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Exchange of gene activity in transgenic plants catalyzed by the Cre-*lox* site-specific recombination system

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Abstract

The Cre-*lox* site-specific recombination system of bacteriophage P1 was used to excise a firefly luciferase (*luc*) gene which had previously been incorporated into the tobacco genome. The excision event was due to site-specific DNA recombination between two *lox* sequences flanking the *luc* gene and was catalyzed by the Cre recombinase introduced by cross-fertilization. Recombination resulted in the fusion of a promoter with a distally located hygromycin phosphotransferase (*hpt*) coding sequence and the excision event was monitored as a phenotypic change from expression of *luc* to expression of *hpt*. The efficiency of recombination was estimated from the exchange of gene activity and confirmed by molecular analysis. The relevance to potential applications of site-specific deletion-fusion events for chromosome engineering are discussed.

Introduction

There are many well characterized procaryotic and lower-eucaryotic site-specific recombination systems which offer immense potential for specific rearrangement of DNA in higher organisms. In a situation where recombination sites flank a segment of the chromosome, the intervening DNA is deleted or inverted depending on whether the two flanking sites are in direct or opposing orientations, respectively. If two sites are placed in different chromosomes, recombination would be expected to generate reciprocal translocation events. Of the many conservative recombination systems [for review, see 4], two have been demonstrated to function in higher eucaryotic cells: the Cre-*lox* system from bacteriophage P1 and the FLP system from the yeast 2 μm plasmid. Both systems use a single-polypeptide recombinase and 34 bp target sequences [1, 2, 8, 18, 19]. In the case of the Cre recombinase, product of the bacteriophage P1 *cre* (*c*ontrol of *re*combination) gene, recombination between specific *lox* (*lo*cus *o*f *x*-over) sites has been reported for mammalian [16, 17] and plant cells [5, 12]. Likewise, the FLP recombinase, product of the *flp* (*flip*) gene, has been shown to catalyze recombination of FLP recognition targets (termed FRT) placed into the *Drosophila* and mammalian genomes [7, 13].

A long-term goal of this laboratory is to use site-specific recombination to generate chromosomal deletion, inversion and reciprocal translocation events. Plant libraries of chromosome deletions or translocations could potentially be

useful for genetic analyses and genome mapping, especially since the cross-over points of recombination are physically tagged. Previously, we [5] and Odell *et al.* [12] reported that the bacteriophage P1 Cre-*lox* system can catalyze site-specific recombination in plant cells. Since the effectiveness of Cre-*lox* recombination in the genome bears upon our efforts to engineer chromosomes, we decided to assess the efficiency of obtaining plants that have undergone recombination between two genomic *lox* sites. Since events that occur late during plant development would produce a chimeric phenotype that is less desirable and may not be readily detectable in genetic screening protocols, we sought to monitor for early recombination events that are less likely to produce chimeric plants.

In this report, we examined a Cre-*lox* site-specific deletion event in tobacco plants that results in the concomitant removal of one gene cassette and activation of another. By scoring for either the loss of one gene function or the gain of the other, we estimated that approximately half of the seedlings which received both the *cre* gene and the *lox*-containing locus exhibited the phenotype expected for an early genomic recombination event.

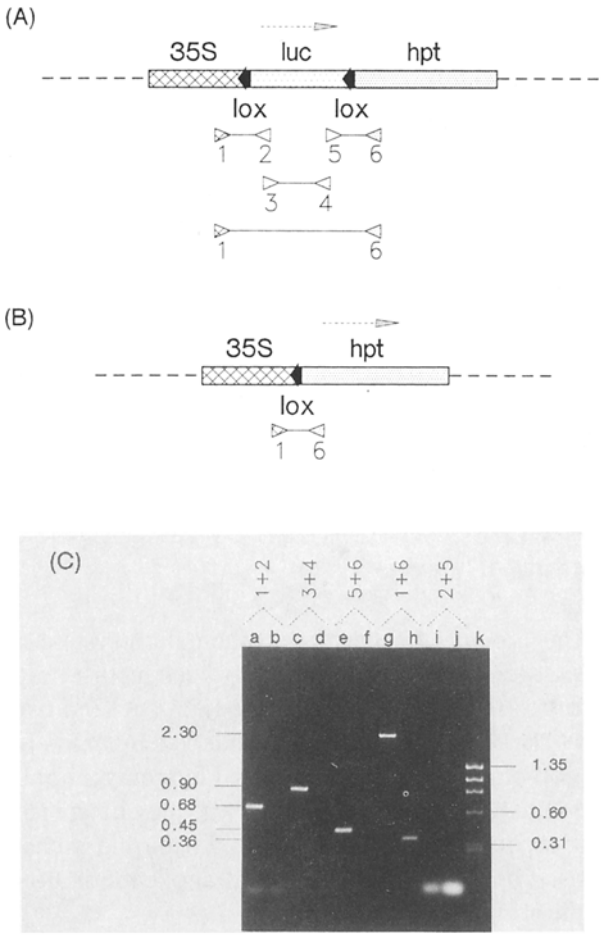

Fig. 1. Schematic representations of the 35S-*luc-hpt* construct transferred from pCB5 to plant ntCB7 (A) and the 35S-*hpt* excision product in ntCB7 × ntED23.1 progeny (B). Triangles below each map indicate the primers used and the expected PCR product for each primer set is shown as a line. PCR results are presented in (C). Primers used are indicated for each pair of reactions containing DNA from either ntCB7.1, lanes a, c, e, g, i, or from a representative ntCB7.1 × ntED23.1 progeny, lanes b, d, f, h, j. Shown in the margins are the sizes (in kb) of the PCR products (left) and the *Hae* III cleaved ϕX174 DNA (lane k) as size standards (right).

Materials and methods

Recombinant DNA

Standard techniques were used throughout [11]. pED23 contains a cauliflower mosaic virus (CaMV) 35S promoter, abbreviated as 35S, fused to the *cre*-coding region and has been described previously [5]. pCB5 contains a 35S-*lox-luc-lox-hpt* fusion as depicted (Fig. 1) and was constructed from the following steps. A 1635 bp *Bam* HI/*Hin*d III fragment, containing the bacterial hygromycin phosphotransferase (*hpt*) coding region linked to a plant polyadenylation site from the *Agrobacterium* nopaline synthase gene, was transferred from pCaMVhygΔ (gift from M. Fromm, unpublished) into the corresponding sites of pUC19 to yield pCB3. Next, a 1940 bp *Kpn* I fragment from pED32 [5] that contains a *luc* cDNA (with its poly(A) signal) [6, 14] preceded by a *lox* site was inserted into pCB3 between the *Kpn* I and *Bam* HI sites upstream of the *hpt* gene. The insertion event was mediated by the use of synthetic *Kpn* I to *Bam* HI adapter which also codes for a second *lox* sequence (5′-

GATCCATATAACTTCGTATAATGTATG-CTATACGAAGTTATTAGGTAC-3′/5′-CTA-ATAACTTCGTATAGCATACATTATACG-AAGTTATATG-3′). The resulting plasmid, pCB4, with a *lox-luc-lox-hpt* configuration has both *lox* sites oriented in the same direction. The *Hin*d III site of pCB4, located at the 3′ border of the *hpt* fragment, was then converted to a *Kpn* I site by blunt end ligation to a synthetic linker (5′-GGGTACCC-3′) to yield plasmid pCB6. The *lox-luc-lox-hpt* fusion was purified as contiguous fragment upon partial cleavage by *Kpn* I. The 1585 bp 35S promoter fragment from pDO432 [14] was transferred as a *Hin*d III to *Bam* HI fragment into pUC19 to produce plasmid pCB1 which can then be linearized at a *Kpn* I site downstream of the 35S promoter. The final product, pCB5, was assembled by combining the *Kpn* I linearized plasmid backbone of pCB1 with the 3623 bp *lox-luc-lox-hpt* fragment of pCB6 obtained by partial *Kpn* I cleavage. pCB5 places transcription of *luc* under the 35S promoter, but promoter fusion with the distal *hpt* gene would occur only upon excision of the intervening *luc* gene.

Transgenic plants

To transfer pUC19-derived constructs into the plant genome, pCB5 or pED23 was linearized at a *Hin*d III site upstream of the 35S promoter and inserted into the corresponding site in the binary vector pBIN19 [3]. The pBIN19::pCB5 (also known as pCB7) or pBIN::pED23 co-integrate plasmids were introduced into the 'disarmed' *A. tumefaciens* strain GV3111 harboring helper Ti plasmid pTiB6S3S3. *Agrobacterium* infection-mediated gene transfer into tobacco was essentially as described [9], using *Nicotiana tabacum* cv. Wi38. Transformed shoots were selected using kanamycin (100 μg/ml), and *Agrobacterium* was removed with cefotaxime (500 μg/ml). *N. tabacum* plants harboring the 35S-*lox-luc-lox-hpt* construct from pCB5 are designated ntCB7. Plants harboring the 35S-*cre* construct from pED23 are designated ntED23.

Cross-pollination

Individual transformants were grown in the greenhouse to maturity. Unopened flowers ca. 4 cm in length were sliced open lengthwise and emasculated with forceps. Mature pollen from the donor plant was transferred by brushing anthers onto the recipient stigmas of emasculated plants. Pollinated flowers were labelled and sealed with tape to prevent further uncontrolled cross-pollination. Self-pollination was achieved by sealing intact flowers with tape prior to opening.

Screen for hygromycin resistance

Seeds were harvested one month after pollination, surface-sterilized with 1.2% sodium hypochlorite/0.1% SDS, rinsed thoroughly with sterile H_2O and sown on MS medium (Gibco) with 3% sucrose and 20 μg/ml hygromycin B. Three weeks later, healthy green seedlings were scored as a fraction of germinating seedlings obtained.

Assay for luciferase activity

Two randomly chosen leaves about 1 cm in length were removed from each 3-week-old seedling, sliced into smaller pieces and placed into an assay tube. Using a disposable plastic pestle, the leaf pieces were gently macerated and mixed with 100 μl of buffer containing 50 mM HEPES buffer pH 7.8, 20 mM $MgCl_2$, 10 mM ATP and 0.5 mg/ml BSA. The amount of light emitted after adding 100 μl of a 0.5 mM luciferin solution was measured using a luminometer (Monolight 2001, Analytical Luminescence Lab) set to integrate for 10 s.

Polymerase chain reaction analysis

Template DNA for each PCR reaction was derived from an entire plant, a vegetative clone of the line, that was aseptically grown to about 8 cm in height, with 4 to 5 leaves. Plant tissues were

ground in liquid N_2, extracted with 100 mM Tris, 1% SDS, 50 mM EDTA, 500 mM NaCl, 10 mM β-mercaptoethanol at 65 °C, 10 min, followed by the addition of potassium acetate to 1.3 M, chilling to 0 °C and removal of debris by centrifugation. The DNA was then precipitated with isopropanol, washed with 70% ethanol and resuspended in 10 mM Tris, 1.0 mM EDTA. The following synthetic oligonucleotide 20 bp primers were used in PCR analysis for parent and progeny plants: Primer 1 (from the 35S promoter sequence), 5′-CACAATCCCACTATCCTTCG-3′; Primer 2 (from the *luc* gene sequence), 5′-CTGAAATCCCTGGTAATCCG-3′; Primer 3 (from the *luc* gene sequence), 5′-CCTTCCGCATAGAACTGCCT-3′; Primer 4 (from the *luc* gene sequence), 5′-GCGGTTGTTACTTGACTGGC-3′; Primer 5 (from the *luc* gene sequence), 5′-CTGTATTCAGCGATGACGAA-3′; Primer 6 (from the *hpt* gene sequence), 5′-ATGCAAAGTGCCGATAAACA-3′. Each PCR reaction [15] contained approximately 1 μg of template DNA, 0.25 μg of each primer, 0.2 mM dNTP, *Taq* polymerase and buffer (Promega). The PCR profile used was 1 min at 94 °C, 2 min at 60 °C and 2 min at 72 °C for a total of 30 cycles (Perkin-Elmer Cetus DNA Thermal Cycler).

Northern blot analysis

A small-scale isolation procedure [21] was used to obtain total RNA from leaves. Approximately 15 μg of total RNA were subjected to electrophoresis in a formaldehyde agarose gel and transferred to Zeta-Probe (Biorad) nylon hybridization membrane. A lane containing RNA size standards (BRL 0.24–9.5 kb RNA ladder) was excised prior to transfer and stained with ethidium bromide to allow approximation of transcript sizes. A 1293 bp *Xba* I-*Eco* RV *luc* fragment from pDO432 and a 990 bp *Pst* I *hpt* fragment from pCaMVhygΔ were the templates in random DNA labelling reactions to prepare ^{32}P-labelled probes. Blotted membranes were prehybridized, hybridized and washed according to the manufacturer's recommendations.

Results and discussion

Cre-mediated generation of Hyg^R, Luc$^-$ plants

The construct pCB5 was designed to test for removal of a promoter-proximal gene and concomitant fusion of a promoter-distal coding region in the plant genome. The relevant segment of this plasmid, as depicted in Fig. 1, consists of a CaMV 35S promoter, followed by the firefly *luc* cDNA and the bacterial *hpt*-coding region [10]. To test Cre-*lox*-mediated removal of *luc*, the recombination sequence of the bacteriophage P1 *lox* site was placed between the 35S promoter and the *luc* cDNA in an orientation that does not contain an ATG codon. A second *lox* site in the same orientation was inserted between the *luc* cDNA and the promoter-distal *hpt* coding region. This orientation of the *lox* sites would facilitate an excision event. The plasmid pCB5 was inserted into the *Agrobacterium* binary vector pBIN19, which confers kanamycin resistance (Kan^R) to plant cells [3]. The Kan^R *Nicotiana tabacum* transformants, obtained from *Agrobacterium*-mediated gene transfer, were indeed positive for the production of luciferase (Luc$^+$) and sensitive to hygromycin (Hyg^S). Two independent *N. tabacum* transformants harboring the pCB5 construct, designated ntCB7.1 and ntCB7.2, were cross-pollinated with ntED23.1, a plant with a 35S-*cre* construct (within pED23) integrated into the genome.

To score for the yield of progeny having undergone recombination, seedlings derived from the cross-fertilization of plants ntCB7.1 or ntCB7.2 with ntED23.1 pollen were examined for the hygromycin-resistant (Hyg^R) phenotype. From the ntCB7.1 × ntED23.1 cross, most of the 125 seedlings that germinated in the presence of hygromycin became bleached and stunted; while only 8 seedlings were unaffected by hygromycin (Table 1A). Of 80 seedlings examined from the ntCB7.2 × ntED23.1 cross, only 9 developed as green Hyg^R seedlings. All 17 Hyg^R seedlings from the two crosses were examined for the expression of *luc*. None scored positive for luciferase activity (Luc$^-$), suggesting that the selection used in

Table 1. Analysis of offsprings from cross-fertilizations of ntCB7 × ntED23.1

	Observed	Predicted if 100% Cre efficiency[1]	Estimated Cre efficiency[1] (%)
A. Number of Hyg^R plants			
ntCB7.1 selfed (65 seedlings)	0	–	–
ntCB7.1 × ntED23.1 (125 seedlings)	8	31	26
ntCB7.2 selfed (118 seedlings)	0	–	–
ntCB7.2 × ntED23.1 (80 seedlings)	9	20	45
B. Number and ratio of Luc$^+$/Luc$^-$ plants			
ntCB7.1 selfed (47 seedlings)	35:12 (3:1)	–	–
ntCB7.1 × ntED23.1 (112 seedlings)	40:72 (1:1.8)	28:84 (1:3)	57
ntCB7.2 selfed (114 seedlings)	82:32 (2.6:1)	–	–
ntCB7.2 × ntED23.1 (104 seedlings)	41:63 (1:1.5)	26:78 (1:3)	42

[1] 100% Cre efficiency would result in excision occurring in all plants containing both Cre and target (*lox*) sites.

the germination assay effectively eliminated seedlings with late somatic excision events that would be partially Luc$^+$ and Luc$^-$. In contrast, none of 183 seedlings from self-pollination of plant ntCB7.1, ntCB7.2 and ntED23.1 were Hyg^R. This indicates that the pED23 construct is required for generating the Hyg^R, Luc$^-$ progeny and is consistent with the interpretation that synthesis of Cre from the pED23 construct catalyzed excision of the *luc* gene (Fig. 1).

In a germination assay, the number of Kan^R to Kan^S (kanamycin-sensitive) progenies from self-fertilized transformants ntCB7.1, ntCB7.2 and ntED23.1 were 97 to 29, 108 to 33 and 101 to 31, respectively. Based on these nearly 3:1 segregation patterns for the Kan^R trait, it was concluded that primary transformants ntCB7.1, ntCB7.2 and ntED23.1 are hemizygous for their transgenic locus (also see below on segregation of *luc* gene). Therefore, 1/4 of the seedlings were expected to harbor both the pED23 and pCB5 constructs. If Cre-mediated excision occurred at 100% efficiency early in plant development, then 25% of the progeny should have been Hyg^R (Table 1A). The data from these two crosses, however, suggested that the Cre-catalyzed excision events that were scored as a Hyg^R phenotype in germinating seedlings occurred at an efficiency no greater than 45%. As noted earlier, the selection conditions used in this Hyg^R germination assay did not favor the detection of late somatic excision events.

The frequency of Cre-mediated excision events was also extrapolated from data on *luc* expression. Luc$^+$ to Luc$^-$ plantlets were scored among seedlings germinated without hygromycin selection (Table 1B). The 3:1 Luc$^+$/Luc$^-$ ratio of seedlings from the self-crosses confirmed a single transgenic locus in ntCB7.1 and ntCB7.2. If Cre-mediated excision of *luc* occurred with an efficiency of 100%, then a a Luc$^+$/Luc$^-$ ratio of 1:3 would be expected from a cross between ntCB7 and ntED23.1 plants. On the other hand, an equal number of Luc$^+$ and Luc$^-$ plants would be expected if deletion of the *luc* gene were not to occur at all or occurred only late in development (such that *luc* expression was still detected). From the data obtained, an estimate of the efficiency of Cre-mediated excision was 42% in one cross and 57% in another, similar in magnitude to the value determined from the Hyg^R data. The interpretation that these Luc$^-$ plants were probably not chimeric in phenotype (Hyg^R/Hyg^S, Luc$^+$/Luc$^-$) rests on the absence of the dominant Luc$^+$ trait.

Molecular analysis of luc *gene excision*

Primers designed to flank the *lox* sites of the pCB5 construct were used in a polymerase chain reaction (PCR) analysis [15]. In the event of Cre-mediated excision, the PCR product from the recombined locus would differ in size from the parental genome. Pairs of primers used for the analysis of the Hyg^S,Luc$^+$ parental and the Hyg^R, Luc$^-$ progeny genomes are depicted in Figs 1A and 1B. Four vegetative clones of individual seedlings were analyzed from each ntED23 cross with ntCB7.1 and ntCB7.2. All eight of these plants

produced PCR products that indicate DNA rearrangement had occurred consistent with site-specific excision of the luciferase gene.

Figure 1C shows the PCR analysis of the Hyg^S,Luc^+ parent ntCB7.1 and a representative Hyg^R,Luc^- seedling resulting from its cross with ntED23.1. Three fragments of the predicted sizes of 0.68 kb, 0.9 kb and 0.45 kb were amplified from the genome of a clonal derivative of the parental plant ntCB7.1 (lanes a, c and e, respectively). The 0.68 kb fragment, generated by primers 1 and 2, represented the 35S-*lox-luc* junction; the 0.45 kb fragment, generated by primers 5 and 6, bridged the *luc-lox-hpt* junction and the 0.9 kb fragment, expected from primers 3 and 4, verified the presence of the *luc*-coding region in the genome of plant ntCB7.1. A 2.3 kb PCR product was observed with ntCB7.1 template DNA using primers 1 and 6, which spanned across the *luc* gene from the 35S promoter to the *hpt* coding sequence (lane g). In contrast, the 0.68 kb and 0.45 kb fragments were not found in the genome of the Hyg^R, Luc^- progeny (lanes b and f). This indicates that the 35S-*lox-luc* and *luc-lox-hpt* junctions no longer existed. The 2.3 kb PCR product was also not observed in the progeny plant and instead, a new 0.36 kb fragment representing a recombinant 35S-*lox-hpt* junction was visible (lane h). Furthermore, a PCR product of the expected size of 0.9 kb or 0.77 kb was not detected from the progeny genome using either the sets of primers 3 plus 4 (lane d) or 2 plus 5 (lane j) respectively (although primers 2 plus 5 produced a ca. 0.15 kb non-specific band from both parental and progeny DNA, lanes i and j). These results indicate that neither the *luc* gene sequence spanning between primers 3 and 4 nor its circular permutation spanning between primers 2 and 5 in the excision product was present. The conclusion can be drawn that Cre-mediated excision of the *luc* gene led to its disappearance from the plant genome.

Transcript analysis

Recombination-mediated de-activation of *luc* and activation of *hpt* gene expression was examined at the RNA level. DNA probes corresponding to *luc* and *hpt* sequences were used for northern blot hybridization to RNA isolated from the parent plants ntCB7.1 and ntCB7.2 as well as from a representative Hyg^R,Luc^- progeny from each cross-fertilization with ntED23.1 (Fig. 2). A *luc*-specific transcript ca. 2 kb in length was found in plants ntCB7.1 and ntCB7.2 (lanes 2, 4); a *hpt*-specific RNA was not detected (lanes 7, 9). This is consistent with transcription terminating at the poly(A) site present in the *luc* cDNA. In contrast, the two Hyg^R,Luc^- progeny derived from ntCB7.1 and ntCB7.2 do not show a *luc*-specific transcript (lanes 3, 5). Instead, a *hpt*-specific transcript of ca. 1.6 kb was found (lanes 8, 10). This is consistent with transcription termination at the *nos* poly(A) site downstream of the *hpt*-coding region. The RNA from non-transformed plants contained neither a *luc*- nor a *hpt*-specific transcript (lanes 1, 6). The transcript data are compatible with the interpretation that the recombination-mediated exchange of gene activity is a result of switching transcription from one gene to another. A recent report in mammalian cells of using the FLP recombinase to delete a promoter-proximal gene to express a distally located one is

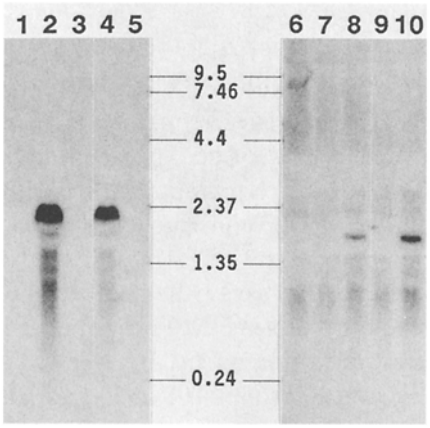

Fig. 2. Northern blot hybridization of RNA isolated from nontransformed *N. tabacum* (lanes 1, 6), ntCB7.1 (lanes 2, 7), ntCB7.2 (lanes 4, 9), a ntCB7.1 × ntED23.1 progeny (lanes 3, 8), and a ntCB7.2 × ntED23.1 progeny (lanes 5, 10). Lanes 1–5 were probed with *luc* DNA and lanes 6–10 were probed with *hpt*-coding sequence. RNA size standards (in kb) are as indicated.

similar to the event described here [13]. The difference, however, is that the distally located gene was activated presumably at the level of translation. Deletion of the upstream gene allowed translational initiation of the previously distal coding region.

The efficiency of recombination

We have shown that Cre-*lox* site-specific recombination can generate a deletion-fusion event that would alter gene transcription from a promoter-proximal to a promoter-distal gene cassette. Since the resolution of bacteriophage P1 dimer chromosomes, with *lox* sites separated by ca. 100 kb, is a highly efficient reaction in *Escherichia coli*, we sought to assess whether it would be comparable in plant cells. To assess the efficiency of a reaction requires setting certain parameters. The time provided for the reaction is critical since all enzymatic reactions will reach equilibrium when sufficient time is allowed. This equilibrium favors excision since the reverse event, integration, is a kinetically slower bimolecular reaction. Hence, if time is not a restriction, excision should approach 100% efficiency. With the gain-of-function assay utilized in this study, selection was applied at the earliest developmental stage. The intention was to eliminate the scoring of progeny that have chimeric phenotypes expected from developmentally late recombination events.

Having chosen a relatively stringent selection of hygromycin (20 μg/ml) during germination, it is possible that some of the seedlings expressing low levels of *hpt* were selected against early in their development. On the other hand, if a lower hygromycin level had been used it is likely that chimeric plants would have been recovered. Although this elevated level of hygromycin facilitated the selection of early-stage excision events, it may also account for the differences observed between the two transgenic lines. Different integration sites are known to cause variability in gene expression and the two transformants, ntCB7.1 and ntCB7.2, might have different levels of *hpt* gene expression. A weaker expression of the *hpt* gene might result in a lower percentage of early recombination events detected as Hyg^R. That may explain the apparent discrepancy between the 26% scored as Hyg^R and 57% scored as Luc^- among the progeny from the same cross (Table 1).

With the loss-of-function assay, we chose *luc* as the marker for excision because the luciferase enzyme can be sensitively detected. Seedlings that produce enzyme activity would indicate that at least a fraction of the cells harbors the *luc* gene. Again, the experimental design was to minimize the scoring of developmentally late recombination events. Overall, the numbers of progeny scored as Luc^- suggest that an early recombination event occurred in approximately half of the progeny that received both elements of the Cre-*lox* system. The lack of luciferase activity in all the Hyg^R plants and the absence of the *luc* gene as demonstrated by PCR analysis in each of the 8 Hyg^R plants tested also support the contention that the excision event occurred early in plant development.

It is not clear why a higher number of progeny with an early recombination event was not achieved. It might be possible that during the early stages of plant development, *cre* or *lox* sequences were partially methylated, or simply that an insufficient amount of the recombinase was supplied to the target sites to catalyze the reaction efficiently. The amount of Cre recombinase available could be affected by the particular *cre*-expression construct, the site of *cre* gene integration, as well as whether the *cre* plant was the maternal or paternal parent. If those were the causes, then a higher efficiency of recombination during germination might be possible with the appropriate combination of *cre*- and *lox*-transformed lines. Given that our data were derived from only two crosses by the same *cre* parent, the estimated efficiency of the Cre-*lox* reaction should be treated as tentative.

Odell *et al.* [12] also reported on the effectiveness of Cre-*lox* recombination using a gain-of-function phenotype (Kan^R) [12]. Transgenic seedlings derived from crosses of *cre*- and *lox*-containing plants were first germinated in the ab-

sence of selection and then leaf tissues were examined for callus formation in the presence of kanamycin. Using this criterion, which selects both early and late events, frequencies up to 100% recombination were reported. However, in a seed germinating assay similar to the one we have employed in this study, the seedlings did not produce roots or true leaves. On the contrary, we were able to obtain antibiotic-resistant plants when selection was imposed directly on germinating seedlings, although this apparent discrepancy could be explained by a number of possibilities. Aside from the obvious difference between using *nptII* (neomycin phosphotransferase gene) and *hpt* as selectable markers, it is also possible that we might have obtained better expression of *cre* at an early developmental stage, or that we had chosen a more appropriate (less stringent) level of antibiotics for selection.

Future prospects

In applying this system to engineering chromosomes, the placement of *lox* sites in *cis* in the plant genome would be a prerequisite for marking the intervening segment of DNA for excision or inversion. Transposable elements can potentially be utilized to translocate a second *lox* site to a nearby locus to bracket the intervening DNA for recombination. Since the reaction depends on the accessibility of the substrates, the frequency of recombination may be affected by the distance between the two sites. Hence, it may be necessary to increase the synthesis of Cre recombinase or the time of the reaction. For recombination achieved via sexual hybridization, that would mean the use of parent plants with strong expression of *cre* or by selection of recombinants during later stages of plant development. For instance, selection could be imposed on cells cultured from chimeric tissue or on the germinating progeny of a subsequent generation.

The controlled deletion-fusion of DNA segments from the genome of plants might give rise to other applications. One possible use is for concomitant removal of a selectable marker and transcriptional fusion with a desired gene. The selectable marker used in detecting stable DNA uptake can also be utilized to select for favorable integration sites that affect the level of gene expression. When Vaeck *et al.* [20] fused the *Bacillus thuringiensis* toxin protein to neomycin phosphotransferase, insecticidal activity and Kan^R were conferred by the chimeric protein. Thus, this protein fusion permitted high-level kanamycin resistance to select efficiently for transformed plants with high-level expression of insecticidal activity. The strategy proposed here would be analogous but without the need to construct fusion proteins. Moreover, upon selection of a favorable integration site, removal of the promoter-proximal selectable marker would accomplish more than to activate a desired transgene. It would also yield transformed cells devoid of DNA markers that are no longer required.

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