Functional characterization of the *rad51* genes in *Zea mays* and their roles in *Mu* transposition

by

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CHAPTER 1. GENERAL INTRODUCTION

Introduction

RecA in *E. coli*

The *recA* gene of *Escherichia coli* (*E. coli*) was discovered by Clark and Margulies. Mutants of *recA* are sensitive to ultraviolet irradiation and deficient in carrying out recombination (Clark and Margulies, 1965). The *recA* gene was subsequently cloned (McEntee, 1976) and purified RecA protein was shown to mediate strand exchange *in vitro* (Shibata *et al.*, 1979; Weinstock *et al.*, 1979; West *et al.*, 1980). RecA protein is a DNA-dependent ATPase (Roca and Cox, 1990) and can bind both single-stranded DNA (ssDNA) and double-stranded DNA (dsDNA) (Bell, 2005).

Homologs of *recA* in *Saccharomyces cerevisiae*

There are four *recA*-like genes (*RAD51*, *RAD55*, *RAD57* and *DMC1*) in yeast (*Saccharomyces cerevisiae*). *RAD51*, a yeast ortholog of *E. coli recA,* was cloned via sequencing the plasmid clone resulting from methyl-methanesulfonate (MMS)-resistant transformants that could complement a *rad51-1* MMS sensitive mutant (Shinohara *et al.*, 1992). Rad51p polymerize on dsDNA to form a helical filament nearly identical in three-dimensional structure to that formed by RecA (Ogawa *et al.*, 1993). Purified Rad51p can mediate DNA strand exchange in an ATP-dependent manner (Sung, 1994 and Figure 1). Rad52p (Hiom, 1999), another DNA binding protein, can interact with Rad51p and greatly stimulates strand exchange mediated by Rad51p *in vitro* (Benson *et al.*, 1998; New *et al.*, 1998; Shinohara and Ogawa, 1998). *DMC1* is a meiosis-specific yeast ortholog of *E. coli recA* (Bishop *et al.*, 1992), and Dmc1p and Rad51p have overlapping functions in mediating

meiotic interhomolog recombination (Shinohara *et al.*, 1997). Two paralogs of Rad51p, Rad55p and Rad57p, form protein complex with Rad51p to function in the repair of double-strand breaks (DSBs) (Hays *et al.*, 1995; Johnson and Symington, 1995; Krogh and Symington, 2004).

Homologs of *recA* genes in vertebrate

There are seven *recA*-like genes (*RAD51*, *RAD51B*, *RAD51C*, *RAD51D*, *XRCC2*, *XRRC3* and *DMC1*) in vertebrate genome (Kawabata *et al.*, 2005). Human and mouse *RAD51* was cloned and showed 83% homology with yeast Rad51p at the amino acid level (Morita *et al.*, 1993; Shinohara *et al.*, 1993) and mouse *RAD51* complemented a yeast *rad51* mutation with sensitivity to MMS, which generates DSBs of DNA (Morita *et al.*, 1993). Human *DMC1* gene is a meiosis-specific *recA*-like gene (Sato *et al.*, 1995). Human *RAD51B* (Albala *et al.*, 1997), *RAD51C* (Dosanjh *et al.*, 1998) and *RAD51D* (Pittman *et al.*, 1998) were cloned based on their sequence homology with human *RAD51*. Human *XRCC2* and *XRCC3* were cloned by their functional complementation in Chinese hamster cell lines irs1 and irs1SF which are sensitive to DNA crossing-linking agents (Cartwright *et al.*, 1998; Liu *et al.*, 1998). Disruption of *RAD51* leads to lethality in embryonic mice (Tsuzuki *et al.*, 1996) and *RAD51*-deficient vertebrate cells accumulate chromosomal breaks prior to cell death (Sonoda *et al.*, 1998). On the other hand, although *RAD51B* (Shu *et al.*, 1999), *RAD51D* (Pittman and Schimenti, 2000) and *XRCC2* (Deans *et al.*, 2000) are required for embryogenesis in mice, none of these five paralogs (*RAD51B*, *RAD51C*, *RAD51D*, *XRCC2* and *XRRC3*) are essential for cell viability (Takata et al., 2001). *In vivo*, five Rad51 paralogs are associated in two protein complexes, one containing Rad51B, Rad51C, Rad51D and Xrcc2, the other containing Rad51C and Xrcc3 (Masson *et al.*, 2001; Liu *et al.*, 2002). Both

human Rad51 and Dmc1 have the ability to catalyze DNA strand exchange *in vitro* (Sigurdsson *et al.*, 2001; Sehorn *et al.*, 2004).

Homologs of *recA* in *Arabidopsis*

Homologs of seven vertebrate *recA*-like genes are all identified in *Arabidopsis* genome (Doutriaux *et al.*, 1998; Bleuyard *et al.*, 2005). Mutant analysis shows that both *AtRAD51* (Li *et al.*, 2004) and *AtDMC1* (Couteau *et al.*, 1999) are required for meiosis. *AtRAD51B* (Osakabe *et al.*, 2005) is important for DSBs repair in mitosis, *AtXRCC3* is essential for meiosis (Bleuyard and White, 2004), and *AtRAD51C* is important for DSBs repair in both mitosis and meiosis (Abe *et al.*, 2005; Li *et al.*, 2005). Among five *AtRAD51* paralogs (*AtRAD51B*, *AtRAD51C*, *AtRAD51D*, *AtXRCC2* and *AtXRCC3*), only AtRad51C/AtXrcc3 protein complex is required to achieve meiosis (Bleuyard *et al.*, 2005).

Homologs of *recA* in maize

Maize (*Zea mays* L.) is one of the most important crops in the world, but less is known about *recA*-like genes in maize as compared to in yeast, vertebrate and *Arabidopsis*. Both *rad51* and *dmc1* have been cloned from maize (Franklin *et al.*, 1999). Maize also contains a *rad51c* homolog (GenBank Accession nos. BT017904 and BD270520). Based on three-dimensional microscopy analysis, it has been suggested that RAD51 plays an important role in homology search phase of meiotic chromosome pairing (Franklin *et al.*, 1999; Pawlowski *et al.*, 2003).

Two competing models for *Mu* transposons in maize

Cloning of the two maize *rad51* genes provides us a means to study the relationship between RAD51-directed homologous recombination (HR) and *Mu* transposition. *Mutator* transposon family of maize, first identified by its high forward mutation rate (Robertson,

1978), consists of an autonomous (*MuDR*) and several non-autonomous elements, all of which share approximately 200-bp conserved terminal inverted repeats (TIRs) (Bennetzen, 1996; Chandler and Hardeman, 1992; Lisch, 2002; Walbot and Rudenko, 2002). In germinal cells, *Mu* transposition frequencies average more than once per element per plant generation (Alleman and Freeling, 1986). However, germinal excision events from *Mu* insertion alleles are recovered only rarely (around 10^{-4}) (Brown *et al.*, 1989; Levy *et al.*, 1989; Schnable *et al.*, 1989). In contrast, somatic excision events occur at high rates (Walbot and Rudenko, 2002). Two major models have been proposed to reconcile different behaviors of *Mu* transposons in germinal and late somatic cells (Chandler and Hardeman, 1992; Lisch, 2002; Walbot and Rudenko, 2002).

In the Model A, "cut-and-paste" is the only transposition mechanism for *Mu* transposons. In germinal cells *Mu*-induced DSBs are repaired via HR using sister chromatid or homologous chromosome as templates, whereas in late somatic cells *Mu*-DSBs are mainly repaired via non-homologous end-joining (NHEJ) (Donlin *et al.*, 1995; Hsia and Schnable, 1996; Lisch, 2002; Walbot and Rudenko, 2002). The recovery of internal deletion derivatives of *MuDR* (Hsia and Schnable 1996) and abortive transposition events (Das and Martienssen, 1995; Levy and Walbot, 1991; Taylor and Walbot, 1985) suggest that gap repair pathway may be involved in the repair of *Mu*-induced DSBs in germinal cells. In the Model B, *Mu* transposons utilize "cut-and-paste" mechanism in late somatic cells (Raizada *et al.* 2001), but replicative transposition in germinal cells (Craig, 1995; Lisch, 2002; Walbot and Rudenko 2002). The bacterial transposon Tn7 is competent to make a switch between "cut-and-paste" and replicative transposition (May and Craig, 1996). Maize *mudrA* produces multiple transcripts that can encode multiple transposases and these naturally multiple

transposases may be able to make a similar switch between "cut-and-paste" and replicative transposition (May and Craig, 1996; Walbot and Rudenko 2002).

Dissertation Organization

This dissertation consists of two journal papers (chapters 2 to 3), a chapter of general conclusion (chapter 4) and an appendix. The manuscript in chapter 2 is to be submitted to Genetics. Jin Li isolated adjacent deletion alleles of *rad51a* and *rad51b*, characterized *rad51* double mutants molecularly and cytologically, and wrote this manuscript under the guidance of Dr. Schnable. Dr. Lisa C. Harper and Dr. W. Zacheus Cande provided FISH and RAD51 immunostaining data. Dr. David Weber assisted with cytological analyses of *rad51* double mutants. Drs. Robert B. Meeley, John McElver and Ben Bowen were involved in the initial screen of TUSC alleles of *rad51a* and *rad51b*. The manuscript in chapter 3 is to be submitted to Genetics. Dr. Tsui-Jung Wen summarized data related to germinal revertants of *a1-m5216* and prepared Figure 1 for this manuscript. Jin Li did the rest of other experiments and wrote this manuscript under the guidance of Dr. Schnable. General Conclusions section of this dissertation summarizes the overall results and findings related to specific questions addressed in the General Introduction. In the appendix, Jin Li isolated two *rad50* mutant alleles, analyzed all data and summarized the significance of this study.

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Figure 1. Yeast Rad51p catalyzes DNA pairing and strand exchange *in vitro*

Purified yeast (*Saccharomyces cerevisiae*) Rad51p can mediate the strand exchange between circular ssDNA and homologous linear dsDNA *in vitro*. In the end, the nicked circular DNA and displaced ssDNA are formed. This diagram is adapted from Sung 1994, Science.

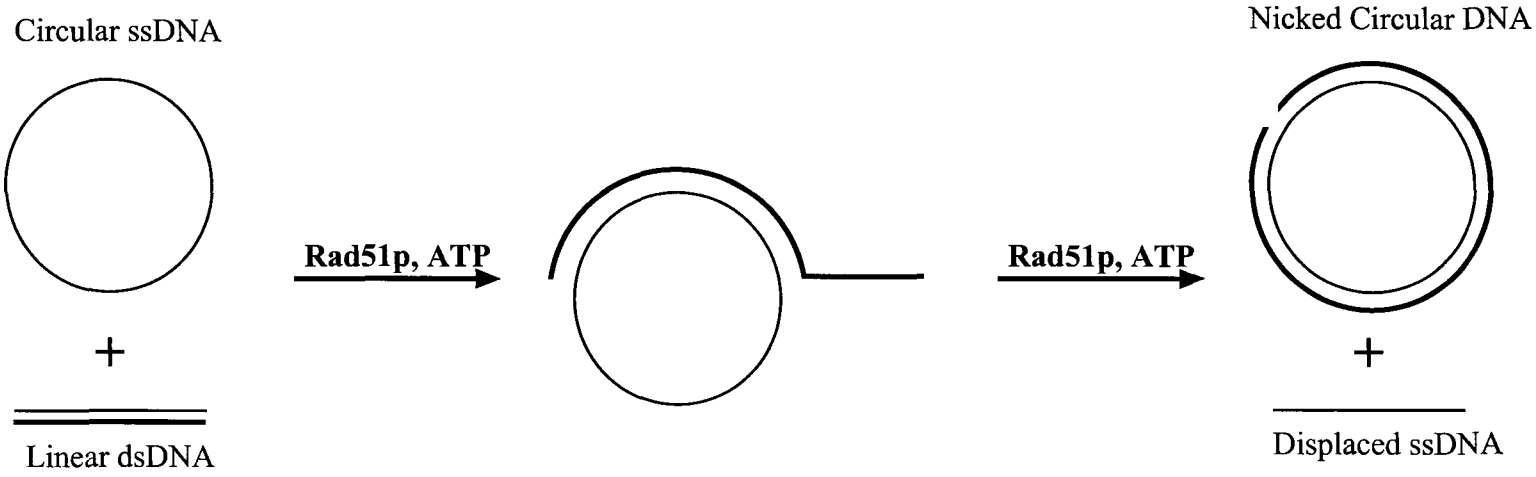
Circular ssDNA
+
Linear dsDNA
Rad51p, ATP
Rad51p, ATP
Nicked Circular DNA
+
Displaced ssDNA

Figure 1

CHAPTER 2. MAIZE RAD51 IS REQUIRED FOR EFFICIENT CHROMOSOME PAIRING AND PROPER CHROMOSOME SEGREGATION IN MEIOSIS AND THE REPAIR OF RADIATION-INDUCED MITOTIC DSBs

A paper to be submitted to *Genetics*

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RAD51 is required for meiosis

Keyword: RAD51, chromosome pairing, chromosome segregation, meiotic crossovers, double-stranded breaks

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ABSTRACT

In *Saccharomyces cerevisiae* (yeast), Rad51p plays a central role in homologous recombination and DNA repair. Double mutants of the two *Zea mays* L. (maize) *rad51* homologs are viable, but male sterile and have ~22% of normal seed set. Light microscopic analyses of male meiosis in these plants reveal: homologous chromosomes pair apparently normally at pachytene, many chromosomes are unpaired at diakinesis, and over 33% of quartets carry cells that either lack a nucleolus or have two nucleoli, indicating that non-disjunction occurs at both meiotic divisions. FISH analysis shows that 70% pachytene cells have paired 5S rDNA loci. Thus, maize RAD51 is required for efficient chromosome pairing and proper chromosome segregation in meiosis. FISH data also indicate that RAD51 is not essential for DNA homology search and chromosome pairing. Consistent with that, surviving female gametes produced by double mutants are euploid and exhibit near-normal rates of meiotic crossovers. These results differ from those of *Arabidopsis* in which a *rad51* mutant is completely male and female sterile, and exhibits completely disrupted chromosome pairing during meiosis. Although maize *rad51* double mutants develop well under normal condition, RAD51 function is critical for the repair of radiation-induced double-stranded breaks (DSBs) during early vegetative development.

INTRODUCTION

Members of the RecA-like protein family are required for homologous recombination and DNA repair (ROCA and COX 1990). Rad51p, a *recA* homolog of yeast, polymerizes *in vitro* on double-stranded DNA to form a helical filament that is nearly identical, at least at low resolution, to the three-dimensional structure formed by RecA (OGAWA *et al.* 1993). Rad51p can also bind single-stranded DNA and mediate DNA strand exchange *in vitro* (BENSON *et al.* 1998; NEW *et al.* 1998; SHINOHARA and OGAWA 1998; SUNG 1994). *In vivo* Rad51p complexes with Rad52p, Rad55p, Rad57p and Dmc1p (BISHOP 1994; HAYS *et al.* 1995). When cultured in sporulation medium, yeast *rad51* mutant diploid cells can produce 10-20% spores relative to wild type. Rarely formed asci contain dyads and triads; very few contain tetrads (SHINOHARA *et al.* 1992). Based on mutant analysis, it appears that Rad51p and Dmc1p have overlapping functions in meiotic crossovers. Only when both proteins are eliminated, do rates of meiotic crossovers decrease substantially as compared to wild type (GRISHCHUK and KOHLI 2003; SHINOHARA *et al.* 1997).

RAD51 homologs have been cloned from several plant species (DOUTRIAUX *et al.* 1998; FRANKLIN *et al.* 1999; TERASAWA *et al.* 1995). Maize has two closely related genes, *zmrad51a* and *zmrad51b,* that encode RecA-related proteins (In this report these two genes will be referred to *rad51a* and *rad51b* except in those instances where the *zm* prefix is required for clarity). The *zmrad51a* and *zmrad51b* are only distantly related to *AtRAD51* paralogs (*AtRAD51B*, *AtRAD51C*, *AtRAD51D*, *AtXRCC2* and *AtXRCC3*) (BLEUYARD *et al.* 2005). It has been suggested that in addition to their role in mediating DNA transfer,

ZmRAD51 may be involved in homology search during meiosis (FRANKLIN *et al.* 1999; PAWLOWSKI *et al.* 2003).

A reverse genetic approach was used to functionally characterize the two maize *rad51* genes. Analysis of the single *rad51a* and *rad51b* mutants and the *rad51a*, *rad51b* double mutant established that although *rad51a* and *rad51b* have at least partially redundant functions, RAD51 function is required for efficient chromosome pairing and proper chromosome segregation in meiosis. Although maize plants that lack RAD51 function survive and even flower, dry seeds of *rad51* double mutants are highly sensitive to irradiation treatment. In addition, *rad51* double mutants are male sterile and exhibit a greatly reduced production of viable female gametes. Surprisingly, the rate of meiotic crossovers in these surviving female gametes does not differ significantly from that of wild type.

MATERIALS AND METHODS

rad51a and *rad51b* Genomic Clones

The *rad51a* cDNA clone (pRAD51A, GenBank Accession no. AF079428) was digested with *Eco*R I and *Xho* I. The resulting insert was purified using a Qiaquick Gel Extraction Kit (Cat. No. 28704, Qiagen, Valencia, CA) and used as a hybridization probe to screen a genomic lambda B73 library prepared by J. Tossberg (Pioneer Hi-Bred International, Inc.). DNA from positive phage clones was isolated using a QIAGEN lambda Midi kit (Cat. No. 12543, Qiagen). Phage DNA was digested with restriction enzymes and subjected to electrophoresis and gel blot analysis using probes derived from the *rad51a* cDNA. These

analyses demonstrated that clones III-1, III-2 and III-3 were apparently identical and derived from *rad51a*; similar analyses also demonstrated that clones III-4, III-5 and III-6 were apparently identical and derived from *rad51b*. A 2.3 kb *Sst* I- and *Xba* I-digested DNA fragment of clone III-1 was ligated into the pBSK vector prepared by double-digestion with *Sst* I and *Spe* I. The resulting subclone, pRAGSS, was fully sequenced using primer walking and sequence data have been deposited with the GenBank data under the following accession no. AY359681.

DNA Gel Blot Analysis of *rad51* Copy Number in the Maize Genome

Genomic DNA was isolated from 2-week old B73 seedlings using a method based on that of DELLAPORTA *et al.*, 1983. The *rad51a* cDNA probe was PCR amplified from pRAD51A using the primer pair rad51a-244 + ra3utr; the *rad51b* cDNA probe was amplified from pRAD51B using the primer pair rb714 + rb14824r. Agarose gel electrophoresis and DNA gel blot hybridizations were performed as described (SAMBROOK *et al.*, 1989), except that the final wash was conducted using 10 mM Tris (pH 7.5) and 0.2% SDS.

Analysis of DNA Sequences

Sequence analyses were performed using the Wisconsin GCG software package Version 10.0-UNIX from the Genetics Computer Group, Inc (Madison, Wisconsin). Sequence contigs were assembled using Sequencher Version 4.1.2 from Gene Codes Corporation (Ann Arbor, MI).

Oligonucleotides

The approximate locations of the following oligonucleotides are shown in Figure 2.

11981: 5' TGC TTC CAG CAG AAG TCT AGA TTG 3'

13219: 5' CTT GAC ATC AGT GAC ACC CTC AGG TGA 3'

Mu-TIR: 5'AGA GAA GCC AAC GCC A(AT)C GCC TC(CT) ATT TCG TC 3'

ra3utr: 5' AAG TTA GTT TTC ACC ACC AGC C 3'

rad51a-244: 5' GGC AGA GGT GAG ACT TGA GA 3'

radel: 5' CAC TTG TAA ATC CCA GTG GAA CTA T 3'

raidp31: 5' TGC ATT AGA TTC CTC ATT TTC AG 3'

rb1482cr: 5' TTC AGG AGT AGA CGA GAT GTT AC 3'

rb3utr: 5' CTCTA CCACT TTACA ACAAT GCC 3'

rb714: 5' CAC GAG ATT TTT TGC CGC TTC G 3'

rbdel: 5' CCA TCC ACT TGA GCC ACT ACT T 3'

RB-98E7: 5' TGT ACT AGA GAA TGT GGC TTA TGC 3'

Note: At positions marked by parentheses, the base composition can be either of the two indicated bases.

Genetic Stocks and Genetic Crosses

The female parent of Cross 1 is a *Mu* stock derived from those of ROBERTSON (1978, 1980, 1981) via crosses to the hybrid B77 x B79 and Q66 x Q67. The inbred line B73 is fixed for functional alleles of *rad51a* (*Rad51a-B73*) and *rad51b* (*Rad51b-B73*), mutant alleles were

maintained via crosses to B73. A Material Transfer Agreement governs the distribution of the *Mu* insertion alleles of *rad51a* and *rad51b*; inquiries should be directed to Dr. Meeley.

Cross 1: *Mu Rad51b/Rad51b* x *rad51b-98E7/Rad51b*

Cross 2: *Mu rad51b-98E7/Rad51b* x B73

Characterization of *Mu* Transposons and Insertion Sites in *rad51* TUSC Alleles

Members of the *Mu* transposon family share approximately 200 bp conserved terminal repeats (TIRs). PCR was performed on each *Mu*-insertion allele using a series of gene-specific primers in combination with a primer (Mu-TIR) located in the highly conserved *Mu* TIR. The resulting PCR products were purified and sequenced. These PCR products contain *Mu* TIR sequences terminal to the Mu-TIR primer annealing site. Comparing the TIR sequences from each of the *Mu*-insertion alleles to the left and right TIRs of each of the previously defined classes of *Mu* transposons established which class of *Mu* transposon was responsible for each of the TUSC alleles. The resulting PCR products also contained *rad51* sequences between the positions of the *Mu* insertion site and the *rad51*-specific primer. Comparison of these PCR sequences to the *rad51* genomic sequences established the position of the *Mu* insertion in each of the TUSC alleles.

Screen for Deletion Derivatives of *rad51b-98E7*

Approximately 1,000 seeds were planted in a 96-well format and tissues from up to 4 seedlings were bulked for DNA extraction in the initial screen. Individual seedlings from a pool that contained a deletion candidate were transplanted to large pots for further growth.

Leaf samples from each plant were recollected and DNA was isolated. The PCR screen was repeated to identify the individual plant that carried a putative deletion derivative.

The PCR screen was performed using the Mu-TIR primer in conjunction with the *rad51b*-specific primer 13219. In the absence of a structural rearrangement at the *rad51b* locus, this primer pair will produce a 395-bp PCR product. In contrast, PCR amplification of DNA from a plant that carries a deletion of DNA between the annealing sites of primers 13219 and Mu-TIR would be expected to yield a smaller product.

The PCR products derived from deletion candidates were purified using the Qiaquick Gel Extraction Kit (Cat. No. 28704, Qiagen) and sequenced using the Mu-TIR primer. As a control, PCR products from *rad51b-98E7* were purified and sequenced at the same time.

Molecular Characterization of Derivative Alleles

Any of three possible types of sequence rearrangements can generate derivative alleles from *rad51b-98E7* that yield smaller PCR products when amplifying with a *rad51b*-specific primer 13219 in combination with a Mu-TIR primer: an intragenic *Mu* transposition, an insertion of an additional *Mu* transposon, or a deletion of *rad51b* sequences adjacent to the original *Mu* insertion. To differentiate an intragenic transposition from an adjacent deletion, a second PCR reaction was performed using the Mu-TIR primer in combination with a *rad51b*-specific primer RB-98E7 whose binding site is on the opposite side of the *Mu* insertion as compared to the primer 13219 used in the initial screen. If a derivative allele arose via an adjacent deletion, the PCR product from the other side should be the same size

between *rad51b-98E7* and the derivative allele. In contrast, if an allele arose via an intragenic transposition, the PCR product from the other side of the *Mu* insertion would be larger than the PCR product obtained from *rad51b-98E7*. To differentiate between the insertion of an additional *Mu* transposon and an adjacent deletion, a primer rbdel was designed to anneal to the region thought to have been deleted. If a derivative allele arose via an adjacent deletion, this primer pair would not be expected to yield a PCR product. In contrast, if a derivative allele arose via the insertion of a second *Mu* transposon, amplification with Mu-TIR and rbdel would be expected to yield a *rad51b*-specific PCR product.

Genotyping

The primer pair Mu-TIR and 11981 amplify *Mu* insertion alleles *rad51a-54F11* and *rad51a-54F11d1*. Because the wild-type *Rad51a* allele in our stock is from inbred line B73, one IDP marker (raidp31) can anneal to *Rad51a-B73* but not to the *rad51a-54F11* and *rad51a-54F11d1*, PCR amplification with the primer pair rad51a-244 and raidp31 will be positive for *Rad51a-B73/rad51a-54F11* and *Rad51a-B73/rad51a-54F11d1* plants and negative for *rad51a-54F11/rad51a-54F11* and *rad51a-54F11d1/rad51a-54F11d1* plants (Figure 2). In addition, the primer radel is located on the region deleted in *rad51a-54F11d1* relative to *rad51a-54F11*, PCR amplification with the primer pair rad51a-244 and radel will be positive for *Rad51a-B73/rad51a-54F11d1* plants and negative for *rad51a-54F11d1/rad51a-54F11d1* plants.

The primer pair Mu-TIR and RB-98E7 can amplify the *Mu* insertion allele *rad51b-98E7d4*. The primer rbdel is located on the region deleted in *rad51b-98E7d4* relative to *rad51b-98E7*.

Hence, PCR amplification with the primer pair RB-98E7 and rbdel will be positive for *Rad51b/rad51b-98E7d4* plants and negative for *rad51b-98E7d4/ rad51b-98E7d4* plants.

RNA Isolation and RT-PCR Analysis

RNA was isolated from meiotic tassels using Trizol Reagent (Cat. No. 15596026, Invitrogen Life Technologies, CA). First-stranded cDNA was synthesized with poly T primer using SuperScript II RNase H$^-$ Reverse Transcriptase (Cat. No. 18064-014, Invitrogen, CA).

Irradiation Treatment of Maize Dry Seeds

Irradiation was conducted at the Linear Accelerator Facility (LAF) at Iowa State University Meat Laboratory. Dry seeds from the self-pollination of plants with the genotype (*Rad51a/rad51a; rad51b/rad51b*) were irradiated by a Circe' IIIR Electron Beam (EB) irradiator (Linac Technologies, SA, Orsay, France) with an energy level of 10MeV and a dose rate of 29 kGy/min. Dry seeds were placed on a conveyor cart in a single layer. Two alanine dosimeters were placed on the top and bottom surfaces of these samples. The irradiation dose was applied by exposing samples to the electron beam using a single-sided pass. The true absorbed doses were measured by placing the alanine dosimeters into the e-scan Electron Paramagnetic Resonance instrument (Bruker BioSpin Corporation, EPR Division, USA). The actual dose that maize dry seeds absorbed was 0.37 kGy for this treatment. Treated seeds were immediately germinated 96-well flats in greenhouse. DNA was isolated from germinated seedlings and their genotypes were determined via PCR.

Preparation and Observation of Microsporocytes

Microsporocyte samples were fixed in a freshly-prepared 3:1 mixture of 95% ethanol and propionic acid (V:V) and then stored at –20°C. Anthers were smeared and stained with propiocarmine following the procedure of DEMPSEY, 1994.

Fixation and preparation of meiocytes for FISH and immunostaining

Anther where prepared as in (GOLUBOVSKAYA *et al.* 2002). Briefly, anthers from developing tassels were staged with the acetocarmine squash technique. Anthers from the same floret and from those in close proximity, and thus close in developmental stage, were fixed at room temperature in 4ml of 4% formaldehyde in Buffer A (15 mM Pipes -NaOH, pH 6.8, 80 mM KCl, 20 mM NaCl, 0.5 mM EGTA, 2mM EDTA, 0.15 mM spermine tetra HCL, 0.05 mM spermidine, 1 mM dithiothreitol, 0.32 M sorbitol (BASS *et al.* 1997) for 45 minutes in a gently shaking 10 ml Petri dish. They were then washed 3 times, 30 min each in fresh Buffer A and stored at 4°C in the buffer. Fixed anthers were cut open at the tip to release the meiocytes into 100-200 ul of Buffer A. 10 ul of meiocytes suspended in the Buffer A were then transferred by micropipette onto a glass cover slip (22x22 mm) followed by the immediate addition of 5 ul of activated acrylamide stock. The activation of acrylamide was done by addition of 5 ul of 20% ammonium persulfate and 5ul of 20% sodium sulfite to 100 ul of a 15% (29:1 acrylamide:bis acrylamide) gel stock in 1X buffer A. The slides were quickly stirred and a second cover slip was placed on top for 45 min, then removed with a razor blade, leaving a thin pad of acrylamide with embedded meiocytes on the slide.

Probes

A 27 bp oligonucleotide, 5'-CCTAAAGTAGTGGATTGGGCATGTTCG-3', labeled with either Cy5 or FITC, was obtained from Genset Inc., Paris and was used to detect the CentC sequence which resides near maize centromeres (ANANIEV *et al.* 1998). Oligonucleotides complementary to the telomere repeat (5'-{CCCTAAA}4-3') and labeled with either Cy5 or FITC (Genset Inc.) were used to detect maize telomeres (BASS *et al.* 1997). A 5S rDNA probe was made by PCR. Approximately 1 to 10 nanograms of a plasmid containing 5S rDNA sequence from maize (ZIMMER *et al.* 1988) was added to a standard PCR reaction mix: 2 ul 10x buffer with 15 mM MgCl2 from Perkin Elmer, 2 ul of forward and reverse primers, 2 ul 1mM dATP, dGTP, dCTP, a mixture of dTTP and dUTP-FITC or dUTP-Cy5, 2 units Amplitaq (Perkin-Elmer) and water to 20 ul. For each labeling reaction, a 20 ul unlabeled control reaction was performed, and an aliquot of equal molar volume was run side by side with the labeling reaction in a 4% gel. Incorporation of fluorescent label could sometimes be seen on the transilluminator, but empirical determination of probe effectiveness by FISH was required for each batch of probe made.

Fluorescence *In Situ* Hybridization (FISH) and indirect immunofluorescence

FISH was performed as in GOLUBOVSKAYA 2002. Briefly, newly polymerized acrylamide pads were washed with 1x PBS, followed by four washes with a prehybridization buffer (50% deionized formamide, and 2x SSC). Cover slips were placed on a slide, then 50 ul of probe in prehybridization buffer was added, then sealed under a second cover slip using rubber cement, and incubated at 37°C for 30-45 min. The slides were denaturated on a PCR block at 96°C for 6 min followed by overnight incubation at 30°C. The slides were then washed for 30 minutes sequentially with 1x PBS and 1x SSC (3 times), 1x PBS and 0.1%

Tween-20 (4 times), 1x PBS (3 times), 1x TBS (1 time). The slides were then stained with 10ug/ml DAPI (4',6-diamidino-2-phenylindole, dihydrochloride) in 1x TBS for 30 minutes at room temperature. Excess DAPI was removed by washing with 1x TBS (3 times) for a total of 30 minutes. Slides were then mounted in 1,4 Diazabicyclo-[2,2,2] octane (DABCO) and sealed with clear fingernail polish and stored at 20°C. The procedure for staining RAD51 foci using the anti-HsRAD51 rDNA antibody was described previously (FRANKLIN *et al.* 1999).

3-Dimensional deconvolution light microscopy and image generation

Images were acquired on a Delta Vision (Applied Precision Inc.) imaging station: an Olympus IX70 inverted microscope with 100X, 1.35 NA oil-immersion lens and a Photometric (Roper Scientific.Inc.) CCD. All images were taken with a Z step size of 0.2 microns, saved as 3-D stacks, and subjected to constrain iterative deconvolution. 3-D Data analysis and 2-D image creation was performed using the DeltaVision/Soft WoRx software package (Applied Precision Inc.) on a Silicon Graphics Workstation. 2-D images were converted to TIFF, and opened in Photoshop on a Macintosh computer. Photoshop was used to manipulate false colors and to covert colors from RGB to CMYK for printing.

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RESULTS

Structures of the *rad51a* and *rad51b* Genes

It has been reported that the maize genome contains two homologs of the yeast *rad51* gene (FRANKLIN *et al.* 1999): *rad51a* and *rad51b*. These two genes are orthologs of *AtRad51*, but only distantly related to *AtRad51B* or *AtRad51C* (Figure 1). Full-length cDNA sequences of each gene have previously been reported (GenBank Accession nos. AF079428 and AF079429). The structures of the *rad51a* and *rad51b* genes were constructed based on alignments of these apparently full-length cDNA sequences with assembled genome survey sequences (GSSs) supplemented by additional sequencing of a *rad51a* genomic clone (Figure 2, Materials and Methods). The splice sites of *rad51a* and *rad51b* are conserved except that exon II of *rad51b* is split into two exons (II and III) in *rad51a*.

Analysis of *rad51* copy number in the maize genome

The coding regions of *rad51a* and *rad51b* are 84% identical at nucleotide level and 90% identical at amino acid level. DNA gel blot analysis was conducted to determine the number of *rad51* genes in the maize genome. When the final wash was performed at 65°C, the *rad51a* (Figure 3, lanes 1 and 4) and *rad51b* (Figure 3, lanes 3 and 6) cDNA probes are gene-specific. Although the *rad51a* cDNA hybridized to three Bgl II fragments (Figure 3, lane 4), analysis of the *rad51a* genomic sequence (Figure 2) establishes that all of these fragments are derived from the *rad51a* gene. Under less stringent wash conditions (55°C) the *rad51a* cDNA probe detects all the fragments derived from the *rad51a* and *rad51b* genes (Figure 3, lanes 2 and 5), but no other fragments. Consistent with DNA gel blot results, the MAGI

(http://magi.plantgenomics.iastate.edu/) and EST databases do not contain another gene that is highly similar to *rad51a* or *rad51b* (cut-off value is 75% identity at nucleotide level). In summary, these results indicate that there are only two *rad51* genes in the maize genome.

Isolation of *Mu*-insertion alleles of *rad51a* and *rad51b*

Three *Mu*-insertion alleles of *rad51a* (*rad51a-42A8*, *rad51a-54F11* and *rad51a-family6*) and two of *rad51b* (*rad51b-63H3* and *rad51b-98E7*) were recovered via the reverse genetic screen, TUSC, Trait Utility System for Corn (BENSEN *et al.* 1995).

Members of the *Mu* transposon family share approximately 200-bp conserved terminal inverted repeats (TIRs). PCR was performed on plants that carry each *Mu*-insertion allele using a series of gene-specific primers in combination with a primer located in the highly conserved *Mu* TIR. Sequence analysis of the resulting PCR products established which class of *Mu* transposon was inserted in each of the TUSC alleles and the positions of the *Mu* insertions (Figure 2).

Out of the five TUSC alleles, only one, *rad51a-54F11*, contained a *Mu* insertion in a coding region. The remainder had insertions in introns or 5' of the apparent transcription start sites. Because such alleles might confer residual gene function and might also be subject to "*Mu* suppression" (BARKAN and MARTIENSSEN 1991; CUI *et al.* 2003; MARTIENSSEN and BARON 1994), they were not considered ideal reagents for the study of RAD51 functions.

Deletion derivatives of *rad51b-98E7*

Although germinal excision events are recovered only rarely from *Mu*-insertion alleles (BROWN *et al.* 1989; LEVY *et al.* 1989; SCHNABLE *et al.* 1989), approximately 1% of gametes carry deletions adjacent to a given *Mu* insertion (DAS and MARTIENSSEN 1995; LEVY and WALBOT 1991; TAYLOR and WALBOT 1985). A PCR-based screen modified from that described by DAS and MARTIENSSEN (1995) was used to identify rare derivatives of *rad51* TUSC alleles that contained deletions adjacent to the *Mu* insertions.

The *rad51b-98E7* allele was selected to screen for deletions because its *Mu* insertion is located near an intron-exon junction. Initially, *rad51b-98E7* was crossed into a *Mu* active line (Cross 1, Materials and Methods). Progeny of this cross that carried *rad51b-98E7* were crossed by the inbred line B73 (Cross 2); approximately 1,000 of the resulting progeny were germinated and analyzed via PCR using the Mu-TIR primer in conjunction with the gene-specific primer 13219. Seedlings that yielded smaller PCR products than those obtained from *rad51b-98E7* were grown to maturity and backcrossed to B73. The progeny from these crosses were genotyped via PCR, and four heritable derivative alleles were recovered (Figures 2, 4 and 5).

Three types of sequence rearrangements could generate derivative alleles that yield smaller PCR products in the screen described above: intragenic transposition of *Mu*, the insertion of an additional *Mu* in *rad51b*, or a deletion of *rad51b* sequences adjacent to the *Mu* insertion. Experiments conducted as described in the Materials and Methods indicated that all four of the heritable derivative alleles arose via adjacent deletions (data not shown). The structures of these alleles are illustrated in Figure 5. Sequence analysis of the PCR products associated

with these alleles established that the sizes of the deletions range from 69 to 179 bp (Figure 5). In the case of deletion derivative *rad51b-98E7d4*, all of exon VII has been deleted. Because only half of the progeny from Cross 2 would be expected to carry *rad51b-98E7*, the rate at which adjacent deletions were recovered from *rad51b-98E7* was ~0.8% (4/~500).

A deletion derivative of *rad51a-54F11*

While genotyping a family segregating for *rad51a-54F11* using primer pair Mu-TIR and 11981, one plant (00-2608-1) with a smaller-size PCR product was identified. PCR-based experiments similar to those described in the Materials and Methods section established that this allele (designated *rad51a-54F11d1*) is a deletion derivative of *rad51a-54F11* (Figures 2 and 5). Sequence analysis of the PCR product established that the 363 bp (1447~1809 in GenBank Accession no. AY359681) that is deleted in *rad51a-54F11d1* includes part of exon II, all of intron II and exon III, and part of intron III.

RT-PCR analysis of *rad51* double mutants

RT-PCR experiments were conducted to test whether the *rad51a* and *rad51b* deletion derivative alleles are null mutants. Consistent with observations of FRANKLIN *et al.* (1999) *rad51a* and *rad51b* transcripts of the expected sizes were detected during meiotic prophase in maize tassels that carry *Rad51a* and *Rad51b,* respectively (Figure 6). In contrast, *rad51a* transcripts were not detected in plants homozygous for *rad51a-54F11d1.* Hence, *rad51a-54F11d1* is an apparently null mutant allele.

Although ethidium bromide staining of the RT-PCR products did not reveal any *rad51b* transcripts in plants homozygous for *rad51b-98E7d4*, RT-PCR products of two transcripts could be detected via hybridization with a *rad51b* cDNA probe (data not shown). Sequence analysis demonstrated that one of these transcripts is missing all of exon VII as expected based on the structure of *rad51b-98E7d4* (Figures 2 and 5). The deletion of exon VII causes a frame shift in this transcript. The second transcript is also missing exon VII but includes 142 bp from the TIR of the *MuDR* transposon. In this transcript, a stop codon has been introduced before exon VIII. Hence, in both transcripts approximately eighty amino acids of the normal RAD51B protein, belonging to the ATP binding site (NARA *et al.* 2000; SHINOHARA *et al.* 1992), are lost due to deletions, frame shift or a premature stop codon. Hence, it is very unlikely that *rad51b-98E7d4* encodes a functional protein.

RAD51 foci in *rad51* double mutants

To further test whether *rad51a-54F11d1* and *rad51b-98E7d4* are null alleles, RAD51 were immunostained for zygotene cells from both B73 and *rad51* double mutant (Figure 7). As expected (FRANKLIN *et al.* 1999), hundreds of RAD51 foci were observed in the B73 cell. In contrast very few, if any, RAD51 foci were observed in the double mutant cell. This dramatic difference, together with RT-PCR results (Figure 6), demonstrates that the *rad51a-54F11d1* and *rad51b-98E7d4* alleles do not encode functional RAD51.

RAD51 is required for the repair of radiation-induced DSBs during vegetative development

To address the role of RAD51 in DNA repair during early vegetative development, around 200 dry seeds among which *rad51* double mutants segregate were irradiated with a dose of 0.37 kGy. Subsequent genotyping results established that *rad51* double mutants were highly sensitive to the irradiation treatment. Indeed, all double mutants (N=31) died within two weeks of germination. In contrast, control plants germinated and survived to develop into apparently healthy seedlings (Table 2). This significantly different survival rates between *rad51* double mutants and controls indicate that RAD51 function is critical to repair radiation-induced DSBs in maize.

Effects of *rad51* double mutants on gamete production

Plants homozygous for *rad51a-54F11, rad51a-54F11d1* or *rad51b-98E7d4* (i.e., single mutants) develop apparently normally, are fully male fertile and have normal seed sets. To determine whether the double mutant exhibits a phenotype, the progeny resulting from the self-pollinations of plants heterozygous for *rad51a-54F11* or *rad51a-54F11d1* and homozygous for *rad51b-98E7d4* were genotyped and scored for male- and female-fertility. Among the progeny of these crosses, all double mutant plants were male sterile and exhibited dramatic reductions in female seed sets (Table 1 and Figure 8). In one experiment, fourteen *rad51* double mutants produced 800 kernels, while six wild-type siblings produced 1,555 kernels when crossed as females (i.e., served as pollen recipients). Hence, in this experiment, seed set of *rad51* double mutants was ~22% ((800/14)/(1555/6) X 100%=22%) of wild type. In all other respects double mutant plants developed apparently normally.

Meiosis in *rad51* double mutants

Meiosis was analyzed in plants that were homozygous for either of the two single mutations (i.e., *rad51a/rad51a; Rad51b/Rad51b* or *Rad51a/Rad51a; rad51b/rad51b*). Diakinesis cells in both genotypes regularly possessed 10 bivalents and quartets of both genotypes had one nucleolus in each microspore. The meiotic sequence appeared to be normal in both of these single mutant genotypes.

Meiosis was also analyzed in microsporocytes of eight plants that were homozygous for both mutations (i.e., *rad51a/rad51a; rad51b/rad51b*). Similar results were obtained using either *rad51a-54F11d1* or *rad51a-54F11*. In both instances, pachytene cells from all double mutant plants appeared to be normal with apparently paired homologous chromosomes (Figure 9A).

At diakinesis in the double mutants, very few paired chromosomes were observed in any cells (Figure 9E-F) and the homologs of "bivalents" that were usually observed appeared to be more weakly associated with each other than in cells from normal plants. In normal cells, the synaptonemal complex, which holds the homologs together during pachytene, breaks down at the beginning of diplotene, and the homologs are held together by chiasmata from diplotene until they disjoin at metaphase I (BASCOM-SLACK *et al.* 1997). The observation that at diakinesis many chromosomes in cells from double mutants are present as univalents indicates that the frequency of chiasmata is low in these plants. Also, lagging univalents were frequently observed in anaphase I cells (Figure 9C).

Quartets of microspores were also examined in eight *rad51* double mutant plants. Many of the quartets contained one or two microspores that lacked an organized nucleolus but contained numerous micronucleoli (smaller bodies that resembled nucleoli, Figure 9H-L). Also, some of the microspores in these abnormal quartets contained two nucleoli (Figure 9J-L). The frequencies of quartet types observed in four of these double mutant plants are provided in Table 3. In normal maize plants, one nucleolus is present in each of the four microspores of each quartet because each microspore contains one and only one copy of chromosome 6, which carries the only nucleolar organizing region (NOR) (McCLINTOCK 1934). McCLINTOCK (1934) found that cells with no NOR lack an organized nucleolus but instead contain multiple micronucleoli. She also found that cells with 2 NORs sometimes have two nucleoli, but frequently have only a single nucleolus due to nucleolar fusion. Because cells with two nucleoli possess two copies of chromosome 6, the abnormal quartet types observed in the *rad51* double mutant in this study could only be produced if chromosome 6 underwent non-disjunction at one of the meiotic divisions. For example, Quartet type VIII (Table 3) was almost certainly produced via disjunction of chromosome 6 during the first meiotic division. Similarly, quartet type III and IV (Table 3) could only be produced via disjunction of chromosome 6 during the second meiotic division. Hence, *rad51* function is required for the normal disjunction of chromosome 6 (and presumably the other chromosomes) during both meiotic divisions.

Homologous synapsis of 5S rDNA locus (2L) decreases in *rad51* double mutant

To study the extent of homologous pairing in *rad51* double mutants, FISH was performed using a probe that hybridizes to the 5S rDNA loci during prophase I. The 5S rDNA is located in the distal end of the long arm of chromosome 2 (bin 2.08). In wild-type plants, 50% of the 5S rDNA loci are paired in zygotene and 100% are paired in pachytene (GOLUBOVSKAYA *et al.* 2002). In contrast, in *rad51* double mutants, only 70% of examined cells (N=26) have paired 5S rDNA loci at pachytene. These results indicate that homologous pairing is reduced in the absence of RAD51. This correlates well with the reduced chiasmata seen in double mutants.

Meiotic crossovers in *rad51* double mutants

Our finding that frequency of chiasmata at diakinesis was low in male gametes of *rad51* double mutants (Figure 9E, 9F) is consistent with the fact that reduced chromosome pairing was observed at pachytene (Figure 10) and only about 62% quartets were normal due to aberrant segregation of chromosome 6 (Table 3). The rates of meiotic crossovers between two genetic markers (*a1* and *et1*) were assayed in double mutants and *rad51b* single mutant controls. In this 10 cM interval (DA COSTA E SILVA *et al.* 2004; GOODMAN *et al.* 1980) no significant differences in the rates of crossovers were detected between *rad51* double mutants and controls (Table 4). The rates of crossovers were also measured at another genetic interval (i.e., between IDP1440 and IDP1983) using an independent population. Similarly, no significant differences in the rates were detected between *rad51* double mutants and wild type controls (Table 5). These results indicate that RAD51 is not essential for meiotic crossovers in female meiosis.

DISCUSSION

Consistent with the hypothesis that maize is a segmental allotetraploid (GAUT *et al.* 2000), the maize genome contains two *rad51* homologs: *rad51a* and *rad51b* (FRANKLIN *et al.* 1999; Figure 3). We have isolated apparently null or severely disrupted alleles of both genes. Plants homozygous for single mutant of either gene develop normally, are fully male fertile and do not display reduced seed set. Cytological observations do not reveal any differences between these single mutants and the inbred line B73, which carries functional alleles of both genes. In contrast, plants that are homozygous for mutant alleles at both loci are completely male sterile (Figure 8A), exhibit dramatically reduced seed set (Figure 8B), and abnormal male meiosis (Figure 9). Hence, *rad51a* and *rad51b* appear to be functionally redundant in maize.

Maize *rad51* double mutants are vegetatively normal, flower (Figure 8A) and can even produce some kernels when crossed as females (Figure 8B). As such, this study extends that of LI *et al.* (2004) which reported that unlike animal *rad51* mutants which are inviable, an *Arabidopsis rad51* mutant is viable. Together these studies demonstrate a significant difference in the roles of RAD51 between plants and animals. The RAD51-dependent homologous recombination repair is the major pathway by which double-stranded breaks (DSBs) are repaired in animals, accounting for 30~50% of repair events (LIANG *et al.* 1998). Consequently, *rad51* disruptions in animal cells lead to lethal embryo and cell death (SONODA *et al.* 1998; TSUZUKI *et al.* 1996) due to the accumulation of unrepaired chromosome breaks (SONODA *et al.* 1998). In contrast, the viability of our *rad51* double

maize mutants (and the *Arabidopsis rad51* mutant of LI *et al.*, (2004)) demonstrate that the RAD51-dependent homologous recombination repair plays at best only a minor role in mitotic cell divisions and DNA repair under normal condition in plants. This is consistent with observations that in somatic cells of higher plants broken chromosomal ends are frequently repaired via the ligation of DNA ends to unrelated sequences or to the sequences with only micro-homologies (RAY and LANGER 2002).

Although *rad51* double mutants develop well under normal condition, RAD51 function is critical for the repair of radiation-induced DSBs (Table 2). Consistent with our data, maize *rad51a* and *rad51b* express in all examined tissues including seedling leaves and roots (FRANKLIN *et al.* 1999). *RAD51* null mutants in yeast are sensitive to methyl-methanesulonate (MMS), which can generate DSBs (SHINOHARA *et al.* 1992). In vertebrates, disruption of *RAD51* leads to lethality in embryonic mice (TSUZUKI *et al.* 1996) and *RAD51*-deficient cells accumulate chromosomal breaks prior to cell death (SONODA *et al.* 1998). Our data demonstrate that maize RAD51 possesses a conserved role in the repair of DSBs during vegetative development as compared to yeast and vertebrates. However, in vertebrates, RAD51 function is more crucial than in plants during vegetative development.

Our data demonstrate that in maize RAD51 function is required for proper chromosome segregation during male meiosis. This conclusion is based on several lines of cytological evidence derived from *rad51* double mutants. First, more than 33% of quartets (Table 3) in *rad51* double mutants are abnormal, likely due to non-disjunction during meiosis I. Second, in these double mutants many univalents are observed during diakinesis (Figure 9E-F) and

metaphase I (Figure 9B) in male meiosis. Third, occasionally one or two lagging chromosomes can be found at anaphase I (Figure 9C). Fourth, the abnormal Quartet Type III and IV (Table 3) observed in the *rad51* double mutants could only be produced via non-disjunction of chromosome 6 during the second meiotic division. Fifth, in metaphase I and anaphase I, broken chromosomes are observed, suggesting that some meiotic-induced DSBs are not repaired. The role of maize RAD51 in chromosome segregation is consistent with previous reports that the RecA protein of *E. coli* (CAMPBELL and DAVIS 1999), the fission yeast Rhp51p (JANG *et al.* 1995), *Arabidopsis AtRad51* (LI *et al.* 2004) and *AtDmc1* (COUTEAU *et al.* 1999) are required for proper chromosome segregation. Our finding that number of chiasmata decreases at diakinesis in the maize *rad51* double mutant suggests that RAD51's role in meiotic recombination may be required for formation or maintenance of chiasmata.

In maize the loss of RAD51 function has a dramatic effect on gamete viability. The *rad51* double mutants are completely male sterile (Figure 8A) and female seed sets are about 22% of wild type (Figure 8B). This result is consistent with previous reports that yeast Rad51p is required for spore viability (MORRISON and HASTINGS 1979; SHINOHARA *et al.* 1992). If it is assumed that chromosome 6 is a good model of the behavior of the other 9 chromosomes and both female and male gametes exhibit similar rates of aberrant chromosome segregation in *rad51* mutants, the expected rate of euploid female gametes can be calculated as followed. Based on the data from 01-2992-5 (Table 3), the rate of euploid female gametes from this plant would be expected to be $(60/89 + 15/89 \times 3/4 + 2/82 \times 2/4 + 3/89 \times 1/4 + 8/89 \times 2/4)^{10}$ = 23%. Based on the data from 01-2996-8 (Table 2), the rate of euploid female gametes

from this plant would be expected to be (50/92 + 21/92 x 3/4 + 3/92 x 2/4 + 2/92 x 2/4 + 3/92 x 1/4 + 10/92 x 2/4 + 1/92 x 2/4)^{10} = 12%. This calculated expected rate of euploid gametes is similar to the observed rate of seed set from *rad51* double mutants (i.e., ~22% of wild type), thus the lethality of female gametes in *rad51* double mutants is likely largely the result of disruption of RAD51-dependent chromosome segregation.

LI *et al.*, 2004 have described an *Arabidopsis rad51* mutant that is both male and female sterile. Although maize *rad51* double mutants are also fully male sterile, they exhibit partial female fertility. There are at least two explanations for the reported differences in the phenotypes associated with the maize and *Arabidopsis rad51* mutants. First, the importance of RAD51 for female fertility may differ between maize and *Arabidopsis*. Second, only 10 crosses were analyzed in *Arabidopsis* and further crossing might reveal small amount of female fertility.

Based on three-dimensional microscopy, it has been suggested that in maize RAD51 plays a role in DNA homology search and chromosome pairing (FRANKLIN *et al.* 1999; PAWLOWSKI *et al.* 2003). Consistent with this hypothesis, only 70% of pachytene cells in *rad51* double mutants have paired 5S rDNA loci, versus 100% in wild type. In addition, counts of bivalents at diakinesis and metaphase I show that there are about 7 bivalents on average per cell from *rad51* double mutants. These data also demonstrate that in maize RAD51 is not essential for meiotic chromosome pairing because there is only a modest reduction in the amount of chromosome pairing in *rad51* double mutants. This conclusion is further confirmed by the following observations. First, *rad51* double mutants produce >20% of

normal seed set (Figure 8B), and all sampled progeny (N=20) from these female gametes have 20 chromosomes in root tip preparations and 10 pairs of chromosomes at diakinesis in male meiosis (data not shown). Second, the near-normal rates of meiotic crossovers observed in two chromosome intervals (Table 4 and 5) in *rad51* double mutants demonstrate that strand invasion occurs between homologous chromosomes 3, at least in the surviving female gametes produced by *rad51* double mutants. This finding provides further evidence that chromosome 3 (at least) can pair in the absence of RAD51. Consistent with our data, yeast *rad51* mutants undergo normal chromosome pairing and nearly perfect synapsis (ROCKMILL *et al.* 1995), perhaps due to the presence of functional Dmc1p, which can also promote chromosome pairing (TSUBOUCHI and ROEDER 2003). Our data differ from the previous report that *Atrad51* plays an essential role in chromosome pairing during meiosis in *Arabidopsis* (LI *et al.*, 2004).

Given the critical role of RAD51 in recombination, it is puzzling that no significant differences in the rates of meiotic crossovers were detected between *rad51* double mutants and controls when two linked intervals were assayed (Table 4 and 5). These double mutants do not accumulate normal transcripts from either *rad51a* or *rad51b* (Figure 6). There are no detectable RAD51 foci at zygotene cells from *rad51* double mutants (Figure 7). In addition, our data indicate that maize does not contain any other *rad51*-like genes that can cross-hybridize to the *rad51a* cDNA at low stringency conditions that allow *rad51a* to detect *rad51b* (Figure 3). Hence, it is unlikely that the observed crossovers are a consequence of residual RAD51 activity. Another hypothesis we considered was that the 22% viable female gametes from *rad51* double mutants are somehow enriched for crossover events, i.e., these

gametes survived because they were among the "lucky few" that experienced close to normal rates of crossovers on chromosome 3 (and the other nine chromosomes). This hypothesis is not consistent with our observation that recombination between sister chromatids is dramatically reduced in the *rad51* double mutants during the repair of *MuDR*-induced DSBs (LI *et al,* submitted). In addition, this "lucky few" hypothesis would not explain results obtained in yeast "return to growth" assays involving *rad51*Δ mutants. Although diploid cells in these experiments undergo meiotic crossovers, they do not undergo meiotic divisions. Like the maize *rad51* double mutants, these yeast *rad51*Δ mutants exhibit close to normal rates of crossovers (SHINOHARA *et al.* 1992; SHINOHARA *et al.* 1997). We can therefore rule out residual RAD51 activity and the "lucky few" hypothesis as explanations for the near-normal rates of crossovers among the surviving female gametes from the maize *rad51* double mutants.

We therefore conclude that the *rad51* genes are not essential for chromosome pairing and crossovers in meiosis. We do not, however, interpret our findings to mean that the RAD51 protein is not involved in these processes. Rather, we hypothesize that in maize (unlike *Arabidopsis*) these roles of RAD51 can be complemented by other RecA homologs. If true, this maize homolog would be predicted to exhibit functional differences relative to the corresponding *Arabidopsis* homolog.

There are multiple *rad51* paralogs in plants, only two of which (viz., *rad51c* and *xrcc3*) are involved in meiosis in *Arabidopsis* (BLEUYARD *et al.* 2005). Although maize contains a *rad51c* homolog (GenBank Accession no BD270520) and two *xrcc3* homologs (GenBank

Accession nos AW225129 and DR795779), proteins encoded by these genes have not been shown to be able to catalyze strand invasion. In contrast, like Rad51p (BENSON *et al.* 1998; NEW *et al.* 1998; SHINOHARA and OGAWA 1998; SUNG 1994), Dmc1p has the ability to mediate strand exchange in *vitro* (SEHORN *et al.* 2004). Yeast, mammalian and plant Dmc1p and Rad51p co-localize to nuclear foci during meiotic recombination (ANDERSON *et al.* 1997; BISHOP 1994; TARSOUNAS *et al.* 1999). In addition, it has been shown that yeast Dmc1p can complement Rad51p during meiotic crossovers (SHINOHARA *et al.* 1997). Hence, our working hypothesis is that in the *rad51* double mutant, one or both of the two maize *dmc1* genes (FRANKLIN *et al.* 1999 and Figure 1) can complement the meiotic chromosome pairing and crossover functions normally provided by RAD51.

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Table 1. Correlation between Male Sterility and the *rad51* Double Mutant

rad51a Genotype[a]	Fractions of Progeny with the Indicated Genotypes that were Male Sterile[b]	
	rad51a-54F11	*rad51a-54F11d1*
rad51a/rad51a	12/12	12/12
Rad51a/rad51a	0/25	0/24
Rad51a/Rad51a	0/11	0/6

[a] All plants are homozygous for *rad51b-98E7d4*.

[b] Progeny obtained via self-pollinations of plants heterozygous for the indicated allele of *rad51a*.

Table 2. RAD51 is required for the repair of radiation-induced DSBs

Genotype	No. of seedlings that die two weeks after germination	No. of surviving seedlings
rad51a/rad51a; rad51b/rad51b	31[a]	0
Rad51a/rad51a; rad51b/rad51b	0	97[b]
Rad51a/Rad51a; rad51b/rad51b	0	55

[a] Five seeds that didn't germinate are not included here.

[b] Among them, two seedlings are very short (less than half length of normal seedlings) and have aberrant-shaped leaves

Table 3. Frequencies of Quartet Types in *rad51* Single and Double Mutants

rad51a	Mutant[a]				Mutant		Wild-type[b]	
rad51b	Mutant				Wild-type		Mutant	
Plant ID / Quartet Type[c]	01-2992-5	01-2996-8	05B-2886-16	05B-2886-4	01-1004-1	01-1002-2	01-1009-1	01-1009-2
I 	60	50	84	71	78	80	80	80
II 	15	21	12	13	0	0	0	0
III 	2	3	6	3	0	0	0	0
IV 	0	2	3	4	0	0	0	0
V 	3	3	4	6	0	0	0	0
VI 	8	10	11	14	0	0	0	0
VII 	0	1	2	2	0	0	0	0
VIII 	1	2	6	8	0	0	0	0
Abnormal Quartets[d]	33%	43%	34%	41%	0%	0%	0%	0%

[a] Homozygous for a mutant allele of the indicated gene. This table reports results using *rad51a-54F11* and *rad51b-98E7d4*. Similar results were obtained when *rad51a-54F11*d1 was substituted for *rad51a-54F11*. All (N=~80) quartets examined from the inbred line B73 (homozygous for *Rad51a* and *Rad51b*) were Quartet Type I.

[b] Homozygous for a wild-type allele of the indicated gene.

[c] Black spots represent organized nucleoli; open circles represent cells with multiple micronucleoli.

[d] "Abnormal Quartets" indicates the total percentage of observed abnormal quartets (Types II-VIII).

Table 4. Effect of RAD51 on Rates of Meiotic Crossing-over across the *a1 et1* Interval

rad51 Genotype	No. Kernels[a]		Genetic Distance (cM) ± standard error
	Parental Genotypes	Recombinant Genotypes	
	A1 Et1/a1 et1	*A1 et1/a1 et1*	
Double Mutant[b]	331	46	12.2 ± 1.7
Single Mutant[c]	655	111	14.5 ± 1.3

[a] From the testcross: *A1 Et1/a1 et1* X *a1 et1/a1 et1*

[b] Female parents of testcrosses were homozygous for *rad51a-54F11d1* and *rad51b-98E7d4.* Data pooled from 12 related testcrosses.

[c] Genotypes for female parents of testcrosses were either *Rad51a/Rad51a; rad51b-98E7d4/rad51b-98E7d4* or *Rad51a/rad51a-54F11d1; rad51b-98E7d4/rad51b-98E7d4.* Data pooled from 6 related testcrosses. These results are similar to those obtained from wild type lines.

Table 5. Effect of RAD51 on Rates of Meiotic Crossing-over across the IDP1440-IDP1983 Interval[a]

rad51 Genotype	No. Kernels		Genetic Distance (cM) ± Standard Error
	No. Parental Genotypes	No. Recombinant Genotypes	
Double[b] Mutant	145	20	12.1 ± 2.5
Wild Type[c]	156	26	14.2 ± 2.6

[a] IDP1440 and IDP1983 are PCR-based IDP markers located on the long arm of chromosome 3. Detailed information about these two markers is available from: http://maize-mapping.plantgenomics.iastate.edu/idp/index.php.

[b] Progeny from three related crosses are analyzed here.

[c] These plants have the genotype (*rad51a/Rad51a*; *rad51b/Rad51b*) and are siblings to the *rad51* double mutants. Progeny from two related crosses are analyzed here.

FIGURE LEGENDS

Figure 1. A phylogenetic tree of selected RecA-like proteins of maize, *Arabidopsis* and yeast.

This average distance tree was generated using ClustalW program from http://www.ebi.ac.uk/clustalW/. Ec=*Escherichia coli*; Zm=*Zea mays*; At=*Arabidopsis thaliana*; Sc=*Saccharomyces cerevisiae.* From top to bottom, GenBank accession numbers are listed as follows: V00328, DT947589, AY820160, AF079428, AF079429, NM_122092, M88470, BM501047, NM_113188, P25453, BD270520, NM_130091.

Figure 2. Structures of the *rad51a* and *rad51b* Genes.

Exons are represented with boxes. *Mu* insertions in TUSC alleles are indicated by triangles. The class of *Mu* element is indicated in each case. The positions of oligonuleotides are indicated with arrows. The horizontal dotted and dot-dash lines represent GSSs obtained from methylation-filtered and high C_0T libraries respectively. GenBank accession numbers are indicated for each genome survey sequence. The portion of the *rad51a* genomic sequence (GenBank Accession no. AY359681) that fills the gap between CC684371 and BZ683021 was subcloned from a *rad51a* genomic phage clone. The thick horizontal lines above exons II and III of *rad51a* and below exon VII of *rad51b* designate the deleted portions of *rad51a-54F11d1* and *rad51b-98E7d4*, respectively. The two *Bgl* II restriction sites in *rad51a* are designated as "B". There is no *Bgl* II site in *rad51b* and no *Bgl* I site in either gene.

Figure 3. DNA Gel Blot Analysis of *rad51* Copy Number in the Maize Genome.

B73 genomic DNA was digested with *Bgl* I (panel A) and *Bgl* II (panel B). Hybridization probes and final washing temperatures are indicated above each lane.

Figure 4. PCR Analysis of *rad51b-98E7* Deletion Derivatives.

A portion of the structure of *rad51b-98E7*, including its *Mu* insertion is illustrated. PCR products obtained using the indicated primers were analyzed via gel electrophoresis. The PCR products obtained from four deletion derivatives (*rad51b-98E7d1*, *rad51b-98E7d2*, *rad51b-98E7d3* and *rad51b-98E7d4*) are compared to that from several siblings that carry *rad51b-98E7*.

Figure 5. Structures of *rad51a-54F11* and *rad51b-98E7* Deletion Derivatives.

Shaded boxes represent exons; *Mu* insertions are designated by triangles. The 5' end of each deletion is located at a *Mu* insertion site. Deleted regions are designated by dashed lines and the size of each deletion is indicated in bp. Each deletion derivative was isolated from an independent cross.

(A) *rad51a-54F11* and its deletion derivative, *rad51a-54F11d1*

(B) *rad51b-98E7* and its deletion derivatives

Figure 6. RT-PCR Analysis of *rad51a* and *rad51b*.

RNA was isolated from tassels undergoing meiotic prophase from plants with the following genotypes: homozygous for *Rad51a* and *Rad51b* (lanes 1-2), homozygous for *rad51a-54F11d1* and *Rad51b* (lanes 3-4), homozygous for *Rad51a* and *rad51b-98E7d1* (lanes 5-6), and homozygous for *rad51a-54F11d1* and *rad51b-98E7d4* (lanes 7-8). Gene-specific PCR

primers that flank the *Mu* insertions and more than one intron were used to amplify first-strand cDNAs. PCR products were resolved via agarose (0.8%, w/v) gel electrophoresis and visualized with ethidium bromide staining.

(A) RT-PCR analysis of *rad51a*. First-strand cDNAs were amplified with the primer pair rad51a-244 and 11981 (Figure 1).

(B) RT-PCR analysis of *rad51b*. First-strand cDNAs were amplified with the primer pair RB-98E7 and rb3utr (Figure 1).

Figure 7. RAD51 Foci at Zygotene Cells

(A) B73 cell

(B) *rad51* double mutant cell

Figure 8. Phenotypes of *rad51* Double Mutant.

(A) Effect on male fertility. The plant shown on the left had the genotype *rad51a-54F11/Rad51a; rad51b-98E7/rad51b-98E7d4* and was fully fertile. The plant shown on the right was homozygous for *rad51a-54F11* and *rad51b-98E7d4*, and was completely male sterile. The same phenotype was observed when *rad51a-54F11d1* was substituted for *rad51a-54F11*. To further check the viability of male gametes from *rad51* double mutants, five anthers were dissected from each male sterile plant. These anthers were then cut into small pieces and directly put on female silks of B73 plants to achieve pollination. Ten similar crosses were done and no seed was recovered.

(B) Effect on female fertility. Ears on the left were generated via self-pollination of plants with the genotype *rad51a-54F11/Rad51a; rad51b-98E7d4/rad51b-98E7d4*. Ears on the

right were generated by crossing plants homozygous for *rad51a-54F11* and *rad51b-98E7d4* as females by plants with the genotype *rad51a-54F11/Rad51a; rad51b-98E7d4/rad51b-98E7d4*. Seed sets on plants with the genotype *rad51a-54F11/rad51a-54F11; rad51b-98E7d4/rad51b-98E7d4* that were open pollinated were similar. The same effects on female fertility were observed when *rad51a-54F11d1* was substituted for *rad51a-54F11*.

Figure 9. Cytological Observations of Microsporocytes from *rad51* Double Mutants. Microspores from plants with *rad51a-54F11/rad51a-54F11; rad51b-98E7d4/rad51b-98E7d4* (panels A-C and E-L) or *rad51a-54F11/rad51a-54F11; Rad51b/Rad51b* (panel D) genotypes were analyzed as described in Materials and Methods.

(A) Normal thread-like chromosomes are present at pachytene

(B) Homologous chromosomes align at metaphase I. Note presence of several univalents. One univalent is indicated by an arrow.

(C) Chromosomes migrate to opposite poles with one lagging chromosome (arrow) at anaphase I.

(D) Chromosomes pair apparently normally at diakinesis. Arrow indicates a chiasma.

(E)-(F) Chromosomes do not pair well during diakinesis. Arrows indicate some of the univalents.

(G)-(L) Quartets: normal (G) and abnormal (H-L). The arrow in G indicates an organized nucleolus. The arrows in H and I indicate microspores missing an organized nucleolus. The arrows in J, K, and L indicate microspores with two organized nucleoli.

Figure 10. FISH analysis of 5S rDNA loci in *rad51* double mutants

Chromosomes are shown in red (DAPI), centromeres are in blue and 5S rDNA loci in green.

(A) One paired 5S rDNA loci are seen at pachytene from *rad51* double mutants.

(B) Two unpaired 5S rDNA loci are visible at pachytene from *rad51* double mutants.

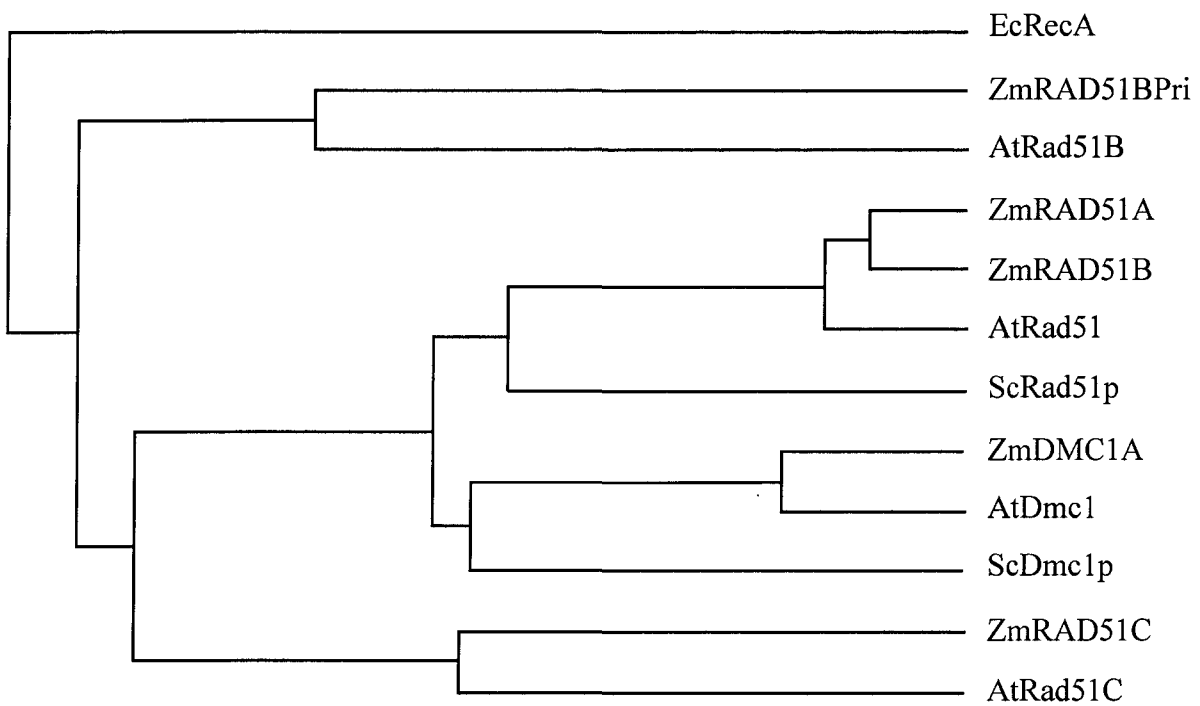

Figure 1. Li *et al.*

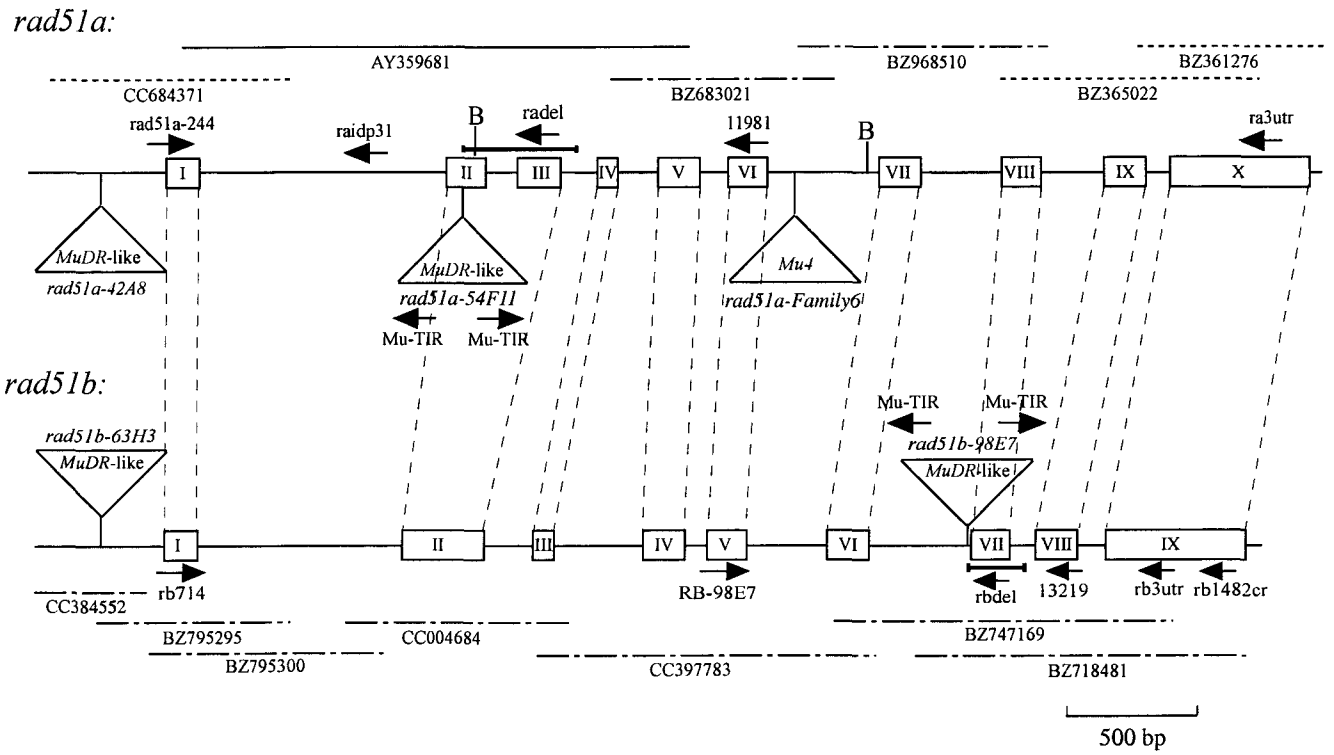

Figure 2. Li *et al.*

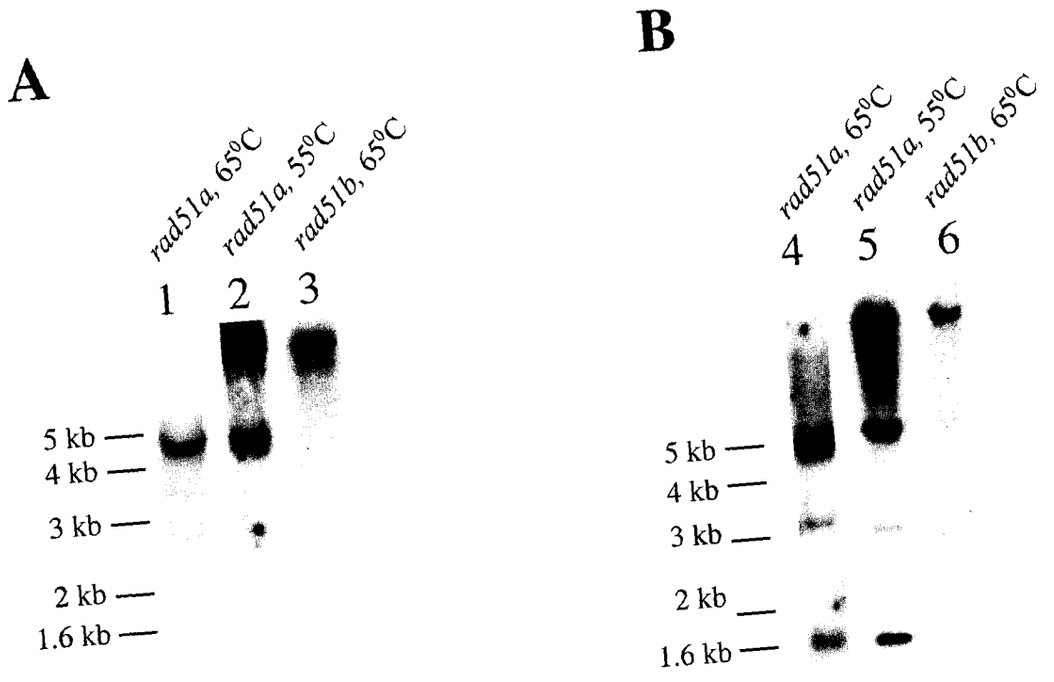

Figure 3. Li *et al.*

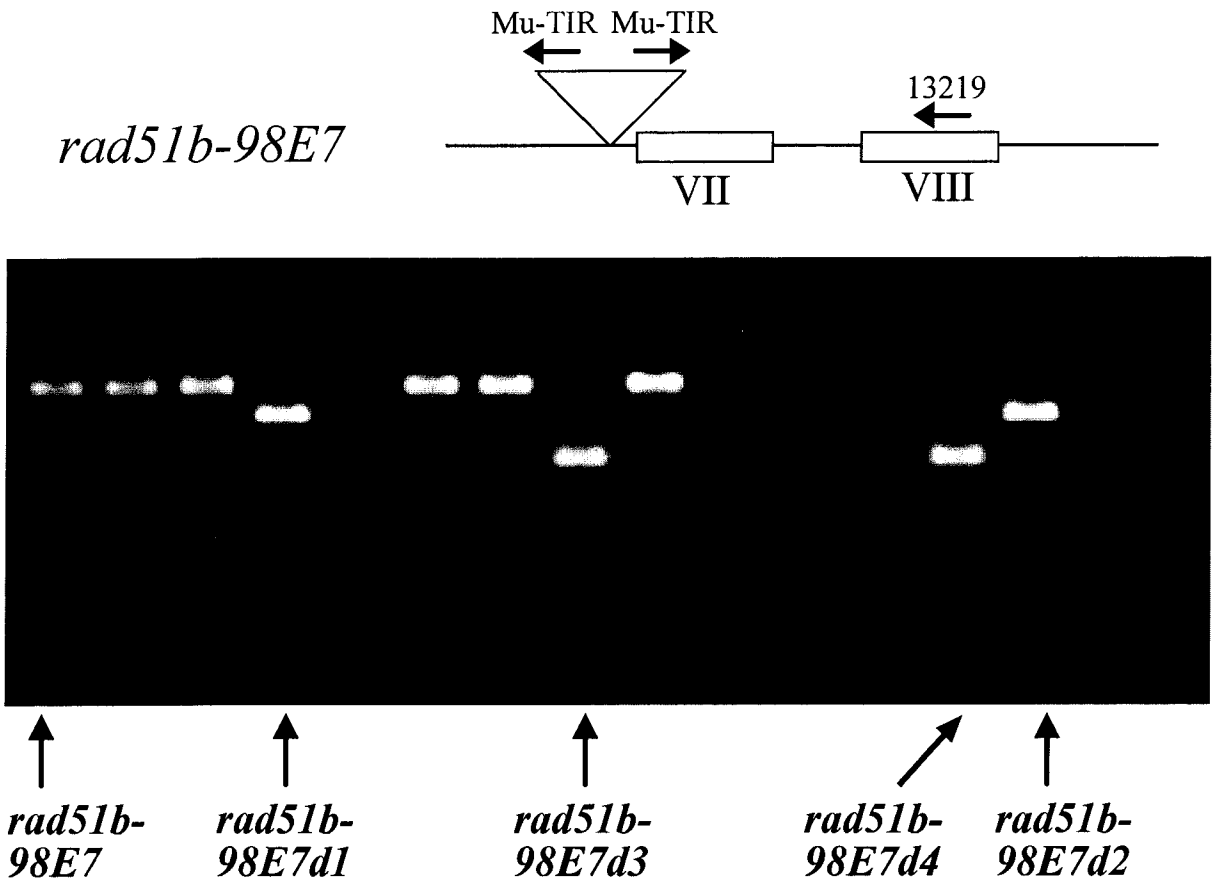

Figure 4. Li *et al.*

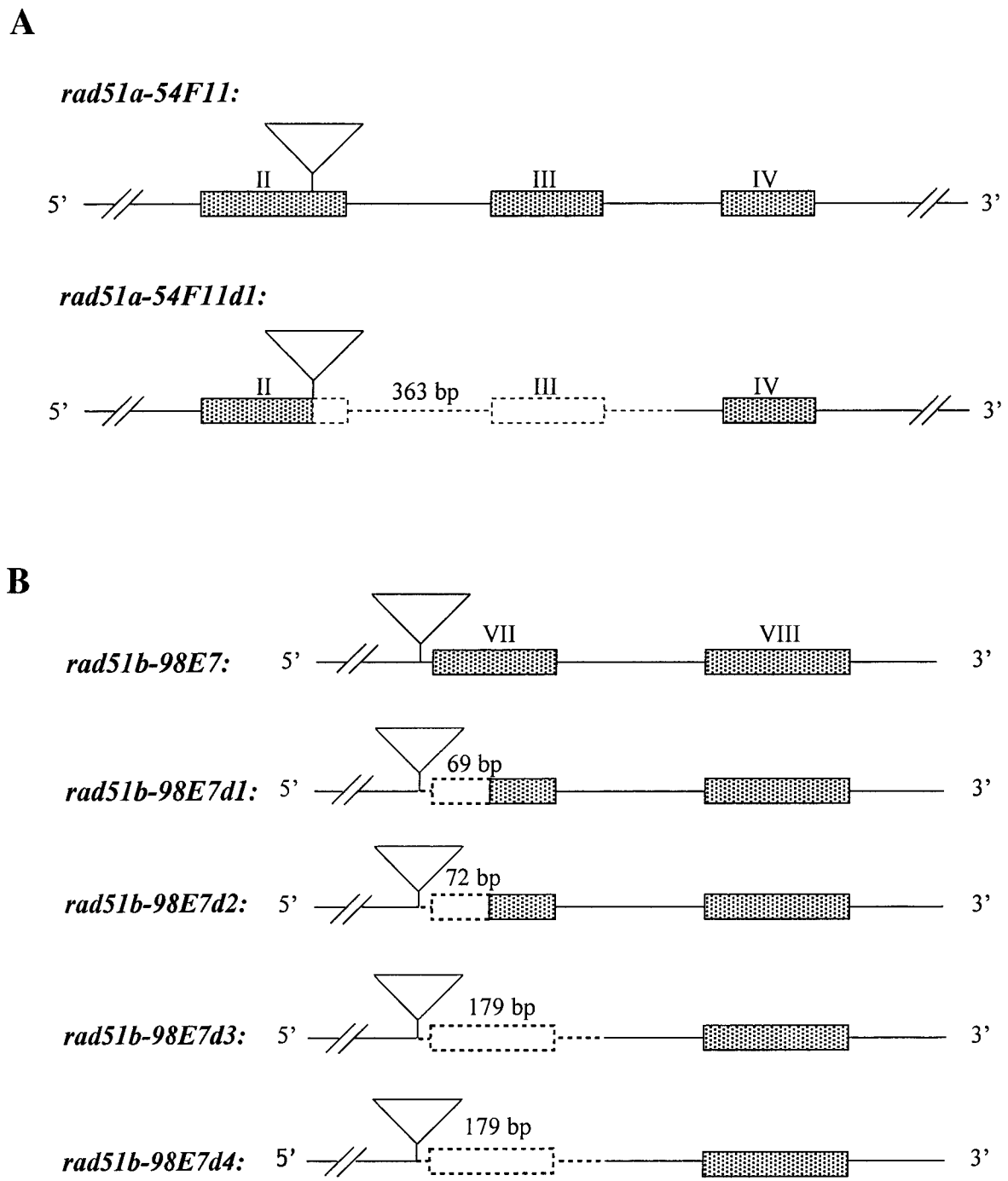

Figure 5. Li *et al.*

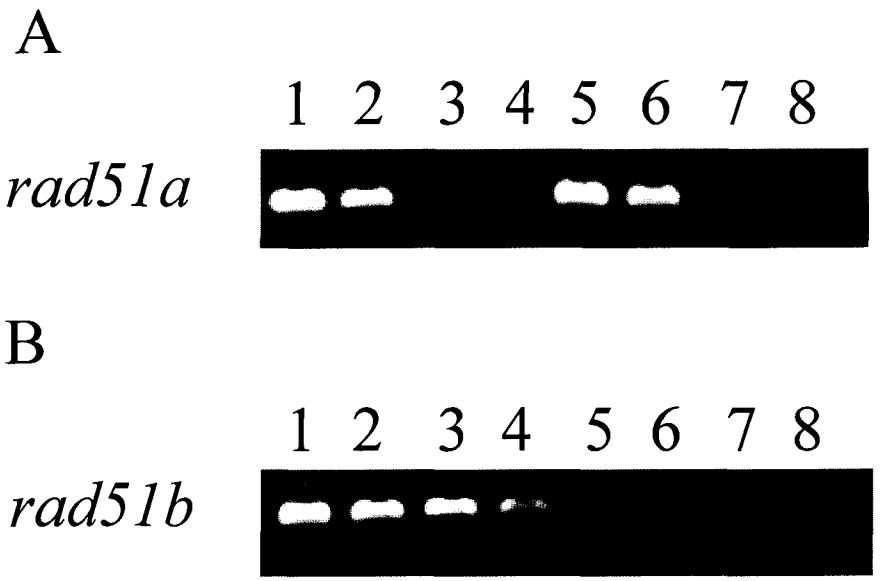

Figure 6. Li *et al.*

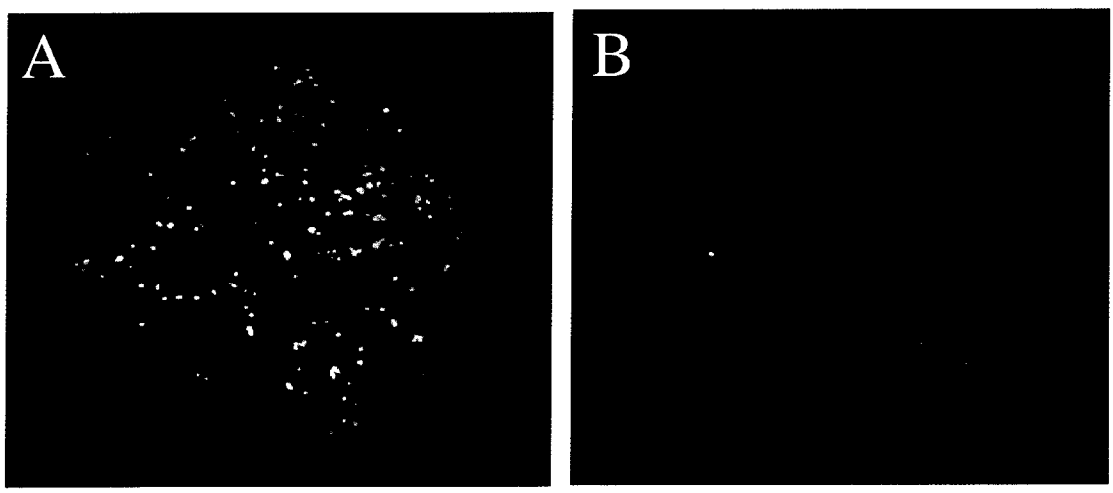

Figure 7. Li *et al.*

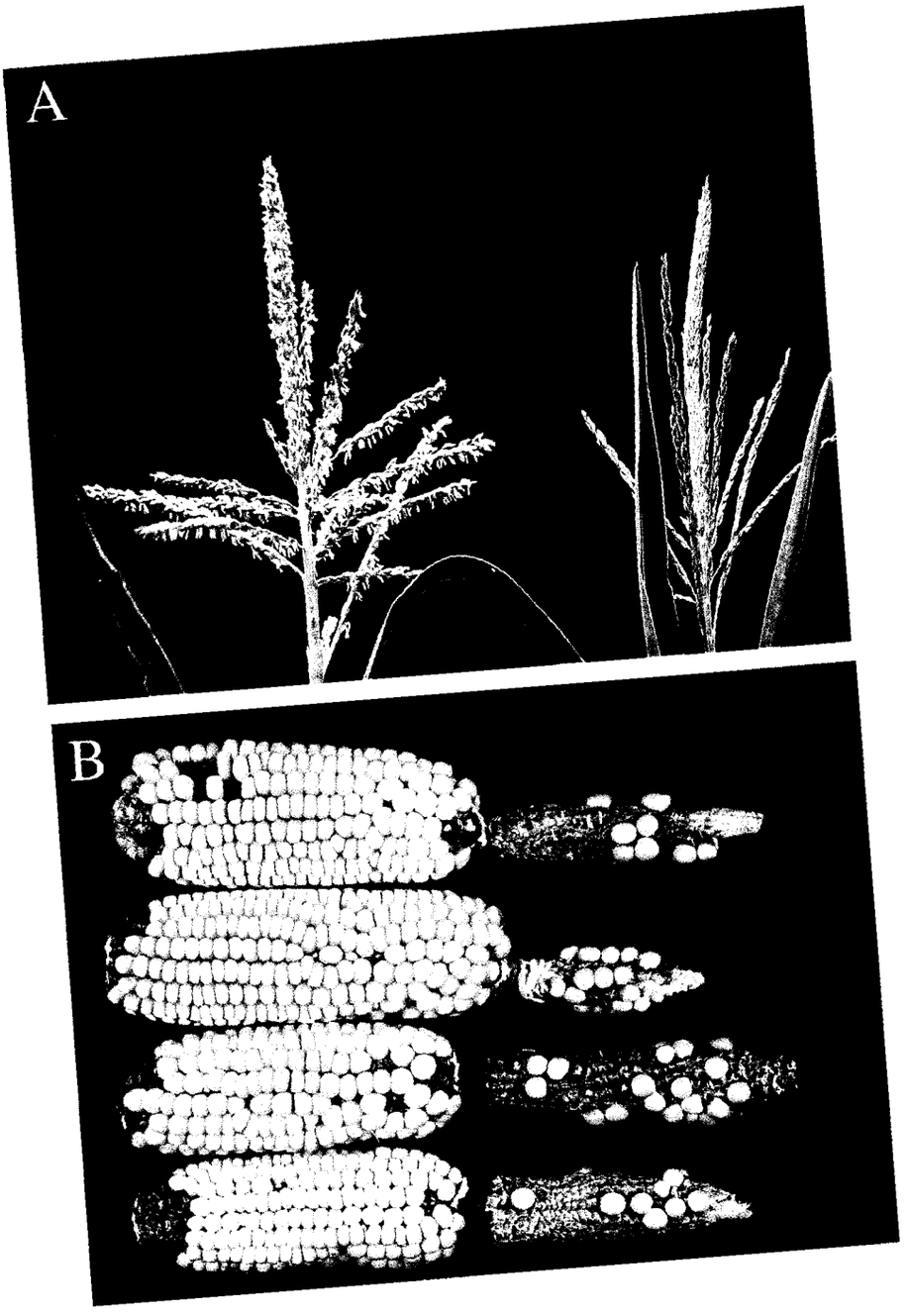

Figure 8. Li *et al.*

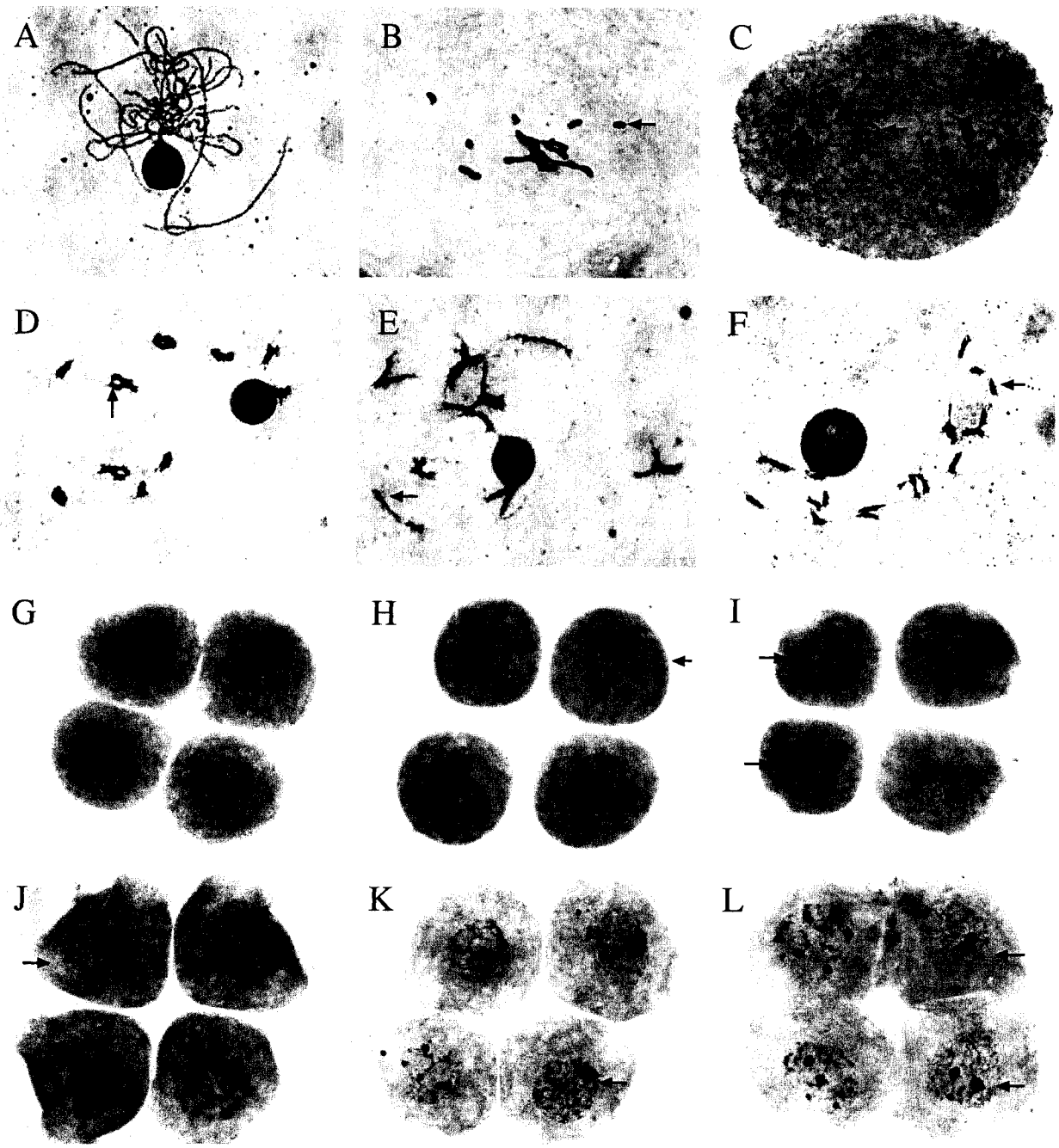

Figure 9. Li *et al.*

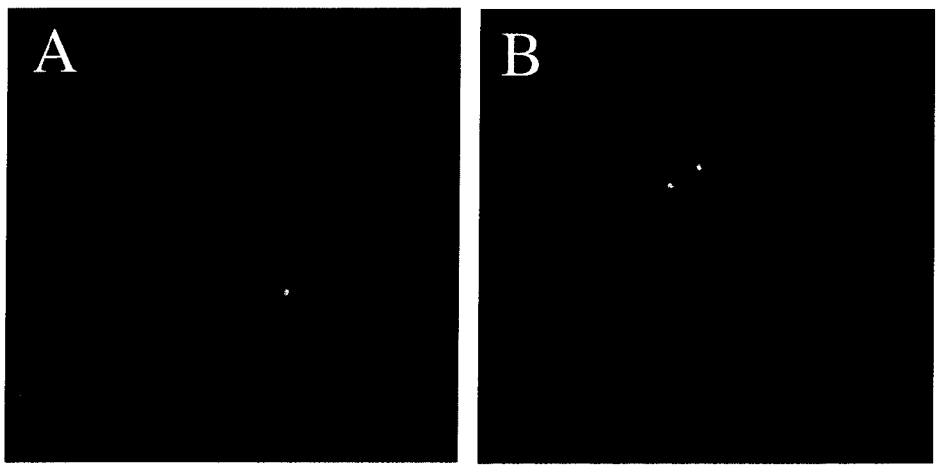

Figure 10. Li *et al.*

CHAPTER 3. THE ROLE OF RAD51 IN THE REPAIR OF *MuDR*-INDUCED DSBs IN MAIZE

A paper to be submitted to Genetics

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RAD51 is involved in DNA repair

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ABSTRACT

In late sporophytic cells rates of *Mu* insertion and excision are both high. In contrast, although high rates of insertion are also observed in germinal cells, germinal excisions are recovered only rarely. We have isolated deletion alleles of the two maize *rad51* genes. In RAD51+ plants germinal revertants are recovered only rarely (2.6×10^{-5}) from the *MuDR*-insertion allele *a1-m5216*. In contrast in RAD51- plants (i.e., *rad51a*, *rad51b* double mutants) the rate of germinal derivatives from *a1-m5216* is at least 40-fold higher than controls. Most of the germinal derivatives involve deletions of the *MuDR* insertion and/or the *a1* gene. Our interpretation is that in wild-type germinal cells *MuDR* excisions are efficiently repaired via RAD51-directed homologous recombination with the sister chromatid, which replaces the excised *MuDR*, resulting in a low rate of germinal derivatives. Two experiments suggest that RAD51 is also required for repairing *Mu*-induced DSBs in early somatic cells. First, a high proportion of *Mu*-active (but not *Mu*-inactive) RAD51- mutants exhibit severe developmental defects. Second, ear sectors of germinal derivatives were recovered at a higher rate from RAD51- mutants than from controls. Together these results suggest that RAD51 is required for repairing *Mu*-induced DSBs during early vegetative development. The high rate at which partial deletions of the *a1* locus were recovered in this study indicates that the *rad51* double mutant stock offers an attractive means to generate knock-out alleles for functional genomic studies.

INTRODUCTION

The *Mutator* transposon family, first identified by its high forward mutation rate (ROBERTSON 1978) consists of an autonomous (*MuDR*) and several non-autonomous

elements, all of which share approximately 200-bp conserved terminal inverted repeats (TIRs) (BENNETZEN 1996; CHANDLER and HARDEMAN 1992; 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). Germinal excision 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 (WALBOT and RUDENKO 2002) and the spectrum of footprints from *Mu*-induced somatic excisions was observed (RAIZADA *et al.* 2001). Two major models have been proposed to reconcile the differential behavior of *Mu* transposons in germinal and late somatic cells (CHANDLER and HARDEMAN 1992; LISCH 2002; WALBOT and RUDENKO 2002).

In Model A, *Mu* transposes exclusively by a "Cut-and-paste" mechanism. In germinal cells, *Mu*-induced double-stranded breaks (DSBs) are repaired via homologous recombination using sister chromatid or homologous chromosome as templates (DONLIN *et al.* 1995; HSIA and SCHNABLE 1996; LISCH 2002; WALBOT and RUDENKO 2002). P-element in *Drosophila melanogaster* transposes via a "cut-and-paste" mechanism but is followed by double-strand gap repair to restore the P element at the donor site (ENGELS *et al.* 1990). The recovery of internal deletion derivatives of *MuDR* (HSIA and SCHNABLE 1996) and abortive transposition events (DAS and MARTIENSSEN 1995; LEVY and WALBOT 1991; TAYLOR and WALBOT 1985) suggest that gap repair pathway may be involved in the repair of *Mu*-induced DSBs in germinal cells.

In Model B, *Mu* transposons utilize a "cut-and-paste" mechanism in late somatic cells but replicative transposition in germinal cells (CRAIG 1995; LISCH 2002; WALBOT and RUDENKO 2002). *Mu* transposons transpose via "cut-and-paste" in late somatic cells (RAIZADA *et al.* 2001). It has been proposed that replicative transposition instead of 'cut-and-paste" occurs in germinal cells (LISCH 2002; WALBOT and RUDENKO 2002). The bacterial transposon Tn7 is competent to make a switch between "cut-and-paste" and replicative transposition (MAY and CRAIG 1996). Maize *mudrA* produces multiple transcripts that can encode multiple transposases and these naturally multiple transposases may be able to make a switch between "cut-and-paste" and replicative transposition (WALBOT and RUDENKO 2002).

DSBs can be repaired via two major pathways: homologous recombination (HR) and non-homologous end-joining (NHEJ) (PASTWA and BLASIAK 2003; WEST *et al.* 2004). RAD51 plays a central role in the HR pathway (BAUMANN and WEST 1998; THACKER 1999). The maize genome contains two *rad51* genes (FRANKLIN *et al.* 1999; LI *et al.*, submitted). RAD51 is required for efficient chromosome pairing and proper chromosome segregation in meiosis (LI *et al.*, submitted).

Because RAD51 play a central role in homologous recombination that is conserved across multiple organisms (BAUMANN and WEST 1998; THACKER 1999), it is likely that RAD51 possesses similarly conserved functions in maize. Although male sterile, maize *rad51* double mutants can still produce 22% seed set when pollinated as female (LI *et al.*, submitted). If RAD51-directed HR is involved in the repair of *MuDR*-induced DSBs, these surviving seeds

from *rad51* double mutants could be used to test Model A as an explanation for the different behaviors of *Mu* transposons in maize.

MATERIALS AND METHODS

Genetic stocks:

Both *rad51a-54F11d1* and *rad51b-98E7d4* were isolated as described (LI *et al.*, submitted). The *a1-m5216* allele contains a *MuDR* transposon insertion in exon III of the *a1* gene and conditions a spotted kernel phenotype (HSIA and SCHNABLE 1996). The *a1::rdt* (BROWN *et al.* 1989a), *a1-mrh* (SHEPHERD *et al.* 1989) and *a1-mr102b* (CUYPERS *et al.* 1988) alleles all contain transposon insertions that disrupt *a1* gene function and condition a colorless kernel phenotype. The *a1-s* allele has an 8-bp insertion in exon III that disrupts the *a1* gene function (GenBank Accession no. U46056). Kernels without functional *Sh2* allele are shrunken (TSAI and NELSON 1966). The *ax-1* allele was obtained from the Maize Genetics Stock Center. DNA gel blot analysis shows that both *a1* and *sh2* genes are physically deleted in the *ax-1* stock (Yandeau-Nelson *et al.*, submitted). The *a1-sh2* interval is located at maize chromosome 3 and serves as a model for recombination (CIVARDI *et al.* 1994; XU *et al.* 1995; YAO *et al.* 2002).

Isolation of germinal revertants from *a1-m5216*:

Cross 1: *a1-s Sh2/a1-s Sh2* X *a1-m5216 Sh2/a1* Sh2*

Cross 2: *a1-m5216 Sh2/a1* Sh2* X *a1-s Sh2/a1-s Sh2*

Cross 3: *a1-m5216 Sh2/ax-1* X *a1-s Sh2/a1-s Sh2*

The symbol *a1** indicates either *a1-mrh* or *a1-mr102b*. Colored kernels from Crosses 1-3 were selected as putative germinal revertants. *A1* alleles carried by these kernels were PCR-amplified using the primer pair (ARRSP + A1.2), purified using Qiagen PCR purification kits (Cat. No. 28106, Valencia, CA) and sequenced.

Strategy I for isolating germinal derivatives of *a1-m5216*:

Cross 4: *rad51a-54F11d1/rad51a-54F11d1*; *rad51b-98E7d4/rad51b-98E7d4*; *a1-m5216 Sh2/a1::rdt sh2* X *a1-s sh2/a1-s sh2*

Cross 5: *rad51a-54F11d1/Rad51a*; *rad51b-98E7d4/Rad51b; a1-m5216 Sh2/a1::rdt sh2* X *a1-s sh2/a1-s sh2*

Cross 5 serves as the control for Cross 4. Female parents of Cross 4 and 5 are siblings. Kernels with the genotype (*a1-m5216 Sh2/a1-s sh2*) confer spotted round phenotype. Colorless, pale and colored round kernels from Cross 4-5 were selected as candidates for germinal derivatives of *a1-m5216*. Because the genetic distance between *a1* and *sh2* is only 0.1 cM (CIVARDI *et al.* 1994), almost all round kernels of the progeny are supposed to originate from *a1-m5216 Sh2* instead of *a1::rdt sh2*.

Strategy II for isolating germinal derivatives of *a1-m5216*:

Cross 6: *rad51a-54F11d1/rad51a-54F11d1*; *rad51b-98E7d4/rad51b-98E7d4*; *a1-m5216 Sh2/ax-1* X *a1::rdt sh2/a1::rdt sh2*

Cross 7: *rad51a-54F11d1/Rad51a*; *rad51b-98E7d4/Rad51b*; *a1-m5216 Sh2/ax-1* X *a1::rdt sh2/a1::rdt sh2*

The *a1-m5216* allele was made hemizygous with *ax-1*. Cross 7 serves as the control for Cross 6. Female parents of Cross 6 and 7 are siblings. Kernels with the genotype (*a1-m5216 Sh2/a1-s sh2*) confer spotted round phenotype. Colorless, pale and colored round kernels from Cross 6-7 were selected as candidates for germinal derivatives of *a1-m5216*.

Oligonucleotides

The approximate locations of oligonucleotides (5216R, A1.2, m567, Mu-TIR and XX231) are shown in Figure 2. The remaining oligonucleotides were designed based on the *MuDR* sequence.

5216R: 5' TAA ATA AAA GGT GTC GTC AGC G 3'

A1.2: 5' GAT TGT TGC TTA AGC GCC AAT CGT 3'

m567: 5' CCT GAG GTA 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' ACA TTT 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 GCG 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'

XX231: 5' GCC AAA CTC TGA TTC GCT CCG TG 3'

Identification of germinal derivatives of *a1-m5216* via PCR:

All colorless and pale round kernels of progeny (Cross 4~7) were germinated in our greenhouse and genomic DNA was isolated from one-week old seedlings using a modified high-throughput CTAB method (DIETRICH *et al.* 2002). PCR was used to check the presence of *MuDR* at the *a1* gene using two primer pairs (Mu-TIR + XX231; Mu-TIR + A1.2) (Figure 2). If PCR is negative for either or both of these two primer pairs, the corresponding seedling will be regarded as a germinal derivative of *a1-m5216*. Footprints of identified germinal derivatives were subsequently determined by directly sequencing of PCR products.

Temperature gradient capillary electrophoresis (TGCE):

TGCE was conducted using the Reveal System, model RVL 9612, rev. 2.0 (SpectruMedix, State College, PA). Sample preparation and TGCE conditions were followed as described (HSIA *et al.* 2005). Due to the large size (4.9 kb) of *MuDR* element, two rounds of PCR reactions were performed to prepare templates for TGCE analysis. The first round of PCR was performed using two primer pairs (XX231 + Mu2332r; 5216R + MuDR2270). These

PCR products were purified using Qiagen PCR purification kits (Cat. No. 28106) and then diluted 1000x using distilled water. If the diluted PCR products were PCR-amplified using the primer pair (XX231 + Mu2332r), four more pairs of primers (m567 + Mu534r; Mu473 + Mu1253; Mu1211 + Mu1936; Mu1805 + Mu2332r) were used for the second round of PCR. On the other hand, if the diluted PCR products were PCR-amplified with the primer pair (5216R + MuDR2270), five more pairs of primers (MuDR2270 + Mu2903; Mu2400 + Mu3106R; Mu3102 + Mu3962; MuDR3960 + Mu4700; Mu4536u + 5216R) were used for the second round of PCR. The comparison was made between female parents and their corresponding progeny.

RESULTS

Germinal revertants from *a1-m5216*:

The product of the *a1* gene is involved in the anthocyanin biosynthesis pathway (WIENAND *et al.* 1990) and *a1-m5216* allele conditions a spotted kernel phenotype due to the insertion of *MuDR* in exon III of the *a1* gene (HSIA and SCHNABLE 1996). Three different crosses (Cross 1~3, Materials and Methods) were performed to isolate germinal revertants from *a1-m5216*. Sixteen independent events were identified and subsequently confirmed by direct sequencing (Figure 1 and Table 1). Rates of germinal revertants of *a1-m5216* are very rare (2.60×10^{-5}), which is consistent with several previous reports (BROWN *et al.* 1989b; LEVY *et al.* 1989; SCHNABLE *et al.* 1989). All but four events are perfect excisions (Figure 1). In all these cases, molecular markers from pollen parents were used to distinguish true germinal

revertants from possible contaminants. The high proportion of perfect excision events is probably due to selection, in that only colored kernels were analyzed in our experiments.

Germinal adjacent deletions of *Mu*-insertion alleles:

Mu transposons have been used to generate loss-of-function alleles in maize (Bensen *et al.*, 1995; LUNDE *et al.*, 2003; May *et al.*, 2003; McCarty *et al.*, 2005; Settles *et al.*, 2004). However, alleles that have *Mu* insertions in non-coding regions (introns, 5' end or 3' end of genes) may not disrupt gene functions, thus these alleles will not be very useful for functional analysis. Germinal adjacent deletions from *Mu*-insertion alleles have been reported from several maize loci: *adh1* (TAYLOR and WALBOT 1985), *bz2* (LEVY and WALBOT 1991) and *hcf106* (DAS and MARTIENSSEN 1995), and the recovery rate of deletion ranges from 0.3% to 2.5% (Table 2). Adjacent deletion screens were performed for eight *Mu*-insertion alleles in our lab and germinal adjacent deletions were identified for four loci: *pdc1*, *rad51a*, *rad51b* and *rth1*. The recovery rates range from 0.02% to 0.8% (Table 2). We also demonstrate that adjacent deletions can be generated from *dMuDR*- and *Mu7*-insertion alleles (Table 2), whereas deletion alleles had previously been obtained only from *Mu1*-insertion alleles (DAS and MARTIENSSEN 1995; LEVY and WALBOT 1991; TAYLOR and WALBOT 1985). The size of deletions can be at least as large as 383 bp (Table 2).

The effect of RAD51-directed HR repair on the rate of germinal derivatives of *a1-m5216*:

To determine the relationship between *MuDR* activity and RAD51-directed HR in maize germinal cells, two crossing strategies were designed (Cross 4~7, Materials and Methods).

The product of the *sh2* is involved in starch biosynthesis and kernels without functional *Sh2* allele are shrunken (TSAI and NELSON 1966). Both *a1* and *sh2* are located on chromosome 3L and their genetic distance is only 0.1 cM (CIVARDI *et al.* 1994)

In strategy I, *a1-m5216 Sh2* was crossed to *a1::rdt sh2* (Figure 2 and Cross 4-5, Materials and Methods). At the same time, both *rad51a* and *rad51b* mutant alleles are also introduced into the same genetic stock. Two different *sh2* alleles serve as convenient phenotypic markers. Kernels with the genotype (*a1-m5216 Sh2/a1-s sh2*) are spotted due to somatic excisions of *MuDR* element from the *a1* locus. In control crosses with functional *Rad51*, 97% of round kernels of the progeny are spotted as expected. In contrast, only 19% of round kernels from *rad51* double mutants are spotted (Figure 3). All non-spotted kernels were subject to PCR analysis using two primer pairs (Mu-TIR + XX231; Mu-TIR + A1.2) (Figure 2). These analyses established that the *a1* alleles carried by most of these non-spotted kernels had undergone genomic rearrangements. Subsequent sequencing analyses demonstrated that the majority of these germinal derivatives from RAD51- plants are missing some portions of the *a1* gene and all or part of the *MuDR* insertion of *a1-m5216* (Class I, II and III in Figure 3). In the absence of RAD51 the HR repair pathway is not available. Hence, these imperfect footprints are likely due to DNA repair introduced via the error-prone NHEJ pathway. The rate of recovering germinal derivatives from *rad51* double mutants is 64 fold [((36 +50+2)/130/(2+7)/855)=64] higher than in wild type control (Figure 3). This significant difference indicates that RAD51-directed HR is required for repairing *MuDR*-induced DSBs at the *a1* locus in germinal cells.

A second strategy was utilized to study the relationship between *MuDR* activity and RAD51-directed HR (Cross 6-7, Materials and Methods). In this genotype, recombination between homologous chromosomes at the *a1* locus is completely blocked due to lack of template at the homologous chromosome. All round kernels from Cross 6-7 should be derived from the *a1-m5216 Sh2* haplotype. Kernels with the genotype (*a1-m5216 Sh2/a1::rdt sh2*) are spotted due to somatic activity of *MuDR* at the *a1* locus. As expected, in control crosses, 98% of round kernels of the progeny were spotted. In crosses involving the *rad51* double mutant, however, only 14% of round kernels were spotted. The majority of round kernels (86%) were either non-spotted or pale. The *a1* haplotypes carried by these kernels (N=36) all underwent genomic rearrangements relative to *a1-m5216* (Figure 3). The rate of recovering germinal derivatives from *rad51* double mutants is 41 fold [((14+22)/42)/(3/143)=41] higher than wild-type control (Figure 3).

Two independent crossing strategies both show that *MuDR* of *a1-m5216* excises very frequently (68% and 86%) in germinal cells of *rad51* double mutants. On the other hand, it indicates that *MuDR*-induced DSBs are mainly repaired via RAD51-directed HR in wild type plants. This can explain why germinal excision events rarely occur in normal maize plants.

TGCE analysis is useful to identify germinal derivatives with small polymorphisms: Two primer pairs (Mu-TIR + XX231; Mu-TIR + A1.2) were used to identify germinal derivatives of *a1-m5216* (Figure 2). If PCR products of the expected size are amplified using both primer pairs, the *MuDR* transposon at the *a1* locus was considered to have intact TIRs and flanking *a1* sequences. Based on these criteria, thirty-nine kernels out of a population

(1,170) were identified, but all were non-spotted. Because the PCR assay will not detect very small polymorphisms, it cannot be determined whether all these thirty-nine kernels still have intact *MuDR* transposon at *a1* locus. Temperature gradient capillary electrophoresis (TGCE) is useful to detect a single nucleotide polymorphism in amplicons of over 800 bp (HSIA *et al.* 2005). TGCE analysis was utilized to compare these 39 kernels, their correspondent female parents and a known DNA sample with intact *MuDR* (for control). Two of thirty-nine kernels are found to be polymorphic versus intact *MuDR*. One plant (04B-277-6) has a 54-bp deletion and the other one (04B-279-2) has a 38-bp deletion. Neither of these small deletions affects the Mu-TIR primer annealing sites. Based on the TGCE analyses the remaining 37 of 39 non-spotted kernels carry intact *MuDR* transposon at the *a1* locus. The loss of somatic excision activity in these kernels is likely due to epigenetic changes at *a1* (CHANDLER *et al.* 1988; CHOMET *et al.* 1991).

Clusters of germinal derivatives from *rad51* double mutants:

The majority (127/136=93%) of germinal derivatives recovered in this study were sequenced, so it is possible to compare their footprints at the nucleotide level. A cluster of germinal derivatives will be considered if the following two criteria are met: first, two or more germinal derivatives have the same footprint at the nucleotide level. Second, all these germinal derivatives come from a single cross. Based on these criteria, eight clusters of germinal derivatives were identified, with cluster sizes of 3, 4, 6, 7, 8, 10, 19, 39 cells respectively. The size of each cluster is determined based on sequence analysis. Clusters of germinal derivatives can be generated if *MuDR* of *a1-m5216* excises and DNA repair occurs before formation of the megaspore mother cell. The rate of recovering clusters is at least 47

fold higher in *rad51* double mutants as compared to wild type (8/172 vs 0/998). This dramatic difference indicates that RAD51-directed HR is required for repairing *MuDR*-induced DSBs in pre-meiotic cells.

***rad51* plays an important role during vegetative development in *Mu* active plants:** Although maize (LI *et al.*, submitted) and *Arabidoposis* (LI *et al.* 2004) plants that lack RAD51 can develop normally, this is not the case in *Mu* active maize plants. Indeed, maize *rad51* double mutants with active *Mu* often exhibit severe developmental defects (Table 3). In contrast, similar defects were not observed among plants that have either functional RAD51 or in which *Mu* is inactive (Table 3). This correlation strongly suggests that RAD51-directed HR is involved in repairing *MuDR*-induced DSBs during early vegetative development. Our results are consistent with gene expression profile results indicating that *mudrA* and *mudrB* are expressed in all developmental stages (LISCH *et al.* 1999; RUDENKO and WALBOT 2001), and that *rad51* transcripts levels are elevated in actively dividing mitotic cells (DOUTRIAUX *et al.* 1998; FRANKLIN *et al.* 1999). *Atrad51* transcriptional induction by gamma irradiation indicates that *Atrad51* may also play a positive role in repair of radiation-induced DSBs (DOUTRIAUX *et al.* 1998).

DISCUSSION

RAD51-directed HR is required for repair of *MuDR*-induced DSBs in germinal cells: Although germinal revertants of a *MuDR*-insertion allele of *a1* are recovered only rarely in RAD51+ plants (2.60×10^{-5}, Table 1), germinal derivatives with various genomic rearrangements arise at ~40-fold higher rates in *rad51* double mutants compared to wild type

controls (Figure 3). This finding indicates that RAD51-directed HR repair is a major reason why germinal derivatives are only rarely recovered in RAD51+ plants. This behavior of *Mu* transposons is similar to that described for P transposons of *Drosophila* (ENGELS *et al.* 1990). Our results are consistent with the gap-repair model to explain the transposition of *Mu* elements in maize germinal and/or pre-germinal cells (DONLIN *et al.* 1995; HSIA and SCHNABLE 1996).

Model A (Figure 4) reconciles different behaviors of *MuDR* in germinal and late somatic cells:

There are two major models to explain different behaviors of *MuDR* in germinal and late somatic cells (Figure 4). Our study provides strong experimental support for Model A to explain different *MuDR* transposition behaviors in maize germinal and late somatic cells (CHANDLER and HARDEMAN 1992; LISCH 2002; WALBOT and RUDENKO 2002). There are several lines of evidence based on our genetic and molecular data. First, the absence of RAD51 leads to significantly increased rate of derivatives recovered in germinal cells (Figure 3). Second, Model B cannot explain the high rate of germinal excisions and one-side deletions of *MuDR* (Class I and II, Figure 3). Finally, the majority of germinal derivatives have deletions of *MuDR* or *a1* genic region. This is consistent with the idea that they result from the error-prone NHEJ repair pathway. Hence, this study provides strong support for the Model A that the bulk of *Mu* transposition in both germinal and somatic tissue occurs via a cut-and-paste mechanism. The differences in outcomes of *Mu* transpositions between germinal and late somatic cells appear to be a consequence of the available repair pathways. In germinal cells *MuDR*-induced DSBs are mainly repaired via the highly accurate RAD51-

directed HR pathway, whereas in late somatic cells *MuDR*-induced DSBs are mainly repaired via the error-prone NHEJ pathway (Figure 4). .

MuDR is active in pre-meiotic cells and RAD51-directed HR is required to repair _MuDR_-induced pre-meiotic DSBs:

Eight germinal clusters of *a1-m5216* were recovered from *rad51* double mutants. The finding of these clusters indicates that *MuDR* is active at pre-meiotic stages. Our results are consistent with the timing of *Mu* activity described by previous reports (ROBERTSON 1980; ROBERTSON 1981). In addition, the significantly higher rate (at least 47-fold higher) of recovering germinal clusters from *rad51* double mutants compared to wild type controls demonstrates that RAD51-directed recombination is required for DNA repair of *MuDR*-induced DSBs at pre-meiosis. The large size of clusters (10, 19 and 39 cells) shows that the *Mu* pre-meiotic activity can occur at the very early stage before meiosis. These sizes of clusters recovered from *rad51* double mutants are much larger than that described in previous reports (ROBERTSON 1980; ROBERTSON 1981). The lack of RAD51-directed HR in *rad51* double mutant may be responsible for recovering large sizes of germinal clusters. The increased rate of meiotic crossovers by *MuDR* transposase establishes that *MuDR* is also functional in meiosis (YANDEAU-NELSON *et al.* 2005). Therefore, *MuDR* is active both immediately prior to and during meiosis.

Two ends of _Mu_ transposon are not cut simultaneously when _Mu_ transposes in germinal cells:

Out of 136 germinal derivatives, eighty-two events (more than 60%) only have one-side of *MuDR* deleted (Class II, Figure 3). These events would not be generated if both ends of *MuDR* were always cut simultaneously. This cutting pattern of *Mu* transpostion is consistent with the recovery of germinal adjacent deletions from several maize loci (DAS and MARTIENSSEN 1995; LEVY and WALBOT 1991; TAYLOR and WALBOT 1985). After one end of *Mu* transposon is cut in germinal cells, the resulting *Mu*-induced DSBs must be repaired or part of a chromosome will be lost. Generally, there are two major DSBs repair pathways: homologous recombination and NHEJ (PASTWA and BLASIAK 2003; WEST *et al.* 2004). Although NHEJ only plays a minor role in germinal cells, the high frequency of one-sided *Mu* transposition may explain the high recovery rate (2.5%) of germinal adjacent deletions (LEVY and WALBOT 1991). In addition, all of the one-sided deletions detected in our study (Class II, Figure 3) lacked intact TIRs and this particular aspect is different from previously reported adjacent deletions (DAS and MARTIENSSEN 1995; LEVY and WALBOT 1991; TAYLOR and WALBOT 1985; Table 2). Therefore, it is unlikely that the *MuDR* transposase is involved in repairing one-side deletions generated in our experiments (Class II, Figure 3) because TIRs are not protected in these cases.

Why almost all the germinal derivatives of *a1-m5216* isolated from *rad51* double mutants arose from the NHEJ pathway:

First, although DMC1 can complement RAD51 during recombination between homologous chromosomes (SHINOHARA *et al.* 1997), RAD51, but not DMC1, can mediate recombination between sister chromatids in yeast (ARBEL *et al.* 1999; SHINOHARA *et al.* 2000). Consistent with this observation, the rate of meiotic crossovers in surviving female gametes produced by

rad51 double mutants is almost the same as in RAD51+ plants (LI *et al.*, submitted). Recombination between sister chromatids will be reduced dramatically in *rad51* double mutants if RAD51 functions are conserved between yeast and maize. Second, pre-meiotic *MuDR*-induced DSBs will not be repaired via recombination using homologous chromosomes because chromosomes do not pair at that stage. Third, recombination between homologous chromosomes may be blocked or inhibited due to different homologous templates used in our study. It is possible that the *rdt* transposon of the *a1::rdt* allele used in strategy I (Cross 4, 5, Materials and Methods) will inhibit the recombination between homologous chromosomes. The *a1* gene is physically deleted in the *ax-1* stock used in strategy II (Cross 6, 7, Materials and Methods). In this case, recombination between homologous chromosomes is completely blocked due to lack of template. Therefore, because recombination between sister chromatids does not function due to lack of RAD51 and recombination between homologous chromosomes is inhibited or blocked due to different timing of *Mu* activity or homologous templates, NHEJ becomes the only available major pathway to repair these *MuDR*-induced DSBs in *rad51* double mutants.

Germinal derivatives of *Mu*-insertion alleles can be recovered at a higher rate in *rad51* double mutants:

Mu insertion alleles are widely used for reverse genetics (BENSEN *et al.*, 1995; LUNDE *et al.*, 2003; MAY *et al.*, 2003; MCCARTY *et al.*, 2005; SETTLES *et al.*, 2004). *Mu*-derived alleles with *Mu* insertions in intron, 5' promoter or 3' end of genes often do not disrupt gene functions. 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; SPRINGER et al., 2004) many

Mu–insertion alleles isolated via reverse genetics are still functional. One approach for dealing with this challenge is to isolate derivative alleles that have lost genic sequences adjacent to the *Mu* insertion. Although germinal adjacent deletion alleles have been recovered from several maize loci (DAS and MARTIENSSEN 1995; LEVY and WALBOT 1991; TAYLOR and WALBOT 1985), rates vary dramatically among loci and it has not been possible to identify adjacent deletion alleles of four loci even after our extensive screening (Table 2). In contrast, mutant derivatives of *Mu*-insertion alleles can be efficiently isolated using *rad51* double mutants. Most gametes (124/162) isolated from *rad51* double mutants have undergone genomic rearrangements in the vicinity of the *MuDR* insertion in the *a1* gene (Figure 3). All but two of these germinal derivatives contain deletions of *a1* sequences. Such alleles would be useful for functional analyses of the function of the *a1* gene (ranging from 30 to 358 bp in size).

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Table 1. Rates and classes of germinal revertants isolated from *a1-m5216*

Cross Strategy	No. Perfect Excisions	No. Imperfect Excisions	Total No. Revertants	Population Size	Rate of Germinal Revertants
a1-s Sh2/a1-s Sh2 X *a1-m5216 Sh2/a1*[a] *Sh2*	7 (Class 5)[b]	3 (Class 1, 2, 3)	10	347,300	2.88×10^{-5}
a1-m5216 Sh2/a1 Sh2 X *a1-s Sh2/a1-s Sh2*	3 (Class 5)	0	3	110,000	2.72×10^{-5}
a1-m5216 Sh2/ax-1 X *a1-s Sh2/a1-s Sh2*	2 (Class 5)	1 (Class 4)	3	157,000	1.91×10^{-5}
Pool[c]	12	4	16	614,300	2.60×10^{-5}

[a] Designates either *a1-mrh Sh2* or *a1-mr102b Sh2*

[b] Different classes of germinal revertants are illustrated in Figure 1.

[c] No significant difference among these three populations was detected based on Chi-square homogeneity test. Therefore, data from three cross strategies are pooled and presented in the last row.

Table 2. Summary of germinal adjacent deletions derived from *Mu*-insertion alleles

Locus	Allele	*Mu* Insertion	No. Adjacent Deletions	Genic Deletion (bp)	Rate	Source
adh1	*Adh1-S3034*	*Mu1*	1	74	ND[a]	(TAYLOR and WALBOT 1985)
bz2	*bz2::mu1*	*Mu1*	3	75~77[b]	~2.5% (3/118)	(LEVY and WALBOT 1991)
dmc1a	*dmc1a-93F11*	*Mu1*	0	0	0/1,250	Unpublished data (LIU and SCHNABLE)
hcf106	*hcf106-mum1*	*Mu1*	1	244	~0.3% (1/350)[c]	(DAS and MARTIENSSEN 1995)
pdc1	*pdc1-mu4365*	*dMuDR*	1	202	~0.2% (1/550)	Unpublished data (FU and SCHNABLE)
rad51a	*rad51a-54F11*	*dMuDR*	1	363	ND	LI et al, submitted
rad51b	*rad51b-98E7*	*dMuDR*	4	69~179	~0.8% (4/500)	
rf2a	*rf2a-m8122*	*Mu1*	0	NA[d]	0/1,200	Unpublished data (SKIBBE and SCHNABLE)
rf2c	*rf2c-m1307*	*Mu1*	0	NA	0/1,500	
rf2d	*rf2d-m1310*	*Mu8*	0	NA	0/1,500	
rth1	*rth1-2*	*Mu7*	1	383	~0.02% (1/5,400)	Unpublished data (WEN and SCHNABLE)

[a] No data

[b] Part of *Mu1* (16-22 bp) is deleted for the allele Δ1 of *bz2*. For the rest of adjacent deletion alleles in this table, *Mu* transposon remains intact.

[c] Two *Mu*-insertion alleles are not included.

[d] Not applicable

Table 3. RAD51 is required for repair of *MuDR*-induced DSBs in vegetative tissues

Genotype	*Mu* activity[a]	No. plants with severe developmental defects[b]	No. normal plants
rad51a/rad51a; rad51b/rad51b	active	10[d]	13
Rad51a/rad51a; Rad51b/rad51b[c]	active	0	30
rad51a/rad51a; rad51b/rad51b[e]	inactive	0	15

[a] Plants all carry *a1-m5216* allele. If the majority of kernels with *a1-m5216* from one ear are highly spotted, their mother plant is regarded as to be *Mu* active.

[b] These developmental defects include short plants (less than one-third the height of normal plants), aberrantly shaped leaves, early emerging and tiny tassels.

[c] Plants from two upper categories have very similar genetic background because they are siblings.

[d] One defective plant died two weeks after germination.

[e] This category of plants possess almost same background to two upper categories. However, they do not have active *Mu* because no spotted kernels were observed for two generations.

FIGURE LEGENDS

Figure 1. Summary of germinal revertants of *a1-m5216*

Five classes of germinal revertants are isolated from *a1-m5216*. TSD stands for "Target Site Duplication". Underlined bases belong to TSD. The number in the parenthesis represents how many times each class of germinal revertants is isolated. Bases in small letters do not belong to either *a1* or *MuDR* transposon. Dashed line represents deleted sequences.

Figure 2. Gene structures of *a1-m5216* and *a1::rdt*

Triangles represent either *MuDR* or *rdt* transposons. *MuDR* is located at exon III of *a1-m5216* (upper allele) and *rdt* transposon is located at exon IV of *a1::rdt* (lower allele). Arrows represent primers and their correspondent positions.

Figure 3. Rates and structures of repair products isolated from RAD51 minus maize

[1] Fraction of non-spotted and pale round kernels that carry the indicated type of derivative of *a1-m5216* are 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 derivative 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: apparently intact *MuDR* elements. Most of these kernels carry apparent intact *MuDRs* and are therefore probably non-spotted due to epigenetic inactivation of *MuDR*.

[8] % non-spotted (non-spotted round/total round)

[9] all are Class II

Figure 4. Two proposed models for *Mu* transposition mechanism

Two competing models are presented. Triangles designate *Mu* transposons. The symbol "( )" represents DSBs. Dash line represents deleted region. In the right column, two single-strand nicks are illustrated in a magnified rectangle, where each line represents a single stranded DNA. Elsewhere each line represents dsDNA. Our data provide strong support for Model A.

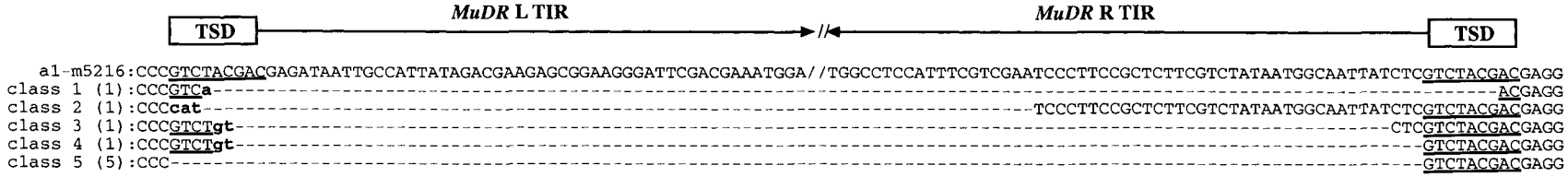


Figure 1. Li *et al.*

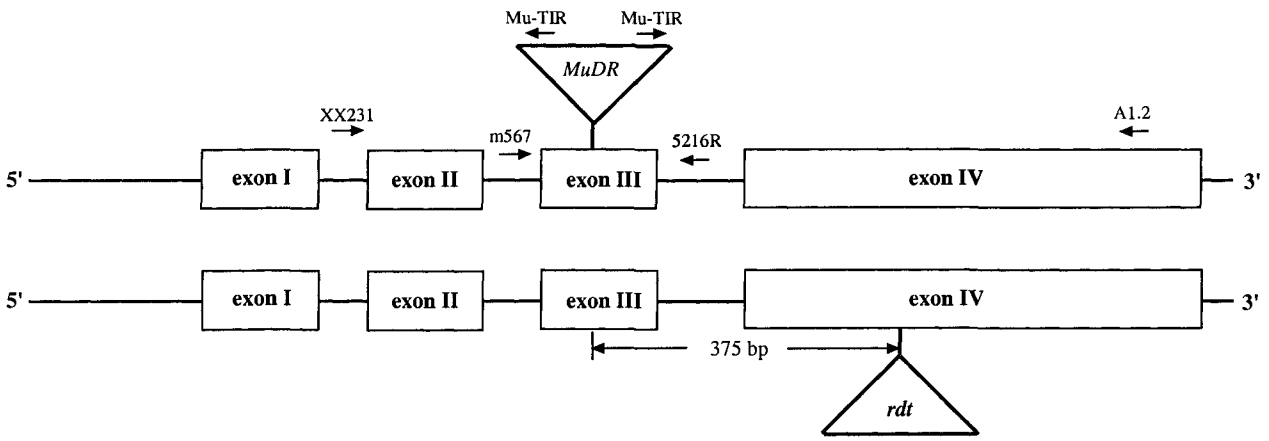


Figure 2. Li *et al.*

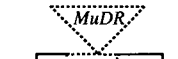
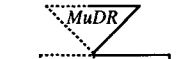
Cross Type	Female Parent[2]	No. Round Kernels[3]					Non-spotted and Pale Round Kernels[1]			
		Spotted	Non-spotted	Pale	Colored	Total	 Class I[4]	 Class II[5]	 Class III[6]	 Class IV[7]
a1-m5216 Sh2/a1::rdt sh2 X *a1-s sh2/a1-s sh2*	RAD51-	25	105 (81%)[8]	0	0	130	36/105	50/105	2/105	17/105
	RAD51+	826	29 (3%)	0	0	855	2/29	7/29	0/29	20/29
a1-m5216 Sh2/ax-1 X *a1::rdt sh2/a1::rdt sh2*	RAD51-	6	28 (67%)	8 (19%)[9]	0	42	13/36	23/36	0/36	0/36
	RAD51+	140	3 (2%)	0	0	143	0/3	3/3	0/3	0/3

Figure 3. Li *et al.*

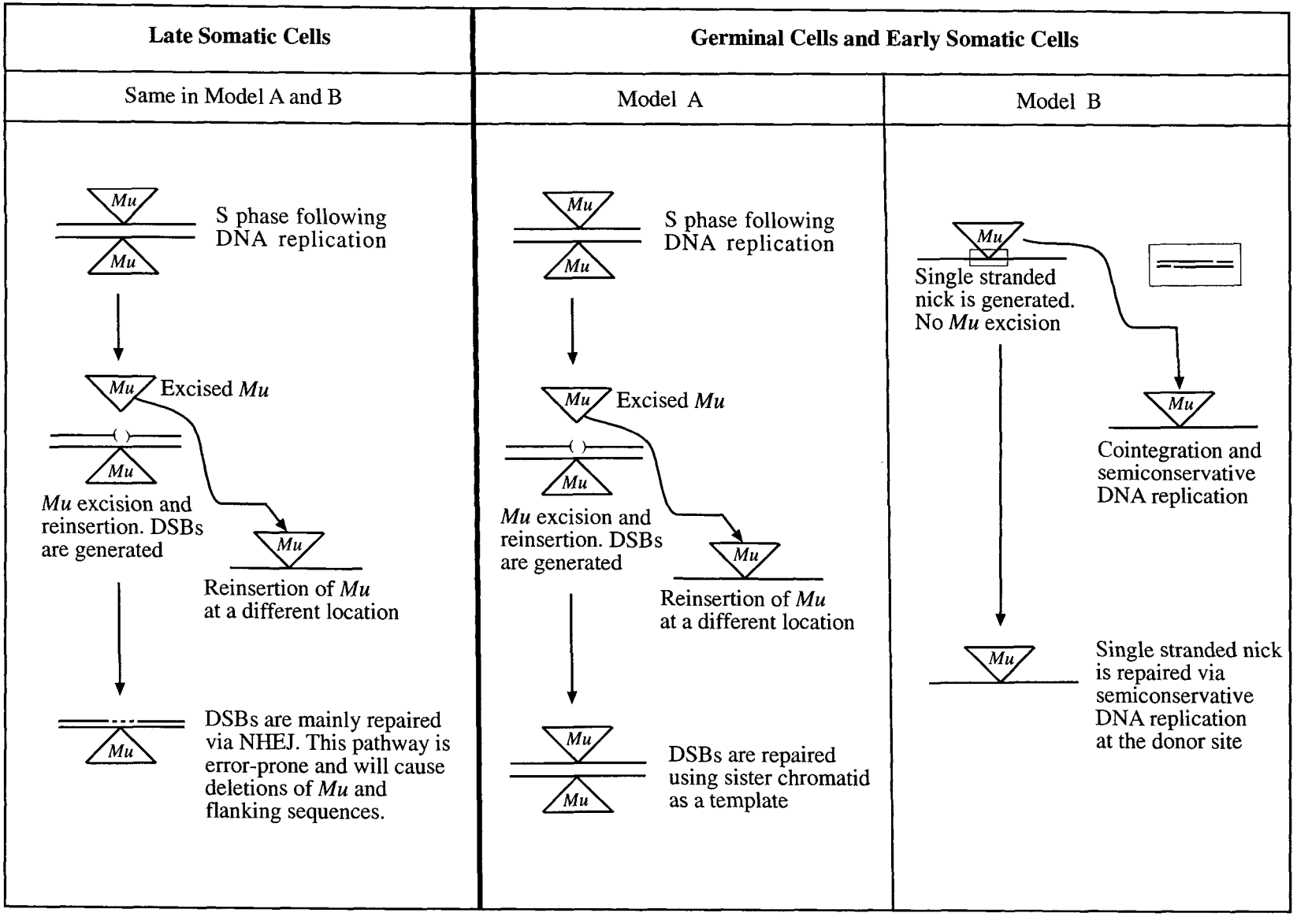


Figure 4. Li *et al.*

CHAPTER 4. GENERAL CONCLUSIONS

Summary and Discussion

There are only two *rad51* genes in maize genome. Isolation of *rad51a* and *rad51b* mutant alleles provides us a means to study their roles in homolog search and chromosome pairing, chromosome segregation, interhomolog recombination during meiosis and in the repair of double-stranded breaks (DSBs) during vegetative development. Although male sterile, *rad51* double mutants can still produce 22% seed set when pollinated as female. These surviving progeny from *rad51* double mutants together with the previously isolated *MuDR*-inserted *a1-m5216* (Hsia and Schnable, 1996) make it possible to study the relationship between RAD51-directed homologous recombination (HR) and *Mu* transposition in maize germinal cells.

Several conclusions can be drawn from our study of the two *rad51* genes in maize: (1) There are only two *rad51* genes in the maize genome and they are functionally redundant. (2) RAD51 is dispensable for vegetative growth under normal condition. (3) RAD51 plays a critical role in the repair of radiation-induced DSBs during early vegetative development. (4) RAD51 is required for proper chromosome segregation in meiosis. (5) Although it has been suggested that in maize RAD51 plays a role in DNA homology search and chromosome pairing (Franklin *et al.*, 1999; Pawlowski *et al.*, 2003), our data demonstrate that RAD51 is not essential for these processes. (6) RAD51 is not essential for meiotic crossovers in surviving female gametes produced from *rad51* double mutants.

The study of RAD51's role in *Mu* transposition provides us insights about *Mu* transposons in maize. (1) RAD51-directed HR is required for repairing *MuDR*-induced

DSBs in germinal cells. (2) Our experiments provide strong experimental support for Model A (Donlin *et al.*, 1995; Hsia and Schnable, 1996; Lisch, 2002; Walbot and Rudenko, 2002) that differences in DNA repair pathways are responsible for the different behaviors of *Mu* transposons in germinal and late somatic cells. (3) The two ends of *Mu* transposon are not cut simultaneously when *Mu* transposes in germinal cells. (4) *MuDR* is active in pre-meiotic cells and RAD51-directed HR is the predominant pathway to repair *MuDR*-induced pre-meiotic DSBs. (5) RAD51 plays a critical role in the repair of *MuDR*-induced DSBs during early vegetative development. (6) Germinal derivatives of *Mu*-insertion alleles can be recovered at a significantly higher rate in *rad51* double mutants as compared to wild-type controls.

Two mutant alleles of *rad50* gene were isolated and characterized in our study. Our molecular and genetic data indicate that RAD50 is essential for early embryogenesis in maize. In contrast, *Arabidopsis rad50* mutants are sterile but viable (Gallego *et al.*, 2001). It has been shown that maize RAD51 is not essential for meiotic chromosome pairing, which is also different from that observed in *Arabidopsis rad51* mutants (Li *et al.*, 2004). These apparently different functions of RAD50 and RAD51 between maize and *Arabidopsis* demonstrate the importance to study gene functions using multiple model organisms even in the plant kingdom.

Our genetic analysis did not detect significant differences in the rates of meiotic crossovers between *rad51* double mutants and controls from two linked intervals (*at1-et1*, IDP1440-IDP1983). In contrast, the recombination between sister chromatids was reduced dramatically based on our study of the relationship between RAD51-directed HR and *Mu* transposition in maize. Thus, we hypothesize that one or two maize *dmc1* genes (Franklin *et*

al.) can complement RAD51 in mediating meiotic crossovers, and similar complementation has been observed in *Saccharomyces cerevisiae* (Shinohara *et al.*, 1997). More importantly, Rad51p, but not Dmc1p, can mediate recombination between sister chromatids in yeast (Arbel *et al.*, 1999; Shinohara *et al.*, 2000). The different roles of Rad51p and Dmc1p in interhomolog and interchromatid recombination in yeast (Arbel *et al.*, 1999; Shinohara *et al.*, 2000) are consistent with our observation that interchromatid recombination was greatly reduced, whereas interhomolog recombination was not affected in *rad51* double mutants. Therefore, it is likely that maize DMC1 and RAD51 function differently during interhomolog and interchromatid recombination in a similar manner to that described in yeast (Arbel *et al.*, 1999; Shinohara *et al.*, 2000). We predict that meiotic recombination pathway will be greatly shifted to interhomolog recombination in *rad51* double mutants. In addition, *MuDR* is active during meiosis and can increase the rate of crossovers up to fourfold at *a1* locus (Yandeau-Nelson *et al.*, 2005). Our future goal is to test if *MuDR*-induced DSBs in meiosis will lead to significantly increased rate of interhomolog recombination in *rad51* double mutants. Functional characterization of *dmc1* in maize is also very critical to test the hypothesis that maize DMC1 and RAD51 can complement each other during interhomolog recombination.

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APPENDIX. RAD50 IS REQUIRED FOR EMBRYOGENESIS IN MAIZE

Introduction

The yeast (*Saccharomyces cerevisiae) RAD50* gene was cloned via the identification of a plasmid that can complement gamma-ray sensitive *rad50* mutants (Kupiec and Simchen, 1984). This gene encodes a predicted 153-kD protein containing a purine nucleotide-binding domain and two large heptad-repeat regions (Alani *et al.*, 1989). Double-strand breaks (DSBs) are unprocessed in a null *rad50* mutant (*rad50S*) and can be physically mapped in yeast (Cao *et al.*, 1990). Rad50p, together with Mre11p (Ajimura *et al.*, 1993) and Xrs2p (Ivanov *et al.*, 1992) form a protein complex, which is involved in DNA repair and meiotic recombination (Johzuka and Ogawa, 1995; Usui *et al.*, 1998; Chamankhah and Xiao, 1999).

The mouse *RAD50* gene is essential for embryogenesis and cell viability (Luo *et al.*, 1999). Nijmegen breakage syndrome (NBS) is an autosomal recessive chromosomal instability syndrome characterized by growth retardation, immunodeficiency and cancer predisposition. *NBS1* was mutated in NBS cells and was isolated using the positional cloning approach (Varon *et al.*, 1998). The Mre11-Rad50-Nbs1 protein complex is required for the repair of cellular DNA damage (Petrini, 1999) in a similar manner as the Mre11p-Rad50p-Xrs2 complex in yeast (Chamankhah and Xiao, 1999).

Arabidopsis RAD50 (*AtRAD50*) is required for plant fertility, resistance to methyl-methanesulfonate (MMS) and telomere maintenance (Gallego *et al.*, 2001; Gallego and White, 2001). More interestingly, somatic homologous recombination significantly increases in the absence of AtRad50 and it has been hypothesized that Non-homologous End-joining pathway (NHEJ) has greater dependence than homologous recombination (HR) on the prior

action of Rad50/Mre11/(Xrs2/Nbs1) complex (Gherbi *et al.*, 2001). *AtMRE11* is required for maintenance of chromosome stability in both mitosis and meiosis (Puizina *et al.*, 2004). The protein interaction between AtRAD50 and AtMre11 has been shown via co-immunoprecipitation (Daoudal-Cotterell *et al.*, 2002) and the ortholog of *XRS2* or *NBS1* has not been identified in *Arabidopsis* genome yet.

To understand the functions of *rad50* in maize, we isolate and functionally characterize two *rad50* mutant alleles in the following study.

Results

Isolation of *Mu*-Insertion Alleles of *rad50*

Five *Mu*-insertion alleles of *rad50* (*rad50-52B7, rad50-66D, rad50-52E2, rad50-109H5* and *rad50-75C11*) were recovered via the reverse genetic screen, Trait Utility System for Corn (TUSC, Bensen *et al.*, 1995).

Members of the *Mu* transposon family share approximately 200-bp conserved terminal inverted repeats (TIRs). PCR was performed on plants that carry each *Mu*-insertion allele using a series of gene-specific primers in combination with a primer located in the highly conserved *Mu* TIR. Sequence analysis of the resulting PCR products established which class of *Mu* transposon was inserted in each of the TUSC alleles and the positions of the *Mu* insertions (Figure 1).

Out of the five TUSC alleles, two of them (*rad50-109H5* and *rad50-75C11*) contained a *Mu* insertion in the coding region (Figure 1). Therefore, these two alleles were used to study functions of *rad50* in maize.

RAD50 is not required for gamete viability in maize

Two *rad50* alleles (*rad50-109H5* and *rad50-75C11*) were chosen to further characterize functions of *rad50*. Genetic analysis indicates that both alleles can transmit normally through male and female gametes (Table 1). Therefore, RAD50 is not essential for gamete development in maize.

RAD50 is essential for embryogenesis in maize

Self-pollination was performed to isolate homozygous plants using both alleles (*rad50-109H5* and *rad50-75C11*). Interestingly, no homozygous *rad50* plant was recovered after all progeny were genotyped via PCR. In contrast, the other two classes of plants with wild type *Rad50* can be recovered at normal rates as expected (Table 2). Because both alleles have been shown to transmit normally through female and male gametes (Table 1), these results demonstrate that RAD50 plays an essential role during embryogenesis in maize.

Discussions

Genetic analysis using two mutant alleles (Table 2) establishes that RAD50 is essential for embryogenesis in maize, which is consistent with the previous report that mouse Rad50 is essential for stem cell viability and early embryogenesis (Luo *et al.*, 1999). RAD50/MRE11/(XRS2/NBS1) protein complex is involved in two major pathways: HR and

NHEJ, which are required for repairing DSBs (Jeggo, 1998; Pastwa and Blasiak, 2003). The indispensable role of RAD50 in maize embryogenesis indicates that repair of DSBs is essential for normal embryo development in maize.

Although *Arabidopsis RAD50* (*AtRAD50*) is required for plant fertility, resistance to methyl-methanesulonate (MMS) and telomere maintenance, it is not essential for viability (Gallego *et al.*, 2001; Gallego and White, 2001). In contrast, maize RAD50 is essential for embryogenesis (Table 2). Functions of RAD50 between maize and *Arabidopsis* are not completely conserved in this aspect. In addition, it has been shown that maize RAD51 is not essential for meiotic chromosome pairing, which is also different from the report that meiotic chromosome pairing is completely disrupted in absence of AtRAD51 (Li *et al.*, 2004). The functional divergence between maize and *Arabidopsis* demonstrates the importance to study gene functions using multiple model organisms even in the plant kingdom.

Mu transposons of five isolated *rad50* TUSC alleles are all inserted in the first exon (Figure 1). Among them, three *Mu* insertions are located in the 5' untranslated region. This *Mu* insertion pattern is similar to the previous report that *Mu* transposons are targeted to the 5' untranslated region of the *gl8* gene (Dietrich *et al.*, 2002). Knowledge of the targeting pattern of *Mu* transposons will be very useful for the initial primer design in reverse genetic experiments using *Mu* transposons.

Table 1. Transmission ratios of *rad50* through male and female gametes

Name of Alleles		Number of Progeny	
		with *rad50*	with *Rad50*
rad50-109H5	*rad50-109H5/Rad50* X inbred[1]	12	13
	inbred X *rad50-109H5/Rad50*[2]	22	20
rad50-75C11	*rad50-75C11/Rad50* X inbred[3]	11	9
	inbred X *rad50-75C11/Rad50*[4]	6	13

[1] Their pedigrees are 03-2567-7/2736-8 and 03-2567-11/2736-1

[2] Their pedigrees are 03-2588/2565-5, 03-2588/2566-3, 03-2559/2568-1 and 03-2553/2568-6

[3] Their pedigrees are 03-2571-4/2710-11, 03-2572-9/2710-11

[4] Their pedigrees are 03-2553/2571-3, 03-2559/2570-5 and 03-2588/2571-3

Table 2. RAD50 is required for early embryogenesis in maize

Cross Strategy	Number of Progeny		
	Rad50/Rad50	*Rad50/rad50*[1]	*rad50/rad50*
Rad50/rad50-109H5⊗[2]	18	48	0
Rad50/rad50-75C11⊗[3]	31	53	0

[1] *rad50* can be either *rad50-109H5* or *rad50-75C11*

[2] These genotyped seedlings come from six related crosses: 03g-1229-1⊗, 03g-1229-2⊗, 03g-1230-5⊗, 03g-1230-6⊗, 03g-1230-9⊗ and 03g-1230-11⊗.

[3] These genotyped seedlings come from seven related crosses: 03g-1231-1⊗, 03g-1231-4⊗, 03g-1231-6⊗, 03g-1231-9⊗, 03g-1230-9⊗, 03g-1232-1⊗ and 03g-1232-10⊗.

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Figure Legends

Figure 1. Structures of five *rad50* TUSC alleles

Exons are represented with boxes and only the first two exons of *rad50* are shown in this figure. The first ATG site is marked with "*". *Mu* insertions in TUSC alleles are indicated by triangles. The *rad50-52B7*, *rad50-109H5* and *rad50-75C11* alleles have a *Mu1* insertion in exon I. *Mu7* and *dMuDR* are inserted in *rad50-52E2* and *rad50-52B7*, respectively. Positions of PCR primers are indicated with arrows.

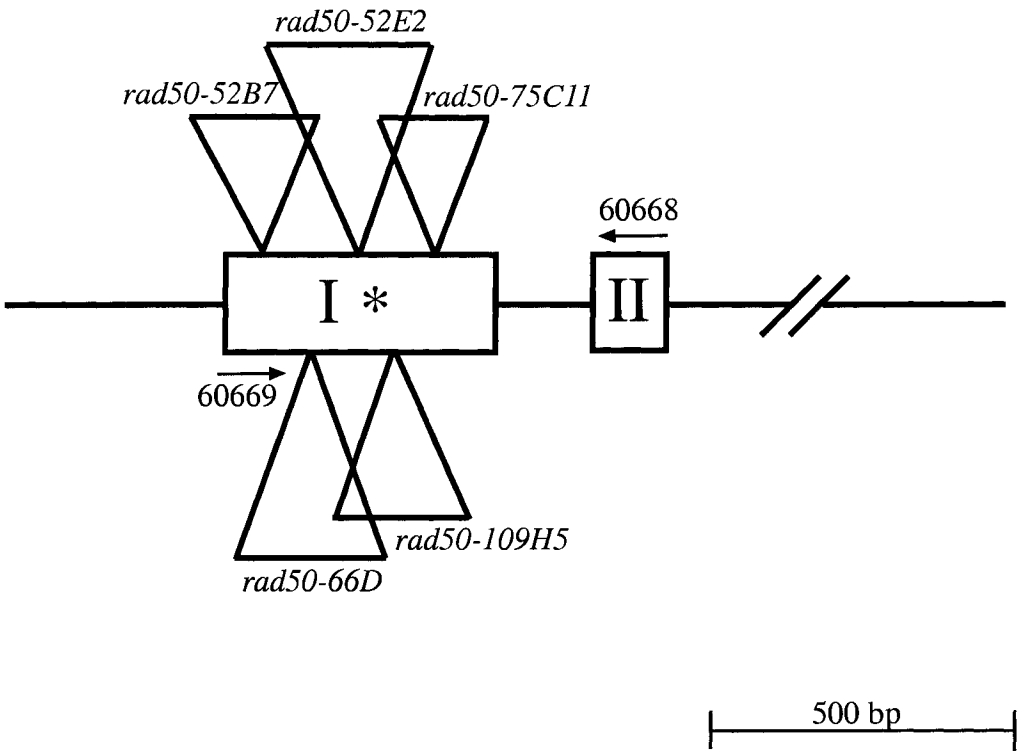
rad50-52E2
rad50-52B7
rad50-75C11
60668
I *
II
60669
rad50-109H5
rad50-66D
500 bp

Figure 1

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