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Visual characterization of recombination at *FRT-gusA* loci in transgenic tobacco mediated by constitutive expression of the native FLP recombinase

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Abstract FLP/*FRT*-mediated site-specific recombination was studied with a recombination-reporter gene system which allows visualization of β-glucuronidase (GUS) expression after site-specific excisional activation of a silent *gusA* gene. This system was used for characterization of the functional activity of the *Saccharomyces cerevisiae* native FLP recombinase driven by the cauliflower mosaic virus (CaMV) 35*s* promoter [linked to the tobacco mosaic virus (TMV) omega translational leader] in mediating site-specific recombination of chromosomal *FRT* sites in tobacco *FLP* × *FRT*-reporter hybrids. Six hybrids were generated from crosses of lines containing either a stably integrated recombination-reporter or a FLP-expression construct. The activated *gusA* phenotype was specific to hybrid progenies and was not observed in either parental plants or their selfed progenies. Recombination efficiency in whole seedlings was estimated by the percent of radioactivity on a Southern blot which was incorporated into the recombined DNA product. Estimated efficiency mean values for the six crosses ranged from 5.2 to 52.0%. Histochemical analysis in hybrid plants visualized GUS activity with variable chimeric patterns and intensities. Recombination efficiency and GUS expression varied both among and within crosses, while higher recombination efficiency coincided with larger and more intense patterns of GUS activity. These data suggest that recombination is induced randomly during somatic developmental stages and that the pattern and intensity generated in a given plant are affected by factors imposing varibility not only between but also within crosses. Additionally, while recombination in a population of FLP/*FRT* hybrids may occur in all plants, recombination efficiency may still be low in any given plant. The activity of the native, as compared to a modified, FLP (Kilby et al. 1995) in the activation of transgenic traits in tobacco is discussed.

Key words Native FLP/*FRT* · Site-specific · Excisional recombination · Transgene activation · β-Glucuronidase · Tobacco

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Introduction

Site-specific recombination systems, such as Cre/*lox* of phage P1, R/*RS* of *Zygosaccharomyces rouxii* and FLP/*FRT* of *Saccharomyces cerevisiae*, function through interactions of a recombinase with its specific target site. Whether intramolecular (excision, inversion) or intermolecular (integration) recombination occurs depends on the relative position and orientation of the recombination target sites (for a review see Craig 1988). Recombination between target sites on separate circular molecules produces a co-integrate. Intramolecular recombination between invertly or directly oriented sites results in inversion of the intervening DNA or its excision as a circular molecule, respectively.

Site-specific recombination systems have been found to function efficiently in various heterologous organisms and provide potentially powerful tools for the manipulation of chromosomal DNA and the control of transgene expression in higher eukaryotic organisms (for reviews see Kilby et al. 1993; van Haaren and OW 1993; Odell and Russell 1994; Sauer 1994; OW and Medberry 1995). Site-specific recombination activity in plant cells has been demonstrated with Cre/*lox* (Dale and Ow 1990, 1991; Odell et al. 1990, 1994; Bayley et al. 1992; Russell et al. 1992), R/*RS* (Onouchi et al. 1991, 1995), a modified Gin/*gix* system of phage Mu (Maeser and Kahmann 1991), and FLP/*FRT* (Lyznik et al. 1993, 1995; Lloyd and Davis 1994; Sonti et al. 1995; Kilby et al. 1995). Excisional recombination in whole plants mediated either loss of a trait (Dale and Ow 1991; Russell et al. 1992; Bayley et al. 1992) or gain of a selectable phenotype which resulted from elimination of

an interrupting target site-bounded DNA from a silent selectable marker gene in the hybrid progeny (Odell et al. 1990; Bayley et al. 1992; Lloyd and Davis 1994). Using the latter strategy, the expression of Cre or FLP under the control of the CaMV 35*s* promoter, mediated the activation of *hygromycin phosphotransferase* (*hpt*) and enabled germination of hygromycin-resistant (Hyg^R) seedling progeny on a selective medium (Bayley et al. 1992; Lloyd and Davis 1994). This indicated that, with both systems, recombination can occur at an early developmental stage in tobacco. In an alternative approach, a cryptic *gusA* recombination-reporter construct was used to visualize the patterns induced by Cre/*lox* under the control of the CaMV 35*s* promoter and seed-specific promoters in tobacco (Odell et al. 1994), R/*RS* and FLP/*FRT* under the CaMV 35*s* promoter in *Arabidopsis* (Onouchi et al. 1995; Sonti et al. 1995) and, more recently, a modified *FLP* (derived from pOG44; O'Gorman et al. 1991) driven by CaMV 35*s* and heat-inducible promoters in tobacco and *Arabidopsis*, respectively (Kilby et al. 1995). Here we have used a similar approach to visually characterize the site-specific recombination activity of the native, non-modified, FLP in populations of cross-hybrid FLP/*FRT* plants. A CaMV 35*s* promoter-omega leader-FLP expression vector (JFL) and an *FRT*-interrupted *gusA* reporter construct (FFG) were brought together into the same plants by crossing the respective transgenic lines. GUS activity was observed in hybrids but not in parental lines or their selfed progenies. Histochemical examination indicated GUS activity in variable patterns and intensities. Recombination efficiency (estimated by Southern analysis of whole-seedling DNA) and GUS activity from the activated *gusA*-reporter varied coincidentally among crosses and, additionally, among individual progeny of each cross. Potential differences in activity between the modified and native FLP in tobacco is discussed.

Materials and methods

DNA modifications and plasmid constructions

Molecular cloning procedures were carried out as described in Maniatis et al. (1982). *Eco*RI-digested pJD330 (in which *gusA* is placed under the control of the CaMV 35*s* promoter and omega enhancer sequences; Gallie et al. 1989) was cloned into a pPCV002 binary plasmid (Koncz and Schell 1986), generating the 11.1-kb pGUS. The *SalI-SmaI* β-glucuronidase (GUS)-coding region of pGUS was then replaced with the 1.3-kb *SalI-FspI* FLP protein-coding region of pFV17 (Volkert and Broach 1986) generating pJFL (see Fig. 1 A).

pNeOβGal containing the *neomycin phosphotransferase*-coding region (*npt*) flanked by two directly oriented *FRT* sites (O'Gorman et al. 1991) was obtained from Stratagene (La Jolla, Calif.). The *FRT-npt-FRT* region of pNeOβGal was amplified by the polymerase chain reaction (PCR) using the primers 5′-GGGGGCTAG-TCGACAGAAGTTCCT-3′ and 5′-GGCCAGTGGTCGACCTT-GAAGTTCC-3′. These primers were designed to direct a new *SalI* at each end of the amplified DNA product. PCRs were peformed using a Thermal Controller (M-J Research Inc., Watertown, Mass.). Cycling conditions were 1 min at 94 °C; 25 cycles of 1 min at 94 °C, 1 min at 45 °C and 4 min at 72 °C; and extension at 72 °C for 10 min. Reactions contained 5–10 ng of DNA template, 0.2 mM of dNTPs, 160 ng of each primer and 1.5 U of *Taq* DNA polymerase (Boehringer-Mannheim, GmbH, Germany) in a final reaction volume of 50 µl. Reaction products were analysed by electrophoresis in 1% agarose gels. The resulting 1.4-kb *FRT-npt-FRT* amplified product was digested with *SalI* at both ends, gel purified, and directly ligated into the *SalI* site of pGUS to generate pFFG (Fig. 1 B). During the construction of both pJFL and pFFG all newly formed 5′ and 3′ junctions were confirmed by DNA sequencing.

Formation and manipulation of transgenic plants

The pJFL and pFFG binary constructions were introduced into *Agrobacterium tumefaciens* GV3101-pMP90RK (Koncz and Schell 1986) with the freeze-thaw procedure (An et al. 1988). *Agrobacterium* clones were used to transform leaf disks of *N. tabacum* L. samsun NN (Horsch et al. 1985). Kanamycin-resistant (Kan^R) plants were regenerated and transferred to the greenhouse. The number of genomic loci of the integrated DNA was evaluated by both Southern-blot analysis and the germination of T_1 seeds from selfed primary transformants. Seeds were surface-sterilized (20 min in a solution of 20% bleach and 0.1% SDS, rinsed three times for 20 min in sterile water) and then sown on MS medium containing 3% sucrose and 100 µg/ml of kanamycin (Duchefa, Haarlem, the Netherlands). After 3–4 weeks green seedlings with developed roots were scored as Kan^R while those with a bleached phenotype and arrested root growth were scored as Kan^S.

Cross-pollination of transgenic plants harbouring a single hemizygous copy of either pJFL [called JFL(72), JFL(74), JFL(201)], or pFFG [called FFG(600), FFG(601), FFG(602), FFG(611)] yielded hybrid JFL × FFG plants. All pollinated flowers were bagged in order to prevent access to other pollen.

Southern blotting and hybridization

Greenhouse seedlings containing 4–5 fully developed true-leaves where frozen in liquid N_2, homogenized to a fine powder and then sampled for total DNA isolation (Doyle and Doyle 1990). Ten micrograms of DNA were digested with *Eco*RV, separated on a 1% agarose gel and blotted onto a Qiabrane membrane. The *gusA*-specific hybridization probe was gel-purified as a 0.6-kb *Eco*RV-*SalI* fragment from pFFG (see Fig. 1B) and ^{32}P-labeled using a random priming kit (Boehringer-Mannheim, GmbH, Germany). Following hybridization in the presence of 50% formamide, according to the manufacturer's instructions, the membranes were washed twice for 15 min in 2 × SSPE (0.18 M NaCl, 10 mM NaH_2PO_4, 1 mM EDTA, pH 7.7) and 0.1% SDS at room temperature, for 30 min in 1 × SSPE and 0.1% SDS at 42 °C, and once for 30 min in 0.1 × SSPE and 0.1% SDS at 65 °C. Southern products were visualized by X-ray autoradiography and scanned by a Personal Densitometer (Molecular Dynamics, Sunnyvale, USA).

Fluorometric GUS activity assay

To score GUS(+) and GUS(−) progeny, a cotyledon from the fully developed 2nd-true-leaf stage of Petri-dish grown seedlings was ground individually in a tube containing 100 µl of GUS assay buffer [1 mM 4-methyl-umbelliferyl-β-glucuronide (MUG) in lysis buffer containing 50 mM NaH_2PO_4, pH 7.0, 10 mM EDTA, 10 mM β-mercaptoethanol]. After centrifugation for 5 min at 12000 g, the supernatent was incubated at 37 °C for 1 h. Than, 60 µl of the reaction was transferred to 1.2 ml of stop solution (0.2 M Na_2CO_3) and fluorescence was measured in a TKO-100 mini-fluorometer (Hoefer Scientific Instruments, San Francisco, Calif.). GUS activity was expressed as nmol of 4-methylumbelliferone (MU) produced per min per mg of protein (Jefferson et al. 1987). Samples with GUS activity of less than 0.02 units were defined as GUS(−) and those with above 0.28 units were scored as GUS (+). No intermediate values were observed.

Histochemical GUS-activity assay

Seedlings, grown under sterile conditions at 26 °C, 16-h light/8-h dark photoperiod cycles, were immersed in ether 10 s, dried briefly and then put in 5 ml of X-Gluc staining solution (0.1 M phosphate buffer, pH = 7.0, 50 mM potassium ferrocyanide, 50 mM potassium ferricyanide, 10 mM EDTA, 0.1% triton X-100, 0.1% sarkosyl, 14 mM β-mercaptoethanol, 1 mM X-glucuronide; Jefferson et al. 1987). Following a 2-min evacuation at 600 mmHg, incubation proceeded for 16–18 h at 37 °C in the dark. Seedlings were then dehydrated through repeated replacements with CLP (2:1:1 mixture of chloral hydrate, lactic acid and phenol) for up to 3 days (Beeckman and Engler 1994). Photographs were taken under a Zeiss stereomicroscope using Fujichrome RD135 100 ASA film.

Statistics

Data was analyzed by a one-way analysis of variance using the general linear model procedure (PROC - GLM) of the SAS package (SAS Institute, 1989). Treatments were compared by Duncan's multiple range test at the 0.05 protection level. Normal distribution of data was confirmed by the Shapiro-Wilk test.

Results

Experimental strategy

The experimental strategy used in this study is schematically described in Fig. 1. The yeast 2μ-plasmid native *FLP* recombinase gene was positioned under the control of the CaMV 35*s* promoter and omega leader in pJFL (Fig. 1A). The recombination-reporter construct, pFFG, contains a 1.31-kb *npt* spacer DNA, bounded by directly oriented *FRT* sites and inserted between the CaMV 35*s* promoter-omega enhancer and the *gusA* structural gene. As a result, *gusA* expression from the 35*s* promoter in the pFFG construct was blocked. After FLP-mediated excision (Fig. 1 B, bottom) the remaining 35*s*-omega-*FRT-gusA* product was expected to permit transcription and the accurate translation of *gusA*. The effectiveness of these constructions to direct excisional *FRT*-specific recombination and activate expression of GUS was studied in FLP/*FRT* hybrid tobacco plants.

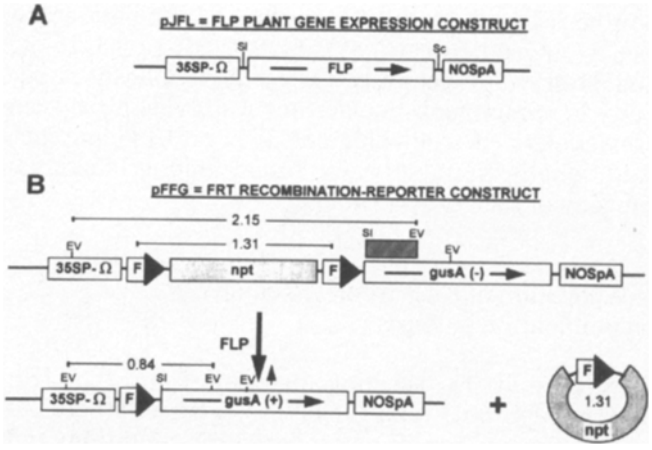


Fig. 1A A schematic diagram of the FLP expression binary-vector construct, pJFL. The yeast 1.3-kb *FLP* recombinase gene is positioned under the control of the CaMV 35*s* gene promoter and the omega translational enhancer sequences of TMV (Gallie et al. 1989) as well as the nopalin synthase polyadenylation signal. **B** A schematic diagram of the *FRT* recombination-reporter construct, pFFG. The *neomycin phosphotransferase* coding region (*npt*) is flanked by *FRT* target sites (*F*) (O'Gorman et al. 1991) and inserted between the 35*s* promoter-omega control sequences and the *gusA* gene. Thus β-glucuronidase is not expressed and the phenotype is gusA(−). Excision of the 1.31-kb intervening *npt* DNA results in an activated β-glucuronidase gene and a gusA(+) phenotype. Positions of the expected *Eco*RV 2.15-kb and 0.84-kb Southern products are indicated. The probe for *gusA* is shown by a *striped bar* and relevant RE sites and expected Southern products (in kb) are indicated. Abbreviations are: *SI SalI*; *Sc SacI* and *EV Eco*RV

Parental JFL and FFG transgenic lines

Both pJFL and pFFG binary vector plasmids were introduced into tobacco plants via *Agrobacterium*-mediated leaf-disk transformation. The copy number of transgene loci was determined by Southern analysis (data not shown) and confirmed by segregation of the Kan^R phenotype in selfing progeny of hemizygous parents. Segregation of Kan^R: Kan^S in a nearly 3:1 ratio (Table 1, top) is indicative of a single transgene locus. Lines that contained a single integrated pJFL or pFFG locus were selected for further study.

JFL and FFG transgenic lines and their selfing progeny exhibited normal developmental phenotypes. Additionally, all selfed FFG progeny expressed no GUS activity (Table 1, top). This suggests minimal or no cryptic *FRTs* or endogenous FLP-like activity in tobacco.

Frequency and efficiency of recombination in F_1 hybrids

To monitor FLP/*FRT* activity in plants, JFL and FFG loci were brought into the same plants by cross-pollination of four hemizygous FFG plants with pollen from three hemizygous JFL plants. After selection on kanamycin-containing media, a cotyledon from 2nd-true-leaf stage Kan^R hybrid seedlings was excised and subjected to a GUS activity assay. The Kan^R seedlings of each cross constituted about three-quarters of the total progeny (Table 1, bottom). GUS activity was positively scored in one-third of the Kan^R population. These numbers are consistent with the expected segregation ratio of progenies theoretically having the JFL/FFG genotype (Table 1, bottom). Southern-blot analysis was used to verify FLP-mediated excisional recombination in the chromosomal FFG locus (Fig. 2). Total genomic DNA, extracted from whole-seedling homogenates of 8–15 individuals from each cross, were digested with *Eco*RV, separated on agarose gels and blot-hybridized to a *gusA* probe (Fig. 1B). A 2.15-kb fragment characteristic of the intact, non-recombined FFG-substrate DNA was visualized in the parental FFG(611) plant (Fig. 2, lane 1). A 0.84-kb DNA fragment, which arises due to

Table 1 Segregation of Kan^R and GUS(+) phenotypes in self-and cross-fertilized progeny

Self-fertilization	Observed numbers of progeny plants				Estimated recombination frequency[b]
	Tested	Kan^R	Kan^R:Kan^S	GUS(+)[a]	
JFL(72)	189	143	3.1:1	0	0
JFL(201)	45	34	3.1:1	0	0
JFL(74)	112	87	3.5:1	0	0
FFG(601)	148	104	2.4:1	0	0
FFG(600)	51	40	3.6:1	0	0
FFG(611)	64	50	3.6:1	0	0
FFG(602)	51	38	2.9:1	0	0
Cross-fertilization[c]					
JFL(72) × FFG(611)	150	120	4.0:1	43	43/40
JFL(72) × FFG(602)	149	107	2.5:1	31	31/36
JFL(72) × FFG(600)	135	95	2.4:1	27	27/32
JFL(72) × FFG(601)	369	279	3.1:1	98	98/93
JFL(201) × FFG(601)	102	79	3.4:1	32	32/26
JFL(74) × FFG(601)	122	88	2.6:1	26	26/29

[a] GUS expression in Kan^R progeny of the JFL × FFG crosses was scored at their 1–2-true-leaf stage

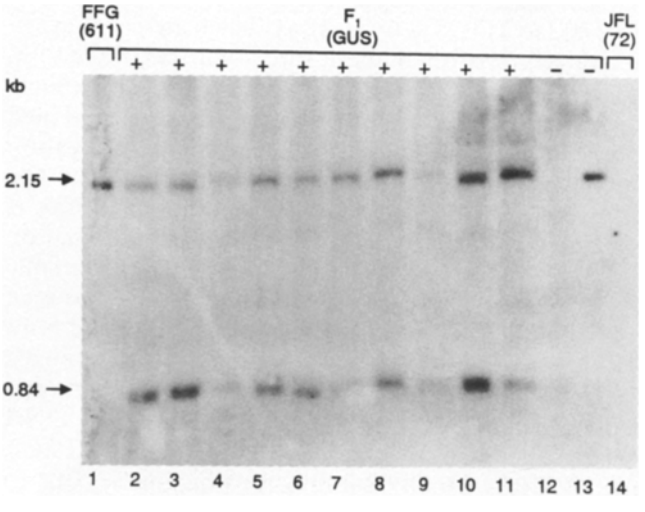
[b] The ratio between the number of seedlings which exhibited a GUS(+) phenotype to the number of seedlings which were predicted to have the JFL/FFG genotype. A chi-square goodness-of-fit test indicated no significant differences between actual GUS(+) and predicted JFL/FFG segregants at $P = 0.05$

[c] Hemizygous FFG plants were cross-pollinated with pollen from hemizygous JFL plants

excision of the intermediate 1.31-kb *FRT-npt-FRT* sequence, characterizes the FLP-mediated recombination product of FFG and was visible only in GUS(+) progeny (lanes 2–11). This signal was not evident in either the JFL(72) parent (lane 14) or in GUS(−) progeny (lanes 12, 13). The presence of both the recombined product (0.84 kb) and the non-recombined substrate (2.15 kb) band reflects the chimeric status of recombination in these plants. The appearance of the predicted 0.84-kb post-excisional signal only in GUS(+) progeny confirms *FRT* site-specific recombination and the re-location of the CaMV 35*s* promoter proximal to *gusA* at the FFG genomic locus.

Recombination efficiency in the hybrid progeny was estimated by the percent of the 0.84-kb product following scanning of the radioactive 2.15-kb and 0.84-kb Southern bands. In the six crosses recombination efficiency ranged between 1–13, 3–28, 6–31, 12–43, 16–35 and 31–65%, respectively, (Table 2). Significant variability in recombination efficiency was evident between crosses derived from different JFL or FFG parents. Additionally, variability was found among individual progeny of each cross (Table 2).

Fig. 2 Southern analysis of FLP-mediated excisional recombination in FFG (611-female) × JFL (72-male) progeny and parental plants. Ten micrograms of *Eco*RV-digested total-plant DNA were fractionated on agarose gels and blot hybridized to the *gusA* probe described in Fig. 1B. Positive and negative scores for GUS activity were determined on a cotyledon from fully developed 2nd-true-leaf stage, Petri-dish-grown seedlings, as described in Materials and methods. The sizes (in kb) of expected products are indicated



Visualization of FLP-mediated chimeric recombination patterns

Control and F_1 seedling progeny from the JFL (201) × FFG(601) and JFL(72) × FFG(611) crosses were grown in Petri dishes under sterile conditions and then incubated with X-Gluc (see Materials and methods). As expected, no staining was detected in parental FFG(611) plants (Fig. 3A) whereas GUS activity was observed in most tissues of pGUS transgenic plants (Fig. 3B). Representative recombination patterns, as reflected by GUS expression in plantlets in their 4th-developed leaf stage, are shown in Fig. 3C–F. In both crosses particularly strong staining was observed in veins, roots and scattered single spots and expression was stronger in cotyledons and older leaves. There was, however, large variation between and within both crosses. From 80 GUS(+) progeny of the JFL(72) × FFG (601) cross, approximately 25% showed small blue spots

Fig. 3A–F Histochemical visualization of somatic recombination in JFL × FFG hybrids. Fourth-leaf stage plantlets were incubated in X-Gluc (See Materials and methods). Negative and positive GUS activity from the 35*s* promoter-omega leader was examined in FFG (611) (**A**) and pGUS (**B**) plantlets, respectively, as well as in progeny from the JFL(72) × FFG(611) cross (**C–F**). The magnificantion is X 3.5

Table 2 Recombination efficiency in JFL × FFG hybrid progenies. a, b, c, d represent significant differences at $P < 0.05$

Cross	Number of plants	Estimated recombination efficiency,[a] [mean ± SE (range)]
JFL(72) × FFG(611)	10	52.0 ± 3.4 (31–65) a
JFL(72) × FFG(602)	12	28.1 ± 2.5 (12–43) cd
JFL(72) × FFG(600)	9	11.9 ± 3.0 (3–28) cd
JFL(72) × FFG(601)	8	25.4 ± 2.4 (16–35) b
JFL(201) × FFG(601)	15	17.1 ± 1.9 (6–31) c
JFL(74) × FFG(601)	9	5.2 ± 1.6 (1–13) d

[a] Relative intensity (%) of the 0.84-kb band after scanning of the radioactive 0.84-kb and 2.15-kb Southern bands of each plant

scattered in cotyledons, leaves and root tips (similar to the type shown in Fig. 3C); 61% showed extensive staining patterns in cotyledons (Fig. 3D), and in 14% this pattern was extended further to the 1st and 2nd leaves (Fig. 3E). Both of the latter types exhibited various additional small GUS-active patches scattered in

younger leaves (Fig. 3D,E). One-hundred and eighteen GUS(+) progeny of the JFL(72) × FFG(611) cross showed a higher staining intensity, as compared to the former cross. The types shown in Fig. 3C, D and E were observed in frequencies of approximately 5%, 24% and 36%, respectively. In the remaining 35% of this population, blue staining appeared mainly in shoot tissues and also in some regions of roots and root tips (Fig. 3F). Histochemical analysis of plants from the 2nd-leaf seedling stage, until flowering, revealed a progressive appearance of GUS-activity in leaves (tested with leaf-disks), sepals and petals (data not shown). To test the recombination status of the FFG locus in the F_2 generation, four F_1 plants from each cross were self-pollinated and the resulting F_2 seedlings were subjected to histochemical staining. Examination of 2107 F_2 seedlings showed an approximately 9:7 ratio of stained to non-stained individuals. This is consistent with independent segregation of hemizygous JFL and FFG loci.

Discussion

In this study, excisional activation of *gusA* was used to visualize the functional activity of the native 2μ-plasmid FLP recombinase under the control of the CaMV 35*s* promoter-omega leader in catalyzing the excisional recombination of chromosomally integrated *FRT* sites in tobacco. Both the recombination-substrate (FFG) and FLP-expressor (JFL) plants exhibited normal phenotypes and upon self-pollination demonstrated simple Mendelian inheritance for their respective transgenic traits. The absence of GUS activity in all seedlings derived from self-pollination of the FFG lines (Table 1, top) indicate minimal, or no, spontaneous recombination of *FRTs* in the tobacco genome. In all six populations of JFL × FFG cross progenies, a GUS(+) phenotype appeared in a frequency consistent with that expected for the segregation of a hemizygous JFL/FFG hybrid genotype (Table 1). Southern analysis of F_1 seedlings derived from these crosses indicated a chimeric status of recombination, and histochemical staining visualized chimeric patterns of GUS expression. These patterns are a direct consequence of FLP-mediated excisional activation of *gusA* in the FFG locus, at different developmental stages. GUS expression at higher intensity in cotyledons and older leaves seems to reflect late, rather than recombinational events of clonal origin. In a few instances, however, the appearance of partial leaf (data not shown) and root sectors of GUS(+) phenotype (Fig. 3C) suggests the clonal development of meristematic recombination events. Both the R/*RS-gusA* (Onouchi et al. 1995) and FLP/*FRT-gusA* (this report) systems generated a large vairability in the GUS expression patterns found both between crosses and among seedling progeny of each cross. Variability among crosses is related mainly to the chromosomal positions of transgenic loci in alternative parents. However, since GUS(+) progeny of a given F_1 cross are genetically identical, variability due to gene dosage or 'chromosomal position' effects are unlikely. Rather, this variability may suggest recombination events taking place at different developmental stages in different sibs. This phenomenon is most likely affected by methylation of either the recombinase or the recombination target-site loci during development.

Accumulating data on the activity of the FLP/*FRT* system in tobacco provides a suitable background for the comparison of different FLP expression constructs and recognition sequences. Constitutive activity of FLP recombinase in tobacco *FLP/FRT* hybrids was previously shown by Lloyd and Davis (1994), who used a selectable reporter system, and more recently by Kilby et al. (1995), who characterized recombination at *FRT-gusA* loci. In the latter report, efficient transfer of the recombined locus through germinal cells was suggested. In the present work, using a similar experimental strategy revealed that recombination activity progressed during plant development up to the flowering stage; yet the inheritance of recombined loci to the next generation was either absent or occurred at a very low rate. Since in both studies the wild-type 48-bp *FRT* site was used, this discrepancy may relate to the different FLP gene constructions employed. While experiments described here were conducted with the native *FLP* structural and immediate 3′ sequences, Kilby et al. (1995) used a modified *FLP* gene derived from pOG44 (O'Gorman et al. 1991). In the latter construct cryptic splicing acceptor sites, that are found immediately 3′ to the native FLP, and three canonical polyadenylation signals (AATAAA), from the 5′ part of the native coding region, have been eliminated without actual alteration of the native amino-acid sequence. These modifications have been shown to elevate FLP activity in mammalian cells (O'Gorman et al. 1991). This suggests that the constitutive expression of the native FLP in tobacco accumulated sufficient protein for the generation of chimeric and sectoral somatic patterns; yet in contrast to pOG44 FLP, it was not sufficient to promote the efficient inheritance of the recombined loci. Comparing different modified forms of FLP/*FRT* and different expression levels of recombinase in whole plants (currently in progress) will contribute to a better understanding of how these factors affect recombination activity for a variety of potential applications (Kilby et al. 1993; van Haaren and Ow 1993; Odell and Russell 1994; Ow and Medberry 1995).

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