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ORIGINAL PAPER

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A test for ectopic exchange catalyzed by Cre recombinase in maize

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Abstract A maize line expressing Cre recombinase as well as the recipient line without the transgene were assayed for evidence of ectopic recombination within the maize genome. Such a test is valuable for understanding the action of Cre as well as for its use to recombine two target *lox* sites present in the chromosomes. Pollen examination and seed set tests of material expressing Cre provided no evidence of ectopic recombination, which would be manifested in the production of translocations or inversions and result in pollen abortion and reduced seed set. Root-tip chromosome karyotype analysis was also performed on material with and without Cre expression. Chromosomal aberrations in Cre+ material were not observed above the background level.

Introduction

The *cre-lox* recombination system is an efficient mechanism used to integrate target DNA sequences into a genome or to excise sequences from transgenic inserts. Cre recombinase (Cre), the *cre* gene product, is an integrase derived from the bacteriophage P1. Cre recognizes two adjacent 34-bp *lox* sites and recombines the DNA within them (Hoess and Abremski 1990). The relative orientation of the *lox* sites (inverted versus directly repeated orientation) dictates the type of recombination that Cre performs, such as deletions or inversions, respectively. At low frequency, Cre may also recombine *lox* sites located on different chromosomes to cause translocations. Cre/*lox* site-specific recombination with target DNA sequences has been applied to a variety of animal and plant systems (Chou and Perrimon 1992; Sauer and Henderson 1988; Golic 1991; Orban et al. 1992; Vergunst et al. 1998; Qin et al. 1994; Hajdukiewicz et al. 2001; Gilbertson 2003: Zhang et al. 2003) The Cre-*lox* system has also been used to induce chromosomal rearrangements at specific locations in genomes (Ow and Medberry 1995; Osborne et al. 1995; Yu and Bradley 2001). Such capabilities will help researchers study in vivo the effects of genome rearrangements on the function of the organism. In addition, *cre-lox* has been used to target transgenes to pre-existing *lox* sites and excise selectable marker genes or other DNA sequences that are no longer required after transformation experiments (Dale and Ow 1991; Russell et al. 1992; Zuo et al. 2001; Hoff et al. 2001 ; Hoa et al. 2002; Zhang et al. 2003).

Evidence from experiments in yeast using a powerful selection regime that allows the detection of rare recombinants shows that Cre recombinase may recognize natural variations of *lox* sites in addition to the 34-bp sites (Sauer 1992). Until recently, evidence was lacking that Cre recombinase may cause ectopic recombination with other sites in a genome. Such apparent promiscuity might lead to chromosomal rearrangements that impact the normal function of the affected cell and its progeny. It would also complicate the use of *cre-lox* for biotechnological applications. Schmidt et al. (2000) have shown that the *cre-lox* P system driven by a very strong promoter in post-meiotic mouse spermatids causes ectopic rearrangements, leading to sterility in all male founders and their descendants. Loonstra et al. (2001) showed

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that expression of Cre recombinase may induce more chromosomal aberrations than normal in cultured mammalian cells. In addition, they found that any toxicity induced by *cre* depends on its level of activity, but consistently low levels of expression seem to allow for normal recombination without the toxicity. Others have shown that Cre activity induced later in development can also obviate the apparent detrimental effects (Hare and Chua 2002).

In plants, the available evidence suggests that Cre recombinase may have no effect on some plants, whereas aberrant phenotypes may be observed in others. For example, Qin et al. (1994) used the *cre-lox* system for site-specific recombination in tobacco and observed no phenotypes that would infer large-scale genomic rearrangements resulting from Cre recombinase activity. On the other hand, aberrant phenotypes have been observed in petunia, tomato, and tobacco that correlate with *cre* activity (Que et al. 1998; Coppoolse et al. 2002). In these cases, *cre* was under the control of the constitutive and strong cauliflower mosaic virus (CaMV) 35S promoter. In an effort to study Cre-mediated chromosome effects using a genetic model in plants, we created transgenic maize inbred lines that express Cre recombinase. Potential aberrant phenotypes and/or genotypes caused by Cre were monitored by the frequency of translocations in meiotic chromosomes as measured by pollen abortion rate or kernel seed set. In a separate analysis, the presence of chromosomal aberrations was tested in mitotic metaphase chromosomes using fluorescence in situ hybridization (FISH) analysis. We also screened the progeny of self-pollinated, *cre*-expressing plants for defective kernel mutations as a measure of any *cre*-induced mutagenic activity. The results show that the *cre* gene tested has no apparent effect in maize.

Materials and methods

The *cre* construct (pMON36136) line, the *lox* tester line (ZM_S27755), and the LH198 inbred were obtained from Monsanto Company (St. Louis, Mo.). The *cre* transgenic line used was 5080-01-01 (Zhang et al. 2003). The *lox* tester line contained a transgenic insert comprising selectable marker 5-enolpyruvylshikimate-3-phosphate synthase from *Agrobacterium tumefaciens* strain CP4 (*EPSPS-CP4*) flanked by *lox511* sites (Hoess et al. 1986) and a chimeric Bt gene (m 11768) as the gene of interest. The material containing *cre* resulted from a cross of plants homozygous for the *cre* construct to the recipient inbred line, LH198. The construct, pMON36136 (Zhang et al. 2003), places *cre* expression under the control of the *act1* promoter from rice and the first *act1* intron (McElroy et al. 1990). The *cre* is interrupted between nucleotides 431 and 432 of its open reading frame by intron 2 of the *ST-LS1* gene in potato (Vancanneyt et al. 1990). The selectable marker gene for transformation was the neomycin phosphotransferase gene (*npt*II), driven by the e35S promoter and a HSP70 intron.

Individual pollen samples were collected separately and preserved in 1.5-ml tubes containing 95% ethanol. Normal pollen is opaque; aborted pollen grains are collapsed and translucent.

Metaphase spreads were prepared according to Kato (1997, 1999) with some modifications. The slides were UV cross-linked to prevent loss of DNA during the denaturing and washing steps. Probes were labeled by nick translation, column-purified and co-precipitated 2 h to overnight with 35 μg salmon sperm DNA. In each labeling reaction, 1 μg or 5 μg of DNA was used. FISH was performed essentially according to Kato et al. (2004).

In order to demonstrate the ability of this technique to recognize translocations, we examined a selection of translocations obtained from the University of Illinois stock center. These translocations have been converged to either inbred M14 or W23. This set involves a series referred to as the T-waxy translocations that all involve chromosome 9 and various other chromosomes. Comparing the normal inbred line to the available converged translocations provides a test of the ability of the chromosome identification kit to discern the presence of translocations. Five different translocations [1-9(4995); 2-9c; 3-9c; 6-9e; 8-9(6673)] were tested and could be distinguished with this technique, thereby demonstrating its effectiveness for recognizing translocations. Some subtle translocations might go undetected, but a comparative approach of Cre+ and Cre− lines will likely reveal most translocations.

Detection of Cre activity in plant crosses

Progeny derived from crosses between *cre* parents and *lox* tester plants were screened by an ELISA assay on four-leaf-stage tissue to detect the EPSPS-CP4 protein. The *lox* tester plants were homozygous, so loss of EPSPS-CP4 protein indicated Cre-mediated excision. Because the Cre parents were hemizygous for the *cre* and *npt*II genes, an NPTII ELISA was used to screen the same progeny for the segregation of *cre* and *npt*II and confirm that loss of EPSPS-CP4 was dependent on the presence of *cre*. Selected progeny were also screened by Southern blot for evidence of Cre-mediated recombination between *lox* sites, leading to excision of the *EPSPS-CP4* gene. DNA extraction and Southern blotting was carried out as previously described (Zhang et al. 2003) using leaf tissue from eight-leaf-stage plants. Details about the specific enzyme used for digests and the DNA fragment used as probes are given in the figure legends or text.

Results

A test for mutagenic effects of Cre recombinase

Three thousand kernels from inbred LH198 containing the *cre* construct and 3,000 kernels without the construct were planted in an isolated field north of Columbia, Missouri, USA on June 1, 2002 using a randomized block design. Each plant that emerged was tagged with a row and plant number. A portion of the plant tag was used to track the ear, and another portion was removed and preserved with the respective pollen sample. When possible, plants were self-pollinated to assay for any heritable mutations generated by the expression of *cre*. If Cre caused any significant mutagenesis, a higher rate of defective kernel mutations relative to the Cre− samples would be observed. Recessive mutations were detected on the basis of a 3:1 ratio for any of the potentially hundreds of endosperm mutations (Neuffer et al. 1997). In order to have a 95% probability of detecting at least one mutant kernel, if the expectation is one in four, 21 kernels must be present on an ear (Hanson 1959). Therefore, only those ears with 21 kernels or more were considered in the analysis. A total of 801 ears fulfilled this criterion and were examined (421 Cre+ and 380 Cre−) for any type of defective kernel (*dek*) mutations segregating in a 3:1 ratio. Candidate ears segregating 3:1 were subject to a chi-square test of significance. Two ears fulfilled the statistical criterion of a segregating *dek* mutation (one Cre+ and one Cre− ear, $P < 0.05$), and both cases involved kernels that were smaller than their siblings but neither exhibited characteristics typical of more distinctive *dek* mutations. To test whether these cases were due to environment effects or truly represented new mutations, we used normal kernels from these ears to test for heritability of the putative mutations. If these cases were true mutations, two-thirds of the normal kernels should be heterozygous for the mutation and would exhibit a 3:1 ratio of normal to mutant when grown to maturity and self-pollinated. Eight normal kernels from each of these ears were planted in the greenhouse and self-pollinated to confirm whether a *dek* mutation was segregating. Only one Cre− ear showed evidence of a 3:1 ratio ($P < 0.05$). Based on this analysis, we concluded that there was no evidence that low expression of Cre induces any heritable mutations at a detectable rate.

Pollen abortion analysis

Pollen samples from 2,114 Cre+ individuals and 2,073 Cre− individuals were collected and preserved in 95% ethanol. An image was taken from an aliquot of each sample; the total number of normal and aborted pollen grains from each sample were recorded. If any rearrangements occurred in the previous generation they would be present as heterozygotes in the present generation. Similarly, we would be able to detect any rearrangements occurring early in development of the plant that result in pollen abortion in the affected tassel sectors. Pollen sample pictures with 25 grains visible were included in the final analysis because this number is required to have a 95% probability of observing at least one aborted grain if the expected frequency is one in nine (Hanson 1959). Theoretically, this cutoff should detect pollen samples with 11% pollen abortion or higher. Of the samples collected, 2,391 samples fulfilled this criterion. Of these, only one plant had pollen abortion greater than 10%, namely 105-26, which was a Cre− plant.

As a control, pollen from 164 translocation heterozygotes was examined. The translocation was between chromosomes 3 and 4 in the W22 inbred line [T3-4 (4726)]. For these samples, 87 pictures contained 25 grains. The abortion rates were routinely higher than our observations in the Cre+ and Cre− pollen but fell lower than the 50% abortion rate expected with a translocation heterozygote. The reason for this is unknown. Cytological examination of root-tip chromosomes, as described below, of the translocation heterozygous material confirmed this genotype. All of the translocation plants examined with a hand microscope during the field season showed high levels of pollen abortion. Random sampling of the Cre+ and Cre− pollen using the hand microscope revealed no case of pollen abortion above the very minimal normal level.

Cre activity assay

The Cre line used in this study has previously been shown to efficiently catalyze recombination and marker gene excision in crosses with lines bearing *lox*-flanked marker genes (Zhang et al. 2003). To confirm that the plants used in this field study possessed levels of Cre activity sufficient to catalyze marker excision, we used selected Cre+ and Cre− plants to pollinate *lox* tester plants in which the *EPSPS-CP4* gene was flanked by *lox* sites. Progeny from these crosses were screened by an ELISA for the EPSPS-CP4 protein. Cre activity would be expected to result in the excision of the *EPSPS-CP4* gene. Since the *lox* tester plants were homozygous and the Cre parents were hemizygous, efficient excision would be reflected as a 1:1 segregation of the *EPSPS-CP4* gene. An additional prediction was that all *npt*II-positive plants (Cre+ lines) would be negative for the *EPSPS-CP4* gene, and vice versa. The data in Table 1 show the results of EPSPS-CP4 and nptII ELISA on progeny from two crosses with two different Cre+ parents (147-2 and 103-2), as well as a control cross with a Cre− parent (104-3). These data indicate complete excision of the *EPSPS*-CP4 gene in the two crosses with Cre+ parents, thereby confirming that our Cre+ line is active.

Table 1 Phenotypic evidence[a] for Cre activity in crosses

Parent	CP4+ NPTII+	CP4− NPTII+	CP4+ NPTII−	CP4− NPTII−
147-2	0	20	22	0
103-2	0	24	16	0
104-3	0	0	82	0

[a]The numbers indicate the number of progeny from the crosses that had the phenotypes indicated at the top of the column. P4 and NPTII were assayed by ELISA (see Materials and methods section)

A total of 27 Cre+ progeny from three additional crosses with three different Cre+ parents were examined by Southern blot methods to confirm that excision had occurred as expected. Digestion with *Mfe*I, combined with the use of a fragment of the M11768 gene as a probe, was expected to produce a single band of approximately 3.1 kb if excision occurred precisely between the two *lox* sites. As seen in Fig. 1, all 27 progeny had the expected size band, indicating that excision had occurred in every progeny plant. A sample from a single plant from a cross with a Cre− control plant had the 6.4-kb band expected for lack of excision. These results of these two tests (EPSPS-CP4 ELISA and Southern blot) confirmed that the Cre plants used in this study had levels of Cre activity sufficient to catalyze a high frequency of recombination between *lox* sites, as previously reported (Zhang et al. 2003).

Screening for translocations with FISH

In addition to screening pollen and kernels, we used FISH to test for any translocations between chromosomes that might be attributable to *cre*. Kato et al. (2004) have developed a system to identify all ten pairs of mitotic metaphase chromosomes using a combination of DNA probes. This procedure also allows one to detect translocations between chromosomes. Three probes are chromosome-specific, whereas the others are found on more than one chromosome but at different locations and intensities. Each probe was chosen because it identifies a particular chromosome or helps distinguish a particular chromosome in combination with the other probes. Taken together, these probes produce a pattern on each chromosome that allows for the clear identification of all ten pairs of chromosomes. Any deviation in the pattern of probes on the chromosomes indicates that a translocation may have occurred.

Seven different probes were used in our FISH experiments (Table 2). We direct-labeled each probe with one of four fluorochromes—Texas Red dUTP, Oregon Green dUTP, Coumarin Blue dUTP, and Cyanine-5 dUTP. Because certain repeats hybridize to different locations on a given chromosome, we labeled them with the same fluorochrome. This enabled us to use more probes without confusing the identity of any one probe. We used all of the filter channels; consequently, the chromosomes were not counterstained. Background and autofluorescence were sufficient to locate the chromosomes.

Because of its intensity, the only sequence we labeled with Cyanine-5 dUTP was the 180-bp knob sequence, which is a highly repeated sequence found in heterochromatic regions (Dennis and Peacock 1984). Knobs vary in intensity and location from chromosome to chromosome and across different inbred lines.

Table 2 Collection of probes and fluorescent labels used

Probe	Label (dUTP)	Color
180-bp knob	Cyanine-5	White
TR-1 repeat	Texas red	Red
5S rRNA genes	Coumarin blue	Blue
Nucleolus organizer region	Coumarin blue	Blue
Centromere repeat (CentC)	Coumarin blue	Blue
Cent4 repeat	Oregon green	Green
Microsatellite repeat (AGT)	Oregon green	Green

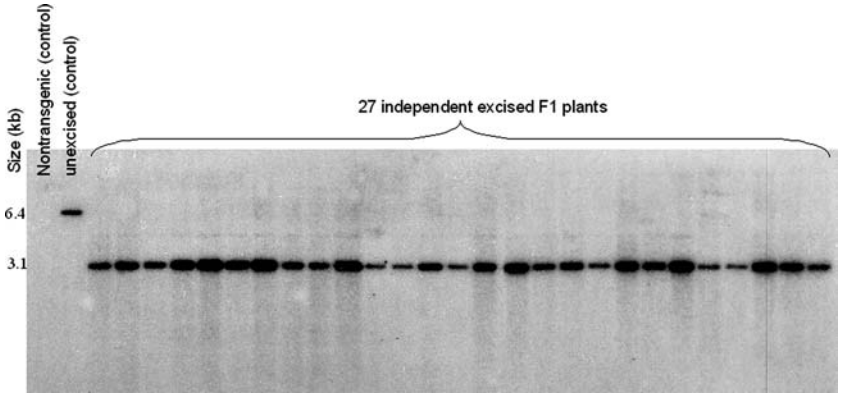

Fig. 1 Southern blot analysis showing evidence for excision in F_1 plants (see text and Materials and methods for details)

Table 3 Chromosome features of inbred line LH198

Chromosome	Knob location	Relative knob intensity	TR-1 location	Satellite location	Relative CentC intensity	Cent4 location	5S rRNA genes	NOR
1	Short	Very low		Long	High			
2				Long, short	Very low		Long	
3					Medium			
4			Long	Short	Very low	Centromere		
5	Long	Medium			Low			
6	Short, long	Very low, extremely low	Short	Short	High			Short
7	Long	High			High			
8	Long, short	High, extremely low			Medium			
9	Short	Medium			Medium			
10					Medium			

We labeled a 180-bp centromeric repeat (CentC), the nucleolus organizer region (NOR), and the 5S rRNA gene region with Coumarin Blue. CentC hybridizes to all the centromeres, but at different intensities (Ananiev et al. 1998). The NOR is found on the short arm of chromosome 6, and the 5S rRNA genes are on the tip of the long arm of chromosome 2 (Mascia et al. 1981; Tai et al. 1999; Li and Arumuganathan 2001). Another heterochromatin-associated repeat, TR-1, was labeled with Texas Red. TR-1 is present on chromosomes 4 and 6 in LH198, but its presence varies across other inbred lines (Ananiev et al. 1998). A centromere 4-specific probe (Cent4) and a microsatellite repeat (AGT) were labeled with Oregon Green dUTP. Cent4 hybridizes only to the primary constriction of chromosome 4 (Page et al. 2001). The microsatellite repeats are found in different locations on chromosomes 1, 2, 4, and 6 (Chin et al. 1996; Taramino and Tingey 1996). As with the knob and TR-1 repeat, the location of microsatellite repeats may vary across inbred lines.

The morphology of the chromosomes is also useful in the identification and detection of translocations. Any perturbation of normal arm ratios indicates that a translocation may have occurred. While the degree of condensation of chromosomes may vary in different preparations, relative size within a spread may help distinguish larger chromosomes from smaller ones.

Chromosome identification in inbred LH198

The LH198 inbred shows variation with respect to the location and intensity of the knob, TR-1, and microsatellite repeats relative to other inbred lines. While CentC positions are fixed, the intensity may also vary slightly across inbred lines. However, these signals within the LH198 inbred line were consistent and allowed for unambiguous identification of all ten pairs of chromosomes at mitotic metaphase (Fig. 2,; Table 3). Chromosome 1 has a small knob on the short arm and a scattered layout of the microsatellite repeat on the long arm, accompanied by a relatively strong CentC signal. Chromosome 2 was identified by the presence of 5S rRNA genes near the tip of the long arm. This chromosome also has microsatellite repeats on the long and short arms, but the intensity was consistently brighter on the short arm. The intensity of CentC was relatively low compared to the other chromosomes.

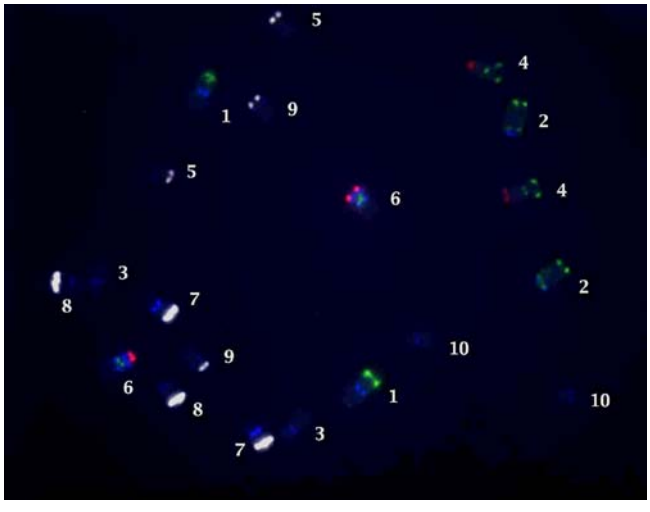

Fig. 2 The karyotype of normal maize inbred line LH198

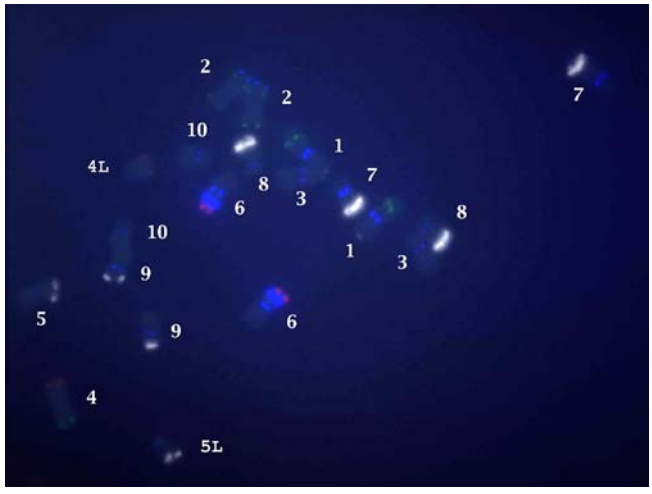

Fig. 3 Karyotype of Cre− seedling (no. 91) with truncated chromosomes 4 and 5

Fig. 4 Karyotype of Cre+ seedling (no. 82), showing a cell representing a sector with a 1–4 translocation

Chromosome 3 showed only a medium CentC signal. Chromosome 4 is distinguished by the presence of Cent4 as well by a TR-1 repeat on the long arm and a visible microsatellite repeat on the short arm. Chromosome 5 showed a medium-sized sub-telomeric knob and had a low CentC signal, while chromosome 6 has a small knob, a TR-1 signal, a satellite repeat, and the NOR on the short arm. The long arm has a very small knob signal that was occasionally detectable in chromosome spreads with very little background. Chromosome 7 had a strong CentC signal and a large knob on its long arm. Chromosome 8 also has a large long arm knob; however it could be distinguished from chromosome 7 because its CentC signal was less intense and because the arm ratio differs. In addition, the short arm had a very small knob signal that was occasionally present in low background hybridizations. Chromosome 9 has a small short arm knob with a moderate CentC signal, while chromosome 10 had a small to moderate CentC signal and no other visible signals.

Karyotypic analysis of Cre+ and Cre− material

We assayed mitotic metaphase chromosomes from 84 *cre*+ and 89 *cre*− root tips for any detectable translocations. One *cre*− individual showed evidence of rearrangements in chromosomes 4 and 5 (Fig. 3). These aberrations were in every cell spread observed. This individual also had a translocation between chromosomes 2 and 7, which was found in only one cell. We observed one other cell-specific translocation between chromosomes 1 and 4 in a *cre*+ sample (Fig. 4); however all of the other cells in metaphase from this individual were normal. All other chromosome spreads examined appeared to be normal. Because root tips were taken 2–3 days after germination, any rearrangements that occurred could only have come from the previous generation or have been generated early in development.

Discussion

We assayed for semi-sterility in pollen and defective kernel (*dek*) mutations in the progeny of plants that express *cre* at levels sufficient to catalyze a high frequency of the expected *lox*-mediated excision and found no evidence that the Cre/*lox* recombination system causes heritable chromosome rearrangements in maize at a detectable rate. We found only one pollen sample (*cre*−) that showed evidence of significant abortion. Further analysis of the two putatively segregating *dek* ears (one *cre*+ and one *cre*−) also revealed that low levels of Cre recombinase probably do not cause any heritable mutations at a detectable rate. We also tested for translocations and other rearrangements in chromosomes using FISH but observed no significant differences in Cre lines.

We did detect a small number of chromosome translocations in both the *cre*+ and *cre*− individuals using FISH analysis. However, it is important to note that our procedure cannot detect every possible translocation that could have taken place. Since the chromosomes came from somatic tissue early in development, any rearrangements that might be attributable to *cre* must have occurred in the previous generation or in the anlagen giving rise to the root tip. These rearrangements would be present as heterozygotes in the test progeny.

Schmidt et al. (2000) present evidence of large-scale chromosome rearrangements that occurred in a *cre*-expressing mouse germ line. Although their study did not examine somatic tissues, any rearrangements that might occur in these tissues would be harder to detect because they are not heritable. Furthermore, cells that are killed as a result of a rearrangement may be replaced if the rearrangement occurred early in development.

Although the plants used in this study express Cre at levels sufficient to catalyze *lox*-mediated recombination at a frequency of nearly 100% (Zhang et al. 2003; this study), Western blots indicated very low levels of stable Cre protein in these plants (data not shown). In the construction of the *cre* gene cassette in pMON36136, an out-of-frame ATG was generated 28 bp upstream of the normal Cre start codon (data not shown), which probably accounts for the low expression level in the Cre line used in this study. Several studies present evidence in plants that correlate high *cre* activity with abnormal phenotypes. These studies used a stronger promoter than the one used in this study (McElroy et al. 1991; Que et al. 1998; Coppoolse et al. 2002). Whether high *cre* expression results in a greater probability for rearrangements and/or adverse effects on the cell is still

unclear and more studies focusing on this question are needed. It has been noted that the previously reported abnormal phenotypes cosegregate with the *cre* gene (Coppoolse et al. 2002; Gilbertson 2003), so if rearrangements are occurring, either they are not transmitted from one generation to the next, or they result in 100% gamete sterility. Zhang et al. (2003) reported that low-level *cre* expression enables the accurate and reliable excision of a target sequence after transformation. One could speculate that there may be a threshold to *cre* activity at which point it may become toxic to the cell. On the other hand, activity below this threshold does not compromise normal cell function. It would be useful to know if such a threshold exists so the *cre-lox* system can be used efficiently and with as few side effects as possible to the organism. The available evidence indicates that at expression levels of Cre that are sufficient for efficient and complete marker excision, the integrity of the maize genome is maintained.

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