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Intra- and intermolecular site-specific recombination in plant cells mediated by bacteriophage P1 recombinase

(Recombinant DNA; Cre-*lox* recombination system of bacteriophage P1; electroporation; gene transfer; luciferase)

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SUMMARY

A site-specific recombination system has many potential uses for rearranging genetic material in higher eukaryotic cells: for example, the control of gene expression by deletion or inversion of DNA segments, the clustering of transgenic constructs via site-specific integration, and the generation of chromosomal translocations. In this report, we describe a first step towards the application of a site-specific recombination system in plant cells. By use of a transient assay, we demonstrate that the bacteriophage P1 *cre* gene can be expressed as a functional recombinase in tobacco cells. Upon expression in tobacco protoplasts, Cre recognizes its target sites, *lox*, and mediates reciprocal genetic crossovers at these sites. When the *lox* sites are present in *cis* to one another, and arranged in either direct or inverted orientations, we detect Cre/*lox*-specific deletion and inversion events, respectively. The placement of *lox* sites in *trans* resulted in the co-integration of the substrates by Cre-mediated intermolecular recombination. These results indicate that the Cre/*lox* site-specific recombination system might be further developed as an additional tool for manipulating DNA in plant cells. Applications relevant to the genetic engineering of higher plants are discussed.

INTRODUCTION

Site-specific recombination is the reciprocal genetic exchange between defined nt sequences present on each participating DNA segment. Strand exchange occurs in a conservative manner by the precise breakage and rejoining of the DNA within the recombination sites. These reactions are catalyzed by a system-specific recombinase which in some cases also requires additional factors. Site-specific recombination plays an important role in diverse biological contexts, effecting gross changes in DNA linkage in cases such as bacteriophage integration and excision (Nash, 1981), resolution of plasmid multimers for proper partitioning (Austin et al., 1981), amplification of the 2 μm plasmid copy number (Futcher, 1986), and the resolution of transposition intermediates (Grindley and Reed, 1985). Excision events can also regulate gene expression. For example, excision of an element within the *nifD* gene of *Anabaena* provides for *nifD* expression during heterocyst formation (Lammers et al., 1986), and inversion events are responsible for the switching of flagellar antigens in *Salmonella typhimurium* (Simon et al., 1980). Site-specific recombi-

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Abbreviations: Ap, ampicillin; bp, base pair(s); BSA, bovine serum albumin; CaMV35S, cauliflower mosaic virus 35S promoter; DTT, dithiothreitol; *lox*, bacteriophage P1 *loxP* site; *luc*, firefly luciferase-encoding gene; Luc$^{+/-}$, proficient/deficient in luciferase activity; *nos3'*, *Agrobacterium tumefaciens* nopaline synthase polyadenylation region; nt, nucleotide(s); *ori*, plasmid ColE1 origin of DNA replication; PolIk, Klenow (large) fragment of *E. coli* DNA polymerase I; ^{R}, resistant/resistance; *tsp*, transcription start point.

nation systems have also been exploited as a method to manipulate nt sequences in homologous and heterologous systems (Backman et al., 1984; Podhajska et al., 1985; Hasan and Szybalski, 1987; Sauer, 1987; Sauer and Henderson, 1988; 1989).

The focus of this work is the utility of the bacteriophage P1 Cre/*lox* site-specific recombination system in plant cells. The 38-kDa product of the bacteriophage P1 *cre* gene is sufficient to catalyze recombination between *lox* sites; additional factors are not required. The *lox* site consists of two inverted 13-bp repeats separated by an asymmetric 8-bp spacer. Each inverted repeat and the contiguous 4 bp of the spacer comprise a binding domain for Cre (Hoess and Abremski, 1984), and strand exchange occurs at a 6-bp staggered cut within the spacer (Hoess and Abremski, 1985). The asymmetry of the spacer gives a directionality to the *lox* site and determines the topological consequences of the recombination event. Abremski et al. (1983) have shown that recombination between two directly repeated sites results in the excision of the DNA segment between the sites, whereas recombination between inverted *lox* sites produces an inversion of the intervening DNA segment. In addition, recombination between unlinked *lox* sites forms cointegrate molecules. The integration of P1 at the *loxB* site on the *E. coli* chromosome is a well characterized example of Cre-mediated intermolecular recombination (Hoess et al., 1982).

Since Cre/*lox* recombination requires only one gene product and a 34-bp nt sequence, this system could provide a simple means to manipulate DNA in eukaryotic cells. Our long-term goal is to develop the Cre/*lox* recombination system as a tool to control plant gene expression via excision and inversion events, to mediate chromosomal translocations, and as a means to target nt sequences into the plant genome via site-specific insertion. The results presented here demonstrate Cre recombinase activity in plant cells and are an initial step toward these ends. While intramolecular excision has been reported in yeast and mammalian cells (Sauer, 1987; Sauer and Henderson, 1988), and more recently demonstrated in tobacco plants (Odell, J.T., Caimi, P.G., Sauer, B. and Russell, S.H., personal communication), we show in addition that Cre is also proficient in catalyzing inversion and insertion events. To our knowledge, this is the first report of intermolecular recombination by Cre in eukaryotic cells.

RESULTS AND DISCUSSION

(a) Excision

The plasmid pED26 carries two *lox* sites in direct orientation. Recombination at the *lox* sites of pED26 would result in an excision of the DNA between the two sites, yielding two circular products (Fig. 1). One product carries the CaMV 35S-*luc* construct but no plasmid replication sequences. The other carries the antibiotic resistance marker and also the plasmid *ori* and would be successfully maintained upon transformation into *Escherichia coli*. To test whether Cre synthesized from the CaMV 35S promoter on pED23 was competent in site-specific recombination in

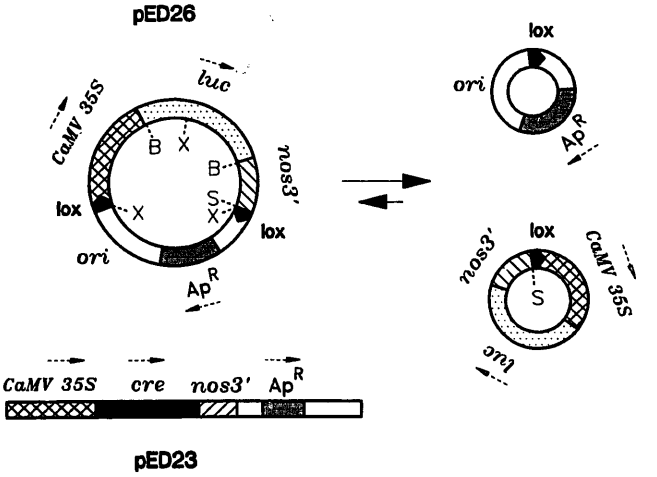

Fig. 1. Schematic map of plasmids pED26, pED23 and the expected Cre mediated recombination products. The Cre expression plasmid, pED23, was linearized with *Hin*dIII. pED26 has a CaMV 35S-*luc* construct flanked by *lox* sites arranged in the same orientation as indicated by the small black arrows. Intramolecular recombination results in two smaller circular molecules as shown. The relative length of the arrows indicates that the more favorable reaction is towards resolution (Austin, 1981; Abremski and Sternberg, 1983). B, *Bam*HI; X, *Xba*I; S, *Sal*I. Only the *Sal*I site, if present, is shown in the recombination products. Plasmid construction: pED13, the source of a 70-bp *Xho*I-*Sal*I fragment containing the *lox* site was constructed as follows. The *Xho*I-*Bam*HI *lox*-containing fragment from pBS30 (Sauer and Henderson, 1989) was subcloned into a pUC19 (Yanisch-Perron et al., 1985) derivative which has a *Xho*I (5'-CCTCGAGG-3') linker inserted into the *Sma*I site. The *Bam*HI site was then removed by treating *Bam*HI staggered ends with PolIk and T4 ligase. pED26 (Fig. 1) is derived from pDO606 (Ow et al., 1987), which carries the firefly *luc* cDNA (de Wet et al., 1987). The 70-bp *Xho*I-*Sal*I *lox* fragment from pED13 was inserted into the *Xho*I site at the 5' end of the CaMV35S promoter, and the 89-bp *Hin*dIII-*Bam*HI CaMV35S promoter fragment of pDO606 was exchanged with the larger 1585-bp *Hin*dIII-*Bam*HI promoter fragment from pDO432 (Ow et al., 1986). A second *lox* fragment (*Xho*I-*Sal*I from pED13) was introduced at the *Sal*I site at the 3' end of the *nos3'* fragment. The relative orientation of the *lox* sites was confirmed by restriction enzyme analysis followed by nt sequence analysis (Sanger et al., 1977; Sequenase DNA Sequencing Kit, US Biochemical). pED23 (Figs. 1, 3, 5) is derived from pDO469, which is essentially pDO446 (Ow et al., 1986) but with a *Kpn*I to *Sal*I conversion by linker (5'-GGTCGACC-3') addition downstream from the *nos3'* region. An additional *Xho*I linker was introduced at the *Bam*HI site near the *tsp* of the CaMV35S promoter. The *Xho*I-*Sal*I *luc* fragment of this plasmid, pED20, was exchanged with an *Xho*I-*Sal*I fragment from pBS7 (from B. Sauer, similar to pBS31 described by Sauer and Henderson, 1988) carrying the *cre* gene to yield pED21. A 304-bp *Sph*I to *Bam*HI deletion within the *nos3'* region of pED21 produced pED23. *E. coli* strain DH5α (*lacZ*ΔM15, *endA*1, *recA*1, *hsdR*17, *thi*-1, *supE*44, *argS*, *relA*1, *gyrA*96) was used to propagate recombinant plasmids.

plant cells, both pED23 and pED26 were introduced by electroporation into tobacco protoplasts. Following a 16–18 h incubation, the cells were harvested, lysed and plasmid DNA was recovered. These DNA samples were used to transform *E. coli* to Ap^R, and the resulting colonies were scored for luciferase activity (light production). The Cre expression plasmid pED23 was linearized with *Hin*dIII

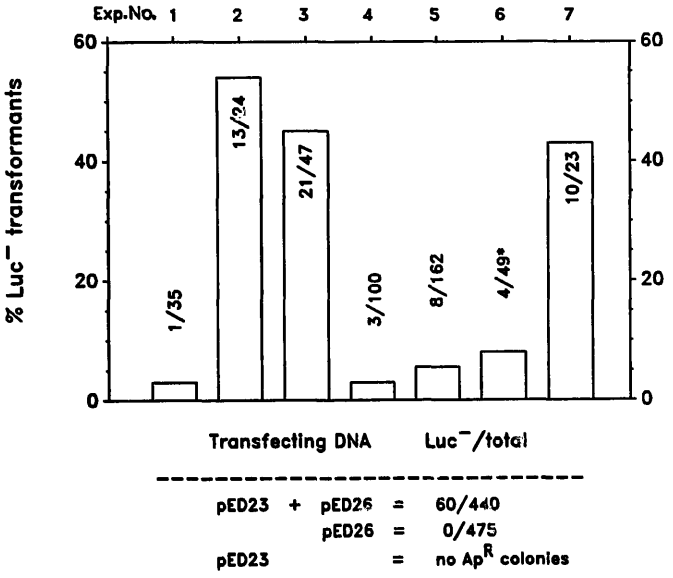

Fig. 2. Cre-*lox* site-specific excision. Protoplasts from the *Nicotiana tabacum* TXD cell line (Horsch et al., 1985) were transfected with CsCl-purified plasmid DNA (Clewell and Helinski, 1970) using a modified electroporation procedure as described previously (Ow et al., 1986). Each electroporation cuvette (Biorad No. 165–2085) contained 1 ml of protoplast suspension at 1–5 × 10^6 protoplasts/ml, 80 μg of sonicated calf thymus 'carrier' DNA and various plasmid DNAs (20 μg each). Plasmid mixtures were adjusted to a final volume of 60 μl before addition to the protoplast suspension. Following electroporation, the cells were diluted into 10 ml of growth media supplemented with 0.2 M mannitol and placed at 26°C overnight (16–18 h). Plant protoplasts were then harvested by centrifugation at 200 × *g* and DNA was extracted by the alkaline method of Birnboim and Doly (1979). The DNA samples were then used to transform *E. coli* DH5α to Ap^R. Luciferase activity present in *E. coli* was tested by mixing a sample of a colony with ATP buffer (50 mM Hepes pH 7.8/20 mM $MgCl_2$/10 mM ATP/0.5 mg per ml BSA) + luciferin (0.1 mM) and measuring light units in the luminometer. A sample with light units of < 1000 was considered Luc^-; one with > 30000 units being Luc^+. Transformants which fell between these two classes were not observed. Alternatively, bacterial colonies, stabbed onto a grid pattern on agar plates, were tested by spotting 15 μl of ATP buffer + luciferin (0.5 mM) onto each colony and placing the agar plate on Kodak T-Mat H film for 3 h. For each of seven independent experiments, a bar indicates the percentage of Luc^- clones among Ap^R *E. coli* transformants recovered using DNA-prepared tobacco protoplasts transfected with pED26 + pED23 (Fig. 1). Also shown are the number of Luc^- colonies per total Ap^R colonies examined for each experiment, and for the total of seven pED26 electroporation experiments, with or without the addition of *Hin*dIII-linearized pED23. Plasmid DNA from all Luc^- colonies (except the four in experiment 6, indicated by an asterisk) were examined by restriction enzyme analysis, and the *lox* sites of two of these DNAs (from experiments nos. 3 and 5) were sequenced.

prior to electroporation to prevent its subsequent transformation into *E. coli*.

The data from a series of seven experiments are shown in Fig. 2. As expected, the linearized Cre expression plasmid alone did not yield any Ap^R transformants, and colonies recovered from the pED26 samples were all proficient in *luc* expression since the CaMV 35S promoter appears to be active in *E. coli* (unpublished observations). The homology shared between the *lox* sites on pED26 is 74-bp. This extent of homology appears to be inefficient in promoting intramolecular homologous recombination in plant cells. Less than 0.2% recombination was found with pED26 alone, which was the background level without Cre. The combination of both pED23 and pED26 during electroporation, however, resulted in the recovery of Luc^- colonies, ranging from 3% to 54% of total transformants examined. This variability is most likely due to different degrees of plasmid segregation following transformation into *E. coli*. Since the primary transformants were scored for the Luc^+ phenotype with a qualitative assay, a parental Luc^+ plasmid would mask the presence of a co-transformed excision product. Thus, the Luc^- phenotype may have been underrepresented in some of the data.

To determine the nature of the Luc^- phenotype, plasmid DNAs from 56 Luc^- transformants were subjected to restriction enzyme analysis (data not shown). In each case, the *Bam*HI *luc* gene fragment, as well as multiple *Xba*I fragments, were absent. The linear size of all recovered plasmids as determined by electrophoresis in a 1% agarose gel was that of the pUC19-derived vector backbone. In contrast to pUC19 or pED26, however, the recovered plasmids were resistant to cleavage by *Sal*I. This is consistent with the restriction pattern of an *ori*-Ap^R recombinant product, if site-specific recombination between *lox* sites had occurred in pED26 (Fig. 1). A representative *ori*-Ap^R recombination product from two experiments, Nos. 3 and 5, was also subjected to sequence analysis (Sanger et al., 1977; Sequenase DNA Sequencing Kit, U.S. Biochemical). The 17-mer universal primer provided by U.S. Biochemical anneals to the polylinker sequence of the pUC19 backbone present on pED26 and the expected recombination products. While the *lox* sites on the pED26 substrate are each flanked by pUC19 sequences on one side and *luc-nos3'* sequences on the other, the single *lox* site present on the predicted product plasmid is flanked on both sides by only pUC19 sequences. The sequence data obtained for two independent experiments demonstrate that the *lox* site carried on the recombination product is intact, and it is also flanked on both sides by pUC19 sequences as predicted for a cross-over event occurring within the *lox* sites of pED26 (data not shown). The recovery of the excision product demonstrates that the CaMV 35S-*cre* construct is expressed in plant protoplasts and that the Cre enzyme is functional.

(b) Inversion

The plasmid pED32 (Fig. 3) carries the *luc* gene inverted with respect to the CaMV35S promoter, and a *lox* site separates the promoter from the 3′ end of the gene. A second *lox* site is present in an inverted orientation at the 5′ end of the *luc* coding sequence (Fig. 3). Inversion of the DNA fragment between the two *lox* sites would lead to the proper orientation of the *luc* gene with respect to the promoter. This plasmid substrate was used to test for Cre function in a transient assay. After a 16–18-h incubation period following electroporation, a crude extract prepared from the DNA-transfected protoplasts was tested for luciferase activity (Fig. 4). The level of luciferase activity in an extract from cells electroporated with pED32 in the absence of the Cre expression plasmid pED23 was essentially background (approx. 300 light units). However, when 50 ng of pED23 were combined with 20 μg of pED32, at a molar ratio of approx. 1 to 400, a fourfold increase in luciferase activity was observed. With increasing quantities of pED23, a proportional rise in luciferase activity was seen and a plateau was reached with 20 μg. The Cre expression plasmid (20 μg) in the absence of pED32 did not yield levels of luciferase activity greater than background (approx. 300 light units).

The inversion of the *luc* gene should be a reversible reaction and maximal luciferase activity would be expected when an equilibrium between the two states had occurred. The observed plateau in luciferase activity may represent

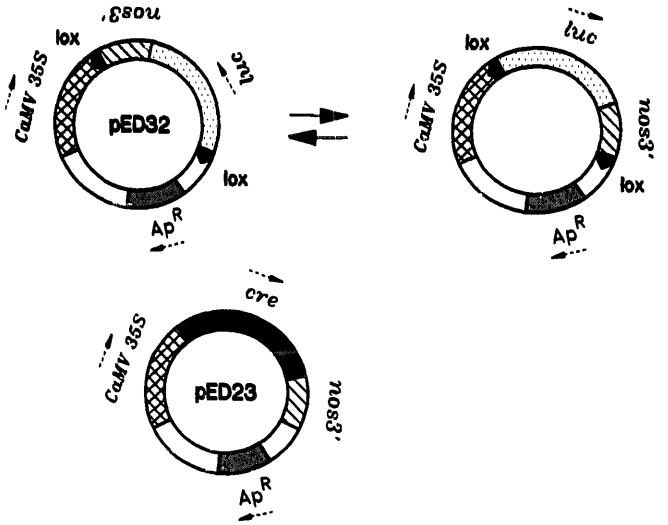

Fig. 3. Schematic maps of plasmids pED32, pED23 and the expected inversion product. The *luc-nos3′* region of pED32 is in an inverted orientation with respect to the CaMV 35S promoter as depicted by the upside down lettering of *luc* and *nos3′*. pED32 was generated by a tri-molecular ligation among (1) a *Bam*HI-*Kpn*I vector-CaMV 35S promoter fragment from pED27 (Fig. 5), (2) a *Bam*HI-*luc-nos3′*-*Sal*I fragment from a partial digest of pED26 (Fig. 1), and (3) a synthetic 48-bp adapter (5′–GATCCATATAACTTCGTATAATGTATGCTATACGAAGTTATTAGGTAC–3′) containing the *lox* sequence flanked by *Bam*HI and *Kpn*I sites. pED23 is as described in Fig. 1, but was used in its circular form.

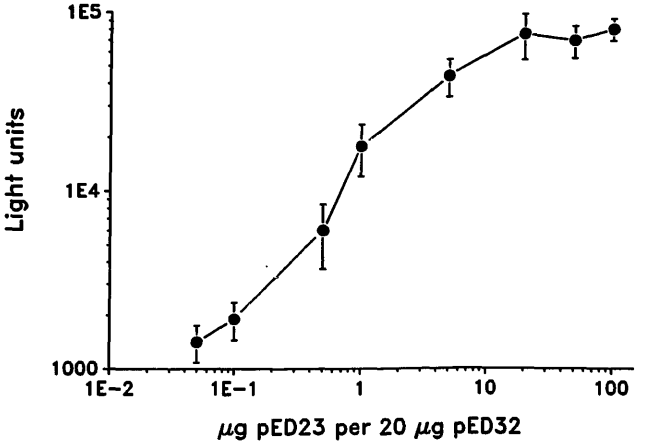

Fig. 4. Cre-*lox* site-specific inversion. Crude extracts prepared from protoplasts transfected by electroporation, as described in Fig. 2, with a constant 20 μg of pED32 plus varying amounts of pED23 (0.05–100 μg) were analyzed for luciferase activity (light units). The total amount of DNA in each sample was held constant by the addition of pUC19 plasmid DNA and the final volume adjusted to 60 μl. The crude extracts were prepared by three rounds of freezing and thawing in extraction buffer (100 mM K · phosphate pH 7.5/1 mM DTT), followed by the removal of cell debris by centrifugation (15000 × *g*, 10 min at 4°C). Luciferase activity in the soluble extract (approx. 500 μl) was determined by mixing a 50-μl sample with 100 μl of ATP buffer (50 mM Hepes pH 7.8/20 mM $MgCl_2$/10 mM ATP/0.5 mg per ml BSA), and initiating the reaction with the injection of 100 μl of 0.5 mM luciferin. Light units were measured with a luminometer (Bioluminescence model 2001) and integrated over a 30-s time period. The luciferase activity of each extract was measured two to three times and an average of these values recorded. Each value represents the mean of three independent experiments; error bars indicate population standard deviation. Average light units obtained from transfection with pED32 only, pED23 only, and no DNA control were 341, 151, and 313, respectively.

such an equilibrium within the pool of plasmid molecules that was accessible to Cre. This maximum, however, may still not reflect the total amount of transcription of the *luc* gene, as the plasmid with the inverted *luc* gene could be expressing antisense mRNA active in reducing translation of the *luc* message (Simons and Kleckner, 1983; Mizuno et al., 1984; Izant and Weintraub, 1984). Nonetheless, with luciferase activity of greater than 200-fold above background level, this inversion experiment demonstrates that Cre can associate with the pED32 substrate and catalyze site-specific inversion in plant cells, leading to a recombination product that is also transcribed and translated. Moreover, this reaction shows that the number of luciferase-producing recombination products is proportional to the number of *cre* plasmids present up to 10 μg of pED23 DNA.

(c) Intermolecular recombination

The reverse of the excision reaction is an intermolecular co-integration event which has not been previously reported in higher eukaryotic cells (Fig. 5). This type of event requires that Cre associates with two distinct molecules and per-

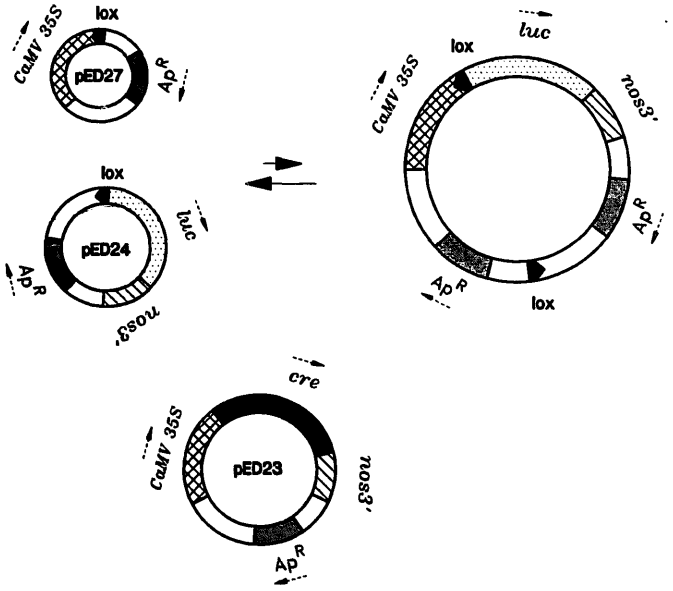

Fig. 5. Schematic representation of plasmid substrates used to detect Cre mediated intermolecular recombination. Recombination between the *lox* sites of pED24 and pED27 produces a cointegrate molecule, and the relative length of the arrows indicates that resolution of the cointegrate is favored (Austin, 1981; Abremski and Sternberg, 1983). pED27 was derived from pED21 (Fig. 1, legend) by exchanging the *XhoI-SalI cre* gene fragment with the *XhoI-SalI lox* fragment of pED13 (Fig. 1, legend). pED24 was constructed by the insertion of both the *KpnI-nos3'-SalI* and the *KpnI-lox-luc-KpnI* fragments from pED32 (Fig. 3) into pUC19 digested with *KpnI* + *SalI*. The Cre expression plasmid pED23 is as described in Fig. 3.

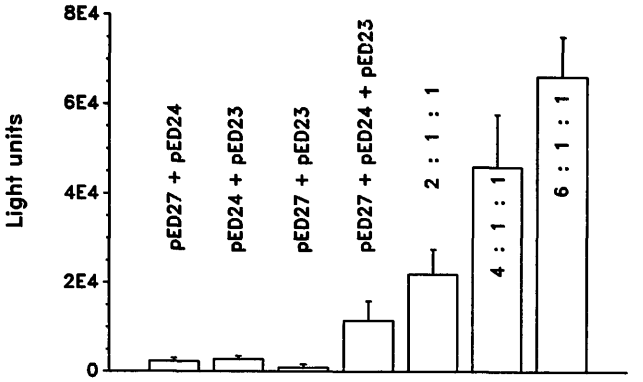

Fig. 6. Cre-mediated intermolecular recombination. Plasmid substrates pED27, pED24 and pED23 (Fig. 5), were introduced into tobacco protoplasts in the various combinations indicated in the figure and crude extracts were analyzed for luciferase activity as described in Fig. 4. In all samples where the plasmid names are shown, 20 μg of each plasmid was used during electroporation. Samples which are indicated by a ratio were transfected with a constant 20 μg of each of pED23 and pED24, plus varying amounts of pED27 (40 μg, 80 μg or 120 μg). In addition, pUC19 plasmid DNA was also added to all samples to maintain a consistent total amount of plasmid DNA (160 μg) introduced to the protoplasts. Each value represents the mean obtained from three independent experiments. Error bars indicate population standard deviation.

forms recombination between these two unlinked *lox* sites. To test for intermolecular recombination, three plasmids in various combinations were introduced by electroporation into tobacco protoplasts. One plasmid, pED24, carries a *luc* coding sequence without a plant promoter, but with a *lox* site at its 5′ end. A second construct, pED27, carries the CaMV 35S promoter immediately followed by a *lox* site; the third, pED23, is the Cre expression plasmid (Fig. 5). Recombination between the *lox* sites on pED24 and pED27 would physically link the *luc* gene to the CaMV 35S promoter and permit expression of this gene. As before, crude extracts prepared from electroporated protoplasts were analyzed for luciferase activity.

The results of these experiments are shown in Fig. 6. The combination of pED23 with pED27 gave the expected background level of luciferase activity (approx. 300 light units). The combination of pED24 with either pED23 or pED27 exhibited an aberrantly high level of luciferase activity above this background level (approx. 2300 light units), presumably due to plant promoter-like sequences upstream of the *luc* gene in pED24. However, inclusion of all three plasmids (pED23 + pED24 + pED27) resulted in a five-fold increase of the luciferase activity above the pED24 + pED27 value, indicating that Cre-dependent intermolecular recombination between pED24 and pED27 had occurred. An increase in the ratio (in μg) of pED27 to pED24 and pED23 gave increasingly higher levels of luciferase activity, up to a 29-fold increase above the pED24 high background level, and when corrected for this pED24 background, the level was as high as 160 times the pED23 + pED27 basal level. This is most likely due to increasing the probability that a given cell received all three plasmids and/or to a higher concentration of one substrate within the cell, hence favoring intermolecular synapse. Recombination of pED27 or pED24 plasmids to form homo-multimers may have also occurred, but the formation of such multimers would not interfere with a subsequent recombination event between pED27 and pED24 to produce a functional promoter-*luc* linkage.

Luciferase activity of 160-fold above the basal pED27 + pED23 background resulting from the co-integration of two plasmids approaches the levels obtained for inversion. Whereas the maximal luciferase activity obtained from the inversion experiment was nearly achieved when pED23 (Cre) and pED32 were added in equal amounts, only a 15-fold increase (corrected for pED24 background) over the basal level was observed when pED23 (Cre), pED24 and pED27 were added in a 1:1:1 ratio. However, we have shown that the intermolecular reaction is sensitive to the concentration of substrates, as increasing one of the substrates, pED27, led to a linear increase in luciferase production. Further optimization of co-integration may be possible by increasing the level of Cre.

(d) Potential applications

The results presented here comprise only a first step in the potential adaptation of this site-specific recombination system for the genetic manipulation of higher plants. While these experiments were performed with transiently introduced DNAs, we do not anticipate that chromatin structure will interfere with Cre function since Sauer (1987), and Sauer and Henderson (1989) have reported Cre/*lox* intrachromosomal excision in yeast and mammalian cells. Recently, Cre-mediated excision events have also been demonstrated in transgenic tobacco plants (Odell, J.T., Caimi, P.G., Sauer, B. and Russell, S.H., personal communication).

There may be many potential uses for a site-specific recombination system in plants. Genes which are regulated by site-specific recombination have been described in prokaryotes (reviewed by Craig, 1988), and these systems provide the basis for constructing similar regulatory circuits in stably transformed plants. For example, the deletion of genes to irreversibly prevent their expression, or the deletion of intervening DNA to restore gene function. Inversion might be used to invert a gene with respect to its promoter, with the intriguing possibility of converting functional genes to their antisense derivatives. Cre/*lox* recombination events are reversible, however, and would continue to occur as long as Cre is functional. Thus it would be desirable to control Cre expression, for example by the use of a regulated plant promoter. The reversibility of Cre/*lox* recombination events may hinder the usefulness of the Cre/*lox* system in some instances. To circumvent this problem, the use of other recombination systems which are less freely reversible such as the *λ att*/Int system could also be explored (Backman et al., 1984; Podhajska et al., 1985). Alternatively, we have recently introduced mutations into *lox* sites which render the recombination between the mutated sites more unidirectional (unpublished data).

Site-specific recombination may also provide a means to target inserts to a predetermined locus within the plant genome. Positional targeting would be particularly useful in the comparison of different molecular constructs, as each alteration made in vitro could be introduced at the same locus in the plant genome, thereby eliminating the 'position effect' thought to cause variability in such studies (Jones et al., 1985). The ability to place a gene at a known site on the plant genome would also be useful for plant breeding. The sequential introduction of genetic materials as they are isolated and/or modified in vitro into the same locus on the plant genome would expedite the passage, via a single genetic cross, of multiple genetically engineered traits from a laboratory cultivar to field varieties. Cre/*lox* intermolecular recombination may also be used to generate reciprocal translocations between nonhomologous chromosomes. This would lead to the generation of genetic diversity in germ plasms and segmental haploid/triploid libraries for classical and RFLP-based mapping.

Recently, gene targeting via homologous recombination has achieved success in animal and plant cells (Thomas et al., 1986; Thomas and Capecchi, 1987; Paszkowski, 1988). While homologous recombination requires a clone of the desired target locus as the source of homology, Cre/*lox* recombination is limited by the necessity of the prior introduction of a *lox* site into the plant genome. The highest efficiency of targeting by homologous recombination has been achieved with the use of linear substrates (Orr-Weaver et al., 1981; Lin et al., 1984). An advantage of site-specific recombination is that the use of a circular substrate along with a single crossover event ensures that the entire DNA molecule introduced becomes incorporated into the genome. Although there are yet insufficient data to compare the relative efficiencies of the two approaches, successful development of the Cre/*lox* system would add an additional dimension to the genetic modification of higher plants.

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