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Determination of Rates and Patterns of Recombination at the Maize Red Color (r1) Locus

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Determination of rates and patterns of recombination at the maize *red color* (*r1*) locus

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University

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Abstract

DETERMINATION OF RATES AND PATTERNS OF RECOMBINATION AT THE MAIZE *RED COLOR* (*R1*) LOCUS

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Homologous recombination has been studied in many plant and animal systems. In maize, most recombination occurs intragenically. The current study assessed the frequency and location of meiotic recombination at the maize *red color* (*r1*) gene. Three independent mutant (colorless seed) alleles derived from the colored seed allele, *R-sc:124,* were made heterozygous with the colored plant allele, *r-r:n142*. Each mutant resulted from the insertion of a *Dissociation* (*Ds*) transposable element into the 3' end of *R-sc:124*. In the presence of *Activator* (*Ac*), each *Ds* insertion allele (*r-sc:mutables*) produced spotted seeds and germinal reversion to fully colored seeds due to the excision of *Ds*. In the absence of *Ac*, each insertion allele stable and produces

colorless or very faintly pigmented seeds. The *r-sc:mutable/r-r:n142* heterozygotes were pollinated with *R-g:8pale*, an *r1* allele that produces pale brown seeds, in the absence of *Ac,* to recover revertant progeny likely resulting from recombination rather than *Ds* excision. Revertant progeny were self-pollinated to verify paternity. Molecular analysis using polymerase chain reaction, Southern blot and DNA sequence analyses showed that 92 out of 94 of the revertant alleles arose via a cross over event between the *r-sc:mutable* and *r-r:n142* chromosomes. The remaining two revertant alleles likely arose via gene conversion, double cross over or cryptic *Ac* activity. The ratio of genetic to physical distance ($1/\rho$) calculated for the 3' end of *r1* is approximately 0.07 cM/kb, which is comparable to $1/\rho$ values calculated for *a1*, *b1*, *bz1* and *wx1* loci. Given the average value for $1/\rho$ throughout the genome is 0.00021 cM/kb, this analysis supports the hypothesis that recombination in maize primarily occurs intragenically and that *r1* serves as a recombination hot spot in the maize genome. Molecular analysis also revealed that the majority of exchanges occurred at the 3' end of *r1* as has been previously observed. The pattern of recombination observed at *r1* is different from those observed at other maize loci. The recovery of substantially more cross over events (92/94) relative to non-cross over events (2/94) or the nature of the recombination event is consistent with previous observations at *a1, b1* and *bz1*. The frequency of potential gene conversion is estimated at 3.97×10^{-5} and the gene conversion

tract length is maximally 2.5 kb. The possibility of the influence of insertional mutations, amount and structure of DNA sequence homology, *cis*- and *trans*-factors and preference for cross over versus non-cross over events could explain the observed pattern at *r1*.

Introduction

Homologous recombination occurring in eukaryotic genomes can effect a change in genetic diversity (Schnable *et al.* 1996) as well as repair double strand breaks in DNA. Two products of recombination in eukaryotic genomes are crossing over and gene conversion. Crossing over involves the reciprocal exchange of information between homologous DNA duplexes, whereas gene conversion is the unidirectional transfer of information without reciprocity (Roeder 1990). Central to these processes is the Holliday Junction and its resolution (Holliday 1974) (Figure 1). Several models (see Stahl 1994 for review) have been postulated to explain the mechanistic basis for recombination and gene conversion; however, most retain the central tenets of Holliday's original model. An important aspect of current models is the recognition of homologous sequences to repair double strand gaps which induce recombination. Several models have proposed the involvement of multiple proteins and/or the synaptonemal complex to effect homology recognition (Roeder 1990; 1995). Recombination models also propose that once recognition occurs, the broken ends are degraded by a 5' to 3' exonuclease leaving overhanging 3' ends which invade the homologous template via the action of Rec-A-like proteins. Each invading strand acts as a primer to initiate DNA synthesis using the intact duplex as template (Figure 1).

Breakage and rejoining of the two resulting Holliday junctions formed yield two possible arrangements, crossing over or gene conversion, depending on whether the inner or outer strands are cleaved and re-ligated.

Recombination has been studied in many plant systems including *Arabidopsis* (e.g., Assaad and Signer 1992; Mourad *et al.* 1994), *Brassica* (e.g., Gal *et al.* 1991), *Zea mays* (Nelson 1968; Moore and Creech 1972; Freeling 1976; Dooner and Kermicle 1971, 1976, 1986; Dooner 1986; Kermicle 1988; Dooner and Ralston 1990; Robbins *et al.* 1991; Sudupak *et al.* 1993; Civardi *et al.* 1994; Hu and Hulbert 1994; Richter *et al.* 1995; Eggleston *et al.* 1995; Patterson *et al.* 1995; Xu *et al.* 1995; Schnable *et al.* 1996; Timmermans *et al.* 1996, 1997; Dooner and Martinez-Ferez 1997; Okagaki and Weil 1997), *Nicotiana tabacum* (Lee *et al.* 1990; Peterhans *et al.* 1990; Tovar and Lichtenstein 1992; Offringa *et al.* 1993) and *Triticum* sp. (Dvorak and Appels 1986). Furthermore, the resolution of the Holliday junction has been proposed to occur preferentially in eukaryotic genic sequences (Thuriaux 1977). While the number of genes remains fairly constant among eukaryotes, Thuriaux (1977) showed that the length of genetic maps among eukaryotes also remains fairly constant despite an increase in genome size. Thus, he concluded that the majority of recombination exchanges occur within, not between, the short DNA segments corresponding to structural genes.

Recombination has been studied at many loci in maize. At the *red color* (*r1*) locus, Robbins *et al.* (1991) determined the position and orientation of

genic elements in *R-r:standard* (*R-r*) by examining patterns of unequal crossing over among tandemly repeated genes in the complex. Southern blot analysis of the presence or absence of restriction site polymorphisms between progenitor and progeny alleles with altered phenotypes determined that *R-r:standard* consists of two genetic components, (P) and (S). The (P) component pigments plant tissue (e.g. coleoptiles and anthers) whereas the (S) complex directs pigment deposition in the aleurone layer of the kernel (Robbins *et al.* 1991). (P) is comprised of a single *r1* gene, *P*, whereas (S) is comprised of several *r1* genes or fragments of genes in the proximal to distal order *q, S1* and *S2* (Robbins *et al.* 1991). These molecular data support previously obtained genetic data on the structure of *R-r:standard* (Dooner and Kermicle 1971). Using similar methods, Eggleston *et al.* (1995) determined the structure and orientation of genic elements in the maize *R-stippled* complex. *R-stippled* consists of four tandemly arranged *r1* genes (*Sc, Nc1, Nc2* and *Nc3*) oriented in the same direction as the *P* gene of *R-r* (Eggleston *et al.* 1995). Both studies demonstrated that the majority of recombination events were intragenic and could be localized to the 3' ends of identified *r1* genes (Robbins *et al.* 1991; Eggleston *et al.* 1995)

Dooner (1986), Civardi *et al.* (1994), Patterson *et al.* (1995), Xu *et al.* (1995) and Okagaki and Weil (1997) hypothesized that regions within genes [e.g. *anthocyaninless1* (*a1*), *booster1* (*b1*)] or entire genes themselves [e.g. *bronze1* (*bz1*), *waxy1* (*wx1*)] may be hot spots for recombination events in

maize. For example, the ratio between genetic and physical distance ($1/\rho$) at *a1* (0.03 cM/kb), *b1* (0.05 cM/kb), *bz1* (0.07 cM/kb), and *wx1* (0.07 - 0.10 cM/kb) loci is considerably higher than the overall average for the entire maize genome (0.00021 - 0.00068 cM/kb), indicating that simple sequences such as genes act as hot spots for recombination. Further study suggested that most if not all recombination in maize occurs within genes (Dooner 1986; Xu *et al.* 1995; Patterson *et al.* 1995; Dooner and Martinez-Ferez 1997; Okagaki and Weil 1997). The pattern or where recombination occurs in a gene varies among different maize loci. Within *a1*, Xu *et al.* (1995) determined that one-fifth of all recombination events occurred in a 377 base pair region at the 5' end of the locus. Similarly, Patterson *et al.* (1995) determined that the 5' end of the *b1* locus is recombinationally more active than 3' sequences. However, Dooner and Martinez-Ferez (1997) determined that the pattern of recombination at *bz1* is dispersed throughout the gene. The *wx1* locus was characterized extensively in search of possible mechanisms to explain why it is a recombination hot spot in the genome. A deletion of promoter sequences (see below) and/or insertion polymorphisms have little effect on recombination frequencies. The data also show that there is no preferred site for recombination within *wx1* (Okagaki and Weil 1997).

Consistent with the hypothesis that recombination may occur preferentially in unique sequences, Dvorak and Appels (1986) observed no homologous crossing over between rDNA repeat units in wheat. Additionally,

Timmermans *et al.* (1996) characterized a recombination event without phenotypic selection (i.e., without a change in gene function) by using a physical mapping approach. The cross over mapped to a 534 bp region of 100 percent homology between the two parental chromosomes. This unmethylated region was located in a 2,773 bp unique sequence embedded in a region of repetitive DNA sequence.

Transposable elements have been used in many studies of recombination. The insertion of transposable elements into genes can create stable mutants. Such mutants can be used as starting materials in a series of genetic crosses to generate "wild type" progeny resulting from recombination (Figures 2 and 3). Transposable elements are units of DNA capable of movement from one region and insertion into another. Certain transposable elements are capable of removing themselves (excision) from pre-existing sites during transposition while others are not. One of the most useful is the *Ac/Ds (Activator/Dissociation)* family of transposable elements in maize first discovered by Barbara McClintock and now well characterized (reviewed in Fedoroff 1989). *Ac/Ds* elements belong to a class of transposable elements with short, terminal inverted repeats (TIRS) and may transpose via a DNA intermediate using a "cut-and-paste" mechanism (reviewed in Fedoroff 1989). *Ac* and *Ds* have similar TIRS which play a role in transposition (reviewed in Fedoroff 1989). *Ac* elements contain an open reading frame (ORF) encoding a transposase which mediates the element's excision as well as that of other

family members; such elements are termed autonomous (Pohlman *et al.* 1984). *Ds* elements typically are deletion derivatives of *Ac* with the central portion missing and are not capable of producing transposase (reviewed in Fedoroff 1989). However, *Ds* elements can transpose in the presence of *Ac* elements, and therefore are termed non-autonomous. As noted above, transposable element insertions can disrupt gene function, resulting in a mutant phenotype (reviewed in Berg and Howe, 1989). Transposable elements often create direct duplications of short sequences, typically 3-9 bp of host DNA (e.g. target site duplications), during insertion (reviewed in Berg and Howe 1989). Loss of a transposable element insertion by excision or recombination can restore gene function, producing progeny (termed revertants) with a wild type or near wild type phenotype.

The insertion of transposable elements has been found to influence the rate of recombination at several loci (Dooner and Kermicle 1986; Dooner and Ralston 1990; Lowe *et al.* 1992). Insertions of a *Mutator1* (*Mu1*) transposable element, in the absence of the autonomous *MuDr* elements, suppress the rate of crossing over in *a1* (Xu *et al.* 1995). Dooner (1986) also noted that relative to a point mutation, a *Ds* insertion in *bz 1* reduced intragenic crossing over. Based on molecular data, Dooner and Martinez-Ferez (1997) further detailed the suppressive effect *Ds* insertions have on recombination. They found that recombination between two *bz 1* alleles with *Ds* insertions separated by 600 bp in a completely homologous region was reduced fourfold relative to point

mutations at the same locations. However, in the presence of an *Ac* element, *Ds* insertions in *r1* did not increase the rate of crossing over in the vicinity of *r1* (J. L. Kermicle, pers. comm.).

Dooner and Kermicle (1986) observed that transposable element insertions in *bz1* and *r1* loci alter the nature of intragenic recombination. Recombination between *r1* alleles with *Ds* insertions at distinct sites and in the absence of *Ac*, produced many different recombinant types. However, revertant progeny with parental flanking genetic markers, indicating that these revertants resulted from a gene conversion or double cross over event, were most common (i.e., approximately 50% of the total number of recombinant types) (Dooner and Kermicle 1986). Revertant progeny derived from plants heterozygous for the same *Ds* insertion mutation alleles and point mutation alleles typically had non-parental flanking marker composition consistent with crossing over (i.e., approximately 92% of the total). Additional data at *a1* suggest that *Mu1* does not appear to play a role in the resolution of recombination events (Xu *et al.* 1995). Recombination data at *b1* using *Ds* insertions also indicate transposable elements may alter the ratio of crossover to non-crossover products (Patterson *et al.* 1995). Similarly, Dooner and Martinez-Ferez (1997) observed that the majority of revertant *bz1* alleles arose via a crossover event as opposed to gene conversion based on flanking marker analysis. Dooner and Ralston (1990) used *Mu* and *Ds* insertion mutations in heterozygous combinations at *bz1* to determine the nature of

recombination events. They found that insertion heterozygosities may not affect the pattern of intragenic recombination (e.g. a change in the ratio of cross over vs. non-cross over events), but rather the nature of the mutation (e.g. insertion vs. point) may cause a change in the pattern of recombination (Dooner and Kermicle 1986; Dooner and Ralston 1990). Athma and Peterson (1991) reported that active *Ac* elements between, but not in, two 5.2 kb repeats flanking the maize *pericarp color* (*p1*) locus increased the frequency of unequal crossing over between the repeats. Lowe *et al.* (1992) also reported that *Mu* element insertions located between the repeats at the *knotted1 (kn1)* locus and in the presence of active *MuDr* elements, increased the frequency 100 - 2000 fold of unequal recombination between repeats.

Additionally, recombination studies at *a1, b1* and *bz 1* have documented that cross over events, as opposed to non-cross over events such as gene conversion or double cross overs, are recovered at a higher frequency (Dooner 1986; Patterson *et al.* 1995; Xu *et al.* 1995; Dooner and Martinez-Ferez 1997). The lack of gene conversion or double cross overs potentially may be explained by the high chiasma interference in certain genomic regions (Dooner 1986; Dooner and Martinez-Ferez 1997).

Recombination events also play a role in generating new disease resistance in plants (Sudupak *et al.* 1993; Hu and Hulbert 1994; Richter *et al.* 1995). Screening of the *Rp1-J* and *Rp1-G* loci, which are part of a complex of rust resistance genes in maize, determined that novel resistance specificities

likely were generated by unequal pairing (intrachromosomal recombination), intragenic crossing over and/or gene conversion (Sudupak *et al.* 1993; Richter *et al.* 1995). Hu and Hulbert (1994) determined that gene conversion occurs at the *Rp1* locus at a rate of approximately 7×10^{-4} gametes per generation and contributes to the meiotic instability of this complex locus.

Gene conversion has been studied in bacteria (reviewed in Klein 1995; see also Yamamoto *et al.* 1996), *Neurospora* (Bowring and Catcheside 1996), *S. cerevisae* (reviewed in Klein 1995; see also Vincent and Petes 1989; Welch *et al.* 1991; Nicolas and Petes 1994; Gangloff *et al.* 1996; Weng *et al.* 1996), *Drosophila* (Engels *et al.* 1990; Gloor *et al.* 1991; Nassif and Engels 1993) and in plants (Lee *et al.* 1990; Peterhans *et al.* 1990; Assaad and Signer 1992; Tovar and Lichtenstein 1992; Offringa *et al.* 1993; Dooner and Martinez-Ferez 1997; Mathern and Hake 1997). As noted above, differential cleavage and resolution of the Holliday Junctions produced during recombination will yield either crossover or gene conversion products (Figure 1).

Gene conversion tracts generally are between 300 and 850 bp long in bacteria (Kowalchuk *et al.* 1995) and between 1 and 2 kb long in yeast (Judd and Petes 1988; Borts and Haber 1989), *Drosophila* (Gloor *et al.* 1991) and plants (Assaad and Signer 1992). However, Voelkel-Meiman and Roeder (1990) reported that some gene conversion tracts lengths induced by *HOT-1*, a recombinationally active sequence in yeast, were between 33 kb and 77 kb in length. *HOT-1* is thought to induce double strand DNA breaks followed by a

replicative repair mechanism which may account for the extended gene conversion tracts at this site (Voelkel-Meiman and Roeder 1990).

Clusters of recombination events in particular intervals at several yeast genes result in higher recombination frequencies at one end of the gene relative to the other end, a condition referred to as polarity. That is, recombination is non-random throughout these genes. Evidence from the yeast *ARG4* gene and other genes indicates that specific sites at the 5' end, possibly promoter sequences, may initiate meiotic gene conversion (Nicolas *et al*. 1989; Fan *et al*. 1995; reviewed in Roeder 1995). Recombination data obtained from the maize *a1* and *b1* loci indicate that hot spots for recombination events exist at the 5' ends of these genes (polarity) (Patterson *et al*. 1995; Xu *et al*. 1995). However, work at *bz1 and wx1* indicates that the recombination pattern is dispersed throughout the gene (little or no polarity) (Dooner 1986; Dooner and Martinez-Ferez 1997; Okagaki and Weil 1997). Gene conversion tracts can extend in both directions from the initial double strand gap, as documented in yeast (Schultes and Szostak 1990) and *Drosophila* (Gloor *et al*. 1991). Preferential breakage at one end of a gene coupled with a greater likelihood that sequences closer to the break will participate in conversion is thought to account for the polarity (reviewed in Lichten and Goldman 1995). Gene conversion tracts typically are continuous, meaning that there is no switching of templates used in copying homologous

sequences from one duplex to another (Voelkel-Meiman and Roeder 1990; Gloor *et al.* 1991; Nassif and Engels 1993; Weng *et al.* 1996).

The use of *Agrobacterium*-mediated transformation of cell cultures has provided an important tool in studying recombination, particularly gene conversion, in plants. Peterhans *et al.* (1990) inserted plasmids containing pairs of nonfunctional antibiotic drug resistance genes into tobacco protoplasts to study recombination. Only crossing over or gene conversion could restore function to these genes. Selection for drug resistance provided a simple and effective means to identify and quantify recombinant cell progeny. Lee *et al.* (1990), Tovar and Lichtenstein (1992) and Assaad and Signer (1992) also used similar constructs to study gene conversion and homologous recombination. All four investigations produced similar recombination frequencies in the range of 10^{-4} and 10^{-6} gametes for germinal events. These events most likely occurred intrachromosomally as opposed to studies involving alleles on homologous chromosomes (interchromosomal) typically tested in maize (see above). Additionally, complex alleles and transgenes often are susceptible to genome silencing mechanisms such as cytosine methylation or condensed chromatin and may complicate these results (Bestor and Tycko 1996; Martienssen 1996; Kass et al. 1997).

Zea mays provides an important tool for recombination research. It has been the subject of intense investigation and a large body of knowledge already exists regarding recombination. The mating system of corn permits

controlled pollinations to be performed easily due to the physical separation of "male" and "female" flowers. A large number of progeny can be generated using maize since each ear can contain 200 - 400 kernel progeny.

The maize *r1* locus belongs to small family of regulatory genes encoding transcriptional activator protein containing a helix-loop-helix motif related to the *myc* family of proto-oncogenes. Other family members include *b 1*, *leaf color* (*lc1*) and *scutellar node* (*sn1*) loci (Kermicle 1980; Ludwig *et al.* 1989; Ludwig and Wessler 1990; reviewed in Dooner *et al.* 1991). *lc1* and *sn1* are displaced duplicated *r1* genes (Dooner and Kermicle 1976; Ludwig *et al.* 1989) and are 98% homologous to *R-sc:124* (Dooner *et al.* 1991; Alleman and Kermicle 1993). Alleles of the *b 1* locus are "highly homologous" to *r1* at the 3' end (Chandler *et al.* 1989; Dooner *et al.* 1991; Radicella *et al.* 1992). Anthocyanins are red, dark purple pigments and most commonly associated with floral tissues but also can be present in leaves, roots and seeds. If maize plant tissues such as leaves or roots lack anthocyanins they are green or white, respectively, and seeds are yellow. In the work performed here, the phenotype of rare revertant kernels (darkly colored) is easily distinguishable from the phenotype of non-revertant kernels (yellow to pale brown). For simplicity, the phenotype of revertant kernels will be termed black.

Tissue specificity for *b1, lc1, r1* and *sn1* alleles is determined by their promoter regions (Kermicle 1980; Ludwig *et al.* 1989; Ludwig and Wessler 1990; Dooner *et al.* 1991). Certain *r1* alleles are described using a two letter

nomenclature with the first letter designating seed color and the second, plant color. For example, *R-g* alleles specify a colored (*R*-) aleurone and green plant (*-g*) parts; similarly, *r-r* alleles indicate a colorless aleurone (*r*-) and red plant (*-r*) parts. However, not all *r1* alleles are described using this system. *R-sc,* a seed color derivative of *R-stippled,* produces darkly pigmented or self-colored seeds and green plant parts (Eggleston *et al.* 1995). *R-sc:124,* a simplex allele derived from *R-stippled* by unequal crossing over between *Sc* and *Nc3* (Alleman and Kermicle 1993; Eggleston *et al.* 1995), directs strong anthocyanin pigmentation in the aleurone and scutellum layers of the maize kernel. *R-sc:124* also directs strong pigmentation in coleoptile tips. In contrast, *r-r:n142,* a simplex allele derived from *R-r:standard* by unequal crossing over (Robbins *et al.* 1991), directs anthocyanin production in plant parts (anthers, coleoptiles and roots) but not in kernels. Screening coleoptiles and roots of germinating seedlings permits easy identification of *R-sc:124* (red coleoptiles tips) versus *r-r:n142* (red coleoptiles and roots) alleles. The coding regions of *R-sc:124* and *r-r:n142* are interchangeable (Kermicle 1980), providing suitable substrates for recombination to occur.

The current study expands recombination work at *r1* in a number of ways. The data were used to test for a polarity gradient of recombination in *R-sc.* Data from Robbins *et al.* (1991) and Eggleston *et al.* (1995) suggest that recombination preferentially occurs between the 3' ends of *r1* genes in *R-r* and *R-st* as opposed to the 5' ends or central regions. However, these are complex

alleles containing multiple tandem *r1* genes and can recombine intrachromosomally. Thus, the pattern of recombination observed for these alleles could be an artifact of intra- versus interchromosomal recombination due methylation at the 5' ends which has been shown to influence recombination.

Restriction fragment length polymorphisms (RFLPs) and DNA sequence data are used to characterize the location of recombination events between the three *r-sc:mutable* alleles, each containing a *Ds* insertion, and *r-r:n142*. Additionally, the frequency of intragenic recombination and the value of $1/\rho$ at *r1* were compared to those calculated at *a1, b1, bz1* and *wx1*. This analysis further tested whether *r1* serves as a recombination hot spot in the maize genome. DNA sequence analysis also was used to characterize the structure of homology with respect to the location of recombination. The value of $1/\rho$ was calculated for different regions of *R-sc* and was used to explain the impact large regions of divergent DNA sequences (in the range of 2 kb or greater) have on recombination. Relative to a single nucleotide mutation, *Ds* insertions or a cluster of single nucleotide polymorphisms that have been reported to reduce intragenic recombination were examined with respect to their influence at *r1*.

Methods

Genetic Stocks

The inbred line W22 was used as a source for all alleles. *r-sc:mutable* alleles containing *Ds* insertions were recovered by cyclically mutagenizing *R-sc* alleles derived from *R-sc:124* with *Ds* insertions using *Ac* as a source of transposase (Figure 2; Kermicle *et al.* 1989; Alleman and Kermicle 1993). Selection for a *r1* mutant phenotype provided a means of recovering novel *Ds* insertion mutations of *R-sc* (Figure 3; Kermicle *et al.* 1989; Alleman and Kermicle 1993). *Ds* transposed via the action of *Ac* into *R-sc:124* resulted in a mutant termed *r-sc:m3*. The yellow, pale brown or spotted kernels on an otherwise colored ears (indicating that *Ds* had transposed into *R-sc*) were selected. In the absence of *Ac*, *Ds* insertions into *R-sc* interrupt *r1* expression, producing yellow to pale brown kernels. In the presence of *Ac*, kernels typically are spotted. *Ds* elements were excised from *r-sc:m3* using *Ac* and fully colored seeds (reversions) were selected. *Ds* elements typically transpose to genetically linked sites but may not be phenotypically observed during this stage and therefore are termed cryptic (Greenblatt and Brink 1962; Greenblatt 1984; Dooner and Belachew 1989; Chen *et al.* 1992; Dooner *et al.* 1994). Through a second round of *Ac* addition, *Ds* was transposed back into *r1* to create a series of insertional mutations of *R-sc* (*r-sc:m300's*) each with *Ds*

located at a different position. Subsequently, *Ac* was removed from the genome to produce germinally and somatically stable mutants (Kermicle *et al.* 1989; Alleman and Kermicle 1993). Thirty-eight stable (-*Ac*) *r-sc:mutable* alleles were recovered using this technique (Kermicle *et al.* 1989) including the three *r-sc:mutable* alleles used in the current study. Each *r-sc:mutable* allele used has a *Ds* insertion at a different position at the 3' end of the *Sc* gene (Figure 3; Alleman and Kermicle 1993; M. Alleman pers. comm.).

R-g:8pale derived from *R-r* is a distinguishable *r1* allele which produces pale brown kernels and green plant parts and was used to pollinate *r-sc:mutable/r-r:n142* heterozygotes.

A recessive mutation making the endosperm appear opaque in the unlinked *wx* gene at the *wx1* locus served as a genetic marker to identify and eliminate pollen contaminates from final analysis.

r-Δ902 is a deletion encompassing the *r1* locus and served as a negative control in Southern blot analyses (Alleman and Kermicle 1993).

Allele Recovery

Three independent *Ds* insertion alleles, *r-sc:m301, r-sc:m302* and *r-sc:m335* were made heterozygous with *r-r:n142* in the absence of known *Ac* elements. *r-sc:mutable/r-r:n142* heterozygotes were allowed to be open pollinated in an isolation plot by interplanted male rows carrying *R-g:8pale* and the *wx1* mutation. Most of the resulting kernel progeny should be heterozygous

combinations of *r-sc:mutables/R-g:8pale* or *r-r:n142IR-g:8pale* and have a pale brown seed color (Figure 4). Revertant black kernels that arise should be heterozygous for a revertant *R-sc* allele lacking the *Ds* element, and *R-g:8pale*.

To verify the paternity of putative revertant alleles, black kernel progeny were selected, germinated and self-pollinated. After self-pollination, only revertant progeny that produced a 3:1 ratio of wild type to mutant *wx1* and black to pale kernels were considered to be bona fide revertants and selected for further study. Of the black kernels on these self-pollinated ears, only one-third were expected to be homozygous for *R-sc*. The remaining two-thirds of black kernels were expected to be heterozygous for *R-sc* and *R-g:8pale*. Black kernel progeny arising from pollen contaminates and subsequently self-pollinated were not expected to segregate for the *R-g:8pale* or mutant *waxy* phenotypes.

As a control for cryptic *Ac* activity in the genetic background used, *r-sc:m301/r-sc:m301* homozygotes were crossed as above to test whether reversion could occur in the absence of the possibility for gene conversion or recombination with a heteroallele.

Reversion rates from *r-sc* to *R-sc* were calculated by dividing the number of true revertant progeny by the effective population size. The latter was determined by multiplying the total kernel population size by a ratio of the number of potential revertants successfully tested to the number of potential revertants initially recovered.

DNA Isolation and Southern Blot Restriction Map Analysis

Revertant progeny were germinated on wet paper towels under constant illumination until coleoptiles emerged. Seedlings were screened for coleoptile tip color due to *R-sc* expression and the absence of root and full coleoptile color due to *r-r* expression. DNA was extracted and purified from 4 - 5 leaf seedlings as described by Eggleston *et al.* (1995) with modifications of Shure *et al.* (1983) and Chen *et al.* 1992. Purified DNA was subjected to restriction endonuclease digestion, fractionated on 1% agarose (Sigma) 0.5 x TBE [1 X TBE = 0.09M Tris-Borate; 0.004 M EDTA] gels. Restriction enzymes were used under conditions recommended by the manufacturers (Promega, Bethesda Research Laboratories (BRL), United States Biochemical (USB), New England Biolabs (NEB)). The 1 kb ladder (BRL) was used as a molecular weight standard in all gels. Reactions were terminated with Stop Mix [final concentrations = 0.09% sodium dodecyl sulfate (SDS), 3.5% sucrose, 8.7 mM EDTA pH 8.0, 0.009% Bromophenol blue, 0.009% Xylene cyanol] as described by T. Hsieh, pers. comm. and Eggleston *et al.* (1995).

Agarose gels were stained with ethidium bromide and photographed under UV illumination using the Eagle Eye photoimaging system (Stratagene). Gel photographs were used to determine fragments sizes of RFLPs using the 1 kb ladder as a reference and compared to autoradiographs. Agarose gels

were washed for 10 minutes with 0.25 M HCl, followed by two 20 minute washes in 0.5 M NaOH, 1.5 M NaCl and two 25 minute washes in 0.5 M Tris (pH 7.5), 1.5 M NaCl. Gels were placed well-side down on three layers of 3MM chromatography paper saturated with, and the edges submerged in, 20 x SSC (1 X SSC = 150 mM sodium chloride, 15 mM sodium citrate). Duralon-UV (Stratagene) membranes first rinsed in deionized water then in 20 X SSC were placed on top of gels. A piece of 3MM rinsed with 20 X SSC and placed on top of the Duralon and covered by a stack of paper towels to facilitate DNA transfer by capillary action (Southern 1975; Eggleston *et al.* 1995). DNA was fixed to Duralon membranes by UV-crosslinking with 1200 µJ/cm2 and baking at 80°C for 2 hours.

Membranes were prehybridized at 42°C for 1 - 2 hours with a solution containing 50% formamide (v/v), 5 x SSC, 0.2% SDS, 2X Denhardt's solution [1X = 0.02% (w/v) Ficoll, polyvinylpyrrolidone and BSA]. DNA fragments used as probes were prepared according to methods described in Feinberg and Vogelstein (1983) using the Prime-a-gene kit (Promega). Fragments were released from plasmid subclones using restriction endonucleases, size fractionated and excised from 1% (w/v) low melting point agarose gels (Sigma) in 1 X TAE gels [1 X TAE = 40 mM Tris-acetate, 1 mM EDTA] and placed in 1.5 ml microcentrifuge tubes. DNA fragments (approximately 25 - 50 ng) were denatured completely for 10 minutes at >90°C and then centrifuged for 5

seconds to reincorporate condensation. The following was added to each labeling reaction: 1µg/µl BSA, 5X Labeling buffer (250 mM Tris pH 8.0, 25 mM $MgCl_2$, 10 mM DTT, 1M Hepes pH 6.6, 26 A260 random hexanucleotides), 500 µM of each non-radioactive dNTP, either α^{32}P-dGTP, dCTP or both (Amersham, Andotek) at >3000Ci/mmole and 5 units of Klenow fragment. Reactions were allowed to incubate for 2 hours at 37°C. Reactions were terminated with Nick translation stop mix (40 mg/ml dextran blue, 20% SDS, 0.5M EDTA pH 8.0, 10 mg/ml Phenol Red). Sephadex G-50-150 chromatography columns poured in 5 ml plastic pipettes were used to separate radiolabeled DNA fragments from unincorporated radionucleotides. Labeled DNA fragments in TE migrating with dextran blue dye were collected and stored at -20°C after the addition of carrier DNA (50 µg/ml sheared salmon sperm DNA). For hybridization, DNA fragment mixtures were boiled at >90°C, chilled on ice for 10 minutes, added directly to the prehybridization solution and allowed to incubate under constant agitation for 12 - 16 hours at 42°C.

Following the removal of hybridization solution, membranes were posthybridized as follows: one room temperature rinse in 2 X SSC, two 10 minute washes in 2 X SSC, 0.2% SDS at 58°C followed by two ten minute washes in 0.2 X SSC, 0.2% SDS at 58°C. Membranes were blotted dry, wrapped in plastic wrap and exposed to X-ray film using intensifying screens

(Dupont) for 5 - 7 days at -80°C. Membranes were stripped of hybridizing probes using two 15 minute boiling water washes, the first with 20% SDS, under constant agitation. Membranes were blotted dry using 3MM paper and rehybridized as necessary.

r1 fragments from the following clones were used as probes in Southern blot analysis: the 5' end fragments in pR-Nj:1 and pR-Sc-1:U and the 3' end fragments in pSc323:I14 and pSc323:J20 (Figure 3; Alleman and Kermicle 1993).

PCR Amplification, Cloning and DNA Sequence Analysis

Synthetic oligonucleotides (Integrated DNA Technologies, IDT) derived from the sequences of *R-sc:124* (M. Alleman, C. Illingsworth, J. Kermicle and W. Eggleston, pers. comm.) and *lc1* (Ludwig *et al.* 1989; S. Ludwig, L. Habera and S. Wessler, pers. comm.) were used in PCR amplifications (Table 1; Figure 3). The approximate location of *Ds* element in *r-sc:m335* was determined previously whereas the *Ds* elements located in *r-sc:m301* and *r-sc:m302* were localized only to the 3' end of *r1* (Alleman and Kermicle 1993; M. Alleman, pers. comm.).

Using *r1* locus oliogonucleotides near the insertion sites together with either the 5' or 3' oligonucleotides directed out from the end of the *Ds* elements, the relative orientation of each *Ds* was determined based on the

predicted PCR fragment size. Because *Ac* and *Ds* have similar TIRS, *Ds* oligonucleotides were derived from the sequence near the end of the canonical *Ac* element (Pohlman and Starlinger 1984, Shure *et al.* 1983) The 5' *Ds* oligonucleotide is located 110 bp from that end and the 3' oligonucleotide is located 104 bp from that end. Depending on the orientation of the *Ds* element in *R-sc:124*, only one combination of primers (*r1* locus with either 5' or 3' *Ds* oligonucleotides) was expected to yield an amplified junction fragment. *r1* and *Ds* primers directed away from each other will not produce amplified fragments from *r1*. However, it is possible that *Ds* elements located elsewhere in the genome and near *r1*-related sequences could be amplified under these conditions. Amplifications were performed using a standard protocol of 30 cycles of 1 min., 94°C denaturing; 1 min., 55°C annealing; 1 min., 72°C (68°C with eLONGase) elongation. All amplifications were performed using 1 mM $MgSO_4$, 1 or 2 units of either *Taq* polymerase (Promega) or eLONGase enzyme mix (BRL). Modifications included: increased denaturing time and temperature, alternative elongation times based on expected fragment size and annealing temperature based on oligonucleotide Tm values (Table 2). Reactions were terminated with stop mix, electrophoresed through 1 - 2% agarose gels in 0.5 x TBE and visualized by ethidium bromide staining. Where appropriate, gels were Southern blotted and hybridized as described above to confirm that amplified fragments were homologous to *r1* probes and derived from *r1*.

When multiple amplified fragments were observed, *r1*-hybridizing fragments were excised from 1 X TAE gels and purified by either AgarACE™ (Promega) or Geneclean™ (BIO 101) as described by the manufacturer. One-fourth PCR amplification volume of 4.4 M NH_4OAc and 3 X the resulting volume of 100% ethanol was used to precipitate amplified fragments when single amplified fragments were observed. Purified fragments were ligated to *Eco*RV-digested ddT-tailed pBlueskript II SK(+) (Stratagene) or pGEM5Z(+/-)(Promega) plasmids (Sambrook *et al.* 1989; Holton and Graham 1991). Ligation reactions were as follows for 50 - 100 ng of plasmid DNA: 0.75 µl of 10 X Ligase Reaction Buffer, 0.5 units of Ligase enzyme (USB), and purified PCR DNA to a total volume of 10 µl. Ligase reactions were incubated at room temperature (21 - 24˚) for a minimum of 30 minutes. After digestion with *Eco*RV, pBlueskript II SK(+) and pGEM5Z(+/-) were tailed using the following protocol: 20 µl of plasmid DNA, 8 µl of 5 X Terminal transferase Buffer, 4 µl of 100 M ddTTP and 6 µl Terminal transferase enzyme (17 U/µl) were added per reaction and allowed to incubate at 37˚ for 2 hours. The ddT-tailed plasmid was purified using ethanol precipitation (see above). The TA TOPO™ cloning kit (Invitrogen) also was used according to the manufacturers instructions to clone PCR fragments.

Following ligation, the phagemid containing the junction fragment was transformed into XL-1 blue cells by directly adding the phagemid to the cells and placing on ice for 30 minutes. Samples were placed in a 42°C water bath

for exactly 45 seconds then chilled on ice for 2 minutes. Luria-Bertani (LB) [1 L deionized water = 10 g bacto-tryptone, 5 g bacto-yeast extract, 10g NaCl] Media was added to the cells and incubated at 37°C for 1 hour at 250 rpm. Cells were concentrated by brief centrifugation and half of the supernatant was discarded. Cells were resuspended and plated on LB agar containing 50 μg/ml ampicillin, 20 μg/ml X-gal and 20 μg/ml IPTG and incubated overnight at 37°C (Sambrook *et al.* 1989). Putative successful transformants (white colonies) were selected and cultured overnight in LB broth under 50 μg/ml ampicillin selection at 37°C at 250 rpm. Cultured cells (1.5 ml) were transferred to microcentrifuge tubes and briefly centrifuged. Supernatants were discarded and pelleted cells were resuspended in STET (8% glucose, 50 mM Tris pH 8.0, 50 mM EDTA pH 8.0, 5% Triton X-100), to which 50 mg/ml lysozyme was added and allowed to incubate for 5 minutes at room temperature. Samples were boiled for 45 seconds, centrifuged at 4°C for 10 minutes and the supernatant collected. Recombinant plasmid DNA was precipitated with an equivalent amount of 1:10 4.4 M NH_4OAc:Isoproanol to the supernatant and centrifuged for 10 minutes at 4°C. Pellets were washed with 80% ethanol and resuspended in TE (K. Brigle, pers. comm.). Plasmid DNA was digested with appropriate restriction endonucleases and size fractionated as described above to verify the presence of plasmid and insert before further purification. Plasmid DNA

was purified further by adding equivalent amount of 1:1 Phenol:Chloroform to TE, vortexing and centrifuging for 10 minutes at 4°C. The aqueous phase containing DNA was removed and precipitated with 1:12 ratio 4.4 M NH_4OAc:Ethanol and centrifuged for 10 minutes at 4°C. Pellets were washed twice with 80% Ethanol and allowed to dry completely before resuspension with water highly purified water (Sigma) (K. Brigle, pers. comm.).

Purified plasmid DNA was sequenced by the dideoxy method using Sequenase v. 2.0 and conditions recommended by the manufacturer (USB) and Zhang *et al.* (1988). Approximately 2 - 5 μg of plasmid DNA was purified using 13% PEG 8000 and 5 M NaCl precipitation, chilled on ice for 1 hour and centrifuged at 4°C for 20 minutes. Pellets were washed with chilled 80% ethanol and allowed to dry completely (S. Taylor, pers. comm.). Pellets were resuspended in 5 μl of highly purified water (Sigma), 2 μl of 5 X Sequenase reaction buffer (USB). Five nanograms of primer were allowed to anneal slowly to plasmids following a 2 minute incubation at 65°C. Elongation was performed for 3 minutes at room temperature (22 - 25°C) and labeling was performed at 37°C for 5 minutes. α^{35}S-labeled fragments were electrophoresed through 5% Long Ranger polyacrylamide (FMC), 1.2 X TBE gels at 70 W in a 0.6 x TBE running buffer. Gels were transferred to 3MM chromatography paper, vacuum dried at 80°C and exposed directly to X-ray film for 3-4 days at room temperature.

The ABI Prism cycle sequencing kit (PE Applied Biosystems) also was used to sequence plasmid DNA. Reaction conditions were followed as directed by the manufacturer with the following modifications: 600 ng of plasmid, 20 ng of primer and 75% of the recommended amount of kit mix was used in each reaction. Amplifications were performed using conditions recommended by the manufacturer of 25 cycles of 30 seconds, 96°C denaturing, 15 sec, 50°C annealing, 4 minutes, 60°C elongation. Polyacrylamide gel electrophoresis analysis was performed by Molecular Biology Core Facility at MCV and/or Commonwealth Biotechnologies, Inc., Richmond, VA. DNA sequence data was assembled and compared using DNASTAR (Laser Gene).

Results

Molecular Analysis of *R-sc:124* and *r-r:n142*.

Southern blot analysis identified numerous restriction site polymorphisms between *R-sc:124* and *r-r:n142* (Robbins *et al.* 1991, Eggleston *et al.* 1995). These sites are illustrated in Figure 3 and provide a means to localize the region of recombination resolution points and gene conversion tracts. For example, there are two *Bam*HI sites present in the 3' end of intron 2 of *r-r:n142* but none in *R-sc:124*. Additionally, there is a *Hin*dIII site present in exon 8 of *r-r:n142* but absent in exon 8 of *R-sc:124*. The location of *Ssp*I sites at the 3' end of intron 2 differs by 200 bp between the two progenitor alleles. Analysis of these diagnostic sites could be used to characterize the pattern of recombination across *R-sc*. There also were nucleotide differences uncovered by DNA sequence analysis between the *R-sc:124* and *r-r:n142* but the differences did not generate useable restriction sites for Southern blot analyses.

The bottom of Figure 5 represents the alignment using DNASTAR (Laser Gene) of DNA sequence data from *R-sc:124* (M. Alleman, C. Illingsworth, J. Kermicle and W. Eggleston, pers. comm.) and *r-r:n142* (M. Alleman and W. Lison pers. comm.; current study) recorded as percent similarity. DNA sequence analysis revealed regions where there is low DNA sequence

homology (i.e., discontinuities) between *R-sc:124* and *r-r:n142*. Multiple large discontinuities exist in intron 2 where similarity between the two alleles ranges from 26 to 62%. Discrete regions of high sequence identity ranging from 20 to 130 bp in length were present within these discontinuities and potentially could facilitate recombination. There is greater than 99.4 percent similarity between the 5' ends *R-sc:124* and *r-r:n142*. Further 5' of this region exists additional promoter region sequences for each allele and the DNA sequence similarity between *R-sc:124* and *r-r:n142* in this region is low, representing the major discontinuity between the two alleles. As noted previously, the 3' coding regions of *R-sc:124* and *r-r:n142* are functionally interchangeable. This analysis demonstrated that the 3' ends of the alleles was characterized by greater than 95% similarity.

Localization of *Ds* insertions

Each of the *r-sc:mutable* alleles has a *Ds* element inserted at a different position within *R-sc:124*. PCR and DNA sequencing analyses were performed to map precisely the nucleotide of insertion. Since the *Ds* primers are located approximately 100 bp from the end of the element, using either the 5' or 3' primer in conjunction with *r1* primers directed toward the element was expected to produce an amplified fragment. Thus, the relative orientation could be determined and the fragment size produced was used to determine the approximate location of each *Ds* insertion.

Primers Lc8180 and Ds3' were used to amplify regions adjacent to the *Ds* element in *r-sc:m301*. Based on ethidium bromide staining, a fragment of approximately 400 bp was produced. Since *Ds* sequences constitute approximately 100 bp of the amplified fragment, the element was localized approximately 300 bp from the *r1* primer which is in the 5' region of intron 8. The combination of Lc8180 and Ds5' did not produce an amplified fragment, consistent with the orientation of the *Ds* element in *r-sc:m301* being reversed with respect to the direction of *R-sc:124* transcription. The primer combination of Lc8681 and Ds5' produced an amplified fragment approximately 300 bp further supporting the initial localization and orientation of the *Ds* element in intron 8. As predicted from the above data, the primer combination of Lc8681 and Ds3' did not produce an amplified fragment.

The same primer combinations were used to determine the location of the *Ds* element in *r-sc:m302*. However, PCR amplifications using Lc8180 and Ds5' produced a fragment of approximately 300 bp whereas using Lc8681 and Ds3' produced fragment of about a 425 bp. No amplified products were observed when Lc8180 and Ds3' or Lc8681 and Ds5' combinations were used in amplifications with *r-sc:m302* DNA. These analyses localized the *Ds* element in *r-sc:m302* to the 3' region of exon 8 in the same orientation as the direction of *R-sc:124* transcription (Figure 3).

The orientation and approximate location of the *Ds* insertion in *r-sc:m335* had been determined previously (Alleman and Kermicle 1993). Using Lc6274

and Ds3' in PCR amplifications, a fragment of about 300 bp was produced. The primer combination of Lc6710 and *Ds5'* produced a 400 bp fragment. Thus, the *Ds* element in *r-sc:m335* was localized to the 3' end of exon 3 (Figure 3).

Using PCR can result in non-specific amplification, therefore reactions were either repeated, Southern blotted and/or DNA sequence analysis of cloned products was performed to test if *r1* DNA was amplified. In all cases, the presence of *r1* was confirmed. DNA sequence analysis placed the *Ds* insertion in *r-sc:m301* at position 8461 bp relative to the genomic sequence of *Lc*. The *Ds* element in *r-sc:m302* is at position 8321 bp relative to the genomic sequence of *Lc*. Thus, these data confirm that *Ds* insertions are in the 5' region of intron 8 and the 3' region of exon 8, respectively, based on the intron and exon assignments of the *Lc* genomic sequence (Figure 3; Ludwig *et al*. 1989; S. Ludwig, L. Habera and S. Wessler, pers. comm.). DNA sequence analysis also revealed that the target site sequence 5'-CTCAGGT-3' was duplicated at the insertion site of the *Ds* element in *r-sc:m301*. No target site duplication was found in DNA sequences adjacent to the *Ds* insertion in *r-sc:m302*. The precise location of *Ds* in *r-sc:m335* could not be determined because amplified fragments could not be cloned following repeated attempts.

Allele Recovery Analysis

All genetic crosses and frequency data were performed and provided by Dr. W. Eggleston (Table 3). No revertant progeny were observed among the progeny from the cross involving *r-sc:m301* homozygotes suggesting that cryptic *Ac* activity was not the basis for reversion of the *r-sc:mutable* alleles (Table 3). Data from Kermicle *et al.* (1989) tested a series of *Ds* insertion alleles, including those used in this study, in homozygous condition and recovered no reversions in kernel populations ranging from 20,140 to 48,460. Thus, the majority of *Ds* insertions from *r-sc:mutable/r-r:n142* heterozygotes likely were lost due to crossing over or gene conversion. One hundred forty-six putative revertant progeny derived from *r-sc:mutable/r-r:n142* heterozygotes crossed to *R-g:8pale* were recovered and tested (Table 3). Of those tested, ninety-four were confirmed as bona fide *R-sc* revertants.

Because the *Ds* insertions are located at distinct sites in the 3' end of *R-sc:124*, the data can be used to determine if a polarity gradient exists (Figures 3 and 5, Table 3). The reversion rate was lowest (3.8×10^{-4}) among alleles arising from *r-sc:m335* which has a *Ds* element inserted in exon 3. Reversion frequencies from *r-sc:m301* and *r-sc:m302* which have *Ds* insertions in intron 8 and exon 8, respectively, were higher (8.2×10^{-4} and 9.3×10^{-4}) than for *r-sc:m335* (Table 3). When the reversion frequency of *r-sc:m335* was compared to that of *r-sc:m301* or *r-sc:m302* using a comparative binomial proportion test, the difference is significant ($t_{0.05,(2),\infty} = 1.96$; $t = 3.118$; $P < 0.001$).

Although reversion frequencies from *r-sc:m302* are slightly higher compared to reversion frequencies from *r-sc:m301*, these two frequencies are not significantly different from each other ($t_{0.05\,(2),\infty} = 1.96$, $t = 0.55$; $P > 0.50$). These data together suggest that there is a preference for resolution or a hot spot of recombination at the 3' end of *r1*. Alternatively, these data could represent additive effects of the number of recombination events increasing with the size of DNA being analyzed due to chance alone. If this were the case, then the relationship between the frequency of reversion as compared to the distance of each *Ds* insertion maps from the major *R-sc:124/r-r:n142* discontinuity, would increase in a linear fashion. Instead, Figure 5 shows that the reversion frequency of *r-sc:mutable* alleles with *Ds* insertions at the 3' end is substantially greater than *r-sc:mutable* alleles with *Ds* insertions elsewhere in the gene.

As mentioned previously, the ratio of genetic to physical distance ($1/\rho$) can be calculated for entire genes as well as particular regions of genes that serve as hot spots. The average value ($1/\rho$) for the entire *r1* gene is 0.02 cM/kb which is about 100-fold higher than the average for the genome (0.00021 - 0.00068 cM/kb) (Civardi *et al.* 1994). Furthermore, the value of $1/\rho$ calculated at the 5' end (0.0022 cM/kb) is about 30 times lower than $1/\rho$ at the 3' end (0.07 M/kb). Within intron 2, $1/\rho$ is lower (0.0045 cM/kb) relative to values calculated at the 3' end. There are discrete regions with intron 2 that are homologous

between *R-sc:124* and *r-r:n142* and may allow recombination to occur, accounting for the slight increase in recombination observed. This analysis further demonstrates for more 3' *Ds* insertions that the 3' end of *r1* is more recombinationally active than the 5' end or central regions.

Molecular Analysis of Revertant Alleles.

DNA isolated from revertant progeny was digested with *Hin*dIII to verify that each revertant allele had the *R-sc:124* promoter region and to detect the presence of the polymorphic *Hin*dIII site orignially present in exon 8 of *r-r:n142* but absent in *R-sc:124*. The membranes first were hybridized with pR-Nj:1 which recognizes the 5' end of *r1* genes. If *R-sc* is heterozygous with *R-g:8pale*, then the autoradiographs should show diagnostic 2.8 and 3.2 kb fragments when hybridized with pR-Nj:1. All revertant alleles tested lacked these fragments characteristic of *R-g:8pale*. These data show that DNA samples tested were homozygous for *R-sc* revertant alleles (Figure 6A). All revertant alleles also had the expected 4.5 kb fragment from *R-sc:124* and lacked the 3.8 kb fragment from *r-r:n142* (Figure 6A). These data show that molecular characteristics of *R-sc:124* at the 5' end are retained in the revertant allele population. When hybridized with pSc323:I14, 92 out of 94 revertant alleles had the 4.0 kb fragment diagnostic for the 3' end of *r-r:n142* (Figure 6B). Figure 7 shows additional data for revertant alleles digested with *Hin*dIII and

hybridized to pSc323:I14 and the two revertant alleles (lanes 6 and 7) had 8 kb fragments diagnostic for the 3' end of *R-sc:124*. In all alleles tested an extra hybridizing 3.9 kb fragment corresponding to the homologous *b1* locus is present (data not shown). If *Ds* insertions are retained by the revertant alleles, then a 6.0 or 10.0 kb fragment is expected at the 3' end of respectively, *r-r:n142* or *R-sc:124*. In all cases this fragment was not observed and additional restriction map data showed that the presence of a *Ds* element was never observed in the revertant allele population. Since all of the revertant alleles lost the *Ds* element, the 3' boundary of recombination is represented by the location of the *Ds* insertion in each *r-sc:mutable* allele. These data show that recombination occurred within *r1* and 5' to the *Ds* insertion.

Digestion of revertant progeny DNA with *Hin*dIII and *Bam*HI was used to test for the presence or absence of the polymorphic *Bam*HI sites originally present in *r-r:n142* but not in *R-sc:124* (Figures 3 and 8). All revertant alleles have the diagnostic 2.1 and 2.3 kb bands characteristic of *R-sc:124* (lane 1) and lack the 1.6 and 2.5 kb fragments characteristic of the 5' end of *r-r:n142* (lane 2). These data confirm that the *R-sc* promoter region is present in all revertant alleles tested. The presence of two hybridizing fragments can be explained by the location of the *Bam*HI cleavage site within the pR-Nj:1 region (Figure 3) thus, yielding two hybridizing fragments. If *R-sc* is heterozygous with *R-g:8pale*, then the autoradiographs also should show diagnostic 1.6, 1.9 and 2.7 kb fragments characteristic of *R-g:8pale*. Instead, these fragments are

absent in all revertant alleles (Figure 8, lanes 4-10). None of the DNA samples tested was heterozygous for *R-g:8pale* and the revertant *R-sc* allele.

If the polymorphic *Bam*HI sites were present, then the resulting hybridizing fragment is expected to be 3.1 kb (Figure 9, lane 2). Instead, a 4.0 kb fragment corresponding to a fragment observed with a digestion with *Hin*dIII alone (lanes 4 - 7, and 10) was observed in 92 out of 94 revertant alleles when hybridized to pSc323:J20 (Figure 9). Therefore, the 5' limit of recombination occurred 3' to the *Bam*HI site in *r-r:n142* for all revertant alleles. However, some alleles (e.g., lane 10 Figure 9) showed an approximate 3.2 kb band which could indicate that recombination occurred 5' to the *Bam*HI sites. Additionally, there were multiple hybridizing fragments on the autoradiograph which only can be explained partially. Weak hybridization of pSc323:J20 to the *b1* locus and partial digestion of *Bam*HI sites due to cytosine methylation also would explain some of the extra hybridizing fragments (Figure 9). If this is the case, then a simple interpretation is that the *Bam*HI sites are retained among the revertant alleles; thus, recombination occurred 5' to the *Bam*HI sites. However, this is inconsistent for the following reasons: (1) All revertant alleles retained the polymorphic *Sun*I site which is located approximately 100 bp 5' to the *Bam*HI sites on the *R-sc:124* chromosome but absent in *r-r:n142* (data not shown). Thus, recombination occurred 3' to this site. (2) Oligonucleotide primers Sc1-996 and Lc6311 were used to amplifiy a region of intron 2 of a subset of revertant alleles using PCR and the amplified product subsequently

were digested with *Bam*HI. In all cases, the presence of *Bam*HI sites were not detected (data not shown). (3) No similarity in the DNA sequences of encompassing the *Dra*III (of *R-sc:124*) to *Bam*HI (of *r-r:n142*) region was detected (Figure 5). The exact nature of the multiple hybridizing fragments cannot be determined at present and it is unlikely that recombination occurred 5' to the *Bam*HI sites.

To map more precisely the region of recombination, revertant alleles arising from *r-sc:m335* were tested with *Hin*cII and *Ssp*I and hybridized with pSc323:J20. The *Ssp*I site located at the 3' end of intron 2 is polymorphic between *R-sc:124* and *r-r:n142*. Both alleles have a common *Ssp*I site located 3' to exon 9 (Figure 3). Using these enzymes, a 3.8 kb fragment is expected from *R-sc:124* and a 3.6 kb fragment is expected from *r-r:n142* (Figures 3 and 10). Fifteen out of 18 revertant alleles retained the 3.8 kb fragment diagnostic of *R-sc:124* rather than the 3.6 kb fragment of *r-r:n142* when hybridized to this probe. Recombination therefore occurred 5' to *Ssp*I site in *r-r:n142* because the *Ssp*I site in *r-r:n142* was not present in these fifteen alleles accounting for the presence of a 3.8 kb fragment. In the remaining three revertant alleles, a 3.6 kb fragment of *r-r:n142* was observed. For these alleles, recombination could have occurred 5' to either of the *Ssp*I sites in *R-sc:124* or *r-r:n142* because both possibilities include acquiring the *Ssp*I in *r-r:n142*. The *Ssp*I site from *R-sc:124* would be present but not detectable if recombination occurred 3' of the *Ssp*I site in *R-sc:124* but 5' of the *Ssp*I in *r-r:n142*. Regardless, the region

of recombination can be mapped using the *Ds* insertion as the 3' boundary and the *Ssp*I site of *r-r:n142* as the 5' boundary, a region of approximately 0.8 kb. A subset of revertant alleles from *r-sc:m301* and *r-sc:m302* was digested with *Hin*cII and *Ssp*I and all retained the 3.8 kb fragment when hybridized to pSc323:J20. The recombination interval for revertant alleles arising from *r-sc:m301* and *r-sc:m302* progenitors could not be mapped more precisely due to a lack of polymorphic sites 3' to the *Ssp*I site at the 3' end of intron 2 in both progenitor alleles. The high degree of sequence homology between the 3' coding regions of *R-sc:124* and *r-r:n142* (Figure 3) precludes the use of Southern blot analysis where restriction site differences are needed to map the location of recombination junctions.

Two revertant alleles have molecular characteristics of *R-sc:124* at both ends (Figures 7 and 9). This suggests that these alleles arose via a gene conversion event, a rare double crossover event or cryptic *Ac* activity. Because no revertant progeny were isolated from genetic crosses involving *r-sc:mutable* alleles in homozygous condition, the excision of *Ds* due to cryptic *Ac* activity therefore seems unlikely as the basis for reversion (current study, Kermicle *et al.* 1989). Revertant alleles *R-sc:e1268* and *R-sc:e1278* both have the 6 kb molecular fragment characteristic of *R-sc:124* when digested with *Bam*HI and *Hin*dIII and hybridized with pSc323:J20 (Figure 7, lanes 8 and 9). Evidence for gene conversion also is shown from digestion with *Hin*dIII alone. Both revertant alleles *R-sc:e1268* and *R-sc:e1278* had the 8 kb fragment

characteristic of the 3' end of *R-sc:124* when hybridized with pSc323:J20 (Figure 9, lanes 6 and 7). These data suggest that the 3' end of gene conversion tract terminated 5' to the polymorphic *Hin*dIII site present in exon 8 of *r-r:n142* (Figure 3). Because both revertant alleles arose from *r-sc:m335*, they were digested with *Hin*cII and *Ssp*I and hybridized with pSc323:J20. Both had the diagnostic 3.8 kb fragment from *R-sc:124* (see Figure 10 for an example). Thus, the 5' end of the conversion tract begins 3' of the *Ssp*I in *r-r:n142*. Taken together these data show that the gene conversion tract length is maximally 2.9 kb. Minimal tract length was not possible to determine.

Discussion

The pattern of recombination observed at several maize loci is different from that observed at *r1*. Recombination at *bz1* and *wx1* is dispersed throughout each gene with no preference for resolution in a particular region (Dooner 1986; Dooner and Martinez-Ferez 1997; Okagaki and Weil 1997). However, at *a1* and *b1* there is preference for resolution at the 5' end of each locus (Xu *et al.* 1995; Patterson *et al.* 1995). Previous data from Robbins *et al.* (1991) and Eggleston *et al.* (1995) localized recombination to the 3' end of the *r1* genes in these alleles. However, each of these alleles is complex and suppression of recombination from methylation at the 5' end could account for the observed pattern.

Several lines of evidence from the current study support the conclusion that recombination occurs primarily in the 3' end of the gene. First, both *r-sc:m301* and *r-sc:m302* have *Ds* insertions located further 3' than the *Ds* insertion in *r-sc*:m335 and the frequency of revertant alleles arising from *r-sc:m301* and *r-sc:m302* is significantly higher than the reversion frequency for *r-sc:m335*. Second, the ratio of genetic to physical distance also supports that the 3' end of *r1* is recombinationally more active relative to the 5' end. At the 5' end of the gene $1/\rho$ is approximately 0.0022 cM/kb for a region of 0.9 kb whereas at the 3' end, it is 0.08 cM/kb for a 1.0 kb region where recombination

could be mapped precisely (i.e., revertant alleles from *r-sc:m335*). The average value of $1/\rho$ determined for recombinant alleles arising from *r-sc:m301* and *r-sc:m302* is 0.06 cM/kb for a region of 2.9 kb. Although slightly lower than $1/\rho$ from *r-sc:m335*, it cannot be ruled out that this value represents an additive effect (thus, dispersed) of the number of cross over events occurring per unit DNA or whether there are recombinationally more active regions within this 2.9 kb region. Regardless, these values show that recombination preferentially occurs at the 3' end of the *r1* gene.

The complexity of *r1* alleles does not appear to be the basis for the observed pattern of recombination since a similar pattern also was observed for the simplex alleles tested here. Recombination exchanges occurred in the 3' end of all *r1* genes (Robbins *et al.* 1991, Eggleston *et al.* 1995, current study).

The average value of $1/\rho$ calculated for the entire *r1* gene is approximately 0.02 cM/kb and is nearly 100-fold higher than the average for the entire genome (0.00021 - 0.00068 cM/kb; Civardi *et al.* 1994). This observation shows that the maize *r1* gene serves as a recombination hot spot within the maize genome. The value of $1/\rho$ calculated for the entire *r1* gene is comparable to those calculated at *a1* (0.03 cM/kb), *b1* (0.05 cM/kb), *bz1* (0.07 cM/kb), and *wx1* (0.07 - 0.10 cM/kb) (Dooner 1986; Patterson *et al.* 1995; Xu *et al.* 1995; Dooner and Martinez-Ferez 1997; Okagaki and Weil 1997).

The amount or structure of homology does not seem to be the determining factor for recombination considering that approximately 115 bp of sequence homology are needed to facilitate recombination (Nassif and Engels 1993). *r-sc:mutable* alleles with *Ds* insertions at the 5' end reverted much less frequently than those with *Ds* insertions at the 3' end even though DNA sequence analysis of *R-sc:124* and *r-r:n142* also revealed over 99% homology for a 1.0 kb region at the 5' end of both genes (Figure 5; J. L. Kermicle pers. comm.). The presence of a large discontinuity further 5' of this 1.0 kb could suppress recombination from occurring in the region of high homology. Additionally, Dooner and Martinez-Ferez (1997) placed the recombination junction in some *Bz* revertants between two single nucleotide heterologies separated by 30 bp, suggesting that the amount or structure of homology is not the determining factor.

Dooner (1986) and Dooner and Martinez-Ferez (1997) also have documented that *Ds* insertions at *bz1* suppress recombination up to 600 bp 5' to the insertion. Whether *Ds* insertions have a suppressive effect on recombination at *r1* could not be ascertained directly in the current study. However, this study provides indirect evidence to support this possibility. In revertant alleles from *r-sc:m335* where recombination could be mapped using fine scale restriction map analysis, all exchanges occurred at least 0.8 kb from the *Ds* insertion. This is consistent with *Ds* suppressing recombination at directly adjacent sites though further analysis is needed. *Ds* insertions into

r-sc:m301 and *r-sc:m302* also could suppress recombination, however, this would require DNA sequence analysis of each revertant allele and documenting single nucleotide polymorphisms present from either *R-sc:124* or *r-r:n142* to localize the crossover region to more than 600 bp upstream of the *Ds* insertion as documented at *bz1*. Direct evidence would entail the generation of stable mutant *r-sc* alleles containing point mutations where each of the *Ds* insertions was located, each mutant *r-sc* allele placed in heterozygous condition with *r-r:n142*, and then pollinated with *R-g:8pale* to recover revertant progeny. Restriction map analysis and DNA sequence analysis then could uncover whether *Ds* insertions relative to a point mutation at *r1* suppress recombination in directly adjacent regions.

The differences in the pattern of recombination observed at different genes in maize suggests that there are a variety of factors influencing recombination. Although not identified specifically, recent evidence points to genetic factors present on the same chromosome (*cis*-factors) as well as genetic factors produced by genes located on other chromosomes (*trans*-factors) affecting the recombination frequency at distinct chromosomal areas (Timmermans *et al.* 1997). Recombination also may be favored to occur in regions of open chromatin and hypomethylated sequences as is characteristic of many genes in both plants and yeast (Roeder 1990, 1995; Dooner and Martinez-Ferez 1997). Finally, different mechanisms for recombination may

exist or the cell's flexible recombination machinery may accommodate the different patterns observed (Dooner and Martinez-Ferez 1997).

Regardless of the pattern of recombination documented at *a1, b1, bz1* and *wx1*, there is a strong preference for crossing over compared to potential gene conversion. This appears to be true at *r1* since out of 94 revertant progeny, 92 were crossover events. The remaining two, both of which originated from *r-sc:m335/r-r:n142* heterozygotes, are assumed to be potential gene conversion or rare double cross over events. Thus, the frequency of gene conversion at *r1* is estimated at approximately 3.97×10^{-5}. Each convertant tract length is maximally 2.9 kb as determined by fine scale restriction map analysis. The size of these tracts is comparable but slightly larger than 1 - 1.5 kb tracts reported at *bz1* by Dooner and Martinez-Ferez (1997). As observed previously, the maize genome may have a mechanism promoting crossover events rather than gene conversion events at meiosis (Dooner and Martinez-Ferez, 1997).

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Figure Legends

Figure 1. Molecular model of homologous recombination between alleles of the *r1* locus. See text for allele descriptions. Model is a modified version of Szostak *et al.* (1983) double strand DNA break repair model for crossing over and gene conversion. Panel (2) shows a double strand break followed by 5' to 3' exonuclease degradation of the broken strands. Strand invasion of single strand 3' ends is followed by DNA synthesis. Differential cleavage and rejoining of the backbones of the Holliday Junctions (asterix in Panel 3) can yield two products: gene conversion (Panel 4A) or crossing over (Panel 4B).

Figure 2. Sequential mutagenesis of *R-sc* alleles adapted from Kermicle *et al.* (1989). See text for details. *Ds* elements are represented as triangles.

Figure 3. Location of *Ds* insertions in *R-sc* and restriction site polymorphisms between *R-sc:124* and *r-r:n142*. *Ds* insertions as denoted by triangles as in Figure 2 are superimposed on *R-sc:124*. The transcription unit is based on analysis of *lc1* (Ludwig *et al.* 1989; M. Alleman, C. Illingsworth, J. L. Kermicle, W. Eggleston, pers. comm.). The arrow below the second darkly shaded box indicates the presumptive translation start site (Ludwig *et al.* 1989). The arrow in each *Ds* indicates its relative presumed transcription orientation with respect

to *R-sc:124*. Arrows (not to scale) flanking insertions represent oligonucleotide primers used in PCR amplification. Darkly shaded boxes represent protein coding regions. Open boxes are non-coding regions. Darkly stippled boxes represent probes used for Southern analysis. Lightly stippled boxes represent transcribed, untranslated 5' and 3' sequences. Restriction sites are abbreviated as follows: B = *Bam*HI; C = *Hin*cII; D = *Dra*III; H = *Hin*dIII; P = *Pvu*II; S = *Ssp*I. Not all sites are shown.

Figure 4. Genetic scheme used to isolate revertants. See text for details. Triangles denote *Ds* insertions. The circled "X" denotes self-pollination. Following self-pollination, each class is expected to segregate for wild type and mutant *waxy* phenotypes.

Figure 5. Combined data from 3 studies on *r-sc:m* reversion rates versus the distance of the *Ds* insertion from the *R-sc:124/r-r:n142* discontinuity. Shown below is the percent homology as determined by DNASTAR (Laser Gene). Alignments were performed using 0.5 kb DNA sequence blocks beginning at the discontinuity. The location of *Ds* insertions were determined previously (Alleman and Kermicle 1993) by restriciton map analysis. Shaded boxes are as described in Figure 4.

Figure 6. Southern blot analysis of revertant progeny alleles derived from *r-sc:m302/r-r:n142* heterozygotes lacking *Ac*. DNAs were digested with *Hin*dIII and fractionated in agarose gels and hybridized to pR-Nj:1 (Panel A) and pSc323:I14 (Panel B).

Figure 7. Southern blot analysis of revertant progeny derived from *r-sc:m335/r-r:n142* heterozygotes lacking *Ac*. DNAs were digested with *Hin*dIII, fractionated in agarose gels and hybridized to pSc323:I14. Revertant alleles *R-sc:e1268* and *R-sc:e1278* represent putative gene conversion events.

Figure 8. Southern blot analysis of revertant progeny alleles derived from *r-sc:m302/r-r:n142* heterozygotes lacking *Ac*. DNAs were digested with *Bam*HI and *Hin*dIII, fractionated in agarose gels and hybridized to pR-Nj:1.

Figure 9. Southern blot analysis of revertant progeny alleles derived from *r-sc:m335/r-r:n142* heterozygotes lacking *Ac*. DNAs were digested with *Bam*HI and *Hin*dIII, fractionated in agarose gels and hybridized to pSc323:J20.

Figure 10. Southern blot analysis of revertant progeny alleles derived from *r-sc:m335/r-r:n142* heterozygotes lacking *Ac*. DNAs were digested with *Hin*cII and *Ssp*I, fractionated in agarose gels and hybridized to pSc323:J20.

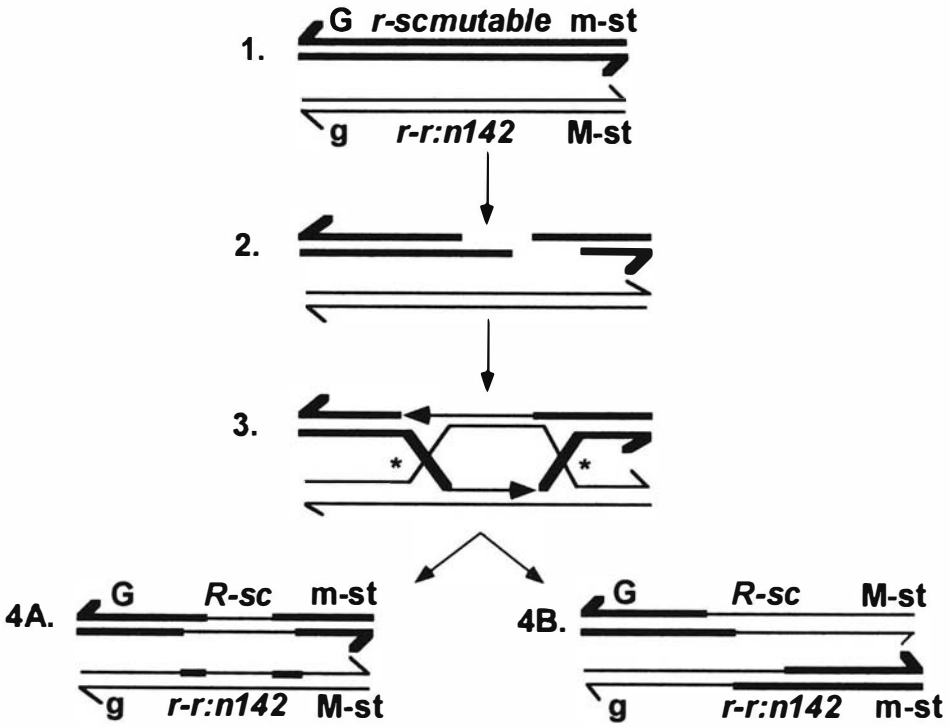

1.
G r-scmutable m-st
g r-r:n142 M-st
2.
3.
*
*
4A.
G R-sc m-st
g r-r:n142 M-st
4B.
G R-sc M-st
g r-r:n142 m-st

Figure 1.

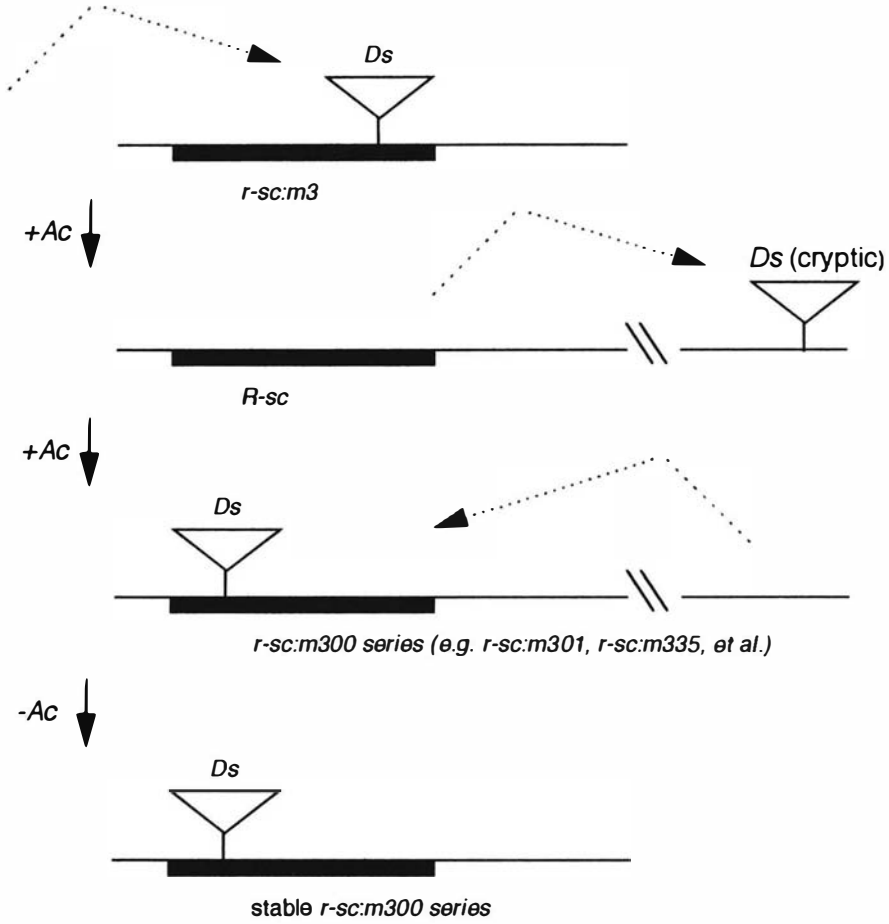

Ds
r-sc:m3
+Ac
Ds (cryptic)
R-sc
+Ac
Ds
r-sc:m300 series (e.g. r-sc:m301, r-sc:m335, et al.)
-Ac
Ds
stable r-sc:m300 series

Figure 2.

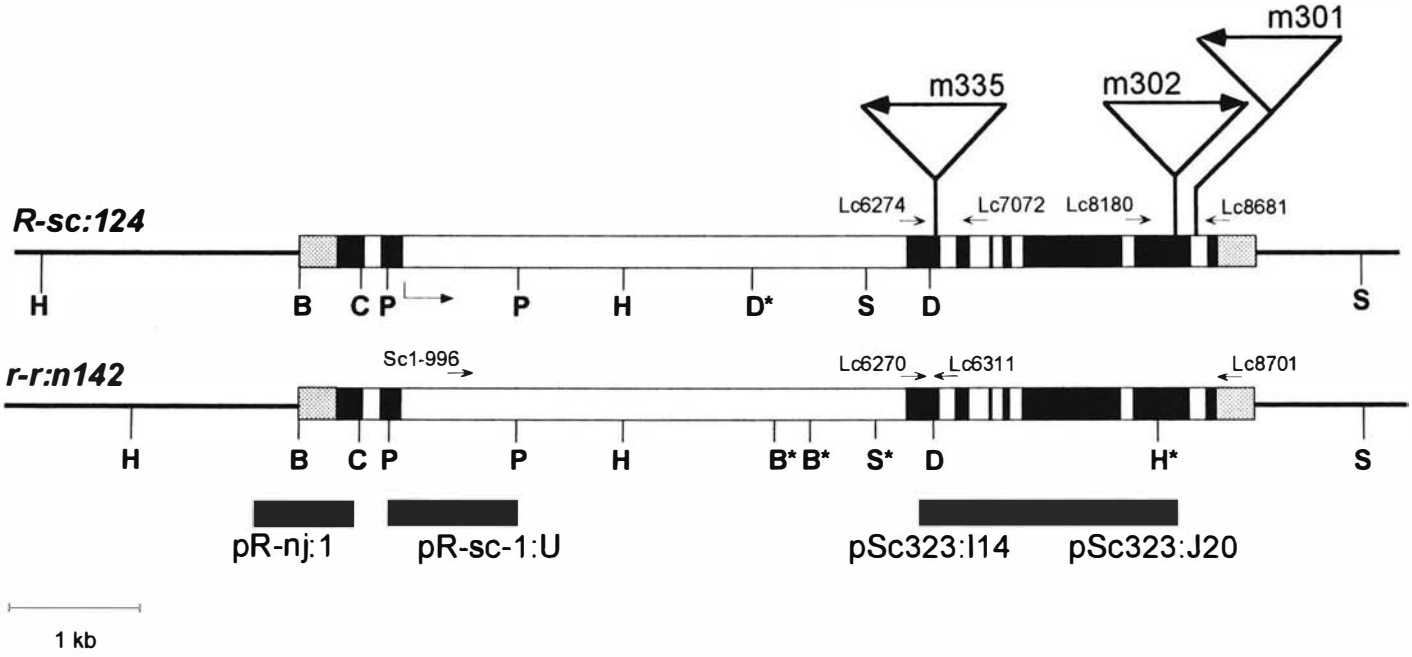

m301
m335
m302
R-sc:124
Lc6274
Lc7072
Lc8180
Lc8681
H
B
C
P
P
H
D*
S
D
S
r-r:n142
Sc1-996
Lc6270
Lc6311
Lc8701
H
B
C
P
P
H
B*
B*
S*
D
H*
S
pR-nj:1
pR-sc-1:U
pSc323:I14
pSc323:J20
1 kb

Figure 3.

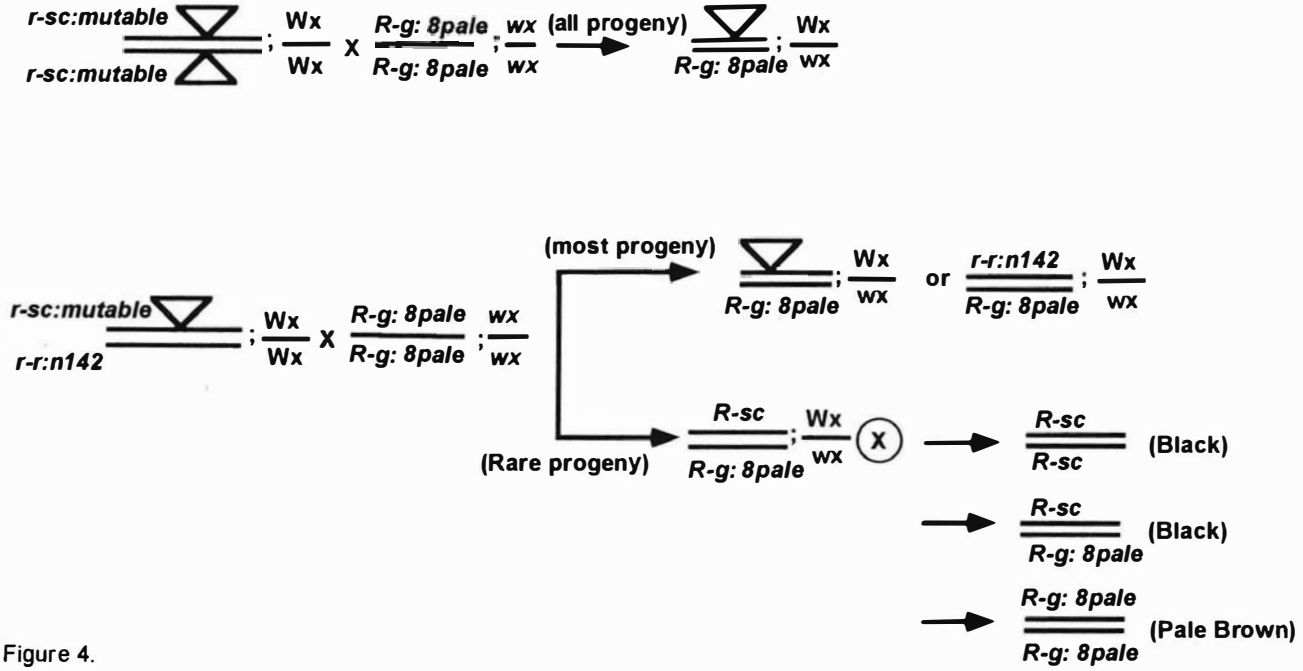
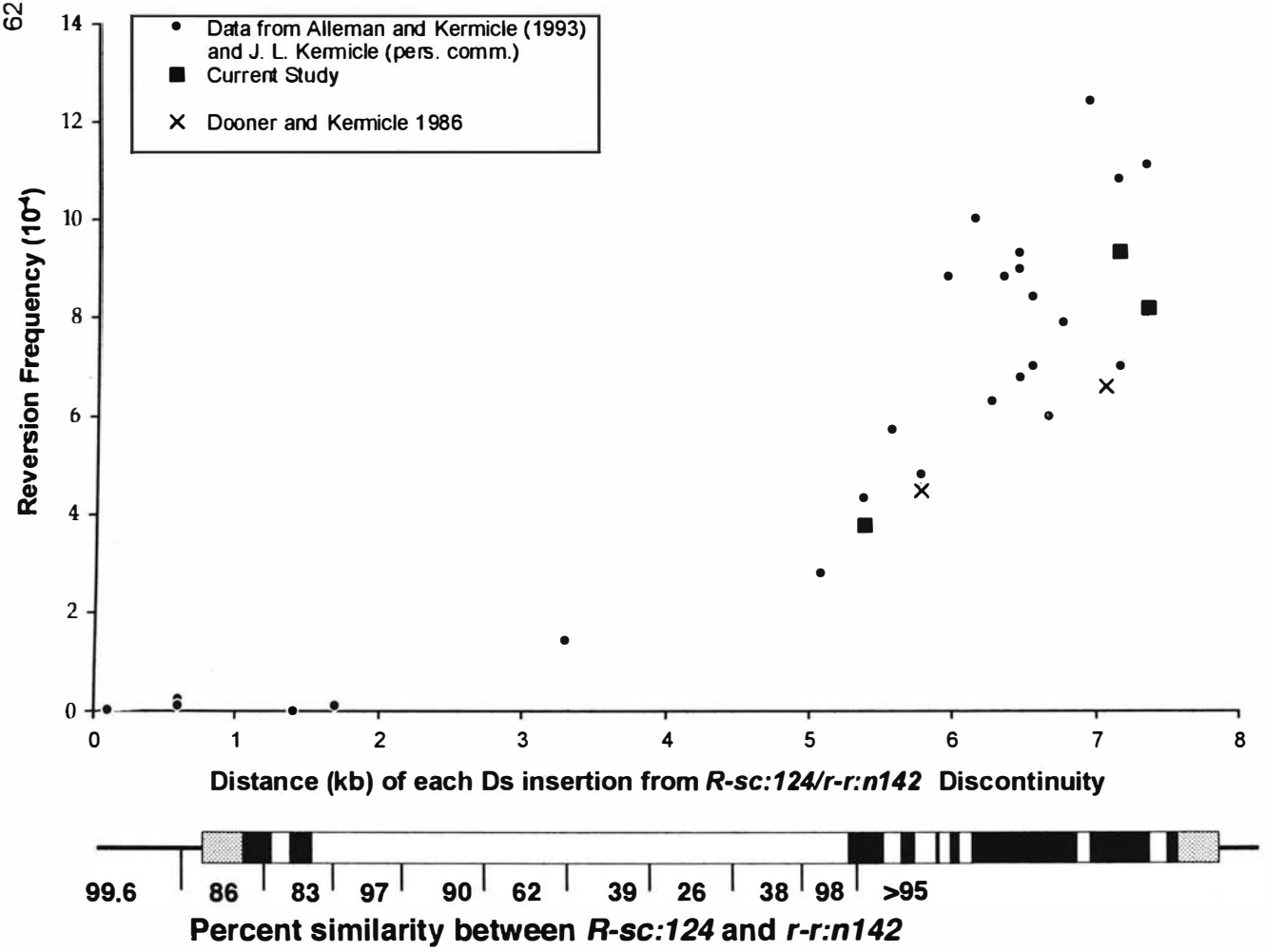

r-sc:mutable
r-sc:mutable
Wx
Wx
X
R-g: 8pale
R-g: 8pale
wx
wx
(all progeny)
R-g: 8pale
Wx
wx
r-sc:mutable
r-r:n142
Wx
Wx
X
R-g: 8pale
R-g: 8pale
wx
wx
(most progeny)
R-g: 8pale
Wx
wx
or
r-r:n142
R-g: 8pale
Wx
wx
(Rare progeny)
R-sc
R-g: 8pale
Wx
wx
X
R-sc
R-sc
(Black)
R-sc
R-g: 8pale
(Black)
R-g: 8pale
R-g: 8pale
(Pale Brown)

Figure 4.


Data from Alleman and Kermicle (1993) and J. L. Kermicle (pers. comm.)
Current Study
Dooner and Kermicle 1986
Reversion Frequency (10^{-4})
0
2
4
6
8
10
12
14
0
1
2
3
4
5
6
7
8
Distance (kb) of each Ds insertion from *R-sc:124/r-r:n142* Discontinuity
99.6
86
83
97
90
62
39
26
38
98
>95
Percent similarity between *R-sc:124* and *r-r:n142*

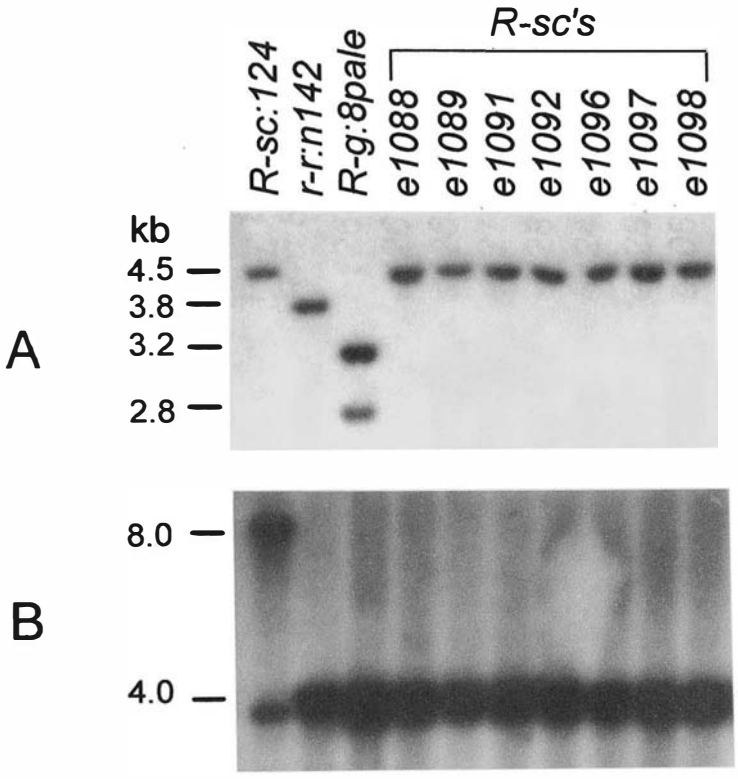

R-sc's
R-sc:124
r-r:n142
R-g:8pale
e1088
e1089
e1091
e1092
e1096
e1097
e1098
kb
4.5
3.8
3.2
2.8
A
8.0
4.0
B

Figure 6

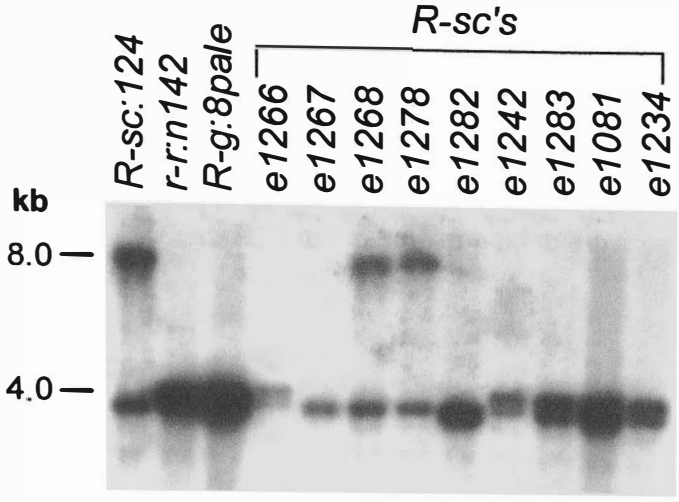

R-sc's
R-sc:124
r-r:n142
R-g:8pale
e1266
e1267
e1268
e1278
e1282
e1242
e1283
e1081
e1234
kb
8.0
4.0

Figure 7

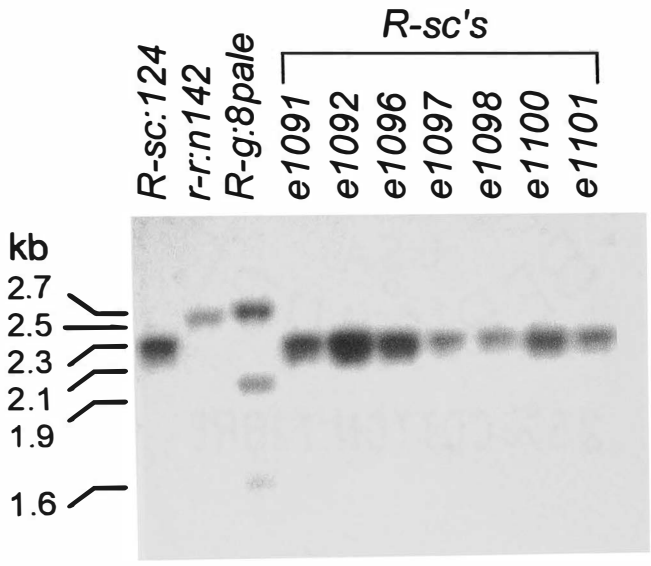

R-sc's
R-sc:124
r-r:n142
R-g:8pale
e1091
e1092
e1096
e1097
e1098
e1100
e1101
kb
2.7
2.5
2.3
2.1
1.9
1.6

Figure 8

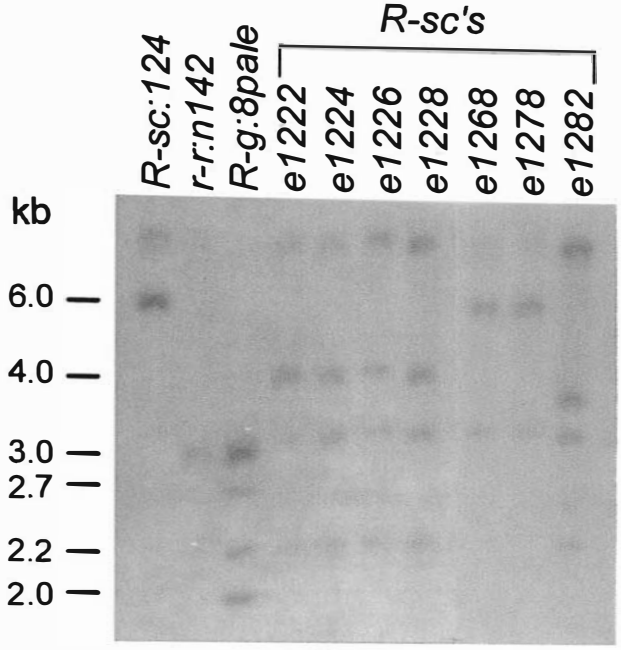

R-sc's
R-sc:124
r-r:n142
R-g:8pale
e1222
e1224
e1226
e1228
e1268
e1278
e1282
kb
6.0
4.0
3.0
2.7
2.2
2.0

Figure 9

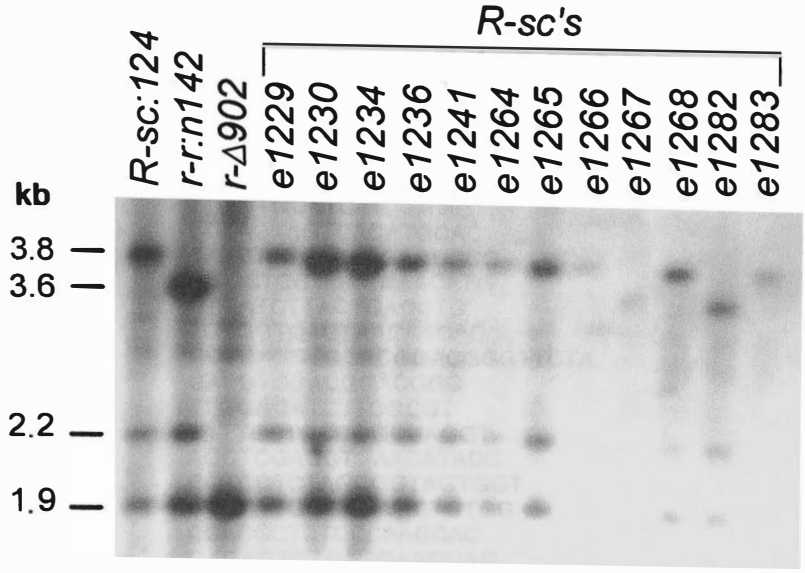

R-sc's
R-sc:124
r-r:n142
r-Δ902
e1229
e1230
e1234
e1236
e1241
e1264
e1265
e1266
e1267
e1268
e1282
e1283
kb
3.8
3.6
2.2
1.9

Figure 10

Table 1. Sequence of oligonucleotides used in PCR amplification and DNA sequence reactions.

Oligonucleotide	Oligonucleotide Sequence (5'-3')
Ds3'	TTCGTTTTTTACCTCGGGTTC
Ds5'	CGTTTTCGTTACCGGTATATC
Lc3242A	AGCGGGAGAATGCTAAGG
Lc3424	CCTTAGCATTCTCCCGCT
Lc3681	GAGGCCCATCCAGATAAC
Lc3876	TGCATGCGACATCGATC
Lc4504A	CCGACCCTCCACCCTC
Lc4504B	GAGGGTGGAGGGTCGG
Lc4804	TTGGTGCATGTGACTACT
Lc4807	CCTAGTAGTCACATGCAC
Lc5155A	CATGTTCCTCACGAGCCCC
Lc5155B	GGGCTCGTGAGGAACATG
Lc5406A	CTGATCTTACTGACCTG
Lc5406B	TCAGGTCAGTAAGATCAG
Lc6270	GCTGACGTGGACGGACGGGTTCTA
Lc6274	GACGTGGACGGACGGG
Lc6311	TTGGAGATCTTCGCGT
Lc6710	CTTTGCTGCCGGCGAGGT
Lc7072	AGCTCGAGCTGAACATACC
Lc7497	CGTCCTCTAGCGGTAGTGGT
Lc7775	GTGCTTGGGAGAGCTGTGG
Lc7775A	CCACGCTCTCCCAAGCAC
Lc8180	GAGAGTGTGAGGAAGGAG
LC8681	TTCCATGCCCGTCATGTCC
Lc8701	GCCTTCCATGCCCGTCGATGTCC
M13	CAGGAAACAGCTATGAC
Sc1-996	GCCGTTGTAGTTGTAGCTA
Sc1-1357	GACCACCACTTGTCACGT
Sc1-1360	TGACAAGTGGTGGTCTT
Sc1-2002	ACCACCATATATGTGTGCT
Sc1-2004	TGGAACACATATATGGT
T7	TAATACGACTCACTATAGGG

Table 2. PCR amplification conditions for *r-sc:mutable* alleles and *r-r:n142*.

Allele	Oligonucleotide Combination	Denaturing [T°/t][1]	Annealing [T°/t]	Elongation [T°/t]	Cycle number
r-sc:m301	Ds3'/Lc8180	94/45	58/60	68/60	30
	Ds5'/Lc8681	94/45	58/60	68/60	30
r-sc:m302	Ds3'/Lc8681	94/45	58/60	68/60	30
	Ds5'/Lc8180	94/45	55/60	68/45	30
r-sc:m335	Ds3'/Lc6274	94/60	55/60	72/45	30
	Ds5'/Lc6710	94/60	60/60	68/60	35
r-r:n142	Lc6270/Lc8701	94/60	62/60	68/180	35
	Sc1-996/Lc6311	94/60	55/60	68/240	35

1 = T° represents temperature in Celsius and t is time in seconds.

Table 3. Reversion frequencies of *r-sc:mutable* alleles.

Female genotype pollinated with *R-g:8pale*	Number of potential *R-sc* revertants recovered	Number of potential *R-sc* revertants successfully tested	Kernel Population	True *R-sc's*	Adjusted kernel population[a]	Reversion frequency (x 10^{-4})
r-sc:m301 / *r-r:n142*	79	60	64,344	40	48,869	8.2[2]
r-sc:m302 / *r-r:n142*	61	49	42,641	32	34,253	9.3[2]
r-sc:m335 / *r-r:n142*	38	37	51,692	17	50,332	3.8[1]
r-sc:m301 / *r-sc:m301*	0	0	11,354	0	11,354	0

a = (# of *R-sc* tested/# of *R-sc* recovered) x total kernel population

1 = Significantly different ($P < 0.001$) when compared to the reversion frequency of *r-sc:m301* or *r-sc:m302*.

2 = Not significantly different ($P > 0.50$)

VITA