Nucleic Acids Research, 1993, Vol. 21, No. 4 969–975

Activity of yeast FLP recombinase in maize and rice protoplasts

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Received October 21, 1992; Revised and Accepted January 16, 1993

ABSTRACT

We have demonstrated that a yeast FLP/FRT site-specific recombination system functions in maize and rice protoplasts. FLP recombinase activity was monitored by reactivation of β-glucuronidase (GUS) expression from vectors containing the *gus*A gene inactivated by insertion of two FRTs (FLP recombination targets) and a 1.31 kb DNA fragment. The stimulation of GUS activity in protoplasts co-transformed with vectors containing FRT inactivated *gus*A gene and a chimeric FLP gene depended on both the expression of the FLP recombinase and the presence and structure of the FRT sites. The FLP enzyme could mediate inter- and intramolecular recombination in plant protoplasts. These results provide evidence that a yeast recombination system can function efficiently in plant cells, and that its performance can be manipulated by structural modification of the FRT sites.

INTRODUCTION

With the development of more efficient DNA transformation procedures, there is an increasing need to specifically control modifications of higher eukaryotic genomes. *In situ* mutagenesis of genes of interest opens new opportunities to study the role of specific genes in basic metabolic or developmental activities of a living cell. DNA recombination systems can serve as a useful means to accomplish this task. Gene targeting strategies take advantage of endogenous homologous recombination activities to introduce foreign DNA into specific sites of a genome (1–5). Site-specific recombination systems may assist gene targeting strategies for more efficient and controlled manipulations of genomic DNA (6).

Among known and characterized site-specific recombination systems, the Cre/lox system of the bacteriophage P1(7,8), the Gin recombinase of phage Mu (9), and the R/RS system of pSR1(10) have been tested and proven to function in plant cells. The FLP/FRT recombination system of yeast has been shown to function in bacteria (11,12), insects (13,14), and animal cells (15). The FLP recombinase encoded by the 2 μm plasmid DNA of *Saccharomyces cerevisiae* is related to the R recombinase encoded by the pSR1 plasmid of *Zygosaccharomyces rouxii* (16). All of these systems are of interest because of their simplicity; they need just one protein (recombinase) and recombination site to be fully operational in contrast to the more complex bacteriophage λ or phage Mu recombination systems (9).

In the yeast FLP/FRT system, the target FRT nucleotide sequence 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. In addition, there is an 8 bp spacer region between the repeats which determines the orientation of the FRT recombination site (17). The FLP recombinase binds to all three repeats, cleaves the sites at the borders of the spacer, and exchanges the DNA strands (18–20). Depending on the orientation of the recombination sites, the DNA fragment between the FRTs can be either inverted or excised. Additionally, the FLP recombinase can act on target FRT sites located on separate DNA molecules. These intermolecular recombinations can lead to integration of foreign DNA into FRT sites in bacterial and mouse genomes (11,15).

In this paper we report the functionality of the FLP/FRT recombination system and describe some of the features of the FLP/FRT recombination activities in maize and rice protoplasts.

MATERIALS AND METHODS

Plasmid constructions and DNA manipulations

All DNA manipulations were conducted according to Sambrook et al. (21). Bacterial transformations were carried out using competent *Escherichia coli* cells, strain DH5α. Plasmid DNA was isolated and purified using CsCl density gradient centrifugation.

Synthesis of FLP expression vectors. A genomic clone of maize *Adh* 1 was described previously (22) as was the construction of the vector pAeiGUS (23). Plasmid pAHC27 was graciously provided by Dr Peter H.Quail, University of California, Berkeley. Plasmids pNEOβGAL and pOG44 were purchased from Stratagene, LaJolla, CA. pUbiGUS was constructed by ligation of the *Xba*I fragment of pAHC27 into *Hin*dIII-*Bam*HI restriction sites of p35SGUS (24). This ligation put the maize ubiquitin gene 5′ controlling elements in front of the *gus*A coding sequence. For the construction of pAeiFLP, both pAeiGUS and pOG44 were digested with *Bgl*II and *Sac*I. The 1.5 kb fragment

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carrying the FLP coding sequence from pOG44 was then ligated directly into the eluted 4.4 kb fragment of pAeiGUS replacing the *gus*A coding sequence. pUbiFLP was obtained by isolating the 1.5 kb *Hin*dIII-*Sac*I FLP fragment from pOG44 and subcloning into the respective sites of pGEM-7Zf(−) (Promega, Madison, WI)). The resulting plasmid was cleaved with *Sac*I and *Sma*I, and the 1.5 kb FLP fragment was then ligated directly into the *Sac*I-*Sma*I digested pUbiGUS replacing the *gus*A fragment with the FLP gene.

Synthesis of FRT containing vectors. Two primers (5′-GTGATCAGAAGTTCCTATTCCGAAGTTCCTATTCTCTAGAAA-3′) and (5′-CTGATCAGAAGTT CCTATACTTTCTAGA-3′) were annealed (4 nmoles each) and incubated with 5 units of T4 DNA polymerase and 50 nmoles of each dNTP in 0.1 ml at 11°C for 3 hrs to form a complete FRT recombination site of 48 bp. The primer-extended fragments contained a *Bcl*I restriction site on each end. Phosphate groups were then added to the terminal nucleotides by incubation with 20 units of T4 DNA kinase and 16 nmoles ATP in 30 μl of 1×kinase buffer. The resulting double-stranded DNA fragments were blunt-end ligated and cleaved with *Bcl*I restriction enzyme. The products were then ligated directly into the *Bgl*II site of pUbiGUS forming pUFRTG. The FRT insertions were into the non-translated leader sequence of the ubiquitin gene and not into coding sequences (Fig.3). For the construction of the pUFRTmG vector, containing a shorter version of the FRT site (37 bp), the primer extended products were blunt-end ligated into the *Bgl*II site of pUbiGUS (the *Bgl*II site of pUbiGUS was filled-in using T4 DNA polymerase). Confirmation of the short FRT sequence was by sequencing the double-stranded plasmid DNA. pU2FRTG and pU2FRTmG were constructed by ligation of the 1.31 kb *Xba*I fragment of pNEOβGAL into pUFRTG and pUFRTmG, respectively. The insertions of the *Xba*I fragment were recovered in both orientations to generate pU2FRTG and pU2FRTG rev (the latter containing nonfunctional FRT sites; Fig.3). The test plasmid pUbiFRT for intermolecular recombination was constructed by *Eco*RI restriction of pU2FRTmG and religation of the resulting plasmid DNA fragments. The other substrate for intermolecular recombination, pFRTGUS, was obtained by digestion of pU2FRTmG with *Eco*RI and *Bam*HI, isolation of the fragment containing the *gus*A coding sequence and the FRT site, and ligation of the resulting fragment into the respective sites of pGEM-7Z f(−).

Molecular Analysis of DNA. Polymerase chain reactions (PCRs) were carried out in a Perkin-Elmer Cetus DNA thermal cycler. Amplification was performed using reagents and protocols as outlined by the Perkin-Elmer Cetus GeneAmp PCR Kit (Perkin-Elmer Cetus, Norwalk, CT). Sequences of primers were: 5′-CCCCAACCTCGTG-3′ for the first exon of the ubiquitin gene and 5′-GGGGTTTCTACAGGACG-3′ for the 5′ end of the *gus*A coding sequence. PCR reactions contained 5 μl of template DNA, 5 μl of primer solution (10 nmoles/μl), 5 μl of 10×PCR buffer II (25), 2 μl dNTPs mix (final concentration 200 μM of each nucleotide), and 0.25 μl of *Taq* polymerase (2.5 units/100 μl) in a final volume of 50 μl. Denaturation, annealing, and extension steps were performed at 94°C for 1 minute, 45°C for 1 minute, and 72°C for 2 minutes, respectively. The extension steps were increased 15 seconds with each cycle. Five microliters of each amplified product were analyzed using 1.0% agarose gel electrophoresis.

Southern blot analysis of the agarose gel containing PCR amplified DNA was performed according to Sambrook *et al.* (21) using capillary transfer to Hybond-N membrane (Amersham, Arlington Heights, IL). DNA was fixed to the membrane by UV irradiation and incubated in pre-hybridization solution (5×SSPE, 5×Denhardt's solution, 0.5% SDS) at 65°C for 4 hours. The radioactive probe (a ^{32}P-labeled *Sma*I−*Bgl*II fragment isolated from pUbiGUS) was prepared using the Multiprime DNA labeling system according to the manufacturer's instructions (Amersham, Arlington Heights, IL). The probe contained the complete ubiquitin intron and part of the first exon. The blot was hybridized overnight in pre-hybridization solution at 65°C and subsequently processed according to standard procedures (21).

The nucleotide sequences of the FRT sites were determined by a modified dideoxy method (26) using Sequenase Version 2.0 (United States Biochemical Cleveland, OH). CsCl purified pUFRTG and pU2FRTmG plasmid DNA was used as template DNA. The primer was the same as that used for PCR which annealed to the 5′ end of the first exon of the ubiquitin gene.

Transformation procedures

A cell suspension culture of maize (*Zea mays* L.) was initiated from A188×BMS type II callus and maintained as previously described (27). Seven days prior to protoplast isolation, 2 ml packed cell volume (PCV) of the suspension culture was transferred into 37 ml of MS medium (28) supplemented with 3.5 mg/l 2,4-D.

Approximately 5 ml PCV of suspension cells were digested for 3 h in 20 ml MS medium containing 0.2 M mannitol, 0.5 mg/l thiamine, 2 mg/l 2,4-D, 80 mM $CaCl_2 \cdot 2H_20$, 2% cellulase, 0.25% pectinase (Worthington Biochemical Co., Freehold, NJ), and 0.1% pectolyase Y-23 (Seishin Pharmaceutical Co., Tokyo, Japan), pH 6.0. Protoplasts were filtered through a 48 μm nylon mesh screen and pelleted by centrifugation at 50×*g* for 15 minutes. The pellet was suspended in 4 ml of protoplast culture medium (PCM) containing 9% Ficoll 400 (Sigma Chemical Co., St. Louis, MO) and overlaid with 4 ml of transformation medium (TM). The transformation medium consisted of 100 mM MES buffer, pH 5.5, 0.2 M mannitol, and 80 mM $CaCl_2 \cdot 2H_20$. Following centrifugation at 75×*g* for 10 minutes, a band of protoplasts was collected from the interface, and the concentration was adjusted to 1.0×10^7 protoplasts/ml with TM.

Protoplast transformation was performed in 12 ml Falcon polystyrene tubes by pipetting 20−25 μl of plasmid DNA (1.0 mg/ml) followed by 0.5 ml of protoplasts and 0.5 ml of 50% PEG (polyethylene glycol, MW=8000; Sigma Chemical Co., St. Louis, MO.) dissolved in F-solution (29). Protoplasts were incubated for 20 minutes at room temperature. After incubation, 330 μl of the protoplast solution were transferred to a 12-well microculture dish containing 2 ml solidified 0.8% low melting point agarose (Betheseda Research Laboratories, Gaithersburg, MD) in PCM. Plates were then wrapped with Parafilm and incubated at 25°C in the dark for approximately 24 hrs.

GUS activity and protein determination

Following the 24 hrs incubation, protoplasts were collected and resuspended in 300 μl of GUS extraction buffer (30) containing 0.1% Triton X-100 (Sigma Chemical Co., St. Louis, MO). After centrifugation at 16,000×g for 5 min., a 25 μl extract of the protoplasts was incubated with 150 μl of 1 mM MUG (4-methyl umbelliferyl β-D-glucuronide) in GUS extraction buffer in a

96-well plate at 37°C. Reactions were stopped at various times by adding 125 μl of GUS stop buffer (0.2 M $CaCO_3$). Fluorescence (excitation at 362 λ and emission at 455 λ) was measured in a Perkin Elmer Luminescence Spectrometer LS50B calibrated with standards of methylumbelliferone (Sigma Chemical Co., St. Louis, MO). GUS activity was calculated from the slope of the line generated from time points and normalized to the protein content determined by the method of Bradford (26).

RESULTS

The FLP/FRT site-specific recombination system used in these studies consists of two elements: plasmid DNA encoding the FLP enzyme and test plasmids containing the FRT recombination sites (Figs. 1 and 2). The FLP recombinase expressed from FLP vectors should recombine test plasmid DNA within the FRT target sites to restore expression of GUS enzyme (Fig. 1). Expression of the FLP protein was driven by the maize *Adh*1 promoter in pAeiFLP plasmid and by the maize ubiquitin promoter in pUbiFLP plasmid (Fig. 2). Both constructs included the first exon and intron of the respective genes. In the pAeiFLP construction, the first intron contained 290 bp of the first *Adh*1 intron fused at the *Bgl*II site to 81 bp of the synthetic intron from pOG44 (Fig. 2B). Both promoters proved to be very effective as determined in transient GUS expression assays of maize protoplasts; however, the ubiquitin promoter was superior (unpublished data).

The *Bgl*II site of the first exon of the ubiquitin promoter in pUbiGUS was chosen for insertion of the FRT sequences following initial screening of other appropriate insertion sites. Two different FRT sites were ligated into the *Bgl*II site—one short 37 bp FRT site referred to as FRTm and one complete 48 bp FRT site referred to as FRT (Fig. 3). The FRTm site lacks the third repeat which includes five additional FLP protein-DNA contact sites; however, a similar FRT deletion (missing one repeat and the same FLP protein-DNA contact sites) has been shown to be as effective as the wild-type FRT site in *in vitro* assays (18). Interestingly, insertion of the FRTm site into the pUbiGUS resulted in higher expression of the GUS enzyme (Table 1). Insertion of the full length FRT site resulted in reduction of GUS expression (Table 1).

A second FRT site for intramolecular recombination of test plasmids was created by insertion of the *Xba*I fragment of pNEOβGAL into the single *Xba*I site of pUFRTG. Insertion of the pNEOβGAL fragment provided supplemental sequences to form an additional FRT site and a 1.31 kb spacer DNA which separated the ubiquitin promoter from the *gus*A coding sequence (Figure 3B). As a result, GUS activity in maize protoplasts transformed with pU2FRTG was substantially reduced (Table 1). The reverse orientation of the ligated *Xba*I fragment of pNEOβGAL should also inactivate GUS expression but should not form functional FRT sites. pU2FRTmG rev. and pU2FRTG rev. vectors were used to demonstrate that fully functional FRT sites were required to obtain activation of GUS expression by the FLP protein (Table 1, Fig. 3B).

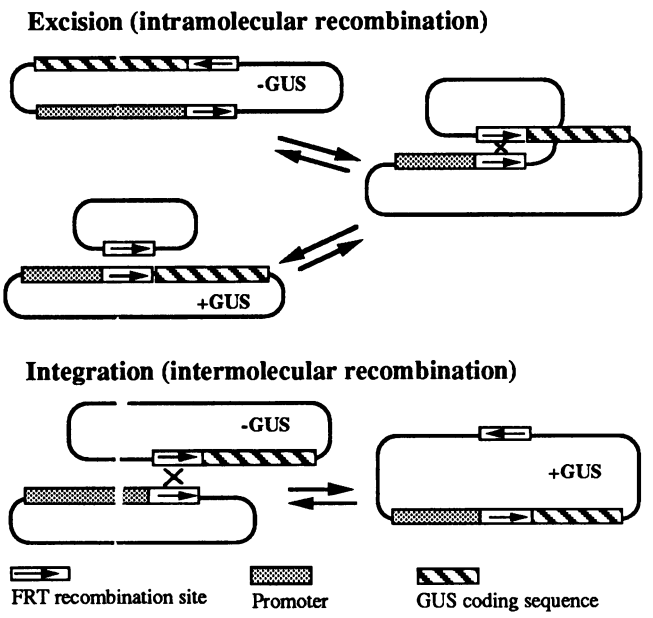

Figure 1. General strategy for observing FLP/FRT mediated intra- and intermolecular recombinations between plasmid DNAs in maize and rice protoplasts. GUS+ and GUS− represent vectors either capable or incapable of expressing the GUS enzyme.

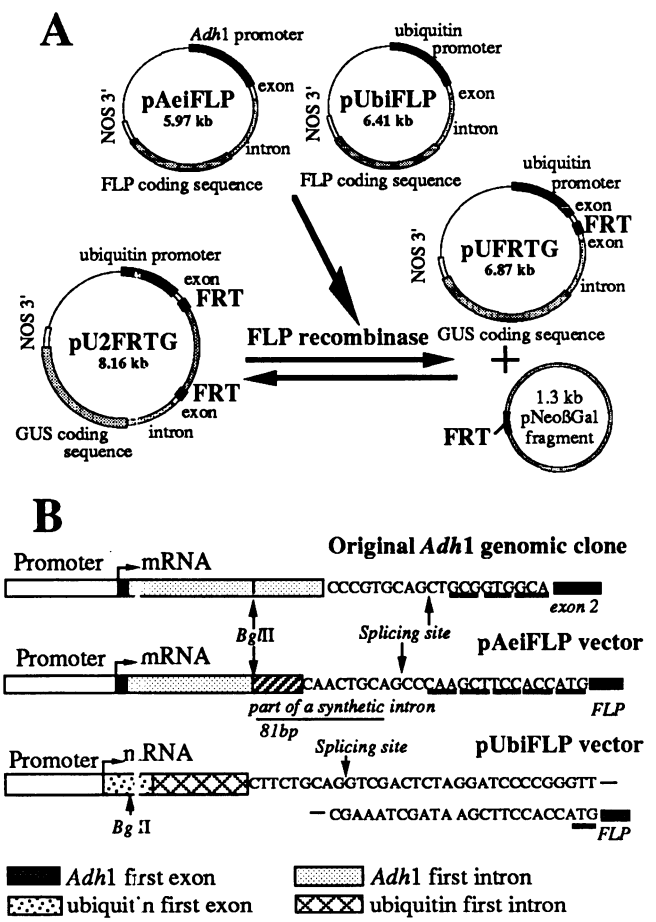

Figure 2. The FLP expression vectors used to study activity of a yeast FLP/FRT system in plant protoplasts. (**A**) Components of the recombination system to test the DNA excision reaction catalyzed by the FLP protein. FLP enzyme can be produced by either pAeiFLP or pUbiFLP. (**B**) Sequence of the junction sites between cloned FLP coding sequence and *adh*1 or ubiquitin maize promoters. Thick lines indicate an open reading frame of *adh*1 gene or the translation start codon for FLP protein synthesis in the pUbiFLP vector. Note that the first exon of the maize ubiquitin gene, in contrast to the *adh*1 first exon, is not translated.

Table 1. GUS activities in maize protoplasts transformed with vectors containing FRT sites and the FLP gene.

Plasmid DNA used for transformation	GUS activity (nmol MU/min/mg protein)
– 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
Co-transformed with pUbiFLP:	
pU2FRTmG	116 ± 26
pU2FRTmG rev.	1.6 ± 0.1
pU2FRTG	19 ± 2
pU2FRTG rev.	0.4 ± 0.1
pUbiFRT+pFRTGUS	26 ± 1

Maize protoplasts were transformed 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 twelve time points) ± standard error.

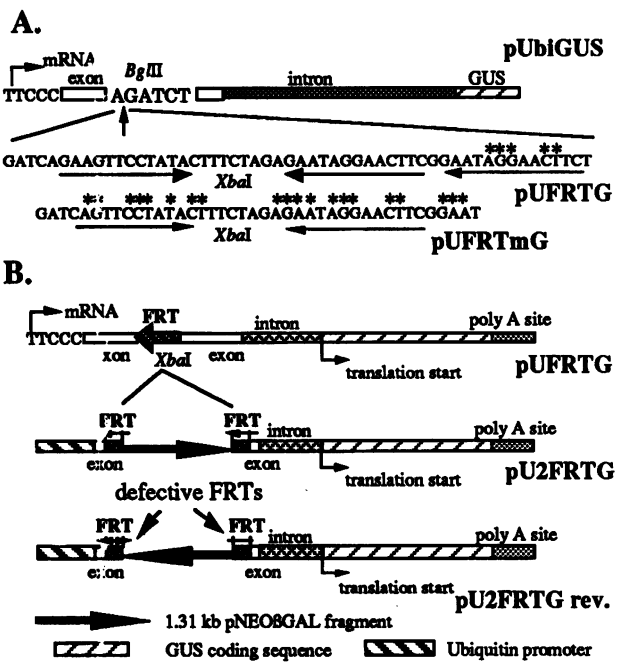

Figure 3. Structure of the FRT site-containing vectors. (A) A single full or short FRT site was ligated into the *BglII* site of the ubiquitin first exon. Asterisks show the FLP binding sites as presented by Senecoff et al., (35). Arrows denote 13 bp inverted repeats. pUFRTmG vector contained only one 13 bp repeat and one shorter 11 bp inverted repeat. This FRT site lacks five FLP protein contact sites. (B) Insertion of the *XbaI* fragment of pNEOβGAL into the *XbaI* site of the FRT vectors provided the second FRT site and led to inactivation of GUS expression. Description of the pNEOβGAL can be found in (15).

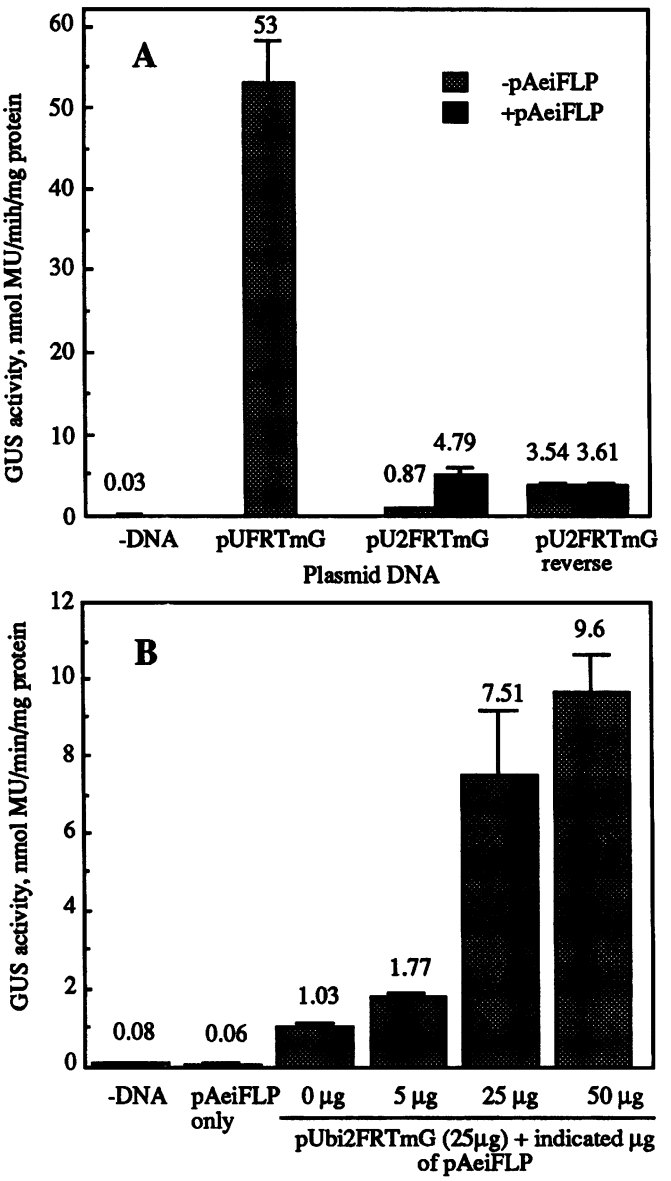

Figure 4. (A) GUS activity in maize protoplasts co-transformed with pAeiFLP (for FLP expression) and pU2FRTmG (the FRT site-containing) vectors. (B) GUS activity in maize protolasts co-transformed with varying amounts (0–50 μg) of pAeiFLP DNA and pU2FRTmG vectors. Total DNA used was adjusted to 100 μg with sonicated salmon sperm DNA. Note that the pAeiFLP vector (25 μg) alone in the transformation medium did not induce expression of GUS in protoplasts above background.

Activity of the FLP protein was monitored by assaying transient GUS activity in maize protoplasts co-transformed with pAeiFLP and pU2FRTmG. GUS activity was higher in co-transformed protoplasts than in protoplasts transformed with only pU2FRTmG (Fig. 4A). The restored GUS expression was approximately 10% of that observed after protoplast transformation with the control pUFRTmG. GUS activity in maize protoplasts transformed with only pAeiFLP was similar to background GUS activity (Fig. 4B). Thus, the FLP/FRT recombination system appeared to be functional in maize protoplasts. This was further substantiated by the increase of GUS activity in maize protoplasts co-transformed with increasing amounts of pAeiFLP DNA (Fig. 4B). This indicated not only that an increase in GUS expression was dependent on the amount of the FLP enzyme present, but also that the amount of the FLP protein might limit the recombination process.

The stronger ubiquitin promoter was used to increase the amount of FLP protein in co-transformed protoplasts. Table 1

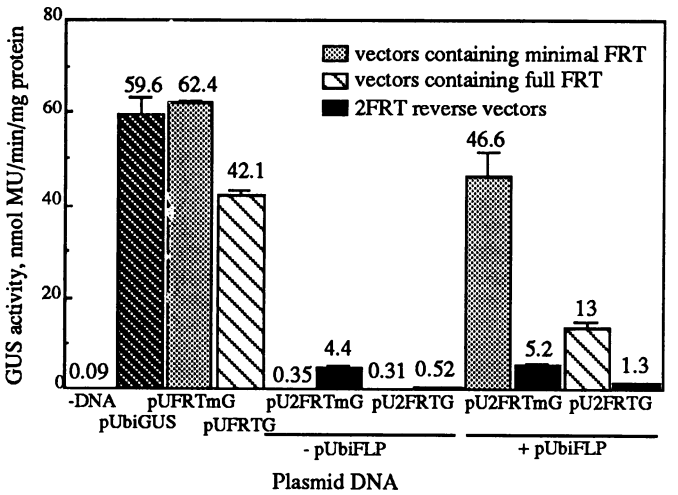

Figure 5. GUS activity in rice protoplasts co-transformed with a set of DNA vectors to test the activity of the FLP/FRT recombination system. Co-transformation conditions and GUS assays were the same as for maize protoplasts (Table 1) except CPW medium was used to resuspend protoplasts before transformation rather than the TM medium used for maize protoplast transformation(37).

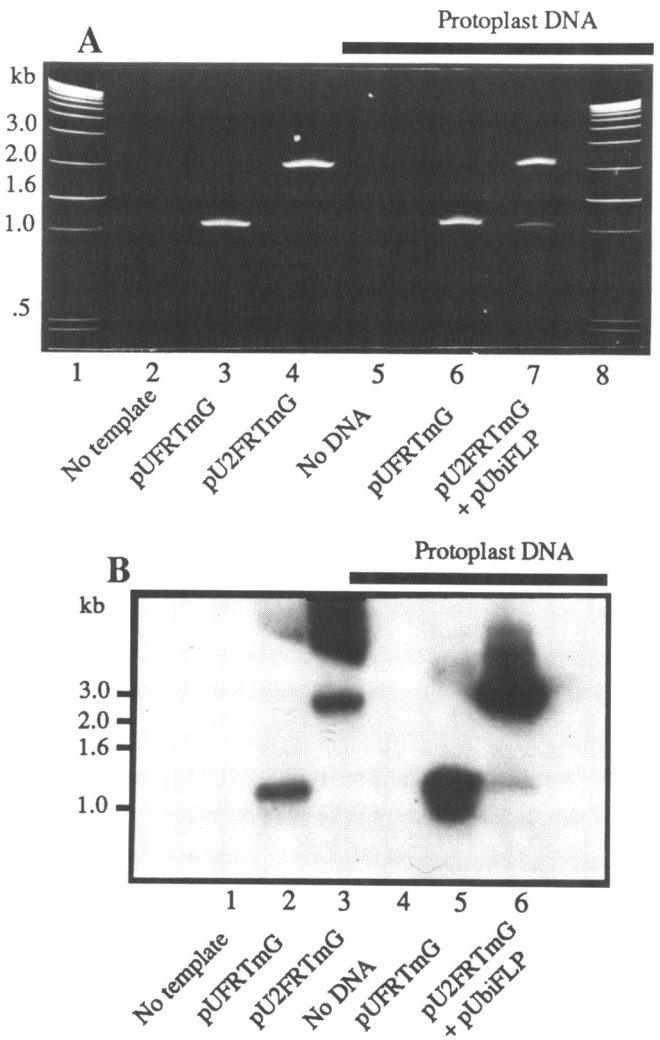

Figure 6. Electrophoretic analysis of PCR amplified products of total DNA isolated from co-transformed maize protoplasts. (**A**) Products of the PCR amplification reactions. The primers used are described in the Materials and Methods section. Lanes 1 and 8 are 1 kb ladders (BRL). Lanes 5–7 represent the amplification products of total DNA isolated from 1×10^7 maize protoplasts treated with no DNA (lane 5), pUFRTmG (lane 6), and co-transformed with pUbiFLP and pU2FRTmG (lane 7). Lanes 3, 4 show the amplification products of pUFRTmG and pU2FRTmG plasmid DNA (5 ngs), respectively. Lane 2 shows negative control of PCR reaction – no template DNA. (**B**) Southern blot analysis of DNA amplified by PCR reactions. After separation of the PCR products on an agarose gel, DNA was transferred onto a nylon membrane and hybridized to the ^{32}P-labeled probe (first intron of the ubiquitin gene). Lanes 4, 5, and 6 represent hybridization patterns of the PCR products from total DNA isolated from transformed protoplasts. The 1.18 kb fragment amplified by the PCR procedure, as a result of FLP-mediated recombination reaction, contains sequences hybridizing to the first intron of the ubiquitin gene.

shows GUS activities in corn protoplasts following transformation using plasmids containing either one or two FRT's and in combination with the pUbiFLP expression vector. When pUbiFLP was introduced into maize protoplasts along with the test plasmids, GUS activities were restored for both pU2FRTmG and pU2FRTG to 81% and 45% of the respective control treatments. Reactivation of GUS in the presence of pUbiFLP and the vectors containing both FRT's indicated that FLP protein catalyzed excision of the 1.31 kb fragment. When the FRT's were mutated by the reverse ligation of the 1.31 kb fragment (pU2FRTmG rev. and pU2FRTG rev.), GUS activity was not restored indicating that these FRT sites were not recognized by the FLP protein (Table 1).

The FLP protein can catalyze recombination between two separate DNA molecules. To observe such intermolecular recombination at the FRT sites, the ubiquitin promoter or the *gus*A coding sequence was removed from pU2FRTmG to form pFRTGUS and pUbiFRT, respectively. Introduction of two FRT sites into protoplasts on two separate DNA molecules (pUbiFRT and pFRTGUS) produced approximately a 4-fold increase in GUS activity above background activity in protoplasts transformed with pFRTGUS alone (Table 1). This activity was 15% of the positive control (protoplasts transformed with pUFRTmG) (Table 1). The relatively high background expression of GUS in pFRTGUS transformed protoplasts (6 ± 1 unit) might be the result of the entire first intron and part of the ubiquitin first exon being present in this vector.

The FLP/FRT recombination system has also been tested in rice protoplasts (Fig. 5). The recombinase-mediated excision of DNA proved to be as effective in rice as in maize protoplasts. The same pattern of response was observed for the different plasmid constructions containing FRTs. Co-transformation of rice protoplasts with pUbiFLP and either pU2FRTmG or pU2FRTG gave 75% and 31% restoration of the control GUS activity. Vectors containing inactive FRT sites gave only 1–2% of the control GUS activity (Fig. 5).

PCR analysis of total DNA isolated from co-transformed maize protoplasts indicated the presence of recombinant plasmid DNA molecules (Fig. 6). The two primers used for the analysis amplify the region between the ubiquitin transcription start and the 5′ end of the *gus*A coding sequence. The length of this region is 2.49 kb in pU2FRTmG and pU2FRTG. After removal of the 1.31 kb fragment as a result of the recombination reaction, the amplified region should be reduced to 1.18 kb and should then be equivalent to the amplification product of pUFRTG DNA. The fragment of 1.18 kb was identified in the products of the

PCR reaction using total DNA isolated from maize protoplasts co-transformed with pU2FRTmG and pUbiFLP as a template (Fig. 6A, lane 7). The identity of this fragment was confirmed through hybridization using a probe specific to the first intron of the ubiquitin gene (Fig. 6B, lane 6).

DISCUSSION

Relatively simple site-specific recombination systems of prokaryotes and eukaryotic yeast provide potential for manipulations of eukaryotic genomic DNA. The Cre/lox system of the bacteriophage P1 has already proven its usefulness. It has been used to remove selectable markers (*hpt* and ALS^r genes) from the genome of transgenic tobacco cells (31,32) and to stabilize expression of β-galactosidase in transgenic mouse cells (6).

Eukaryotic recombination systems (from yeast plasmids), however, may be more effective for operation in the nucleus of eukaryotic organisms. Due to the complexity of eukaryotic chromatin, prokaryotic recombinases may be less efficient in recognizing specific sites and performing the recombination reactions. O'Gorman et al. (15) observed greater efficiency of the FLP/FRT recombination system *versus* the Cre/lox system in mouse cells; however, the observed effect might have been related to either the activity of the recombinases or differences in the vector constructions. For higher plant cells, the choice of site-specific recombination systems is limited. Despite the advanced research on the prokaryotic Cre/lox system (32,33) and successful expression of a mutant Gin recombinase in *Arabidopsis thaliana* and tobacco protoplasts (9), only one eukaryotic site-specific recombination system (the R/RS system of yeast) has been demonstrated to function in plant cells prior to this report (10).

The results presented here show that the FLP recombinase of yeast can promote the site-specific recombination between FRT sites in both maize and rice cells. This conclusion is based on the following evidence. Transient expression of the GUS enzyme from recombination test vectors increased in protoplasts co-transformed with functional FLP expression vectors, and the magnitude of this increase depended on the amount of co-transformed plasmid DNA containing the FLP gene. Additionally, higher expression of GUS protein in co-transformed protoplasts was observed when a stronger promoter was used to drive expression of the FLP protein. The increase in GUS expression was abolished if the FRT sites in test vectors were mutated. Lastly, the products of the site-specific recombination reaction were identified in co-transformed protoplasts.

When the stronger maize ubiquitin promoter was placed in the FLP gene vectors instead of the *Adh*1 promoter, the restored GUS activity increased from approximately 10% to 60–90% of the corresponding positive control (protoplasts transformed with pUFRTmG). This indicated that, when using pAeiFLP, the amount of the FLP protein expressed was too small to saturate the recombination system at the time of GUS activity assays. A large excess of FLP expression vector DNA relative to test plasmid DNA in a transformation mixture could increase the efficiency of the recombination reaction (15, Fig. 4B). However, as demonstrated in this paper, the use of a stronger promoter to drive expression of the FLP protein would appear to be a better solution for obtaining high recombination signals.

The high efficiency of FLP catalyzed DNA excision found in our experiments when using the short FRT recombination sites may be related to the reversibility of the recombination reaction. The FLP/FRT recombination system can catalyze both intra- and intermolecular reactions (14, 36, Table 1). Alteration of the structure of the FRT sites was one approach to alter the rates of the recombination reactions in each direction (35). Jayaram (36) showed that the FRT site when composed of only two repeats (comparable to the FRTm site used in this study) was virtually a non-substrate for the intermolecular recombination in bacteria cells, but the DNA excision reaction was still detectable. Morris et al. (14), using similar synthetic FRT sites, observed 60–100% efficiency of excisions and very low number of integration events in insect embryos. In contrast, the complete FRT sites used by O'Gorman et al. (15) yielded low efficiency of excisions (8–47%) but a high number of integration events in mouse and monkey cells. We have demonstrated varying efficiencies of accumulation of excision products based upon the structure of the FRT sites integrated into otherwise identical DNA vectors. Under the same conditions in either maize or rice protoplasts, plasmid DNA with the full length FRT sites gave only a modest increase in GUS expression (25–50% of the control treatment) as compared to the short FRT sites (60–90%).

The obvious next step in research on the yeast FLP/FRT system in plants is to show its interactions with genomic DNA. We do not anticipate major difficulties in this search since a similar R/RS recombination system (10), as well as other prokaryotic recombination systems (8,9), were able to recognize their target sites once integrated into genomic DNA.

ACKNOWLEDGMENTS

The authors thank Dr V.Chowdhury for providing rice protoplasts. This work was supported by a grant from NRICGP of the Agricultural Research Service, U.S. Department of Agriculture. Jon Mitchell would like to express his appreciation to DowElanco Company for their support of his educational leave assignment at Purdue University. This is journal paper XXXX of the Purdue University Agriculture Experimental Station.

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