The Plant Cell, Vol. 13, 1587–1608, July 2001, www.plantcell.org © 2001 American Society of Plant Biologists

Somatic and Germinal Mobility of the *RescueMu* Transposon in Transgenic Maize

Manish N. Raizada,[1] Guo-Ling Nan, and Virginia Walbot[2]

Department of Biological Sciences, Stanford University, Stanford, California 94305-5020

***RescueMu*, a *Mu1* element containing a bacterial plasmid, is mobilized by *MuDR* in transgenic maize. Somatic excision from a cell-autonomous marker gene yields >90% single cell sectors; empty donor sites often have deletions and insertions, including up to 210 bp of *RescueMu*/*Mu1* terminal DNA. Late somatic insertions are contemporaneous with excisions, suggesting that "cut-and-paste" transposition occurs in the soma. During reproduction, *RescueMu* transposes infrequently from the initial transgene array, but once transposed, *RescueMu* is suitable for high throughput gene mutation and cloning. As with *MuDR*/*Mu* elements, heritable *RescueMu* insertions are not associated with excisions. Both somatic and germinal *RescueMu* insertions occur preferentially into genes and gene-like sequences, but they exhibit weak target site preferences. New insights into *Mu* behaviors are discussed with reference to two models proposed to explain the alternative outcomes of somatic and germinal events: a switch from somatic cut-and-paste to germinal replicative transposition or to host-mediated gap repair from sister chromatids.**

INTRODUCTION

MuDR/*Mu* transposons are responsible for maize Mutator activity: high forward mutation frequency and the somatic instability of reporter genes late in development (Robertson, 1978). *MuDR* encodes the MURA transposase required for *Mu* transposition (Chomet et al., 1991; Hershberger et al., 1991; Qin et al., 1991; Hsia and Schnable, 1996; reviewed by Walbot and Rudenko, 2001) and MURB, a helper protein implicated in insertion (Lisch et al., 1999; Raizada and Walbot, 2000). All *Mu* elements share ~215-bp terminal inverted repeat (TIR) sequences (reviewed by Bennetzen et al., 1993), and the mobile *Mu* elements contain a highly conserved 32-bp MURA transposase binding site (Benito and Walbot, 1997). Characteristic 9-bp host sequence duplications are generated during *MuDR*/*Mu* germinal insertion (reviewed in Bennetzen et al., 1993).

MuDR/*Mu* elements are widely used for maize gene tagging because of their high copy number (Chandler and Hardeman, 1992), preferential insertion into single copy DNA (Cresse et al., 1995), late germinal insertion ensuring gametes with independent mutations (Robertson, 1981, 1985; Robertson and Stinard, 1993), and germinal insertions into both linked and unlinked sites (Lisch et al., 1995). An intriguing attribute of the *MuDR*/*Mu* family is that the germinal insertion frequency is up to 100% per element (Alleman and Freeling, 1986; Walbot and Warren, 1988), yielding a typical forward mutation frequency of 10^{-3} to 10^{-5} per locus (Bennetzen et al., 1993). In contrast, the germinal reversion frequency is $<10^{-4}$ per tagged allele per generation (Schnable et al., 1989; Walbot and Rudenko, 2001). Alternative models to explain the lack of germinal insertions are (1) element excision followed by gap repair from a sister chromatid (Donlin et al., 1995; Hsia and Schnable, 1996) and (2) true replicative transposition (Walbot and Rudenko, 2001).

In dramatic contrast to *MuDR*/*Mu* behavior in germinal cells, these elements excise at a high frequency during somatic development. Excision alleles often contain deletions and/or insertions (Britt and Walbot, 1991; Doseff et al., 1991). The timing of excisions has been monitored by scoring the restoration of anthocyanin pigment from reporter alleles during the nearly synchronous cell divisions generating the aleurone (epidermis) of the endosperm. Levy and Walbot (1990) used the non–cell-autonomous marker *bronze2*::*mu1* and reported that excisions started after cell division number 10; the most common sector sizes corresponded to cell divisions 13 to 14. McCarty et al. (1989) used a cell-autonomous marker, *Vp1*, and found mainly single cell revertant sectors. It has not been resolved whether this timing represents an allele-specific phenotype or a more general property of *Mu* elements. Furthermore, it is unknown whether the somatically excised *Mu* elements are programmed to reinsert or are lost (reviewed in Bennetzen, 1996). Sundaresan and Freeling (1987) characterized extrachromosomal, circular

[1] Current address: Laboratory of Crop Genomics, Department of Plant Agriculture, University of Guelph, Guelph, Ontario, Canada N1G 2W1.

[2] To whom correspondence should be addressed. E-mail walbot@stanford.edu; fax 650-725-8221.

Mu elements that might be formed by excision without insertion ("cut-only" transposition). Other plant transposons are "cut-and-paste" elements in which the excised element reinserts into the genome (reviewed in Walbot and Rudenko, 2001). Rarely, *Mu* somatic insertion events have been recovered (Hu et al., 1998) by observing large sectors with a dominant gain-of-function phenotype. If most somatic insertions occurred as late as somatic excisions (SEs), however, they would be difficult to detect.

To determine whether somatic *MuDR/Mu* excision is routinely coupled to insertion and to explore the mechanism(s) ensuring different transposition outcomes in somatic and germinal cells, we constructed a modified *Mu1* element, *RescueMu*. Transgenic maize was generated to exploit two key features of the construct design. First, *RescueMu* was inserted into *Lc* (Leaf color), a cell-autonomous pigmentation marker encoding a transcription factor of the *R* family (Ludwig et al., 1990), to permit direct observation of excision timing. Second, *RescueMu* contains a bacterial origin of replication and antibiotic marker to permit plasmid rescue from a small population of maize cells. In our experiments, we used *Lc*::*RescueMu* transgenic maize to study the excision timing of transposon activities, to demonstrate that SE is accompanied by insertion, and to examine the spectrum of germinal and somatic insertion alleles to test the assumption that *Mu* elements insert preferentially into genes.

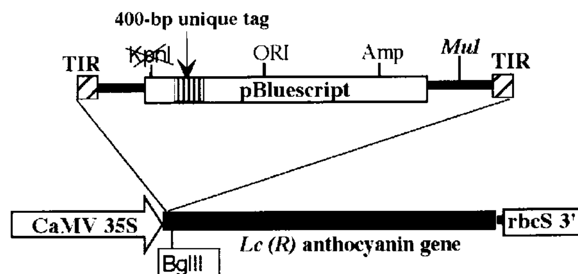

Figure 1. Structure of the *RescueMu* Vector.

The 4.7-kb mobile element, *RescueMu*, consists of a plasmid inserted into an intact *Mu1* nonautonomous element. *RescueMu* is inserted downstream of a cauliflower mosaic virus (CaMV) 35S promoter in the 5′ untranslated leader of maize *Lc*, a transcription factor of the *R* family required for anthocyanin production. Excision of *RescueMu* can restore tissue pigmentation. The integrated transgene locus is defined as *Lc*::*RescueMu*, and five independent *Lc*::*RescueMu* loci are presented in this article: R3-4, R3-8, R3-13, R3-15, and R3-17. Two *RescueMu* elements differ by the presence of unique 400-bp heterologous tags of *Rhizobium meliloti* DNA. These permit easier mutant allele–transposon cosegregation analysis in a background with multiple mobile *RescueMu* elements. There is no KpnI restriction site inside the *RescueMu* element, which is flanked by a unique BglII site in maize *Lc*. These restriction sites are used during plasmid rescue of new *RescueMu* insertion alleles. The pea rbcS 3′ region contains the polyadenylation sites. ORI, origin of replication; Amp, ampicillin.

RESULTS

Transgenic Stocks

Three plasmids, pRescueMu2 and pRescueMu3 (Figure 1) plus pAHC20, were cobombarded into A188 × B73 (HiII hybrid) embryogenic callus (Armstrong and Green, 1985; Gordon-Kamm et al., 1990; Armstrong, 1994). pAHC20 is a maize ubiquitin promoter *Bar* plasmid that encodes resistance to the herbicide Basta. Using herbicide selection, we recovered 17 independent transformants. DNA hybridization blot and subsequent segregation analyses demonstrated that each callus line inherited one or both *RescueMu* plasmids and that all transgenes contained multiple linked copies of pRescueMu (data not shown). The anthocyanin regulatory genotype of the primary transformants (T0) was *r-r/r-g C1/c1*, and these plants lacked a source of active MURA transposase (Raizada and Walbot, 2000; G.N. Rudenko and V. Walbot, unpublished results). Regenerated plants were crossed to active *MuDR* lines in an *r-g C1* background to permit scoring of SE in the aleurone of the T1 and T2 generations. A detailed analysis of five *RescueMu* transformants (lines R3-4, R3-8, R3-13, R3-15, and R3-17) is presented in this report.

RescueMu Elements Excise Preferentially after Cessation of Somatic Cell Division

Because we disrupted a 1.4-kb *Mu1* element with an ~3.4-kb plasmid and placed a *Mu* element at a location not yet found in nature, the first task was to determine if *RescueMu* was mobile. Upon crossing to a *Mutator* transposase source, progeny kernels of 7 of 17 independent lines had small purple sectors on the aleurone, indicative of SE. The somatic mutability of two representative transgenic lines, R3-13 and R3-17, is shown in Figure 2A. We conclude that a large internal addition to *Mu1* permits high frequency SE.

Because *RescueMu* disrupted the expression of a cell-autonomous marker, we were able to analyze the timing of excision. As shown in Figure 2A, in six of seven highly mutable *RescueMu* transformants (R3-3, R3-4, R3-7, R3-13, R3-15, and R3-17), the vast majority of excision sectors were single cells. As shown in Figure 2B, 90 to 96% of reversions were single cells in four independent transformants analyzed. An additional 3 to 7% of sectors contained two cells, although these may have included single-cell sectors in close proximity. Rarer sector sizes, each less than 0.5% of the total, ranged from 8 to 64 cells. Because aleurone sectors were not observed in the absence of transcriptionally active *MuDR* elements, purple sectors were judged to be transposase dependent.

Transformant R3-8 had the most complex integration pattern (>10 copies of pRescueMu), and its kernels displayed an unusual mixture of small and large sectors, with the fol-

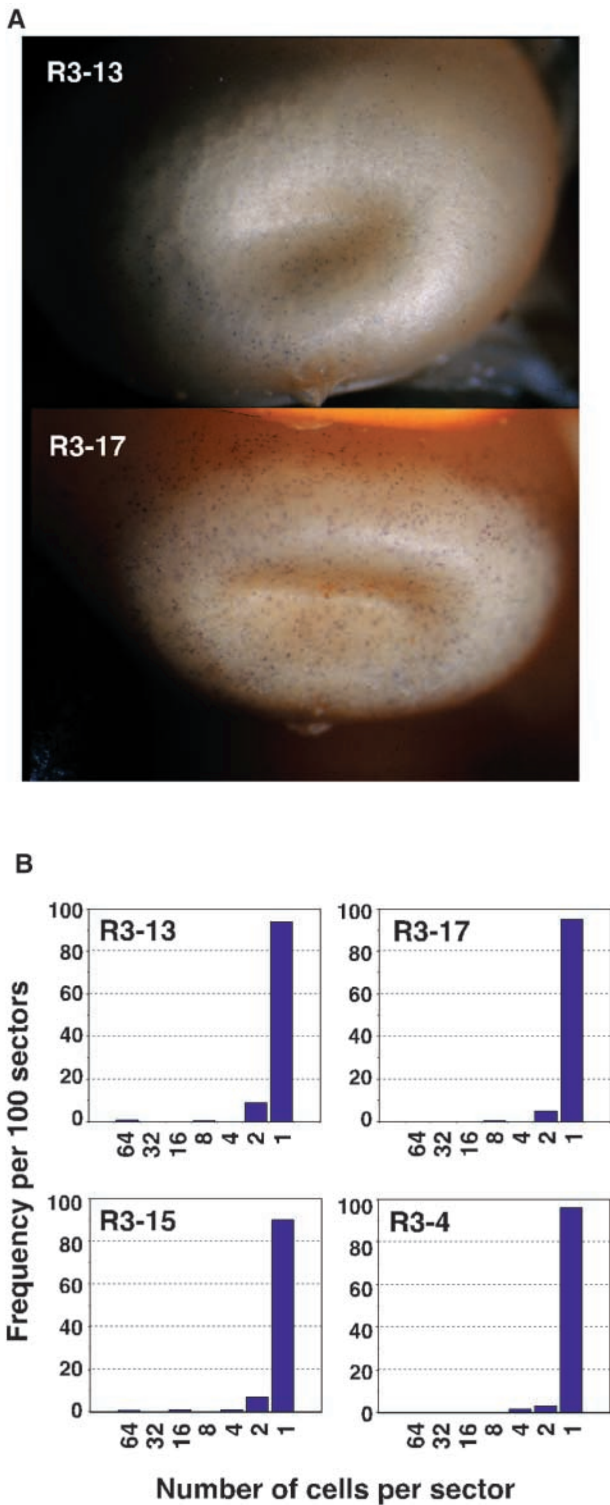

Figure 2. Developmental Timing of *RescueMu* Excisions in the Aleurone at Four Independent *Lc*::*RescueMu* Loci.

Lc::*RescueMu* transgenic plants were crossed with plants expressing the *MuDR* transposase in an *r C1* background to score the size of revertant sectors. The size of each *Lc* revertant sector is an accurate indicator of *RescueMu* excision timing because *Lc* is a cell-autonomous marker (Ludwig et al., 1990).

(A) *Lc*::*RescueMu* revertant aleurone sectors. Two independent transformants are shown, R3-13 and R3-17. Most purple sectors consist of single cells.

(B) Quantitative analysis of *Lc*::*RescueMu* aleurone excision timing. The number of cells in 200 revertant sectors from four kernels was measured randomly for four independent *Lc*::*RescueMu* loci, R3-13, R3-17, R3-15, and R3-4. Despite different chromosomal locations and possible transgene position effects, all four lines exhibit the same late excision timing.

lowing sector size distribution: 1 cell (79%), 2 cells (12%), 4 cells (3%), 8 cells (4%), 16 cells (1%), and ≥32 cells (1%). Within the transgene array, transposase-dependent recombination could generate a functional copy of *Lc* (Lowe et al., 1992; Harris et al., 1994) in large sectors. Alternately, what we observed could be epigenetic activation of the maize *Lc* gene. In suppressible alleles, methylated *Mu1* termini in the promoter or 5′ untranslated region of a gene can program read-out transcription (Barkan and Martienssen, 1991).

Each *Lc*::*RescueMu* allele has a unique transgene integration pattern, and each array is likely located in a unique chromosomal map position. Because all but one *RescueMu* transformant showed primarily single-cell revertant sectors, we conclude that *RescueMu* excises at or after the last cell division during aleurone development, irrespective of the local chromatin context.

RescueMu SE Footprints

Deductions about the biochemistry of transposition reactions and of host repair mechanisms are based on analysis of DNA excision footprints. As shown in Figure 3, we used polymerase chain reaction (PCR) to amplify and clone 115 empty *RescueMu* donor alleles at four independent loci. The PCR primers were located 109 bp to the left and 509 bp to the right of *RescueMu*, and fragments were cloned without size selection. Previous analyses of 44 footprint sequences were based on two *Mu1* insertions 4 bp apart in *bronze1* (Britt and Walbot, 1991; Doseff et al., 1991); size selection prevented recovery of alleles with size changes, deletions, or fillers greater than ∼60 bp on either side of the *Mu* element. We report a total of 45 unique SE events (SE1 to SE45) sequenced from three PCRs; a few clone types (SE40, SE41, and SE44) were recovered many times, possibly as a result of preferential amplification or the presence of early SE or rearrangement events. The three major classes of apparent excision alleles are described below.

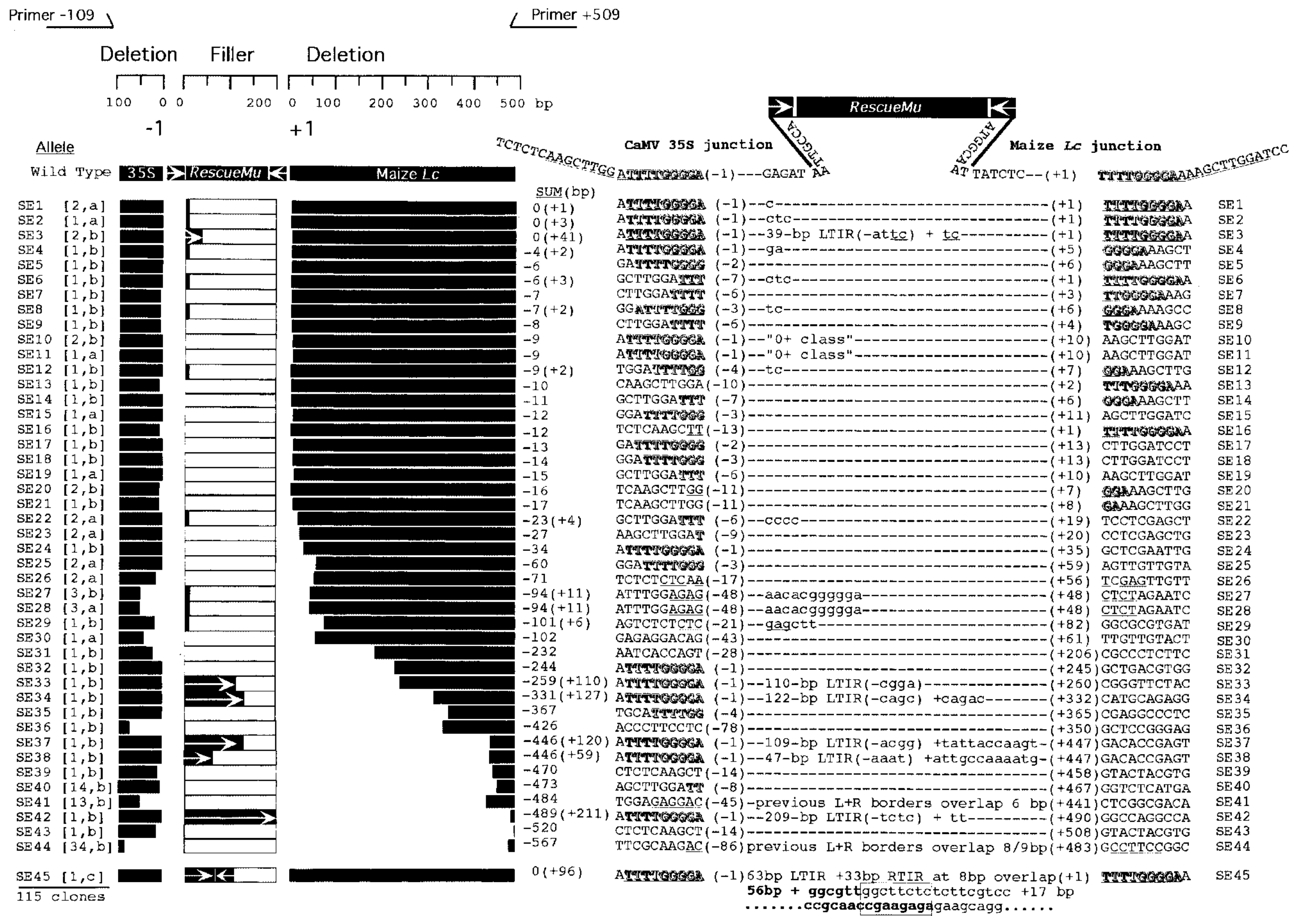

Figure 3. Molecular Confirmation of *RescueMu* SEs and Analysis of DNA Repair Products.

PCR primers flanking *RescueMu* were used to amplify empty *CaMV35S-Lc* sites in mature leaves. The wild-type allele and original *Lc*::*RescueMu* sequences are both shown at the top. The original 9-bp host duplication (TTTTGGGGA) is shown in outlined letters. Flanking this duplication is an overlapping 10-bp direct repeat (AAGCTTGGAT, underlined). After *RescueMu* excision, broken DNA ends appear to be digested variably by exonuclease followed by either blunt ligation (simple deletion) or DNA synthesis, resulting in a fill-in of *Mu1* or other ectopic sequence (lowercase letters). Vector arrows indicate *Mu1* TIR sequences. End points of long filler sequences are shown in parentheses. Nucleotide locations are relative to the original *RescueMu* insertion site. Underlined sequences indicate direct or inverted repeats. Next to the allele name (SE1 to SE45), the number of identical clones recovered and the plant source (a, b, or c; see below) are indicated in square brackets. The primers used for PCR amplification are indicated at the top. Clone SE45 was recovered by plasmid rescue, not PCR. Plant sources: a, MrG157.2; b, pooled MrG157, MrG158.2, MrG158.6, MrGH110-70, MrGH110-71, MrGH110-94, MrGH147-2, and MrGH148-2; c, MrG158.2. L, left; R, right.

Short Deletions with Short Fillers

Half of all empty donor sites (SE1 to SE24) are wild type or have short deletions affecting the 9-bp host sequence duplication and extending up to 34 bp into the cauliflower mosaic virus (CaMV) 35S promoter and/or the 5′ untranslated region of *Lc*. Eight of these alleles also have filler sequences; six filler sequences can be attributed to the terminal 1 to 3 bp of *Mu* (the sequence or its complement), and SE3 contains 39 bp of the outer TIR. The overall range of allele types is similar to that of the footprints at mutable *bz1* alleles, in which deletions as large as 44 bp and short *Mu* termini fillers were observed (Britt and Walbot, 1991; Doseff et al., 1991). These data demonstrate that both short deletions and filler DNA resulting preferentially from the *Mu* termini are common outcomes of host DNA repair after *Mu* excision.

Large Deletions

Approximately 44% of SE types (SE25 to SE44) would not be expected to express *Lc* because large deletions, >60 bp

up to 567 bp (SE44), eliminated the ATG or a substantial component of the CaMV 35S promoter. If these events represent excision products rather than rearrangements within the complex transgene loci, *MuDR/Mu* excisions can result in much more host DNA damage than was reported previously. Of alleles containing large deletions, seven are associated with short (4 to 12 nucleotides) filler sequences (SE22, SE27, SE28, SE29, SE34, SE37, and SE38); at least three are likely derived from the *RescueMu/Mu1* element itself. First, the sequence AACACGGGGGA (SE27 and SE28) is found as AACA at +1057 of *Mu1* adjacent to CGGGGGA at +1045 (*Mu1* numbering according to Barker et al., 1984). Second, the sequence CAGAC (SE34) is found at position +1212 of the *Mu1* right TIR. Finally, the sequence ATTGCC-AAAATG (SE38) is found as ATTGCCA at +7 near AAATG at +44 of the *Mu1* left TIR.

Long Filler DNA

Seventeen of 45 empty *RescueMu* donor sites contained filler DNA (Figure 3). Seven of these were long filler sequences (41 to 211 bp) derived from the *Mu1* TIR at the left edge of the element; some were combined with short (<12 bp) 3′ sequences of diverse origins (i.e., SE3 and SE33). We predicted that PCR would not detect alleles in which both TIRs were present, because amplification of the self-annealing ends is extremely difficult. By plasmid rescue, however, we did recover one allele, SE45, that retained perfect (although incomplete) left and right *Mu1* TIRs. With the exception of this allele, none of the other long filler DNAs were flanked by direct repeats, as would be expected if the *Mu1* filler sequence was generated by intrachromosomal recombination between the *Mu1* TIR and the flanking CaMV 35S or *Lc* sequence. It is more likely, therefore, that large filler sequences are created after excision by homology-dependent gap repair (reviewed in Weaver, 1995; Haber, 2000).

The final observation on footprints is that a subset (29%, 13 of 45 footprint types) had 3- to 9-bp direct repeats (e.g., SE8, SE41, and SE44) or inverted repeats (e.g., SE26, SE27, and SE28) at the new junction. These findings suggest that short sequence homologies may be used as sites of end joining and ligation of 5′ and 3′ broken ends, as has been found in yeast cells (Haber, 2000). In the large deletion class (>100 bp), however, only 3 of 17 footprints contained a terminal short direct repeat, suggesting that most broken DNA ends are repaired by a nonhomologous mechanism (Gorbunova and Levy, 1997).

RescueMu Transposes to New Sites in Somatic Cells

Unlike excisions that are obvious by eye, data on *Mu* somatic insertions are fragmentary. Because SEs occur so late, we predicted that most somatic insertions would be present in tiny sectors. To exclude rearrangements as the source of new flanking DNA, proof of a new *Mu* somatic insertion requires identifying the hallmark 9-bp host sequence duplication. Because *RescueMu* encodes resistance to ampicillin and contains a bacterial origin of replication, we hypothesized that we could use the power of bacterial antibiotic selection to separate bulk maize genomic DNA from those fragments containing *RescueMu*. A significant technical challenge was that only a fraction of maize cells would be predicted to carry a new *RescueMu* insertion, but all should contain both multiple copies of *RescueMu* and the herbicide resistance plasmid at the original donor locus. These constructs also have a bacterial origin of replication and encode resistance to ampicillin, allowing their recovery in *Escherichia coli* by plasmid rescue.

Our strategy to enrich for new *RescueMu* somatic insertions is shown in Figure 4A. From an F1 plant of a cross between active *MuDR* and *Lc*::*RescueMu*, we isolated seedling leaf genomic DNA. We used KpnI to digest outside of the *RescueMu* element. Linear genomic fragments were then circularized in vitro by DNA ligase. To prevent recovery of *CaMV35S-Lc* plasmids, we partially digested circularized genomic DNA with BglII. *RescueMu* lacks BglII sites; however, there is a BglII site in the donor *CaMV35S-Lc*::*RescueMu* allele, which is located ~100 bp downstream of the *RescueMu* insertion, and there also is a site in the herbicide resistance plasmid. Therefore, we predicted that BglII digestion would discriminate against recovery of plasmids at the donor loci and allow selective recovery of new *RescueMu* insertions. To permit recovery of new insertions with closely linked BglII sites, a partial digestion was performed. The efficacy of the restriction digestion strategy was demonstrated by quantifying the recovery of ampicillin-resistant bacterial colonies. In a transformation of highly electrocompetent DH10B *E. coli*, we typically recovered 100 to 800 colonies per microgram of genomic DNA without BglII digestion but 20 to 300 colonies after BglII linearization. After testing several parameters, we formulated an optimized plasmid rescue procedure for maize (see Methods).

As a second step of enrichment, we hybridized colonies in duplicate to either a *RescueMu*-specific probe or to a mixture of CaMV 35S and maize *Lc* probes. We identified colonies that hybridized to the *RescueMu* probe to eliminate recovery of the *Bar* herbicide resistance plasmid. For DNA sequencing, we selected only those colonies that hybridized to the *RescueMu* probes but not to CaMV 35S and maize *Lc* probes.

To test for the presence of *RescueMu* somatic insertions, and to analyze the timing of insertions, if present, we isolated DNA from a small leaf segment from each of two seedlings, plants MrG157.2 and MrG158.2 (Figure 4B). These seedlings are the F1 progeny of a cross between a transgenic *RescueMu* line and an active Mutator stock; therefore, if insertions were detected, they would be somatic, because *MuDR* had just been introduced. After the enrichment protocol, we analyzed plasmids from 12 candidate colonies from plant MrG157.2 and nine candidate colonies from plant

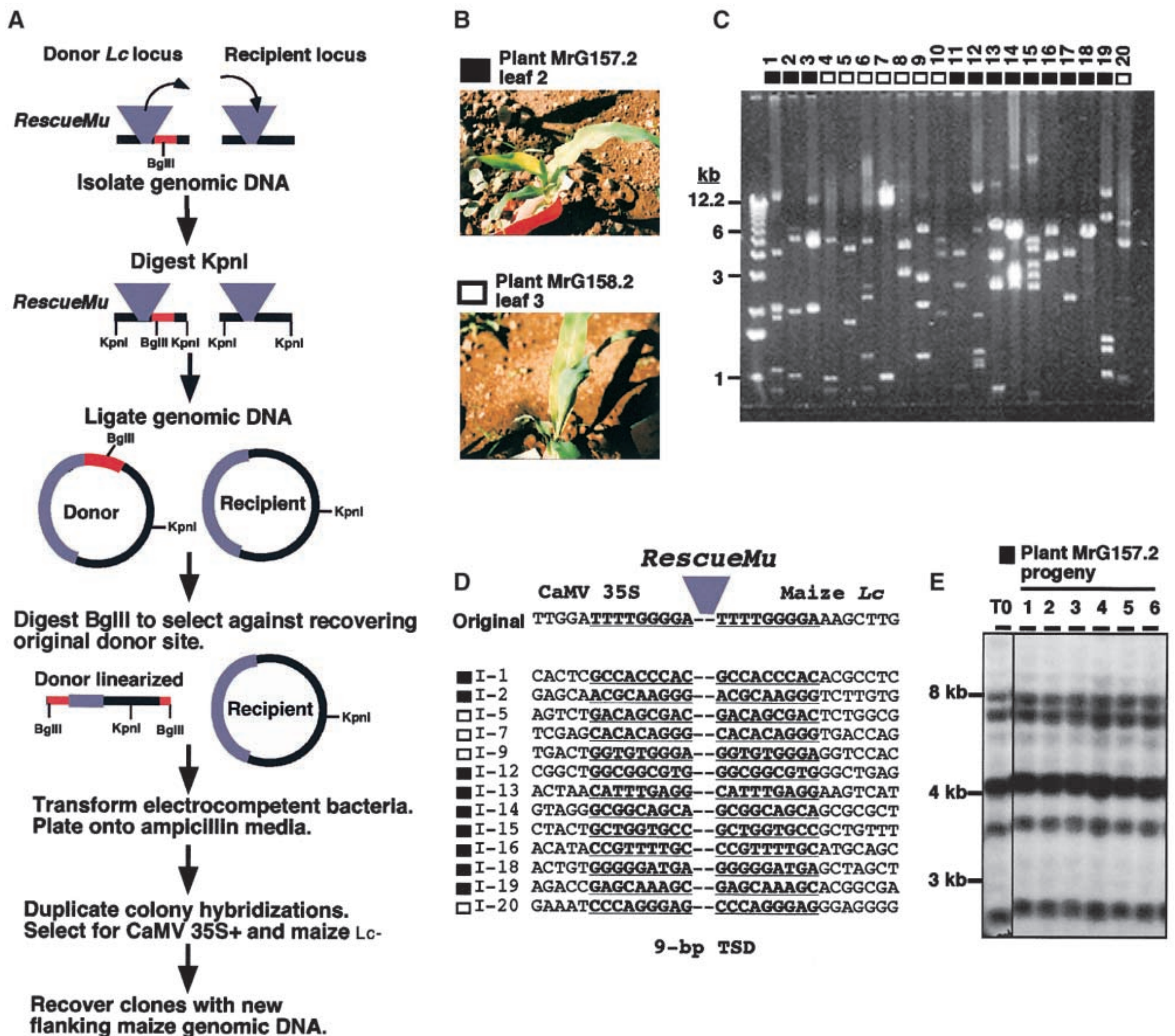

Figure 4. Evidence That *RescueMu* Elements Routinely Transpose to New Loci during Somatic Development.

(A) The strategy to selectively plasmid rescue new somatic insertions of *RescueMu* (recipient loci) while preventing recovery of the original integrated transgene at *Lc* (donor locus). *RescueMu* is shown as purple triangles. Black bars represent flanking maize chromosomal DNA. Red bars represent the *Lc*::*RescueMu* allele. The strategy relies on using a unique BglII site flanking *Lc*::*RescueMu*. After genomic DNA is digested outside of *RescueMu* at KpnI sites, it is self-ligated to form circles. Before bacterial transformation, circles containing a BglII site are linearized to prevent replication in bacteria.

(B) Tissue sources of genomic DNA used for plasmid rescue. Plants MrG157.2 and MrG158.2 are the F1 progeny of a cross between an *Lc*::*RescueMu* (no *MuDR*) plant and a *MuDR* transposase-containing (nontransgenic) plant. Therefore, these plants did not inherit *RescueMu* insertions from either parent. Only a small portion of a seedling leaf was used to isolate genomic DNA. Open (MrG158.2) and closed (MrG157.2) boxes are used in **(B)** to **(E)** to indicate the plant source.

(C) Ethidium bromide–stained agarose gel of maize chromosomal DNA recovered as plasmids in bacteria on ampicillin-containing medium. Genomic DNA was subjected to the protocol shown in **(A)**. Plasmids were digested with KpnI and HindIII. Plasmids range in size from ~10 to 27 kb. Duplicate restriction patterns were not observed, suggesting that new *RescueMu* insertions occurred as small leaf sectors.

(D) Sequence analysis of rescued plasmids from maize chromosomal DNA. The clone names (I-1 to I-20) correspond to the lane names in **(C)**. PCR primers were used to sequence from the left and right borders of the *RescueMu* element. Each sequence carries a new 9-bp host duplication (boldface underlined letters), the hallmark of a new transposition event.

(E) DNA gel blot analysis of the progeny of plant MrG157.2, a source of several somatic insertions. Pollen from plant MrG157.2 was outcrossed

MrG158.2. On the basis of ethidium bromide staining after agarose gel electrophoresis (Figure 4C), rescued plasmids ranged in size from 10 to 27 kb. No plasmids of the same size were recovered; this finding suggested that if these plasmids represented new *RescueMu* insertions, they occurred late during leaf development. To determine if these plasmids represented new *RescueMu* insertion loci, we partially sequenced a subset of them. As shown in Figure 4D, each plasmid possessed a unique 9-bp host target site duplication (TSD) and novel flanking maize DNA sequence. These results confirmed that we had successfully plasmid rescued new *RescueMu* insertion alleles from maize and demonstrated that *Mu* elements do insert in somatic tissues.

If any of these insertions represented large somatic insertion events, they might have been transmitted to the progeny. We crossed pollen from MrG157.2 to a nontransgenic tester and performed DNA gel blot analysis on six progeny. As shown in Figure 4E, although we had recovered at least nine *RescueMu* somatic insertions in leaf 2 of MrG157.2, its progeny inherited only the original *RescueMu* transgene donor locus. This and other data (not shown) indicate that no identified somatic insertions were transmitted to progeny. From these findings, we infer that *RescueMu* elements routinely transpose to new sites late in somatic development. Because excisions also occur late, we propose that *MuDR*/*Mu* elements transpose by a cut-and-paste mechanism in somatic cells in which each insertion is preceded by a corresponding excision. Although this is the simplest explanation, we must caution that our data cannot distinguish between cut-and-paste transposition versus *RescueMu* excisions and insertions occurring in neighboring cells late in development.

RescueMu **Duplicates without Excision in Germinal Cells**

DNA gel blot surveys were used to identify putative germinal *RescueMu* insertions in the progeny of active *MuDR* × *CaMV35S-Lc*::*RescueMu* individuals. Multiple progeny from several individuals were screened; in Figure 5A, lanes 1 to 3 and lanes 4 to 11 show data from two sibling tassels. In this survey, we found one singular late germinal insertion event of 11.5 kb (lane 3) and one or more putative premeiotic or meiotic insertions shared by siblings (10.2-kb band in lanes 1 and 3, 10.5-kb band in lanes 4 and 8). Of >2000 progeny examined by DNA gel blot analysis, we observed no case in which a new insertion was associated with an excision (Figure 5 and extensive data not shown). Instead, as shown in Figure 5A, we observed rare deletion events (lane 6) at the *RescueMu* donor locus in progeny that lacked any new *RescueMu* insertions. The majority of insertions segregated independently of the transgene locus (data not shown). We conclude that *RescueMu* behaves like other *MuDR*/*Mu* elements in germinal cells: it inserts in late germinal cells, without excision of a preexisting element, to both linked and unlinked loci. Consequently, single individuals or small groups of siblings inherit each new insertion (Robertson, 1985; Robertson and Stinard, 1993).

A novel *RescueMu* band could represent a rare large somatic sector that is not inherited or a rearrangement that is not a true insertion. The procedure to formally confirm an authentic *RescueMu* germinal insertion is shown in Figure 5B. We performed plasmid rescue by DNA gel blot hybridization using genomic DNA from a candidate plant (SH4713) that contained a new *RescueMu*-hybridizing fragment. Unlike with somatic insertion, we found that multiple colonies contained plasmids with the same restriction pattern. In our experience, multiple recovery is a reproducible difference between somatic insertions and heritable events. As shown in Figure 5B, two plasmids were sequenced and found to represent the same allele, sharing identical 9-bp TSDs, with strong homology with a rice panicle cDNA. We called this allele Insertion 55 (I-55). We then used PCR to generate two DNA gel blot probes from the genomic DNA to the right and left of the element. As shown in Figure 5C, each probe detected the same band shift in the parent and segregating progeny, confirming that I-55 was a germinal insertion.

In standard Mutator lines with multiple copies of *MuDR*, *Mu1* elements maintain their copy number through successive outcrosses to non-Mutator lines (Alleman and Freeling, 1986; Walbot and Warren, 1988). Therefore, one element must amplify or all elements must, on average, be duplicated to maintain copy number. After screening >300 F1 and F2 plants with the *CaMV35S-Lc*::*RescueMu* transgene in an active Mutator stock, we found that the germinal insertion frequency was abnormally low. As shown in Table 1, the most active families had a germinal insertion frequency in the range of 4 to 24% per progeny; the average was only 11%. This low frequency was true for all *CaMV35S-Lc*::*RescueMu* transformants and was unaffected by *MuDR* copy number or by whether the transgene parent was male or female (data not shown).

Because each transgene locus is complex, with multiple complete and partial *RescueMu* elements in several spacings and orientations, we hypothesized that epigenetic silencing of the transgenes or the transgene locus structure

Figure 4. (continued).

to a nontransgenic tester. The probe used hybridized to both *RescueMu2* and *RescueMu3* elements. Lane T0 corresponds to the primary transformant plant and shows a complex transgene array. In the progeny, only bands of the original transgene locus are present; no new bands are observed, indicating that none of the *RescueMu* insertions recovered in leaf 2 occurred early enough to be contained in the tassel.

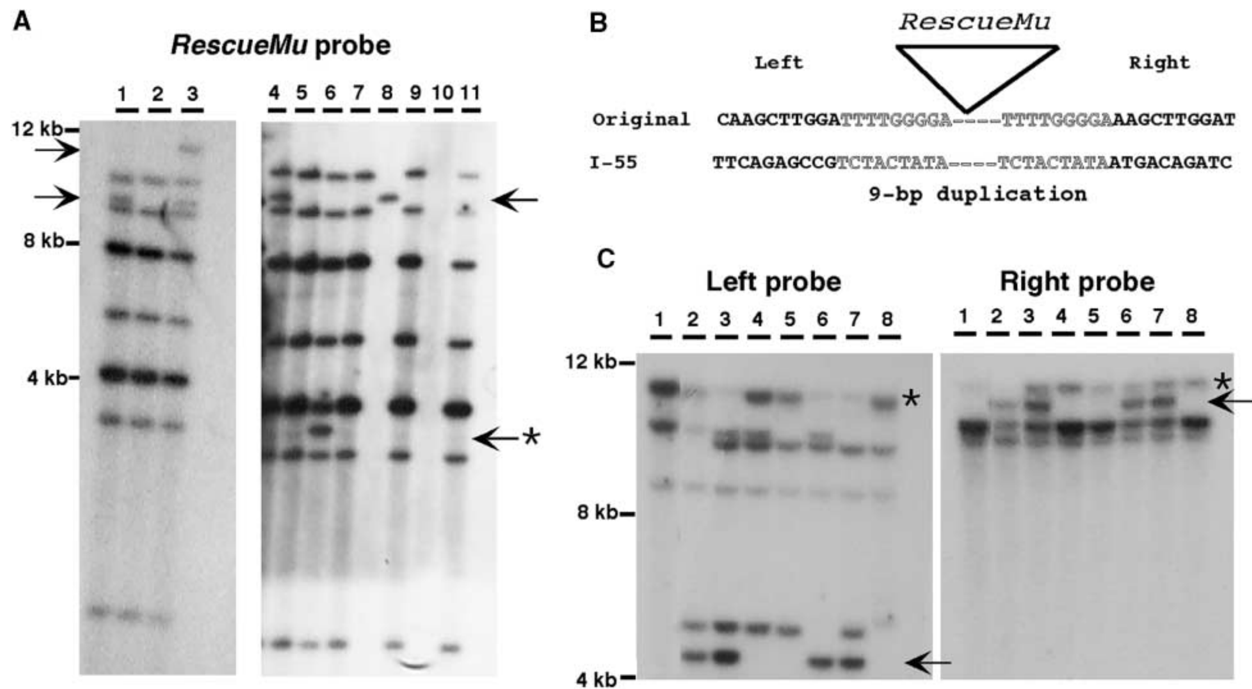

Figure 5. Evidence for *RescueMu* Duplicate Germinal Insertions in the F2 Progeny of *Lc*::*RescueMu* × Active *MuDR* Parents.

(A) DNA gel blot evidence for new *RescueMu* insertions in the outcross progeny of a transgenic plant (allele R3-13). Novel bands are indicated by arrows. Insertions are not correlated with excisions, indicating that *RescueMu* elements duplicate in germinal cells. The single band present in lane 8 indicates that *RescueMu* inserted into a locus not linked to the original donor site. A rare deletion in lane 6 is indicated by an asterisk.
(B) Sequence of I-55, a putative germinal insertion. DNA gel blot analysis showed the presence of a new *RescueMu* band in plant SH4713, which is not related to the plants shown in **(A)**. After plasmid rescue, multiple colonies were found to have the same restriction fragment pattern. Two were sequenced and found to be identical. Because somatic insertions have been recovered only as unique colonies, the rescued I-55 allele was assumed to be germinal. Both the left and right flanking sequences showed strong homology with a rice panicle cDNA.
(C) Confirmation of the germinal inheritance of *RescueMu* insertion allele I-55 in the progeny of plant SH4713. After sequencing of the rescued plasmid, flanking PCR primers were designed and used to generate an ~520-bp left probe and an ~400-bp right probe to *RescueMu*. Each probe was hybridized to the SH4713 parent and its outcross progeny. Lane 1, sibling of *RescueMu* parent; lane 2, parent plant SH4713; lanes 3 to 8, progeny (cross A188 × SH4713). In the left panel, the arrow shows the appearance of a new ~4.5-kb band in the parent segregating in the progeny. Concurrently, as indicated by the asterisk, an ~10.5-kb band is diminished. Genomic DNA was digested with XbaI. The decrease in band size is likely the result of *RescueMu* contributing a more proximal XbaI site present inside the element. The faint ~10.5-kb band (asterisk) in lanes 2, 3, 6, and 7 is likely the intact wild-type homolog copy in combination with variable late somatic reversions of the mutant copy. Because all plants represent hybrid lines, other bands are found to be segregating. In the right panel, in the same progeny shown in the left panel, the right probe of plasmid I-55 detects the appearance of a novel ~10-kb band, indicated by the arrow. This band is present in the SH4713 parent (lane 2) but not in its nontransgenic sibling (lane 1). As in the left panel, the appearance of the novel band coincides with a diminished high molecular weight band (~11 kb, asterisk), although only in the parent (lane 2). The size decrease likely reflects the introduction of a more proximal HindIII site present within *RescueMu*. The genomic DNA was digested with HindIII. Because all of the progeny appear to inherit the ~11-kb band (asterisk), the nontransgenic parent appears to be homozygous for this allele.

itself might inhibit transposition. To test this hypothesis, the germinal insertion frequency of elements that had transposed away from the array was examined. As shown in Table 1, transposed *RescueMu* elements exhibit an enhanced germinal insertion frequency, ranging from 0 to 120% per progeny, with eight families exhibiting an insertion frequency of ~40% or greater (our results combined with unpublished data from V. Chandler, S. Hake, L. Smith, R. Schmidt, L. Roy, K. Slotkin, C. Lunde, and C. Napoli). Hence, *RescueMu* elements can insert at high frequencies after leaving the original transgene array.

RescueMu Preferentially Inserts into Genes

There is already substantial evidence that *Mu* elements selectively insert into genes (McLaughlin and Walbot, 1987; Cresse et al., 1995; Hanley et al., 2000). To perform an unbi-

ased test of the target preference of *RescueMu* elements, we plasmid rescued and sequenced 127 candidate somatic and germinal insertions. Because some alleles were recovered multiple times or represented the donor *CaMV35S-Lc* allele, the data set contains 83 unique, nondonor site inserts. The predicted open reading frames flanking the candidate insertions were translated and compared with those in the databases. A complete list of these insertions is available at http://www.stanford.edu/~walbot/wl_table2_dataset_rmu.html.

As demonstrated by examples shown in Table 2 and summarized in Table 3, although the maize genome contains >65% highly repetitive retrotransposon DNA (SanMiguel et al., 1996), >69% of *RescueMu* insertion sites had high similarity ($E \leq 1e^{-03}$) with genes, either cDNAs or proteins, in GenBank. *RescueMu* detected several novel maize genes, including one strongly homologous with an Arabidopsis lipase (I-40), a squid voltage-dependent potassium channel (I-56), and protein kinases (I-45 and I-86). In many cases, the best homology was with a maize expressed sequence tag (EST). Nine insertion sites were near retroelements or other transposons on either the left or right border or on both, but only three events (4%) appeared to be associated exclusively with retroelement sequences. We did not classify 27% of events because of weak sequence similarity to GenBank entries. Therefore, of alleles with reasonably high similarity to published sequences, 93% of *RescueMu* insertions (57 of 61) were associated with putative maize genes. We must note that some sequence matches may be to unknown small transposons embedded in ESTs and gene sequences. Nevertheless, it is clear that *RescueMu* is an effective mutagen with which to selectively target maize genes (Table 3) for direct recovery in *E. coli*. The size distribution of a subset of the plasmids described in Table 3 is shown in Figure 6. Fifty-eight rescued plasmids ranged in size from 5.1 to ~27 kb. Because *RescueMu* is 4.7 kb, up to 22 kb of flanking maize genomic sequence was obtained.

Unexpected Insertion Events

One *RescueMu* insertion (I-36) occurred in a *MuDR* gene, *mudrA*. Consistent with a recent observation by Hanley et al. (2000), this suggests that unlike some bacterial mobile elements, *MuDR*/*Mu* elements do not possess an absolute immunity mechanism to prevent self-insertion. Of the nine rescued plasmids with CaMV 35S, *Lc*, *Rhizobium*, or other transgene sequences, one plasmid (I-66) was flanked by a novel 9-bp TSD (CTCTGTACC) on both the left and right sides. This must represent a reinsertion event into the original transgene array. Three additional alleles (I-17, I-46, and I-51) contain novel transgene sequences at one border beginning precisely at the *RescueMu* element terminus; the original 9-bp TSD present in the *CaMV35S-Lc*::*RescueMu* construct was lost in all cases. Because we obtained high quality sequence from only one of the TIR host borders for two of these events, these plasmids could represent additional examples of local transposition or element-mediated deletions during abortive transposition (Taylor and Walbot, 1985; Levy and Walbot, 1991). An additional aberrant insertion (I-21) was found to be flanked by an apparent new 9-bp TSD, adjacent to an inverted *Mu1* element TIR, and followed by a novel gene sequence; we do not know how this allele was generated. Collectively, the observations on unusual insertion events indicate that *RescueMu* elements likely are capable of a low frequency of very tightly linked transposition. We cannot estimate the frequency of this phenomenon, because our plasmid rescue involved a selection against recovery of the transgene locus.

Analysis of Target Site Preference

Some transposons have very specific target site requirements; others, such as *MuDR*/*Mu*, insert at diverse sites (reviewed by Bennetzen et al., 1993). A recent analysis of 450 TSDs of randomly sequenced *Mu* insertions yielded the weak consensus 5′-G-T/C-T/C-T/G-G/C-A>T-G-A>G-G>C-3′ (Hanley et al., 2000). As summarized in Table 4, to determine if *RescueMu* exhibits any target specificity, we analyzed 50 9-bp TSDs (raw data not shown) combined with 44 TSDs recovered previously in targeted mutagenesis of known maize genes (Chandler and Hardeman, 1992; Cresse et al., 1995). The nucleotide frequency at a given base is similar between *RescueMu* and the previous *MuDR*/*Mu* insertions and to the target site analysis in the larger compilation (Hanley et al., 2000). Maize coding regions have a GC content of 56 to 67%, whereas introns have a lower content of 40 to 48% (Carels and Bernardi, 2000); consequently, the paucity of A and T bases in the consensus may indicate a slight preference for insertions into exons. We conclude that because there is such a weak consensus target sequence and *Mu* preferentially inserts into genes, other characteristics, such as chromatin configuration, likely are more important than specific bases in determining where *Mu* insertions occur within genes.

DISCUSSION

The *Lc*::*RescueMu* transgene serves two purposes: it is a mutable reporter allele for the study of SE, insertion, and DNA repair, and it is a starting point for maize gene mutagenesis. Using the cell-autonomous *Lc* cDNA as an excision marker at multiple transgene loci, we found that >90% of *RescueMu* excisions occur at or after the terminal cell division in the aleurone. By sequence analysis of 115 alleles representing 45 unique empty donor sites, we observed that approximately half of *RescueMu* excisions were associated with short (<34 bp) deletions. In addition, we found deletions

Table 1. Germinal Insertion Frequency of Active *RescueMu* Families at Original[a] and Transposed Donor Loci

Family	Source[b]	*RescueMu* Donor Loci	Number of *RescueMu* Donor Loci[c]	Number of Plants Tested	Number of New Insertions	Percent Insertion Frequency per Plant
MrHH-1, 2, 3, 8, 9	A	R3-4, original	1	37	4	11
MrHH-4, 16, 17, 18, 19	A	R3-8, original	1	42	7	17
MrHH-5, 6, 10, 11, 12	A	R3-13, original	1	48	6	12
MrHH-14, 15	A	R3-17, original	1	16	0	0
MrI-130	A	R3-4, original	1	14	1	7
MrI-154,158,164,165	A	R3-8, original	1	21	3	14
MrI-178,179, 182,184	A	R3-13, original	1	17	4	24
MrI-206, 210, 211, 212, 213, 214, 215	A	R3-17, original	1	59	7	12
MrI-71 × MrI-184.1	B	R3-13, original	1	15	2	13
MrI-158.1, 165.1, 169.60	B	R3-8, original	1	45	2	4
MrI-212B, 209.9, 213.1/6, 210.7	B	R3-17, original	1	75	5	7
M805	B	Transposed	2	23	9	39
M815	B	Transposed	1	22	4	18
M816	B	Transposed	1	20	0	0
M817	B	Transposed	1	8	1	12
M818	B	Transposed	1	14	0	0
M819	B	Transposed	2	17	0	0
M820	B	Transposed	1	16	3	19
M821	B	Transposed	1	12	6	50
M822	B	Transposed	1	20	0	0
M823	B	Transposed	1	22	1	4
M825	C	Transposed	1	13	0	0
M826B, C	C	Transposed	1	8	1	12
M828A	C	Transposed	1	2	2	100
M828B	C	Transposed	1	22	0	0
M829	C	Transposed	1	41	15	37
M830	C	Transposed	1	14	0	0
M831	C	Transposed	1	1	1	100
M832B	C	Transposed	1	21	0	0
M833	B	Transposed	1	20	4	20
M834	B	Transposed	1	13	8	62
M835A	B	Transposed	1	9	3	33
M835B	B	Transposed	1	10	4	40
M835C	B	Transposed	1	4	1	25
Grid G1.21-25	A	Transposed	1	5	6	120
Grid G5.1-25	A	Transposed	2	25	14	56

[a] In many original donor families (>300 plants), the insertion frequency was 0 to 5%. Only those original families with more active *RescueMu* elements are shown.
[b] Sources of DNA gel blot data: A, Walbot laboratory; B, L. Roy, L. Smith, R. Schmidt, K. Slotkin, and V. Chandler (unpublished results); C, C. Lunde, V. Chandler, and S. Hake (unpublished results).
[c] At original *RescueMu* donor sites, multiple *RescueMu* copies are present in each transgene array.

as large as 567 bp and insertions of up to 209 bp of *Mu* filler DNA; these alleles may represent excision events or rearrangements within the complex transgene arrays. Using plasmid rescue, we demonstrated that *RescueMu* frequently transposes to new loci late during leaf development, suggesting that *MuDR/Mu* elements transpose by a cut-and-paste mechanism rather than a cut-only mechanism. Whereas late excision and insertion in somatic cells may be contemporaneous, we demonstrated that *RescueMu* element insertions during germinal development are not accompanied by excisions. This behavior parallels conclusions and inferences drawn from endogenous *MuDR/Mu* elements, leading to the hypothesis that there is a developmental switch in transposition outcome. Finally, by recovering and sequencing 127 *RescueMu* plasmids representing 83 unique nondonor insertions, we have demonstrated that *RescueMu* is an effective and novel tool for functional genomics in maize.

RescueMu Elements Excise Late in Somatic Development

Our observation (Figure 2) that *RescueMu* is programmed to excise mainly at or after the last of 18 cell divisions in the aleurone is consistent with the findings of a previous study. McCarty et al. (1989) reported that *Mu1* excisions at the cell-autonomous *Vp1* locus are mostly single-cell revertant sectors. Because the only previous quantitative analysis of *Mu* excision timing was performed using a non–cell-autonomous marker, *Bronze2*, in which larger (1- to 64-cell) revertant sectors were observed, it was hypothesized that *Mu* element excisions were developmentally correlated with aleurone differentiation (Levy and Walbot, 1990). Our results and those of McCarty et al. (1989) strongly suggest that *Mu* element excision correlates with the cessation of cell division.

Because the signals that regulate the termination of tissue development are not known, the molecular signal that triggers or permits *Mu* excision is of great interest. There are three models to explain late excision timing (Donlin et al., 1995; Raizada and Walbot, 2000; G. Rudenko, personal communication): (1) competent MURA transposase, MURB, or a required host factor is not present until late in development; (2) the required proteins are present but cannot assemble on the TIRs; and (3) host gap repair masks early SEs by copying *Mu* element sequences from the sister chromatid, a template that is not present after the last anaphase. Because *MuDR* is expressed ubiquitously (Joanin et al., 1997) and MURA (Rudenko and Walbot, 2001) and MURB proteins (Donlin et al., 1995) are abundant in developmentally early cells, it is unlikely that transcriptional or translational regulation of *MuDR* explains late timing. A transgene expressing the 823–amino acid form of MURA is sufficient to result in the demethylation of silenced *Mu* elements early in development, suggesting that the transposase has access to the TIRs throughout development (Raizada and Walbot, 2000). However, because putative cell cycle factor binding motifs overlap the MURA binding site, it is possible that the assembly of a stable transposition complex may be prevented in dividing cells (Raizada et al., 2001a). With regard to the hypothesis that a sister chromatid mediates gap repair in dividing somatic cells, such a mechanism would have to occur at a frequency close to 100%, given that large somatic reversions are exceedingly rare (Walbot and Rudenko, 2001).

RescueMu Excision Footprints Are Diverse

In contrast to the *Ac*-, *Spm*-, and *Tam3*-element families, whose excision alleles contain only short deletions and zero or a few filler bases (Schwarz-Sommer et al., 1985; Coen et al., 1989; Scott et al., 1996), it has long been recognized that *Mu* excision alleles are very diverse (Britt and Walbot, 1991; Doseff et al., 1991). Previously, deletions up to 44 bp and fillers up to 19 bp were observed at the *Bronze1* locus (Britt and Walbot, 1991; Doseff et al., 1991). Using an unbiased recovery strategy, we found that approximately half of the footprints were consistent with these earlier studies; that is, we found deletions of up to 34 bp and short filler DNA sequences of up to 4 bp at *Lc*::*RescueMu* (Figure 3). Because *RescueMu* is in the 5′ untranslated region of the *Lc* gene, this group of alleles should restore gene expression and explain the high frequency of purple somatic sectors in the aleurone (Figure 2).

In addition, *RescueMu* generated deletions as large as 567 bp (Figure 3), indicating that *Mu* excisions can cause much more damage to host genes than suspected previously. We must be cautious, however, when extrapolating *RescueMu* data to *MuDR*/*Mu* elements. The *Lc*::*RescueMu* loci are arranged in tandem arrays that may promote recombination or unusual DNA repair. On the other hand, all information about *Mu*-generated broken chromosome ends indicates that they must be vastly more susceptible to exonuclease than is chromosomal DNA after excision of other well-studied plant transposons. *Ac*, *Spm*, and *Tam3* elements have germinal reversion frequencies several orders of magnitude greater than *MuDR*/*Mu* elements, and early SEs are frequent (reviewed by Walbot, 1991). Because *MuDR*/*Mu* elements are restricted to late somatic events or to gametophytes, there may have been little evolutionary selection on the *Mu* transposition reaction to protect broken DNA ends via end binding proteins or the formation of single-stranded hairpin structures, as has been proposed for *Ac* and *Tam3* elements (Coen et al., 1989; Gorbunova and Levy, 2000).

Structure of Filler DNA Sequences

Figure 3 demonstrates that at 15 empty donor sites, which contain an intact left or right border junction, alleles contain either 1 to 3 bp of *Mu1* terminal sequence (CTC/GAG) or no filler nucleotides at all. These observations confirm and extend previous analysis of mutable *bronze1* alleles: of 10 footprints in which at least one border junction was intact after *Mu1* excision, four contained *Mu1* terminal sequences of 1 to 5 bp (C, CTC, and CTCTA) (Britt and Walbot, 1991; Doseff et al., 1991). The simplest explanation is that these sequences are left behind by the *RescueMu*/*Mu1* elements themselves. We hypothesize that the MURA transposase generates a staggered nick inside the element during excision; DNA repair or strand inversion of such short overhangs could then generate sequences of *Mu* or the inverted complement of the TIR termini.

In all known transposable elements, the 3′ nicks are precisely at the termini, whereas the 5′ nicks, if they occur, can be inside or outside of the element (reviewed in Gorbunova and Levy, 2000). In Drosophila *P* elements, the 5′ nick occurs 17 bp inside the element as a result of the endonucleolytic activity of the *P* transposase (Beall and Rio, 1997). In contrast, an analysis of *Ac*/*Ds* transposition intermediates

Table 2. Examples of *RescueMu* Insertions with High Sequence Similarity[a] to GenBank Sequences

Clone and Border[b]	Somatic or Germinal[c]	Plant Source	No. bp[d]	Search	GenBank Similarity				Distance from *Mu*[f]
					Match	GenBank Name	Similarity Region[e]	Similarity E Value	
I-1R	S(1)	MrG157.2	196	BlastX	Arabidopsis ascorbate peroxidase	emb/CAA06823.1	aa 5–38	$9e^{-06}$	+1
I-6L	S(1)	MrG158.2	236	BlastX	Arabidopsis putative aldolase	pir/T01902	aa 87–137	$3e^{-17}$	>+82
I-9R	S(1)	MrG158.2	375	BlastN	Maize mixed adult 707 cDNA	gb/AW399991.1	nt 2–254	$1e^{-114}$	+57
I-16L	S(1)	MrG157.2	561	BlastX	*Schizosaccaromyces pombe rad16* homolog	pir/T40569	aa 378–417	$5e^{-08}$	+146
I-19L	S(1)	MrG157.2	627	BlastN	Maize PHYT1 acidic phytase	emb/AJ223470.1	nt 1105–1269	$4e^{-34}$	+337
I-21R	S(1)	MrG158.6	245	BlastN	Maize *Mu1* terminal inverted repeat[g]	emb/X00913.1	nt 152–14	$4e^{-72}$	+1–139
I-29R	S(1)	MrGH108-148	514	BlastX	Arabidopsis glucose-regulated repressor	gb/AAD20708.1	aa 182–348	$1e^{-50}$	>+1
I-34R	S(1)	MrGH108-148	598	BlastX	*Phalaenopsis* cysteine proteinase	gb/AAB37233.1	aa 26–75	$4e^{-09}$	>+420
I-36R	S(1)	MrGH108-148	513	BlastX	Maize *mudrA* protein	pir/S59141	aa 455–489	$3e^{-12}$	>+115
I-39R	S(1)	MrGH110.94	567	BlastX	Soybean *dnaK* chaperonin BiP-B	pir/TO6358	aa 245–420	$4e^{-75}$	>+39
I-40R	S(1)	MrGH110.94	614	BlastX	Arabidopsis putative lipase	dbj/BAA94236.1	aa 249–363	$3e^{-43}$	>+186
I-41R	S(1)	MrGH110.94	558	BlastN	Maize ear tissue 606 cDNA	gb/A1691294.1	nt 340–171	$3e^{-59}$	>+261
I-42R	S(1)	MrGH148.2	611	BlastN	Maize leaf primordia 486 cDNA	gb/A1622241.1	nt 178–28	$1e^{-64}$	>+121
I-45R	S(1)	MrGH110.70	511	BlastX	Arabidopsis putative protein kinase	gb/AAC23760.1	aa 79–243	$3e^{-06}$	>+1
I-52R	G(2)	SH-C9.40	656	BlastX	Arabidopsis membrane carrier protein	gb/AAF27035.1	aa 2–49	$4e^{-12}$	+47
I-55L	G(2)	SH4713	693	tBlastX	Rice flowering panicle cDNA ORF	dbj/C98637.2	nt 431–285	$8e^{-33}$	+166
I-55R	G(2)	SH4713	653	tBlastX	Rice flowering panicle cDNA ORF	dbj/C72506.1	nt 27–155	$2e^{-25}$	+3
I-56L	G(2)	VC-M807	536	BlastX	*Loligo opalescens* K^+ channel SqKv1A	gb/AAB02884.1	aa 255–418	$2e^{-69}$	>+8
I-57R	G(2)	VC-E10-4B.1	666	BlastN	Maize endosperm 605 cDNA	gb/A1665158.1	nt 448–243	$1e^{-107}$	+400
I-64L	ND[h](1)	VC-M835 B3	279	BlastX	*Drosophila Ariadne-2* zinc finger protein	gb/AJ010169	aa 332–416	$7e^{-09}$	+2
I-65L	ND[h](1)	VC-M833.7	760	BlastX	*Saccharum* membrane protein	gb/AAA02747.1	aa 32–96	$1e^{-19}$	>+133
I-66R	ND[h](1)	VC-M833.7	296	BlastN	*Rhizobium nodPQ* (vector)	emb/Z14809.1	nt 1983–1706	$1e^{-141}$	+1
I-66L	ND[h](1)	VC-M833.7	549	BlastN	pBluescript vector	emb/X52326.1	nt 693–278	0	+135
I-68R	ND[h](1)	VC-M833.7	704	BlastX	Arabidopsis putative esterase	db/AAD17422.1	aa 248–312	$8e^{-09}$	>+44
I-68L	ND[h](1)	VC-M833.7	130	BlastN	Maize anther/pollen 660 cDNA	gb/AW313235.1	nt 352–475	$3e^{-28}$	>+6
I-72R	G(6)	SD-B40.3	495	BlastX	Maize *copia* retroelement pol polyprotein	gb/AAD20307.1	aa 897–1030	$7e^{-71}$	>+94
I-76R	ND[h](1)	VC-E10-4	729	BlastN	Maize ear tissue 606 cDNA	gb/AI714482	nt 52–434	$6e^{-35}$	+283
I-77R	ND[h](1)	SH-C99-9-40	282	BlastX	Arabidopsis mitochondrial carrier-like protein	gb/AAF27035.1	aa 2–49	$2e^{-12}$	+39
I-83L	ND[h](1)	SH4712	743	BlastX	Arabidopsis CorA-like Mg^{2+} transporter	gb/AAF14678.1	aa 45–94	$1e^{-05}$	+277
I-83R	ND[h](1)	SH4712	766	BlastX	Maize *copia*-type pol protein	gb/AAD20307.1	aa 599–663	$2e^{-30}$	+445
I-84R	ND[h](1)	SH4713	723	BlastN	Sorghum pathogen-induced cDNA	gb/BE596140.1	nt 51–525	$2e^{-77}$	+162
I-86L	ND[h](1)	GN 673005A1	498	BlastX	Maize Ca^{2+}-dependent protein kinase	pir/T03023	aa 51–183	$4e^{-48}$	+66
I-87L	ND[h](1)	GN673005A2	506	BlastX	Arabidopsis pherophorin-like protein	emb/CAA16736.1	aa 425–540	$3e^{-28}$	+3
I-87R	ND[h](1)	GN673005A2	543	BlastX	Arabidopsis pherophorin-like protein	emb/CAA16736.1	aa 392–440	$2e^{-12}$	+94
I-90R	ND[h](1)	GN6730005C1	810	BlastN	Maize immature ear cDNA	gb/AI065431	nt 8–326	$2e^{-21}$	+259

[a] Arbitrarily defined as $E \leq 1e^{-03}$

[b] L, left border; R, right border.

[c] The number in parentheses refers to the number of colonies in which the allele was recovered from a single plasmid rescue. A germinal insertion was confirmed by DNA gel blot inheritance and/or by multiple allele recoveries. G, germinal; ND, not determined; S, somatic.

[d] Number of nucleotides sequenced.

[e] aa, amino acid; nt, nucleotide.

[f] The greater than symbol (>) is used when the 9-bp target site duplication was not sequenced. The precise location of the *RescueMu* insertion is not known.

[g] Immediately flanking the *Mu1* TIR starting at +170 is a region similar to a Sorghum tissue cDNA (gb/BE361626.1; $E = 2e^{-05}$).

[h] This plant carried germinal *RescueMu* insertions by DNA gel blotting, but the allele was not tested for transmission to the progeny.

suggests that the 5′ nick occurs 1 bp outside of the element (Gorbunova and Levy, 2000). Because 1 bp is always lost, this likely explains why none of 621 sequenced *Ds* footprints at the maize *waxy* locus contain two intact flanking sequences (Scott et al., 1996). In contrast, of 45 *Lc*::*RescueMu* alleles, we recovered four independent footprints (SE1, SE2, SE3, and SE45) in which both flanking sequences were intact (Figure 3). In 38% of footprints (27 of 72) in this and previous studies, one or both of the flanking 9-bp duplications were intact (Britt and Walbot, 1991; Doseff et al., 1991). These data suggest that the 5′ nick by MURA occurs inside the element. An important implication is that *MuDR*/*Mu* elements whose overhangs are not repaired properly would not be competent to reinsert into the genome. If true, this may explain the abundance of extrachromosomal *Mu1* element circles characterized previously by Sundaresan and Freeling (1987). Although all data are consistent with the same model, it is possible that the terminal 1- to 3-bp nucleotides may not be left behind by *RescueMu* but instead could be generated by homology-dependent DNA synthesis after excision (reviewed by Yan et al., 1999; Haber, 2000).

Indeed, seven short filler alleles are associated with larger deletions that may be caused by DNA repair after excision. Alleles SE22, SE27, SE28, and SE29 contain 4- to 11-bp filler nucleotides; three additional alleles (SE34, SE37, and SE38) contain five to 12 nucleotides adjacent to larger *Mu1* TIR fillers of up to 211 bp. Of these seven short fillers, we have identified three as likely derived from the *RescueMu*/*Mu1* element itself (SE27/28, SE34, SE38). Perhaps most striking, an additional seven alleles consist of large fillers (41 to 211 bp) derived from the *Mu1* TIR. These alleles provide strong evidence that *Mu*-induced breaks stimulate homology-dependent gap repair in somatic cells (reviewed in Haber, 2000), the small fillers perhaps involving slipped mispairing of template repeat sequences during DNA synthesis (reviewed by Yan et al., 1999). Filler sequences rarely are flanked by direct repeats, suggesting that DNA replication repair after excision, not intrachromosomal recombination within the complex transgene array, generated them. Because the *RescueMu* loci in this study were hemizygous, the template cannot be the homologous chromosome but could be the sister chromatid present after DNA replication (Donlin et al., 1995).

RescueMu Routinely Transposes to New Loci Late during Somatic Development

Using plasmid rescue recovery, we have shown that *RescueMu* routinely transposes to new chromosomal positions in leaf cells (Figure 4; Tables 2 and 3). *RescueMu* elements in these clones were flanked by novel 9-bp TSDs, a characteristic feature of *Mu* element insertions in germinal cells. From a leaf segment of a few square centimeters, at least nine independent *RescueMu* insertions were isolated (Figures 4C and 4D). No duplicate clones were recovered, suggesting that insertion sectors in this leaf were small. None of these insertions were transmitted to the progeny (Figure 4E). On the basis of this and additional experiments (Table 2), we infer that *RescueMu* inserts late during somatic development. Because *Mu* elements also excise late during somatic development in addition to their late insertion behavior, the

Table 3. Summary of DNA Sequences Flanking *RescueMu* Insertions

Category	Number	Percentage
Total number of plasmids sequenced	127	
Total number of unique clones	92	
Number of CaMV 35S-*Lc* donor alleles recovered	9[a]	
Total number of nondonor site clones	83	
Clones with insignificant[b] GenBank sequence similarity	22/83	27
Insertions into identifiable expressed DNA[c]		
DNA similarity to EST/cDNA clones	42/83	
Protein homology		
Existing proteins	29/83	
cDNA translations	23/83	
Total insertions into expressed DNA	57/83	69[d]
Number of clones with non-mRNA DNA homology only	1/83	1
Insertions in or near retroelements and transposons		
Adjacent to expressed DNA	9/83	
Retroelement sequence only	3/83	4

[a] Includes one or more examples of local transposition.
[b] Insignificant sequence similarity is defined as $E > 1e^{-03}$.
[c] Several alleles were not sequenced at both the left and right borders; hence, this may be an underestimate. Some regions of mRNA sequence similarity may represent small, embedded retroelements that have not been identified.
[d] Of 61 insertions into known sequences, 93% are into putative expressed portions of the genome.

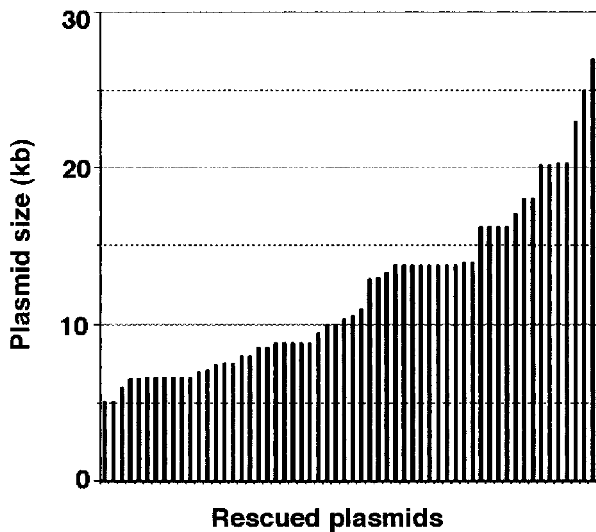

Figure 6. Plasmid Size Distribution of 58 Transposed *RescueMu* Alleles Recovered in *E. coli*.

simplest explanation is that *Mu* elements transpose by a cut-and-paste mechanism in somatic cells (reviewed by Craig, 1995). We cannot exclude the possibility that slightly earlier *Mu* element somatic insertions occur or that excisions and insertions can occur independently in somatic cells. Clearly, however, germinally transmissable early *Mu* somatic insertions into the *y1* locus were not observed (Robertson, 1985; Robertson and Stinard, 1993). Although some later reports of early somatic insertions may reflect differential epigenetic suppression of certain *Mu* alleles in the male and female cell lineages (Martienssen and Baron, 1994), further experiments will be needed with *RescueMu* to determine the frequency and mechanism of transposition of true early somatic *Mu* insertions. Given the apparent rarity of early somatic insertions, the view that gap repair masks developmentally early *Mu* transposition events by copying precisely from a *Mu* template on the sister chromatid seems very unlikely unless such early SE events are unaccompanied by insertion.

A Switch in Transposition Outcome

Consistent with previous studies of *MuDR/Mu* elements, we found that *RescueMu* insertions occur late in germinal development and are not associated with excisions (Figure 5) (Alleman and Freeling, 1986; Walbot and Warren, 1988; Lisch et al., 1995). To explain the lack of germinal excisions, it has been proposed that *Mu* elements transpose in germinal cells by a cut-and-paste mechanism and then use the sister chromatid as a template for homology-dependent DNA synthesis to replace the excised element (Donlin et al., 1995; Lisch et al., 1995; Hsia and Schnable, 1996). The evidence that supports this model is the existence of infrequent, internally deleted *MuDR* elements, sometimes flanked by short direct repeats (Lisch and Freeling, 1994; Hershberger et al., 1995; Hsia and Schnable, 1996). It has been suggested that these deletions arise from incomplete sister chromatid–dependent repair synthesis after *MuDR* excision (Donlin et al., 1995; Hsia and Schnable, 1996).

There are two problems with the gap repair hypothesis. First, using *RescueMu*, we found that somatic events most likely attributable to gap repair resulted in $<$210 bp of *Mu1* filler DNA rather than the majority of the element (Figure 3). Although we note that our PCR assay was biased against amplification of both TIRs, which can form intramolecular duplexes, it was not difficult to find multiple examples of short *Mu* filler sequences. Therefore, our data suggest that *MuDR/Mu*-associated gap repair, if it occurs, is an inefficient process in somatic cells. In contrast, the *MuDR/Mu* germinal reversion frequency is extremely low ($<10^{-4}$ per gamete per generation). Furthermore, a low frequency of *MuDR* internal deletions has been reported; however, internal deletions of the other *Mu* element families (*Mu1* to *Mu8*) are extremely rare (reviewed in Walbot and Rudenko, 2001). Because a *Mu1* element can germinally duplicate at frequencies approaching 100% (Alleman and Freeling, 1986; Walbot and Warren, 1988), any germinal gap repair would need to occur at $>$99.99% efficiency with an extremely high frequency of nearly complete DNA strand synthesis. In contrast, Dooner and Martinez-Ferez (1997) have argued that double-stranded breaks created by *Ac* excision in meiotic cells are repaired by either simple end joining of the broken ends or incomplete gap repair from a sister chromatid. There is an additional caveat. After the last pollen S-phase, *MuDR* transcript products increase 10- to 30-fold compared with that in leaf cells (Raizada et al., 2001a, 2001b), and 20% of new insertions occur in only one of the two sperm in a pollen grain (Robertson and Stinard, 1993). Only empty donor sites created by cut-and-paste transpositions that occur after S-phase until early anaphase in the generative nucleus would have a sister chromatid available as a template for DNA synthesis repair. Although all of these conditions are possible, the gap repair model requires substantial experimental evidence to determine if *MuDR/Mu* germinal transposition is associated with such a remarkable degree of developmental and biochemical precision.

An alternative is that *MuDR* programs a replicative mode of transposition in pregerminal and postmeiotic cells. In this mechanism, no excisions occur and elements duplicate using semiconservative DNA replication (reviewed in Craig, 1995). Internal deletions may arise from early termination of DNA replication during replicative transposition or from occasional cut-and-paste transposition events that occur in the late somatic tissues that give rise to meiotic cells (Robertson, 1981). May and Craig (1996) demonstrated that a single amino acid change in the bacterial Tn*7* transposase causes it to switch from cut-and-paste transposition to replicative transposition; the amino acid change causes a block

in 5′ DNA strand cleavage but not 3′ cleavage or strand transfer. As a consequence, the double-stranded break required for cut-and-paste transposition does not occur. Characterization of the MURA transposase(s) and interacting factors in somatic and germinal cells will be required to determine if the MURA transposase undergoes a similar switch in biochemical competence.

Summary of *Mu* Element Transposition in Somatic and Germinal Cells

In Figure 7, we summarize new and existing data to present a framework for *Mu* element transposition behavior in somatic and germinal cells. In somatic cells, late *RescueMu* insertions are associated with excisions, suggesting that cut-and-paste transposition occurs in these cells (Figures 7A and 7B). Because excised element termini may be damaged, some elements instead may exist extrachromosomally before their degradation. Empty somatic donor sites can be associated with very large flanking deletions and insertion of partial copies of *RescueMu*/*Mu1* filler DNA, suggestive of homology-dependent DNA synthesis; this gap repair likely depends on a sister chromatid. We found that most *RescueMu* revertants are limited to single cells. To explain this timing, either cut-and-paste transposition is inhibited earlier in somatic development or gap repair cannot operate after the last S-phase and chromosome separation at anaphase because a sister chromatid is not available (Figure 7A). In flowers, there is a high frequency of pregerminal (very late somatic) and postmeiotic insertions (Figure 7C). After the last S-phase in the male gamete, there is a large increase in *MuDR* promoter transcription, and insertions occur in individual sperm nuclei. However, unlike late somatic cells, germinal insertions rarely are associated with excisions, extensive flanking DNA deletions, or incomplete *RescueMu* DNA gap repair. One possibility is that cut-and-paste transposition in pregerminal and postmeiotic cells is associated with enhanced, high fidelity repair followed by complete suppression of all activities in postanaphase gametes (Figure 7D). Alternately, *Mu* elements in germinal cells may switch to a replicative mode of transposition (Figure 7E).

Implications of *Mu* Biology for Transposon Tagging

Our discovery that *Mu* elements routinely insert into new locations in somatic cells has a practical implication for reverse genetics tagging strategies involving *Mu* elements. If PCR is used to identify plants that carry *Mu* element insertions at a known sequence, it is likely that false positives will be recovered that correspond to nontransmissible somatic insertions. This has been observed frequently (C. Schmid and V. Walbot, unpublished results). On the basis of the distribution of *RescueMu* insertions, two tissue samples not likely to share the same recent clonal lineage should be analyzed.

Second, by random plasmid recovery of *RescueMu* inserts, we demonstrated that 69% of 83 nondonor site inserts exhibited high similarity to ESTs and virtual translation products (Tables 2 and 3). Of inserts with strong homology

Table 4. Sequence Analysis of 9-bp Target Site Duplications (TSDs) Flanking *RescueMu* and Previous[a] *MuDR*/*Mu* Insertions

	50 *RescueMu* Target Sites[c]				Sum of 94 *RescueMu* and *MuDR*/*Mu* Target Sites								
Position[b]	% A	% C	% G	% T	% A	% C	% G	% T	% GC	Consensus	% AG	% Purine Trinucleotide	% Pyrimidine Trinucleotide
+1	8	40	40	12	13	30	45	12	75	G or C	58		
+2	22	28	24	26	22	26	21	31	47	N	43	11	20
+3	8	40	32	20	15	30	24	20	54	N (low A)	39		
+4	24	18	28	30	28	17	34	21	51	N	62		
+5	14	24	38	24	15	30	36	18	66	G or C	51	23	12
+6	22	32	28	18	39	26	22	13	48	A (low T)	61		
+7	22	14	54	10	22	12	58	9	70	G (low CT)	80		
+8	28	16	42	14	32	12	40	16	52	A or G	72	38	1
+9	22	30	32	16	18	30	38	13	68	G or C	56		
Mean	19	27	35	19	23	24	35	18	59		58	57[d]	35[d]

[a] From Cresse et al. (1995) and Chandler and Hardeman (1992). Includes three independent insertions at the same location at the *bronze1* locus.
[b] We define position +1 as the first base flanking the element at the right border junction.
[c] Thirty-six of fifty TSDs were sequenced at both the left and right sides. The remainder were sequenced at only one junction, but were flanked by novel (nondonor site) sequences.
[d] Percentage of TSDs that carry three purines or three pyrimidines in a row.

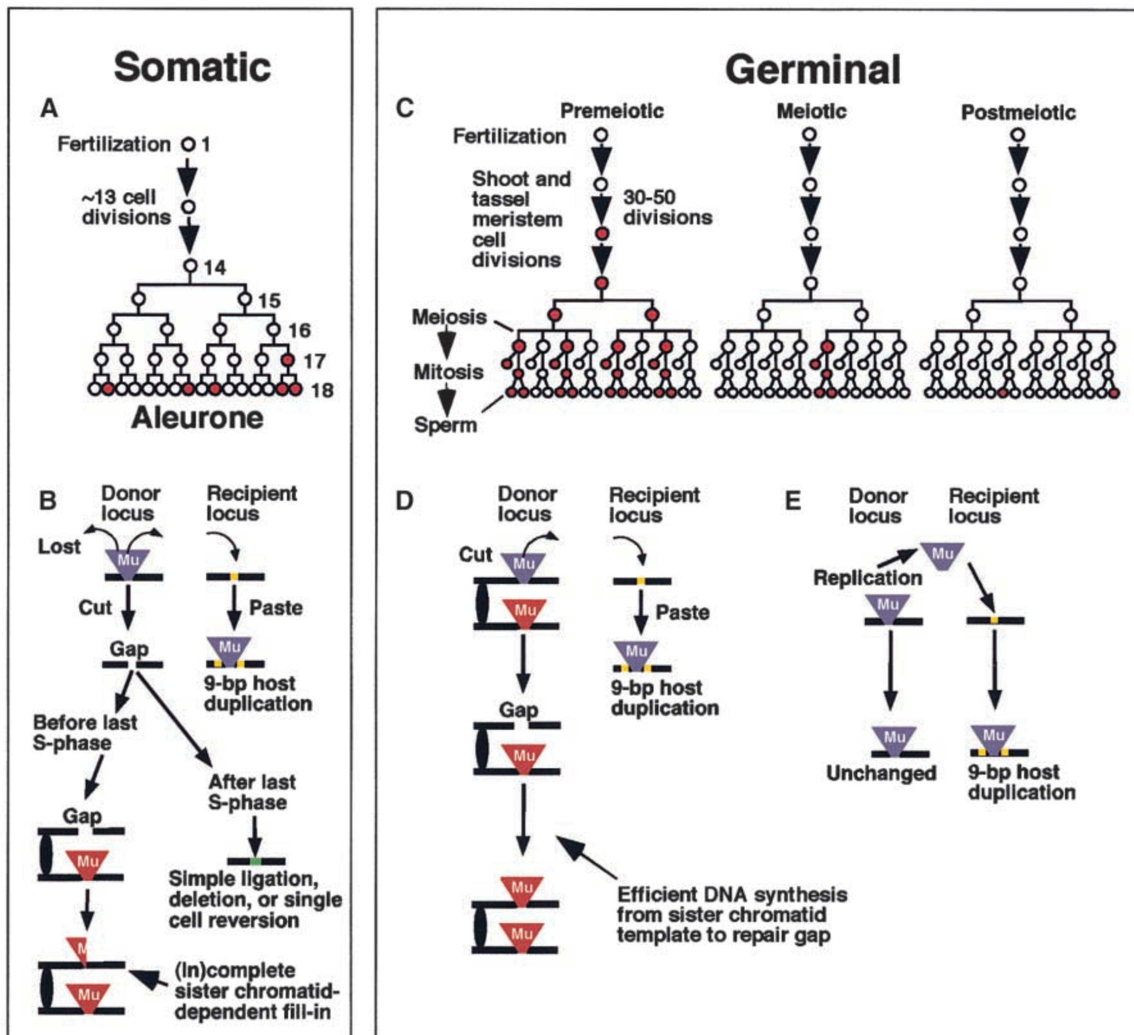

Figure 7. Models Proposed for *RescueMu* and *MuDR/Mu* Element Insertion Activities in Somatic and Germinal Lineages.

(A) *RescueMu* elements excise just before or after the last cell division in the aleurone. Red circles indicate an excision event. The diagram shows the developmental lineage of the aleurone after fertilization (Levy and Walbot, 1990). Numbers to the right of each cell population are with respect to the zygote (cell 1).

(B) A model of *Mu* somatic transposition. Because both *RescueMu* excisions and insertions occur developmentally late, we propose that *Mu* transposes by a cut-and-paste mechanism in terminally dividing somatic cells. When transposition reactions start, double-strand breaks are subject to exonuclease and/or blunt ligation. Before the last S-phase, the homologous *Mu1* template on the sister chromatid (red triangles) may be used to fill in some or perhaps all of the missing *Mu1* sequence. Hence, few revertants of two or more cell sectors are seen. After the last S-phase, because a sister chromatid is not present, single cell revertants are abundant. An alternative model to explain late excision timing is that cell cycle factors may bind to the TIRs to prevent transposition during cell proliferation (Raizada et al., 2001a). Some excised *RescueMu* elements may not reinsert because the TIRs are damaged. These could exist as extrachromosomal circles before degradation (Sundaresan and Freeling, 1987). Those *Mu* elements that do reinsert are associated with a 9-bp host duplication (yellow bars).

(C) *RescueMu* and other *Mu* elements insert but rarely excise in premeiotic, meiotic, and postmeiotic germinal cells. Red circles indicate an insertion event. Shown are cell lineages from the zygote to sperm nuclei located within pollen. After meiosis, each haploid nucleus divides mitotically to produce a vegetative cell nucleus and a generative cell nucleus. The generative cell further divides to produce two sperm nuclei. The majority of *Mu* insertions occur late during development. Up to 20% of *Mu* insertions occur after the last postmeiotic mitosis (data summarized from Robertson, 1981, 1985; Robertson and Stinard, 1993). Insertions occur after the last gamete S-phase, but germinal revertants are rare.

(D) A gap repair model to explain how *Mu* elements insert in germinal cells but generate no reversions at the donor allele. *Mu* continues to transpose by a cut-and-paste mechanism as in the soma. However, there is enhanced and more efficient sister chromatid–dependent DNA synthesis (gap) repair to completely replace the missing *MuDR/Mu* element at the empty donor site in germinal cells (summarized from Donlin et al., 1995; Hsia and Schnable, 1996). Transposition is inhibited in individual sperm, which lack a sister chromatid to use as the template for gap repair.

with any sequence in the database, 93% (57 of 61) were in putative genes. Only 4% of inserts were flanked exclusively by retroelement sequence, which is remarkable given that >80% of the maize genome consists of repetitive DNA (SanMiguel et al., 1996). Because *Mu* elements target genes, genome size is irrelevant. The nonrandom insertion behavior of *RescueMu* is consistent with previous observations with *Mu* elements (McLaughlin and Walbot, 1987; Cresse et al., 1995). Confirming previous studies with *MuDR*/*Mu* elements (reviewed in Bennetzen et al., 1993; Lisch et al., 1995), we also observed that *RescueMu* elements insert at both linked and unlinked sites (Figure 5A and Table 2, and data not shown). In contrast, up to ~50% of *Ac*/*Ds* insertions occur within 1 to 10 centimorgans of the donor site (reviewed in Parinov et al., 1999). We also found an example of a *Mu* element inserting into itself (Table 2).

As summarized in Table 4, an analysis of 50 9-bp *RescueMu* TSDs and 44 previous *Mu* TSDs (Chandler and Hardeman, 1992; Cresse et al., 1995) revealed only a weak consensus sequence, (G/C)NNN(G/C)AG(A/G)(G/C), similar to the consensus sequence G(T/C)(T/C)(T/G)(G/C)(A>T) G(A>G)(G>C) reported recently by Hanley et al. (2000). The consensus sequence is not a good predictor of the insertion site, although the average GC content of 94 *RescueMu*/*Mu* TSDs is 58%, close to that of maize coding regions (56 to 67%) (Carels and Bernardi, 2000). This is in strong contrast to the 3-bp TSDs of maize *Spm* elements in Arabidopsis, which have an AT content of 73% and a complete absence of GC trinucleotides (Speulman et al., 1999).

Despite the lack of a consensus TSD, there are cases of insertion preference within a gene; for example, three independent *Mu* insertions were recovered at the same position in the *Bronze1* gene (reviewed in Chandler and Hardeman, 1992). Local secondary structure or host proteins may play an important role. We observed that there is an asymmetrical distribution of purines and pyrimidines within the 9-bp *Mu* TSD: positions +2 and +3 are high in CT nucleotides, and positions +7 and +8 are high in AG nucleotides. This may promote the formation of a stem–loop structure as a result of internal DNA base pairing.

As for *RescueMu* insertion preference between exons and introns, because much of the maize sequence in GenBank consists of ESTs, our sequence similarity is biased for exons rather than introns. As more maize genomic DNA sequence becomes available, the exact distribution of *RescueMu* insertions with respect to coding regions, untranslated sequences, and introns will become clear.

***RescueMu* Is an Effective Tool for Maize Functional Genomics**

Finally, we have demonstrated that *RescueMu* will be a useful tool for functional genomic studies in maize. Despite the large size of the maize genome, we have shown that plasmid rescue from maize can be efficient. We recovered 5- to 27-kb segments of maize genomic DNA as plasmids (Figure 6). In many cases, the entire target gene would be recovered in *E. coli*, in contrast to PCR screens, in which only a segment is recovered (Das and Martienssen, 1995; Hanley et al., 2000). Plasmid rescue is successful for both somatic and germinal insertions; however, recovery more than once is a good indicator of a germinal insertion (Table 2). The class of rare, early somatic insertions also should be recovered multiple times.

Second, we found that *RescueMu* can amplify and be transmitted in multiple copies to progeny (Table 2 and data not shown). Although we did not distinguish between hypothesized very early somatic insertions and true germinal cell insertions, an important lesson is that the transposon transgene tandem array appeared to inhibit the generation of inherited insertions (Table 1) such that there were far fewer transposed *RescueMu* elements than expected. After an element had transposed away from the original donor transgene locus, however, the apparent germinal insertion frequency increased dramatically. Already noted are native genomic position effects that influence the transposition frequency of *MuDR* elements (Lisch et al., 1995); the “minimal line” containing a single *MuDR* on chromosome 2L exhibits an 11 to 24% germinal insertion frequency per generation. Once this copy of *MuDR* transposes to new chromosomal locations, however, the insertion frequency is >70%, consistent with the frequency required to maintain its copy number in most progeny.

Because the outcome of *Mu* germinal events results in the transmission of donor alleles and new insertion sites, selective recovery of new *Mu* insertions is almost impossible with standard *Mu* elements prepared from genomic libraries or by PCR strategies. With a combination of restriction enzyme digestion at donor sites and DNA hybridization screens, new *RescueMu* insertions can be recovered selectively in *E. coli* (Figure 4 and Table 2). The diversity of genomic sequences flanking *RescueMu* insertions and their similarity to ESTs (Tables 2 and 3) indicate that *RescueMu* will be a useful gene discovery tool in maize. These methods are suitable for high throughput functional genomics research

Figure 7. (continued).

(E) An alternative replicative transposition model to explain the lack of germinal revertants. *Mu* elements switch from a cut-and-paste transposition mechanism in somatic cells to a replicative mechanism in pregerminal and postmeiotic cells. Hence, no excisions occur, because only a single strand of the donor allele is transferred to the new insertion site. DNA synthesis at the donor and recipient sites generates the complementary strands, followed by ligation of the transposon to the host chromosome (reviewed in Craig, 1995).

and have been implemented for maize genomics research (http://zmdb.iastate.edu).

METHODS

Vectors

pRescueMu2 and pRescueMu3 were constructed as follows. Vector pKYLX71, based on published vector pKYLX7 (Schardl et al., 1987) with a modified polylinker, was obtained from Chris Schardl (University of Kentucky, Lexington, KY). It contains a 900-bp cauliflower mosaic virus (CaMV) 35S promoter fragment from position +6500 to +7460 (Franck et al., 1980), including a 25-bp 5′ untranslated leader. The native leader was ligated to a polylinker containing the following sites: HindIII, BamHI, XhoI, PstI, SacI, and XbaI. The vector also contains a 700-bp 3′ rbcS transcriptional terminator (rbcS 3′). Into the polylinker, the maize *Lc* (*Leaf color*) cDNA from vector pSRL349 (Ludwig et al., 1989) was ligated as a 2.2-kb XbaI–XbaI fragment. The *Lc* cDNA fragment was missing the first three ATGs and began at position +197 (Ludwig et al., 1989). At the HindIII polylinker site between CaMV 35S and maize *Lc*, a complete 1.4-kb *Mu1* element was inserted after being adapted with HindIII linkers. This *Mu1* subclone was from pALMH25 (Luehrsen and Walbot, 1990) and was derived from the *AdhI-S3034 Mu1* insertion allele (Barker et al., 1984); it is flanked by its native 9-bp host duplication TTTTGGGGA. There are 90 bases from the right terminal inverted repeat (TIR) to the ATG codon of *Lc*. The *CaMV35S-Mu1-Lc*-rbcS 3′ construct is called pAL197-7 and was kindly provided by Alan Lloyd (University of Texas, Austin, TX).

The *CaMV35S-Mu1-Lc*-rbcS 3′ region was cut from pAL197-7 as an EcoRI–ClaI fragment and inserted into pOK12, a 2.1-kb kanamycin-encoding plasmid with a p15A origin of replication (Vieira and Messing, 1991). This resulted in clone pMR31. The DNA backbone of this construct was expanded by the addition of a 2.4-kb tetracycline subclone from pACYC184 (GenBank accession number X06403), which was inserted as a BstB1 fragment into the ClaI site of pMR31 to create a larger plasmid, pMR34. This was done to favor the plasmid breakpoint occurring outside of the *RescueMu* element and the *CaMV 35S-Lc* reporter.

A modified pBluescript KS+ plasmid (Stratagene, La Jolla, CA) was inserted into the *Mu1* element of pMR34. By site-directed mutagenesis, the unique KpnI site in pBluescript KS+ was changed to a BstEII site to permit its insertion into the middle of *Mu1*. A second NotI site was created to permit inverse polymerase chain reaction (PCR); the unique SmaI site was mutagenized to NotI, creating a novel SacI site in the process. This clone was called pMRΔ7B1. To be able to distinguish between different *RescueMu* plasmids, unique 400-bp tags were inserted into the BamHI site of the polylinker of pMRΔ7B1 from the *nod* genes of *Rhizobium meliloti*; these are highly rich in GC to facilitate efficient hybridization on DNA gel blots. To create the future *RescueMu2*, a 400-bp BstY1 *NodPQ* fragment (+1832 to +2229; GenBank accession numbers M68868 and J03676) was inserted to create pMR15 (also known as p173-3). To create the future pRescueMu3, a 200-bp BclI–BamHI *NodPQ* fragment (+1204 to +1014, inverted; GenBank accession numbers M68858 and J03676) was inserted as a direct repeat to create pMR17 (also known as p192-1).

To create pRescueMu2, plasmid pMR15 was ligated as a BstEII fragment into the BstEII site of *Mu1* in pMR34 to create pMR36 (also known as p738-4), an ~13-kb plasmid. To create pRescueMu3, plasmid pMR17 was ligated similarly to create pMR37 (also known as p743-2), an ~13-kb plasmid. pAHC20 is the maize ubiquitin promoter *Bar* herbicide resistance plasmid kindly provided by P. Quail (Plant Gene Expression Center, Albany, CA) (Christensen and Quail, 1996).

Maize Transformation and Plant Material

Embryogenic A188 × B73 (HiII hybrid) embryogenic calli were cotransformed biolistically with plasmids pRescueMu2, pRescueMu3, and pAHC20 as described previously (Raizada and Walbot, 2000). A detailed transformation protocol is available at http://www.stanford.edu/~walbot/StableMaizeTransf.html. Because the *Lc*::*RescueMu* alleles are linked to pAHC20, resistance to Basta (Hoescht, Montreal, Canada) was used to follow the transgene array (Spencer et al., 1990). To test for Basta resistance, a 5-cm-diameter marked leaf surface was painted with 0.75% glufosinate ammonium (Ignite 600, 50% solution; Hoescht) with 0.1% Tween 20 using a Q-tip. The area was scored visually for the presence or absence of necrosis 5 to 7 days later. Primary transformants (genotype *r-r/r-g C1/c1*) were outcrossed to A188, W23, and K55 inbred or mixed hybrid backgrounds with the genotype *r-g* or *r-r C1* before or after crossing to low copy *MuDR* (*a1-mum2/a1 R C1*) or standard higher copy *MuDR* lines (*bz2*::*Mu1/bz2 R C1*). *RescueMu* elements were somatically mutable in both *MuDR* backgrounds.

Hybridization Probes and DNA Gel Blot Analysis

To determine transgene array complexity or to search for new *RescueMu* insertions, *RescueMu2*- and *RescueMu3*-specific probes were prepared. The *RescueMu2*-specific probe was obtained as a 520-bp XhoI–XbaI fragment from pMR15. The *RescueMu3*-specific probe was obtained as a 478-bp XhoI–SacI fragment from pMR17. Alternately, PCR was used to amplify these fragments. To amplify *RescueMu2*, the primers were 5′-GCGAATTCGACAGCCGGCAGGGCATTC-3′ (primer p173+155F) and a T7 primer, 5′-CGCGTAATACGACTCACTATAGGGC-3′. To amplify *RescueMu3*, the primers were 5′-TTCCTGCAGCGGCCGCGGATCAGC-3′ (primer p192+130F) and the T7 primer. PCR cycle conditions were 94°C for 45 sec, 50°C for 45 sec, and 72°C for 60 sec (30 to 35 cycles) in the presence of 2 mM $MgCl_2$. PCR products were purified with agarose gel. Instead of using *RescueMu*-specific probes to detect new *RescueMu* insertions, an ampicillin probe was used. It was isolated as an ~1-kb BspHI fragment from pBluescript KS+ (Stratagene).

CaMV 35S and maize *Lc* probes were used to select against the recovery of the original *Lc*::*RescueMu* alleles after plasmid rescue. The CaMV 35S probe extends from +7072 to +7565 (Franck et al., 1980) and was isolated as an XbaI–PstI fragment from plasmid pR (Ludwig et al., 1990). The maize *Lc* probe was isolated as an ~800-bp PstI fragment from pR. Ten to 50 ng of probe DNA was prepared using a DecaPrimeII random primer kit (Promega, Madison, WI) and ^{32}P-radiolabeled dCTP (Amersham, Piscataway, NJ), incubated at 37°C for more than 3 hr, and then purified on a NucTrap push column (Stratagene). Genomic DNA was isolated from leaves using the protocol of Dellaporta (1994), blotted, and hybridized to ^{32}P-radiolabeled probes as described previously (Warren and Hershberger, 1994).

Analysis of *RescueMu* Excision Alleles

To recover *RescueMu* empty donor sites, nested PCR was used to amplify leaf DNA. In the first round of PCR, the 5′ primer was 5′-GCAAGTGGATTGATGTGATATCTCCACTGAC-3′ (primer 35S+7325) and the 3′ primer was 5′-CGTGTCAGTTGTACCAAGCTCAAG-CACGC-3′ (primer R+1090). PCR was performed using 100 ng of genomic DNA, 2.5 mM $MgCl_2$, 10 pmol of each primer, 1 × Taq buffer, 0.1 mM deoxynucleotide triphosphates, and 2.5 units of AmpliTaq (Perkin-Elmer, Foster City, CA) in a final volume of 100 μL. Before the addition of polymerase, the mixture was kept at 95°C for 5 min and then cooled to 80°C while enzyme was added. The reaction was performed at 95°C for 45 sec, 52°C for 1 min, and 72°C for 2 min (35 cycles). For nested PCR, 2 μL of the first reaction was used directly; all PCR conditions were the same as in the first round except that the annealing temperature was 55°C and the primers used were 5′-GCGGTACCACTGACGTAAGGGATGACGCAC-3′ (primer 35S+7360) and 5′-CGCGAATTCGTCGCTCGCGAAACTCCTGCCG-3′ (primer in *Lc*). The internal 5′ primer was located 99 bp upstream of the left 9-bp host duplication, whereas the 3′ primer was located 499 bp upstream of the right 9-bp host duplication. Because of introduced restriction sites inside the nested primers, amplified fragments were subcloned as KpnI–EcoRI fragments. For DNA sequencing, the primer used was 5′-CAGCAGTTCTTCCGCCTGCTGAAC-3′ (primer R+260) or T3/SK cloning vector primers.

Plasmid Rescue Procedure and Sequencing of Insertion Alleles

To prevent contamination by foreign ampicillin-encoding plasmids, all mortars, pestles, enzymes, and other materials were segregated from general laboratory use. Solutions were purchased directly from Sigma (St. Louis, MO) where possible and divided into single use batches. Genomic DNA was isolated from leaves using the protocol of Dellaporta (1994). Ten micrograms of genomic DNA was digested with 50 units of KpnI and 15 μL of 10 × React 4 buffer in the presence of RNaseA (Bethesda Research Laboratories, Rockville, MD) in a volume of 150 μL for 90 min at 37°C. After two phenol:chloroform extractions and a final chloroform extraction, DNA was ethanol precipitated in the presence of 0.3 M sodium acetate, centrifuged for 20 min at 10,000*g* at 4°C, washed with 1 volume of 70% ethanol, air dried, and dissolved in 20 μL of water. DNA fragments were self-ligated at 14°C for 16 hr with 10 units of T4 DNA ligase (Bethesda Research Laboratories) and 100 μL of previously unthawed 5 × ligation buffer (Bethesda Research Laboratories) in a final volume of 500 μL. The ligation mixture was then extracted twice in phenol:chloroform and once in chloroform. DNA was precipitated with 1 volume of isopropanol with 0.3 M sodium acetate, centrifuged for 20 min at 10,000*g* at 4°C, washed with 1 volume of 70% ethanol, and air dried. The pellet was then dissolved in 10 μL of water.

An optional BglII selection step was performed as follows. DNA was digested with 30 units of BglII and 1 × React 3 buffer (Bethesda Research Laboratories) in a final volume of 100 μL for 1 hr at 37°C. The mixture was extracted once in phenol:chloroform and once in chloroform followed by ethanol precipitation in the presence of 0.3 M sodium acetate. The mixture was centrifuged for 20 min at 10,000*g* at 4°C, washed with 1 volume of 70% ethanol, and then dissolved in 10 μL of water. Electroporation was used to transform ElectroMAX DH10B cells (Bethesda Research Laboratories), a highly competent strain (>10^{10} colony-forming units/μg plasmid DNA) suitable for cloning large, methylated plasmids containing direct repeats. For electroporation, 2 μL of DNA (~1 μg) was used to transform 30 μL of DH10B cells exactly according to the manufacturer's instructions. After electroporation, the bacterial mixture was resuspended immediately in 1 mL of SOC medium (Bethesda Research Laboratories) and then allowed to recover at 37°C for 1 hr with shaking. The cells were centrifuged gently at 2500*g* in a tabletop centrifuge at room temperature for 5 min and resuspended in 200 μL of SOC. Aliquots (20 and 100 μL) were plated onto ampicillin/carbenicillin–containing medium.

To identify plasmid contamination, colonies were usually hybridized to a mixture of two *RescueMu*-specific probes to confirm colony identity using the Grunstein-Hogness filter colony lift method. An optional pair of colony hybridizations was used to confirm that only new *RescueMu*-containing plasmids were picked; a subset of positive colonies from the first hybridization screen was numbered and arrayed on duplicate agarose plates. Colonies from one plate were hybridized to a mixture of CaMV 35S− and maize *Lc*-specific probes; colonies from the second plate were hybridized again to the mixture of *RescueMu*-specific probes. Colonies that were positive with the *RescueMu* probes but negative with CaMV 35S and *Lc* were then selected.

For sequencing of flanking genomic DNA, plasmids were first linearized with EcoRI to obtain cleaner sequences. The primers were located just inside of the *Mu1* TIRs. The *RescueMu* right border primer was 5′-CGCGTGACTGAGATGCGACGGAG-3′ (primer *Mu1* R out), and the *RescueMu* left border primer was 5′-AGCACCGCCGTG-CTGCCGTAGAGCG-3′ (primer *Mu1* L out).

ACKNOWLEDGMENTS

We thank H. Bailey, K. Brewer, and R. Swinney for laboratory and field assistance. We thank J. Fernandes for help with database analysis. We thank Y. Cho, B. Nakao, G. Randhawa, K. Sarsour, and B. Schneider for assistance with DNA sequencing. We thank V. Chandler, S. Hake, C. Lunde, C. Napoli, L. Roy, R. Schmidt, L. Smith, and K. Slotkin for providing us with *RescueMu* germinal insertion materials and for allowing us to present unpublished results. We thank A. Lloyd, S. Long, P. Quail, and C. Somerville for materials or equipment used in this study. We thank D. Pareddy, P. Lemaux, R. Williams Carrier, Y. Wan, D.B. Walden, and C. Armstrong for advice on maize transformation. We thank G. Rudenko, A. Ono, and a thoughtful anonymous reviewer for comments on the manuscript. M.N.R. was the recipient of a Natural Sciences and Engineering Research Council Centennial Science and Engineering Predoctoral Fellowship from the Canadian Government and the Joseph R. McMicking Graduate Fellowship from Stanford University. This work was supported by National Institutes of Health Grant GM49681 and by National Science Foundation Grant 98-72657 to V.W.

Received January 3, 2001; accepted April 20, 2001.

REFERENCES

Alleman, M., and Freeling, M. (1986). The *Mu* transposable elements of maize: Evidence for transposition and copy number regulation during development. Genetics **112,** 107–119.

Armstrong, C.L. (1994). Regeneration of plants from somatic cell cultures: Applications for in vitro genetic manipulation. In The Maize Handbook, M. Freeling and V. Walbot, eds (New York: Springer-Verlag), pp. 663–671.

Armstrong, C.L., and Green, C.E. (1985). Establishment and maintenance of friable, embryogenic maize callus and the involvement of L-proline. Planta **164,** 207–214.

Barkan, A., and Martienssen, R.A. (1991). Inactivation of maize transposon *Mu* suppresses a mutant phenotype by activating an outward-reading promoter near the end of *Mu1*. Proc. Natl. Acad. Sci. USA **88,** 3502–3506.

Barker, R.F., Thompson, D.V., Talbot, D.R., Swanson, J., and Bennetzen, J.L. (1984). Nucleotide sequence of the maize transposable element *Mu1*. Nucleic Acids Res. **12,** 5955–5967.

Beall, E.L., and Rio, D.C. (1997). Drosophila *P*-element transposase is a novel site-specific endonuclease. Genes Dev. **10,** 921–933.

Benito, M.-I., and Walbot, V. (1997). Characterization of the maize *Mutator* transposable element MURA transposase as a DNA-binding protein. Mol. Cell. Biol. **17,** 5165–5175.

Bennetzen, J.L. (1996). The *Mutator* transposable element system of maize. Curr. Top. Microbiol. Immunol. **204,** 195–229.

Bennetzen, J.L., Springer, P.S., Cresse, A.D., and Hendrickx, M. (1993). Specificity and regulation of the *Mutator* transposable element system in maize. Crit. Rev. Plant Sci. **12,** 57–95.

Britt, A.B., and Walbot, V. (1991). Germinal and somatic products of *Mu1* excision from the *Bronze-1* gene of *Zea mays*. Mol. Gen. Genet. **227,** 267–276.

Carels, N., and Bernardi, G. (2000). Two classes of genes in plants. Genetics **154,** 1819–1825.

Chandler, V.L., and Hardeman, K.J. (1992). The *Mu* elements of *Zea mays*. Adv. Genet. **30,** 77–122.

Chomet, P., Lisch, D., Hardeman, K.J., Chandler, V.L., and Freeling, M. (1991). Identification of a regulatory transposon that controls the *Mutator* transposable element system in maize. Genetics **129,** 261–270.

Christensen, A.H., and Quail, P.H. (1996). Ubiquitin promoter-based vectors for high level expression of selectable and/or screenable marker genes in monocotyledonous plants. Transgenic Res. **5,** 213–218.

Coen, E.S., Robbins, T.P., Almeida, J., Hudson, A., and Carpenter, R. (1989). Consequences and mechanisms of transposition in *Antirrhinum majus*. In Mobile DNA, D.E. Berg and M.M. Howe, eds (Washington, DC: American Society of Microbiology), pp. 413–436.

Craig, N.L. (1995). Unity in transposition reactions. Science **270,** 253–254.

Cresse, A.D., Hulbert, S.H., Brown, W.E., Lucas, J.R., and Bennetzen, J.L. (1995). *Mu1*-related transposable elements of maize preferentially insert into low-copy-number DNA. Genetics **140,** 315–324.

Das, L., and Martienssen, R. (1995). Site-selected transposon mutagenesis at the *hcf106* locus in maize. Plant Cell **7,** 287–294.

Dellaporta, S. (1994). Plant DNA miniprep and microprep: Versions 2.1–2.3. In The Maize Handbook, M. Freeling and V. Walbot, eds (New York: Springer-Verlag), pp. 522–525.

Donlin, M.J., Lisch, D., and Freeling, M. (1995). Tissue-specific accumulation of MURB, a protein encoded by *MuDR*, the autonomous regulator of the *Mutator* transposable element family. Plant Cell **7,** 1989–2000.

Dooner, H.K., and Martinez-Ferez, I.M. (1997). Germinal excisions of the maize transposon *Activator* do not stimulate meiotic recombination or homology-dependent repair at the *bz* locus. Genetics **147,** 1923–1932.

Doseff, A., Martienssen, R., and Sundaresan, V. (1991). Somatic excision of the *Mu1* transposable element of maize. Nucleic Acids Res. **19,** 579–584.

Franck, A., Guilley, H., Jonard, G., Richards, K., and Hirth, L. (1980). Nucleotide sequence of cauliflower mosaic virus DNA. Cell **21,** 285–294.

Gorbunova, V., and Levy, A.A. (1997). Non-homologous DNA end joining in plant cells is associated with deletions and filler DNA insertions. Nucleic Acids Res. **25,** 4650–4657.

Gorbunova, V., and Levy, A.A. (2000). Analysis of extrachromosomal *Ac/Ds* transposable elements. Genetics **155,** 349–359.

Gordon-Kamm, W.J., et al. (1990). Transformation of maize cells and regeneration of fertile transgenic plants. Plant Cell **2,** 603–618.

Haber, J.E. (2000). Partners and pathways, repairing a double-strand break. Trends Genet. **16,** 259–264.

Hanley, S., Edwards, D., Stevenson, D., Haines, S., Hegarty, M., Schuch, W., and Edwards, K.J. (2000). Identification of transposon-tagged genes by the random sequencing of *Mutator*-tagged DNA fragments from *Zea mays*. Plant J. **22,** 557–566.

Harris, L.J., Currie, K., and Chandler, V.L. (1994). Large tandem duplication associated with a *Mu2* insertion in *Zea mays B-Peru*. Plant Mol. Biol. **25,** 817–828.

Hershberger, R.J., Warren, C.A., and Walbot, V. (1991). *Mutator* activity in maize correlates with the presence and expression of the *Mu* transposable element *Mu9*. Proc. Natl. Acad. Sci. USA **88,** 10198–10202.

Hershberger, R.J., Benito, M.-I., Hardeman, K.J., Warren, C., Chandler, V.L., and Walbot, V. (1995). Characterization of the major transcripts encoded by the regulatory *MuDR* transposable element of maize. Genetics **140,** 1087–1098.

Hsia, A.-P., and Schnable, P.S. (1996). DNA sequence analyses support the role of interrupted gap repair in the origin of internal deletions of the maize transposon, *MuDR*. Genetics **142,** 603–618.

Hu, G., Yalpani, N., Briggs, S.P., and Johal, G.S. (1998). A porphyrin pathway impairment is responsible for the phenotype of a dominant disease lesion mimic mutant of maize. Plant Cell **10,** 1095–1105.

Joanin, P., Hershberger, R.J., Benito, M.-I., and Walbot, V. (1997). Sense and antisense transcripts of the maize *MuDR* regulatory transposon localized by *in situ* hybridization. Plant Mol. Biol. **33,** 23–36.

Levy, A.A., and Walbot, V. (1990). Regulation of the timing of transposable element excision during maize development. Science **248,** 1534–1537.

Levy, A.A., and Walbot, V. (1991). Molecular analysis of the loss of somatic instability in the bz2::mu1 allele of maize. Mol. Gen. Genet. **229,** 147–151.

Lisch, D., and Freeling, M. (1994). Loss of *Mutator* activity in a minimal line. Maydica **39,** 289–300.

Lisch, D., Chomet, P., and Freeling, M. (1995). Genetic characterization of the *Mutator* system in maize: Behavior and regulation of *Mu* transposons in a minimal line. Genetics **139,** 1777–1796.

Lisch, D., Girard, L., Donlin, M., and Freeling, M. (1999). Functional analysis of deletion derivatives of the maize transposon *MuDR* delineates roles for the MURA and MURB proteins. Genetics **151,** 331–341.

Lowe, B., Mathern, J., and Hake, S. (1992). Active *Mutator* elements suppress the Knotted phenotype and increase recombination at the *Kn1–0* tandem duplication. Genetics **132,** 813–822.

Ludwig, S.R., Habera, L.F., Dellaporta, S.L., and Wessler, S.R. (1989). *Lc*, a member of the maize *R* gene family responsible for tissue-specific anthocyanin production, encodes a protein similar to transcriptional activators and contains the myc-homology region. Proc. Natl. Acad. Sci. USA **86,** 7092–7096.

Ludwig, S.R., Bowen, B., Beach, L., and Wessler, S.R. (1990). A regulatory gene as a novel visible marker for maize transformation. Science **247,** 449–450.

Luehrsen, K.R., and Walbot, V. (1990). Insertion of *Mu1* elements in the first intron of the *Adhl-S* gene of maize results in novel RNA processing events. Plant Cell **2,** 1225–1238.

Martienssen, R., and Baron, A. (1994). Coordinate suppression of mutations caused by Robertson's *Mutator* transposons in maize. Genetics **136,** 1157–1170.

May, E.W., and Craig, N.L. (1996). Switching from cut-and-paste to replicative *Tn7* transposition. Science **272,** 401–404.

McCarty, D.R., Carson, C.B., Stinard, P.S., and Robertson, D.S. (1989). Molecular analysis of Viviparous1: An abscisic acid–insensitive mutant of maize. Plant Cell **1,** 523–532.

McLaughlin, M., and Walbot, V. (1987). Cloning of a mutable *bz2* allele of maize by transposon tagging and differential hybridization. Genetics **117,** 771–776.

Parinov, S., Sevugan, M., Ye, D., Yang, W.-C., Kumaran, M., and Sundaresan, V. (1999). Analysis of flanking sequences from *Dissociation* insertion lines: A database for reverse genetics in Arabidopsis. Plant Cell **11,** 2263–2270.

Qin, M., Robertson, D.S., and Ellingboe, A.H. (1991). Cloning of the *Mutator* transposable element *MuA2*, a putative regulator of somatic mutability of the *a1-Mum2* allele in maize. Genetics **129,** 845–854.

Raizada, M.N., and Walbot, V. (2000). The late developmental pattern of *Mu* transposon excision is conferred by a cauliflower mosaic virus 35S-driven MURA cDNA in transgenic maize. Plant Cell **12,** 5–21.

Raizada, M.N., Benito, M.-I., and Walbot, V. (2001a). The *MuDR* transposon terminal inverted repeat contains a complex plant promoter directing distinct somatic and germinal programs. Plant J. **25,** 79–91.

Raizada, M.N., Brewer, K.V., and Walbot, V. (2001b). A maize *MuDR* transposon promoter shows limited autoregulation. Mol. Genet. Genomics **265,** 82–94.

Robertson, D.S. (1978). Characterization of a *Mutator* system in maize. Mutat. Res. **51,** 21–28.

Robertson, D.S. (1981). *Mutator* activity in maize: Timing of its activation in ontogeny. Science **213,** 1515–1517.

Robertson, D.S. (1985). Differential activity of the maize *Mutator Mu* at different loci and in different cell lineages. Mol. Gen. Genet. **200,** 9–13.

Robertson, D.S., and Stinard, P.S. (1993). Evidence for *Mu* activity in the male and female gametophytes of maize. Maydica **38,** 145–150.

Rudenko, G.N., and Walbot, V. (2001). Expression and post-transcriptional regulation of maize transposable element *MuD*R and its derivatives. Plant Cell **13,** 553–570.

SanMiguel, P., Tikhonov, A., Jin, Y.K., Motchoulskaia, N., Zakharov, D., Melakeberhan, A., Springer, P.S., Edwards, K.J., Lee, M., Avramova, Z., and Bennetzen, J.L. (1996). Nested retrotransposons in the intergenic regions of the maize genome. Science **274,** 765–768.

Schardl, C.L., Byrd, A.D., Benzion, G., Altschuler, M.A., Hildebrand, D.F., and Hunt, A.G. (1987). Design and construction of a versatile system for the expression of foreign genes in plants. Gene **61,** 1–11.

Schnable, P.S., Peterson, P.A., and Saedler, H. (1989). The *bz-rcy* allele of the *Cy* transposable element system of *Zea mays* contains a *Mu*-like element insertion. Mol. Gen. Genet. **217,** 459–473.

Schwarz-Sommer, Z.A., Gierl, A., Cuypers, H., Peterson, P.A., and Saedler, H. (1985). Plant transposable elements generate the DNA sequence diversity needed in evolution. EMBO J. **4,** 591–597.

Scott, L., LaFoe, D., and Weil, C.F. (1996). Adjacent sequences influence DNA repair accompanying transposon excision in maize. Genetics **142,** 237–246.

Spencer, T.M., Gordon-Kamm, W.J., Daines, R.J., Start, W.G., and Lemaux, P.G. (1990). Bialaphos selection of stable transformants from maize cell culture. Theor. Appl. Genet. **79,** 625–631.

Speulman, E., Metz, P.L.J., van Arkel, G., Hekkert, B.L., Stiekema, W.J., and Pereira, A. (1999). A two-component *Enhancer-Inhibitor* transposon mutagenesis system for functional analysis of the Arabidopsis genome. Plant Cell **11,** 1853–1866.

Sundaresan, V., and Freeling, M. (1987). An extrachromosomal form of the *Mu* transposons of maize. Proc. Natl. Acad. Sci. USA **84,** 4924–4928.

Taylor, L.P., and Walbot, V. (1985). A deletion adjacent to the maize transposable element *Mu1* accompanies loss of *AdhI* expression. EMBO J. **4,** 869–876.

Vieira, J., and Messing, J. (1991). New pUC-derived vectors with different selectable markers and DNA-replication origins. Gene **100,** 189–194.

Walbot, V. (1991). The *Mutator* transposable element family in maize. In Genetic Engineering, J.K. Setlow, ed (New York: Plenum Press), pp. 1–37.

Walbot, V., and Rudenko, G.N. (2001). *MuDR/Mu* elements of maize. In Mobile DNA II, N.L. Craig, R. Craigie, M. Gellert, and A. Lambowitz, eds (Washington, DC: American Society of Microbiology). (in press).

Walbot, V., and Warren, C.A. (1988). Regulation of *Mu* element copy number in maize lines with an active or inactive Mutator transposable element system. Mol. Gen. Genet. **211,** 27–34.

Warren, C.A., and Hershberger, R.J. (1994). Southern blots of maize genomic DNA. In The Maize Handbook, M. Freeling and V. Walbot, eds (New York: Springer-Verlag), pp. 566–568.

Weaver, D. (1995). What to do at an end: DNA double-strand-break repair. Trends Genet. **11,** 388–392.

Yan, X., Martinez-Ferez, I.-M., Kavchok, S., and Dooner, H.K. (1999). Origination of *Ds* elements from *Ac* elements in maize: Evidence for rare repair synthesis at the site of *Ac* excision. Genetics **152,** 1733–1740.