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Intra-chromosomal rearrangements generated by Cre–*lox* site-specific recombination

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ABSTRACT

Chromosomal rearrangements are useful genetic and breeding tools but are often difficult to detect and characterize. To more easily identify and define chromosome deletions and inversions, we have used the bacteriophage P1 Cre–*lox* site-specific recombination system to generate these events in plants. This involves three steps: (i) the introduction of two *lox* sites into one locus in a plant genome, including one site within a modified *Ds* transposon; (ii) *Ac* transposase-mediated transposition of the *Ds–lox* element to a new locus on the same chromosome; (iii) Cre-mediated site-specific recombination between the two *lox* sites that bracket a chromosome segment. We report the production of a deletion and three inversion events in tobacco. The utility of chromosomal segments bracketed by *lox* sites for targeted manipulation and cloning is discussed.

INTRODUCTION

Chromosomal rearrangements alter the normal linear relationship of genetic loci. Plants containing chromosomal rearrangements such as deletions, inversions and translocations often have reduced fertility, and the presence of such rearrangements in closely related species is thought to be a barrier to inter-specific hybridization (1). Chromosomal rearrangements are used by geneticists to assign linkage groups, to study chromosomal position effects on gene expression and as genetic stocks (2,3). Typically, rearrangements are generated using chemical mutagens or ionizing radiation (4,5). However, events generated in this manner are often difficult to detect, since they must produce distinguishable cytogenetic or morphogenetic phenotypes, and are difficult to characterize, since the endpoints are not tagged with unique molecular markers (5).

An alternative method for generating chromosome rearrangements is through the use of site-specific recombination systems. A number of simple site-specific recombination systems have been shown to operate in yeast (6,7), animals (8–17) and plants (18–23). These systems include Cre–*lox* from bacteriophage P1, FLP–*FRT* from *Saccharomyces cerevisiae* and R–*RS* from *Xygosaccharomyces rouxii*. Each system consists of a recombinase, Cre, FLP or R, that catalyzes recombination between recognition sites *lox*, *FRT* or *RS*, respectively (reviewed in 24). In numerous instances, these systems have been used to mediate rearrangements within a transgenic locus. By the appropriate placement of these recombination sites within or between chromosomes, rearrangement of chromosomal segments can also occur, as shown with the R-mediated translocation, inversion and deletion of chromosome segments in yeast (25). In higher eukaryotes, recombination between non-contiguous transgenes was reported for the FLP-mediated recombination of homologous chromosomes in *Drosophila* (26–28) and the Cre-mediated reciprocal translocation of chromosomes in tobacco (29).

In this article, we describe the generation of tobacco intra-chromosomal rearrangements mediated by the Cre–*lox* site-specific recombination system. A plant transposable element was used to move a second *lox* site from the first *lox* site at the primary transgenic locus. Cre-mediated recombination between the two separated *lox* sites generated a chromosome deletion and three chromosome inversion events. In one of the four recombination events, the rearrangement (an inversion) was stably transmitted to progeny. The potential applications of the system described here are discussed.

MATERIALS AND METHODS

Recombinant DNA

Plasmid pED204 was constructed using standard methods (30) and the relevant structure is as depicted in Figure 1A. Abbreviations used are: *35S* (cauliflower mosaic virus 35S RNA promoter), *Ac* (*Association* transposable element), *Ds* (*Dissociation* transposable element), *hpt* (hygromycin phosphotransferase coding region), *luc* (firefly luciferase cDNA) and *npt* (neomycin phosphotransferase coding region). The left and right *Ds* ends in pED204 (as depicted in Fig. 1A) were derived from *Ac7* bp 4563–4265 and bp 337–1, respectively (31). For brevity, the nopaline synthase gene polyadenylation sites, which were placed downstream of *npt* and *hpt*, are not shown in Figure 1A. The plasmid pED53204 was formed from a co-integration between pED204 and a modified pBIN19 (32) plasmid in which the plant-active kanamycin resistance gene had been deleted. Details of plasmid construction are available by request.

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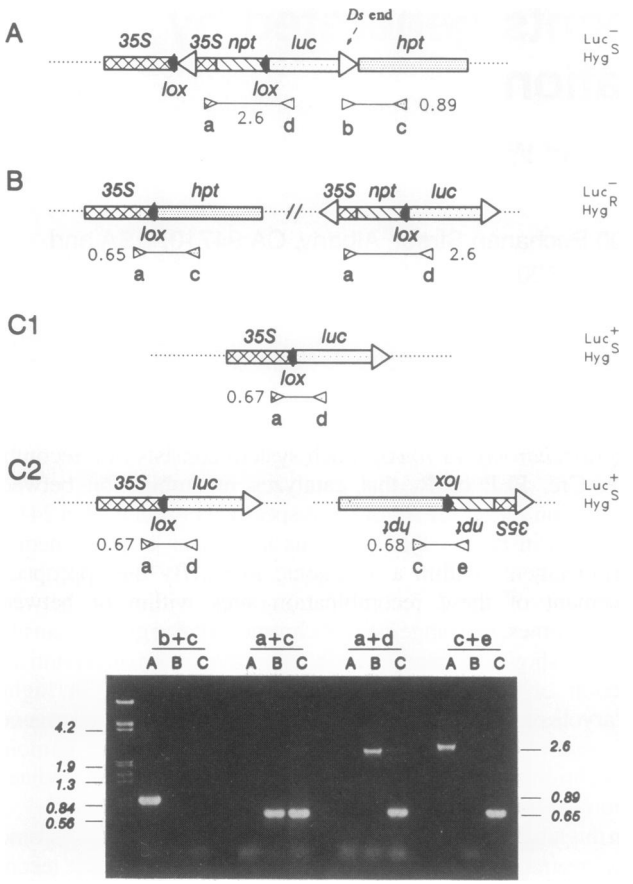

Figure 1. Strategy for generating chromosomal rearrangements. (**A**) The original transgene at the primary integration locus. (**B**) *Ac*-mediated *Ds* transposition to a new locus. (**C1**) Cre-mediated deletion generated if the *Ds* element transposed to a linked site and both *lox* sites are in the same relative orientation. (**C2**) Cre-mediated inversion generated if the *Ds* element transposed to a linked site and *lox* sites are in opposite relative orientation or a reciprocal translocation generated if the *Ds* element transposed to a separate chromosome and the *lox* sites are in the same relative orientation with respect to the centromere. The expected phenotype of plants containing each construct is noted to the right of the construct. Bottom panel: PCR detection of the transposition and recombination junctions. Lanes A, B and C contain PCR-amplified DNA from tobacco plants 512 (containing a transgene as in A), 9 (a Hyg^R progeny of a 512 × *Ac* cross) and 9.9 (a Luc^+ progeny of a 9 × *cre* cross), respectively. The location and predicted amplification product sizes in kb are indicated in parts A–C2 of this figure for the pairs of synthetic oligonucleotide primers (b+c, a+c, a+d and c+e). For brevity, the 2.9 kb PCR product produced from primers e and c (*npt* to *hpt*) is not shown in part A.

Transgenic plants

Transformation of *Nicotiana tabacum* Wi38 was carried out with *Agrobacterium tumefaciens* strain LBA4404 harboring pED53204 using standard protocols (33). The transformants were grown to flowering and cross-pollinated by wild-type plants or the *Ac* transposase-containing plant JJ2853 (34). Resulting seed was germinated on MS medium containing 20 μg/ml hygromycin to identify plants containing a transposition event. To regenerate hygromycin-resistant (Hyg^R) plants, a leaf piece was placed on shoot-inducing medium. The Hyg^R plants were allowed to flower and were cross-pollinated with the *cre*-expressing plant ntCB34.4 (containing a heterologous *35S–cre* gene and a plant-selectible Kan^R marker; M. Morgan and D. Ow, unpublished). The resulting progeny were germinated and a leaf piece analyzed for luciferase activity (35).

Molecular analysis

Primers used for PCR analysis were: a, 5′-CACAATCCCAC-TATCCTTCC-3′; b, 5′-GTGCTACATTAAACTATGTGTGC-3′; c, 5′-CCAGTGATACACATGGGGATC-3′; d, 5′-CTGAAATC-CCTGGTAATCCG-3′; e, 5′-AGCCTCCTCATCTCCCAGTT-3′. PCR was performed using conditions suggested by the polymerase supplier. For Southern analysis, DNA was isolated using a CTAB procedure (36), digested with *Dra*I, fractionated by gel electrophoresis, blotted to nylon filter (Zeta-Probe) and hybridized and washed using conditions suggested by the membrane supplier.

RESULTS

Experimental design

To generate chromosome rearrangements, we tested a strategy where *Ds*, the non-autonomous derivative of the maize *Ac* transposon, is used to move a *lox* site to a new location in the genome. Since *Ac* and *Ds* move preferentially to linked sites in an orientation-independent manner (37–39), obtaining deletions and inversions should be more common than reciprocal translocations. Figure 1A shows diagrammatically the transgenes on pED53204 where one *lox* site is within and another *lox* site outside the *Ds* element. Excision of this '*Ds–lox*' element allows transcription of *hpt* by *35S* which should then confer a Hyg^R phenotype. Figure 2 shows that *Ds–lox* transposition can lead to six possible configurations depending on where the element has re-inserted: (A) downstream, *lox* in the same orientation; (B) upstream, *lox* in the same orientation; (C) downstream, *lox* in the opposite orientation; (D) upstream, *lox* in the opposite orientation; (E) to another chromosome, *lox* in the same orientation with respect to centromere; (F) to another chromosome, *lox* in the opposite orientation with respect to centromere. Cre-mediated recombination between the separated *lox* sites would fuse *35S* to the luciferase cDNA (*luc*) in all six possibilities. However, in the situation shown in Figure 2B, the *35S–luc* junction is on the excision product and would presumably be lost. In another situation shown in Figure 2F, Cre–*lox* recombination leads to dicentric and acentric chromosomes, which would likely cause lethality. Hence, a luciferase-positive (Luc^+) phenotype would indicate one of two possible deletions (Fig. 2A) or reciprocal translocations (Fig. 2E), or either type of inversion (Fig. 2C and D).

Genetic analysis

The construct shown in Figure 1A was transferred into the tobacco genome by *Agrobacterium*-mediated transformation. Transgenic plant 512 was found by Southern blotting to harbor a single non-rearranged copy of this construct. Plant 512 was crossed to plants expressing *Ac* transposase to promote transposition of the non-autonomous *Ds–lox* element. Hyg^R progeny resulting from *Ds–lox* excision were identified and nine seedlings were grown and crossed with a *cre* donor. Progeny of these nine crosses (~40 progeny from each cross) were then screened for luciferase activity to detect plants containing a recombination between the two *lox* sites. Luc^+ progeny were found from Hyg^R plant lines 5, 7, 8 and 9 (Table 1).

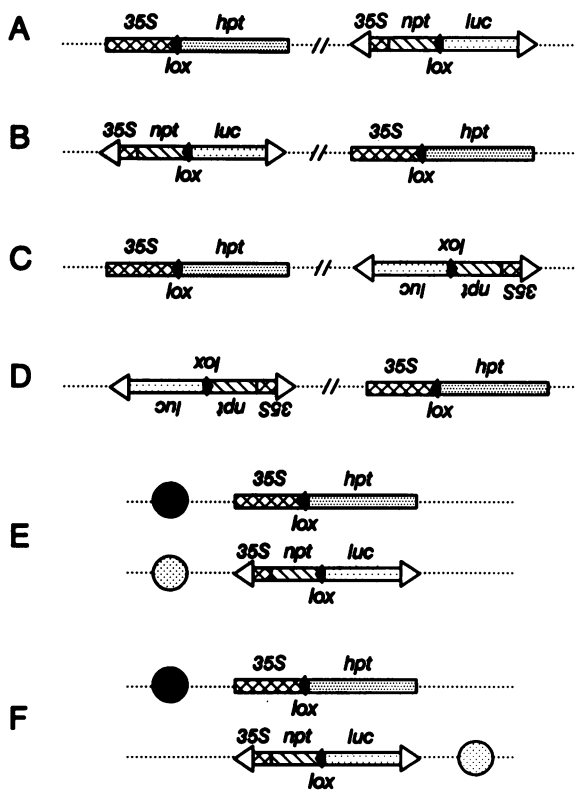

Figure 2. Possible locations the *Ds–lox* element can move to in relationship to the T-DNA locus. (**A** and **B**) transposition to a linked site with *lox* sites in the same relative orientation. (**C** and **D**) transposition to a linked site with *lox* sites in the opposite relative orientation. (**E** and **F**) transposition to a separate chromosome with *lox* sites in the same and opposite orientation relative to the centromere, respectively. Deletions, inversions, and reciprocal translocations will result from Cre-mediated recombination of the arrangements depicted in A and B, C and D, and E and F, respectively.

To distinguish between intra- and inter-chromosomal recombination in the Luc$^+$ plants, a linkage analysis between the transposed *Ds* element and the transgene was performed. Plants 5, 7, 8 and 9 were each cross-pollinated by a wild-type (WT) plant. In 20 Hyg^R progeny from each cross, the co-transmission of both the primary integration locus and the *Ds–lox* locus was examined by Southern blot analysis. If the loci are unlinked, 50% of the Hyg^R progeny should contain the *Ds–lox* element; if the two loci are linked, the co-transmission frequency should exceed 50%. As summarized in Table 1, *Ds–lox* relocated to a linked site in each of the four lines, indicating that the Luc$^+$ progeny from Cre-mediated recombination could only represent inversions or deletions.

One representative Luc$^+$ plant from each of the four lines (5.16, 7.70, 8.10 and 9.9) was cross-pollinated by a WT plant and the resulting progeny were analyzed for the inheritance of the putative inversion or deletion. Out of 1000 outcross progeny of 5.16 and 7.70, screened in pools of 10 for luciferase activity, none were found to be Luc$^+$. This indicated that the rearrangements did not transmit to the next generation (Table 1). In contrast, Luc$^+$ progeny were found from plants 8.10 and 9.9. The three possible genotypes of the Luc$^+$ progeny are: *35S–luc* without *cre*, *35S–luc* plus *cre* and *35S–hpt* plus *cre*. Plants of the latter two types are expected to be both Luc$^+$ and Hyg^R due to Cre-mediated recombination. In the 8.10-derived progeny, 2/3 of the Luc$^+$ 8.10 progeny were Hyg^R. In contrast, all of the Luc$^+$ progeny of 9.9 were Hyg^R, indicating that the *35S–luc* without *cre* genotype was not recovered.

Molecular analysis

To confirm that the four Hyg^R lines were due to *Ds–lox* transposition and that their Luc$^+$ progeny were generated through Cre–*lox* recombination, the genomic DNA of the parental line 512, the Hyg^R progeny lines 5, 7, 8 and 9, and their representative Luc$^+$ progeny 5.16, 7.70, 8.10 and 9.9 were examined by PCR. A representative PCR analysis is shown in the lower portion of Figure 1 for a three generation set of plants (512, 9 and 9.9). With primers b+c, which prime amplification across the *Ds–hpt* junction, the expected 0.89 kb fragment was produced in the original transformed plant line 512. This junction could not be detected in the other plants. When primers a+c were used, which amplify across the *35S–lox–hpt* junction formed after *Ds* excision, the expected 0.65 kb fragment was detected in the Hyg^R line 9 and the Luc$^+$ progeny 9.9, but not in the original transformed line 512. After Cre–*lox* recombination to form the Luc$^+$ phenotype, two new *lox* junctions should be formed, a 0.67 kb *35S–lox–luc* junction detectable using primers a+d and a 0.68 kb *npt–lox–hpt* junction detectable using primers c+e. While both junctions are formed regardless of the type of rearrangement produced, the *npt–lox–hpt* junction would be on the excised molecule in the case of a deletion event and hence would not be maintained. As shown in Figure 1, both junctions were detected in the Luc$^+$ plant 9.9. Along with the co-segregation data for the two *lox* sites, this suggests that the rearrangement is an inversion. Analysis of plants 7 and 7.70, and 8 and 8.10 yielded similar results (data not shown). Analysis of plants 5 and 5.16 detected the same junctions as the other sets of plants except that 5.16 lacks a detectable *npt–lox–hpt* junction. This suggests that a deletion may have occurred in 5.16.

Table 1. Linkage of the *Ds–lox* and T-DNA loci, recovery frequency of Luc$^+$ plants after introduction of *cre* and transmission of the Luc$^+$ phenotype to progeny

Hyg^R plant line	Transposon and T-DNA co-transmission frequency (%) (χ^2)[a]	Cre–*lox* recombination frequency[b] (%)	Luc$^+$ transmission frequency[c] (%)
5	90 (12.8*)	5	0
7	95 (16.2*)	10	0
8	90 (12.8*)	5	54
9	100 (20.0*)	25	41

[a]Co-transmission determined by Southern blot analysis of 20 progeny from an outcross to a WT plant by Hyg^R plants 5, 7, 8 or 9. χ^2 calculated on the basis of independent segregation. *Significant at the 0.1% level.

[b]Luc$^+$ plants recovered from screening 40 progeny from an outcross to the *cre*-expressing plant ntCB34.4 by Hyg^R plants 5, 7, 8 or 9.

[c]100 progeny from 8.10 or 9.9 × WT and 1000 progeny from 5.16 or 7.70 × WT.

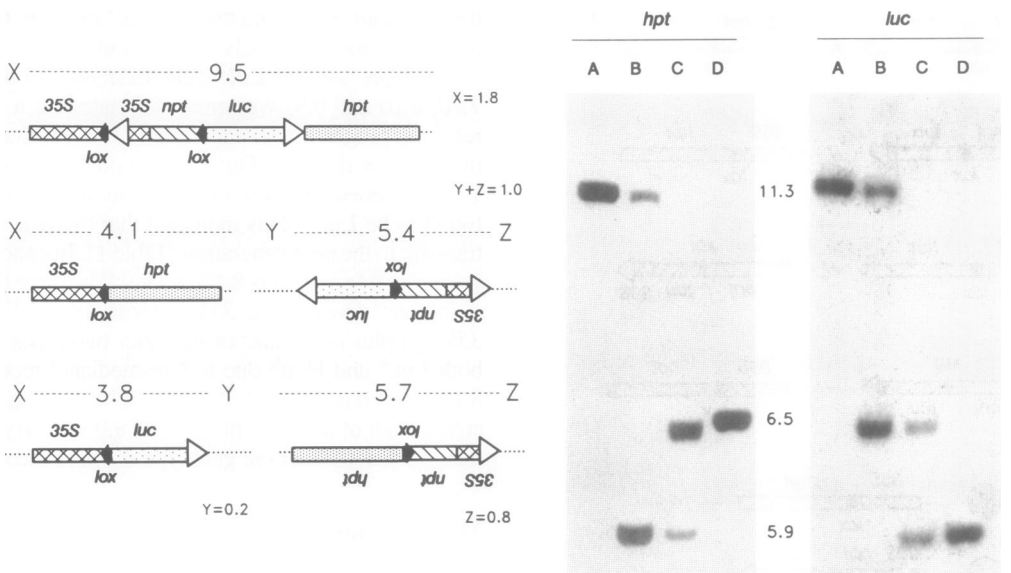

Figure 3. Southern blot analysis of a chromosome rearrangement. DNA was digested with *Dra*I and probed sequentially with *hpt* and *luc*. Lanes A–D correspond to DNA from plants containing the original transgene (512, diagrammed in the upper left), the transposed transgene (8, middle left), the recombined transgene (8.10, lower left) and the rearranged chromosome segregated away from the Cre source (8.10.40), respectively. On the left side of the figure is a demonstration of the conservation of genetic material after transposition and recombination. Using both the sizes of the constructs (indicated in kb above each) and the size of bands detected in the autoradiogram, the size (labeled X, Y and Z) of the regions between the genomic *Dra*I sites and the constructs was deduced. For example, the *Ds* excision appears precise, since the size of the chromosomal DNA flanking the T-DNA is the same after transposition (5.9–4.1 = 1.8) as before transposition (11.3–9.5 = 1.8).

The molecular structures in these plants were also examined by Southern blotting. Genomic DNA was digested with *Dra*I, which cuts once at the end of the T-DNA insertion, and the blot was probed sequentially with *luc* and *hpt* DNA. Figure 3 shows a representative blot for the set of plants 512, 8, 8.10 and 8.10.40. Both the *hpt* and *luc* probes detect an 11.3 kb band (lane A) in the 512 DNA corresponding to the original transgenic locus (9.5 kb from the T-DNA plus 1.8 kb of chromosomal DNA labeled X, Fig. 3). After *Ds* transposition (lane B), *hpt* DNA detected a 5.9 kb band representing the T-DNA locus after excision of the 5.4 kb *Ds–lox* element. The original T-DNA locus is also detected in the chimeric plant. The new locus of the relocated *Ds–lox* element was detected by *luc* DNA as a 6.4 kb band consisting of the 5.4 kb element plus flanking chromosomal DNA of 0.2 and 0.8 kb (labeled Y and Z, respectively, in Fig. 3). After Cre-mediated recombination (lane C), *hpt* DNA hybridized to a band of ~6.5 kb that resulted from moving *hpt* next to a different chromosomal *Dra*I site. A correspondingly smaller 5.8 kb band was detected by the *luc* probe, the result of bringing *luc* next to the *Dra*I site that was previously next to *hpt*. Both *hpt* and *luc* probes detected only the newly generated rearranged bands in the 8.10.40 plant (lane D). These results are consistent with the stable transmission of an inversion event from plant 8.10 to 8.10.40. Similar Southern blotting results were obtained for the sets of plants 512, 9 and 9.9; and 512, 7 and 7.70 (data not shown), which agrees with the PCR analysis that lines 7, 8 and 9 all yielded inversion events.

For plant 5.16, no rearrangement could be detected by Southern blot analysis. This indicates that the putative deletion in 5.16, deduced from PCR analysis, is present in only a small fraction of the cells in the plant. Consistent with this, the Luc$^+$ phenotype in 5.16 is rather 'weak', with ~50-fold less luciferase activity than in plants 7.70, 8.10 and 9.9.

DISCUSSION

We have shown that the Cre–*lox* system can generate intra-chromosomal recombination events. We conclude that three plant lines gave rise to inversions since: (i) *Ds–lox* moved to a linked site in each case; (ii) all introduced DNA elements are present after recombination; (iii) the rearrangements caused a reciprocal exchange of flanking chromosomal DNA. The inversions appear to be simple and precise events with predictable new junctions. From our data, we found no evidence of unexpected rearrangements.

A major factor limiting the recovery of chromosomal rearrangement events appears to be their transmissibility. Plant 8.10 produced Luc$^+$ progeny that can be either Hyg^R or Hyg^S. In the latter case, such as in 8.10.40, the *cre* gene is not detectable in the genome. Hence, the Hyg^S plant 8.10.40 exemplifies a stable transmission of a Cre–*lox*-generated chromosome inversion. Although plant 9.9 transmitted its inversion locus to progeny, these Luc$^+$ progeny were invariably Hyg^R. Southern blot analysis showed that these Luc$^+$ plants maintained a copy of the *cre* gene and that the *lox*-flanked DNA was present in both the inverted and the non-inverted state. As *cre* was introduced from another genome, it could not be linked to the *lox*-flanked DNA. Hence, this pseudo-linkage between these two loci suggests preferential selection of gametes. It is possible that gametes harboring the inversion in 9.9 are inviable and that the Luc$^+$ progeny resulted from the co-segregation of *cre* and a *lox*-flanked non-inverted locus. Cre-mediated recombination at a later stage of development subsequently produced the chimeric Luc$^+$ Hyg^S/Luc$^-$ Hyg^R phenotype. Alternatively, since 9.9 appears to be a chimeric plant, the reproductive tissue of this plant might have contained only the chromosome without an inversion. In this case, Luc$^+$ progeny

could only result from co-segregation of *cre* and a *35S–hpt* locus followed by somatic *cre*-mediated generation of a *35S–luc* junction.

In contrast to 8.10 and 9.9, we failed to observe transmission of the deletion in 5.16 and the inversion in 7.70. The simplest interpretation would be that gametes harboring these rearrangements are not viable. Deletions are particularly prone to lethality, as they can lead to loss of an essential function in the haploid gametophyte (40). Lethality could result from transmission of a rearranged locus or a combination of the *cre* gene along with a non-rearranged locus. In the latter case, an assumption would be that Cre-mediated recombination occurs efficiently during the haploid stage. If that were the case, then viable gametes with the *lox*-flanked locus would only be those without a co-segregating *cre* gene. It is interesting to note that Luc^+ progeny could be formed by combining separate gametes carrying *cre* or the *lox*-flanked DNA, but could not be formed from a gamete harboring the same two loci. This type of selection for hybrid plants could have applications in commercial seed production.

The co-transmission frequencies for *Ds–lox* and T-DNA (Table 1) in lines 7 and 9 suggest that the *Ds–lox* element is ≤5 cM from the primary T-DNA locus, whereas in line 8, the *Ds–lox* element appears further away, at ~10 cM. It is interesting that the inversion derived from line 8 transmitted stably to progeny, whereas the ones from lines 7 and 9 did not. This suggests that factors other than size may be important for transmissibility of the rearrangement at this chromosomal locus.

This system for generating chromosomal rearrangements has several advantages over other methods: (i) rearrangements can be identified through the phenotypes caused by reporter genes; (ii) the same rearrangement can be generated repeatedly from the same *Ds–lox* transposed parental lines; (iii) in the case of inversions and translocations, the rearrangements can be reversed upon re-introduction of *cre*; (iv) the boundaries of the rearrangements are molecularly marked by *lox* sites. These *lox* sites can essentially be treated as restriction endonuclease sites with a 34 bp recognition sequence, since inter-molecular recombination between chromosomal and exogenously provided *lox* oligonucleotides can split the chromosome at that site. With that in mind, it should be possible to isolate megabase size DNA from plants containing the rearrangement by reacting the DNA with Cre and a *lox* oligonucleotide *in vitro* and separate the *lox*-flanked chromosomal segment from the rest of the genomic DNA by pulsed-field gel electrophoresis. This would allow for a direct estimation of the genetic and physical distances between the rearrangement breakpoints and for the cloning of specific chromosomal segments into large DNA vectors such as YACs. We are currently working on developing this technique with plant DNA.

While this method has several advantages, we feel there are drawbacks that limit its practical implementation on a large scale (41). An important limitation is the time required to generate each rearrangement. The method requires a transformation followed by a minimum of two cross-pollinations (four crosses if one avoids using chimeric plants). A considerable amount of space and effort is also required. For each transformed line, several independent transformants must be isolated. Each transformant must then be cross-pollinated and the resulting progeny screened for a rearrangement. Improvements to this system are possible. If targeted insertion becomes practical in plants, as it currently is in some animals and fungi, then *lox* sites can be targeted into specific regions of the chromosome. In that instance, the targeted *lox* sites would pre-select regions for Cre-mediated rearrangements *in vivo* as well as the possible cloning of the *lox*-flanked DNA *in vitro*.

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