

Site-specific integration of DNA into wild-type and mutant *lox* sites placed in the plant genome

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Summary

The bacteriophage P1 Cre-*lox* site-specific recombination system has been used to integrate DNA specifically at *lox* sites previously placed in the tobacco genome. As integrated molecules flanked by wild-type *lox* sites can readily excise in the presence of Cre recombinase, screening for mutant *lox* sites that can resist excisional recombination was performed. In gene integration experiments, wild-type and mutant *lox* sites were used in conjunction with two strategies for abolishing post-integration Cre activity: (i) promoter displacement of a *cre*-expression construct present in the target genome; and (ii) transient expression of *cre*. When the promoter displacement strategy was used, integrant plants were recovered after transformation with constructs containing mutant *lox* sequences, but not with constructs containing wild-type *lox* sites. When *cre* was transiently expressed, integrant plants were obtained after transformation with either mutant or wild-type *lox* sites. DNA rearrangements at the target locus were less frequent when mutant *lox* sites were used. DNA integration at the genomic *lox* site was usually without additional insertions in the genome. Thus, the Cre-*lox* site-specific recombination system is useful for the single-copy integration of DNA into a chromosomal *lox* site.

Introduction

With current plant transformation methods, insertion of DNA into the genome occurs randomly and in many instances at multiple sites (for review, see Rogers, 1991; Weising *et al.*, 1988). Associated position effects, copy number differences and multigene interactions can make gene expression experiments difficult to interpret and plant phenotypes less predictable. Gene integration via homologous recombination, though successful in fungal

and animal systems, is not yet practical in plants. DNA integration into the plant genome through homologous recombination occurs at very low frequencies compared with the high background of illegitimate recombination events (Halfter *et al.*, 1992; Lee *et al.*, 1990; Miao and Lam, 1995; Offringa *et al.*, 1990; Paszkowski *et al.*, 1988).

An alternative means to achieve site-directed integration of DNA into the plant genome may be through the use of site-specific recombination systems. In yeast and mammalian cells, this integration has been reported for the bacteriophage P1 Cre-*lox* (Fukushige and Sauer, 1992; Sauer and Henderson, 1990) and the *Saccharomyces cerevisiae* FLP-*FRT* systems (O’Gorman *et al.*, 1991). Each system utilizes a small single-polypeptide recombinase, Cre or FLP, and a minimal 34 bp recombination site, *lox* or *FRT*. The simplicity of the two systems has a drawback in that recombination events are freely reversible. In site-specific DNA integration, recombination between a site on a circular molecule and a site on a chromosome results in the insertion of the circular DNA into the chromosome (Figure 1). However, the integrated DNA is flanked by two recombination sites of the same orientation and can therefore excise if the recombinase is present. Since intramolecular excision is kinetically favored over bi-molecular integration, insertion products are inherently unstable in the presence of the recombinase.

In yeast and mammalian cells, a successful strategy used to obtain stable insertion products was to curtail post-integration recombinase activity through the transient provision of recombinase (Baubonis and Sauer, 1993; Fukushige and Sauer, 1992; O’Gorman *et al.*, 1991; Sauer and Henderson, 1990). A second attempted strategy was to introduce selective base changes into recombination sites such that the product sites of a recombination reaction are less likely to undergo further recombination (Senecoff *et al.*, 1988).

Both *lox* and *FRT* sites are composed of an asymmetric 8 bp spacer flanked by 13 bp inverted repeats as depicted in Figure 1. Each of the 13 bp elements of *lox* or *FRT* is a binding site for Cre or FLP, respectively, with cooperative binding of two recombinase molecules per 34 bp site (Argos *et al.*, 1986; Bruckner and Cox, 1986; Mack *et al.*, 1992). Here we abbreviate the left and right 13 bp elements that flank the 8 bp spacer as LE and RE, respectively. If nucleotide changes were made in one of the 13 bp elements as depicted in Figure 1, recombination between a site with a mutation in the LE (LE mutant site) and a site with a mutation in the RE (RE mutant site) would produce two recombination product sites: one with mutations in neither

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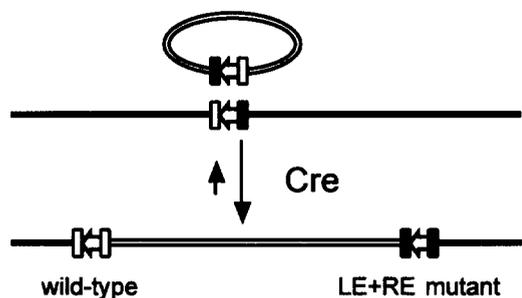


Figure 1. Site-specific recombination between a *lox* site on a plasmid and a *lox* site on a chromosome inserts the circular molecule into the chromosome.

The *lox* site is depicted as a tripartite structure with an asymmetric 8 bp spacer (arrowhead) and two 13 bp elements (rectangles). Filled rectangles indicate mutant elements. Recombination between a LE (left element) mutant site and a RE (right element) mutant site produces a wild-type and a LE+RE mutant site. If the LE+RE mutant site is poorly recognized by Cre, then the inserted molecule is less likely to excise (depicted by a shorter arrow).

LE nor RE (wild-type site) and the other with mutations in both LE and RE (LE+RE mutant site). If the LE+RE mutant site has reduced binding affinity for the recombinase, subsequent recombination between the wild-type site and the LE+RE mutant site would be less probable. In effect, the 'forward direction' of recombination (between LE mutant and RE mutant sites for DNA integration) would be favored over the 'reverse direction' (between wild-type and LE+RE mutant sites for DNA excision).

In this paper, we report a series of experiments which identified three sets of mutant *lox* sites that favor the forward over the reverse direction of recombination and therefore might be useful for DNA integration. Two of these sets were tested in gene integration experiments. In addition to using mutant *lox* sites to stabilize integration events, two strategies for abolishing post-integration Cre activity were also examined: the insertional inactivation of *cre* expression and the transient expression of *cre*. From these gene integration experiments, we conclude that:

- (i) the Cre-*lox* system can direct the site-specific integration of exogenous DNA into the plant genome;
- (ii) the mutant *lox* sites tested yielded site-specific integration events at frequencies comparable to or better than those obtained with wild type *lox* sites;
- (iii) DNA rearrangements were less frequently associated with mutant sites than with wild-type sites; and
- (iv) more often than not, a single copy insertion at the chromosomal target was found without additional integrations in the same genome.

Results

In vivo analysis of altered *lox* sites

The effects of mutant *lox* sequences on recombination were assessed using two oppositely oriented *lox* sites in a

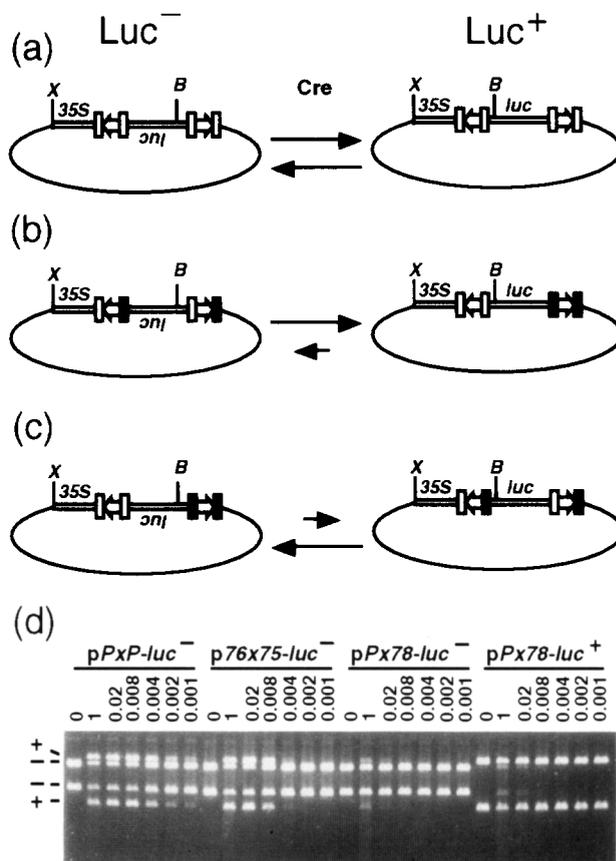


Figure 2. Schematic representation of *in vivo* and *in vitro* analyses of mutant *lox* sites.

The *lox* site is depicted as a tripartite structure with an asymmetric 8 bp spacer (arrowhead) and two 13 bp elements (rectangles). Filled rectangles indicate mutant elements. Inverted lettering indicates inverted orientation of *luc* with respect to 35S. Restriction sites shown are *Xho*I (X) and *Bst*EII (B). Cre-*lox* recombination converts a *Luc*⁻ construct to a *Luc*⁺ configuration which produces luciferase activity. Length of arrow indicates relative efficiency of recombination. Shown are: (a) *pPxP-luc*⁻ (left) converting into *pPxP-luc*⁺ (right). (b) The *Luc*⁻ construct (left), representing *p66x71-luc*⁻, *p76x75-luc*⁻ or *p43x44-luc*⁻, with a LE mutant site and a RE mutant site used to test the forward reaction. The *Luc*⁺ recombination product (right), representing *pPx72-luc*⁺, *pPx78-luc*⁺, or *pPx65-luc*⁺, carries a wild type and a LE+RE mutant site. (c) The *Luc*⁻ construct (left), representing *pPx72-luc*⁻, *pPx78-luc*⁻ or *pPx65-luc*⁻, carries the two *lox* sites of the *Luc*⁺ product shown in (b) and was used to test the reverse reaction. (d) Representative *in vitro* analysis of Cre-*lox* recombination. Designated plasmid substrates were incubated with the indicated units of Cre and cleaved with *Xho*I and *Bst*EII to detect the *Luc*⁻ (-) or *Luc*⁺ (+) configuration.

plasmid. Recombination between two sites in opposite orientations inverts the intervening DNA. Figure 2(a, left) shows *pPxP-luc*⁻, a *Luc*⁻ plasmid with wild-type *lox* sites (designated as *loxP*). The luciferase coding sequence (*luc*) is in the anti-sense direction with respect to the cauliflower mosaic virus 35S RNA promoter (35S) and is surrounded by two oppositely oriented *loxP* sites. When transiently introduced into tobacco protoplasts along with *p35S-cre* (a construct that expresses *cre*), Cre-mediated recombination in *pPxP-luc*⁻ inverts *luc* to form the *Luc*⁺ construct

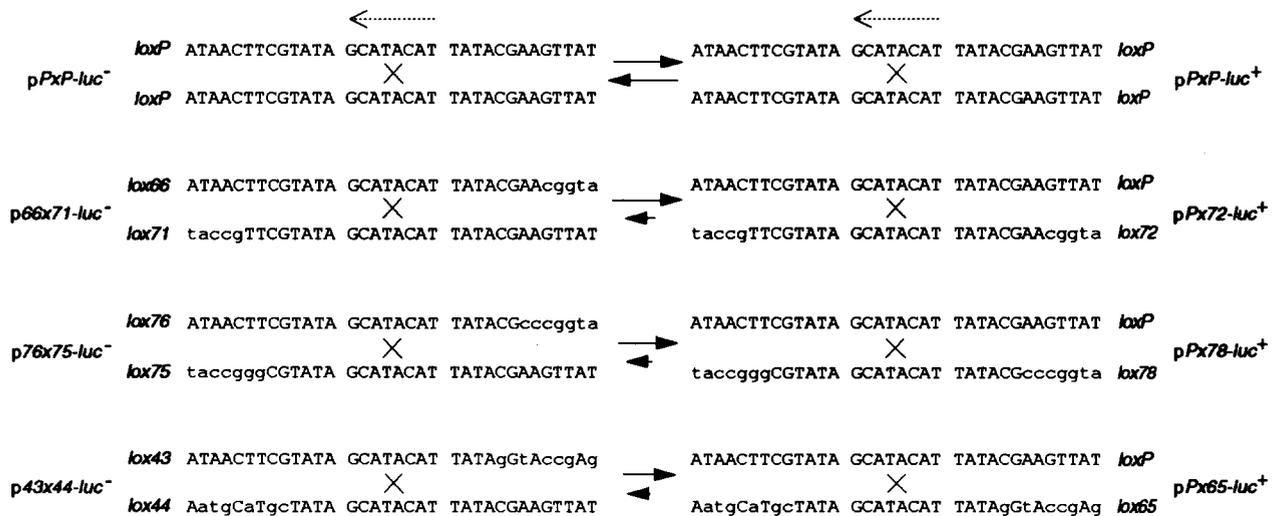


Figure 3. 5' to 3' (left to right) nucleotide sequence of lox sites within constructs described in Figure 2, Tables 1 and 2.

For example, p66x71-luc⁻ (see Figure 2b) harbors lox66 and lox71, while pPx72-luc⁺ or pPx78-luc⁺ (see Figure 2c) harbors loxP and lox72, the products of recombination between lox66 and lox71. Nucleotide base changes are denoted by lower case letters. Solid arrows between pairs of lox sites indicate the relative recombination efficiencies in the forward and reverse directions. 'X' indicates recombination between two lox sites. Arrows with dotted lines depict the orientation of the 8 bp spacer as drawn in the other figures and as defined by Hoess *et al.* (1982).

pPxP-luc⁺ (Figure 2a, right) which produces luciferase activity. Since both lox sites are wild-type, product sites are indistinguishable from substrate sites and the recombination reaction is freely reversible.

Plasmids isogenic to pPxP-luc⁻, but with a pair of one LE and one RE mutant site, were tested for the formation of the Luc⁺ configuration (Figure 2b). The two product sites of the Luc⁺ construct are loxP and a LE+RE mutant site. Since reversion of Luc⁺ to a Luc⁻ configuration produces a loss-of-function phenotype, the reverse reaction was assessed indirectly with another Luc⁻ construct that harbors the loxP and the LE+RE mutant sites as shown in Figure 2(c). If the LE+RE mutant site in this construct is indeed poorly bound by Cre, then few Luc⁺ derivatives would arise from recombination to produce luciferase activity. Sets of 'forward and reverse constructs' were screened by this transient expression assay. Pairs of mutant lox sites were not considered further if the mutations abolished the forward reaction or if they failed to show a more efficient forward than reverse reaction. In this paper, we report only the three sets of plasmid constructs and their mutant lox sequences that were found to have a forward reaction more efficient than the corresponding reverse reaction. Specifically, the forward constructs p66x71-luc⁻, p76x75-luc⁻, and p43x44-luc⁻ (Figure 2b), produced greater luciferase activity than did their corresponding reverse constructs pPx72-luc⁻, pPx78-luc⁻, and pPx65-luc⁻ (Figure 2c). Figure 3 shows the wild-type and mutant lox sequences with their allelic designations.

Cre-lox recombination for these three sets of mutant lox sites was analyzed over a range of p35S-cre DNA concentrations. As shown in Table 1, maximal difference in

luc expression between the forward and reverse constructs depended on the amount of recombinase provided by p35S-cre. At low Cre concentrations (0.1–1 μg p35S-cre), p66x71-luc⁻ and p76x75-luc⁻ produced six- to sevenfold more luciferase activity than did pPx72-luc⁻ and pPx78-luc⁻, respectively. In contrast, higher amounts of Cre (10–20 μg p35S-cre) were required to achieve a four- to five fold difference between p43x44-luc⁻ and pPx65-luc⁻. Compared with pPxP-luc⁻, lower luc expression was observed with p43x44-luc⁻ and p76x75-luc⁻, indicating that the recombination between lox43 and lox44, or between lox76 and lox75, is less efficient than between two loxP sites. By contrast, p66x71-luc⁻ consistently produced greater luciferase activity than pPxP-luc⁻. The higher luciferase activity, however, does not necessarily indicate that the recombination between lox66 and lox71 is more efficient than between loxP sites. The efficiency of the forward reaction minus the efficiency of the reverse reaction would determine the net pool of Luc⁺ molecules and thus the level of luciferase activity observed.

In vitro analysis of mutant lox sites

It is possible that the *in vivo* effects observed with the mutant lox sites were caused by steric hindrance of a nearby DNA binding protein, and not by the introduced base changes. Thus, recombination of the plasmids was also analyzed *in vitro* with purified Cre recombinase. Plasmid substrates were incubated with a range of Cre concentrations followed by cleavage with endonucleases XhoI and BstEII to determine the orientation of the luc fragment. Figure 2(d) shows a representative analysis of

Table 1. *In vivo* analysis of *lox* site activity

p35S- <i>cre</i> (μ g DNA)	Relative luciferase activity of test construct (%) ^a			
	Control	Forward	Reverse	Relative efficiency ^b
	pPxP- <i>luc</i> ⁻	p66x71- <i>luc</i> ⁻	pPx72- <i>luc</i> ⁻	
0.1	9.3	17.6	2.6	6.8
1	62.0	104	13.8	7.5
10	100	152	26.9	5.7
20	120	284	77.2	3.7
50	143	283	104	2.7
	pPxP- <i>luc</i> ⁻	p76x75- <i>luc</i> ⁻	pPx78- <i>luc</i> ⁻	
0.1	12	5.8	0.9	6.4
1	67.5	34.8	5.4	6.4
10	100	35.4	12.6	2.8
20	112	38.7	29.1	1.3
50	147	39.0	51.4	0.8
	pPxP- <i>luc</i> ⁻	p43x44- <i>luc</i> ⁻	pPx65- <i>luc</i> ⁻	
0.1	8.7	7.0	3.4	2.1
1	39.6	7.2	4.0	1.8
10	100	21.1	4.9	4.3
20	147	36.6	6.5	5.6
50	184	43.7	12.7	3.4

^aTest construct (10 μ g) co-transformed with p35S-*cre* (0.1–50 μ g). Relative luciferase activity (mean from three independent experiments, standard error less than 25%) is normalized to the value obtained from 10 μ g each of pPxP-*luc*⁻ and p35S-*cre*.

^b Activity of the forward construct over the activity of the reverse construct.

one set of plasmids. The minimum amount of Cre at which recombination ('+' configuration product bands) was detected from p76x75-*luc*⁻ was 0.004 Cre units, fourfold higher than the 0.001 units required for pPxP-*luc*⁻ and fivefold lower than the 0.02 units needed for pPx78-*luc*⁻ ('+' configuration product bands) and pPx78-*luc*⁺ ('-' configuration product bands). This agrees with the *in vivo* data that recombination of *lox76* and *lox75* is more efficient than the reverse reaction between *loxP* and *lox78*, but less efficient than the recombination between two *loxP* sites.

In vitro recombination experiments for the other mutant sites (data not shown) were also consistent with results from transient expression assays. *In vitro* recombination of constructs with *lox43* and *lox44*, or with *loxP* and *lox65*, needed a minimum of 0.008 or 0.04 Cre units, respectively. Detectable recombination between *lox66* and *lox71*, or between *loxP* and *lox72*, required 0.002 or 0.008 Cre units, respectively. With each pair of mutant *lox* sites, the forward reaction was favored over the reverse reaction by roughly fivefold. Compared with recombination with *loxP* sites, the recombination efficiencies are approximately 50% for *lox66* and *lox71*, approximately 25% for *lox76* and *lox75*, and approximately 12% for *lox43* and *lox44*.

Stable transformation of plants through DNA integration into genomic *lox* sites

After identifying three sets of *lox* sites with directional recombination activity, the two most promising sets (*lox66*/

lox71 and *lox76*/*lox75*) were incorporated into genomic target constructs and corresponding insertion plasmids (Table 2). The *lox43*/*lox44* set was not used because it has the least efficient forward reaction and might therefore integrate infrequently (Table 1). Two types of target constructs, reflecting the two strategies for controlling Cre activity, were placed into the tobacco genome through *Agrobacterium*-mediated gene transfer. Protoplasts derived from these transgenic 'parent' plants were used for direct DNA transformation by the insertion plasmids.

DNA integration with cre expression terminated through promoter displacement

One strategy tested to abolish post-integration recombinase activity was the insertional inactivation of *cre*. A transgenic parent plant harboring 35S-*lox-cre* was transformed with a plasmid carrying a promoterless *lox-hpt* construct, where *hpt* encodes hygromycin phosphotransferase (Figure 4a). The transgenic parent synthesizes Cre, which mediates insertion of the circular molecule into the genomic *lox* site to produce a 35S-*lox-hpt-lox-cre* linkage. Integration of *lox-hpt* displaces 35S from *cre* to terminate *cre* transcription and fuses 35S to *hpt* to confer a hygromycin-resistant (Hyg^R) phenotype. This strategy was tested using *loxP* and two sets of mutant *lox* sequences, *lox66*/*lox71* and *lox76*/*lox75* (Figure 3).

The 35S-*lox66-cre*, 35S-*lox76-cre* and 35S-*loxP-cre* constructs were introduced into *Nicotiana tabacum* to

Table 2. Efficiency of Cre-mediated versus random DNA integration

Plant line	Experiment no.	Hyg ^R calli per 10 ⁶ protoplasts obtained from transformation with construct		Relative efficiency (%) ^a
		p35S-hpt	plox71-hpt	
nt35S-lox66-cre.5	1	200	16	8
"	2	789	756	96
nt35S-lox66-cre.M	3	40	< 0.1	
"	4	29	< 0.1	
"	5	23	6.6	29
"	6	414	238	58
		p35S-hpt	plox75-hpt	
nt35S-lox76-cre.3	7	44	0.7	1.6
"	8	1000	790	79
"	9	82	< 0.13	
nt35S-lox76-cre.A	10	15	< 0.04	
"	11	557	318	57
"	12	453	429	95
		p35S-hpt	ploxP-hpt	
nt35S-loxP-cre.3	13	2.7	< 0.07	
"	14	20	< 0.1	
"	15	67	< 0.1	
"	16	31	< 0.13	
"	17	25	< 0.13	
nt35S-loxP-cre.5	18	870	< 0.05	
"	19	24	< 0.05	
"	20	19	< 0.13	
		p35S-hpt	plox75-hpt	
nt35S-lox76-luc	21	< 0.5	6.8	
"	22	58	< 0.04	
"	23	86	< 0.1	
"	24	146	24	16
"	25	262	136	52
"	26	238	< 0.13	
"	27	14	318	2270
		p35S-hpt	ploxP-hpt	
nt35S-loxP-luc	28	625	< 0.14	
"	29	200	13	6.5
"	30	5.9	< 0.05	
"	31	58	< 0.1	
"	32	497	112	23
"	33	168	111	66
"	34	< 0.13	72	

^a Efficiency of Cre-lox integration over random integration by p35S-hpt.

generate transformed lines nt35S-lox66-cre, nt35S-lox76-cre and nt35S-loxP-cre, respectively. Two independently transformed lines of each type were chosen for DNA integration (Table 2). The selection of these lines was based on Southern analysis in which a single junction fragment hybridized to the T-DNA right border probe. While the lone fragment suggested a single hemizygous copy of the transgene, subsequent analysis revealed that one of the lines, nt35S-lox66-cre.5, harbored an additional but rearranged copy of the 35S-lox66-cre construct (data not shown).

Leaf mesophyll protoplasts from vegetatively propagated parent plants were transformed with plox71-hpt, plox75-hpt or ploxP-hpt. The transformed protoplasts were regenerated in the presence of hygromycin. As transformation efficiencies varied widely among independent experiments, no attempt was made to average the data (Table 2). To compare random versus Cre-lox mediated integration, aliquots from each batch of protoplasts were also transformed with p35S-hpt. This plasmid lacks a lox site and does not require integration into the 35S-lox-cre target to confer a Hyg^R phenotype.

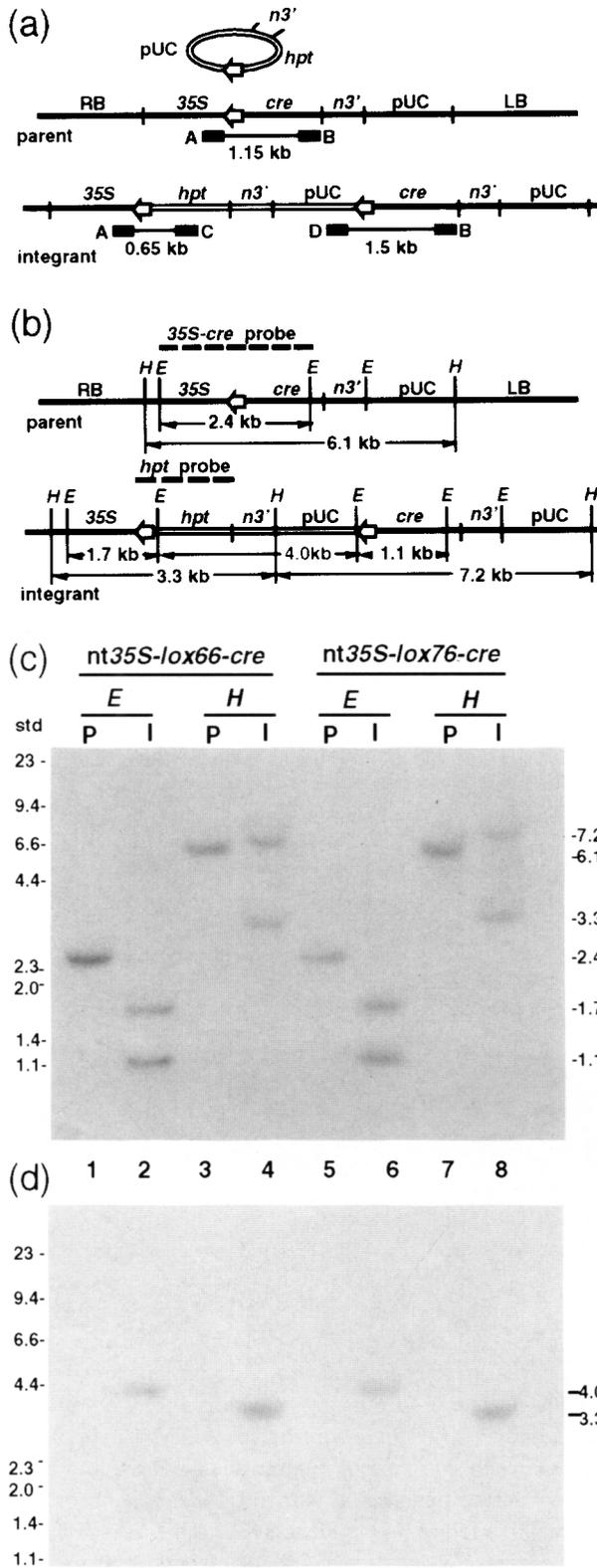


Figure 4. Molecular analyses of *plax-hpt* integration into *nt35S-lox-cre* targets.

(a) Schematic maps representing the *nt35S-lox66-cre*, *nt35S-lox76-cre* or *nt35S-loxP-cre* target construct in the parent and the expected arrangement in the integrant after insertion of *plox71-hpt*, *plox75-hpt* or *ploxP-hpt*, respectively. Shown are PCR primers (filled boxes A-D), expected sizes of the PCR products, *lox* sites (arrowheads), T-DNA right (RB) and left (LB) borders, and the *nos3'* transcription termination region (*n3'*).

(b) *EcoRI* (E) and *HindIII* (H) maps of the genomic targets before and after integration of the *lox-hpt* plasmid.

(c) DNA from parent (P) and representative integrant (I) plants was cleaved with either *EcoRI* (E) or *HindIII* (H) and probed with a 2.4 kb *EcoRI* 35S-*cre* fragment (b, dashed line) from *p35S-cre*.

(d) To determine *hpt* transgene copy number, a similar blot was probed with a 1.35 kb *SaI* *hpt* fragment (b, dashed line) from *p35S-hpt*. Numbers on the left of the autoradiograms correspond to size standards and numbers on the right correspond to sizes of predicted fragments (in kb).

Despite repeated efforts with two independently transformed *nt35S-loxP-cre* lines, calli with a stable *Hyg^R* phenotype were not recovered after transformation with *ploxP-hpt* (Table 2, experiment no. 13–20). Microcalli were observed in some transformation experiments, but they did not sustain viability in the presence of selection. These protoplasts were competent for transformation as *Hyg^R* calli were recovered from the same *nt35S-loxP-cre* protoplasts transformed with *p35S-hpt*. With the mutant *lox* site constructs, *Hyg^R* calli were obtained after introducing *plox71-hpt* into either *nt35S-lox66-cre* line (experiment no. 1, 2, 5 and 6). Likewise, both *nt35S-lox76-cre* lines yielded *Hyg^R* calli when transformed with *plox75-hpt* (experiment no. 7, 8, 11 and 12). In five of 12 experiments (experiment no. 2, 6, 8, 11 and 12), Cre-*lox*-mediated transformation efficiencies were comparable to those obtained with the *p35S-hpt* reference construct. From representative *Hyg^R* calli, plants were regenerated for molecular analyses.

Analysis of integration into 35S-lox-cre targets

Thirty-one *Hyg^R* plants derived from transformation of plants harboring either *35S-lox66-cre* or *35S-lox76-cre* were examined by polymerase chain reaction (PCR) analysis. Figure 4(a) diagrams the expected PCR products derived from primer pairs A-B, A-C and D-B. Unlike parent plants, the 1.15 kb PCR product, representing the 35S-*lox-cre* junction, was absent from all integrant plants. With primer pairs A-C and D-B, PCR products representing the upstream 35S-*lox-hpt* and downstream pUC-*lox-cre* junctions were not detected from parental DNA. The 0.65 kb A-C PCR product was found in all integrants, but the 1.5 kb D-B PCR product was detected in only 25 of 31 integrant plants (13 of 15 *nt35S-lox66-cre*.5-, two of two *nt35S-lox66-cre*.M-, six of 10 *nt35S-lox76-cre*.3- and four of four *nt35S-lox76-cre*.A-derived integrants). The lack of a D-B

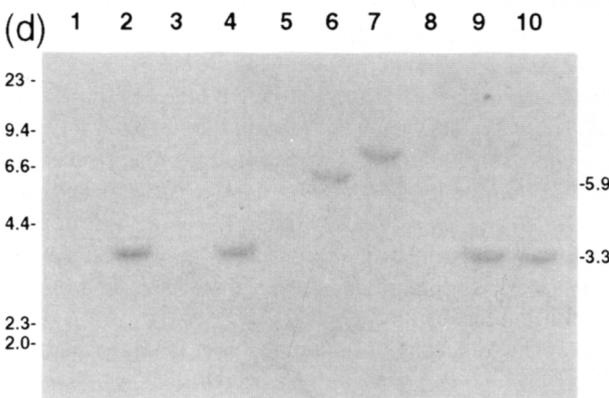
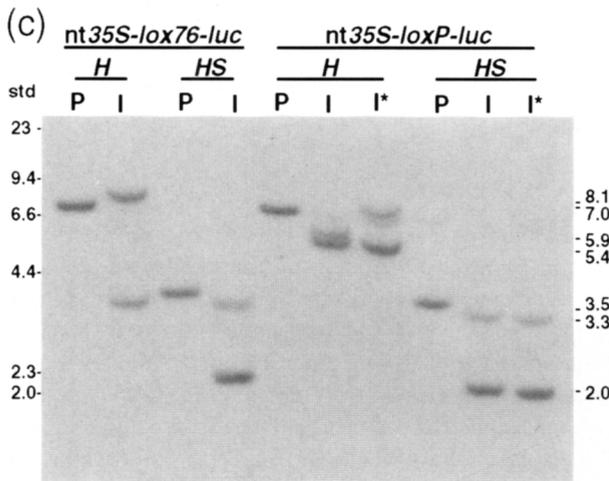
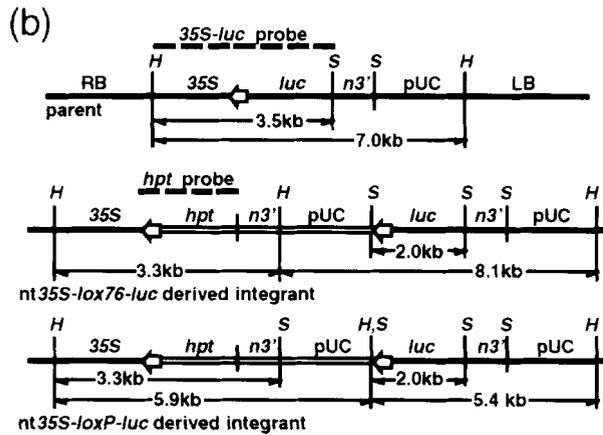
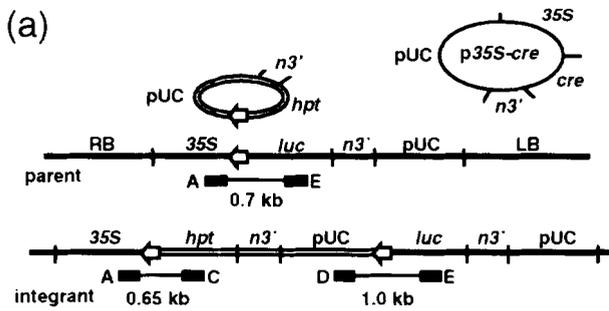


Figure 5. Molecular analyses of *plox-hpt* integration into *nt35S-lox-luc* targets.

(a) Schematic maps representing the *nt35S-lox76-luc* or *nt35S-loxP-luc* target construct in the parents and the expected arrangement in the integrants after insertion of *plox75-hpt* or *ploxP-hpt*, respectively. Shown are PCR primers (filled boxes A, C-E), the expected sizes of the PCR products, *lox* sites (arrowheads), T-DNA right (RB) and left (LB) borders, and the *nos3'* transcription termination region (*n3'*).

(b) *HindIII* (H) and *SacI* (S) maps of the genomic targets before and after integration of the *lox-hpt* plasmid.

(c) DNA from parent (P), precise integrant (I) or imprecise integrant (I*) plants was cleaved with *HindIII* alone (H) or in combination with *SacI* (HS). The Southern blot was probed with a 3.5 kb *HindIII-SacI* 35S-*luc* fragment (b, dashed line) from pDO432 (Ow *et al.*, 1986).

(d) The same blot was reprobed with a 1.35 kb *SacI* *hpt* fragment (b, dashed line) from p35S-*hpt*. Numbers on the left of the autoradiograms correspond to size standards and numbers on the right correspond to sizes of predicted fragments (in kb).

PCR product suggests the possibility of a DNA rearrangement at or around the pUC-*lox-cre* junction.

Southern analysis was performed on parent (P) and representative integrant (I) plants that produced PCR products representing both upstream and downstream junctions (Figure 4b and c). With *EcoRI* (E) cleaved DNA, a 35S-*cre* probe hybridized to a single band of 2.4 kb in *nt35S-lox66-cre* and *nt35S-lox76-cre* parental DNA (Figure 4c, lanes 1 and 5), and two bands of 1.1 and 1.7 kb in integrant DNA (lanes 2 and 6). With *HindIII* (H) cut DNA, this same probe detected a 6.1 kb fragment from parent genomes (lanes 3 and 7), but 3.3 and 7.2 kb fragments from integrant genomes (lanes 4 and 8). These are the expected hybridization patterns for precise integration events.

DNA integration with transient production of recombinase

Transient *cre* expression was tested as another method to limit post-integration Cre activity. In this strategy, the genome harbored a 35S-*lox-luc* target and Cre was provided through the transient expression of p35S-*cre* (Figure 5a). The integration of the *lox-hpt* plasmid would be stably maintained if Cre production were terminated through the loss of p35S-*cre*. As before, the transcriptional fusion between 35S and *hpt* would confer a Hyg^R phenotype. This strategy was examined with mutant *lox76/lox75* and wild type *loxP* sites. A parent line harboring a genomic copy of either 35S-*lox76-luc* or 35S-*loxP-luc* was co-transformed with p35S-*cre* and either *plox75-hpt* or *ploxP-hpt*, respectively. Comparable numbers of transformants were obtained from the use of either mutant (Table 2, experiment no. 21, 24, 25 and 27) or wild-type *lox* sites (experiment no. 29 and 32-34). This contrasts with the results from the promoter displacement strategy, where stable integration events were not recovered using wild-type sites (experiment no. 13-20). Consistent with results from the promoter displacement strategy, Cre-*lox* insertions occur almost as frequently as random insertions by p35S-*hpt*.

Analysis of integration into 35S-lox-luc targets

As before, DNA from representative Hyg^R plants were analyzed by PCR. Figure 5(a) shows the primer pairs used and the expected sizes of the PCR products. Primer pair A-E amplified the 0.7 kb 35S-lox-luc junction fragment from parent plants, but from none of the 29 integrant plants examined. With primer pair A-C, the 0.65 kb PCR product representing the upstream 35S-lox-hpt junction was detected in all 29 integrant plants. With primer pair D-E, however, the 1.0 kb PCR product representing the downstream pUC-lox-luc junction was found in only 11 of 13 nt35S-lox76-luc- and eight of 16 nt35S-loxP-luc-derived integrants. Neither primer pairs A-C nor D-E produced a PCR product with parental DNA.

Figure 5(c) shows a representative Southern blot of nt35S-lox76-luc- and nt35S-loxP-luc-derived integrants considered to have precise upstream and downstream junctions, and of an nt35S-loxP-luc-derived integrant that failed to produce a downstream-junction PCR product. A 35S-luc probe hybridized to a single HindIII band of 7.0 kb (lanes 1 and 5) and a single HindIII-SacI (HS) band of 3.5 kb (lanes 3 and 8) in nt35S-lox76-luc or nt35S-loxP-luc parent plants. An insertion into the genomic target separates 35S from luc such that they reside on different restriction fragments. In a 'precise' nt35S-lox76-luc-derived integrant, HindIII bands of 3.3 and 8.1 kb were observed (lane 2). When cleaved with a combination of HindIII and SacI, bands of 2.0 and 3.3 kb were found (lane 4). In a 'precise' nt35S-loxP-luc-derived integrant, the same 2.0 and 3.3 kb bands were produced from cleavage with HindIII and SacI (lane 9). As the HindIII site in ploxP-hpt and therefore in the nt35S-loxP-luc-derived integrant genome is in a different location (see Figure 5b), HindIII bands of 5.4 and 5.9 kb were found (Figure 5c, lane 6). These hybridization patterns are expected for precise insertions.

In an 'imprecise' nt35S-loxP-luc-derived integrant (I*), the 5.4 kb HindIII fragment (lane 7) containing the luc-nos3'-pUC elements was present, but the expected 5.9 kb HindIII fragment representing the 35S-lox-hpt-nos3'-pUC elements was not. Instead, a new fragment of approximately 8 kb was found (lane 7). As the two fragments produced by cleavage with HindIII and SacI showed no change in size (lane 10), the rearrangement must lie outside the 35S-lox-hpt-nos3' and luc elements and within the intervening pUC sequence. This deduction is consistent with the DNA rearrangement suggested by the lack of an amplifiable PCR product from the pUC-lox-luc junction.

Infrequent additional insertion of DNA in the parent genome

As Cre-lox site-specific integration events do not occur more frequently than random insertions by a 35S-hpt

construct (Table 2), we wanted to determine whether there were additional copies of the lox-hpt plasmid inserted elsewhere in integrant genomes. Blots similar to those shown in Figures 4(c) and 5(c) were hybridized to hpt DNA. Figures 4(d) and 5(d) show that hpt hybridized only to integrant DNA and to a single prominent EcoRI, HindIII or HindIII-SacI fragment of the integration locus (Figure 4d, lanes 2, 4, 6 and 8; Figure 5d, lanes 2, 4, 6, 7, 9 and 10). The 1.7 kb 35S-lox EcoRI fragment in nt35S-lox66-cre- and nt35S-lox76-cre-derived integrants (Figure 4c) extends roughly 300 bp into the hpt coding sequence (Figure 4b) and consequently hybridized weakly with the hpt probe (Figure 4d, lanes 2 and 6). The single hpt-hybridizing HindIII fragment from the imprecise nt35S-loxP-luc-derived integrant is roughly 8 kb, the same size found using the 35S-luc probe (Figure 5c and d, lane 7). As this fragment spans the 35S-lox-hpt junction, it is not due to a random (non-Cre-lox) integration event. From a total of 14 integrant plants examined with the hpt probe (two nt35S-lox66-cre.5-, one nt35S-lox66-cre.M-, one nt35S-lox76-cre.3-, five nt35S-lox76-cre.A-, two nt35S-lox76-luc- and three nt35S-loxP-luc-derived integrants), only three nt35S-lox76-cre.A- and one nt35S-lox76-luc-derived integrants showed additional hpt-hybridizing fragments (data not shown). Hence, these four plants harbor both site-specific and random insertions.

Discussion

Mutant lox sites

In an effort to make the Cre-lox system an effective tool for site-directed integration of transgenes, we identified three pairs of mutant lox sites that have a forward reaction more favorable than the corresponding reverse reaction. The approach of using LE and RE mutant sites was first reported by Senecoff *et al.* (1988) for the FLP-FRT system, where single-base changes were introduced into the 13 bp elements of the FRT site. In an *in vitro* recombination assay, a set of LE and RE mutant sites was identified with a forward reaction 10-fold more favorable than its reverse reaction. Unfortunately, when compared with wild-type FRTs, the efficiency of recombination between the LE and RE mutant sites was fivefold lower. When tested for gene targeting in *Escherichia coli*, this set of mutant sites produced roughly 100-fold fewer integrants than did wild-type FRTs (Huang *et al.*, 1991). Thus, the enhanced stability conferred upon integrated molecules was outweighed by the far fewer integration events that occurred from an inefficient forward reaction.

For the three sets of mutant lox sites we described, the forward and reverse recombination reactions were studied by both *in vivo* and *in vitro* assays over a range of recombinase concentrations. As the lox43/lox44 pair of

sites showed the least efficient forward reaction, we focused our gene targeting efforts on the two pairs of mutant sites that might be more proficient in DNA integration.

Abolishing post-integration Cre activity

When the recombinase was supplied by transient *cre* expression, both mutant (*lox76/lox75*) and wild-type *lox* sites yielded integration events at comparable frequencies. However, when Cre was produced by the parent genome, stable integrants were obtained with only mutant *lox* sites. One possible explanation may be an incomplete inactivation of Cre activity. Although integration of the insertion plasmid results in promoter displacement and the presumed termination of *cre* transcription, prior constitutive expression of *cre* could have accumulated a large amount of recombinase which was slow to deplete. Alternatively or additionally, promoter displacement might have failed to abolish Cre production completely, but might have left a residual level of *de novo* synthesis. As utilization of the LE+RE mutant site requires higher Cre concentrations, low residual Cre activity would be more likely to delete inserts flanked by wild-type sites than those flanked by a combination of wild-type and LE+RE mutant sites.

Construct fidelity of the integration locus

PCR analysis showed that a number of integrant plants failed to maintain an intact downstream *lox* junction. Alterations of the downstream junction were also shown by representative Southern analysis (Figure 5c). These rearranged junctions may represent mutations that stabilized the inserts through inactivation of a *lox* site. The upstream junction was invariably intact, most likely because hygromycin selection imposes the need for a functional *35S-lox-hpt* linkage.

For integration into the *35S-lox-luc* target, comparable numbers of insertions were obtained using wild-type or mutant *lox* sites (Table 2, experiment nos 21–34). However, the number of precise insertions differed. From the PCR data, only 11 out of 13 nt*35S-lox76-luc*-derived integrants, and eight out of 16 nt*35S-loxP-luc*-derived integrants had the expected upstream and downstream junctions. By this criterion, most (85%) of the integration events using mutant sites and only half of the events involving wild-type sites were precise. Although the sample size is small, the difference in the precision of integration is statistically significant ($P < 0.05$). Perhaps, as excisions at *loxP* sites were more frequent, then spontaneous DNA rearrangements that prevented excision (such as rearrangements resulting in non-functional *lox* sites) would be recovered as a larger fraction of stable transformants.

Transgene copy number in Hyg^R integrants

In our experiments, transformants were identified based on the Hyg^R phenotype, which resulted from insertion of *lox-hpt* behind *35S* or some other promoter. Random insertion of *lox-hpt* into the genome without fusion to a promoter would not be detected. Thus, we do not know how frequently these silent illegitimate integration events occurred. All 60 Hyg^R plants analyzed by PCR had the *35S-lox-hpt* junction. This indicates that random insertion of *lox-hpt* behind another promoter is rare compared with Cre-lox-mediated integration.

A single-copy insertion at the target locus is not unexpected as additional copies would generate tandemly repeated *lox* sites that would be readily resolved by Cre-mediated excision. Integration at the target locus, however, does not preclude the insertion of additional copies of *lox-hpt* elsewhere in the genome. Indeed, four out of 14 plants revealed the presence of additional *hpt*-hybridizing fragments. With Cre-lox gene targeting experiments in mammalian cell cultures, Fukushige and Sauer (1992) reported only two out of 54 site-specific integrants had additional illegitimate integration events. The frequency that we observed for an accompanying random insertion is higher; none the less, a 70% rate of single-copy site-specific integration should be acceptable for most gene transfer experiments.

Future prospects

There are several potential uses of this system in plant research. Studies of gene expression involving transformation of plants with modified regulatory elements are often confounded by the variability of gene expression among independent transformants. This variability could be caused by chromosomal position effects at the sites of transgene integration (An, 1986; Odell *et al.*, 1987; Peach and Velten, 1991). Inserting test constructs into the same genomic target might control this variable and facilitate valid comparisons of expression levels. Since the substrate for integration is circular, one can maintain the positive selection strategy presented here while other transgenes can be included on the insertion plasmid. Independently transformed mammalian cell colonies with DNA inserted into the same *lox* site showed similar levels of reporter gene expression (Fukushige and Sauer, 1992). However, it remains to be shown whether this reduced variability in gene expression occurs in whole organisms where developmental and tissue-specific controls may exert their effects.

Multiple insertions of transgenes can also produce highly variable levels of gene expression in transgenic plants through 'co-suppression' (Kooter and Mol, 1993; van der Krol *et al.*, 1990; Linn *et al.*, 1990; Matzke and Matzke, 1991;

Napoli *et al.*, 1990). Use of the Cre-lox system should minimize the number of transformants with multiple transgenes and thereby reduce the efforts required to identify single-copy transformants. Further development of this system, possibly in combination with other site-specific recombination systems, might allow successive rounds of transformation with different genes directed to the same locus. The clustering of transgenes into a known locus could facilitate the introgression of new traits into elite field varieties.

While Cre-lox site-specific integration has many potential utilities, there are two drawbacks to the present system. First, a target site must be placed into the genome through conventional means, and currently this first integration event is not site-selective. Second, the system as described in this work requires direct DNA delivery. In many plant species, this is less convenient than the use of *Agrobacterium*-mediated gene transfer. Combining T-DNA delivery with Cre-lox integration may be a possibility. Alternatively, it may be possible to translocate the insertion construct from a random-integration site into the desired location through Cre-lox recombination. In this approach, the insertion construct would first be placed randomly into the plant genome, such as through *Agrobacterium*-mediated gene transfer. If the insertion construct is flanked by two lox sites of the same orientation, the introduction of Cre would then excise the insert and at some frequency integrate it into another lox site. With an appropriate selection scheme, it should be possible to identify the plant with the translocated molecule at the desired target locus. For example, if a plant with a lox-hpt-lox transgene is crossed to a plant with a 35S-lox-cre target, Cre-mediated recombination in the F₁ progeny should release a lox-hpt circular molecule *in planta*. Integration of the molecule into the 35S-lox-cre target would generate a 35S-lox-hpt-lox-cre linkage and a Hyg^R phenotype.

To conclude, DNA can be delivered into genomic lox sites at a workable frequency approaching that of random integration. The transgene is usually integrated as a single copy into the genomic target without additional insertions elsewhere in the same genome. Finally, the transgene retains construct fidelity in a large percentage of cases, particularly when mutant sites are used. Taken together, Cre-lox recombination offers a method for precise insertion of single-copy DNA into genomic targets.

Experimental procedures

DNA constructs

The 35S-cre construct pMM23 is called p35S-cre in this paper. It is isogenic to pED23 (Dale and Ow, 1990; Morgan and Ow, 1995), but the cre transcript lacks a prokaryotic ribosome binding site. The Luc⁻ plasmids shown in Figure 2 were made by inserting

synthetic lox sequences (mutant or wild-type, listed in Figure 3) on both sides of the luc fragment. pPx72-luc⁺, pPx78-luc⁺, and pPx65-luc⁺ were isolated from tobacco protoplasts transiently transformed with p35S-cre and p66x71-luc⁻, p76x75-luc⁻ or p43x44-luc⁻. The 35S-hpt construct pCaMvhygΔN is called p35S-hpt in this paper and has been described (Bayley *et al.*, 1992). The insertion plasmids plox71-hpt, plox75-hpt and ploxP-hpt contain one lox site followed by a promoterless hpt. The target constructs p35S-lox66-cre, p35S-lox76-cre, p35S-loxP-cre, p35S-lox76-luc and p35S-loxP-luc were linearized with HindIII and inserted into the HindIII site of the *Agrobacterium* vector pBIN19 (Bevan, 1984) such that 35S was proximal to the T-DNA right border. Further details of the above plasmids are available upon request.

In vivo and in vitro assays of recombination

Protoplasts from *Nicotiana tabacum* TXD suspension cell line were electroporated with CsCl-purified DNA, incubated for 16–18 h, and the cell extracts assayed for luciferase activity as described (Ow *et al.*, 1986). For *in vitro* analysis, plasmid DNA (0.35 µg) was treated with varying units of Cre (NEN Cat no. NEE-158) in 20 µl reaction buffer (50 mM Tris-HCl, pH 7.5, 33 mM NaCl, 10 mM MgCl₂, 25 µg ml⁻¹ BSA) at 37°C for 30 min and terminated at 65°C for 10 min. NaCl and DTT concentrations were adjusted to 100 mM and 1 mM, respectively, and the samples were incubated with XhoI and BstEII prior to gel electrophoresis.

Stable transformation

Co-integrate plasmids pBIN19::p35S-lox66-cre, pBIN19::p35S-lox76-cre, pBIN19::p35S-loxP-cre, pBIN19::p35S-lox76-luc and pBIN19::p35S-loxP-luc were mobilized into *Agrobacterium tumefaciens* GV3111(pTiB6S3SE) for leaf disc infection of *N. tabacum* (Wi38) (Horsch *et al.*, 1985) to produce transgenic lines nt35S-lox66-cre, nt35S-lox76-cre, nt35S-loxP-cre, nt35S-lox76-luc and nt35S-loxP-luc, respectively. Kanamycin-resistant plants were scored for the ability to form shoots from leaf explants on Murashige-Skoog (MS) medium containing 100 µg ml⁻¹ kanamycin sulfate. T-DNA copy number was assessed by Southern analysis of genomic DNA cleaved with HindIII (unique site in pBIN19) and hybridized to a T-DNA right border probe.

Polyethylene glycol (PEG)-mediated DNA transformation was performed on protoplasts isolated from young leaves essentially as described (Morgan and Ow, 1995; Paszkowski and Saul, 1986) except that after transformation, the protoplast-containing PEG solution was diluted very slowly by the dropwise addition of five 2 ml aliquots of K₃A at 5 min intervals. Protoplasts were further diluted with K₃A to approximately 10⁵ cells ml⁻¹ and incubated in the dark at 26°C. After 1 week protoplasts were imbedded in low melting agarose and the gel was cut into segments, placed in liquid K₃A medium containing 20 µg ml⁻¹ hygromycin sulfate and incubated in the dark at 26°C on a rotary shaker at 60 r.p.m. Liquid medium with hygromycin sulfate was replaced weekly. Hyg^R calli of 2–3 mm in diameter (4–6 weeks after transformation) were placed on solid MS shoot-inducing medium containing 20 µg ml⁻¹ hygromycin sulfate and incubated at 26°C with a 16 h light period. Shoots that developed were transferred to solid MS medium and incubated under the same conditions.

Molecular analyses

For Southern analysis, 20 µg of DNA were cleaved with endonucleases, separated by electrophoresis through a 0.7% agarose gel

and blotted to Hybond-N+ membrane (Amersham) by alkaline transfer. Hybridization conditions at 65°C were as described by Church and Gilbert (1984). Probes were labeled with ^{32}P by the method of Feinberg and Vogelstein (1983). PCR conditions were described previously (Dale and Ow, 1991). The significance of the differences observed in the frequency of precise (with correct upstream and downstream junctions) and imprecise (lacking correct downstream junction) integrants among various constructs was determined using the χ^2 test for k-independent samples.

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