*Proc. Natl. Acad. Sci. USA*
Vol. 88, pp. 10558–10562, December 1991
Applied Biological Sciences

# Gene transfer with subsequent removal of the selection gene from the host genome

(transgenic plant/transformation/antibiotic resistance/Cre/*lox* recombination/luciferase)

EMILY C. DALE AND DAVID W. OW*

Plant Gene Expression Center, U.S. Department of Agriculture/Agricultural Research Service, 800 Buchanan Street, Albany, CA 94710; and Department of Plant Pathology, University of California, Berkeley, CA 94720

*Communicated by W. James Peacock, September 4, 1991*

**ABSTRACT** **A general method of gene transfer that does not leave behind a selectable marker in the host genome is described. A luciferase gene was introduced into the tobacco genome by using the hygromycin phosphotransferase gene (*hpt*) as a linked selectable marker. Flanked by recombination sites from the bacteriophage P1 Cre/*lox* recombination system, the *hpt* gene was subsequently excised from the plant genome by the Cre recombinase. The Cre-catalyzed excision event in the plant genome was precise and conservative—i.e., without loss or alteration of nucleotides in the recombinant site. After removal of the Cre-encoding locus by genetic segregation, plants were obtained that had incorporated only the desired transgene. Gene transfer without the incorporation of antibiotic-resistance markers in the host genome should ease public concerns over the field release of transgenic organisms expressing such traits. Moreover, it would obviate the need for different selectable markers in subsequent rounds of gene transfer into the same host.**

Recent advances in the genetic engineering of plants have led to the production of a large variety of crop plants with agriculturally desirable traits (reviewed in refs. 1–3). Notable examples are engineered plants that exhibit enhanced tolerance to plant pathogens. Protection from insect pests was achieved through the engineered synthesis in transgenic plants of either an insect-specific toxin from *Bacillus thuringiensis* (4–7) or a trypsin inhibitor from cowpea (8). The discovery of enhanced viral resistance as a result of expression of a viral coat protein gene (9) has made possible the engineering of resistance to a wide variety of viral agents (10–19). In addition to coat protein genes, protection against plant viruses was also obtained with the engineered synthesis of satellite RNA (20, 21) and antisense RNA (12, 13). More recently, high-level resistance to tobacco mosaic virus was reported due to the transgenic expression of a nonstructural viral product (22).

Despite the scientific advances made in crop improvement, the commercialization of genetically engineered plants has been slowed by public concerns on the issue of the environmental safety of genetically engineered organisms. One such concern is with the genes that code for antibiotic resistance. Cotransformed into the host genome as selectable markers, they serve a necessary role in the detection of genetic transformants. Hence, agriculturally desirable characteristics are invariably cointroduced with resistance to antibiotics or herbicides. The products of these selection genes are not necessarily harmful, but their presence in transgenic plants does increase the environmental uncertainty of their distribution. This uncertainty factor can have a highly negative effect on the progress of plant improvement. At a minimum, reservations expressed at various levels of intensity have political effects that retard the progress of obtaining improved plant species.

In this communication, we describe a strategy for engineering plants free of selectable markers. A representative antibiotic-resistance gene, flanked by recombination sites and used for selection during transformation, is shown to be removed subsequently from the plant genome by enzyme-mediated site-specific recombination. Of the many site-specific recombination systems that have been characterized, several are simple systems requiring only a single-polypeptide enzyme and short specific DNA sequences as substrates for recombination (reviewed in ref. 23). Among these, we chose the bacteriophage P1 Cre/*lox* recombination system, which consists of the 38-kDa product of the *cre* gene (the Cre recombinase) and the asymmetric 34-base-pair (bp) *lox* sites (reviewed in ref. 24). No additional factors are required for Cre-catalyzed recombination between *lox* sites, and this system has recently been reported to be functional in plant cells (25, 26).

## MATERIALS AND METHODS

**Enzymes and Chemicals.** Restriction enzymes were purchased from New England Biolabs and Fisher. Kanamycin sulfate was purchased from Sigma, hygromycin sulfate from Calbiochem, and luciferin from Analytical Luminescence (San Diego). Oligonucleotide synthesis was performed with an Applied Biosystems 380B DNA synthesizer as suggested by the manufacturer.

**Plasmid Constructions.** Plasmid pED23 (25), containing a 35S promoter–*cre*–*nos*3′ fusion in pUC19 (27) and the *Agrobacterium* gene-transfer vector pBIN19 (28) have been described. Plasmid pED53 was produced by the deletion of a 1.3-kilobase (kb) *Pst* I fragment of pBIN19. The deletion removes part of the T-DNA of pBIN19 including the neomycin phosphotransferase (*npt*II) coding region [also referred to as the kanamycin-resistance gene (*kan*)] along with the *Hin*dIII and *Sph* I sites. Plasmid pED37 was constructed by the addition of a 2.0-kb *Hin*dIII fragment carrying the 35S promoter–*hpt*–*nos*3′ chimeric gene (from M. Fromm, Monsanto; *hpt* is described in ref. 29) into the *Hin*dIII site of pED26 (25). The cointegrate plasmid pED53::pED37 was formed by linearizing pED37 at an *Xho* I site adjacent to one of the *lox* sites and inserting it into the *Sal* I site of pED53 in the orientation shown in Fig. 1. The cointegrate plasmid pBIN19::pED23 was formed by ligating the *Hin*dIII-linearized pED23 into the corresponding site in pBIN19 in the orientation such that transcription of the *cre* gene is directed toward the *Agrobacterium* T-DNA left border. Selection for cointegrate plasmids, which confer on bacteria resistance to both kanamycin ($Km^R$) and ampicillin ($Ap^R$), is facilitated by

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

*To whom reprint requests should be addressed at: Plant Gene Expression Center, U.S. Department of Agriculture/Agricultural Research Service, 800 Buchanan Street, Albany, CA 94710.

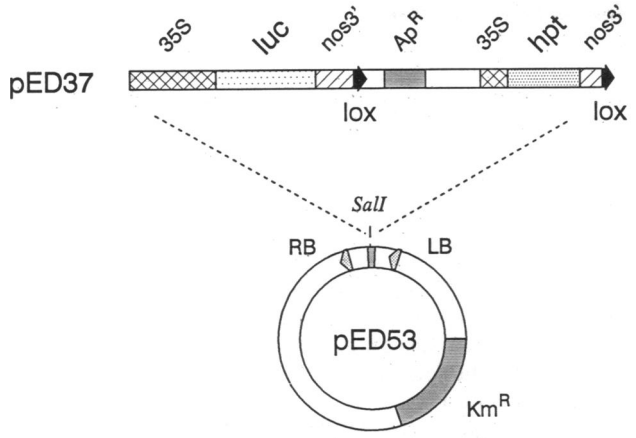


FIG. 1. Schematic representation of cointegrate plasmid pED53::pED37. Plasmid pED37, cleaved at its single *Xho* I site, which separates the chimeric *luc* gene (35S promoter–*luc*–*nos*3′) and one of the *lox* sites (black arrows), was inserted into the *Sal* I site between the T-DNA right and left borders (RB and LB) carried on pED53. In this configuration the antibiotic-resistance genes that are transferred to the plant genome, encoding resistance to ampicillin in bacteria ($Ap^R$) and to hygromycin in plant cells (35S–*hpt*–*nos*3′), are flanked by *lox* sites that have the same orientation.

transformation into a DNA polymerase I-deficient host, *Escherichia coli* JZ294 (*argH, strA, polA*::Tn*10*), which permits replication of the wide host-range replicons of pED53 and pBIN19 but not the ColE1 replicons of pED37 and pED23.

**Transgenic Plants and Phenotype Analysis.** The cointegrate plasmids pED53::pED37 and pBIN19::pED23 were mobilized into *Agrobacterium tumefaciens* GV3111(pTiB6S3SE) for infection of *Nicotiana tabacum* (Wisconsin-38 cultivar) leaf explants as described (30). $Hyg^R$ and $Kan^R$ plants were scored for the ability of leaf explants to form shoots on shoot-inducing Murashige–Skoog medium containing hygromycin sulfate (20 μg/ml) or kanamycin sulfate (100 μg/ml). $Luc^+$ plants were assayed for luciferase activity as described (31).

**Molecular Analysis of Plant DNA.** Plant DNA was prepared by grinding leaf tissues in liquid $N_2$, extracting with 100 mM Tris, pH 7.6/1% SDS/50 mM EDTA/500 mM NaCl/10 mM 2-mercaptoethanol at 65°C for 10 min, adding potassium acetate to 1.3 M, chilling to 0°C, and removing the debris by centrifugation. The DNA was then precipitated with 2-propanol, washed with 70% ethanol, and resuspended in 10 mM Tris, pH 8.0/1 mM EDTA. Polymerase chain reactions (PCRs) were carried out under standard conditions (32) with denaturation, annealing, and extension at 94°C, 55°C, and 72°C, respectively, for 1 min each during 30 cycles. Reaction products were resolved by electrophoresis in a 1.5% agarose gel. The sequences of the PCR primers in Fig. 2 are as follows: A, 5′-GAGCTCGGTACCCGGGGATC-3′; B, 5′-GAGTGCACCATATGCGGTGT-3′; C, 5′-GACGCCCCAGCACTCGTCCG-3′; D, 5′-GGTACCCGGGATCCTCTAG-3′; E, 5′-GTTCATTTCATTTGGAGAGG-3′; F, 5′-CAGTGATACACATGGGGATC-3′. The two primers for detection of the *cre* gene (5′-ATGTCCAATTTACTGACCGT-3′ and 5′-CTAATCGCCATCTTCCAGCA-3′) represent the N- and C-terminal *cre* coding sequence and the expected PCR product size is 1.0 kb. To determine the sequence of the *lox* sites, fragments from PCR (as shown in Fig. 2*C*) were purified, digested with appropriate restriction enzymes (A+B, *Bam*HI/*Nde* I; C+D, *Pst* I/*Bam*HI; A+D, *Cla* I/*Bam*HI) and ligated into either pUC19 (for A+B and C+D) or pBR322 (33) (for A+D). The nucleotide sequences of the regions surrounding the *lox* sites derived from PCR from plants ntED5337 and ntED5337-23 were determined by the dideoxy method (34) as modified by United States Biochemical (Sequenase kit).

## RESULTS AND DISCUSSION

**Demonstration of a General Strategy.** A chimeric firefly luciferase (*luc*) gene was chosen as a representative gene to engineer into tobacco. As with a conventional gene-transfer experiment, the chimeric *luc* gene (31) was inserted into an *Agrobacterium* gene-transfer vector that contains a selectable marker as a cotransferred gene—in this case, a chimeric *hpt* gene encoding hygromycin phosphotransferase (29). An important and unique feature of this construct is that two *lox* sites of the same orientation flank the selectable marker. Recombination between the two sites would result in excision of the intervening *hpt* selectable marker.

Fig. 1 shows the configuration of the constructs used. The wide-host-range shuttle plasmid pED53 contains the *Agrobacterium* T-DNA right (RB) and left (LB) borders, where DNA transfer initiates and terminates, respectively. A selectable marker for plant cell transformation is not included between the RB and LB sequences, permitting the option of inserting different selection-conferring genes. The primary construct pED37 contains the *hpt* selectable marker and the *luc* gene separated by 34-bp *lox* sequences of the same orientation. Both the *hpt* and *luc* coding sequences are expressed under the control of the cauliflower mosaic virus 35S promoter (35S) and nopaline synthase gene polyadenylylation signals (*nos*3′). At an appropriate restriction site, pED37 was linearized and inserted into pED53 to form the cointegrate plasmid pED53::pED37. Upon mobilizing the cointegrate into a nontumorigenic ("disarmed") strain of *Agrobacterium*, transfer of the DNA between the RB and the LB of pED53::pED37 into the tobacco genome was carried out by *Agrobacterium* infection of leaf explants (30). Transformed shoots selected as resistant to hygromycin ($Hyg^R$) all tested positive for luciferase activity ($Luc^+$), indicating stable incorporation of the *luc* gene.

**Expression of *cre* Leads to a Hygromycin-Sensitive ($Hyg^S$) Phenotype.** To excise the chimeric *hpt* gene from the genome of the $Luc^+$ $Hyg^R$ transformants (plants ntED5337), a chimeric *cre* gene expressed from the 35S promoter and *nos*3′ terminator was introduced by a second round of *Agrobacterium*-mediated transformation. As ntED5337 plants are $Hyg^R$, the plasmid pED23 (25) with the chimeric *cre* construct was ligated into pBIN19, a shuttle vector carrying a chimeric *npt*II gene that confers kanamycin resistance ($Kan^R$) on plant cells (28). From this secondary transformation of ntED5337 leaf explants, $Kan^R$ shoots were reexamined for the $Hyg^R$ phenotype. Consistent with Cre-mediated excision of the *hpt* marker, 10 of the 11 $Kan^R$ plants (ntED5337-23) were found to be $Hyg^S$. The $Hyg^R$ plant was not examined further. Introduction of the chimeric *cre* gene into ntED5337 was also achieved via pollination by ntED23, a plant transformed with the pBIN19::pED23 cointegrate plasmid. Of 316 progeny examined, 78 were $Luc^+$ $Kan^R$, and among these, 42 were $Hyg^S$, indicating that the efficiency of Cre-catalyzed excision was ≈50% when determined by scoring for antibiotic resistance in the $F_1$ seedlings.

**Conservative Site-Specific Recombination in the Plant Genome.** Molecular data from five of the $Luc^+$ $Hyg^S$ $Kan^R$ plants derived from secondary transformation with pBIN19::pED23 confirmed site-specific recombination at the *lox* sites. Fig. 2*C* shows the PCR analysis of the genomes of a parental ntED5337 and a derivative ntED5337-23 plant. Corresponding to the structures shown in Fig. 2 *A* and *B*, a fragment of the predicted size of 1.1 kb or 0.71 kb was produced from the ntED5337 genome, but not from ntED5337-23 DNA, using primers A and B or C and D, respectively (lanes 1–4). In contrast, primers A and D pro-

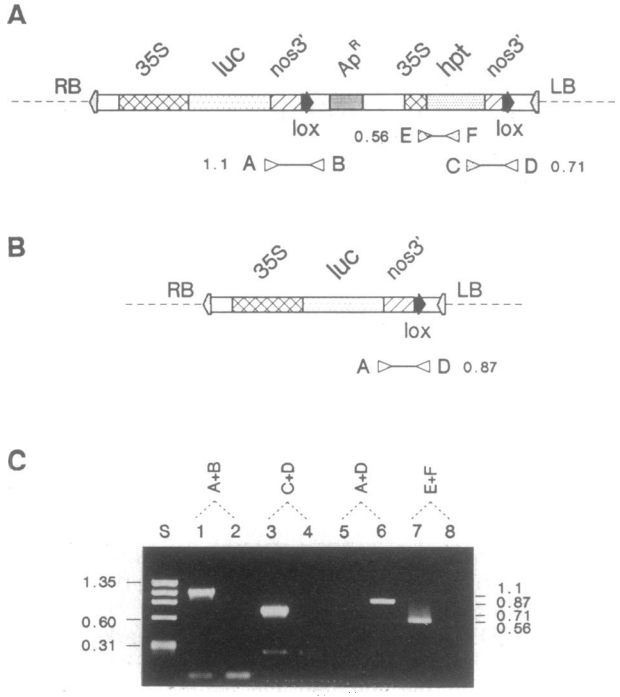


FIG. 2. (*A* and *B*) Diagrams of the genomic copies of the pED37 construct carried by ntED5337 plants (*A*) and the predicted excision product of ntED5337-23 plants (*B*). Indicated by small triangles below each map are the 20-bp primers used for PCR analysis of DNA prepared from these plants. The expected PCR product for each set of primers is indicated by a line, and the expected size (kb) of the product is also shown. (*C*) Results of the PCR analysis. The primers used are indicated for each pair of reactions containing DNA from either ntED5337 (lanes 1, 3, 5, and 7) or from ntED5337-23 (lanes 2, 4, 6, and 8). Lane S, size markers obtained from *Hae* III digestion of $\phi$X174 DNA.

duced a 0.87-kb band from ntED5337-23 DNA, but not from ntED5337 DNA (lanes 5 and 6). This band corresponds to the fragment expected from the joining of the chimeric *luc* gene with sequence adjacent to the LB. To examine whether an excised copy of the *hpt* gene might have translocated elsewhere in the genome, primers internal to the flanking *lox* sites were used. Primers E and F indeed produced a fragment corresponding in size to the predicted 0.56-kb band with ntED5337 DNA, but no such fragment was observed with ntED5337-23 DNA (lanes 7 and 8), indicating that the excised *hpt* gene is not present elsewhere in the genome. In addition to the *hpt* gene, the excision event also removes the silent $Ap^R$ marker from the plant genome. Of the five plants examined, the PCR profiles also showed no indication that they harbor both excised and intact copies of the locus, which suggests that the excision events must have occurred early between introduction of the *cre*-expressing construct and plant organogenesis.

Evidence for a precise recombination event was obtained by determining the nucleotide sequence surrounding the *lox* sites of both the parental and the product (ntED5337-23) genomes. Plant ntED5337-derived fragments produced from PCR amplification by primers A and B and primers C and D, as well as the ntED5337-23 derived fragment from primers A and D, were cloned into plasmid vectors. Fig. 3 shows the experimentally obtained sequences of the three different *lox*-containing regions. The *lox* regions derived from primers A and B and primers C and D share 80 bp of identity that includes the 34-kb *lox* sequence, but the sequences diverge outside of this segment. Each parental *lox* sequence is adjacent to one characteristic restriction site, either *Sal* I or *Bam*HI, but on opposite sides of the *lox* sequence. The *lox* sequence of the PCR product derived from primers A and D from ntED5337-23 DNA, however, is flanked both by restriction sites and by sequences found in opposing sides of each of the parental ntED5337 *lox* sites (Fig. 3). As in bacteria (35) the recombination event within plant chromatin was conservative—i.e., without loss or alteration of the *lox* sequence or its flanking DNA. To our knowledge, this is the first description at the nucleotide sequence level of Cre/*lox*-catalyzed recombination in eukaryotic chromosomal DNA.

**Segregation of the *cre–nptII* Locus.** Two $Luc^+$ $Hyg^S$ $Kan^R$ ntED5337-23 plants were self-pollinated to allow segregation

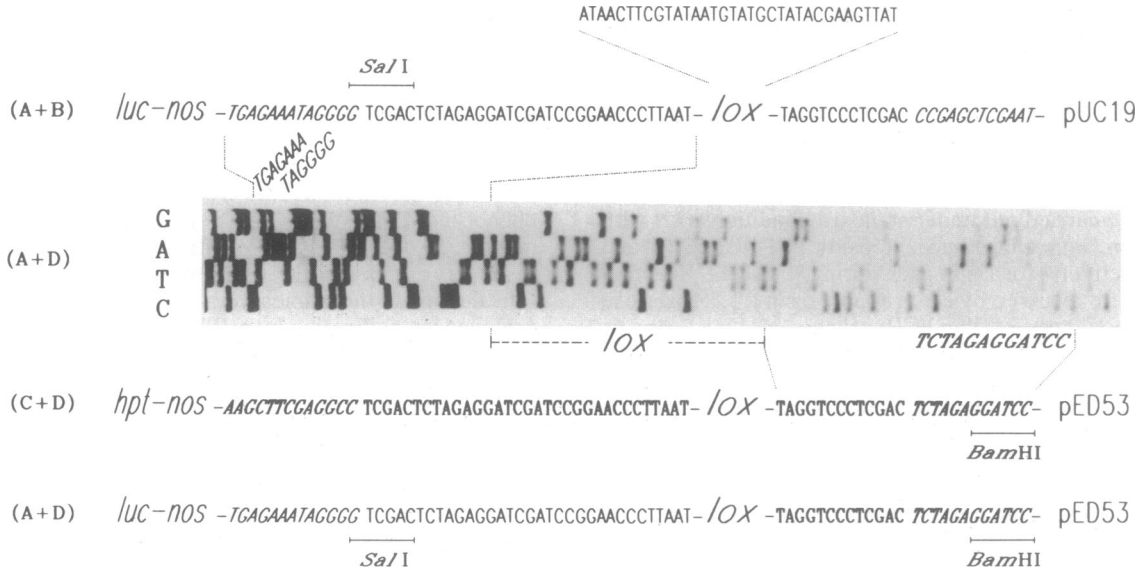


FIG. 3. Experimentally determined sequence of the *lox* sites carried by ntED5337 and ntED5337-23 plants. The sequences shown as A+B and C+D are derived from the PCR products of Fig. 2, lanes 1 and 3, respectively. The structure of the recombinant *lox* site present on ntED5337-23 plant DNA, shown both as a linear sequence and as an autoradiograph, was determined from the PCR fragment derived from primer set A+D (Fig. 2, lane 6). As indicated by the dotted lines, the A+D sequence is composed of the left arm of the A+B *lox* site and the right arm of the C+D *lox* site. Sequences unique (italic) to the A+B site (TGAGAAATAGGGG) and the C+D site (TCTAGAGGATCC) are shown corresponding to the autoradiograph. Also indicated are upstream (*luc–nos* or *hpt–nos*) and downstream DNAs (pUC19 or pED53) flanking the *lox* regions.

of the *luc* gene from the *cre* locus, which also harbored the linked *npt*II selectable marker. Approximately 100 $R_1$ germinated seedlings from each self-pollinated plant were scored for luciferase activity. In both cases, about three-fourths of the total progeny (72 of 104; 95 of 130) were $Luc^+$, and among these, approximately one-fourth were expected to be sensitive to kanamycin ($Kan^S$). This was the case with the progeny from one plant (17 of 72). From the second plant, however, only 1 out of approximately every 16 $Luc^+$ progeny (7 of 95) was $Kan^S$. This indicated either genetic linkage between the *cre–npt*II and *luc* loci or that there were two unlinked copies of the *cre–npt*II construct. It is conceivable that more than a single gene-transfer event occurred during the second round of *Agrobacterium* infection. These possibilities were not further investigated. PCR analysis of DNA prepared from $Luc^+$ seedlings, using primers internal to the *cre* gene sequence, also showed independent segregation of the *cre–npt*II locus at frequencies similar to those obtained in the screen for the $Kan^S$ phenotype (Fig. 4). The loss of the *cre–npt*II locus, as determined by the PCR analysis, was confirmed phenotypically by an inability of leaf explants to form shoots in the presence of kanamycin. A more extensive PCR analysis of the *luc* locus (as described above) of three representative $Luc^+$ $Hyg^S$ $Kan^S$ $R_1$ plants showed that they have the same profile of fragments as described for the $R_0$ ntED5337-23 plants (data not shown). Hence, in two generations ($R_0$ and $R_1$), we have shown the feasibility of transferring a gene into the plant genome without incorporating a selectable marker.

**Conclusions and Prospects.** We have described a method for gene transfer with the subsequent removal of transformation markers from the plant genome. Cre/*lox*-mediated excision of DNA from the yeast and mammalian genomes has also been reported (36, 37). Hence, the strategy presented here should be applicable to these and other systems as well. An obvious advantage in removing the marker gene used to select transformants is that those cells can be retransformed with additional traits in a stepwise process using the same resistance gene. This can reduce the restriction imposed on experimental designs by the limited availability of selectable markers.

This work, conducted in plants, should prove valuable because a large number of transgenic crop plants with agriculturally desirable traits will likely become consumer products in the coming decade. Regulatory agencies and commercial interests are concerned about the environmental impact and public perception of widespread release of organisms expressing genes that confer resistance to antibiotics or herbicides. These concerns can be alleviated by employing the presented strategy to remove selection markers from the host genome. While deletion of the resistance gene removes one potential problem, other issues concerning the environmental safety of transgenic materials must still be addressed. For example, with the recent success in the genetic engineering of male sterility (38), a reduction in the probability of sexual transmission of transgenic materials could be combined with the removal of selection markers. Such efforts should expedite consumer acceptance of genetically modified crop plants.

The approach described in this work should also be relevant to the engineering of animals and human cell lines. In the case of cell lines, where loss of the *cre* locus cannot be achieved by sexual segregation, transient expression of the *cre* gene or direct introduction of purified Cre protein could be used. With the anticipation of transgenic cell lines being employed for the clinical treatment of genetic defects (39), the ability to remove or inactivate antibiotic-resistance genes from those cells will be an option well worth considering.

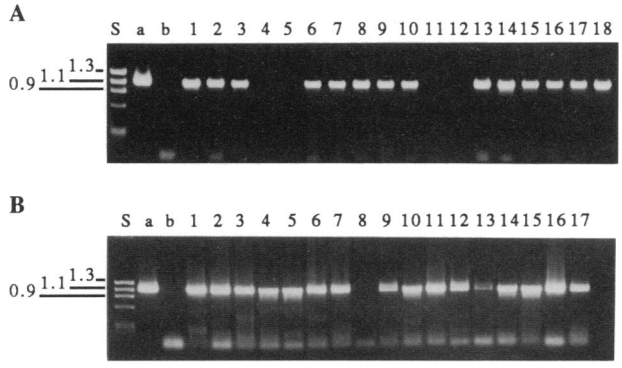


FIG. 4. PCR analysis of $Luc^+$ progeny from two self-pollinated ntED5337-23 plants. Segregation of $Kan^S$ progeny was approximately 1 in 4 (*A*) or 1 in 16 (*B*) of the seedlings tested. Shown are the reaction products obtained using *cre*-specific primers and DNA prepared from ntED5337-23 (lane a), ntED5337 (lane b), and individual $Luc^+$ progeny seedlings from each self-pollinated nt5337-23 plant (lanes 1–18 and 1–17). The expected 1.0-kb PCR product is not detected in lanes 4, 5, 11, and 12 of *A* and lane 8 of *B*. Relevant sizes (kb) of $\phi$X174 *Hae* III fragments are shown in lane S. Leaves from each $Luc^+$ seedling were also placed on shoot-inducing medium containing kanamycin to verify that an absence of the *cre*-specific PCR product corresponded to a $Kan^S$ phenotype (data not shown).

We thank Agnes Soriano for assistance in the propagation of plant materials. This work was funded by the Agricultural Research Service, U.S. Department of Agriculture (D.W.O.), and by a National Science Foundation Postdoctoral Fellowship in Plant Biology (E.C.D.).

1. Goodman, R. M., Hauptli, H., Crossway, A. & Knauf, V. C. (1987) *Science* **236,** 48–54.
2. Weising, K., Schell, J. & Kahl, G. (1988) *Annu. Rev. Genet.* **22,** 421–477.
3. Gasser, C. S. & Fraley, R. T. (1989) *Science* **244,** 1293–1299.
4. Vaeck, M., Reynaerts, A., Hofte, H., Jansens, S., De Beuckleleer, M., Dean, C., Zabeau, M., Van Montagu, M. & Leemans, J. (1987) *Nature (London)* **328,** 33–37.
5. Fischoff, D. A., Bowdish, K. S., Perlak, F. J., Marrone, P. G., McCormick, S. M., Niedermeyer, J. G., Dean, D. A., Kusano-Kretzmer, D., Mayer, E. J., Rochester, D. E., Rogers, S. G. & Fraley, R. T. (1987) *Bio/Technology* **5,** 807–812.
6. Delannay, X., LaVallee, B. J., Proksch, R. K., Fuchs, R. L., Sims, S. R., Greenplate, J. T., Marrone, P. G., Dodson, R. B., Augustine, J. J., Layton, J. G. & Fischhoff, D. A. (1989) *Bio/Technology* **7,** 1265–1269.
7. Perlak, F. J., Deaton, R. W., Armstrong, T. A., Fuchs, R. L., Sims, S. R., Greenplate, J. T. & Fischhoff, D. A. (1990) *Bio/Technology* **8,** 939–943.
8. Hilder, V. A., Gatehouse, A. M. R., Sheerman, S. E., Barker, R. F. & Boulter, D. (1987) *Nature (London)* **330,** 160–163.
9. Powell Abel, P., Nelson, R. S., Barun, D., Hoffmann, N., Rogers, S. G., Fraley, R. T. & Beachy, R. N. (1986) *Science* **232,** 738–743.
10. Tumer, N. E., O'Connel, K. M., Nelson, R. S., Sander, P. R., Beachy, R. N., Fraley, R. T. & Shah, D. M. (1987) *EMBO J.* **6,** 1181–1188.
11. Loesch-Fries, L. S., Merlo, D., Zinnen, T., Burhop, L., Hill, K., Krahn, K., Jarvis, N., Nelson, S. & Halk, E. (1987) *EMBO J.* **6,** 1845–1851.
12. Hemenway, C., Fang, R. X., Kaniewski, W., Chua, N.-H. & Tumer, N. E. (1988) *EMBO J.* **7,** 1273–1280.
13. Cuozzo, M., O'Connell, K. M., Kaniewski, W., Fang, R.-X., Chua, N.-H. & Tumer, N. E. (1988) *Bio/Technology* **6,** 549–557.
14. Van Dun, C. M. P. & Bol, J. F. (1988) *Virology* **167,** 649–652.
15. Hoekema, A., Huisman, M. J., Molendijk, L., van den Elzen, P. J. M. & Cornelissen, B. J. C. (1989) *Bio/Technology* **7,** 273–277.
16. Stark, D. M. & Beachy, R. N. (1989) *Bio/Technology* **7,** 1257–1262.
17. Lawson, C., Kaniewski, W., Haley, L., Rozman, R., Newell,

C., Sanders, P. & Tumer, N. E. (1990) *Bio/Technology* **8,** 127–134.
18. Kaniewski, W., Lawson, C., Sammons, B., Haley, L., Hart, J., Delannay, X. & Tumer, N. E. (1990) *Bio/Technology* **8,** 750–754.
19. Angenent, G. C., van den Ouweland, J. M. W. & Bol, J. F. (1990) *Virology* **175,** 191–198.
20. Harrison, B. D., Mayo, M. A. & Baulcombe, D. C. (1987) *Nature (London)* **328,** 799–802.
21. Gerlach, W., Llewellyn, D. & Haseloff, J. (1987) *Nature (London)* **328,** 802–805.
22. Golemboski, D. B., Lomonossoff, G. P. & Zaitlin, M. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 6311–6315.
23. Craig, N. (1988) *Annu. Rev. Genet.* **22,** 77–105.
24. Hoess, R. H. & Abremski, K. (1990) in *Nucleic Acids and Molecular Biology*, eds. Eckstein, F. & Lilley, D. M. J. (Springer, Berlin), Vol. 4, pp. 99–109.
25. Dale, E. C. & Ow, D. W. (1990) *Gene* **91,** 79–85.
26. Odell, J. T., Caimi, P. G., Sauer, B. & Russell, S. H. (1990) *Mol. Gen. Genet.* **223,** 369–378.
27. Yanisch-Perron, C., Vieira, J. & Messing, J. (1985) *Gene* **33,** 103–119.
28. Bevan, M. (1984) *Nucleic Acids Res.* **12,** 8711–8721.
29. Kaster, K. R., Burgett, S. G., Rao, R. N. & Ingolia, T. D. (1983) *Nucleic Acids Res.* **11,** 6895–6911.
30. Horsch, R. B., Fry, J. E., Hoffmann, N. L., Eichholtz, D., Rogers, S. G. & Fraley, R. T. (1985) *Science* **227,** 229–1231.
31. Ow, D. W., Wood, K. V., DeLuca, M., de Wet, J. R., Helinski, D. R. & Howell, S. H. (1986) *Science* **234,** 856–859.
32. Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B. & Erlich, H. A. (1988) *Science* **239,** 487–491.
33. Bolivar, F., Rodrigues, R. L., Greene, P. J., Betlach, M. C., Heyneker, H. L., Boyer, H. W., Crosa, J. H. & Falkow, S. (1977) *Gene* **2,** 95–113.
34. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 5463–5467.
35. Hoess, R. H., Ziese, M. & Sternberg, N. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 3398–3402.
36. Sauer, B. (1987) *Mol. Cell. Biol.* **7,** 2087–2096.
37. Sauer, B. & Henderson, N. (1989) *Nucleic Acids Res.* **17,** 147–161.
38. Mariani, C., De Beuckeleer, M., Truettner, J., Leemans, J. & Goldberg, R. B. (1990) *Nature (London)* **347,** 737–741.
39. Weatherall, D. J. (1991) *Nature (London)* **349,** 275–276.