

# Role of RAD51 in the Repair of *MuDR*-Induced Double-Strand Breaks in Maize (*Zea mays* L.)

Jim Li,<sup>\*,†,1</sup> Tsui-Jung Wen<sup>†,2</sup> and Patrick S. Schnable<sup>\*,†,‡,§,3</sup>

<sup>\*</sup>Department of Genetics, Development, and Cell Biology, <sup>†</sup>Interdepartmental Genetics Graduate Program,  
<sup>‡</sup>Department of Agronomy and <sup>§</sup>Center for Plant Genomics, Iowa State University, Ames, Iowa 50011

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## ABSTRACT

Rates of *Mu* transposon insertions and excisions are both high in late somatic cells of maize. In contrast, although high rates of insertions are observed in germinal cells, germinal excisions are recovered only rarely. Plants doubly homozygous for deletion alleles of *rad51A1* and *rad51A2* do not encode functional RAD51 protein (RAD51<sup>-</sup>). Approximately 1% of the gametes from RAD51<sup>+</sup> plants that carry the *MuDR*-insertion allele *a1-m5216* include at least partial deletions of *MuDR* and the *a1* gene. The structures of these deletions suggest they arise via the repair of *MuDR*-induced double-strand breaks via nonhomologous end joining. In RAD51<sup>-</sup> plants these germinal deletions are recovered at rates that are at least 40-fold higher. These rates are not substantially affected by the presence or absence of an *a1*-containing homolog. Together, these findings indicate that in RAD51<sup>+</sup> germinal cells *MuDR*-induced double-strand breaks (DSBs) are efficiently repaired via RAD51-directed homologous recombination with the sister chromatid. This suggests that RAD51<sup>-</sup> plants may offer an efficient means to generate deletion alleles for functional genomic studies. Additionally, the high proportion of *Mu*-active, RAD51<sup>-</sup> plants that exhibit severe developmental defects suggest that RAD51 plays a critical role in the repair of *MuDR*-induced DSBs early in vegetative development.

**T**HE *Mutator* transposon family of maize (*Zea mays* L.), first identified by its high forward mutation rate (ROBERTSON 1978), consists of an autonomous (*MuDR*) and several nonautonomous elements, all of which share ~200-bp conserved terminal inverted repeats (TIRs). The *MuDR* element carries the *mudrA*, which is required for transacting transposase activity (reviewed by CHANDLER and HARDEMAN 1992; BENNETZEN 1996; LISCH 2002; WALBOT and RUDENKO 2002). In germinal cells, *Mu* transposition frequencies can be as high as more than once per element per plant generation (ALLEMAN and FREELING 1986; WALBOT and WARREN 1988). Germinal revertant events from *Mu*-insertion alleles are, however, recovered only rarely (BROWN *et al.* 1989b; LEVY *et al.* 1989; SCHNABLE *et al.* 1989). In contrast, somatic excision events occur at high rates (RAIZADA *et al.* 2001; WALBOT and RUDENKO 2002). Double-strand breaks (DSBs), including those generated by the excision of transposons, can be repaired via two major pathways: homologous recombination (HR) and non-

homologous end joining (NHEJ) (PASTWA and BLASIAK 2003; WEST *et al.* 2004).

Two models have been proposed to reconcile the differential behavior of *Mu* transposons in germinal and late somatic cells (Figure 1). In both models *Mu* transposes via a “cut-and-paste” mechanism in late somatic cells. According to model A, *Mu* transposes exclusively via the cut-and-paste mechanism, *i.e.*, this mechanism is also utilized in germinal cells. To explain the low rate of germinal revertants, this model states that in germinal cells, but not in late somatic cells, *Mu*-induced DSBs are repaired via HR using the sister chromatid or homologous chromosome as a template (DONLIN *et al.* 1995; LISCH *et al.* 1995; HSIA and SCHNABLE 1996). The proposed role of HR in the repair of *Mu*-induced DSBs was based on the recovery of internal deletions of *Mu* elements thought to have arisen via a gap repair model (DONLIN *et al.* 1995; LISCH *et al.* 1995; HSIA and SCHNABLE 1996), which was originally described to explain the behavior of *Drosophila P* elements (ENGELS *et al.* 1990).

According to the alternative model B, although *Mu* transposons utilize a cut-and-paste mechanism in late somatic cells, replicative transposition is used in germinal cells, thereby explaining the low rate of germinal revertants (CRAIG 1995; RAIZADA *et al.* 2001; WALBOT and RUDENKO 2002). This model is based on the finding that the bacterial transposon Tn7 is competent to make a switch between cut-and-paste and replicative transposition

<sup>1</sup>Present address: Department of Biology and Rosenstiel Center, Brandeis University, Waltham, MA 02254.

<sup>2</sup>Present address: 6416 E. Lake, Sammamish Parkway NE, Redmond, WA 98052.

<sup>3</sup>Corresponding author: 2035B Roy J. Carver Co-Lab, Iowa State University, Ames, IA 50011. E-mail: schnable@iastate.edu

(MAY and CRAIG 1996) and the fact that the maize *mudrA* gene produces multiple transcripts that could at least potentially enable the switch between cut-and-paste and replicative transposition (RAIZADA *et al.* 2001; WALBOT and RUDENKO 2002).

RAD51, the RecA homolog in eukaryotes, plays a central role in the HR pathway (BAUMANN and WEST 1998; THACKER 1999), including that of maize (FRANKLIN *et al.* 1999; LI *et al.* 2007). We have compared the behavior of a *MuDR* transposon in RAD51<sup>+</sup> and RAD51<sup>-</sup> maize plants. Our data establish that RAD51-directed HR plays a major role in the repair of *MuDR*-induced DSBs in germinal cells. As such this study provides strong experimental support for the excision of *Mu* transposons in cell lineages that are inherited (germinal events), as well as in those that occur late in somatic cell development. This finding suggests that developmental differences in the regulation of endogenous DSBs repair pathways are responsible for the different behaviors of *Mu* transposons in germinal and late somatic cells.

## MATERIALS AND METHODS

**Genetic stocks:** The *a1-sh2* interval of chromosome 3 serves as a model for the study of meiotic recombination (CIVARDI *et al.* 1994; XU *et al.* 1995; YAO *et al.* 2002; YANDEAU-NELSON *et al.* 2005, 2006; YAO and SCHNABLE 2005). The *a1* gene encodes dihydroflavonol 4-reductase (O'REILLY *et al.* 1985) and is necessary for accumulation of anthocyanins in several plant tissues, including the aleurone (WIENAND *et al.* 1990). The *a1-m5216* allele contains a *MuDR* transposon insertion in exon III of the *a1* gene and conditions a spotted kernel phenotype due to somatic excision of *MuDR* (HSIA and SCHNABLE 1996; Figure 2). The *a1::rdt* (BROWN *et al.* 1989a; Figure 2; GenBank accession no. AF072704), *a1-mrh* (SHEPHERD *et al.* 1989) and *a1-mr102b* (CUYPERS *et al.* 1988; GenBank accession no. AY687856) alleles all contain transposon insertions that disrupt *a1* gene function and condition a nonspotted kernel phenotype in the absence of appropriate active transposases. The *a1-dl* allele has an 8-bp insertion in exon III that disrupts *a1* gene function (HSIA and SCHNABLE 1996; GenBank accession no. U46056). Kernels that lack a functional *Sh2* allele are shrunken (TSAI and NELSON 1966). The *a1* and *sh2* genes are both physically deleted in the *ax-1* allele (YANDEAU-NELSON *et al.* 2006). The functional characterization of *wx1* locus was described previously (SHURE *et al.* 1983; BARAN *et al.* 1992).

The maize genome contains only two *rad51* genes (FRANKLIN *et al.* 1999; LI *et al.* 2007). Deletion derivatives (*rad51A1-54F11d1* and *rad51A2-98E7d4*) of both of these genes were isolated as described (LI *et al.* 2007). Plants that are homozygous for both of these mutant alleles are referred to as RAD51<sup>-</sup>; all other genotypes with functional RAD51 are referred to as RAD51<sup>+</sup>. A material transfer agreement governs the distribution of *rad51* alleles; inquiries should be directed to Robert Meeley, Pioneer Hi-Bred, Johnston, Iowa.

**Oligonucleotides:** The following oligonucleotides were used in this study: 5216R, 5' TAA ATA AAA GGT GTC GTC AGC G 3'; A1.2, 5' GAT TGT TGC TTA AGC GCCAAT CGT 3'; ARRSP, 5' GAC TAG TTG CAG CGT GTG GTG TT 3'; IDPa1-dl, 5' CGT CGG TCC AGC ACT CCA 3'; m109, 5' AGC AGC AGC TAA AGA AGC AAG TC 3'; m567, 5' CCT GAG GAC GAT CAG TCT TGG C 3'; Mu-TIR, 5'AGA GAA GCC AAC GCC A(AT)C GCC TC(CT) ATT TCG TC 3'; Mu1211, 5' GTG GAA

GGA GGA GGA CTA CT 3'; Mu1253, 5' ATG AGC AAG GGT TTA GCG TGG AAT G 3'; Mu1805, 5' AGG TAT TTC CGT ATG CTG AGA G 3'; Mu1936, 5' ACATTT CTG ACC TTG CTA AC 3'; Mu2332r, 5' TGC CAT TCC TCA CAA GAA CAC TG 3'; Mu2400, 5' CCT CTG CTA CGT CTG GCT GTA CTG G 3'; Mu2903, 5' CCT CTG CTA CGT CTG GCT GTA CTG G 3'; Mu3102, 5' CCA AGA AAA GAC TGA GGA TTA 3'; Mu3106r, 5' GAG CAC TAA TCC TCA GTC TTT TC 3'; Mu3962, 5' CGA CAA CCC TTC CGT AGA T 3'; Mu4536u, 5' GAA CAC AGA ACA CGG GCT AGG 3'; Mu4700, 5' ATC TTC CGT CGC CGA ATT GGA CTG C 3'; Mu534r, 5' ATT AAA CTC ACC TCA CTG CCA CC 3'; MuDR2270, 5' TGG CAG AGG TAC GAG ACA GC 3'; MUDR3960, 5' TCA TCT ACG GAA GGG TTG TC 3'; rdt107, 5' AGC GGT CAC CAA GCA ATA G 3'; wx2481, 5' TAC CAG TCC CAC GGC ATC TAC A 3'; wx2659r, 5' GGT AGG AGA TGT TGT GGA TGC AG 3'; and XX231, 5' GCC AAA CTC TGA TTC GCT CCG TG 3'.

The approximate locations of some oligonucleotides are shown in Figure 2. All of the remaining oligonucleotides were designed on the basis of the *MuDR* sequence or *wx1* gene.

### Isolation of colored germinal revertants from *a1-m5216*:

Cross 1: *RAD51<sup>+</sup>; a1-dl Sh2/a1-dl Sh2 Wx1/Wx1 × a1-m5216 Sh2/a1\* Sh2; wx1/wx1*

Cross 2: *RAD51<sup>+</sup>; a1-m5216 Sh2/a1\* Sh2 × a1-dl Sh2/a1-dl Sh2*

Cross 3: *RAD51<sup>+</sup>; a1-m5216 Sh2/ax-1 × a1-dl Sh2/a1-dl Sh2.*

In all crosses female parents are listed first. The term *a1\** indicates either *a1-mrh* or *a1-mr102b*. To rule out possible pollen contamination, PCR was used to test for the presence of *wx1* or *a1* alleles contributed by the male parents. When amplified with primer pair wx2481 + wx2659r, the *wx1* allele present in the male parent of cross 1 yields a distinctive (smaller) PCR product than produced by any tested *Wx1* alleles. Because primer IDPa1-dl anneals to the 8-bp insertion in *a1-dl*, the primer pair ARRSP and IDPa1-dl amplifies the *a1-dl* allele contributed by the male parent of crosses 2–3, but not any other tested *a1* alleles. Colored kernels from crosses 1–3 that carried the pollen markers were selected as putative germinal revertants from *a1-m5216*. These candidate kernels were germinated and self-pollinated to make homozygous stocks. These newly arisen *A1* alleles were PCR-amplified from DNA extracted from homozygous seedlings using primers ARRSP and A1.2 (Figure 2), purified using QIAGEN (Valencia, CA) PCR purification kits (cat. no. 28106) and directly sequenced.

### Isolation of nonspotted and pale germinal deletions from *a1-m5216*:

Cross 4: *RAD51<sup>-</sup>; a1-m5216 Sh2/a1::rdt sh2 × a1-dl sh2/a1-dl sh2*

Cross 5: *RAD51<sup>+</sup>; a1-m5216 Sh2/a1::rdt sh2 × a1-dl sh2/a1-dl sh2*

Cross 6: *RAD51<sup>-</sup>; a1-m5216 Sh2/ax-1 × a1::rdt sh2/a1::rdt sh2*

Cross 7: *RAD51<sup>+</sup>; a1-m5216 Sh2/ax-1 × a1::rdt sh2/a1::rdt sh2.*

Two strategies were used to isolate germinal deletions from *a1-m5216*. In strategy I, the *a1-m5216* was heterozygous with *a1::rdt* (crosses 4–5); in strategy II, it was made hemizygous using *ax-1* (crosses 6–7). Because the genetic distance between *a1* and *sh2* is only 0.1 cM (CIVARDI *et al.* 1994), almost all round kernels from crosses 4–7 are derived from the *a1-m5216 Sh2* haplotype. If no genomic or epigenetic changes occur at *a1*, all round kernels from progeny (crosses 4–7) will be spotted due to the somatic excision of *MuDR* from *a1-m5216*. Nonspotted round kernels from crosses 4–7 were selected as candidate germinal deletions of *a1-m5216*. The female parents of crosses 4 and 5 are siblings, as are the female parents of crosses 6 and 7. As such, crosses 5 and 7 are appropriate positive controls for crosses 4 and 6, respectively. Crosses 4 and 6 were conducted

using approximately half of the RAD51<sup>-</sup> plants with active *Mu* that do not exhibit severe developmental abnormalities.

Nonspotted round kernels from crosses 4–7 were germinated and genomic DNA was isolated from 1-week-old seedlings using a modified high-throughput CTAB method (DIETRICH *et al.* 2002). To rule out pollen contamination as a source of the nonspotted round kernel phenotype, PCR was used to test the presence of the expected *a1* alleles from male parents. In progeny from crosses 4–5 (which should carry *a1-dl*) this was accomplished as described above; in progeny from crosses 6–7 (which should carry *a1::rdt*) that was accomplished using the primer pair XX231 and rdt107 (Figure 2). To rule out rare recombination events as the origin of nonspotted round kernel phenotype, progeny from crosses 4–5 were also tested for the presence of *a1::rdt*.

Plants that carried pollen markers and that did not arise via recombination between *a1* and *sh2* were further analyzed via PCR using two pairs of primers (Mu-TIR and XX231, Mu-TIR and A1.2) that anneal to *MuDR* and flanking *a1* sequences (Figure 2). For those alleles that failed to amplify with both pairs of primers, two *a1*-flanking primers (m109 and A1.2) were used to directly amplify deletion products (Figure 2). It was possible to specifically amplify (and subsequently sequence) the *a1-m5216* deletions from heterozygous progeny (crosses 4–7) because the primer m109 anneals to *a1-m5216* but not to either *a1::rdt* or *a1-dl*. For those alleles that were amplified with only one of the two primer pairs (Mu-TIR and XX231, Mu-TIR and A1.2), additional PCR was conducted using internal *MuDR* primers plus the appropriate *a1*-flanking primer. The resulting PCR products were subsequently sequenced to determine the deletion endpoints associated with each allele. Those alleles producing apparently normal PCR products using both primer pairs (Mu-TIR and XX231, Mu-TIR and A1.2) were subjected to temperature gradient capillary electrophoresis (TGCE) assays.

**TGCE:** Because the PCR assay described above cannot detect very small deletions, nonspotted round kernels with apparently normal PCR products using two primer pairs (Mu-TIR and XX231, Mu-TIR and A1.2) were analyzed via TGCE, which is capable of detecting deletions as small as a single base (HSIA *et al.* 2005). Due to the large size (4.9 kb) of *MuDR*, two rounds of PCR were performed to prepare templates for TGCE analysis. Initially all haplotypes were analyzed with two pairs of primers (XX231 and Mu2332r, 5216R and MuDR2270). The resulting PCR products were purified using the QIAGEN PCR purification kit (cat. no. 28106) and then diluted 1000× with distilled water for a second round of PCR. If the first reaction was conducted using the primer pair XX231 and Mu2332r, four additional pairs of primers (m567 and Mu534r, Mu473 and Mu1253, Mu1211 and Mu1936, Mu1805 and Mu2332r) were used for the second round of PCR. If the first reaction was conducted with the primer pair 5216R and MuDR2270, five additional pairs of primers (MuDR2270 and Mu2903, Mu2400 and Mu3106R, Mu3102 and Mu3962, MuDR3960 and Mu4700, Mu4536u and 5216R) were used for the second round of PCR. PCR products from the second round of PCR reactions were subjected to TGCE analysis *vs.* the intact *a1-m5216* control. TGCE was conducted using the Reveal System, model RVL 9612, rev. 2.0 (SpectruMedix, State College, PA). Sample preparation and TGCE conditions were as described previously (HSIA *et al.* 2005).

**Crossing strategies for the observation of developmental defects associated with RAD51<sup>-</sup> in a *Mu* active genetic background:**

Cross 8: *rad51A1-54F11d1/Rad51A1*; *rad51A2-98E7d4/Rad51A2*, *A1 sh2/a1-dl sh2* X *rad51A1-54F11d1/Rad51A1*; *rad51A2-98E7d4/Rad51A2*, *a1-m5216 Sh2/a1-dl sh2*

Cross 9: *rad51A1-54F11d1/Rad51A1*; *rad51A2-98E7d4/Rad51A2*, *A1 sh2/a1-dl sh2* X *rad51A1-54F11d1/Rad51A1*; *rad51A2-98E7d4/Rad51A2*, *a1-m5216\* Sh2/a1-dl sh2*.

The male parent of cross 8 carries an active *MuDR* transposon, as demonstrated by the observation that >90% of its *Sh2* progeny (*i.e.*, those that carry *a1-m5216*) are spotted due to somatic excisions from *a1* (HSIA and SCHNABLE 1996; Figure 2). In contrast, the male parent of cross 9 exhibits little or no *Mu* activity because progeny carrying *a1-m5216* are nonspotted. In this cross, the asterisk designates an inactive form of *a1-m5216*.

## RESULTS

**Colored germinal revertants from *a1-m5216*:** Kernels homozygous for stable mutant *a1* alleles are colorless. The *a1-m5216* allele conditions a spotted kernel phenotype due to somatic excisions of *MuDR* from *a1* (HSIA and SCHNABLE 1996). Sixteen independent confirmed, germinal colored revertants were isolated from normal RAD51<sup>+</sup> plants carrying *a1-m5216* (crosses 1–3, MATERIALS AND METHODS) and sequenced (Figure 3A). Consistent with previous reports about rates of germinal revertants from *Mu*-insertion alleles (BROWN *et al.* 1989b; LEVY *et al.* 1989; SCHNABLE *et al.* 1989), germinal revertants arose only rarely ( $2.60 \times 10^{-5}$ ) from *a1-m5216* (Table 1). All but four events (types 1–4, Figure 3A) were perfect germinal excisions (type 5, Figure 3A).

**The effect of RAD51-directed HR repair on the rate of germinal deletions from *a1-m5216*:** Germinal revertants of *Mu*-insertion alleles arise via the excision of *Mu* transposons, which can subsequently insert (*i.e.*, transposon) elsewhere in the genome. In contrast to the low rate of germinal reversions, germinal insertions of *Mu* transposons occur frequently (CHANDLER and HARDEMAN 1992; BENNETZEN 1996; LISCH 2002; WALBOT and RUDENKO 2002). Model A explains this apparent discrepancy in germinal excision and insertion rates by invoking highly efficient HR-mediated repair of the *MuDR*-induced DSBs associated with the excision of *Mu* transposons (Figure 1). Specifically, following *Mu* excision, the sister chromatid or homologous chromosome is used as a template to replace the excised transposon via HR. This model predicts that in the absence of HR, *Mu*-insertion alleles will give rise to high rates of germinal deletions caused by alternative, error-prone DSBs repair pathways. To test this model, the rates at which germinal deletions arose from *a1-m5216* in stocks with or without RAD51 (LI *et al.* 2007), which is central to HR, were compared via two crossing strategies (crosses 4–7, MATERIALS AND METHODS).

In strategy I, plants heterozygous for *a1-m5216* and a recessive *a1* allele were crossed by plants homozygous for another, distinguishable, recessive *a1* allele (crosses 4–5). In strategy II, plants hemizygous for *a1-m5216* were crossed by plants homozygous for a recessive *a1* allele (crosses 6–7). From all crosses, progeny kernels

**TABLE 1**  
**Rates and classes of germinal revertants isolated from *a1-m5216* in RAD51<sup>+</sup> plants**

Cross <sup>a</sup>	No. perfect excisions	No. imperfect excisions	Total no. revertants	Population size	Rate of germinal revertants
Cross 1	7 <sup>b</sup>	3 <sup>c</sup>	10	347,300	$2.88 \times 10^{-5}$
Cross 2	3 <sup>d</sup>	0	3	110,000	$2.72 \times 10^{-5}$
Cross 3	2 <sup>e</sup>	1 <sup>f</sup>	3	157,000	$1.91 \times 10^{-5}$
Total <sup>g</sup>	12 <sup>h</sup>	4	16	614,300	$2.60 \times 10^{-5}$

<sup>a</sup> Refer to MATERIALS AND METHODS.

<sup>b</sup> Type 5 in Figure 3A (plant identification nos. 03B-17-3, 03B-38-6, 03B-47-4, 03B-49-6, 03B-93-5, 03B-66-7, 02B-1132-L).

<sup>c</sup> All three types are shown in Figure 3A [plant identification nos. 03B-16-2 (type 1), 03B-45-1 (type 2), 03B-68-3 (type 3)].

<sup>d</sup> Type 5 in Figure 3A (plant identification nos. 03B-56-1, 03B-60-4, 03B-61-4).

<sup>e</sup> Type 5 in Figure 3A (plant identification nos. 02B-1185 and 02B-1188).

<sup>f</sup> Type 4 in Figure 3A (plant identification nos. 02B-1181).

<sup>g</sup> No significant differences were detected among these three populations based on the chi-square homogeneity test. Therefore, data from the three crosses were pooled.

<sup>h</sup> The high proportion of perfect excision events is probably due to selection, in that only colored kernels from crosses 1–3 were analyzed.

that carried *a1-m5216* were spotted due to somatic excisions of the *MuDR* transposon at *a1*. Partial or complete deletions of the *MuDR* insertion and/or *a1* sequences resulted in a nonspotted kernel phenotype. Such germinal deletions were distinguished from nonspotted sibling kernels with the genotypes (*a1::rdt sh2/a1-dl sh2* or *ax-1/a1::rdt sh2*) via the *a1-m5216*-coupled *Sh2* marker that conditions round kernels (and subsequent PCR genotyping). As expected, only a small percentage of round kernels from RAD51<sup>+</sup> plants (3% from cross 5 and 2% from cross 7) were nonspotted. In contrast, most round kernels from RAD51<sup>-</sup> plants (81% from cross 4 and 67% from cross 6) were nonspotted (Figure 4).

All of the 165 nonspotted kernels from crosses 4–7 were subjected to PCR and sequencing analyses using two pairs of primers that anneal to *MuDR* and flanking *a1* sequences (MATERIALS AND METHODS; Figure 2). These analyses established that 134 of the nonspotted kernels carried alleles that had undergone deletions of *MuDR* and/or *a1* sequences (classes I and II, Figure 4). The remaining 39 nonspotted kernels without apparent genomic rearrangements were subjected to TGCE, which is capable of detecting polymorphisms as small as a single base (HSIA *et al.* 2005). Using this assay and subsequent sequencing, two additional nonspotted round kernels were identified as carrying internal deletions of 54 bp (04B-277-6; GenBank accession no. 825704) and 38 bp (04B-279-2; GenBank accession no. 817897) of *MuDR* sequences as compared to *a1-m5216* (class III, Figure 4). These two deletions likely arose via interrupted gap repair (HSIA and SCHNABLE 1996).

Hence, in strategy I (*a1-m5216* is heterozygous), class I, II, and III germinal deletions were recovered from RAD51<sup>-</sup> plants 64-fold more frequently than from RAD51<sup>+</sup> controls  $\{[(35 + 51 + 2)/130/(2 + 7)/855] =$

64}. Similarly, in strategy II (*a1-m5216* is hemizygous), germinal deletions are recovered from RAD51<sup>-</sup> plants 41-fold more frequently than from RAD51<sup>+</sup> controls  $[(12 + 24)/42/(3/143) = 41]$ . When considering progeny from both strategies, all but two of the deletions responsible for the nonspotted kernel phenotypes included both *MuDR* and *a1* sequences flanking the *MuDR* insertion site (Figure 3, B and C; class I and II, Figure 4). These results strongly suggest that in RAD51<sup>+</sup> germinal cells, *MuDR*-induced DSBs occur frequently, but are usually successfully repaired via RAD51-directed HR. Because low rates of germinal deletions were also observed in control crosses 5 and 7, *MuDR*-induced DSBs are repaired via NHEJ at low rates even in the presence of RAD51. The loss of somatic excision activity in the 37 kernels that did not contain deletions (class IV, Figure 4) may be due to epigenetic changes at *a1-m5216* (CHANDLER *et al.* 1988; CHOMET *et al.* 1991).

**Ear sectors of germinal deletions from RAD51<sup>-</sup> plants:** Ear sectors of germinal deletions could be generated if *MuDR* excises prior to meiosis. Two or more kernels from an ear were considered to be part of a sector if they had the same deletion endpoints. Based on this criterion, nine ear sectors were identified from RAD51<sup>-</sup> plants. These sectors contained 2, 3, 4, 7, 7, 8, 10, 19, and 39 kernels. All kernels from the sectors that contained 2–19 kernels were sequenced. For the sector of 39 kernels, 17 kernels were sequenced; DNA from the remaining 22 kernels yielded the same-sized PCR product as from the sequenced kernels. These excision ear sectors recovered from RAD51<sup>-</sup> plants are much larger than the *Mu*-insertion sectors described previously (ROBERTSON 1980, 1981). The rate of recovering ear sectors is at least 52-fold higher in RAD51<sup>-</sup> plants as compared to RAD51<sup>+</sup> controls (9/172 *vs.* 0/998). This

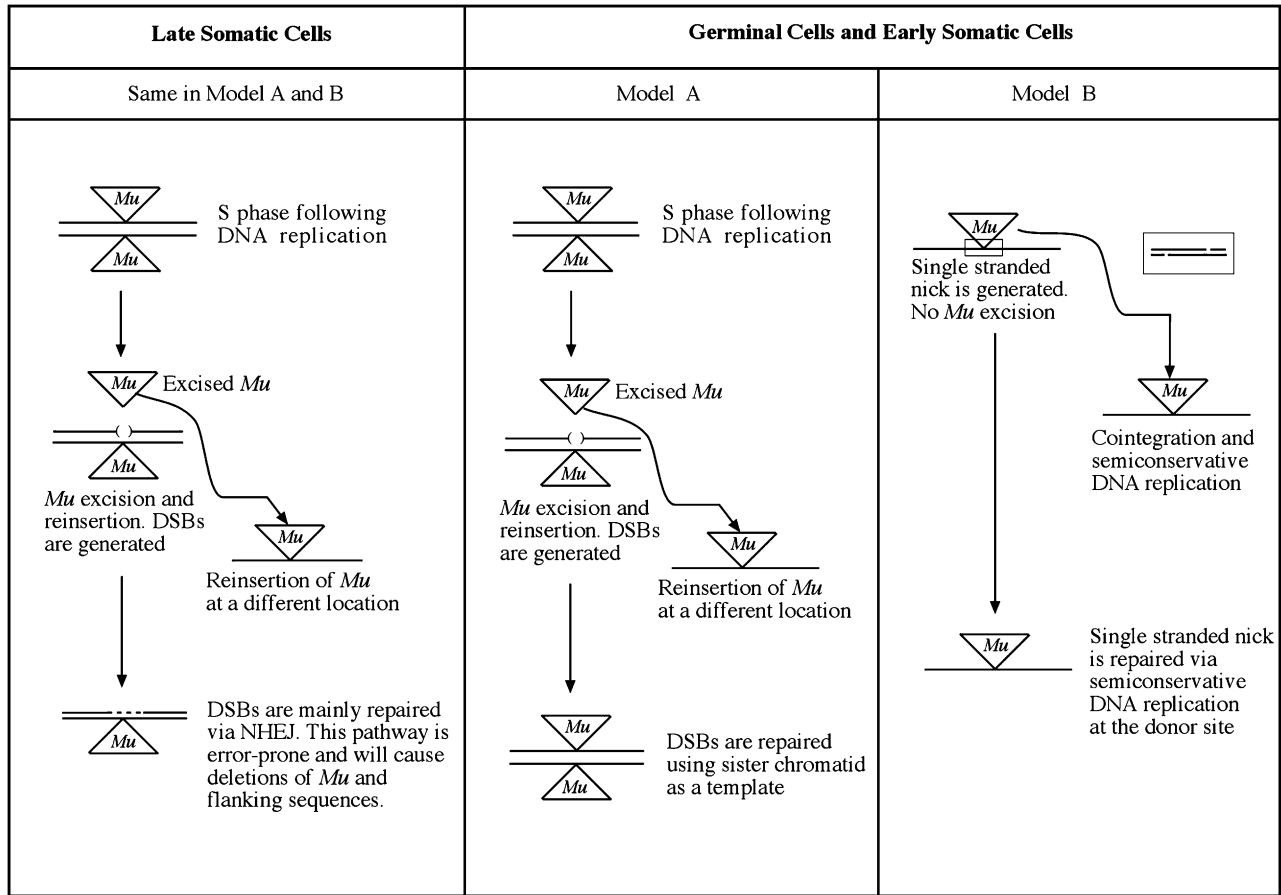


FIGURE 1.—Two competing models for *Mu* transposition mechanism are presented. Triangles designate *Mu* transposons. The parentheses represent DSBs. The dashed line represents a deletion. In the right column, two single-strand nicks are illustrated in an enlarged rectangle, where each line represents a single-strand of DNA. Elsewhere each line represents double-strand DNA. Our data provide strong experimental support for the view that repair of *Mu*-induced DSBs in germinal cells and early somatic cells occurs via the mechanism outlined in model A.

indicates that RAD51-directed HR is required for repairing *MuDR*-induced DSBs in premeiotic cells.

**RAD51 plays a critical role early in vegetative development of *Mu* active plants:** Maize RAD51<sup>-</sup> plants develop well under normal conditions, but are highly sensitive to radiation treatment, presumably due to the absence of RAD51-mediated repair of radiation-induced DSBs (Li *et al.* 2007). To test the role of

RAD51 in the repair of *MuDR*-induced DSBs in early vegetative development, an active *MuDR* was introduced into RAD51<sup>-</sup> plants (cross 8, MATERIALS AND METHODS). Approximately half of the *Mu*-active, RAD51<sup>-</sup> plants exhibit severe developmental defects (Table 2 and Figure 5). These defects were not observed among sibling plants that have functional RAD51 or related plants that lack active *MuDR* (Table 2). This finding strongly

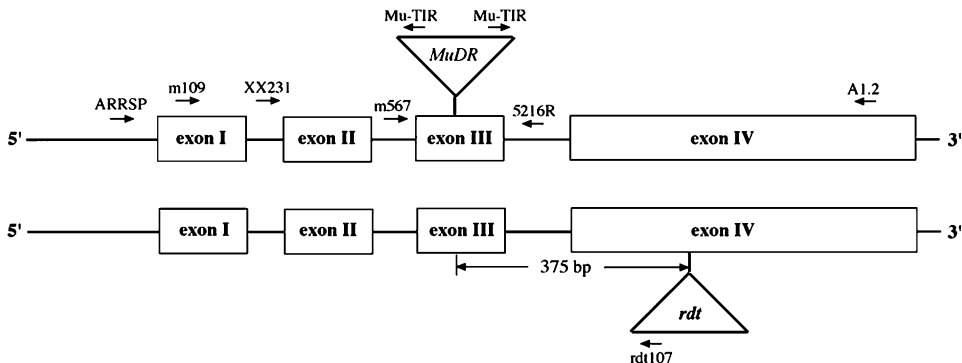


FIGURE 2.—Structures of the *a1-m5216* and *a1::rdt* alleles. Triangles represent *MuDR* or *rdt* transposons. The *a1-m5216* (top) and *a1::rdt* (bottom) alleles contain *MuDR* and *rdt* transposon insertions, respectively. Primer positions are designated by arrows.

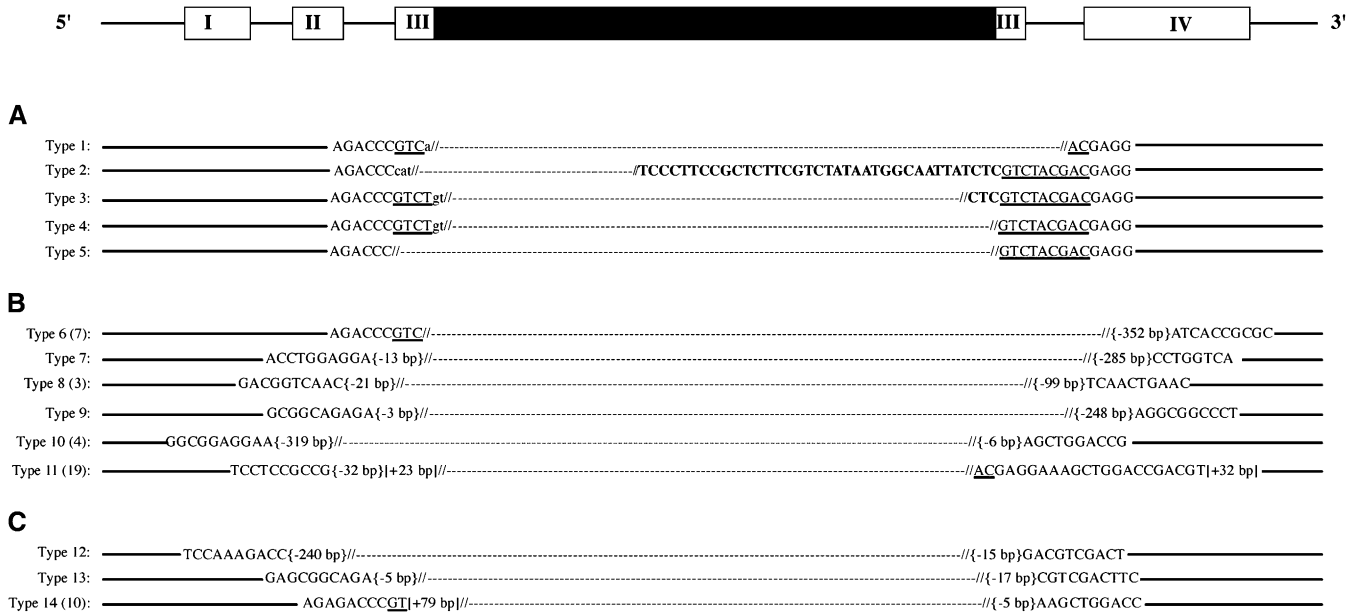


FIGURE 3.—Sequence analysis of germinal revertants and class I germinal derivatives from *a1-m5216*. Open and solid boxes represent exons of the *a1* gene and the *MuDR* insertion in *a1-m5216*, respectively. Retained *a1* sequences flanking deletions are shown for reference in regular type. Sequences derived from the 9-bp target site duplication (TSD) of *a1* are underlined. When nonzero, the number of base pairs of *a1* sequences adjacent to *MuDR* that were deleted is presented in braces. Dashed lines between two double slashes designate deleted sequences. In most cases, the entire *MuDR* element was deleted. Retained portions of *MuDR* are shown in boldface type. Short filler sequences of the type described by WESSLER *et al.* (1990) in spontaneous mutations are shown in lowercase letters, and the sizes of large fillers are indicated within brackets. Numbers of kernels in ear sectors are listed in parentheses. (A) Five types of germinal revertants from Table 1. One case of each of footprint types 1–4 was recovered. Twelve cases of the footprint type 5 (perfect excision) were recovered. (B) Class I events from RAD51<sup>-</sup>. Cross: *a1-m5216 Sh2/a1::rdt sh2 X a1-s sh2/a1-s sh2* (Figure 4). (C) Class I events from RAD51<sup>-</sup>. Cross: *a1-m5216 Sh2/ax-1 X a1::rdt sh2/a1::rdt sh2* (Figure 4).

suggests that RAD51-directed HR is also involved in the repair of *MuDR*-induced DSBs during early vegetative development.

## DISCUSSION

**The different behaviors of *Mu* transposons are a consequence of differential developmental regulations of DSB repair pathways:** High rates of *Mu* excisions and insertions are often observed only in somatic tissues formed late in development. In contrast, one of the long-standing puzzles associated with *Mu* biology has been the low rate of germinal reversions, even though germinal insertions are frequent. Two models have been proposed to explain this apparent discrepancy (Figure 1). Model A proposes that different DSB repair pathways are utilized in germinal and late somatic cells (DONLIN *et al.* 1995; LISCH *et al.* 1995; HSIA and SCHNABLE 1996). In contrast, model B proposes that different *Mu* transposition mechanisms are utilized in germinal and late somatic cells (RAIZADA *et al.* 2001; WALBOT and RUDENKO 2002). In yeast, Rad51p-mediated recombination requires ~70–100 bp homology on each side of the DSB (IRA and HABER 2002). Because RAD51 is not involved in the search for microhomologies, model B predicts

that the absence of RAD51 would not have an effect on the outcome of *Mu* transposition in germinal or early somatic cells.

In the absence of RAD51, the *a1-m5216* allele frequently gives rise to germinal deletions surrounding the *MuDR* insertion site (Figure 3, B and C, and Figure 4). Our findings strongly suggest that *MuDR*-induced DSBs occur frequently, but that in RAD51<sup>+</sup> germinal cells these DSBs are usually repaired via RAD51-directed HR (model A, Figure 1). Most of germinal derivatives recovered from the RAD51<sup>-</sup> plants are nonspotted (Figure 4) as a consequence of deletions of *a1* sequences that range from 5 to 352 bp (Figure 3, B and C). This finding indicates that the *MuDR*-induced DSBs are extensively resected, at least in the RAD51<sup>-</sup> background. The association of severe developmental defects with *Mu* activity in RAD51<sup>-</sup> plants (Table 2, Figure 5) indicates that RAD51 plays a critical role in the repair of *MuDR*-induced DSBs not only in germinal cells but also early in vegetative development (model A, Figure 1).

Hence, this study strongly suggests that the bulk of *Mu* transposition in both somatic and germinal cells occurs via a cut-and-paste mechanism, and the difference in outcomes of *Mu* transpositions is a consequence of the differing availabilities of DSB repair pathways during development (model A, Figure 1). Specifically, in late

Cross Type	Female Parent <sup>2</sup>	No. Round Kernels <sup>3</sup>				Non-spotted and Pale Round Kernels <sup>1</sup>				
		Spotted	Non-spotted	Pale Colored	Total	Class I <sup>4</sup>	Class II <sup>5</sup>	Class III <sup>6</sup>	Class IV <sup>7</sup>	
<i>a1-m5216 Sh2/a1::rdt sh2</i> <i>X a1-s sh2/a1-s sh2</i>	RAD51 <sup>-</sup>	25	105 (81%) <sup>8</sup>	0	0	130	35 [6]/105 <sup>9</sup>	51 [8]/105	2/105 <sup>10</sup>	17/105
	RAD51 <sup>+</sup>	826	29 (3%)	0	0	855	2/29	7/29	0/29	20/29
<i>a1-m5216 Sh2/ax-1</i> <i>X a1::rdt sh2/a1::rdt sh2</i>	RAD51 <sup>-</sup>	6	28 (67%)	8 (19%) <sup>11</sup>	0	42	12 [3]/36 <sup>12</sup>	24 [9]/36	0/36	0/36
	RAD51 <sup>+</sup>	140	3 (2%)	0	0	143	0/3	3/3	0/3	0/3

FIGURE 4.—Rates and structures of repair products isolated from RAD51<sup>+</sup> and RAD51<sup>-</sup> plants. (1) Fraction of nonspotted and pale round kernels that carry the indicated type of deletion of *a1-m5216* is listed. (2) For a given cross type, the female parents are siblings that differ as indicated in their *rad51* genotype. (3) Numbers of kernels within a family of this type are derived from multiple ears. (4) Class I events: loss of *MuDR* and part of *a1* flanking sequences. Structures of deletion alleles were determined via PCR and sequencing of selected PCR products. (5) Class II events: deletions of terminal portions of *MuDR* and loss of flanking *a1* sequences. Only deletions from one side are illustrated but deletions can occur on either side of *MuDR*. (6) Class III events: internal deletions of *MuDR*. (7) Class IV events: based on TGCE results these kernels carry apparently intact *MuDR* elements and therefore are nonspotted probably due to epigenetic inactivation. (8) Non-spotted (non-spotted round/total round). (9) Numbers in brackets indicate the total numbers of independent events (after correcting for ear sectors). Relevant plant identification numbers and their associated footprint types (Figure 3B) are: 04B-276-1, -2, -6, -14, -16, -18, -23 (type 6); 04B-276-21 (type 7); 04B-283-2, -4, -6 (type 8); 04B-283-8 (type 9); 05B-120-4, -7, -14, -15 (type 10); 05B-123-1–19 (type 11). (10) Relevant plant identification numbers are: 04B-277-6 (54-bp deletion) and 04B-279-2 (38-bp deletion). (11) All are class II. (12) Relevant plant identification numbers and their associated footprint types (Figure 3C) are 04B-279-1 (type 12), 04B-279-5 (type 13), 04B-128-1–10 (type 14).

somatic cells, *MuDR*-induced DSBs are primarily repaired via the error-prone NHEJ pathway (resulting in somatic sectors of “empty sites” which may restore gene function and in the case of *a1-m5216* result in colored spots on a colorless aleurone); in germinal cells and early somatic cells, usually (but not always) repaired via the highly accurate RAD51-directed HR pathway (resulting in the restoration of the *Mu* transposon; model A, Figure 1).

**Sister chromatids serve as the major template for the repair of *MuDR*-induced DSBs:** Substantially higher rates of germinal deletions were obtained from RAD51<sup>-</sup>

than from RAD51<sup>+</sup> plants. The majority of these deletions removed portions of both *MuDR* sequences and *a1* sequences that flank the *MuDR*-insertion site (Figure 3, B and C; classes I and II, Figure 4). This observation and the central role of RAD51 in HR strongly suggest that these germinal deletions in RAD51<sup>-</sup> plants arise via the error-prone NHEJ pathway.

In yeast, Rad51p is required for equal sister chromatid exchange (GONZALEZ-BARRERA *et al.* 2003), but not for HR between homologous chromosomes (SHINOHARA *et al.* 1997, 2000). If these specialized functions are conserved between yeast and maize, rates of recombination between sister chromatids, but not between homologous chromosomes, would be expected to be significantly reduced in RAD51<sup>-</sup> maize plants. Evidence for at least partial conservation of this functional specialization was obtained from our observation that rates of meiotic crossovers in surviving female gametes produced by RAD51<sup>-</sup> maize plants were not significantly lower than those in RAD51<sup>+</sup> controls (LI *et al.* 2007).

In strategy I, *a1-m5216* was heterozygous with the *a1::rdt* allele (crosses 4–5, MATERIALS AND METHODS). Hence, the template for HR-mediated repair of the *MuDR*-induced DSBs could have been either the sister chromatid or the homologous chromosome. All round progeny from cross 4 carry either *a1-m5216* or imperfect footprints derived from *a1-m5216* (Figure 4), suggesting that the homologous chromosome carrying *a1::rdt* was not used as a template to repair *MuDR*-induced DSBs. In contrast, in strategy II only the sister

TABLE 2

**RAD51 is required for repair of *MuDR*-induced DSBs in vegetative tissues**

Genotype	<i>Mu</i> activity	No. plants with severe developmental defects <sup>a</sup>	No. normal plants
RAD51 <sup>-b</sup>	Active	10 <sup>c</sup>	13
RAD51 <sup>+b</sup>	Active	0	30
RAD51 <sup>-d</sup>	Inactive	0	15

<sup>a</sup> The severe developmental defects are described in Figure 5.

<sup>b</sup> Progeny of cross 8 (MATERIALS AND METHODS). Because plants from these two categories are siblings, they can be directly compared.

<sup>c</sup> One defective plant died 2 weeks after germination.

<sup>d</sup> Progeny of cross 9. With the exception of *rad51* genotypes and *Mu* activity, this category of plants possesses almost the same genetic background as the other two categories.

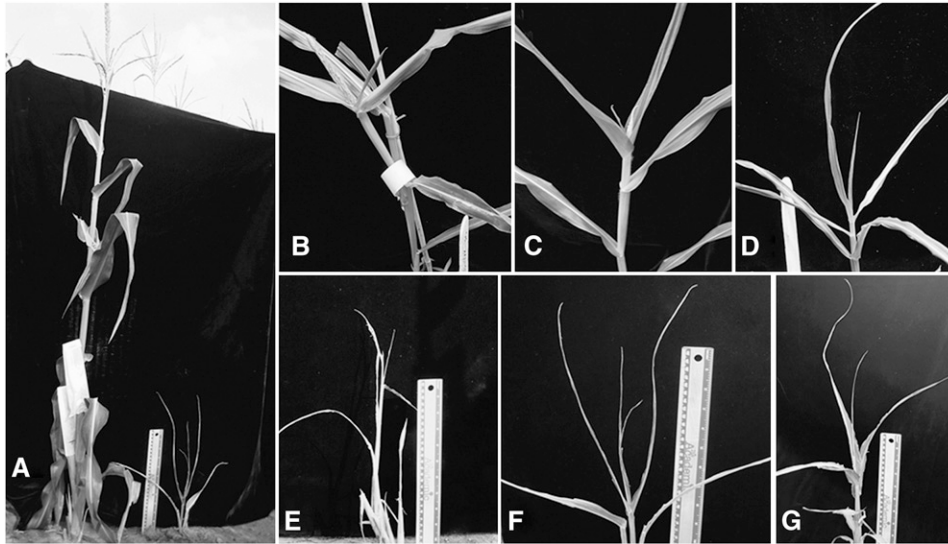


FIGURE 5.—Developmental defects associated with RAD51<sup>-</sup> plants and *Mu* activity. Many RAD51<sup>-</sup> plants with *Mu* activity are short (less than one-third the height of nonmutant siblings), have tiny tassels or no tassels, and do not produce ears (Table 2). These developmental defects were observed in multiple families and planting seasons including our 2005 greenhouse, 2006 (S. LIU and P. S. SCHNABLE, unpublished data), and 2007 summer nurseries. (A) Two *Mu* active siblings from the 2007 summer nursery (plant identification nos. 07-3616-1 and 07-3616-2). Left and right plants are RAD51<sup>+</sup> and RAD51<sup>-</sup>, respectively. The ruler is 12-inches long. (B–D) RAD51<sup>-</sup> plants with *Mu* activity from 2005

greenhouse season (plant identification nos. 05B-1594-25, 05B-1594-66, 05B-1597-36; Table 2). The white stake is 6-inches long. (E–G) RAD51<sup>-</sup> plants with *Mu* activity from the 2007 summer nursery (plant identification nos. 07-1238-5, 07-3616-2, 07-3617-2). The ruler is 12 in. long.

chromatid could have been used as the repair template because *a1-m5216* was hemizygous (crosses 6–7). Similarly increased rates of germinal deletions were observed in both strategies from RAD51<sup>-</sup> plants (Figure 4). We, therefore, conclude that sister chromatids are at least the major template for the repair of *MuDR*-induced DSBs via RAD51-directed HR.

On the other hand, our results do not demonstrate that the homologous chromosome is never used as a template for the repair of *MuDR*-induced DSBs. Indeed, the fact that the presence of an active *Mutator* increases rates of intragenic crossovers in a heterozygote involving

a *Mu*-insertion allele, suggest that the homologous chromosome can at least sometimes be used as a repair template during meiosis (YANDEAU-NELSON *et al.* 2005). Our finding that germinal revertants are recovered only rarely from RAD51<sup>+</sup> plants that are heterozygous for *Mu*-insertion alleles (*e.g.*,  $2.60 \times 10^{-5}$  for *a1-m5216*, Table 1) indicates that either the homologous chromosome is not often used as the repair template (perhaps because these events arise in premeiotic cells) or that the transposon insertions in the *a1* allele (crosses 1–2 and similar to strategy I) inhibit recombination between homologous chromosomes.

TABLE 3

Summary of germinal adjacent deletions derived from *Mu*-insertion alleles

Locus	Allele	<i>Mu</i> insertion	No. adjacent deletions	Genic deletion (bp)	Rate	Source
<i>adh1</i>	<i>Adh1-S3034</i>	<i>Mu1</i>	1	74	ND <sup>a</sup>	TAYLOR and WALBOT (1985)
<i>bz2</i>	<i>bz2::mu1</i>	<i>Mu1</i>	3	~75–77 <sup>b</sup>	~2.5% (3/118)	LEVY and WALBOT (1991)
<i>dmc1a</i>	<i>dmc1a-93F11</i>	<i>Mu1</i>	0	0	0/1,250	S. LIU and P. S. SCHNABLE (unpublished data)
<i>hcf106</i>	<i>hcf106-mum1</i>	<i>Mu1</i>	1	244	~0.3% (1/350) <sup>c</sup>	DAS and MARTIENSSSEN (1995)
<i>pdcl</i>	<i>pdcl-mu4365</i>	<i>dMuDR</i>	1	202	~0.2% (1/550)	Y. FU and P. S. SCHNABLE (unpublished data)
<i>rad51A1</i>	<i>rad51A1-54F11</i>	<i>dMuDR</i>	1	363	ND	LI <i>et al.</i> (2007)
<i>rad51A2</i>	<i>rad51A2-98E7</i>	<i>dMuDR</i>	4	~69–179	~0.8% (4/500)	
<i>rf2a</i>	<i>rf2a-m8122<sup>d</sup></i>	<i>Mu1</i>	0	NA <sup>e</sup>	0/1,200	D. SKIBBE and P. S. SCHNABLE (unpublished data)
<i>rf2c</i>	<i>rf2c-m1307</i>	<i>Mu1</i>	0	NA	0/1,500	
<i>rf2d</i>	<i>rf2d-m1310</i>	<i>Mu8</i>	0	NA	0/1,500	
<i>rth1</i>	<i>rth1-2</i>	<i>Mu7</i>	1	383	~0.02% (1/5,400)	WEN <i>et al.</i> (2005)

<sup>a</sup> No data.

<sup>b</sup> Includes a partial deletion of *Mu1* (16–22 bp). All other adjacent deletion alleles listed in this table have intact *Mu* transposons.

<sup>c</sup> Two recovered *Mu*-insertion alleles are not included.

<sup>d</sup> This allele was described by CUI *et al.* (2003).

<sup>e</sup> Not applicable.

**The two termini of *Mu* transposons are not cut and/or repaired simultaneously:** For *Mu* transposons to excise, DSBs must be introduced adjacent to both termini. Over 60% (82/136) of the germinal deletions recovered in this study have lost only one terminus of the *MuDR* transposon (class II, Figure 4). Similar observations have been observed in somatic events (RAIZADA *et al.* 2001). Because these “one-sided” deletions would not be expected in RAD51<sup>-</sup> plants (where RAD51-directed HR is blocked) if both *MuDR* termini are cut or repaired simultaneously, we hypothesize that the two termini of *Mu* transposons are not cut and/or repaired simultaneously during *Mu* transposition. Consistent with this hypothesis, class I events (Figure 3, B and C, and Figure 4) show polarity of deletion sizes from the two sides.

**Germinal deletions of *Mu*-insertion alleles can be recovered at a higher rate in RAD51<sup>-</sup> plants:** In RAD51<sup>+</sup> germinal cells, a DSB on one side of a *Mu* transposon has at least three possible fates. First, following the introduction of a second DSB, the *Mu* transposon could excise and the empty site will be mainly repaired via HR or rarely via NHEJ. Second, it is possible that the first DSB could be repaired directly via HR without the introduction of a second DSB. Third, the one-sided DSB could be repaired directly by the error-prone NHEJ. If this repair occurs after nucleotide resection, a partial deletion of the host gene could result. Germinal adjacent deletions are recovered only at low rates from RAD51<sup>+</sup> plants, and it has not been possible to identify adjacent deletions of some loci even after extensive screening (Table 3). In contrast, germinal deletions of the host gene are readily recovered from RAD51<sup>-</sup> plants (classes I and II, Figure 4). These results suggest that RAD51 is required to repair *MuDR*-induced DSBs and prevent deletion of flanking DNA.

*Mu* transposons are widely used for reverse genetics (BENSEN *et al.* 1995; LUNDE *et al.* 2003; MAY *et al.* 2003; SETTLES *et al.* 2004; MCCARTY *et al.* 2005). Even so, *Mu*-insertions in promoters, introns, and 3' ends may not disrupt gene function. Coupled with the preference of *Mu* transposons to insert in the 5' ends of at least some genes (DIETRICH *et al.* 2002; MAY *et al.* 2003), many *Mu*-insertion alleles isolated via reverse genetics are at least partially functional and not ideal substrates for functional genomic studies. One approach for dealing with this challenge is to isolate derivative alleles that have lost genic sequences adjacent to the *Mu* insertion (Table 3). The high rate at which partial deletions of the *a1* gene were recovered in this study suggests that RAD51<sup>-</sup> stocks may offer an attractive means to generate knock-out mutants from *Mu*-insertion alleles.

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