

FLP/*FRT*-mediated manipulation of transgenes in the plant genome

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Site-specific recombinases catalyze recombination reactions between two nucleotide sequences referred to as recombination sites. If such recombination sites are integrated into genomic DNA, depending on the orientation of these sites relative to each other, i.e., direct or inverted, the intervening genomic DNA sequence can be either inverted or excised by certain site-specific recombinases. If the recombination sites are on two different chromosomes, some of the recombinases can catalyze the exchange of chromosomal fragments. Thus, site-specific recombination reactions have the potential to have substantial practical applications in genetic engineering. We have investigated one of these site-specific recombinases, the FLP/*FRT* system from the 2- μ m plasmid of *Saccharomyces cerevisiae*, with respect to its function in cells of monocots, especially maize and rice. We have shown that the FLP recombinase can recognize and recombine *FRT* sites located in a plasmid molecule in maize or rice protoplasts, and if the *FRT* sites are on two different plasmids, they can be recombined by FLP. A recombinase test target vector containing *FRT* sites (pUFNeoFmG), the complete sequence being ubiquitin promoter/*FRT* site/*neo* gene/a mutated *FRT* site/*gusA* gene, was introduced into protoplasts. Calli were selected on kanamycin and suspension cultures were made. Protoplasts of these suspension cultures were treated with a FLP expression vector (pUbiFLP) containing the maize ubiquitin promoter driving FLP. β -glucuronidase (GUS) activity resulting from the recombination-mediated excision of *neo* (resulting in activation of *gusA*) was observed in 3-4% of all treated protoplasts. Southern blot analyses of putatively retransformed calli (GUS⁺) indicated that all of the calli contained the product of the site-specific recombination reaction. Protoplasts from the same suspension culture were also treated with pUbiFLP and pHyg (containing the hygromycin resistance gene), and calli were selected on hygromycin. A recombined *FRT* site in the genome of

one of the selected GUS⁺ callus lines was sequenced, which confirmed that the recombination reaction indeed produced the chimeric *FRT/FRTm* site. To eliminate the second retransformation step in the genomic DNA excision procedure, the target vector, pUFNeoFmG, was cointruded into maize protoplasts together with the *FLP* gene driven by a soybean heat-shock promoter. *FLP* expression was activated (based on Western blots) in transgenic calli by a heat-shock treatment. Some of these heat-shocked transgenic calli had also lost NPTII activity and gained GUS activity. Southern blot analyses indicated that the expected recombination product had been produced; however, not all the *neo* genes had been excised from the genome. This was due to the initial integration of multiple copies of the target vector (intact or fragmented) into the genome.

Manipulations of transgenes after incorporation into the genome of a host have both practical and fundamental applications in genetic engineering. In plants, it would be desirable, for example, to remove antibiotic resistance genes or herbicide resistance genes after they have been used for their primary purpose, i.e., following selection of transformed cells or after providing resistance to specific herbicides, respectively. Other applications utilizing transgene excisions or gene inversions could be useful for controlling developmental processes. Such manipulations or modifications of transgenes in the plant genome will soon be possible using certain DNA site-specific recombinase systems (Odell and Russell 1994, Ow and Medberry 1995).

Site-specific recombinases are enzymes that recognize short DNA sequences, and in the presence of two such recombination sites, they catalyze the recombination of DNA strands. With some of these recombinases, the orientation of the recognition sites relative to each other (i.e., direct or inverted) affects whether the recombination results in the intervening strand of DNA being either excised or inverted. Some site-specific recombinase systems do not require other factors for their function; thus, they represent relatively simple systems that are capable of functioning accurately in heterologous systems.

Examples of site-specific recombination systems that have been shown to be functional in plants include 1) *Cre/lox* from *Escherichia coli* phage P1, where the Cre (control of recombination) recombinase recognizes *lox* (locus of χ -over) sites (Sauer 1987, Sauer and Henderson 1989, Hoess and Abremski 1990); 2) *FLP/FRT* from the 2- μ m plasmid of *Saccharomyces cerevisiae*, where the FLP recombinase acts on the *FLP* recombination target, *FRT* sites (Cox 1989, Huang et al 1991); 3) *R-RS* from *Zygosaccharomyces rouxii*, where R and RS are the recombinase and recombinase site, respectively (Araki et al 1985, Matsuzaki et al 1990); and 4) a mutant Gin protein (G inversion) and the *gix* site from enteric bacteriophage Mu (Kahmann et al 1985). Dale and Ow (1990) and Odell et al (1990) first reported the *Cre-lox* function in transgenic tobacco subsequently followed by Dale and Ow (1991), Bayley et al (1992), Russell et al (1992), Odell et al (1994), and Qin et al (1994). *FLP-FRT* was first reported to function in rice and maize cells by Lyznik et al (1993) and then in tobacco

(Lloyd and Davis 1994, Kilby et al 1995) and in *Arabidopsis* (Kilby et al 1995, Sonti et al 1995). The mutant *Gin-gix* (Maeser and Kahmann 1991) and the *R-RS* (Onouchi et al 1991, 1995) systems have been found to function in tobacco and/or *Arabidopsis thaliana*.

In this presentation, we will summarize our progress on analyzing the performance of the FLP/*FRT* system in maize and rice cells.

FLP/*FRT* site-specific recombination in plant cells

General description of the FLP/*FRT* system

The FLP enzyme is a 48-kDa protein that covalently binds as four monomeric units to two *FRT* sites and catalyzes the cleavage and ligation of these sites (Cox 1989). FLP-mediated DNA excision or inversion occurs depending on the orientation of the *FRT* sites and when they are in *cis* (Fig. 1). Also shown in Figure 1 is the FLP-mediated

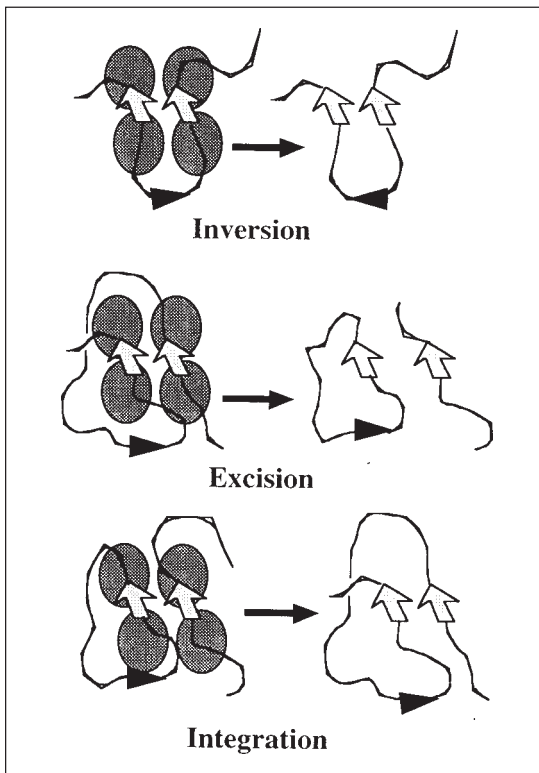


Fig. 1. Diagram depicting reactions catalyzed by FLP upon interaction with *FRT* sites. Depending on the orientation and location of the *FRT* sites, either excision, inversion, or integration of DNA fragment occurs.

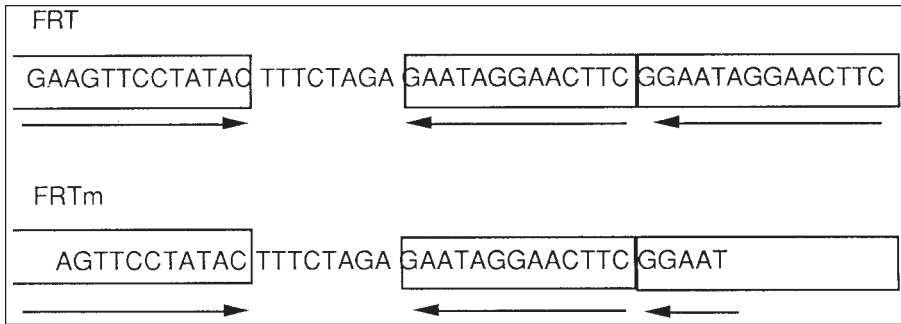


Fig. 2. Nucleotide sequence of a full-length I and a truncated *FRTm* site. The truncated *FRTm* site is referred to in the text as either *FRTm* or *Fm*.

integration (combining) of two DNA molecules when the *FRT* sites are in *trans*. The *FRT* site consists of three repeated DNA sequences of 13 bp each; two repeats in a direct orientation and one repeat inverted relative to the other two (Fig. 2; Futcher 1988). In addition, there is an 8-bp spacer region between the repeats that determines the overall orientation of the *FRT* recombination site. The mechanism of DNA exchange and the requirements of the FLP-catalyzed reaction have been studied extensively (Qian et al 1992, Serre and Jayaram 1992, Luetke and Sadowski 1995). The first step in the recombination reaction involves FLP binding to the recognition target sites followed by cleavage of the phosphodiester bond at the border of the spacer region by the tyrosine hydroxyl group of the FLP active site (Evans et al 1990, Pan et al 1993). The subsequent strand exchange reaction generated a transient Holliday intermediate (Dixon and Sadowski 1993). The exchange of the second pair of DNA strands completes the recombination reaction.

Intramolecular recombination of plasmids in protoplasts

To test whether the FLP/*FRT* system would function in plant cells, FLP-mediated recombinations were investigated in maize and rice protoplasts. A target vector was constructed that contained two *FRT* sites flanking a 1.2-kb DNA fragment inserted between the maize ubiquitin promoter and *gusA* (Lyznik et al 1991). One *FRT* site consisted of 48 nucleotides (denoted as *FRT* or *F*), and the other *FRT* site was truncated to consist of 39 nucleotides (denoted as *FRTm* or *Fm*) (Fig. 2). This vector was cointroduced into maize (hybrid A188 × BMS) or rice (*IR54*) protoplasts with an FLP expression vector (pUbiFLP) containing the maize ubiquitin promoter driving FLP. The activity of the FLP recombinase, which would lead to excision of the 1.2-kb DNA fragment, thus removing the block in *gusA* expression, was monitored by determining GUS activity 24 h after the pUbiFLP treatment (Table 1). The results demonstrated that the FLP recombinase could function in plant cells to catalyze the excision of a segment of DNA from the target vector. In addition, FLP catalyzed the reversible or cointegration event (the treatment involving all three plasmids—pUbiFRT + pFRTGUS + pUbiFLP). Interestingly, the combination of a full-length and a truncated version of the *FRT* sites (pU2FRTmG) was more effective than two full-length *FRT*s

Table 1. GUS activities in maize protoplasts treated with vectors containing *FRT* sites and the *FLP* gene.^a

Plasmid DNA	GUS activity (nmole MU min ⁻¹ mg protein ⁻¹)
-DNA	0.11+0.05
pUbiGUS	70+8
pUFRTmG	136+32
pUFRTG	40+3
pU2FRTmG	1.5+0.1
pU2FRTmG rev.	1.9+0.1
pU2FRTG	0.3+0.05
pU2FRTG rev.	0.14+0.04
pUbiFRT	0.1+0.05
pFRTGUS	6+1
Cotransformed with pUbiFLP:	
pU2FRTmG	116+26
pU2FRTmG rev.	1.6+0.1
pU2FRTG	19+2
pu2FRTG rev.	0.4+0.1
pUbiFRT + pFRTGUS	26+1

^aMaize protoplasts were treated with 20 µg of test plasmid DNA and 25 µg of pUbiFLP DNA where applicable. Values represent average GUS activity of four independent measurements (total of 12 time points) + standard error. Taken from Lyznik et al (1993).

(p2FRTG). Also, if the *FRT* sites had been altered (indicated as rev in Table 1), *FLP* would not have been able to recombine these altered *FRT* sites. Thus, the results shown in Table 1 demonstrate that the *FLP/FRT* system can function in plant cells. Furthermore, the *FLP/FRT* system in plant cells appeared to be as efficient as in animal cells (O’Gorman et al 1991), indicating that in both of these heterologous systems, no additional transacting factors are necessary. Based upon these results, we then carried out experiments to determine whether *FLP* could find and catalyze *FRT* sites after they had integrated into the plant genome.

Intramolecular recombination via retransformation

Experimental system. A target vector was constructed (pUFNeoFmG, Fig. 3), which contained the maize ubiquitin promoter driving a fully functional *neo* gene whose coding region was flanked by *FRT* sites followed by a promoterless *gusA* gene. This vector was introduced, via polyethylene glycol (PEG), into maize protoplasts derived from a suspension culture (identified as PCE). Calli were selected in the presence of kanamycin, and suspension cultures of these callus lines were established and confirmed to contain the intact 5.5-kb *XhoI-SacI* fragment containing the *neo* and *gusA* genes (Fig. 4).

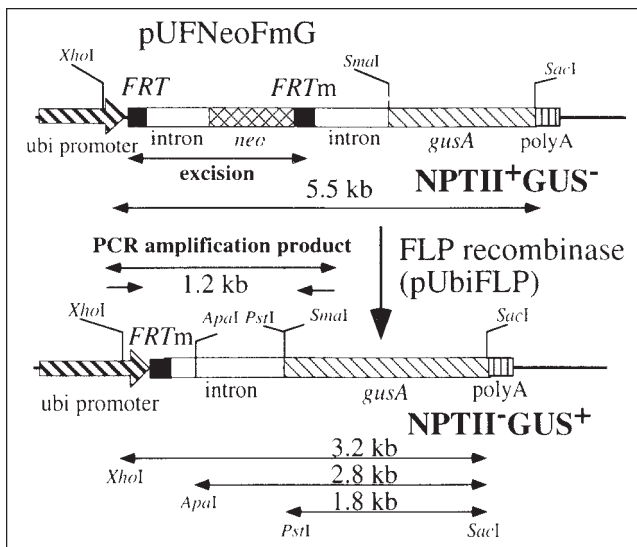


Fig. 3. Diagram of the recombination test vector, pUFNeoFmG, and the potential recombination product having a phenotype that is NPTII⁺GUS⁺. Only restriction sites pertinent to this study are indicated. *XhoI* and *SacI* restriction enzymes would yield a 5.5-kb fragment before FLP-mediated recombination and a 3.2-kb fragment after recombination. From Lyznik and Hodges (1996).

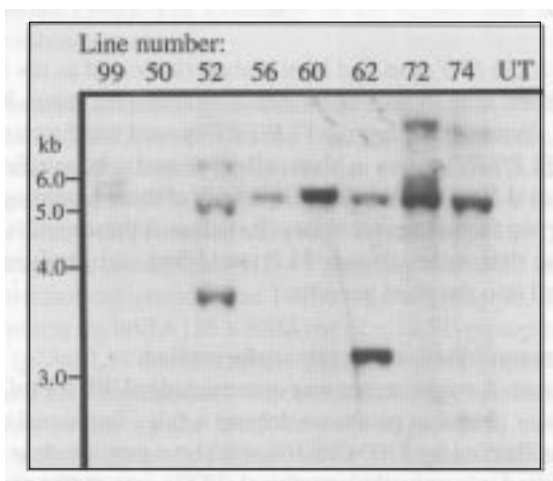


Fig. 4. Southern blot of maize callus DNA to test for the presence of the target vector (pUFneoFmG—see Fig. 3) sequence. The DNA was restricted with *XbaI* and *SacI* restriction enzymes and probed with the *gusA* coding sequence. The expected 5.5-kb fragment was seen in all lines, plus some modified fragments in certain lines. From Lyznik and Hodges (1996).

Excision of chromosomal neo from protoplasts by pUbiFLP. Three suspension cultures (56, 60, and 302), which contained the functional *neo* gene and were negative for GUS (i.e., the NPT⁺GUS⁻ phenotype), were chosen for further study. Protoplasts isolated from these three suspension cultures were then incubated with pUbiFLP in the presence of PEG and assayed for GUS within 48 h. Protoplasts from two of these lines, 56 and 60, exhibited an activation of GUS, indicating that FLP recombinase had catalyzed excision of the *neo* gene. The parental suspension culture line (PCE) as well as one of the Kan^r lines (302) did not exhibit GUS activity after treatment with pUbiFLP. These results indicate that transient expression of *FLP*, as well as the levels and activity of the FLP recombinase, is sufficient to recombine the genomic *FRT* sites resulting in excision of the *neo* gene, and simultaneously positioning the ubiquitin promoter proximal to *gusA* resulting in expression of *gusA* and GUS activity.

In similar experiments, protoplasts of line 56 (NPT⁺GUS⁻) were treated with pUbiFLP and then cultured for 4 wk with no selection on kanamycin. Individual microcalli were then assayed for GUS activity using MUG as the substrate (Jefferson et al 1987), and the frequency of GUS expressing calli was in the order of 3-4% (Fig. 5). As expected, GUS⁺ lines G8, E11, and E2 (all derived from line 56, which had the *neo* gene, expressed NPTII), did not exhibit NPTII activity while some of the others still retained NPTII activity (Fig. 6b). The strong GUS activity of one of the lines (G8) is illustrated in Figure 6a. A polymerase chain reaction (PCR) analysis of DNA using primers that would amplify only the recombination product (i.e., ubiquitin promoter to *gusA*—the 1.2-kb fragment shown in Figure 3) confirmed that lines E11, G8, and E2 (lanes 3, 4, and 5, respectively) had the expected fragment, indicating that excision of the *neo* gene had occurred (Fig. 7a). A Southern blot of genomic DNA

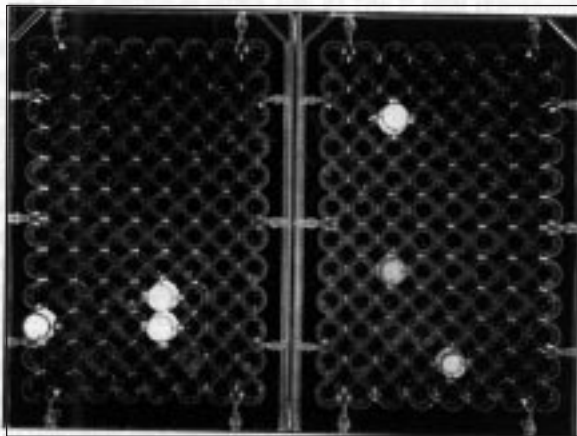


Fig. 5. A screening procedure for determining GUS activity from several callus lines derived from treating protoplasts of line 56 with pUbiFLP. Young calli were chosen randomly and grown for 4 wk without selection and stained with X-gluc according to Jefferson et al 1987.

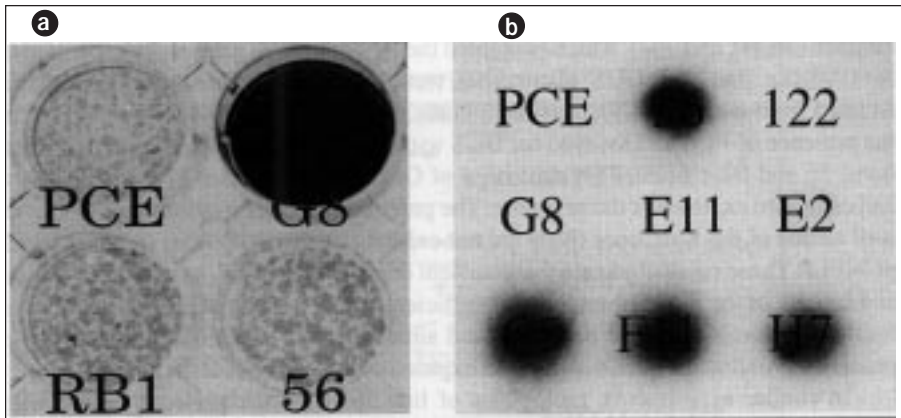


Fig. 6. a) GUS and b) NPTII activities in calli obtained from protoplasts of callus line 56 (NPTII⁺GUS⁻) transformed with pUbiFLP. PCE is the original parent callus of line 56. a) Callus stained with X-gluc according to Jefferson et al (1987) for demonstrating GUS activity. b) NPTII activity in various cell lines assayed according to Peng et al (1993).

from these lines that had been restricted with *XhoI-SacI* and hybridized with *gusA* contained a 3.25-kb band—the expected size following *neo* excision (Fig. 7b). Expected size fragments were also obtained for line G8 when other restriction enzyme treatments were used (Fig. 7c). All GUS⁺ lines derived from line 56 possessed the 3.25-kb band (Fig. 7b); however, some of the lines also still contained a 5.5-kb band. The latter results indicate that not all of the target sites had been recombined to remove the *neo* gene or that these callus lines may have been chimeric, or possibly that a reintegration might have occurred after the excision. Nevertheless, these experiments illustrate that the frequency of transient FLP-catalyzed excisions are quite high and easy to detect. However, since it is possible that these calli could have been chimeric, i.e., some of the cells might have had the *neo* gene excised and thus expressed GUS⁺ while other adjacent protoplasts/calli may not have had the *neo* gene excised, and we wished to do a molecular analysis to confirm that the recombination had indeed occurred, these experiments were repeated by treating the protoplasts not only with pUbiFLP, but also with pHyg, and then selected the calli on hygromycin.

Protoplasts from line 56 (NPT⁺GUS⁻), which had been cotreated with pUbiFLP and pHyg, were cultured and allowed to grow on medium containing 100 mg hygromycin L⁻¹. These lines were screened initially for GUS activity to provide an indication that FLP had excised the *neo* gene, and then they were tested for the loss of NPTII activity. To obtain additional evidence that the *neo* gene had been excised, the genomic DNA from the initial cell line used in this study (PCE), the daughter line (56) containing the target vector (pUFneoFmG), and a granddaughter line (122) retransformed with pUFLP were restricted with *XhoI* and *SacI* and hybridized to either the *neo* gene or the *gusA* gene (Fig. 8). In line 56, the *neo* and *gusA* genes banded at

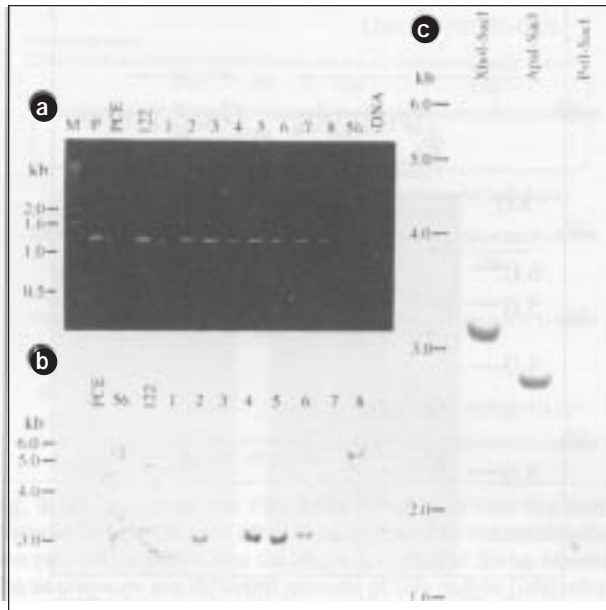


Fig. 7. a) PCR and b,c) Southern blot analysis of genomic DNA isolated from callus of the original parent line never transformed (PCE), line 56 transformed with pUFNeoFmG, line 122 contains the product of the recombination reaction (positive control), and a sample of GUS⁺ callus lines selected after transient retransformation with pUbiFLP is shown in lanes 1-8. a) The P lane represents the amplification product of the pUFRTG vector and M lane indicates molecular markers. DNA was isolated from the callus 4 wk after treatment with pUbiFLP. The PCR amplification product (1.2 kb) is shown in Figure 3. DNA was isolated from the callus 4 wk after treatment with pUbiFLP. b) DNA was digested with *XhoI-SacI* restriction enzymes. The length of the restriction fragment hybridizing to the *gusA* probe in line 56 (5.5 kb) should be reduced to a fragment size of 3.2 kb after a successful recombination event (compare lanes 56 and 122). c) DNA from transgenic maize suspension present in lane 4 (part B of this figure) was additionally digested with *XhoI-SacI*, *ApaI-SacI*, and *PstI-SacI* to verify the recombination event. The size of the fragments hybridizing to the *gusA* probe (3.2, 2.8, and 1.8 kb, respectively) was as expected from the product of the site-specific recombination event, see Figure 3. From Lyznik and Hodges (1996).

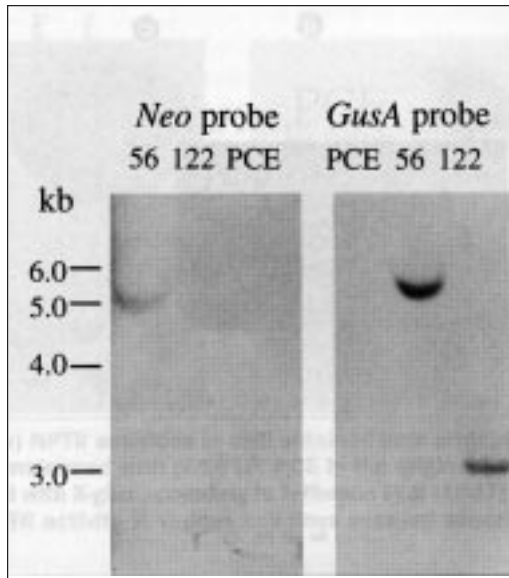


Fig. 8. Southern blot analysis of genomic DNA from the grandmother callus line PCE, the daughter line 56 (transformed with pUFNeoFmG), and granddaughter line 122 (line 56 retransformed with pUbiFLP) and probed with either the *neo* (left panel) or the *gusA* (right panel) genes. The DNA was digested with *XhoI-SacI* restriction enzymes and $10 \mu\text{g lane}^{-1}$ was added. A 5.5-kb band hybridized to both probes as predicted; a band of 3.2 kb, which is the product of a site-specific recombination reaction mediated by the FLP protein, hybridized only with the *gusA* probe. From Lyznik and Hodges (1996).

5.5 kb. In the derivative line 112, the *neo* gene was missing and the *gusA* gene was present as the expected 3.25-kb band.

To prove that FLP mediated the recombination, as the above results indicated, the sequence of the recombination product was determined (Fig. 9). This was achieved by amplifying a DNA fragment consisting of the 5' untranslated region of the *gusA* gene from line 122 via PCR. This fragment was subcloned into the pGEM7(z) vector and its 5' end, containing the putatively recombined *FRT*, was sequenced. The results confirmed that the recombined *FRT* site was chimeric between the original full-length *FRT* and the original mutated *FRTm* (Fig. 9).

Based on Southern analyses (Fig. 8) and finally on sequencing of the recombined DNA (Fig. 9), we have shown that chromosomal DNA containing the selectable marker gene *neo* flanked by *FRT* sites can be cleansed of the *neo* gene by the expression of the *FLP* site-specific recombinase. Thus, this procedure provides an effective way of

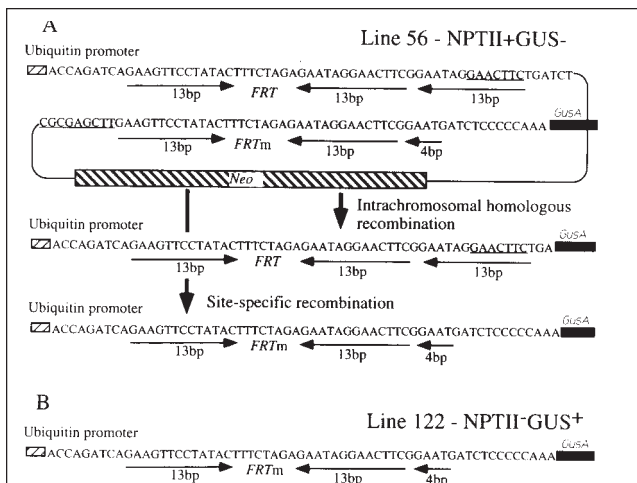


Fig. 9. Sequence of the *FRT* sites integrated into the maize genome before (a) and after (b) site-specific recombination. The two *FRT* sites in line 56 share a region of 39-bp identity. The sequences are different outside of this region (underlined nucleotides). Any conservative DNA recombination within the 39-bp identical regions of the *FRT* sites, including the site-specific recombination, should exchange flanking DNA segments producing a chimeric *FRT* site shown at the bottom of (a). The actual sequence of the *FRT* site in line 122 is shown in (b). This sequence corresponded exactly to the predicted product of the site-specific recombination reaction. From Lyznik and Hodges (1996).

removing a previously integrated gene (*neo* in this case) and simultaneously activating another gene (*gusA* in this case).

Excision of chromosomal neo via inducible expression of chromosomal FLP. In addition to being able to excise DNA fragments from the genome by using retransformation treatments, it would be desirable to achieve this result by a more efficient method. An alternative approach that has worked for the Cre/*lox* site-specific recombination, but one that is rather slow, is to put the two components (Cre and the *lox* sites) in separate plants and then cross breed the plants (Dale and Ow 1991, Russell et al 1992). Although this procedure is useful, it is time-consuming and somewhat difficult. Another approach we have pursued is to integrate the recombinase gene (*FLP*) into the genome under the control of an inducible promoter. With the entire site-specific recombinase system (*FLP/FRT*) integrated into the genome, but with the recombinase gene itself controlled by an inducible-promoter, one should be able to utilize the target gene, such as a selectable marker gene, and then activate expression of *FLP* by an external signal to excise the target gene. To achieve this, we employed a heat-shock promoter to control expression of *FLP* (Lyznik et al 1995), which had proven to be effective in *Drosophila* (Golic and Lundquist 1989, Golic 1991, Konsolaki

et al 1992). Our results in maize have recently been substantiated by Kilby et al (1995) in *Arabidopsis*.

We used a soybean heat-shock promoter (Czarnecka et al 1985, Ainley and Key 1990) to drive expression of *FLP* and this gene (in pHsFLP) was integrated into the genome in association with the target vector (pUFneoFmG) (Lyznik et al 1995). Following selection of calli on kanamycin, a heat-shock treatment of Kan^r-calli activated expression of FLP as determined by Western blotting, which then recombined the DNA to apparently excise some of the integrated *neo* genes and activate *gusA* expression (+/-heat shock). Southern blot and PCR analyses (Lyznik et al 1995) indeed illustrated that some of the *neo* genes (but not all) had been excised and the expected recombined product was produced. In these experiments, the results were complicated by the integration of more than one vector molecule (intact or fragmented). Clearly, the FLP enzyme was capable of locating and recombining some of the *FRT* sites, but not all of them. This illustrates the need to screen callus initially for single-copy or simple-gene integrations for the *FLP* to be most effective, which should now be easily attained in rice using *Agrobacterium tumefaciens* (Hiei et al 1994, Aldemita and Hodges 1996). These results illustrate that it is possible to have the entire FLP/*FRT* recombination system integrated into the genome and functional, i.e., capable of excising a transgene and simultaneously activating a second one.

Summary

The FLP/*FRT* site-specific recombinase system was shown to catalyze DNA recombinations between two plasmid molecules when introduced into maize or rice protoplasts. It was also shown that *FRT* sites integrated into the maize genome could be located and recombined by FLP following transient expression of the *FLP* gene, and that this occurred in nonselected, randomly chosen calli at a frequency of 3-4%. Molecular evaluations involving both Southern analyses and nucleotide sequencing of the recombined chimeric *FRT* site proved that the expected DNA recombinations had occurred. Finally, it was demonstrated that a heat-shock promoter driving expression of genomic *FLP* was effective in controlling the expression and activity of FLP such that it could locate and recombine *FRT* sites within the genome.

In addition to our studies of the FLP/*FRT* system in maize and rice (Lyznik et al 1993, 1995; Lyznik and Hodges 1996). Lloyd and Davis (1994), Kilby et al (1995), and Sonti et al (1995) have shown this site-specific recombinase to operate in tobacco and *Arabidopsis*, respectively. In tobacco, Lloyd and Davis (1994) used a cross breeding experiment to introduce *FLP* from one plant into a receptor plant containing previously inserted *FRT* sites into the genome and FLP-catalyzed excisions occurred at a frequency of about 70%. Kilby et al (1995) used a heat-shock promoter to drive expression of FLP, and they demonstrated that cell fate could be determined in *Arabidopsis* using this system.

Use of the FLP/*FRT* and the other site-specific recombinase systems for the excision of unwanted transgenes, the inversion of either active or quiescent transgenes, the removal of blocking genes to activate other genes, the development of plant hybrids

by excision or inversion of sterility or fertility genes, the control of the recombinase genes by environment-sensitive (temperature, chemical) or tissue-specific (endosperm, floral) promoters, etc. provides the opportunity to manipulate the plant genome for the development of transgenic plants in a very sophisticated fashion.

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Notes

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