

Cre recombinase-mediated site-specific recombination between plant chromosomes

(Cre-lox/genome rearrangement/chromosome translocation)

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Communicated by Ronald L. Phillips, November 12, 1993 (received for review, June 7, 1993)

ABSTRACT We report the use of the bacteriophage P1 Cre-lox system for generating conservative site-specific recombination between tobacco chromosomes. Two constructs, one containing a promoterless hygromycin-resistance gene preceded by a lox site (lox-hpt) and the other containing a cauliflower mosaic virus 35S promoter linked to a lox sequence and the cre coding region (35S-lox-cre), were introduced separately into tobacco plants. Crosses between plants harboring either construct produced plants with the two constructs situated on different chromosomes. Plants with recombination events were identified by selecting for hygromycin resistance, a phenotype expressed upon recombination. Molecular analysis showed that these recombination events occurred specifically at the lox sites and resulted in the reciprocal exchange of flanking host DNA. Progenies of these plants showed 67–100% cotransmission of the new transgenes, 35S-lox-hpt and lox-cre, consistent with the preferential cosegregation of translocated chromosomes. These results illustrate that site-specific recombination systems can be useful tools for the large-scale manipulation of eukaryotic chromosomes *in vivo*.

Chromosome rearrangements, which can have a wide range of effects on genome structure, chromosome behavior, and gene expression, are useful for a variety of genetic experiments (1–4). Deletions, inversions, and translocations are commonly induced by ionizing radiation, which has some disadvantages. Irradiation often causes additional mutations, and mapping the breakpoints requires cytogenetic analysis, which is not readily amenable in some systems. For example, chromosomes of *Caenorhabditis elegans* and *Arabidopsis thaliana* are too small to be identified with ease under the microscope.

To develop alternative means to generate chromosome rearrangements, we explored the use of the bacteriophage P1 Cre-lox site-specific recombination system. Cre, the product of the cre gene, is a 38.5-kDa recombinase that can catalyze reciprocal exchange of DNA at the 34-bp lox sites (5). Cre has been shown in numerous instances to mediate lox-site-specific recombination in animal (6–8) and plant (9–13) cells. Recombinase-mediated chromosome recombination events have been reported in yeast (14, 15), and in *Drosophila*, the FLP/FRT system has caused site-specific recombination between homologous chromosomes (16, 17). We now report the interchromosomal recombination of lox sites in tobacco. Recombination yields the reciprocal exchange of flanking DNA segments and, by inference, balanced chromosome translocations. The strategy described is applicable in organisms with a DNA transformation system.

MATERIALS AND METHODS

DNA Constructs. To construct plasmid pCB9, a Sac I fragment from pCB5 (12), containing a lox sequence (same as

loxP) linked to a hygromycin-resistance gene coding region (hpt) and a nopaline synthase gene terminator (nos3'), was inserted into the Sac I site of pUC19 with nos3' proximal to the HindIII site. To construct pCB32, a HindIII-Kpn I fragment of pED74 (E. Dale, this laboratory) carrying the cauliflower mosaic virus 35S promoter (35S) linked to a lox sequence was ligated to a Kpn I-HindIII fragment from pMM23 (M. Morgan, this laboratory) that has cre-nos3' on a pUC19 backbone. pCB9 and pCB32, linearized with HindIII, were ligated into the HindIII site of pBIN19, an Agrobacterium vector with the neomycin phosphotransferase II gene (nptII) marker (18), in the orientation where 35S transcription is toward the T-DNA left border, to produce pCB11 (pBIN19:pCB9) and pCB36 (pBIN19:pCB32).

Transgenic Plants. Transformation of *Nicotiana tabacum* Wi38 was mediated by infection of leaf explants with *Agrobacterium tumefaciens* GV3111(pTiB6S3S3) carrying pCB11 or pCB36 (19). Independent kanamycin-resistant (Kan^R) primary transformants hemizygous for the constructs pCB11 (Ph plants) and pCB36 (Pc plants) were cross-pollinated (Pc as pollen donor) and self-pollinated. Plants whose selfed progenies segregated ≈3:1 for resistance to kanamycin were considered to have a single transgenic locus. Southern blot analysis using Xho I, which cuts outside the transgenes, was used to determine whether there was a single band. Four Ph (11.1, 11.4, 11.5, and 11.6) and four Pc (36.3, 36.4, 36.9, and 36.10) plants met these two criteria.

Selection of Recombinant Products. Surface-sterilized seeds were germinated on Murashige-Skoog (GIBCO) medium containing hygromycin B at 20 μg/ml (germination stage, or selection G). Approximately 500 germinated seedlings were scored; green, as opposed to bleached, seedlings were scored as hygromycin resistant (Hyg^R). For selection during later stages of plant development, seeds were germinated on Murashige-Skoog medium containing kanamycin at 100 μg/ml to select against segregants with neither construct. After 2–3 weeks, 50 Kan^R seedlings were transferred onto the same medium containing hygromycin at 20 μg/ml (seedling stage, or selection S). A plant was scored as Hyg^R if a green sector developed. Kan^R seedlings from each cross were also transferred to Magenta boxes and grown for 2–3 months. Two leaf explants (≈1 cm²) taken from different top leaves were placed on shoot-inducing Murashige-Skoog medium containing hygromycin at 20 μg/ml (late stage, or selection L). Plants from which leaf explants gave rise to shoots were considered as having Hyg^R cells within the leaf explants. Shoots were cultured into small plants prior to molecular analyses.

Abbreviations: 35S, cauliflower mosaic virus 35S promoter; Hyg^R, hygromycin-resistant; Kan^R, kanamycin-resistant.

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Molecular Analyses. Primers used for polymerase chain reaction (PCR) analysis (20) were a, 5'-CCTCTTCGCTAT-TACGCCAG-3'; b, 5'-CAGTGATACACATGGGGATC-3'; c, 5'-GTTTCATTTTCATTTGGAGAGG-3'; and d, 5'-CTAA-TCGCCATCTTTTCAGCA-3'. PCR products were fractionated in a 1.5% agarose gel and, when necessary, were purified by gel electrophoresis prior to endonuclease cleavage. For Southern analysis, plant DNA was isolated (21) from young leaves, cleaved with *Xho* I, and blotted onto Zeta-Probe (Bio-Rad). The filter was probed with a combination of the *hpt* (*Bam*HI-*Bgl* II fragment of pCB9) and *cre* (*Hinc*II fragment of pMM23) coding regions. For contour-clamped homogeneous electric field (CHEF) gel analysis, DNA of >2 megabase pairs in size was prepared as described (22) except that protoplasts were embedded in agarose beads (23). Following enzyme digestion, DNA fragments were separated in a Bio-Rad CHEF DRII gel apparatus (1% agarose gel, 200 V, 24 hr, switch time ramp from 20 sec to 40 sec). The gel was treated with 0.25 M HCl for 30 min with a change of buffer prior to DNA transfer.

RESULTS

Experimental Design. The *lox* site consists of an 8-bp asymmetric sequence between 13-bp inverted repeats. The 8-bp spacer gives the site directionality. If two *lox* sites are on different chromosomes in the same orientation with reference to the centromeres, recombination between them would generate a reciprocal exchange of chromosome arms. Since genetic materials are conserved after recombination, plants harboring balanced translocations of chromosome arms are likely viable. If the two *lox* sites are of opposite orientation with reference to the centromeres, site-specific recombination would produce dicentric and acentric chromosomes. Cells harboring this abnormality are most likely inviable.

Two constructs were used in these experiments. The first, *lox-hpt*, consists of the coding region of a hygromycin-resistance gene (*hpt*) preceded by a *lox* site incorporated into the transcriptional leader sequence, but devoid of a gene promoter (Fig. 1A). The second construct is *35S-lox-cre*, where the coding region of the *cre* gene is preceded by a *lox* site in the leader sequence and then by *35S*. These two constructs were introduced separately into tobacco along with a kanamycin-resistance marker. Plants containing the *lox-hpt* transgene (Ph plants) are sensitive to hygromycin (*Hyg*^S) due to the lack of *hpt* transcription. The two constructs were then brought into the same genome by cross fertilization between Ph and Pc plants. The prediction was

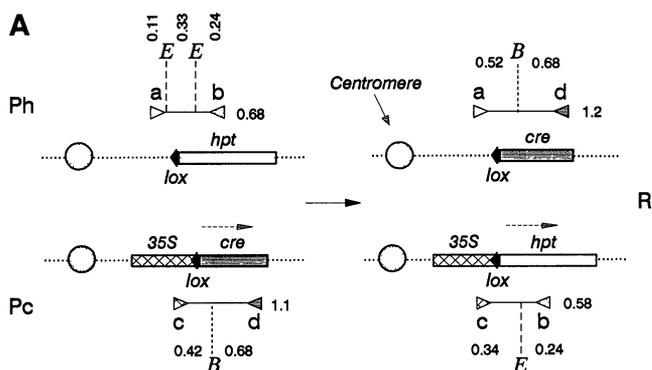


FIG. 1. Experimental design and PCR analysis of a Cre-mediated recombination event. (A) Schematic representation of two chromosomes (derived from Ph and Pc parents) each carrying a transgene before and after Cre-mediated recombination (in R progeny). In this instance, the two *lox* sites are in the same orientation with respect to their centromeres. PCR primers (a, b, c, and d) are shown as triangles, with the numbers indicating sizes in kilobases of the expected PCR products and the DNA fragments after digestion with *Eco*RI (E) and *Bam*HI (B). (B) A representative PCR analysis. (C) Results of restriction analysis of the PCR products. Size markers are indicated in kilobases.

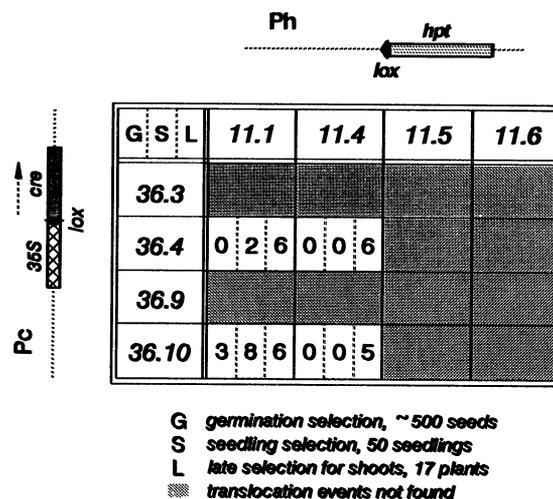
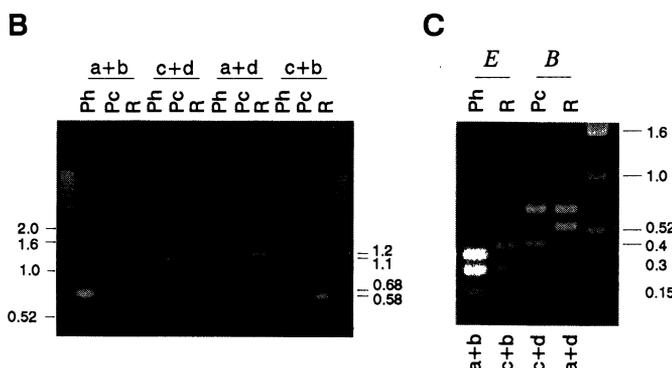


FIG. 2. Number of *Hyg*^R plants derived from selection of progeny of crosses between Ph and Pc plants. Shaded boxes indicate that not a single *Hyg*^R plant was recovered after screening ~500 seeds (selection G), 50 seedlings (selection S), and 17 plants (selection L).

that expression of the *cre* gene would promote recombination between the two *lox* sites, with the *35S* promoter disengaged from the *cre* coding region and fused to the *hpt* coding sequence. The lack of a functional promoter in front of the *cre* gene would terminate *de novo* synthesis of Cre recombinase and would eventually curtail further recombination of the *lox* sites. The formation of a functional *hpt* gene would confer resistance to hygromycin, a phenotype that can be used to select for the recombination event.

Hygromycin Resistance via Recombination. Crosses were made between four Ph and four Pc plants (Fig. 2). The eight Ph and Pc plants chosen for this analysis were previously determined to contain a single transgenic locus (*Materials and Methods*). In progeny where the two *lox*-containing chromosomes are brought into the same genome, recombination could occur. We reasoned that the efficiency of recombination could be lower than that observed between closely linked sites. To enhance recovery of the potentially rare recombination products, selection for hygromycin resistance was performed at three different stages of plant development.

For early recombination events, *Hyg*^R plants were selected for at the onset of seed germination (selection G). Approximately 500 seedlings from each of the 16 pairwise crosses were germinated in the presence of hygromycin. Three progeny, all from cross 11.1 × 36.10, exhibited the *Hyg*^R



phenotype (Fig. 2). Since parent plants are hemizygous for their transgene, a quarter of the progeny would be expected to receive both the *lox-hpt* and the *35S-lox-cre* chromosomes. Therefore, the 3 Hyg^R plants represent 2.4% of the seedlings in which recombination had occurred at this early stage of plant development. A second screening was conducted on Kan^R seedlings that were several weeks old (selection S). From this screening, 10 bleached seedlings exhibited green sectors (Fig. 2). This suggests that a portion of the cells had undergone a somatic recombination event. As only a third of the Kan^R seedlings were expected to harbor both constructs, the frequency of obtaining recombinant products at this later stage of plant development approached 50% in the best case, where 8 Hyg^R plantlets were recovered out of 50 screened (from cross 11.1 × 36.10). A third screening was conducted by taking leaf explants from Kan^R progeny and placing them onto hygromycin-containing shoot-inducing medium (late selection L). Shoot formation in the presence of hygromycin would be indicative of a recombination product derived from a previously Hyg^S progenitor cell. Of the 17 plantlets screened from each of the 16 sets of crosses, a total of 23 Hyg^R plants were obtained from the offspring of 4 pairwise combinations. Since a third of the 17 Kan^R plants were expected to harbor both constructs, the 5 or 6 Hyg^R plants obtained by this late selection of recombinants suggest that recombination between the two *lox* sites situated on separate chromosomes can proceed to completion, provided that time is not a restrictive factor.

New Junctions Formed Upon Recombination. Hyg^R plants were analyzed at the DNA level. Fig. 1B shows the PCR analysis of genomic DNA from a representative Ph (11.1) and Pc (36.10) parent and a Hyg^R progeny (R) recovered from selection G. A specific band was amplified by using primers a and b (0.68 kb) or c and d (1.1 kb) from the Ph or Pc parent genome, respectively, but not from the progeny R genome. Two new bands, obtained by using primers a and d (1.2 kb) or c and b (0.58 kb), were amplified from the R genome but from neither parental DNA. The pattern of PCR products was consistent with a reciprocal recombination event between these two loci. Furthermore, these PCR products harbored the characteristic *Bam*HI and *Eco*RI sites. *Eco*RI cleaved the 0.68-kb a–b product into three fragments of 0.33, 0.24, and 0.11 kb (Fig. 1C). The 1.1-kb c–d product was cleaved by *Bam*HI into two fragments of 0.68 and 0.42 kb. If recombination had occurred in the *lox* sequence, *Bam*HI cleavage of the 1.2-kb a–d product should have yielded the same 0.68-kb fragment as from the parental c–d band, while producing a new fragment of 0.52 kb. Likewise, in the new c–b band, *Eco*RI cleavage should have produced the same 0.24-kb fragment as from the a–b band, in addition to a longer, 0.34-kb fragment. Fig. 1C shows that the *Eco*RI and *Bam*HI cleavage patterns met these expectations. The same analysis shown in Fig. 1B (but not in Fig. 1C) was performed with at least three randomly chosen Hyg^R plants (from selection L) from each of the four crosses. In all cases, the DNA from these Hyg^R plants produced the expected PCR pattern (data not shown).

Exchange of Flanking Genomic Sequences. Southern blot analysis was used to examine a representative Hyg^R plant isolated by the late selection scheme (L) from each of the four combinations of crosses. Genomic DNA was isolated from the parents, a Hyg^S progeny harboring both transgenes (before recombination), and its Hyg^R somatic derivative (R) plant. DNA was cleaved with endonuclease *Xho* I, which does not cut within the two transgenic loci. The Southern blot was probed with a combination of *hpt* and *cre* coding sequences that were expected to hybridize to the *lox-hpt*, *35S-lox-cre*, *lox-cre*, and *35S-lox-hpt* loci. Fig. 3A shows the analysis of all four sets of plants. DNAs in lanes a (m is the same as a) and g are from two Ph parents, 11.1 and 11.4, respectively, and lanes d and j represent two Pc parents, 36.4

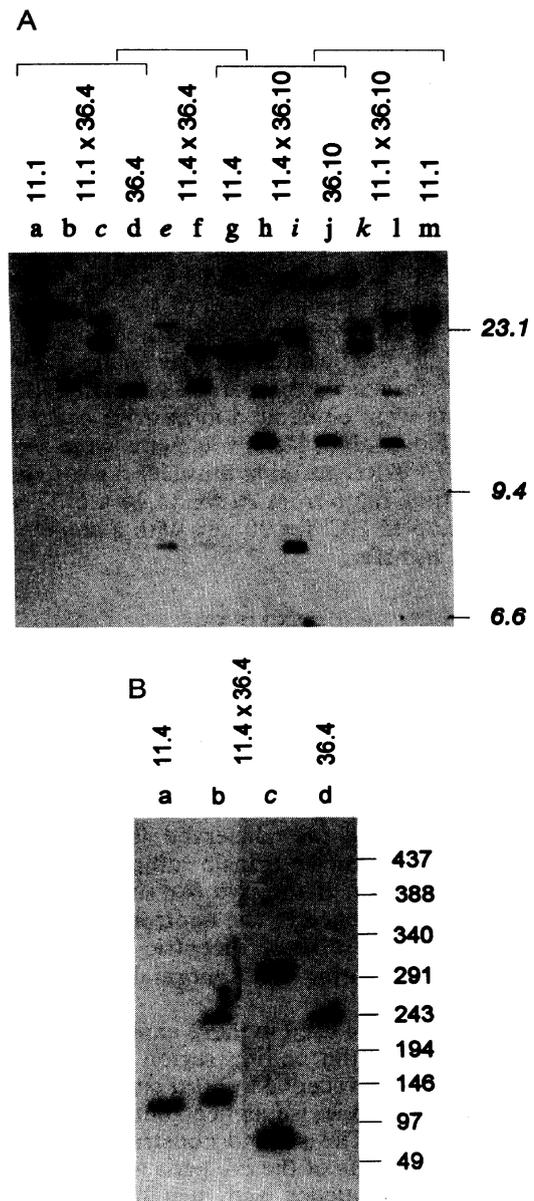


FIG. 3. (A) Autoradiogram of a Southern blot analysis of four sets of plants. Each set, indicated by brackets, consists of the Ph (lanes a, g, and m) and Pc (lanes d and j) parents and a progeny plant harboring both transgene-containing chromosomes before (lanes b, f, h, and l) and after (lanes c, e, i, and k) recombination. Size markers (kb) are *Hind*III-cleaved λ DNA. (B) Autoradiogram of a CHEF gel analysis of a set of plants showing the swapping of large flanking DNA segments before (lane b) and after (lane c) recombination. Size markers (kb) are multimers of λ DNA.

and 36.10, respectively. With the exception of 36.10, each of the parent plants shows a single band that hybridized with the probes, consistent with the segregation data that the transgene is at a single locus. In 36.10, a second band that has a weaker hybridization signal can also be seen. This minor band also shifts mobility after recombination (see below) and probably represents sequences linked to the intact pCB36 construct.

In plants generated in the absence of hygromycin, the Southern blot reveals the presence of both parental loci (lanes b, f, h, and l). This indicates that the vast majority of cells in these progeny plants had not participated in DNA recombination at that point. Leaf tissues from these plants, subjected to shoot formation in the presence of hygromycin, produced the R plants whose DNAs were analyzed and shown in lanes c, e, i, and k. In each case, two new bands were seen in the

R plants in place of the *lox-hpt* and *35S-lox-cre* loci in the somatic progenitor plants. Moreover, the combined sizes of these new bands were approximately the sum of the two original transgene-containing *Xho* I fragments. For example, in the cross between 11.4 and 36.4, the two parental bands before recombination were approximately 20 and 17 kb, respectively. After recombination, two new bands of approximately 25 and 8 kb were seen. The sum of the new fragments (33 kb) is close to the sum of the two parental bands (37 kb). Given the nonlinear relationship between size and mobility in this range of the gel, the 10% discrepancy of the two estimated figures is not significant. This same analysis holds true for all of the other three combinations. These results confirm that in each case, recombination at the *lox* sites led to the swapping of flanking genomic DNA sequences.

To investigate whether the flanking DNA that is being exchanged extended beyond the several kilobases shown by the above analysis, Southern analysis of a set of plants from cross 11.4 × 36.4 was performed on DNA separated by pulsed-field gel electrophoresis. DNA of >2 megabase pairs was prepared from Ph, Pc, and R plants and the somatic progenitor from which the R plant was derived. The DNAs were cleaved with a rare-cutting endonuclease, *Sfi* I, for which there is no restriction site within the transgenes. As before, the membrane filter was probed with the combination of *hpt* and *cre* coding sequences. Two *Sfi* I fragments of approximately 130 and 240 kb were found in Ph and Pc plants (Fig. 3B, lanes a and d, respectively). These two bands were present in the somatic progenitor (lane b) but absent from the Hyg^R derivative (lane c). In the latter, two new bands of approximately 300 and 80 kb were seen. As before, the sum of the new bands is approximately equivalent to the sum of the parental bands. This shows that the recombination event exchanged a large segment of DNA beyond the point of reciprocal cross over.

Progeny Analysis. As T-DNA insertions are random and there are 24 chromosome pairs in the amphidiploid *N. tabacum*, it is unlikely that the two transgenes in all of the four combinations are on homologous chromosomes. Therefore, some or all of the four events are likely to be the recombination between two nonhomologous chromosomes, suggesting that the R plants could represent reciprocal translocation heterozygotes. In such individuals, cosegregation of a normal chromosome with a translocated chromosome will produce gametes with duplications and deficiencies. If the unbalanced genomes do not give rise to viable gametes or zygotes, then the preferential transmission of a balanced genome will result in the pseudolinkage of markers present on the two translocated chromosomes.

We examined the transmission of the new transgenes, *lox-cre* and *35S-lox-hpt*, which represent the two putative translocated chromosomes. R plants were the maternal donors in an outcross with wild-type tobacco and the progeny were germinated in the presence of hygromycin. We asked whether the *lox-cre* transgene was associated with the Hyg^R phenotype, an indication that the chromosome harboring *lox-cre* had cosegregated with the chromosome harboring *35S-lox-hpt*. Because the *lox-cre* transgene does not exhibit a readily scorable phenotype, Southern blotting was performed on Hyg^R progeny derived from each of the four R plants. The same enzyme and DNA probes as in Fig. 3A were used for this analysis.

As shown in Table 1, the frequency of cosegregation of the *lox-cre* and *35S-lox-hpt* transgenes ranged from 67% for R3 crossed with wild type to 100% for R1 crossed with wild type. A 90–100% cotransmission of the two transgenes, by inference the cosegregation of the two translocated chromosomes, is what would be expected if the gametes or zygotes with unbalanced genomes have reduced viability. The 67% and 80% cotransmission frequencies for the progeny of R3

Table 1. Number of Hyg^R progeny from an outcross of R plants that showed the *35S-lox-hpt* and *lox-cre* transgenes on Southern blots probed with *hpt* and *cre* DNA

Parent lines	Number of progeny that harbor:		Co-transmission frequency	χ^2
	<i>35S-lox-hpt</i>	<i>lox-cre</i>		
R1 (11.1 × 36.4) × WT	15	15	100%	15.0*
R2 (11.1 × 36.10) × WT	21	19	90%	13.8*
R3 (11.4 × 36.4) × WT	18	12	67%	2.0
R4 (11.4 × 36.10) × WT	20	16	80%	7.2*

WT, wild type. Origin of the R plants is indicated in parentheses. χ^2 values were calculated on the basis of independent segregation. *Deviation from independent segregation is highly significant.

and R4 with wild type could indicate that the particular chromosome duplications and deficiencies are tolerated.

DISCUSSION

We have shown that Cre can mediate recombination between *lox* sites integrated into separate chromosomes and that there is a reciprocal exchange of DNA segments adjacent to the two *lox* sites. It has been reported that the FLP system from yeast can mediate site-specific recombination between two homologous chromosomes in *Drosophila* (16, 17). In those studies, it is not known to what extent homologous pairing played a role in the recombination process. We do not believe that homologous pairing is involved in the events that we described here. Based on the lack of site preference in T-DNA integration and the 24 pairs of chromosomes in the *N. tabacum* genome, we have made the argument that the two *lox* sites are not likely to be on homologous chromosomes, and even less likely to be within homologous regions. Consistent with this contention, we found that the newly formed *35S-lox-hpt* and *lox-cre* were readily cotransmitted (67–100%) to the same gamete. As homologous chromosomes segregate to different gametes, this high rate of cotransmission is consistent with the deduction that the two loci are most likely on nonhomologous chromosomes.

When the plant R1 was used as the maternal donor in a test cross, we observed 100% cosegregation of the two new transgenes. This is consistent with the general interpretation that in reciprocal translocation heterozygotes, the duplication and deficiency generated by the cosegregation of a normal chromosome with a translocated chromosome may not be tolerated by the gamete or zygote. However, exceptions have been reported where plants missing a small chromosome segment are viable (4). This can occur, for instance, if the translocation breakpoint is close to the telomere. In addition, chromosome losses are not necessarily lethal. In *N. tabacum*, an allotetraploid, it has been reported that depending on the particular chromosome that is missing, gametes show great differences in viability (24). Hence, it would not be unexpected to find that from certain cases of reciprocal translocations, an unbalanced genome can be tolerated. With the progeny from R2, R3, and R4, 10–33% did not show cotransmission of the two transgenes. This suggests that although there may be a bias in favor of a balanced genome, these particular imbalances of genetic information can nonetheless be tolerated.

Reciprocal chromosome translocations that result in two chromosomes, each with a single centromere, occur only when two *lox* sites are situated in the same orientation with respect to their centromeres. Interestingly, only 4 of the 16 combinations of crosses produced Hyg^R plants. The lack of recoverable Hyg^R plants might be due to *lox* sites that were oppositely oriented with respect to the centromeres. However, this argument cannot apply to all 12 combinations.

Other possible explanations for not recovering Hyg^R plants include (i) less efficient expression of the *cre* gene in Pc 36.3 and Pc 36.9 plants, (ii) poor expression of the newly formed 35S-*lox-hpt* transgene after recombination, (iii) less efficient recombination reaction between particular pairs of *lox* loci, and (iv) that the newly formed products are inviable.

Whether recombination between the introduced *lox* sites represents the only event or that other rearrangements have also occurred due to endogenous identical or cryptic *lox* sites in the tobacco genome is unknown. The frequent occurrence of identical 34-bp *lox* sequences in eukaryotic genomes is not likely. However, Cre-promoted chromosome recombination events among cryptic *lox* sites in the yeast genome have been reported (15). Nonetheless, the presence of cryptic *lox* sites, if any, should not pose a critical problem for site-directed chromosome engineering. No obvious aberrant phenotype was observed in the *cre*-expressing Pc or R plants, which suggests a lack of gross rearrangement of the genome. Moreover, cryptic *lox* sites would most likely recombine with lower efficiency than wild-type sites. The probability of having a recombination event of cryptic *lox* sites in the same cell should be much lower than that between wild-type sequences, which themselves are rare events.

Advantages of the system described for generating chromosome rearrangements are that the recombination events are site-specific, selectable, and reproducible. As the translocation breakpoints are physically tagged, these sites can be mapped like restriction fragment length polymorphisms and retrieved from the genome for molecular analysis. If *lox* sites are carried on transposable elements, it should be possible by DNA transposition to saturate a genome with *lox* sites and generate a variety of translocation, inversion, and deletion events (25). Most importantly, this is a general strategy that is adaptable to other organisms. Although the *lox* sites introduced in this study were random insertions, it should be possible, and practical at least in animal systems, to preselect a chromosome location by targeted insertion of a *lox* site through homologous recombination (26). Chromosome translocations in predetermined sites would provide unprecedented opportunities to study the effects of chromosome rearrangements on gene expression and to dissect regions of chromosomes in a selective manner.

We thank E. Dale and M. Morgan for unpublished constructs and S. Medberry and H. Koshinsky for critical reading of the manuscript. Funding for this work was made possible by grants from the U.S.

Department of Agriculture (Agricultural Research Service Project 5335-23000-005-00D and National Research Initiative Competitive Grant 91-37300-6381) and the McKnight Foundation (M1084).

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