74.01	<0.005
1004-03	2339-1-37	1096-01	2344-2-04	166	27	1:3	379.9	<0.005
1006-04	2339-1-38	1094-04	2344-2-03	56	187	1:3	0.40	0.5–0.9
937-05	2339-1-01	1101-02	2344-2-06	65	185	1:3	0.09	0.5–0.9
1004-05	2339-1-37	1101-01	2344-2-06	88	74	1:1	1.20	0.1–0.5
1004-04	2339-1-37	1094-03	2344-2-03	97	116	1:1	1.75	0.1–0.5
1006-01	2339-1-38	1089-05	2344-2-01	52	59	1:1	0.32	0.5–0.9
1006-02	2339-1-38	1096-03	2344-2-04	47	33	1:1	2.11	0.1–0.5
943-03	2339-1-05	1096-02	2344-2-04	65	91	1:1	4.01	0.025–0.05
R_1 (male)		R_1 (female)						
936-01	2339-1-01	1089-01	2344-2-01	40	50	1:3	17.13	<0.005
1004-01	2339-1-37	1089-02	2344-2-01	26	29	1:3	13.39	<0.005
937-01	2339-1-01	1089-04	2344-2-01	15	87	1:3	5.23	0.01–0.025
1004-03	2339-1-37	1096-01	2344-2-04	11	30	1:3	0.01	0.9–0.975
943-03	2339-1-05	1096-02	2344-2-04	15	29	1:3	1.48	0.1–0.5
1004-05	2339-1-37	1101-01	2344-2-06	41	50	1:1	0.70	0.1–0.5

For $R_1 \times R_1$ crosses, the R_1 seeds both from Cre and *lox* lines were planted in the greenhouse at different times to match flowering times for pollination. A total of 22 crosses representing four *lox* events and five Cre events were made, and the resulting embryos isolated were observed for GFP activity (Table 4).

In crosses between R_1 plants, three possible combinations of parental zygosity exist: both parents could be hemizygous, both parents could be homozygous or one parent could be hemizygous and one homozygous. Assuming Mendelian segregation, GFP would segregate 1+:3–, 1+:0– or 1:1, respectively. Evidence for all three segregation ratios was observed, and most of the progeny fit one of the expected ratios. Two crosses (943-02 × 1103-02 and 937-02 × 1103-03) apparently failed to excise the marker gene, even though the parents were derived from events that showed evidence for excision in other crosses. In the crosses 1004-01 × 1089-03 and 1004-03 × 1096-01 (Table 4), *gfp* segregation fell short of the 100% expected if both parents were homozygous, but exceeded the 1:1 ratio expected if one parent was homozygous and the other hemizygous. The predicted ratios assume both transgenes are present as simple, single inserts.

F_1 embryos from crosses between *lox* and Cre lines were isolated onto MS media or Medium D without auxin 7–14 days after pollination and observed under the fluorescent microscope. Some of the embryos showed very strong GFP activity (bright), indicating Cre-dependent excision of the marker (Fig. 2A). Some of the other embryos showed no GFP activity (dull). These were later shown to be azygous segregants. There was another set of embryos (leaky) which showed faint GFP activity, and this was later shown to be due to leakiness of *gfp* gene expression within the unexcised *lox* construct (pMON36133), possibly due to readthrough of the *nos* polyadenlylation signal. Nevertheless, it was possible to distinguish between bright and leaky *gfp* expression. The negative controls, in which a Cre line (5080-01-01) was crossed with non-transgenic plants (H99), showed no GFP activity in the F_1 embryos.

Molecular confirmation of marker excision from transgenic maize plants

Southern blot analysis was carried out on select F_1 plants to determine if marker removal was complete. Genomic DNA was digested with *Bgl*II and probed with the *gfp* gene coding sequence. Plants in which excision occurred are expected to have a 2.1-kb band, while plants in which excision has not occurred would have a 3.4-kb band. As shown in Fig. 3, all plants which were derived from embryos that expressed *gfp* (bright) had a 2.1-kb band, while those that were scored as leaky for *gfp* expression had a 3.4-kb band. This confirmed that the marker gene was removed in the plants that expressed *gfp*, while all the "leaky" plants had a 3.4-kb band, indicating that the marker gene was not removed. Other plants without GFP activity (dull) did not give a hybridization band on the blot. Furthermore, the Southern in Fig. 3 shows that excision appears complete, in that there is no trace of a band indicative of the unexcised construct in the "bright" samples. These data are well correlated with GFP

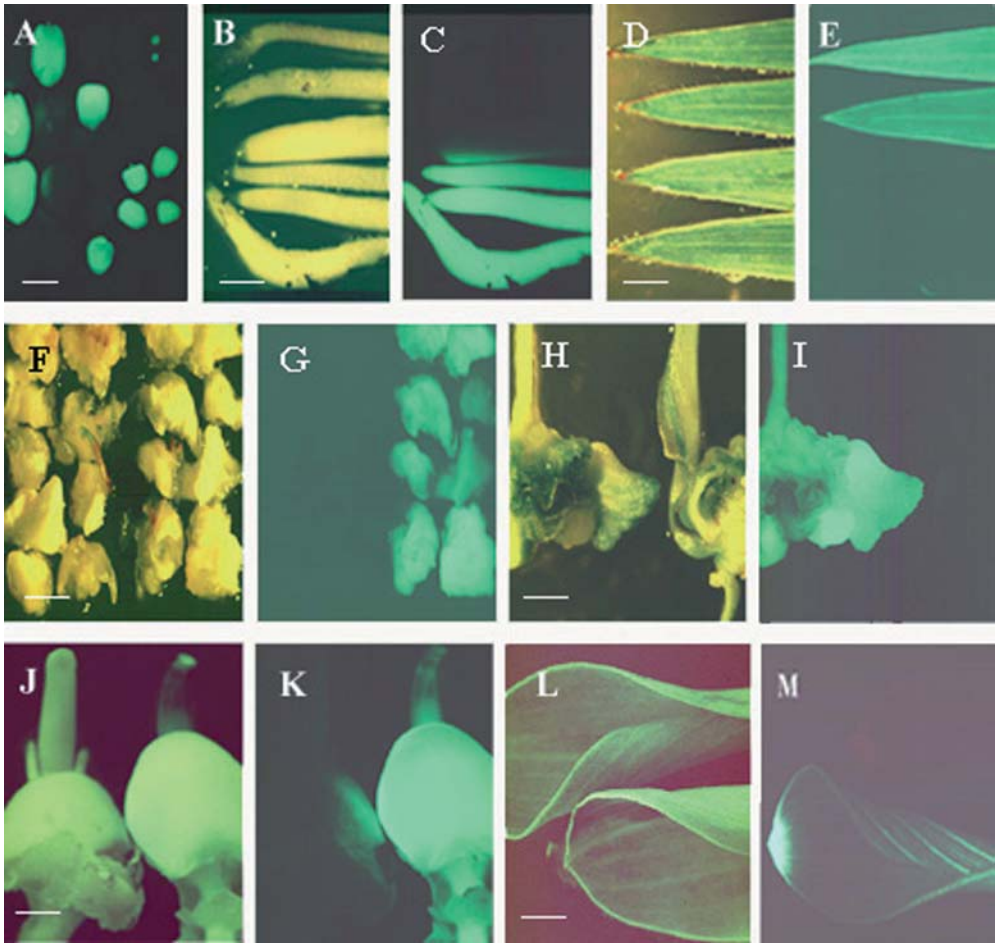

Fig. 2A–M *gfp* expression in different stages of transgenic plant development. **A–E** F_1 embryos and plant tissues are from the cross of Zm-1005-01 (pMON36133) × Zm-5080-01-01 (pMON36136). **A** Immature embryos were photographed under UV light (embryos 7 days after pollination are shown in *upper right corner*; those 16 days after pollination are on *left side*). *Bar*: 2 mm. **B** F_2 plant roots photographed under white light. *Bar*: 3 mm. **C** The same roots as in **B** but photographed under UV light. **D** F_2 leaves under white light. *Bar*: 3 mm. **E** The same leaves as in **D** but under UV light. **F–I** GFP activity in transgenic callus after heat shock and in R0 plantlets generated from heat shock-treated callus. **F** Calli were photographed under white light (calli on *left*, without heat shock; on *right* heat shock-treated). *Bar*: 5 mm. **G** The same calli as in **F** photographed under UV light. **H** Regenerated shoots photographed under white light (shoot on *left* from heat shock-treated callus; on *right*, from non-heat shock-treated callus). *Bar*: 5 mm. **I** The same shoots as in **H** but photographed under UV light. **J–M** Heat shock treatment of embryos results in autoexcision. **J** Germinating embryos under white light (R_2 embryos from R_1 plant Zm-S12223-01). The *right* embryo was treated with HS and the left one was untreated. *Bar*: 3 mm. **K** The same embryos as in **J** photographed under UV light. **L** R_2 plants derived from embryos (the leaf on top was from an untreated control and the one on bottom was from a heat shock-treated embryo. They were photographed under white light. *Bar*: 5 mm. **M** The same leaves as in **L** but photographed under UV light

segregation data. This result also confirmed that marker removal is stable, and marker gene reintegration is not detected in Southern blot analysis. A second Southern blot was conducted using the *nptII* gene as the probe (data not shown) to test if any part of the selectable marker remained at the original locus, or if it had reintegrated somewhere else in the genome. For the plants tested, reintegration did not occur, and no trace of the *nptII* gene at the original locus was detected.

To better assess the precision of marker excision, a DNA fragment comprising the junction produced by excision was cloned by PCR from five GFP-positive F_1 plants from independent crosses using five different *lox* events selected from Tables 3 and 4. The PCR products were 171 bp in size, extending from 72 bp upstream of the *lox511* site to 65 bp downstream of the *lox511* site. Sequence analysis of the PCR fragments revealed that the junction was identical in all five recombinants and that it matched the predicted junction sequence perfectly (data not shown).

Marker excision is stable and heritable

In order to test if marker excision is heritable, we examined F_2 progeny from 15 randomly selected, self-pollinated F_1 plants for GFP activity. The results are shown in Table 5. If excision was complete in the F_1

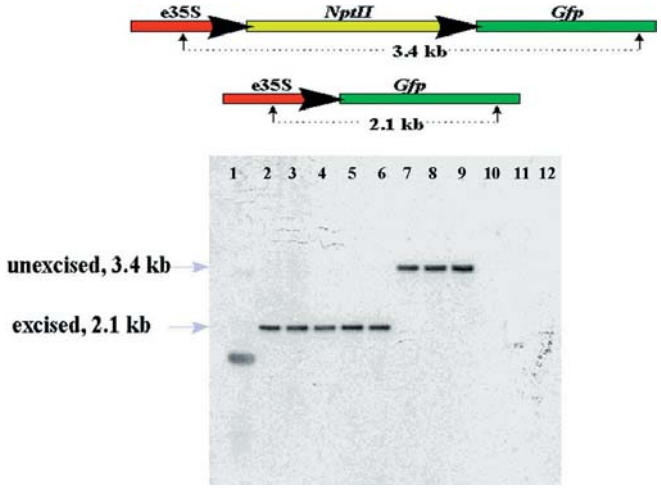

Fig. 3 Southern blot analysis of marker excision of F_1 plants (Table 3, cross of ZM no. 995-01 × ZM no. 5080-01-07). *Lane 1* Molecular weight marker (1-kb DNA ladder,Gibco BRL), *lanes 2–6* genomic DNA from five F_1 individual plants with GFP+, *lanes 7–9* three individual plants with leaky GFP activity, *lanes 10–12* three other individual plants without GFP activity (dull) digested with *Bgl*II and probed with the *gfp* gene coding sequence

plant, including the germline, *gfp* expression should segregate 3+:1– in the F_2 progeny. Plants nos. 11 and 23, derived from F_1 plants with no GFP activity, were selected as negative controls. Eight populations (derived from specific *lox* × Cre event combinations) out of nine had the expected 3+:1– ratio of *gfp* expression. Plant no. 51 (from 997-01 × 5080-01-04) was the only exception. This plant may represent excision that was not complete in the germline. All embryos from plants nos. 11 and 23 were GFP-negative as expected. This indicates that marker excision was generally complete in the F_1 plant and that marker removal in transgenic plants is stable. Figure 2B–E shows GFP expression in roots and leaves from young F_2 seedlings.

Marker gene removal by the autoexcision strategy

To test the autoexcision strategy, pMON36133 was modified by adding a *cre* cassette in which the *cre* gene is expressed by an inducible heat shock protein promoter (HSP17.5E) from soybean (Ainley and Key 1990) to generate pMON36159 (Fig. 1C). In transformation experiments to produce transgenic plants with pMON36159, we observed that the transformation frequency was relatively low (1.0%, Table 1).

After selection but before plant regeneration, callus samples from a single event containing pMON36159 were divided into two parts. One part was treated with a heat shock (HS; 42 °C for 5 h) and the other was used as a control (NHS). The heat shock treatment of transgenic calli at 42 °C for 5 h was shown to be sufficient to induce HSP17.5E promoter activity, to switch on *cre* gene expression and for the Cre protein to remove the *nptII* and *cre* genes between two *lox* sites. Calli treated with heat shock showed *gfp* expression, and shoots generated from those calli also showed strong GFP activity (Figure 2F–I).

R_0 plants were generated from heat shocked or non-heat-shocked calli. In most cases, NPTII ELISA results on R_0 plants (Table 6) were correlated with HS treatment (absence of NPTII activity in HS-treated calli; see plants # ZM-12215–12226), which is expected if the marker gene is removed by autoexcision after HS. Some plants,

Table 5 GFP segregation in corn F_2 embryos from crosses of pMON36133 and pMON36136 (F_1 plant expressing GFP were selfed)

F_1	*lox* transformants		Cre transformants		Observed segregation		Expected segregation ratio	Chi-square	*P* value
Plant no.	Zm no.	Event no.	Zm no.	Event no.	GFP +	GFP -			
	Original cross								
	R_1 (female)		R_0 (male)						
01	1005-01	2339-1-37	5080-01-01	2344-2-01	247	75	3:1	0.41	0.5–0.9
16	1009-01	2339-1-39	5080-01-01	2344-2-01	190	70	3:1	0.42	0.5–0.9
21	1009-01	2339-1-39	5080-01-01	2344-2-01	49	11	3:1	0.42	0.5–0.9
22	1009-01	2339-1-39	5080-01-01	2344-2-01	39	21	3:1	2.69	0.1–0.5
48	997-01	2339-1-33	5080-01-04	2344-2-04	181	60	3:1	0.00	>0.975
77	995-01	2339-1-32	5080-01-07	2344-2-07	169	57	3:1	0.00	>0.975
50	997-01	2339-1-33	5080-01-04	2344-2-04	51	9	3:1	2.69	0.1–0.5
80	995-01	2339-1-32	5080-01-07	2344-2-07	46	16	3:1	0.00	>0.975
51	997-01	2339-1-33	5080-01-04	2344-2-04	22	37	3:1	42.76	<0.005
11	1005-01	2339-1-37	5080-01-01	2344-2-01	0	60	3:1	176.02	<0.005
23	1009-01	2339-1-39	5080-01-01	2344-2-31	0	60	3:1	176.02	<0.005
N/A	R_1 (male)		R_0 (female)						
	995-01	2339-1-32	5080-01-07	2344-2-07	47	19	3:1	0.32	0.5–0.9
	R_1 (female)		R_1 (male)						
106	943-01	2339-1-05	1089-02	2344-2-01	98	43	3:1	1.99	0.1–0.5
94	1004-05	2339-1-37	1101-01	2344-2-06	46	17	3:1	0.05	0.5–0.9
103	936-01	2339-1-01	1089-01	2344-2-01	81	26	3:1	0.00	>0.975
117	1004-01	2339-1-37	1103-01	2344-2-07	227	72	3:1	0.09	0.5–0.9
96	1004-02	2339-1-37	1096-01	2344-2-04	108	30	3:1	0.62	0.1–0.5

[a] F_2 was defined as the progeny from self-pollinated F_1 plants

Table 6 Effect of heat-shock treatment of callus on *NPTII* expression in R_0 plants

Event no.	Plant no.	Heat shock (HS) or non-HS (NHS)	NPTII bioassay	ELISA
2689-4-11	12215	HS	–	–
2689-4-11	12216	HS	–	–
2689-4-12	12217	NHS	+	+
2689-4-12	12218	NHS	+	+
2689-4-21	12219	HS	–	–
2689-4-21	12220	HS	–	–
2689-4-22	12221	NHS	+	+
2689-4-22	12222	NHS	+	+
2689-4-31	12223	HS	–	–
2689-4-31	12224	HS	–	–
2689-4-32	12225	NHS	+	+
2689-4-32	12226	NHS	+	+
2689-4-41	12227	HS	–	–
2689-4-41	12228	HS	–	+
2689-4-42	12229	NHS	+	+
2689-4-42	12230	NHS	+	–
2689-4-51	12231	HS	–	+
2689-4-51	12232	HS	–	+
2689-4-52	12233	NHS	+	+
2689-4-52	12234	NHS	+	+
2689-4-61	12235	HS	–	+
2689-4-61	12236	HS	–	–
2689-4-62	12237	NHS	+	+
2689-4-62	12238	NHS	+	+

Table 7 Analysis of GFP activity in R_1 immature embryos derived from calli with and without heat-shock treatment

Plant ZM no.	Event no.	HS or NHS[a]	Observation segregation		Expected ratio	Chi-square	*P* value
			GFP+	GFP–			
12215	2689-4-11	HS	0	119	3:1	353.0	<0.005
12218	2689-4-12	NHS	2	360	–	–	–
12220	2689-4-21	HS	111	30	3:1	0.85	0.1–0.5
12221	2689-4-22	NHS	1	300	–	–	–
12223	2689-4-31	HS	79	34	3:1	1.30	0.1–0.5
12225	2689-4-32	NHS	1	320	–	–	–
12227	2689-4-41	HS	113	47	3:1	1.41	0.1–0.5
12229	2689-4-42	NHS	0	300	–	–	–
12231	2689-4-51	HS	89	21	3:1	1.75	0.1–0.5
12234	2689-4-51	NHS	0	39	–	–	–
12236	2689-4-61	HS	79	30	3:1	0.25	0.5–0.9
12238	2689-4-62	NHS	0	350	–	–	–
H99	(Wild type)	HS	0	300	–	–	–

[a] HS, Heat-shocked; NHS, non-heat-shocked

however, were still positive in an NPTII ELISA assay after HS treatment (12228, 12231, 12232 and 12235). On the other hand, one plant (12230) was negative in the ELISA assay even without HS treatment.

Genetic segregation in R_1 plants and molecular characterization

The analysis of the segregation of *gfp* expression in R_1 embryos is summarized in Table 7. One ear was taken from each treatment for embryo analysis and the other was saved for seeds. As shown in Table 7, most R_1 populations (derived from R_0 plants 12220, 12223, 12227, 12231 and 12236) generated from HS-treated calli had the expected Mendelian genetic segregation of *gfp* expression (3:1), indicating that the marker excision that occurred in the R_0 callus was heritable. However, two R_1 populations derived from R_0 plants 12215 and 12224 respectively showed no GFP segregation, despite the fact that the R_0 plants did not express *nptII* (Table 6). No population derived from NHS-treated calli showed Mendelian segregation of *gfp* expression, suggesting that the majority of the plants from NHS-treated calli still contained the marker gene. A few GFP-positive segregants were observed in populations derived from NHS calli, however, suggesting that expression of the *cre* gene might be occasionally induced during plant development, resulting in loss of the NPTII gene and activation of *gfp* expression in some embryos.

To provide physical evidence for autoexcision, Southern blot analyses were carried out. R_0 plants derived from

both HS and NHS calli from six independent transformation events were selfed, and six R_1 progeny from each of six R_0 plants were examined by several assays. The R_1 plants were first assayed for GFP and NPTII activity, followed by Southern blots. As shown in Fig. 4A, unexcised plants would have an 8.1-kb band (event 3 with NHS; plant 12225), and a 1.7-kb band (events 3 and 4 with HS; plant 12223 and 12231) would be produced on the Southern blot if autoexcision occurred as expected. For most of the plants derived from the heat shock treatment, a 1.7-kb band was observed as expected, indicating that marker excision had occurred. For most of the samples, the presence of the 1.7-kb band was correlated with the absence of the 8.1-kb band from the unexcised construct, indicating that excision was occurring as expected. Furthermore, event 3 and event 4 shared a similar digestion pattern, suggesting that some of the putative independent events were actually clones from a single event. This is possible since all of the plants used in the Southern blots originated from events from a single transformation experiment and treatment.

R_1 plants from 12215 and 12224 expressed neither the *nptII* gene nor the *gfp* gene. This suggests that either these plants were generated from untransformed cells, or that excision did not produce the expected product. For 12215, Fig. 4B shows that while the 8.1-kb band is absent there is no 1.7-kb band indicative of the expected excision product. For 12224, on the other hand, there is a 1.7-kb band, suggesting that excision did occur properly, despite the absence of *gfp* expression.

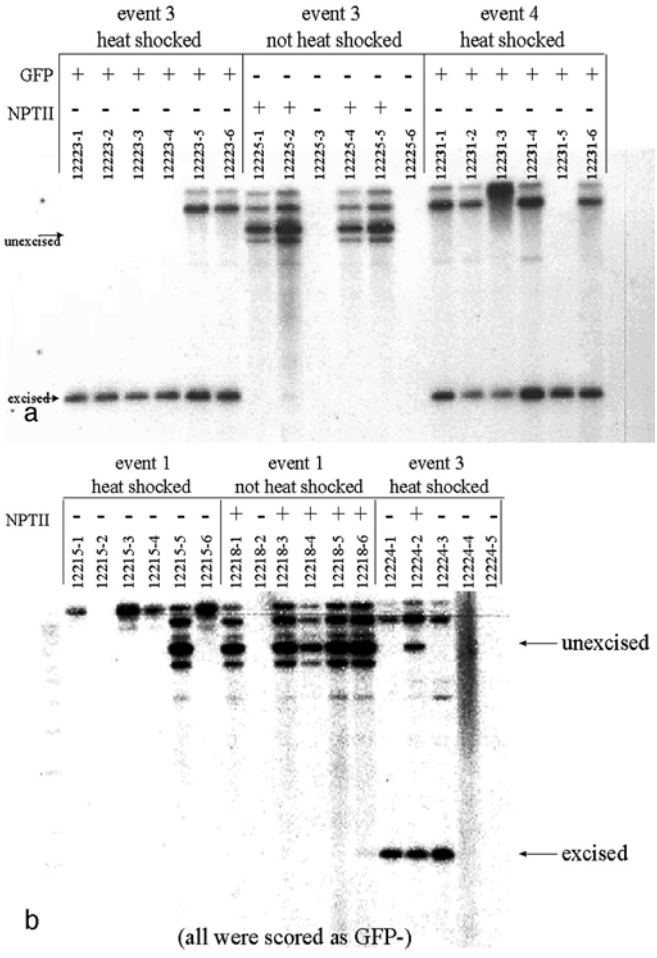

Fig. 4A, B Southern blot analysis on transgenic plants containing pMON36159. Genomic DNA from R_1 plants was digested with *Bgl*II. The *gfp* open reading frame was used as a probe. Lanes with no bands are assumed to represent azygous segregants. **A** DNA samples were isolated from six individual plants, events 2689-4-3 (ZM no. 12223-1 to -6) and 2689-4-4 (ZM no. 12231-1 to -6), respectively, derived from heat shock-treated calli while another six plants from event 2689-4-3 (ZM no. 12225-1 to -6; callus non-heat shock-treated) as a control. **B** GFP negative plants from event 2689-4-1 (ZM no. 12215-1 to -6) and event 2689-4-3 (ZM no. 12224-1 to -5) were derived from heat shock-treated calli, while plants from event 2689-4-1 (ZM no. 12218-1 to -6) were from non-heat shock-treated callus as control

Genetic analysis in R_2 embryos

To track GFP activity in the next generation, four R_1 plants from each of S12215 (HS but GFP-), 12218 (NHS), 12223 (HS), and 12231 (HS) populations were grown for R_2 progeny analysis. Immature R_2 embryos were isolated and assayed for GFP activity. The results are summarized in Table 8. The results correlated well with what was observed in the R_1 generation. 12215-derived R_1 embryos did not show GFP activity, although treated with HS in callus, and its R_2 embryos also lacked GFP activity. All

Table 8 Analysis of GFP activity in R_2 embryos

R_1 plant Zm no.	HS or NHS[a] (callus)	Observed		Correlation to R_1 (Inheritance)
		GFP+	GFP–	
12215-02	HS	0	14	High
12215-05	HS	0	200	High
12215-04	HS	0	169	High
12218-01	NHS	1	299	High
12218-04	NHS	0	250	High
12218-05	NHS	7	245	High
12223-01	HS	260	0	High
12223-02	HS	195	0	High
12223-04	HS	240	0	High
12231-01	HS	82	0	High
12231-02	HS	115	0	High
12231-03	HS	96	0	High

[a] HS, Heat-shocked NHS, non-heat-shocked

R_2 embryos derived from plants 12223 and 12231 (HS) showed strong GFP activity. It is likely that the R_1 plants that were selfed to produce the R_2 populations were homozygous. There was little GFP activity in the R_2 population derived from plant 12218 (NHS), although there were some GFP-positive kernels, due to leakiness of the heat shock promoter.

Autoexcision in immature embryos

To test if the marker gene can be excised from embryos by autoexcision, 15 R_2 embryos from a transgenic pMON36159 plant (R_1 plant 12223-01, regenerated without HS) were treated at 42 °C for 3 h. As shown in Fig. 2J–M, the embryos after HS treatment showed strong GFP activity, while those without HS treatment were all GFP-negative. Leaf and root tips collected from the plants generated from heat-shock-treated embryos also showed strong GFP activity (root picture not shown), demonstrating that the marker gene removal was stable through at least initial plant development. R_3 seed from these plants was harvested and grown in the greenhouse. GFP activity was detected in the seedlings, confirming that excision was heritable.

Discussion

There are at least three strategies for using the Cre/*lox* system to remove marker genes from transgenic plants: re-transformation, crossing and autoexcision. The crossing and autoexcision strategies were evaluated in maize plants in this report.

In the crossing strategy, plants in which the marker gene is flanked by *lox* sites are crossed with plants which express the *cre* gene. Marker excision occurs in F_1 progeny of the cross. The crossing strategy works well for maize plants because of the ease of cross pollination. This strategy has an advantage of avoiding another round of transformation and selection, but it requires the selection of transgenic plants in the F_2 segregating population that contain only the gene of interest without any marker genes. The autoexcision strategy has the *cre* gene with an inducible promoter, together with a marker gene, flanked by two directly repeated *lox* sites. Once the inducible promoter is turned on, the *cre* gene is expressed and excised together with the selectable marker gene (Sugita et al. 2000; Zuo et al. 2001).

For the crossing strategy, the genetic segregation data of crosses between *lox* and Cre lines demonstrated that the marker gene can be effectively removed in F_1 immature embryos and stably inherited in the following generation. Southern blot results correlated well with the *gfp* gene segregation and demonstrated that marker excision is complete, or nearly complete, in F_1 populations. In one population (Table 2: 2089 × 2133) from an $R_0 \times R_0$ cross, GFP segregated approximately 1:1. It is possible that one of the two parental plants used in the cross became homozygous sometime during its development. This occurs occasionally in plants derived from tissue culture due to a somatic recombination event between the transgene and the centromere of the chromosome into which it is inserted. Two populations (Table 4: 943-02 × 1103-02; 937-02 × 1103-03) from an $R_1 \times R_1$ cross had no GFP+ progeny. It may be that in these crosses azygous plants were inadvertently used. In the rest of the 35 crosses in Tables 2–4, excision occurred efficiently.

In two crosses (Table 4: 1004-01 × 1089-03 and 1004-03 × 1096-01), *gfp* segregation fell short of the 100% expected if both parents were homozygous, but exceeded the 1:1 ratio expected if one parent was homozygous and the other hemizygous. The predicted ratios suggested both transgenes were present as simple, single inserts. A Southern blot (data not shown) confirmed this, although it is possible that more detailed Southern analysis would have revealed a more complex insertion pattern, in which case segregation ratios such as what we observed could be expected. Note, however, that the plants used in these crosses came from events that showed expected segregation in other crosses. Another possibility is that both of the parents used in these crosses were homozygous, in which case the small number of progeny that did not express *gfp* provide direct evidence for excision failure. This is noteworthy because it would represent the only evidence in our experiments for incomplete excision at the population level. It is possible that excision sometimes fails in all crosses, but since segregation ratios from crosses involving at least one hemizygous parent are usually used, slight decreases in excision efficiency would go undetected. It is also possible excision occurred in all progeny, but that the *gfp* gene was not expressed.

When F_1 embryos were isolated and evaluated under the fluorescent microscope, three types of GFP activity (bright, leaky and dull) were observed (Fig. 2A). When excision occurred, bright GFP activity could be detected in immature embryos dissected as early as 7 days after pollination, indicating that marker gene excision occurred at a very early stage in embryo development. In addition, we saw no evidence for partial or chimeric *gfp* expression, suggesting that excision occurred very early the developing embryo.

Further analyses on F_2 embryos (Table 5) and their seedlings under fluorescent microscope (Fig. 2B–E) indicate that marker excision was generally complete in the F_1 plant, including the germline, and that marker removal in transgenic plants is stable.

There are several advantages of the autoexcision strategy over the crossing approach. First, it can be used for vegetatively propagated crops, such as potato, or crops that are tedious to cross, such as soybean. Secondly, it does not require outcrossing to bring in the *cre* gene, nor a second generation to eliminate the *cre* gene, saving at least one plant generation. Additionally, it allows one to control the timing for marker removal. The autoexcision strategy was recently demonstrated in tobacco with a chemically inducible promoter and the R/*R*S site-specific recombination system (Sugita et al. 2000) and in *Arabidopsis* using an estrogen receptor-based fusion

transactivator XVE that is β-estradiol-inducible and Cre/*lox* (Zuo et al. 2001).

The autoexcision strategy requires that the *cre* gene be carefully regulated so that it is not expressed during the tissue culture stages in which selection is applied. Expression of the *cre* gene can occur anytime after selection is no longer required, either by induction or through tissue-specific control. The only requirement for *cre* expression is that it occur either in the germline or upstream of it. In our autoexcision experiments, we used an inducible heat shock protein promoter (HSP17.5E) from soybean, that had previously been shown to regulate the *flp* gene in BMS cells (Lyznik et al. 1995). However, it was also shown that expression of the *cre* gene can be leaky without heat shock treatment. This leaky expression probably accounted for the low transformation frequency with pMON36159.

There was a high correlation between HS treatment and GFP activity in transgenic calli. All calli treated with HS showed strong GFP activity. However, some plants (Table 6: 12228, 12231, 12232, and 12235) still expressed the *nptII* gene after HS treatment, suggesting that marker removal was not 100% complete in the callus material. These plants may have been regenerated from cells with an unexcised marker gene even after heat shock. On the other hand, one plant (S12230) was negative in the NPTII ELISA assay without HS treatment, possibly due to leakiness of the HPS17.5E promoter.

The genetic segregation data (Table 7) of R_1 and subsequent R_2 generations demonstrated that the marker excision in HS-treated calli is stably heritable. Plants 12215 and 12224 are exceptions in that the R_0 and R_1 plants showed no GFP activity after heat shock. For 12215, Fig. 4B showed that there was no expected band (1.7 kb) present in the Southern blot for R_1 plants, indicating that the *gfp* gene was absent. It is possible that *gfp* was deleted along with the *nptII* gene during autoexcision. On the other hand, R_1 plants derived from plant 12224 clearly showed an expected 1.7-kb band in Southern blot, suggesting that the *gfp* gene was still present in plants but not expressed. There were additional bands of sizes other than those predicted for excised or unexcised products in transgenic plants. These are likely to represent additional fragments of the pMON36159 T-DNA. It is worth noting that the same number and sizes of additional bands appear in all R_1 families, even though some of the families were thought to be derived from independent R_0 events. This suggests that some of the putative independent events are probably clones from a single event. If the extra bands on the Southern blots represent additional non-intact fragments, some of them are probably excised along with the intact genes, since they are correlated with the presence or absence of the 8.1-kb band. It can be expected that, depending on their configuration, extra copies or fragments would be deleted by Cre-mediated recombination, as has been shown recently in wheat (Srivastava et al. 1999). Other fragments behave as if they are unlinked with the intact genes.

We were able to efficiently excise the marker gene from immature embryos when the heat shock protein promoter was employed in our experiments, and the marker removal was precise, stable and heritable (Fig. 2J–M). Thus, the autoexcision strategy enables removal of the marker gene from embryos during their germination. The autoexcision approach, however, will need to use a tightly controlled promoter which, whether inducible or tissue specific, will ensure a high transformation efficiency.

The results in this report confirm that marker genes used for genetic transformation of maize can be efficiently removed by Cre/*lox* recombination either via crossing or autoexcision. Previous work with Cre/*lox*-mediated excision in plants has generally demonstrated partially excised F_1 plants, suggesting late or inefficient Cre activity. In our experiments in maize, marker gene excision was generally complete using either crossing or autoexcision, and the marker gene excision events were both stable and heritable. Crossing between Cre and *lox* lines is an effective method for removing markers or any other segment of DNA from transgenic maize plants, while autoexcision has the advantage of saving time and eliminating the need for cross fertilization.

Acknowledgements Shengzhi Pang produced the progenitor clone of the *cre* gene that was used in this work. Ron Nagao (University of Georgia) kindly provided the pMA406 plasmid. We thank Ken Barton and Roy Fuchs for critical input on the manuscript.

References

Ainley WM, Key JL (1990) Development of a heat shock inducible expression cassette for plants: characterization of parameters for its use in transient expression assays. Plant Mol Biol 14:949–967

Armstrong CL, Rout J (2001) A novel *Agrobacterium*-mediated transformation method. PCT Patent Application W00109302-A2

Bayley CC, Morgan M, Dale EC, Ow DW (1992) Exchange of gene activity in transgenic plants catalyzed by the Cre -lox site-specific recombination system. Plant Mol Biol 18:353–361

Bevan MW, Flavell RB, Chilton MD (1983) A chimeric antibiotic resistance gene as a selectable marker for plant cell transformation. Biotechnology 24:367–370

Brown SM, Santino C (1999) Enhanced expression in plants. US Patent no. 5859347

Cheng M, Fry JE, Pang S, Zhou H, Hironaka CM, Duncan DR, Conner TW, Wan Y (1997) Genetic transformation of wheat mediated by *Agrobacterium tumefaciens*. Plant Physiol 115:971–980

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

Dale EC, Ow DW (1991) Gene transfer with subsequent removal of the selection gene from the host genome. Proc Natl Acad Sci USA 88:10558–10562

Daley M, Knauf V, Summerfelt KR, Turner JC (1998) Co-transformation with one *Agrobacterium tumefaciens* strain containing two binary plasmids as a method for producing marker-free transgenic plants. Plant Cell Rep 17:489–496

De Block M, Debrouwer D (1991) Two T-DNA's co-transformed into *Brassica napus* by a double *Agrobacterium tumefaciens* infection are mainly integrated at the same locus. Theor Appl Genet 82:257–263

de Frammond A, Back E, Chilton W, Kayes L, Chilton M-D (1986) Two unlinked T-DNAs can transform the same tobacco plant cell and segregate in the F_1 generation. Mol Gen Genet 202:125–131

De Neve M, De Buck S, Jacobs A, Van Montagu M, Depicker A (1997) T-DNA integration patterns in co-transformed plant cells suggest that T-DNA repeats originate from co-integration of separate T-DNAs. Plant J 11:15–29

Depicker A, Herman L, Jacobs A, Schell J, Van Montagu M (1985) Frequencies of simultaneous transformation with different T-DNAs and their relevance to the *Agrobacterium*/plant cell interaction. Mol Gen Genet 201:477–484

Duncan DR, Williams ME, Zehr BE, Widholm JM (1985) The production of callus capable of plant regeneration from immature embryos of numerous *Zea mays* genotypes. Planta 165:322–332

Hoa TTC, Bong BB, Huq E, Hodge TK (2002) Cre/*lox* site-specific recombination controls the excision of a transgene from the rice genome. Theor Appl Genet 104:518–525

Hoess RH, Wierzbicki A, Abremski K (1986) The role of the *lox*P spacer region in P1 site-specific recombination. Nucleic Acids Res 14:2287–2300

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

Howe AR, Feng PCC (2003) Assay for the detection of NPTII expression in Plants. US Patent Application 20030017599A1

Ishida Y, Saito H, Ohta S, Hiei Y, Komari T, Kumashiro T (1996) High efficiency transformation of maize (*Zea mays* L.) mediated by *Agrobacterium tumefaciens*. Natl Biotechnol 14:745–750

Kay R, Chan A, Daly M, McPherson J (1987) Duplication of CaMV 35S promoter sequences creates a strong enhancer for plant genes. Science 236: 1299–1302

Kilby NJ, Davies GJ, Snaith MR (1995) FLP recombinase in transgenic plants: constitutive activity in stably transformed tobacco and generation of marked cell clones in Arabidopsis. Plant J 8:637–652

Komari T, Hiei Y, Saito Y, Murai N, Kumashiro T (1996) Vectors carrying two separate T-DNAs for co-transformation of higher plants mediated by *Agrobacterium tumefaciens* and segregation of transformants free from selection markers. Plant J 10:165–174

Koncz C, Schell J (1986) The promoter of TL-DNA gene 5 controls the tissue specific expression of chimaeric genes carried by a novel type of *Agrobacterium* binary vector. Mol Gen Genet 204:383–396

Lichtenstein CPJHB, Pazkowski J, Hohn B (1994) Intrachromosomal recombination between genomic repeats. In: Paszkowski J (ed) Homologous recombination and gene silencing in plants. Kluwer, Dordrecht, pp 95–122

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

Lu H-J, Zhou X-R, Gong Z-X, Upadhyaya NM (2001) Generation of selectable marker-free transgenic rice using double right-border (DRB) binary vectors. Aust J Plant Physiol 28:241–248

Luo H, Lyznik LA, Gidoni D, Hodges TK (2000) FLP-mediated recombination for use in hybrid plant production. Plant J 23:423–430

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

Lyznik LA, Hirayama L, Rao KV, Abad A, Hodges TK (1995) Heat-inducible expression of FLP gene in maize cells. Plant J 8:177–186

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

McCormac AC, Fowler MR, Chen DF, Elliott MC (2001) Efficient co-transformation of *Nicotiana tabacum* by two independent T-DNAs, the effect of T-DNA size and implications for genetic separation. Trans Res 10:143–155

McElroy D, Zhang W, Cao J, Wu R (1990) Isolation of an efficient actin promoter for use in rice transformation. Plant Cell 2:163–171

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

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497

Odell J, Caimi P, Sauer B, Russell S (1990) Site-directed recombination in the genome of transgenic tobacco. Mol Gen Genet 223:369–378

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

Pang SZ, DeBoer DL, Wan Y, Ye G, Layton JG, Neher MK, Armstrong CL, Fry JE, Hinchee MA, Fromm ME (1996) An improved green fluorescent protein gene as a vital marker in plants. Plant Physiol 112:893–900

Russell SH, Hoopes JL, Odell JT (1992) Directed excision of a transgene from the plant genome. Mol Gen Genet 234:49–59

Sambrook J, Fritsch EF, ManiatisT (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor

Sonti RV, Tissier AF, Wong D, Viret JF, Signer ER (1995) Activity of the yeast FLP recombinase in Arabidopsis. Plant Mol Biol 28:1127–1132

Srivastava V, Anderson OD, Ow DW (1999) Single-copy transgenic wheat generated through the resolution of complex integration patterns. Proc Natl Acad Sci USA 96:11,117–11,121

Sugita K, Kasahara T, Matsunaga E, Ebinuma H (2000) A transformation vector for the production of marker-free transgenic plants containing a single copy transgene at high frequency. Plant J 22:461–469

Vancanneyt G, Schmidt R, O'Connor-Sanchez A, Willmitzer L, Rocha-Sosa M (1990) Construction of an intron-containing marker gene: splicing of the intron in transgenic plants and its use in monitoring early events in *Agrobacterium*-mediated plant transformation. Mol Gen Genet 220:245–250

Wang Y, Zhang W, Cao J, McElroy D, Wu R (1992) Characterization of *cis*-acting elements regulating transcription from the promoter of a constitutively active rice actin gene. Mol Cell Biol 12:3399–3406

Xing A, Zhang Z, Sato S, Staswick P, Clemente T (2000) The use of the two T-DNA binary system to derive marker-free transgenic soybeans. In Vitro Cell. Dev Biol – Plant 36:456–463

Zhang W, McElroy D, Wu R (1991) Analysis of rice *Act1* 5′ region activity in transgenic rice plants. Plant Cell 3:1155–1165

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

Zuo J, Niu QW, Moller SG, Chua NH (2001) Chemical-regulated, site-specific DNA excision in transgenic plants. Nat Biotechnol 19:157–161