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FLP-mediated recombination of *FRT* sites in the maize genome

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ABSTRACT

Molecular evidence is provided for genomic recombinations in maize cells induced by the yeast FLP/*FRT* site-specific recombination system. The FLP protein recombined *FRT* sites previously integrated into the maize genome leading to excision of a selectable marker, the *neo* gene. NPTII activity was not observed after the successful recombination process; instead, the *gusA* gene was activated by the removal of the blocking DNA fragment. Genomic sequencing in the region of the *FRT* site (following the recombination reaction) indicated that a precise rearrangement of genomic DNA sequences had taken place. The functional FLP gene could be either expressed transiently or after stable integration into the maize genome. The efficiency of genomic recombinations was high enough that a selection for recombination products, or for FLP expression, was not required. The results presented here establish the FLP/*FRT* site-specific recombination system as an important tool for controlled modifications of maize genomic DNA.

INTRODUCTION

The FLP recombinase of the 2 μm plasmid of yeast, being a member of the Int family of site-specific recombinases (1), shares a number of structural and functional features with the other well characterized recombinases including bacteriophage P1 Cre protein, phage λ integrase, or yeast R protein (2). Oligomerization of protein monomers, each containing the invariant arrangement of Arg-His-Arg-Tyr amino acid residues (3,4), is required to form an active site. The first step in the recombination reaction involves protein binding to the recognition target sites (these *FRT* sites consist of dyad symmetry elements and the core region) followed by cleavage of the phosphodiester bond at the border of the core region by a nucleophilic attack of the active site tyrosine hydroxyl group (5–7). The subsequent strand exchange reaction generates a transient Holliday intermediate—another common feature of the Int protein family (8,9). The exchange of the second pair of DNA strands completes the recombination reaction.

The FLP/*FRT* system of yeast and the Cre/*lox* system of bacteriophage P1 are the primary candidates for applications in genetic studies of higher eukaryotes. They represent a simple two-component (recombinase and its target site) recombination system which does not discriminate between the integrative and excisional recombination activities unlike, for example, the members of the Tn3/Hin family of the site-specific recombinases (10,11). The Cre recombinase has been successfully used to activate, or inactivate, genes that had been integrated into genomic DNA of plant cells (12–15) or mouse cells (16,17). Seed-specific gene activation mediated by the Cre/*lox* system has also been demonstrated in transgenic tobacco (18). The same recombination system was used to assure uniform expression of foreign DNA in mouse cells (19), or to assist in gene targeting experiments by removing unwanted DNA sequences from a targeted locus (20–22). The Cre protein can recombine *lox* sites located on separate chromosomes thus rearranging the chromosomal structure of a eukaryotic genome (23).

FLP recombinase was shown to work in higher eukaryotic cells including *Drosophila*, mouse, maize, rice, *Arabidopsis* and tobacco cells (24–29). But subsequent application of the FLP recombinase for gene targeting experiments in mouse cells was only partially successful (30,31). Research groups have experienced difficulties in the isolation of recombination products resulting from the excisional activity of the FLP protein. It has not been, however, clearly established whether this difficulty was because of the intrinsic properties of the FLP recombinase or whether it was due to other factors affecting the overall efficiency of the recombination process.

Here, we report on genomic recombination activities of the FLP/*FRT* site-specific recombination system in maize cells. We show that re-transformation of maize protoplasts with the FLP expression vector can lead to successful deletion of a selectable marker previously integrated into genomic DNA. The fidelity and efficiency of the process is high; thus, this observation validates the use of the FLP/*FRT* system for future genomic DNA rearrangements in maize plants.

MATERIALS AND METHODS

Plasmid constructions and transformation procedure

Construction of the pUbiFLP vector was described previously (27). The vector pUFNeoFmG, containing a *neo* gene bordered by a full-length and a modified *FRT* site and the promoterless *gusA* gene (a recombination marker), was constructed from pUFRTG (27) by replacement of the *gusA* coding sequence (the *Sma*I–*Sac*I fragment) with the *neo* coding sequence (*Bam*HI fragment of pTO77) to give pUFRTNeo vector. The *Bam*HI–*Eco*RI fragment of the pU2FRTmG (27) comprising a promoterless *gusA* gene, the first intron of maize *Ubi-1* gene, and a modified *FRT* site was

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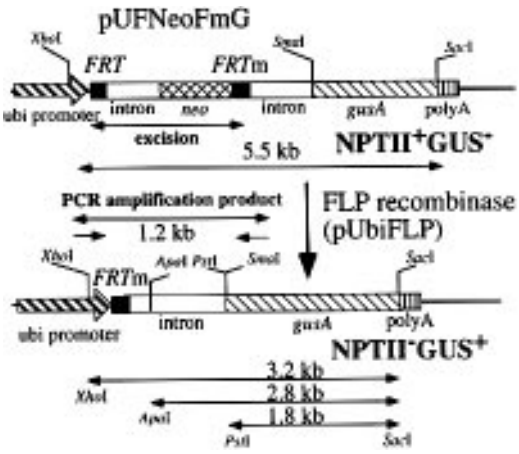

Figure 1. Strategy for testing FLP-mediated genomic deletions. The fragment of the pUFNeoFmG vector to be excised contains the intron and the *neo* coding sequence flanked by two *FRT* sites (black boxes). A successful recombination should generate the active *gusA* gene, changing the phenotype from NPTII-positive and GUS-negative ($NPTII^+GUS^-$) to NPTII-negative and GUS-positive ($NPTII^-GUS^+$). pUbiFLP is the FLP-expression vector that provides FLP protein both transiently and after stable integration into the genome. The original *Xho*I–*Sac*I fragment of 5.5 kb in length is truncated after recombination to 3.25 kb. Restriction sites only relevant to this study are indicated.

subsequently blunt-end ligated into the *Eco*RI site of the pUFRTNeo vector to form pUFNeoFmG. A diagram of this vector is presented in Figure 1 and the sequences of the full-length and modified *FRT* sites are provided in Figure 4. All plasmids used for transformation were purified by CsCl equilibrium density gradient centrifugations (32).

The detailed description of procedures used for maize A188xBMS protoplast isolation, transformation, culture, and selection can be found in (33). Briefly, 1×10^7 protoplasts in 1 ml of transformation medium [100 mM MES (pH 5.5), 0.2 M mannitol, 80 mM $CaCl_2$] were mixed with 50 µl of plasmid DNA (1 mg/ml) and 1 ml of 50% PEG (MW = 8000; Sigma Chemical Co., St Louis, MO) in F-solution (34). After 30 min incubation at room temperature followed by the PEG dilution and protoplast washing in protoplast culture medium, transformed protoplasts (0.2 ml of protoplast suspension at 1×10^6 viable protoplasts per 1 ml) were plated onto Millipore filters (0.8 µm pore size) that overlay feeder cells. One week later, the Millipore filters were transferred onto fresh feeder plates amended with 100 µg/ml kanamycin sulfate for selection of transgenic calli. After one week, the microcalli were transferred onto Murashige and Skoog medium containing 2 mg/l 2,4-dichlorophenoxyacetic acid supplemented with 100 µg/ml kanamycin sulfate for further selection.

Southern blot analysis, PCR and DNA sequencing

Genomic DNA was isolated from callus tissue by grinding ~500 mg of tissue in 5 ml of DNA extraction buffer (35). After 15 min incubation at 60°C, an equal volume of phenol was added, and the homogenate was centrifuged to separate aqueous and organic layers. DNA was precipitated from the aqueous phase by adding an equal volume of isopropanol, and after centrifugation the DNA pellet was dissolved in TE buffer. Subsequent steps of CsCl density gradient centrifugation were performed as previously described (32). Genomic DNA (5 µg) was digested and electrophoresed in a 0.8% agarose gel. Southern blot analysis was performed using vacuum transfer to Hybond-N membrane (Amersham, Arlington Heights, IL), UV membrane irradiation, and hybridization to the radioactive *gusA* coding sequence probe according to standard procedures (32). The probe was prepared using the Rediprime Random Primer labeling kit (Amersham, Arlington Heights, IL). PCR analysis for detection of the recombination products was performed using the primers complementary to the 3′ end of the ubiquitin promoter (5′-CCCCAACCTCGTGTTG-3′) and to the 5′ end of the *gusA* coding sequence (5′-CGCGATCCAGACTGAATGC-3′). The length of the amplified fragment should be 1.2 and 2.8 kb for the product and substrate of the recombination reactions, respectively. Because of its size, efficient amplification of the 2.8 kb fragment was not expected. DNA (100–200 ng) was subjected to 30 cycles of amplification of three steps each (94°C, 1 min; 60°C, 1 min; 72°C, 2 min) in PCR buffer (10 mM Tris–HCl, pH 8.4; 50 mM KCl; 1.5 mM $MgCl_2$; 0.01% gelatin) containing 0.2 mM of each dNTP, 0.1 nM of each primer, and 1.25 U native *Taq* DNA polymerase (Perkin-Elmer, Norwalk, CT). PCR products were analyzed by gel electrophoresis in 1.0% agarose gels.

For DNA sequencing, the 1.2 kb PCR amplified fragment was phosphorylated with T4 DNA kinase (New England BioLabs, Beverly, MA) and then blunt-end ligated into the *Sma*I site of the pGEM7Zf(+) vector (Promega, Madison, WI). The insert was sequenced using the T7 promoter primer (Promega, Madison, WI) by modification to the Sanger dideoxy method (36) and fluorescent chain terminating reactions (37). Sequence data were analyzed using a DuPont Genesis 2000 DNA Analysis System.

GUS and NPTII activity assays

Samples of transgenic calli were sonicated for 5–10 s in GUS extraction buffer containing 0.1% Triton X-100 (38). After centrifugation for 5 min at 16 000 *g*, the supernatant was used directly for GUS activity and protein assays. GUS activity was assayed using a fluorogenic substrate (MUG; 4-methylumberiferyl β-D-glucuronide) and a Perkin Elmer LS50B fluorometer essentially as described in (38). Reactions were terminated at timed intervals, and GUS activity was calculated from the slope of the line generated from time points and normalized to the protein content. NPTII activity was assayed using the dot-blot method as previously described (39). Callus extracts (prepared as for the GUS activity assay) were incubated in a reaction buffer containing 67 mM Tris–HCl (pH 7.1), 42 mM $MgCl_2$, 0.4 M NH_4Cl, 0.01 mM ATP, 0.03 mM neomycin, 10 mM NaF, 1–2 mCi/ml [^{32}P]ATP. Aliquots of the reaction mixture were blotted onto Whatman P81 paper. The blot was washed with 10 mM phosphate buffer (pH 7.5), dried, and washed again with the same buffer at 80°C for 10–15 min. The P81 paper was exposed to X-ray film from one to several hours (exposure time depended on NPTII activity) at room temperature.

RESULTS

Integration of recombination test vector into maize genomic DNA

A vector, pUFNeoFmG, used to assay the FLP activity in maize cells is shown in Figure 1. It provides a fully functional *neo* gene to select stably transformed cells and a promoterless *gusA* gene whose subsequent activation should indicate an FLP-mediated

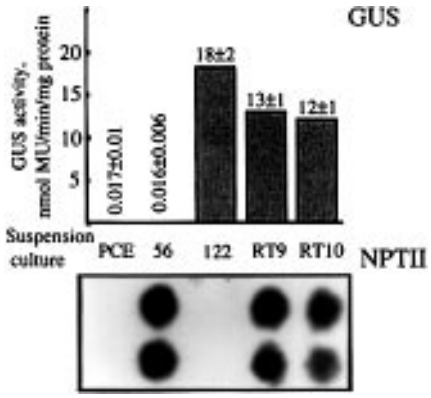

Figure 2. GUS and NPTII activities in hygromycin-resistant line number 122. Line number 122 was generated from the original line number 56 after re-transformation with the pUbiFLP vector. PCE (Protoplast Culture E) is the untransformed suspension cell line. Lines RT9 and RT10 are derivatives of line 122 that were re-transformed with the pUbiFRTNeo vector (the *neo* gene expression vector).

excision of the *neo* gene. The two *FRT* sites flanking the *neo* gene are different. The modified *FRT* site (*FRT*m) contains only two symmetry elements. This modification does not substantially affect the function of the FLP recombinase (27,40,41). However, as discussed later, two structurally different *FRT* sites provided a means to clearly distinguish site-specific recombination products from possible artifacts generated by other genomic DNA modifications. Transgenic kanamycin-resistant maize calli were screened for a simple vector integration pattern and GUS activity to identify $NPTII^{+}GUS^{-}$ phenotypes. One of the callus lines, No. 56, containing a single 5.5 kb *Xho*I–*Sac*I genomic DNA fragment hybridizing to the *gusA* probe was selected (Fig. 3).

Introduction of the FLP recombinase into maize cells

A suspension culture of line 56 was established. Protoplasts of this line were re-transformed with equimolar amounts of pUbiFLP and pHyg (a vector containing the *hpt* gene driven by the 35S CaMV promoter). One hundred and ninety hygromycin-resistant calli were selected and screened for activation of GUS expression. Most of the hygromycin-resistant re-transformed calli showed GUS activity at the level of 0.063 ± 0.003 fluorescence units (corresponding to the background GUS activity in line 56 of 0.016 ± 0.006 nmol MU/min/mg protein), while 58 calli showed GUS activity >0.1 fluorescence unit. Activation of the *gusA* gene expression should indicate the excisional activity of the FLP protein. These callus lines were not screened for the presence of the FLP protein, thus only ~50% of the hygromycin-resistant calli were expected to express FLP—the average co-transformation efficiency in our system (42). The DNA excision reaction in line 122 led to the $NPTII^{-}GUS^{+}$ phenotype (Fig. 2). Protoplasts of line 122 re-transformed with the *neo* expression vector (lines RT9 and RT10 in Fig. 2) regained NPTII activity indicating that the absence of the NPTII activity in line 122 was not related to changes in physiological status of these cells.

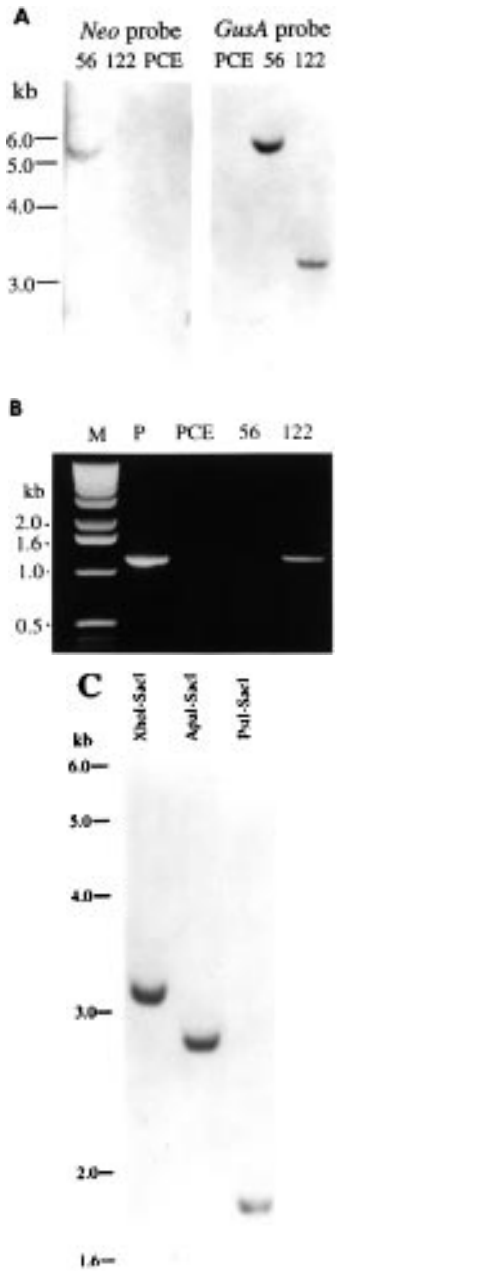

Figure 3. (**A**) Southern blot analysis of FLP-mediated recombination in hygromycin-resistant maize callus. Genomic DNA (10 µg) was digested with *Xho*I and *Sac*I and probed with the *neo* coding sequences (left panel) or the *gusA* coding sequences (right panel). The DNA from untransformed callus is described as PCE. Line number 122 was generated from the original line number 56 after re-transformation with the pUbiFLP vector. 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 (Fig. 1). Numbers on the right indicate the sizes of DNA molecular markers. (**B**) PCR analysis. DNA sample designations are analogous to the Southern blot (Fig. 3A). Lane P shows the product of the pUbiFRTGUS vector DNA (expected product of site-specific recombination reaction) and lane M indicates position of DNA markers. The primers used are described in Materials and Methods and shown in Figure 1. The recombination product should generate a 1.2 kb amplification fragment. (**C**) Southern blot analysis of genomic DNA isolated from the GUS-positive/FLP transiently-transformed maize callus lines. DNA from transgenic maize suspension presented in lane 4 (Fig. 5) was additionally digested with *Sac*I and *Xho*I, *Apa*I and *Pst*I to verify the recombiantion 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 (Fig. 1).

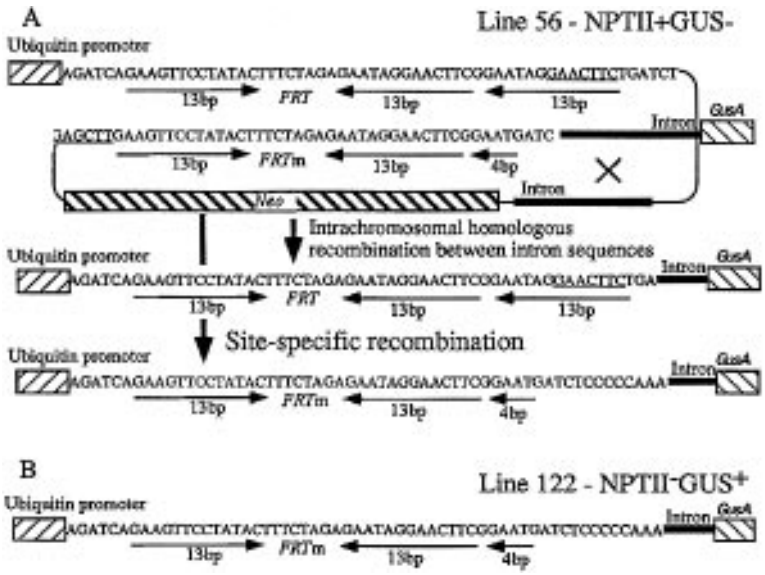

Figure 4. Sequence of the *FRT* sites integrated into the maize genome before (**A**) and after (**B**) site-specific recombination. The two *FRT* sites in line 56 share a region of 39 bp identity. The sequences are different outside of this region (underlined nucleotides). Any conservative DNA recombination within the 39 bp identical regions of the *FRT* sites, including the site-specific recombination, should exchange flanking DNA segments producing 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.

Analysis of GUS-positive clones

Several GUS-positive clones were selected to analyze the FLP-mediated excision process. In *Xho*I–*Sac*I digests of genomic DNA from these clones, the expected 3.2 kb fragment hybridizing to the *gusA* probe was detected (data not shown). This is illustrated for the callus line designated as 122 (derived from line 56) which shows only the 3.2 kb band hybridizing to *gusA* and no DNA sequences hybridizing to the *neo* probe (Fig. 3A). PCR analysis further confirmed the presence of the recombination product in genomic DNA of line 122 (Fig. 3B).

Verification of the FLP-mediated recombination reaction

Although very unlikely, there was a possibility that the *neo* coding sequence could be removed by a spontaneous recombination process involving the repeated ubiquitin intron sequences in front of both the *neo* and the *gusA* coding sequences. Intrachromosomal homologous recombinations between intron sequences could yield a product analogous to the site-specific recombination reaction product (Fig. 4A). If this was the case, however, the product of recombination would contain the original *FRT* site positioned in front of the ubiquitin intron sequence, whereas the product formed by FLP-catalyzed site-specific recombination reaction would contain a chimeric *FRT* that originated from the recombination of the *FRT* and the *FRT*m sites. A genomic DNA fragment amplified by the PCR reaction (Fig. 3B), consisting of the 5′-untranslated sequence of the *gusA* gene in line 122, was subcloned into pGEM7(z) vector and its 5′-end containing the *FRT* site was sequenced. The structure of the integrated *FRT* site was indeed chimeric and exactly as expected from FLP-mediated site-specific recombination reaction (Fig. 4B).

Removal of a selectable marker

Transient expression of the *FLP* gene might provide sufficient FLP protein to recombine *FRT* sites that were previously integrated into chromatin structures (fig. 6 in ref. 43). Accordingly, protoplasts isolated from pUFNeoFmG stably transformed line were re-transformed only with the FLP expression vector (pUbiFLP). Protoplasts were allowed to grow without selection and resulting mini-calli were randomly picked up for GUS activity analysis. In these experiments, a frequency of FLP-mediated activation of the GUS expression was 2–3% (24 GUS-positive calli among 940 analyzed). The high frequency of recombinase-mediated excisions makes it easy to find the events by PCR (100 PCR assays could provide 2–3 positive samples), although we have not tested such a possibility directly. Activation of GUS expression was also correlated with the rearrangement of the DNA fragment containing the *neo* and the *gusA* genes in a similar manner as in stably transformed line 122 (Fig. 3C). A Southern blot analysis of DNA from two GUS-positive calli did not show evidence of the FLP coding sequences in genomic DNA suggesting that, indeed, site-specific recombinations were the result of transient FLP gene activities (Fig. 5). In comparison, genomic DNA from line 122, selected on hygromycin-containing medium, contained sequences hybridizing to the FLP probe (Fig. 5, lane 122). The other hygromycin-resistant, GUS-positive line 61 was apparently not stably co-transformed with the pUbiFLP vector, indicating that the FLP-mediated excision also occurred without the FLP gene integration into the genome.

DISCUSSION

Predictable modifications of the genome of higher eukaryotes have become reality due to the applications of homologous and site-specific recombinations—for recent reviews see refs 44–47. The results presented in this paper prove that genomic recombinations can be efficiently induced in maize cells by application of the yeast FLP/*FRT* site-specific recombination system. Recombinations take place when the FLP gene was stably integrated and

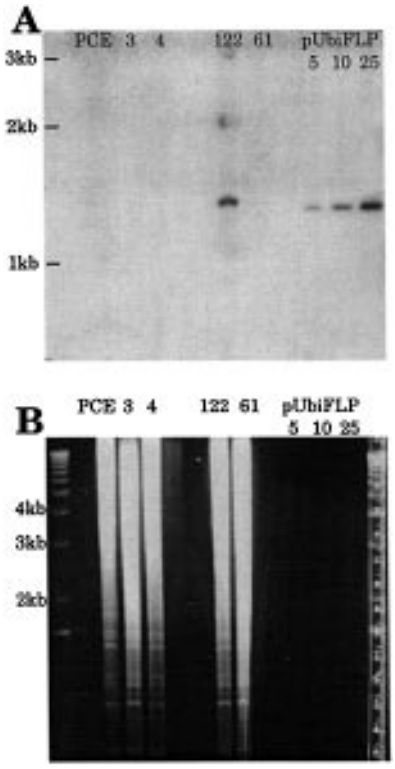

Figure 5. (**A**) Genomic Southern blot of DNA isolated from callus that had been treated with the pUbiFLP vector. Transformation resulted in the *neo* gene excision by transient expression of the FLP gene. Lanes 3 and 4 show that the FLP gene had not integrated into the genome. Lanes 122 and 61 show DNA isolated from calli stably transformed with pUbiFLP. A DNA fragment, 1.4 kb in length, comprising the FLP coding sequence was expected after digestion of genomic DNA (10 µg) with *Sma*I/*Sac*I restriction enzymes. The same FLP gene fragment was used as a probe. PCE indicates DNA isolated from untransformed suspension line. The pUbiFLP vector DNA, digested with *Sma*I/*Sac*I restriction enzymes, was loaded into lanes 5, 10 and 25 (5, 10 and 25 pg, respectively). (**B**) The picture of agarose gel, used for the Southern blot, stained with ethidium bromide to verify the amount of DNA loaded. The lane designations correspond to the Southern blot.

expressed in maize cells, or under conditions when only transient expression of the FLP gene occurred (Figs 3 and 4).

Re-transformation with the FLP-expression vector was used to obtain molecular evidence of site-specific genomic recombination events in maize cells. Approximately one per four hygromycin-resistant calli showed GUS activity, indicative of the site-specific recombination process. The product of site-specific recombination was identified in all GUS-positive maize cells tested. Most of them, however, contained additional DNA rearrangements which resulted in novel DNA fragments hybridizing to the *gusA* or the *neo* probe. This could indicate that recombination was not complete, or that the reaction took place after the first mitotic division of re-transformed cells (producing chimeric material), or that the excised fragment integrated again into another chromosomal location. Such 'experimental noise' is an intrinsic property of all site-specific recombination systems tested (13,18,25) and needs to be taken into account if the experimental objective is to completely eliminate a selectable marker rather than just to activate a silent gene. Nevertheless, complete DNA excision events were easily identified within a pool of re-transformed material. Based on experiments presented here and earlier studies (43), the FLP protein functions effectively in maize cells. In tobacco cross-breeding experiments, 17.5% of progeny seedlings showed the hygromycin-sensitive phenotype instead of the expected 25% (i.e. 70% efficiency) if the introduced FLP gene had been 100% effective (26). These results correspond to ~50–90% efficiency for the Cre-mediated excision in tobacco plants (12,15).

The fidelity and precision of site-specific recombinations is high because of the nature of the catalytic mechanism and the conservative character of this reaction. Indeed, it has been demonstrated that Cre-mediated chromosomal recombinations yield predominantly accurate recombination products (12,16). Based upon genomic sequencing, we found a high fidelity for the recombination reaction mediated by the FLP recombinase in maize cells as well (Fig. 4). This precision, although expected, is vital in order to further utilize FLP in practical applications of genomic engineering.

O'Gorman *et al.* (25) reported 70–80% genomic recombination events in mammalian cells transiently transformed with the *FLP* gene. But subsequent application of the FLP/*FRT* system to eliminate a selectable marker in gene targeting experiments produced only one deletion out of 192 colonies tested (with a transient transformation efficiency of ~5%) (31). The use of the same pOG44 FLP recombinase expression vector by Fiering *et al.* (30) did not produce a single deletion in 548 transiently transformed mouse clones. However, in the plant system studied here, transient expression of the *FLP* gene produced deletions of the *neo* gene in 2–3% (24 out of 940) of the screened calli. These results are comparable with the Cre/*lox* site-specific recombination system which yielded 2–4% deletions of the *neo* gene from mouse targeted interleukin 2 receptor gene (21). We used the same modified coding region of the FLP gene from the pOG44 vector in maize experiments; although, it seems that these modifications are not necessary for achieving a high activity of the FLP gene in plant cells (26). We did use altered *FRT* sites, however. Transient expression assays indicated a lower yield of excision products when two full-length *FRT* sites were used as compared with one full-length and one modified *FRT* site (27). The modified FRT site contained only two binding sites for the FLP protein. Indeed, it has been implied that the presence of additional FLP protein units at the site of crossing-over may adversely affect the resolution of recombination intermediates (7).

It seems that a strong expression of the FLP gene controlled by the ubiquitin maize promoter (one of the strongest promoters available for monocot cell transformations) significantly contributed to the efficient recovery of recombinant events (27). FLP-mediated recombinations depend on the amount of FLP protein produced in cells. This has been demonstrated in transient assays both in mouse and plant cells as well as in transgenic FLP-expressing animals (48). It is also possible that the chromosomal location of the target *FRT* site in line 56 was readily accessible to the FLP enzyme. The location of the target sites was observed to have an effect on recombination rates in other recombination systems (14).

Application of site-specific recombination systems will lead to more sophisticated control of the genetic transformation process, and an important consequence could be the production of environmentally safer transgenic plants with the antibiotic- or herbicide-resistance genes removed. Current procedures, however, require time-consuming methods such as cross pollination and subsequent genetic segregation of transgenic loci (11,12). On the other hand, the use of transient expression of the FLP recombinase

procedure presented in this paper uses a re-transformation step and depends on a highly efficient transformation protocol. In the future, this can be simplified further by using regulated expression of the recombinase gene (43,49–51). After successful transformation and selection, the activated recombinase could cleanse the transgenic genome from any unwanted foreign DNA sequences, including the recombinase gene itself.

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