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Kansas State University, 1992

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UNEQUAL RECOMBINATION AND MEIOTIC INSTABILITY OF *Rp1* REGION DISEASE RESISTANCE GENES IN MAIZE

by

MEHMET ALI SUDUPAK

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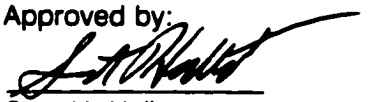

Scot H. Hulbert
Major professor

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1992

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CHAPTER ONE

Fine Structure Analysis of Complex Disease Resistance Genes and Unequal Recombination between Repeated Genes

INTRODUCTION

Race-specific resistance in plants is usually inherited in a Mendelian fashion. The same mode of inheritance is also common for specific virulence of a plant pathogen. Flor's pioneering studies established the Mendelian inheritance of traits involved in host-parasite association for the first time. His classical genetic analysis of both the host plant and pathogen demonstrated that resistance and avirulence traits are generally dominant and monogenically inherited. Interactions occur with gene-for-gene specificity. In the gene-for gene concept, each gene conferring resistance in the host plant has a corresponding gene conditioning avirulence in the pathogen (Flor 1971; Hooker and Saxena 1971; Crute 1985; Day 1985; Keen 1990).

In maize, *Zea mays* L., more than 25 genes conferring resistance to the maize common rust fungus, *Puccinia sorghi*, occur in at least six loci, *Rpl*, *rp2*, *Rp3*, *Rp4*, *Rp5*, and *Rp6*, (Hooker and Russel 1962; Hagan and Hooker 1965; Saxena and Hooker 1968; Hooker 1969). Most of the 25 genes identified by Hooker and co-workers clustered to two genomic areas, *Rp1* and *Rp3*. *Rpl* contains at least 14

resistance factors and is linked to *Rp5* and *Rp6*. The *Rp1* area is located on the short arm of chromosome 10 while the other *Rp* genes segregate independently of *Rpl* and have different chromosomal locations (SAXENA and HOOKER 1968; HOOKER 1969).

Saxena and Hooker (1968) demonstrated that some of the *Rp1* heterozygotes could recombine and reciprocal products could be recovered from certain test cross populations. Similarly, extensive studies of the *M* and *N* loci in flax, which contains seven and three closely linked disease resistance factors respectively, indicated that these loci also recombined reciprocally. Some of the genes at the *M* locus were combined in cis and fractionated into their original specificities (MAYO and SHEPHERD 1972, 1980). It was, therefore, concluded that genes at these loci reside at separate, closely linked loci which are independent of each other. The existence of such gene clusters implies that disease resistance determinants are usually arranged in groups in certain areas of plant genomes (FLOR 1965; SHEPHERD and MAYO 1972; SAXENA and HOOKER 1968). The observations of complexity in plant genes conferring resistance to fungal pathogens have stimulated investigators to propose models in order to explain their structure and origin. Three models have been suggested. One model is a locus with multiple alleles. The second model is that of a locus that contains a series of tandem duplications each carrying a different resistance gene. A third possibility is a combination of both models (SHEPHERD and MAYO 1972; BENNETZEN *et al*. 1990). These models are useful for the analysis of allelism and in construction of genetic

maps.

Several phenomena observed at *Rp1* could be interpreted as evidence that it is composed of multiple loci. One line of evidence supporting a multiple locus model is the reciprocal nature of recombination at *Rp1*. The products of reciprocal recombination between certain *Rp1* alleles have been observed (SAXENA and HOOKER 1968). Another line of evidence is the meiotic instability of some *Rp1* determinants. Susceptibles spontaneously arising in test cross progenies of most of the *Rp1* homozygotes implied that *Rp1* genes are meiotically unstable. The instability could have a number of mechanisms. Some of the well characterized instabilities are associated with unequal crossing-over (STURTEVANT 1925; LAUHNAN 1952; DOONER 1971; GOLDBERG 1983). Unequal crossing-over requires duplicated homologous sequences for mispairing and recombination. Since multiple specificities map to the *Rp1* area, and some of these genes recombine readily, it is likely that *Rp1* consists of a gene family. It is therefore possible that the different resistance genes in this area retain synaptic homology and can recombine by unequal crossing-over. However, since most unstable traits in maize have been associated with transposable elements, this is also a definite possibility.

The *Rp1* genes were originally identified by screening large number of maize lines from diverse sources of germplasm. DNA restriction fragment length polymorphism (RFLP) is therefore abundant at genomic sites flanking the *Rp1* locus in this collection. The *Rp1* genes have been transferred to the maize inbred R168 by backcrossing, creating a series of near isogenic differential lines.

Variation has been maintained among the differential lines at RFLP loci which are closely linked to the *Rp1* locus. These RFLP markers can be used to assay recombination at *Rpl* (Bennetzen *et al.* 1990; Hulbert and Bennetzen 1991).

The maize rust system has certain advantages for genetic analysis. Maize can be easily selfed and out crossed, and the biotypes of the common maize rust can be cultured and maintained during the identification and analysis of *Rpl* genes. Therefore, this host-parasite association is a good model system to study the genetics of host-parasite interactions and to analyze the complex structure of disease resistance genes (Saxena and Hooker 1968).

One object of this study will be to analyze the meiotic instability of $Rp1^J$ and $Rp1^G$ in F_1 individuals that are homozygous for these *Rp1* genes with heterozygous flanking markers at both distal and proximal RFLP loci. In addition, the recombination and mispairing in some *Rp1* heterozygotes will be analyzed.

LITERATURE REVIEW

Studies of disease resistance genes and the "Gene-for gene-concept": Deployment of disease resistance genes has been a valuable means of controlling pathogen epidemics and an important tool in crop improvement programs. However, little is known about their fine structure or the molecular basis of disease resistance. Because of their economical and biological importance, it is essential to analyze the structure of disease resistance genes.

Beginning with the Biffen's (1905, 1912) reports of Mendelian the inheritance of a single recessive gene conditioning resistance to yellow rust, *Puccinia striiformis*, in wheat, many single dominant resistance genes have been identified in various plant species. The majority of disease resistance genes are dominant, and they confer resistance only to specific races of their respective pathogen. The genetic basis of the interaction between host plant and pathogen was formally studied for the first time by Flor (1955). Flor studied the host-parasite association of flax, *Linium usitatissimum* L. and the flax rust, *Melamspora lini* Lev. Based on the analysis of inheritance of resistance and avirulence, respectively, in the two partners, he hypothesized that this relationship was controlled by a gene-for-gene specificity in which each gene conferring resistance in the host plant had a corresponding gene conditioning avirulence in the pathogen (FLOR 1971).

In flax, 29 genes conditioning resistance to flax rust occur at five different loci, *K*, *L*, *M*, *N*, and *P*. They contain 1, 14, 7, 3 and 5 alleles, respectively (FLOR 1971; ISLAM *et al.* 1989). Flor observed avirulence as a dominant trait, and F_2 cultures of hybrids between avirulent and virulent races segregated for virulence on differential lines of flax according to the number of genes for resistance in the host. On host lines carrying one resistance gene, F_2 cultures of the rust isolates segregated in a monofactorial ratio. On the other hand, on differential lines that carried two different resistance genes, F_2 cultures segregated in a bifactorial ratio. In this complementary relationship, the absence of either of the dominant genes (avirulence or resistance genes) results in susceptibility (Table 1). The resistance

gene (*R*) can not be detected unless challenged with a pathogen isolate carrying a complementary avirulence gene (*A*) (FLOR 1971). Multiple pathogen isolates are therefore necessary to detect and differentiate a large number of resistance genes. Some resistance genes, however, condition characteristic reaction types that can be distinguished from each other by the patterns and the extent of colonization.

TABLE 1

Specific interactions between resistance genes in the host plant and avirulence genes in the pathogen

Pathogen genotype		A_	aa
Plant	R_	R	S
genotype	rr	S	S

R = resistance phenotype.
S = susceptible phenotype.

Flor's findings were then applied to several other host-parasite interactions. In barley, resistance to the powdery mildew fungus (*Erysiphe graminis*) occurs at four different loci, namely *Mla*, *Mlg*, *Mlk*, and *Mlp*. One of these loci, *Mla*, contains 12 closely linked genes or alleles. The genetic organization of this locus has been analyzed using marker genes. A linear order was suggested for six of these resistance genes based on recombination frequencies (WISE and ELLINGBOE 1985). Close linkage and multiple allelism are also common characteristics of genes in lettuce which control resistance to downy mildew (HULBERT *et al.* 1985).

Aspects of fine structure analysis of complex disease resistance genes: "Cis-trans test", "Close linkage", and "Meiotic instability": Gene-for-gene specificity is a very useful model for fine structure analysis of complex disease resistance genes. However, when disease resistance genes are clustered at the same genomic area, it is difficult to differentiate between close linkage and multiple allelism by means of recombination alone. The normal *cis-trans* test, widely used to determine allelism, is not applicable to certain groups of genes, including genes conferring plant disease resistance whose expression is co-dominant. Hence, the modified *cis-trans* test was described by Shepherd and Mayo (1972) to differentiate close linkage from functional allelism. According to this test, the *trans* and *cis* arrangements of closely linked resistance genes will have the same phenotype because each gene conditions its own resistance independently. However, if the genes are allelic, they exhibit an interaction such that *cis* and *trans* arrangements are different. Therefore, in practice attempts to combine two genes in *cis* could provide the diagnostic information for the presence of close linkage versus allelism. In test cross progeny from a cross between lines carrying two closely linked genes, the two reciprocal products of recombination between these genes are individuals with the resistance of neither parent, and individuals with the combined resistance of both parents. Combining two different heteroallelic forms of a gene in the *cis* configuration requires an intragenic recombination event. An intragenic recombination event leading to an allele with the combined resistance of both parents would require that the single

gene product of this allele could express the specificities of both parental alleles. The basis of the modified *cis-trans* test is that this recombinant gene product is either impossible or unlikely to occur. Particularly in disease resistance genes whose expression is influenced by numerous biotic and abiotic factors, the expression of the new allele might represent a phenotype that may or may not be distinguishable (SHEPHERD and MAYO 1972).

An interesting aspect of the *cis-trans* test or the modified *cis-trans* test is the difficulty of their application to genes which are closely linked and can undergo unequal crossing-over. A classic example of this is the *Rp1* genes. A tentative map of some *Rp1* determinants was constructed by means of two point crosses. Most of the *Rp1* genes mapped within 0.4 cM (SAXENA and HOOKER 1968). Analysis of allelism between different *Rp1* genes was based on the frequency of recombination and recovery of reciprocal products of recombination in *Rp1* heterozygotes. When the two *Rp1* determinants recombined, they were considered as different genes. On the other hand, when the two *Rp1* determinants failed to recombine, they were considered as alleles of same gene. They were also able to obtain both products of recombination in three pairs of *Rp1* genes. More recently three RFLPs were positioned with respect to the *Rp1* area, and some of the *Rp1* determinants were mapped with respect to these RFLPs and to each other using these RFLPs as flanking markers. In addition, both non-parental combinations of flanking markers were observed for one of the *Rp1* heterozygotes. This was interpreted as a possible unequal crossing-over event (HULBERT and

BENNETZEN 1991). This implied that unequal crossing-over may occur in the *Rp1* area and demonstrated the importance of using flanking markers in elucidation of the nature of recombination at *Rp1*. Unequal crossing-over requires tandemly duplicated sequences (STURTEVANT *et al.* 1925). Since mispairing is common among tandem duplications, the determination of the order and allelism of genes carried on or linked to these duplications might present complications even if flanking markers are used. The conclusion of this study is that recombination in *Rp1* heterozygotes is not directly correlated with allelism and it should not be assumed that genes are not allelic because they can recombine if these genes are closely linked and able to undergo unequal crossing-over.

Another problem in fine structure analysis of disease resistance genes is their meiotic instability. *Rp1* area genes, which have been postulated to contain a number of serial tandem duplications, many of which are clustered within a 0.4 cM, appear to be very unstable. In agreement with the predictions made by Saxena and Hooker, recent observations of spontaneously arising susceptibles in test cross progenies of *Rp1* homozygotes suggested that unequal crossing-over might be the mechanism. Some *Rp1* alleles such as $Rp1^G$ give susceptibles up to 0.68% in test cross progenies of homozygotes (PRYOR *et al.* 1987; BENNETZEN *et al.* 1988). Genetic instabilities might also arise from transposon-induced instability or paramutation. However, many well characterized instabilities are associated with unequal crossing-over as elaborated in following section. Closely linked flanking markers are required to detect unequal crossing-over. Since genetic fine

structure analysis of disease resistance genes is based on the frequency of recombination which gives rise to susceptibles, it is crucial to determine the patterns of recombination in these complex loci. If the susceptibles can arise by mechanisms other than simple crossing-over between the two genes, the frequency of susceptibles might be misleading. The analysis of meiotic instability is also clearly required for determination of the correct order of the host genes conferring resistance. The analysis of meiotic instability will also provide important information about the overall structure and organization of closely linked genes for molecular analysis.

Unequal recombination between repeated genes in eukaryotes: Although substantial evidence indicates that gene duplication has played a major role in the evolution of complexity, little is known about the molecular mechanism of its formation. The *Bar* locus in *Drosophila* is the first example of the occurrence of tandem duplications. It leads to phenotypically (position effect) and cytologically detectable changes in subsequent progenies of unequal cross-over derivatives. Another locus in which unequal crossing-over was studied in great detail is the *white* locus (*w*) of *Drosophila*. The molecular analysis of the structure of reciprocal duplications and deletions produced by unequal crossing-over at the *w* locus in females heterozygous for different white alleles was done by Goldberg *et al.* (1983). They found that a transposable element (BEL) was present at different locations, at about 60 kb apart from each other in direct orientation, in the w^a

(apricot) and w^{a4} alleles. These derivatives were characterized as products of unequal cross-over by the genetic exchange of flanking markers. Further molecular analysis indicated that the transposable element provided the DNA sequence homology for mispairing and crossing-over in these derivatives (GOLDBERG *et al.* 1983). The recent cloning and molecular analysis of the *Bar* locus derivatives also demonstrated that a *P* element, *B104*, was inserted at the break point of the *bar* duplication, indicating that original duplication was probably initiated by the mispairing between two *B104* elements (TSUBOTA *et al.* 1989). Although the original duplication might have been started by misalignment of transposons, it has been shown that subsequent unequal crossing-over events occur within the direct tandem repeats rather than between transposons (TSUBUTA S.I. *et al.* 1991). Similar observations were also made with human color vision genes (red and blue-cone opsin genes); (NATHANS *et al.* 1986).

Genetic instability due to recombination between duplicated sequences has also been studied in plants. The best examples of such studies in maize are the *R* locus, a regulatory locus of the anthocyanin biosynthetic pathway which encodes for a transcriptional activator protein, and the *A* locus which also functions in the anthocyanin pigmentation pathway. Both loci have alleles which are carried on tandem duplications. Unequal crossing-over events were observed in both loci by using flanking morphological marker genes (DOONER *et al.* 1971-1974; LAUGHNAN *et al.* 1961). The former locus was studied in great detail at the classical and molecular level because of the availability of a number of different allelic forms of

the *R* locus and the readily detectable phenotype of unequal cross-over derivatives. The *R* locus contains three tandem repeats; *P*, the plant pigmenting component which regulates anthocyanin pigmentation of plant parts, *S*, the seed pigmenting component which controls the pigmentation of the aleurone layer of the seed, and *Q*, a truncated repeat homologous to *P* with a null phenotype. Components of the *R* locus retain enough synaptic homology to mispair in meiosis and give rise to derivatives that can only pigment either seed or plant parts. The first DNA fragment was isolated from the *R* locus by transposon tagging (Dellaporta *et al.* 1988). In addition, *Sn*, another regulatory locus related to *R*, has been cloned by transposon tagging (Tonelli *et al.* 1991). By using a full length cDNA clone from this locus, coding regions of the components of the *R* locus have been characterized. Unequal crossing-over derivatives from a maize line that carries both components in *cis* have been shown to carry either one of the components which corresponds to its phenotype. These results indicated that one of the components has been deleted from the derivative individuals via unequal crossing-over. Further molecular characterization of the *R* locus derivatives based on their restriction length fragment constitution revealed that 8 out of 9 cross-over derivatives have exchange events right in the coding region. These observations suggest that nearly all unequal cross-over events occur within the repeat, consistent with data from the *bar* locus (Robbins *et al.* 1991).

Recent molecular analysis of naturally occurring deletions causing some human genetic disorders, such as the Low-density-lipoprotein-receptor gene,

(Horsmthemke *et al.* 1987), the Duchenne muscular dystrophy gene, (Hu *et al.* 1991), and the α-globulin gene cluster have indicated the presence of a family of repetitive elements, *Alu*, right in the break points. The human genome contains about 3-5 x 10^5 unevenly distributed copies of *Alu* repeats which are homologous to each other. It has been proposed that *Alu* repeats provide homologous regions for misalignment of meiotic chromosomes, and recombination in misaligned regions results in deletions and duplications of whole genes or parts of genes. These findings suggest that repetitive elements may also provide genetic redundancy for mispairing, which results in formation of deletions and duplications (Nicholls *et al.* 1987). Artificial tandem repeats were constructed from single copy genes in lower eukaryotes such as yeast, using recombinant DNA techniques and yeast transformation systems. The first artificial duplication resulted from homologous integration of the *LEU2*$^+$ gene into the *LEU2* locus carrying a mutant *leu2* gene. These two copies of the *LEU2* gene were separated by plasmid sequences. In similar experiments, multiple tandem duplications were generated. Artificially constructed repeats gave extremely important information about the behavior of tandemly duplicated repeats in recombination. Some of the striking results obtained from studies of duplicated yeast genes include the demonstration of gene conversion between unequally paired duplicated genes (Maloney and Seymour 1987; Welch *et al.* 1990), and the observation of high frequencies of unequal sister chromatid exchanges in the rDNA array of yeast (Thomas *et al.* 1980).

Studies of recombination in tandemly duplicated genes in maize, Drosophila,

and yeast have indicated that the mispairing may occur as frequently as normal pairing (Dooner 1971; Goldberg *et al.* 1983). This indicates that pairing of homologous chromosomes has a significant effect on frequency of unequal crossing-over. From the studies in yeast, it has been proposed that the initiation of pairing between homologous chromosomes in meiosis starts at more than one point, and multiple initiation points might result in mispairing at regions that carry tandem repeats (Maloney and Fogel, 1985). However, it has not been experimentally demonstrated how chromosome pairing is initiated and how it contributes to mispairing. Another factor that could effect the frequency with which recombinants resulting from mispairing and crossing-over are recovered is the orientation of the repeats (Green 1967). Mispairing and recombination between inverted duplications may not be recovered when they result in acentric or dicentric chromosomes. Nevertheless, there are some models that genetic exchange might be a prerequisite for chromosome pairing at meiosis. That is, the initiation of genetic exchange might establish the pairing of homologous chromosomes. Testing these ideas has revealed that mutations impairing recombination do not disrupt the synapsis of homologous chromosomes and chromosome pairing proceeds independently of genetic exchange. However, mutations that effect primarily the formation of the synaptonemal complex have been shown to reduce the recombination frequency to some extent (Rockmill and Roeder 1990; Steele, Morris, and Robertson, 1991; Haber *et al.* 1991).

Multigene families which control complex developmental and defensive

processes arise from two types of duplications; one of which results in multiple copies of uniform members of the original gene, for example, rDNA genes. In the other type of duplications, copy number increase is followed by subsequent differentiation in nucleotide sequence of regulatory and coding regions, for example, the hemoglobins and chorion. As gene family members become more differentiated in both coding and regulatory regions, they may acquire different functions (OHTA *et al.* 1991). Duplication of a whole gene with subsequent differentiation is presumably the simplest and most advantageous way to acquire new functions, because the presence of the undifferentiated original gene allows the duplicated copy to differentiate and escape from natural selection. New variants may be created via unequal crossing-over between the two diverged copies (WILLIAMS 1990).

CHAPTER TWO

Unequal Recombination and Meiotic Instability of Rp1 Region Disease Resistance Genes in Maize

ABSTRACT

The *Rp1* region of maize was originally characterized as a complex locus which conditions resistance to the fungus *Puccinia sorghi*. the causal organism in the common rust disease. Subsequent studies have indicated that more than one *Rp* gene may be present in the *Rp1* region. For instance, we have shown that two presumed alleles of *Rp1*, $Rp1^J$ and $Rp1^G$, map roughly two centiMorgans apart. Some alleles of *Rp1* are meiotically unstable, but the mechanism of instability is not known. We have studied the role of recombination in meiotic instability in maize lines homozygous for either $Rp1^J$ or $Rp1^G$. Test cross progenies derived from a line that was homozygous for $Rp1^J$, but heterozygous at flanking markers, were screened for susceptible individuals. Five susceptible individuals were derived from 9772 progeny. All five had nonparental combinations of flanking markers; three had one combination of recombinant flanking markers while the other two had the opposite pair. In an identical study with $Rp1^G$, twenty susceptible seedlings were detected out of 5874 test cross progeny. Nineteen of these were associated with flanking marker exchange, eleven and eight of each recombinant marker

combination. Our results indicate that these disease resistance genes are carried on duplicated fragments which retain synaptic homology, and that unequal exchange is the main mechanism of their meiotic instability.

INTRODUCTION

Genes controlling resistance to plant pathogens have been identified in numerous plant species. Most of these genes are dominant and confer resistance to specific races of a pathogen (Hooker and Saxena 1971; Crute 1985; Keen 1990). Race-specific resistance genes interact with corresponding genes for avirulence in bacterial and fungal pathogens in a gene-for-gene manner (Flor 1955, reviewed by Keen, 1990). Genes that condition resistance to biotrophic fungi are often tightly clustered in the genome (Flor 1971; Shepherd and Mayo 1972; Saxena and Hooker 1968; Hulbert and Michelmore 1985; Wise and Ellingboe 1985). The *Rp* genes of maize, which condition resistance to the common rust fungus, *Puccinia sorghi*, provide a classic example of this clustering. Most of the 25 *Rp* genes identified by Hooker and co-workers mapped to two genomic areas (Hooker and Russel 1962; Lee *et al.* 1963; Hagan and Hooker 1965; Saxena and Hooker 1968). Each gene could be distinguished by the spectrum of rust isolates to which they conferred resistance. Six of the genes mapped to a locus on chromosome 3 ($Rp3^A$ - $Rp3^F$) and 16 genes mapped to an area on the short arm of chromosome 10. The lack of recombination between 14 of these genes in small test cross families suggested that they might be allelic and they were given the *Rp1* designation ($Rp1^A$ - $Rp1^N$). Two other genes were designated *Rp5* and *Rp6* as they mapped roughly one and two map units from $Rp1^C$ and three map units from each other. More extensive analysis of *Rp1* has indicated that susceptible individuals, and individuals with the

combined resistance of both parents, can be generated from test crosses of certain *Rp1* heterozygotes (SAXENA and HOOKER 1968). A similar analysis with flanking RFLP markers verified that such changes in resistance were generated by recombination and suggested that the *Rp1* locus consisted of more than a single cistron (HULBERT and BENNETZEN 1991). Most of the *Rp1* genes mapped within 0.3 map units of each other, but $Rp1^G$ maps 1-3 cM distally, near *Rp5*.

Another interesting aspect of *Rp1* is that some alleles are meiotically unstable. This instability has been observed as a high frequency of susceptibles in test crosses of certain *Rp1* homozygotes (PRYOR 1987; BENNETZEN *et al.* 1988). The mechanism of this instability is unknown. We have constructed lines homozygous for two *Rp1* area genes, $Rp1^J$ and $Rp1^G$, which were heterozygous for flanking markers. Test crosses of these lines were used to analyze the role of recombination in *Rp1* instability. We report here the characterization of the mechanism of meiotic instability of $Rp1^J$ and $Rp1^G$.

MATERIALS AND METHODS

Genetic stocks: All of the *Rp1* alleles were originally identified and backcrossed into the R168 genetic background by A. L. HOOKER and co-workers at the University of Illinois, Champaign-Urbana. Many of the *Rp1* genes were also transferred to the B14 inbred background. The maize lines carrying $Rp1^J$, $Rp1^G$, and $Rp1^I$ used in this study were in the R168 genetic background. The $Rp1^D$

gene employed was in the B14 inbred background. The inbreds OH43 and H95 carry no known *Rp* genes and were used as male parents in test crosses with the *Rp1* homozygotes. Maize line 1291, carrying the *oy* seedling morphological marker, was obtained from the Maize Genetics Stock Center, University of Illinois, Champaign-Urbana, and also carries no known resistance genes.

Flanking RFLP markers: Several restriction length fragment polymophisms (RFLPs) have been mapped to the short arm of chromosome ten, where the *Rp1* locus resides (Weber and Heletjaris 1989; Burr *et al.* 1991; Beavis and Grant 1991). Three RFLP loci were genetically positioned with respect to the *Rp1* genes by Hulbert and Bennetzen (1991) to provide flanking markers for the studies of the recombinational behavior of *Rp1*. Two RFLP loci, *NPI285* and *NPI422*, mapped proximally to the *Rp1* area. *NPI422* was the closest proximal RFLP marker, mapping roughly one cM from *Rp1* in most crosses. *NPI285* mapped three to ten cM proximal to *Rp1* depending on the cross. *BNL3.04*, was positioned one to two cM distal to $Rp1^G$. Both $Rp1^G$ and *BNL3.04* map distally to the other *Rp1* genes (Hulbert and Bennetzen 1991).

An alternative probe, *KSU3a,* was used to detect the *NPI422* locus. The *KSU3a* clone is a 3.5 kb *Eco*RI-*Hin*dIII fragment that was subcloned from a recombinant maize : lambda clone selected by hybridization to the *NPI422* probe. The *KSU3a* and *NPI422* probes often hybridize to the same genomic fragments upon Southern analysis, but *KSU3a* detects additional fragments. These additional

bands also cosegregate with the *Rp1* region (data not shown). The additional cosegregating bands detected by this probe makes it a more informative marker in most crosses. The probe used to detect the *BNL3.04* locus was a 1.2 kb *Pst*I-*Bam*HI fragment in the 2.2 kb *Pst*I insert of the *BNL3.04* clone (HULBERT and BENNETZEN 1991)

The *Rp1* genes were originally identified by screening large numbers of maize inbred lines from diverse sources of germplasm. The *Rp1* genes have been transferred to the maize inbred R168 by backcrossing, creating a series of near isogenic lines. Variation has been maintained among the differential lines at RFLP loci which are closely linked to the *Rp1* locus (HULBERT and BENNETZEN 1991). Hence, these RFLP markers can be used to assay recombination at *Rp1*.

Construction of *Rp1*^{J} homozygotes with heterozygous flanking markers: To assess the role of recombination in the instability of *Rp1*^{J} homozygotes, it was necessary to construct F_1 hybrids that were homozygous at *Rp1* but heterozygous at the flanking DNA markers. Two such F_1s were made for *Rp1*^{J} using three different recombinant maize lines as parents. Line J1 (Table 2) was used as a parent in both hybrids. The *Rp1*^{J}-R168 line carries the RFLP alleles *NPI285-6* and *BNL3.04-2*. Line J1 was derived from a recombination event within the cross (*Rp1*^{J}-R168/*Rp1*^{D}-B14)/H95 and has the *BNL3.04-1* allele from the *Rp1*^{D}-B14 line. Lines J2 and J3 were derived from recombination events between *Rp1* and *NPI285* and carried *Rp1*^{J} with the *NPI285-3* allele. Line J2 was derived from

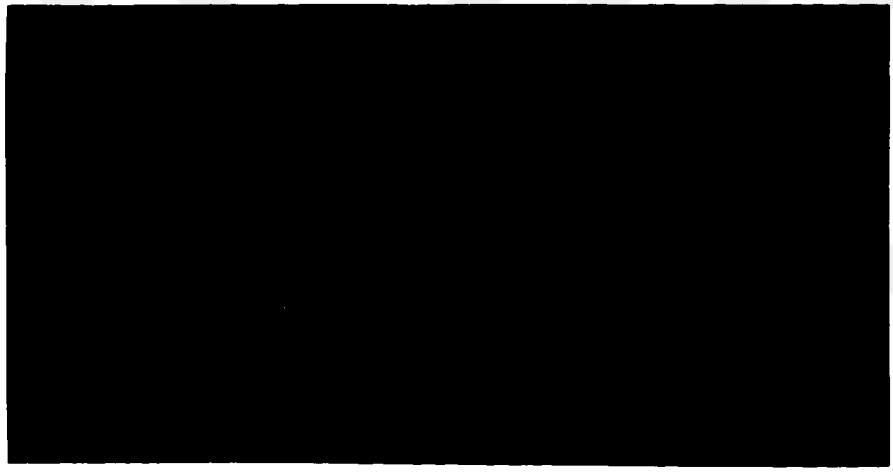
the above cross, while line J3 was derived from the cross (*Rp1*^{J}-R168/*Rp1*^{F}-R168)/1291. Recombinants from these populations were self-pollinated to obtain parental lines which were homozygous at *Rp1*^{J} and the flanking RFLP markers. Two separate populations were derived by test crossing hybrids of the lines J1 crossed to J2 and J1 crossed to J3 (Table 2).

Construction of *Rp1*^{G} homozygotes with heterozygous flanking markers: An F_1 hybrid was constructed which was homozygous for *Rp1*^{G} but heterozygous at flanking markers, essentially the same way the *Rp1*^{J} F_1s were assembled; from recombinant maize lines which carried *Rp1*^{G} but had either the distal or proximal marker alleles exchanged with alternate alleles. The *Rp1*^{G}-R168 line has the *NPI422-10* and *BNL3.04-2* alleles. Line G1 was selected from the cross (*Rp1*^{G}-R168/*Rp1*^{I}-R168)/B14. It was derived from a recombination event between *Rp1*^{G} and *BNL3.04* and had *Rp1*^{G} with the *BNL3.04-1* allele. Line G2 was derived from the cross (*Rp1*^{G}-R168 X 1291) X 1291 and carries *Rp1*^{G} with the *NPI422-9* allele. As with the *Rp1*^{J} recombinants, the original recombinants in these two populations were self-pollinated to obtain the G1, and G2 lines which were homozygous at *Rp1*^{G} and the recombinant flanking markers (Table 3). The hybrid between G1 and G2 was test crossed to a family to screen for susceptible derivatives.

The test cross families of both the *Rp1*^{G} and *Rp1*^{J} homozygotes were constructed by using resistant F_1s as the female parent so that susceptible

individuals would not result from pollen contamination.

Selection and analysis of susceptible test cross individuals: The test cross population from the *Rp1*^{J} lines were screened for susceptible individuals using the rust isolate KS1 that is avirulent on maize lines carrying *Rp1*^{J} (Hulbert *et al.* 1991). The *Rp1*^{G} test cross population was screened with the rust isolate 1-4 which is avirulent on maize lines carrying *Rp1*^{G} (Bennetzen *et al.* 1988; Hulbert *et al.* 1991). Test cross families were screened by planting 100-120 seeds in a 38x61x8 cm flat and inoculation with the appropriate rust isolate. Inoculated seedlings were incubated for 16 h in a mist chamber and scored for susceptible individuals seven days later. Susceptible seedlings from *Rp1*^{J} test crosses were covered with sporulating pustules (Figure 1) while the other seedlings in the flat showed a typical *Rp1*^{J} response; chlorotic spots with occasional small pustules. Susceptible individuals from the *Rp1*^{G} test cross were also fully susceptible while resistant individuals had very small necrotic spots typical of the *Rp1*^{G} response. Susceptible individuals were then transplanted into large pots, and seed was subsequently obtained following self fertilization. The progeny of each seedling were tested to verify susceptibility.



Rp1^J J1 x J3 # 1 J1 x J3 # 2 J1 x J3 # 3 J1 x J2 # 1

FIGURE 1.- Phenotypes of *Rp1^J* and susceptible progeny derived from test cross populations of *Rp1^J* homozygotes. Susceptible individuals correspond to those listed in Table 2.

Flanking marker analysis: Total genomic DNA extraction and gel blot analysis was performed as described previously (HULBERT and BENNETZEN 1991). *Sca*I digested genomic DNA of susceptible individuals derived from $Rp1^J$ populations and *Eco*RI digested genomic DNA of susceptible individuals derived from $Rp1^G$ populations were fractionated in 0.8% agarose gels and transferred to MSI blotting membranes. Membranes were hybridized to the ^{32}P dCTP-labeled RFLP probes *NPI285*, *KSU3a*, and *BNL3.04* for 24 hours at 65°. Hybridized membranes were washed with 0.1 x SSC (1 x SSC = 0.15 M sodium chloride and 0.015 M sodium citrate pH 7.0) and 0.1% SDS solution at 65° for at least 2 hours. The membranes were then autoradiographed for 24 - 48h at -80°.

RESULTS

Analysis of test cross progeny from $Rp1^J$ and $Rp1^G$ homozygotes: The screening of test cross progeny of $Rp1^J$ homozygotes yielded five susceptible seedlings out of 9772 tested. All five had nonparental combinations of flanking RFLP markers (Table 2). Four of the recombinants (out of 6414) were derived from the J1 x J3 population while the J1 x J2 population yielded only one recombinant out of 3358 seedlings screened. Three of the recombinants had the *NPI285-6* allele of J1 at the proximal RFLP locus and the *BNL3.04-2* allele of J3 at the distal RFLP locus (Table 2). The other two recombinants had the opposite nonparental combination of flanking markers (SUDUPAK and HULBERT 1992).

The test cross families of *Rp1*^{G} homozygotes yielded 20 susceptible individuals out of 5874 progeny. Nineteen of these were associated with flanking marker exchange (CO type), whereas one susceptible individual arose on a parentally marked chromosome (NCO type; Table 3). Of the 19 susceptible individuals associated with crossing-over, eight had the *NPI422-9* allele of G2 at the proximal RFLP locus together with the *BNL3.04-1* allele of G1 at the distal RFLP locus, whereas 11 carried the *NPI422-10* allele of G1 along with the *BNL3.04-2* allele of G2 (Figure 2). The single NCO type individual carried both proximal and distal flanking markers of the G1 parent.

Susceptibility of the recombinant individuals from the *Rp1*^{J} and *Rp1*^{G} populations was verified by progeny testing with the appropriate rust isolate. Progeny from all of the recombinants were found to be completely susceptible, including the individual from the *Rp1*^{G} population which originated on a parentally marked chromosome.

FIGURE 2.- Southern blot analysis to determine flanking marker combinations of susceptible progeny derived from test cross populations of *Rp1*^{G}. DNA from susceptible *Rp1*^{G} individuals was digested with *Eco*RI and probed with either the distal RFLP marker, ***BNL3.04*** (A) or the proximal RFLP marker, ***NPI422*** (B). Lanes: 1) tester parent, H95; 2) line G2; 3) line G1; lanes 4-14 are susceptible progeny, numbers (listed in Table 2) 1, 2, 3, 4, 12, 13, 14, 15, 16, 17, 18, 19, respectively.

TABLE 2

Susceptible test cross progeny from *Rp1*^{J} homozygotes

		Genotypes at Flanking RFLP markers[1]	
Test crosses	Origin[2]	Proximal marker	Distal marker
Parent J1		*NPI285-6*	*BNL3.04-1*
Parent J2 and J3		*NPI285-3*	*BNL3.04-2*
Susceptible progeny			
(J1 x J3) x H95 # 1	CO	*NPI285-6*	*BNL3.04-2*
# 2	CO	*NPI285-6*	*BNL3.04-2*
# 3	CO	*NPI285-6*	*BNL3.04-2*
# 4	CO	*NPI285-3*	*BNL3.04-1*
(J1 x J2) x H95 # 1	CO	*NPI285-3*	*BNL3.04-1*

1. Only the flanking marker alleles from the F_1 parent are given for the recombinants; the tester parent, H95, is homozygous for *NPI422* and *BNL3.04*.

2. CO implies that the derivative had nonparental combinations of flanking markers.

TABLE 3

Susceptible test cross progeny from *Rp1*^{G} homozygotes

		Genotypes at Flanking RFLP markers[1]	
Test cross	Origin[2]	Proximal marker	Distal marker
Parent G1		*NPI422-10*	*BNL3.04-1*
Parent G2		*NPI422-9*	*BNL3.04-2*
Susceptible progeny			
(G1 x G2) x H95 # 1	CO	*NPI422-10*	*BNL3.04-2*
# 2	CO	*NPI422-10*	*BNL3.04-2*
# 3	CO	*NPI422-10*	*BNL3.04-2*
# 4	CO	*NPI422-10*	*BNL3.04-2*
# 5	CO	*NPI422-10*	*BNL3.04-2*
# 6	CO	*NPI422-10*	*BNL3.04-2*
# 7	CO	*NPI422-10*	*BNL3.04-2*
# 8	CO	*NPI422-10*	*BNL3.04-2*
# 9	CO	*NPI422-10*	*BNL3.04-2*
# 10	CO	*NPI422-10*	*BNL3.04-2*
# 11	CO	*NPI422-10*	*BNL3.04-2*
# 12	CO	*NPI422-9*	*BNL3.04-1*
# 13	CO	*NPI422-9*	*BNL3.04-1*
# 14	CO	*NPI422-9*	*BNL3.04-1*
# 15	CO	*NPI422-9*	*BNL3.04-1*
# 16	CO	*NPI422-9*	*BNL3.04-1*
# 17	CO	*NPI422-9*	*BNL3.04-1*
# 18	CO	*NPI422-9*	*BNL3.04-1*
# 19	CO	*NPI422-9*	*BNL3.04-1*
# 20	NCO	*NPI422-9*	*BNL3.04-2*

1. Only the flanking marker alleles from the F_1 parent are given for the susceptible progeny; the tester parent, H95, is homozygous for *NPI422* and *BNL3.04*.

2. CO implies that the derivative had nonparental combinations of flanking markers while NCO implies that it did not.

DISCUSSION

The role of recombination in meiotic instability was examined in two different *Rp1* region genes, $Rp1^J$ and $Rp1^G$. Previous mapping experiments have indicated that most *Rp1* genes, including $Rp1^J$, are clustered within about 0.4 cM of each other (SAXENA and HOOKER 1968, HULBERT and BENNETZEN 1991). $Rp1^G$ mapped up to three cM distally in some crosses. Estimates of meiotic instability have been reported for many of the *Rp1* genes (PRYOR 1987, BENNETZEN *et al.* 1988). As with the present study, previous estimates of instability were conducted by screening test cross populations of *Rp1* homozygotes. It was not possible to test the role of recombination in previous studies because the flanking markers were not available. No previous estimate of $Rp1^J$ instability is available but the present frequency (5×10^{-4}) was similar to that reported for a number of different *Rp1* homozygotes. The previous estimates of instability of $Rp1^G$ varied from 1.8 to 7 $\times 10^{-3}$, the highest observed for any *Rp1* area gene (PRYOR 1987, BENNETZEN *et al.* 1991). The frequency of $Rp1^G$ instability in the present population (3.4×10^{-3}) lies within this range.

All five susceptible derivatives from $Rp1^J$ homozygotes and 19 of the 20 derivatives from $Rp1^G$ homozygotes were associated with recombination of closely linked flanking markers. Furthermore, both nonparental combinations of flanking markers were obtained from both types of populations. This indicates that the susceptible derivatives arose by unequal crossing-over. Unequal crossing-over

requires sequence duplications that retain synaptic homology. Studies of recombination between duplicated sequences in maize (Dooner and Kermicle 1971; Robbins *et al.* 1991), Drosophila (Davis, Shen and Judd 1987) and yeast (Maloney and Fogel 1987) have indicated that mispairing occurs frequently, possibly as frequently as normal pairing. The high level of instability in some *Rp1* genes is consistent with this observation. The recovery of both possible nonparental combinations of flanking markers in both $Rp1^{J}$- and $Rp1^{G}$-derived susceptible progeny is evidence of mispairing in both possible directions (Figure 3).

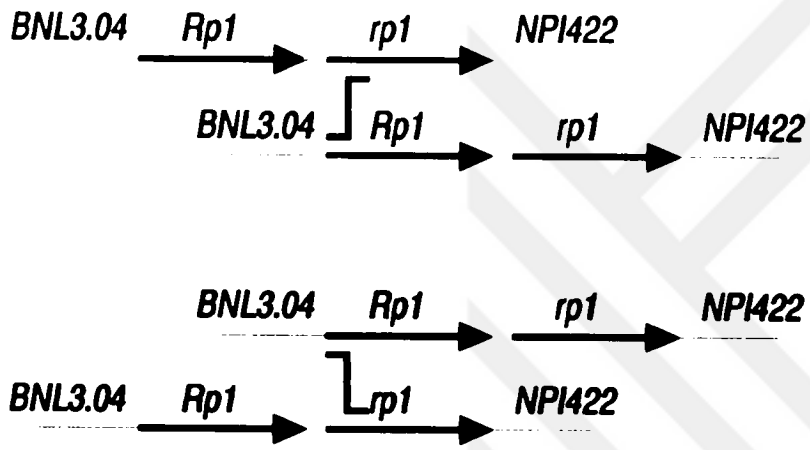


FIGURE 3.- An unequal crossing-over model (modified from Dooner and Kermicle 1971) which explains the instability in the *Rp1* region. The large arrows represent direct repeats carrying the *Rp1* alleles. *NPI422* and *BNL3.04* are the flanking RFLP markers used to assay recombination. Possible mispairing configurations and unequal exchanges are shown. The result of such exchanges will produce susceptible individuals that do not bear detectable *Rp1* alleles and have either of two possible combinations of recombinant flanking markers.

In a previous analysis of recombination in various *Rp1* heterozygotes, virtually all of the susceptible recombinants exhibited recombinant flanking markers (HULBERT and BENNETZEN 1991). Only one recombinant, from an $Rp1^E/Rp1^F$ heterozygote, was reported with parentally marked chromosomes, and this individual died without producing seed which could be progeny tested to verify it's susceptibility. It is likely that this individual was not a true susceptible since the $Rp1^F$ phenotype can be difficult to score unambiguously. In one of the heterozygotes analyzed, $Rp1^D/Rp1^F$, both nonparental combinations of flanking markers were obtained, indicating that mispairing and recombination had occurred. The propensity of derivatives from both *Rp1* homozygotes and heterozygotes to exhibit flanking marker exchange indicates that recombination at *Rp1* generally results from interchromosomal events. This varies somewhat from what has been observed at the *R* and *A* loci of maize (LAUGHNAN 1961; ROBBINS *et al.* 1991). Both loci have alleles which carry duplicated sequences capable of mispairing and recombination, and derivatives with altered patterns of pigmentation are often associated with recombinant flanking markers. Both loci, however, have complex alleles in which derivatives that are not associated with flanking marker exchange (NCO types) make up nearly 50% of the total derivatives (LAUGHNAN 1961; ROBBINS *et al.* 1991). The mechanism by which the NCO derivatives arise is not known. Possibilities include mutation, intrachromosomal recombination and gene conversion following mispairing. The latter events have been documented in Drosophila (PETERSON and LAUGHNAN 1963; HIPEAU-JACQUATE *et al.* 1989) and yeast

(Maloney and Seymour 1987; Jackson and Fink 1985). NCO type derivatives from the *R* locus do not appear to have lost a copy of the duplication, indicating an absence of intrachromosomal events such as unequal sister chromatid exchange or intrachromatid recombination which would result in deletion of a copy of the duplication (Robbins *et al.* 1991). It is not clear whether NCO derivatives from complex alleles of the *A* locus have lost a copy of the duplication.

The unequal crossing-over model in Figure 3 assumes that the unstable *Rp1* area genes lie on the duplicated sequences which mispair. An alternative model is that $Rp1^{J}$ and $Rp1^{G}$ do not lie on duplicated sequences but are flanked by repetitive sequences which can mispair and recombine. This type of recombination has been documented at a number of human loci (Horshemke *et al.* 1987, Ariga *et al.* 1990, Hu *et al.* 1991), Nicholls *et al.* 1987) and at the *white* locus of Drosophila (Goldberg *et al.* 1983). While mispairing and recombination between dispersed repeated sequences are thought to be important in the creation of the initial duplications of complex loci (Goldberg et al 1983; Tsubota *et al.* 1989), these are thought to be rare events. The high frequency of unequal crossing-over in the *Rp1* area, therefore, argues against this model. Furthermore, the high rate of intragenic recombination observed at loci such as *waxy* or *bronze 1* (Dooner *et al.* 1986; Nelson *et al.* 1962) indicates that most recombination in the maize genome lies in low copy sequences. The only example of unequal crossing-over in maize in which derivatives have been analyzed at the molecular level is the *R* locus (Robbins *et al.* 1991). Molecular analysis indicated that most of the unequal

recombination events occurred intragenically. Another indication that *Rp1* instability results from exchange between sequence duplications is that recombination studies have indicated that the *Rp1* area carries more than a single disease resistance locus, some of which (e.g. *Rp5*, *Rp1*^{G}) are easily separable by recombination. Complex disease resistance loci which carry multiple genes may provide crucial variation to plant species which are coevolving with biotrophic pathogens. Multiple genes can be combined in a single haplotype and new combinations can be generated by recombination. In addition, mispairing and intragenic recombination may add to the arsenal of resistance genes by generating novel genes.

The unusually high level of instability of *Rp1*^{G} indicates that the duplicated sequences involved in recombination, recombine frequently. Our frequency of susceptibles with recombinant flanking markers was 3.2×10^{-3}. The reciprocal class of recombination events, those with two copies of *Rp1*^{G}, was not detectable in our assay. In addition, since a *Rp1*^{G} homozygote was used in the experiment, mispairing is required for the recombination event to be observed. Furthermore, if the duplication that carries the *Rp1*^{G} genes is involved in the recombination event, not all cross-overs will be detectable, depending on the proximity of the cross-over to the *Rp1*^{G} gene carried on the duplication (Figure 3). Since the number of duplicated sequences adjacent to *Rp1*^{G}, and the frequency in which they freely mispair is not known, it is impossible to estimate the genetic size of the duplications. Collectively, they appear to span over one half a map unit or more

of recombination in the current population.

Another interesting aspect of *Rp1*^{G} recombination is the variable frequencies of instability in different *Rp1*^{G} homozygote and heterozygote populations. As stated above, the frequency of susceptible individuals in progeny of *Rp1*^{G} homozygotes varies from 1.8 to 6.8 x 10^{-3}. The frequency from heterozygotes in which *Rp1*^{G} was one of the parents is also very variable (1.8 x 10^{-2} to 9 x 10^{-4}; SAXENA and HOOKER 1968; HULBERT and BENNETZEN 1991), and, in some cases, may be lower than the homozygote frequencies. Only three susceptible recombinants were observed from 3450 test cross progeny of an *Rp1*^{G}/*Rp5* heterozygote (SUDUPAK and HULBERT unpublished data). Since *Rp1* recombination and instability requires interchromosomal cross-over events, the variable frequency of recombination in heterozygotes might be expected if the duplication(s) which mispair and recombine in *Rp1*^{G} homozygotes do not pair as well with related sequences in lines such as the *Rp5* line. Similarly, differences in recombination rates from *Rp1* homozygotes may be due to structural differences that have occurred in the different *Rp1*^{G} stocks. Since unequal crossing-over events themselves would alter the tandem copy number of duplications involved, the structural variation that might inhibit further effective pairing could be rapidly generated.

CHAPTER THREE

Recombination and Mispairing in *Rp1* Heterozygotes

ABSTRACT

The *Rp1* locus of maize conditions resistance to races of the maize common rust fungus, *Puccinia sorghi*. Closely flanking restriction fragment length polymorphisms (RFLPs) were used to assay recombination in *Rp1* heterozygotes. Susceptible recombinant progenies were obtained from test cross progenies of several *Rp1* heterozygotes. Recombinants, carrying resistance phenotypes of neither parents or the combined resistance phenotypes of both parents were recovered from test cross progenies of both $Rp1^{J}/Rp1^{F}$ and $Rp1^{D}/Rp1^{J}$ heterozygotes, and all of the recombinants arose on nonparentally marked chromosomes. Both combinations of flanking markers were observed in recombinants derived from the test cross progenies of $Rp1^{J}/Rp1^{F}$ heterozygotes, which suggest that these genes are probably allelic, but mispairing allows them to recombine. As previously observed, most of the *Rp1* genes mapped closely together. $Rp1^{G}$ and *Rp5*, which were positioned 1-3 cM distally to the other *Rp1* genes, mapped approximately 0.2 cM from each other, with $Rp1^{G}$ appearing to be distal to *Rp5*. The close linkage and distal position of $Rp1^{G}$ to *Rp5* suggest that there might be another cluster of *Rp* genes in the *Rp1* area.

INTRODUCTION

A common feature of genes conferring resistance to obligate fungal parasites in plants is that they are often clustered in the plant genome; either as multiple alleles at a single locus or as closely linked multiple loci (Shepherd and Mayo 1972; Saxena and Hooker 1968). Another interesting feature of disease resistance genes is their recognition of specific races of a biotrophic pathogen as first described by Flor. Flor's genetic analysis of the factors interacting in the flax and flax rust fungus has established the genetic basis of host and pathogen association (Flor 1955-1971). Since then a number of gene complexes have been studied by utilizing large numbers of progenies. Fine structure mapping studies have been attempted in three host-parasite associations; maize rust (Saxena and Hooker 1968, 1971; Bennetzen *et al.* 1988_{a-b}; Hulbert and Bennetzen 1991), flax rust (Flor 1955, 1971; Shepherd and Mayo 1972, 1980; Islam *et al.* 1989-1991), and barley powdery mildew (Wise and Ellingboe 1985)

Rp1, the disease resistance locus conferring resistance to *Puccinia sorghi* Schw. in maize, *Zea mays* L., has been studied extensively. Following the identification of a series of disease resistance factors by Hooker and co-workers from various germplasm in the 1960s (Hooker and Russel 1962; Hagan and Hooker 1965; Saxena and Hooker 1968), most of these factors were mapped to the same genomic area by screening large numbers of test cross populations (Saxena and Hooker 1968). These closely linked factors were designated as *Rp1* alleles since

they were initially considered to be allelic. However, in subsequent mapping studies, the recovery of reciprocal recombinants from test cross progenies of some *Rp1* heterozygotes implied that these genes are probably closely linked and that *Rp1* is a complex disease resistance locus (Saxena and Hooker 1968).

The fine structure analysis of closely clustered resistance factors requires a precise analysis of recombinational data along with testing of allelism in heterozygotes. Closely linked flanking markers are essential for both testing of allelism and mapping (Hulbert and Bennetzen 1991). Genetic markers are also useful for guarding against pollen contamination. This is particularly important when screening for rare recombinants. Closely linked RFLP markers flanking the locus have been identified. Recombination was recently analyzed in several *Rp1* heterozygotes using these flanking markers. Susceptibles arising from test crosses were always associated with flanking marker exchange indicating that they arose by crossing-over. This mapping experiment indicated that most of the *Rp1* genes mapped within 0.2 map units. $Rp1^G$ and *Rp5*, however, mapped 1-3 map units distally depending on the cross (Hulbert and Bennetzen 1991).

The *Rp1* genes were extracted from germplasm collected from different parts of the world and transferred to the R168 genetic background. Since the *Rp1* genes were derived from diverse sources, restriction site polymorphism is common among different *Rp1* isogenic lines at RFLP loci which are closely linked to *Rp1*. Therefore, these RFLP markers can be used to detect recombination and to determine the order of genes in recombinant individuals arising in test cross

progenies of *Rp1* heterozygotes.

Aims of this study include the analysis of instability in the *Rp1* heterozygotes and the mapping of some *Rp1* genes using flanking RFLP markers.

MATERIALS AND METHODS

Genetic stocks: The *Rp1* alleles used in this study were obtained from A. Hooker, who originally extracted these genes from maize germplasm collected from Europe, Africa and America, and transferred them into the R168 background, (A. HOOKER, University of Illinois, Champaign, Urbana). A few of these alleles were also transferred to the B14 genetic background. The list of *Rp1* alleles used in this experiment, and their origin and reaction to different rust isolates is shown below (Table 4), (HULBERT *et al.* 1991).

Table 4

Specific virulence phenotypes of *Puccinia sorghi* isolates on *Rp1* differential lines.

Rp1 gene	Source[1]	Background[2]	Rust isolates					
			1-4	AF1	IN1	IN2	IN3	KS1
Rp1^{A}	GG208R, G. King	R168	-	+	+	-	-	+
Rp1^{B}	B38	B14	I	I	+	-	-	+
Rp1^{C}	K148	R168	+	+	+	-	+	+
Rp1^{D}	Cuzco	R168	-	+	-	-	-	+
Rp1^{F}	PI 172332	R168	-	+	+	-	-	+
Rp1^{G}	PI 163558	R168	-	+	-	+	-	+
Rp1^{I}	PI 163558	R168	-	-	+	-	+	-
Rp1^{J}	Queretaro VI 366	R168	+	+	-	+	-	-
Rp1^{K}	Queretaro V 231-5	R168	-	-	-	-	+	-
Rp1^{L}	PI 163558	R168	+	-	+	-	+	+
Rp5	PI 186191	R168	I	+	-	+	+	-

+ : Isolate is virulent, compatible interaction.
- : Isolate is avirulent, incompatible interaction.
I : Intermediate interaction.
1 : Progenitor maize lines from which *Rp1* genes were isolated
2 : Genetic background.

Construction of test cross populations for fine structure mapping: Test cross populations were constructed to determine the recombination frequency between *Rpl* alleles and to map these alleles with respect to each other. Heterozygotes carrying two different *Rpl* alleles were crossed to susceptible (*rpl*/*rpl*) inbred lines. The resulting progenies were screened with appropriate rust isolates to which the *Rp1* alleles carried in the heterozygotes provide resistance. We used two different testing methods to isolate recombinant individuals from the test cross progenies. In one of these methods, the test cross progenies were screened with one rust isolate to which both genes of the *Rp1* heterozygote provide resistance. From this type of screening only the susceptible recombinants could be isolated because the other reciprocal product of recombination carrying resistance of both parents would have the same phenotype as nonrecombinant progeny. In the other method, test cross populations were screened with two complementary rust isolates each of which detects one *Rp1* allele. Since recombinant individuals derived from a simple recombination in *Rp1* heterozygotes would be either susceptible or resistant to both isolates, both types of recombinants can be identified. In all test crosses, the susceptible inbred parent was always used as a pollen source in order to reduce the susceptibles arising from pollen contamination. Individuals from self pollination would be resistant and susceptible individuals could not result from pollen contamination.

The rust cultures used in this study were collected from different geographic regions of United States (Hawaii, Indiana, Texas), and Africa (Hulbert *et al* 1991).

In addition, a rust isolate, KS1, was collected from Rocky Ford experiment farm, Kansas State University, Manhattan in 1991. Rust isolates were purified from a single colony and maintained on an isogenic line which prevented contamination from other isolates. Before the screening of the test cross progenies, uredospores of these rust cultures were mixed with talc. This mixture was then dusted over seven day-old seedlings. This was done by using a separate greenhouses for each rust culture in order to avoid cross-contamination. Dusted plants were placed in a moist chamber for 16 hours and scored after seven days. The same procedure was followed for test crosses inoculated with two complementary rust isolates; three days after inoculation with the first isolate, the same seedlings were inoculated with the second rust isolate. These were then scored for double resistants (recombinants carrying resistance phenotypes of both parents in *cis*) or double susceptibles seven days later. Three thousands to five thousands seedlings were screened for each *Rp1* heterozygote depending upon the recombination frequency. After scoring the test cross progenies, susceptible recombinants and resistant recombinant individuals were transplanted into pots to obtain seed following self pollination in order to progeny test and to characterize RFLP marker loci constitutions.

Map distances were calculated on the assumption that reciprocal crossing-over between two dominant genes generates equal numbers of susceptible individuals and double resistants when only susceptible individuals were assayed. By doubling the frequency of susceptible recombinants, recombination distances

between two genes were estimated (Saxena and Hooker 1968; Dooner 1971, 1974). Confidence intervals of recombination frequencies were calculated by Steven's method (Stevens *et al.* 1942).

Flanking RFLP markers: Several RFLP loci have been mapped to the short arm of chromosome 10, where the *Rp1* locus resides (Weber and Heletjaris 1989; Burr *et al.* 1991; Beavis and Grant 1991). Three RFLP loci were genetically positioned with respect to the *Rp1* genes by Hulbert and Bennetzen (1991) to provide flanking markers for studies on recombinational behavior of *Rp1*. Two RFLP loci, *NPI285* and *NPI422*, mapped proximally to the *Rp1* area. *NPI422* was found to be the closest proximal RFLP marker, mapping roughly 1 cM from *Rp1* in most crosses. *NPI285* mapped 3-10 cM proximal to *Rp1* depending on the cross. *BNL3.04*, was positioned 1-2 cM distal to $Rp1^G$. Both $Rp1^G$ and *BNL3.04* map distally to the other *Rp1* genes (Hulbert and Bennetzen 1991).

Flanking marker analysis: Total genomic DNA extraction and gel blot analysis was performed as previously described (Hulbert and Bennetzen 1991). Genomic DNA of susceptible individuals derived from test cross populations of *Rp1* heterozygotes were digested with appropriate restriction enzymes and fractionated in 0.8% agarose gels and transferred to MSI blotting membranes. Membranes were hybridized to the [^{32}P]dCTP-labeled RFLP probes *NPI285*,

NPI422, and *BNL3.04* for 24 hours at 65°. They washed with 0.1 x SSC (1 x SSC = 0.15 M sodium chloride and 0.015 M sodium citrate pH 7.0) and 0.1% SDS solution at 65° for at least 2 hours. The membranes were then autoradiographed for 24 - 48h at -80°.

RESULTS

Susceptible recombinants were recovered from the test cross populations of several *Rp1* heterozygotes (Table 5). Additionally, recombinants carrying the resistance of both parents (doubly resistant) were also recovered along with recombinants that located resistance from either parent (double susceptibles) in two heterozygotes (Table 6; 7). The recombination frequency in *Rp1* heterozygotes was generally less than 0.2%. All susceptible individuals had the nonparental combinations of flanking markers. This indicates that the susceptibles originated from simple reciprocal recombination.

Screening the test cross progenies of $Rp1^J/Rp1^D$ and $Rp1^J/Rp1^F$ with two complementary rust isolates revealed both types of recombinants arising from a simple reciprocal recombination. From the heterozygotes of $Rp1^J/Rp1^D$, one recombinant individual with combined resistance of both parents and three susceptible individuals lacking resistance phenotypes of either parents were recovered. The three susceptibles had the genotype of $Rp1^D$ at the proximal flanking marker *NPI422* together with the genotype of $Rp1^J$ at the distal flanking

marker site *BNL3.04*. Thus, the combinations of flanking markers in these individuals indicated that *Rp1*^{J} maps proximally to *Rp1*^{D}. Consistent with this gene order, one double resistant individual had the genotype of *Rp1*^{J} at the proximal flanking marker site together with the genotype of *Rp1*^{D} at the distal flanking marker site (Table 6). Similarly, screening the test cross population of *Rp1*^{J}/*Rp1*^{F} revealed four recombinant individuals, three of which lacked resistance phenotypes of either parent while one recombinant had the combined resistance phenotypes of both parents. Among susceptible individuals, one had the genotype of *Rp1*^{F} at the proximal flanking marker *NPI422* along with the genotype of *Rp1*^{J} at the distal marker *BNL3.04*. However, the other two susceptibles had the genotype of *Rp1*^{J} at *NPI422* with the genotype of *Rp1*^{F} at the distal flanking RFLP site, *BNL3.04*. These flanking marker combinations indicates two different gene orders for *Rp1*^{J} and *Rp1*^{F}. One resistant recombinant with the combined resistance phenotypes of both parents had the genotype of *Rp1*^{J} at the *NPI422* flanking RFLP site with the genotype of *Rp1*^{F} at the distal flanking RFLP site *BNL3.04*. These bidirectional flanking marker combinations of recombinants derived from test cross families of *Rp1*^{J}/*Rp1*^{F} heterozygotes suggest that *Rp1*^{J} and *Rp1*^{F} undergo unequal crossing-over and they are probably allelic genes (Table 7).

Three susceptible recombinants derived from test cross progeny of *Rp5*/*Rp1*^{G} were obtained at a low frequency indicating that two genes map closely to each other. All of the recombinants had the genotype of *Rp1*^{G} at the

proximal RFLP site *NPI422* site. The population was not polymorphic for the distal flanking marker. Since all of the recombinants had the genotype of $Rp1^G$ at the proximal RFLP site, $Rp1^G$ should be distal to the *Rp5* (Table 5). It could not be verified, however, that these three susceptibles all arose by simple crossing-over.

TABLE 5

The frequency of recombination in test cross populations of different *Rp1* genes.

F_1s of Test crosses	Susceptible/ total	Frequency (%)	Limits (95%)	Marker recombination[2]	Map distance (cM)	Proximal gene
$Rp1^F/Rp1^{A'}$	3/3687	0.081	0.016-0.23	3/3	0.16	$Rp1^F$
$Rp1^F/Rp1^A$	1/2167	0.046	0.001-0.25	1/1	0.09	$Rp1^A$
$Rp1^C/Rp1^B$	0/2218					
$Rp1^B/Rp1^I$	1/5888	0.016	0.00-0.094	1/1	0.03	$Rp1^B$
$Rp1^D/Rp1^B$	2/5423	0.036	0.004-0.13	2/2	0.07	$Rp1^B$
$Rp1^F/Rp1^C$	1/4251[1]	0.023	0.000-0.13			
$Rp1^J/Rp1^D$	4[3]/3548	0.08[4]	0.017-0.24	5/5	0.16	$Rp1^J$
$Rp1^L/Rp1^D$	5/5512[1]	0.090	0.029-0.21	4/5	0.18	$Rp1^L$
$Rp1^H/Rp1^F$	0/2362					
$Rp1^G/Rp5$	3/3450	0.09	0.018-0.25	3/3	0.18	*Rp5*
$Rp1^K/Rp1^I$	0/3081					
$Rp1^J/Rp1^K$	0/2509					

1 : One susceptible individual died before verifying its flanking marker constitution
2 : Number of susceptibles accompanied by recombinant flanking marker.
3 : Includes both double resistant and double susceptible individuals.
4 : Only the frequency of susceptibles is given.

TABLE 6

Flanking marker combinations of recombinants recovered from test cross progenies of *Rp1*^{J}/*Rp1*^{D}

Rp1^{J}/*Rp1*^{D} test crosses[1]	Phenotypes of recombinants[2]	Genotypes at flanking RFLP markers[3]		Rust isolates[4]
		proximal	Distal	
JD # 3	SS	D	J	IN2, KS1
JD # 4	RR	J	D	
JD # 16B	SS	D	J	
JD # 18	SS	D	J	

1 : These heterozygotes were crossed to tester parents A188, H95 and OH43.
2 : SS; lacking resistance phenotypes of both parents, RR; bearing resistance phenotypes of both parents in *cis*.
3 : NPI422 was used as the proximal flanking marker, BNL3.04 was used as the distal flanking marker.
4 : IN2 and KS1 rust isolates are avirulent on maize lines carrying *Rp1*^{D} and *Rp1*^{J}, respectively.

TABLE 7

Flanking marker combinations of recombinants recovered from test cross progenies of *Rp1*^{J}/*Rp1*^{F}

Rp1^{J}/*Rp1*^{F} test crosses[1]	Phenotypes of recombinants[2]	Genotypes at flanking RFLP markers[3]		Rust isolates[4]
		Proximal	Distal	
JF # 1	SS	F	J	IN2, KS1
JF # 2	SS	J	F	
JF # 7	SS	J	F	
JF # 11	RR	J	F	

1 : These heterozygotes were crossed to tester parents BX, H95 and 1291.
2 : SS; lacking resistance phenotypes of both parents, RR; bearing resistance phenotypes of both parents in *cis*.
3 : NPI422 was used as the proximal flanking marker, BNL3.04 was used as the distal flanking marker.
4 : IN2 and KS1 rust isolates are avirulent on maize lines carrying *Rp1*^{F} and Rp1^{J}, respectively.

DISCUSSION

The recombinational relationships of the *Rp1* genes indicate a consistent gene order as determined by the flanking RFLP markers. However, determination of gene order was not possible in some *Rp1* heterozygotes due to mispairing. Since we have shown that *Rp1* genes are probably carried on tandemly duplicated repeats and that several of the *Rp1* lines probably carry a number of duplications linked to the functional gene, the occurrence of mispairing in *Rp1* heterozygotes indicates that different *Rp1* alleles may also retain synaptic homology for mispairing.

Analysis of recombination in *Rp1* heterozygotes revealed that $Rp1^{A'}$ mapped distally and $Rp1^{A}$ mapped proximally to $Rp1^{F}$, which was previously positioned in the *Rp1* region (HULBERT and BENNETZEN 1991). Recombinants derived from test cross progenies of the heterozygotes $Rp1^{B}/Rp1^{L}$ and $Rp1^{B}/Rp1^{D}$, indicated that $Rp1^{B}$ mapped proximally to both genes and that $Rp1^{L}$ mapped distally to $Rp1^{B}$ but proximally to $Rp1^{D}$. One recombinant derived from the test cross progeny of $Rp1^{B}/Rp1^{I}$ indicated that $Rp1^{I}$ is also distal to the $Rp1^{B}$ (Figure 4). One susceptible recombinant has been observed from the test cross progeny of $Rp1^{C}/Rp1^{F}$, but it died before RFLP loci characterization, and the susceptibility of this individual could not be verified. Similarly, $Rp1^{C}/Rp1^{B}$ did not give any susceptibles. Thus, we are unable to position $Rp1^{C}$ in the *Rp1* area. We are also unable to map $Rp1^{H}$

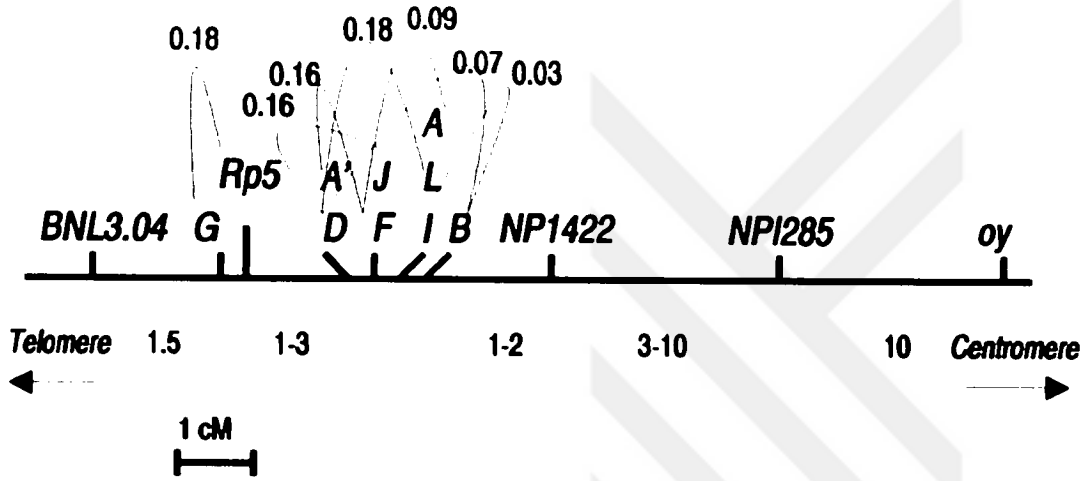


FIGURE 4.- Fine structure recombinational map of *Rp1* and *Rp5* disease resistance determinants on the short arm of chromosome ten. *BNL3.04*, *NPI422* and *NPI285* are flanking markers. *oy*, the recessive seedling marker, maps approximately 15 cM proximally to *Rp1* region. Map orders are consistent between different crosses except for the $Rp1^J/Rp1^F$ population as discussed in the text. The numbers shown above the *Rp1* alleles are map distances obtained from specific test cross populations of *Rp1* heterozygotes (see Table 7). The linear orders of *Rp1* alleles which mapped to same position are unknown. Recombination needs to be analyzed between these *Rp1* alleles to assign definite linear orders.

and *Rp1*^{K} since no susceptibles have been recovered from the test cross progenies of *Rp1*^{H}/*Rp1*^{F}, *Rp1*^{K}/*Rp1*^{I} and *Rp1*^{K}/*Rp1*^{J} (Table 5).

The observation of mispairing in the *Rp1*^{J}/*Rp1*^{F} heterozygotes suggests that they belong to a locus with multiple alleles. Mispairing was also observed in *Rp1*^{D}/*Rp1*^{F} heterozygotes suggesting that both could be allelic (Hulbert and Bennetzen 1991). In addition, we have recovered reciprocal recombinants from the test cross populations of *Rp1*^{J}/*Rp1*^{D} heterozygotes. However, these two genes recombined unidirectionally without mispairing. Hence, the recombination data from these test cross populations indicated neither a clear multiple allelism nor a close linkage for *Rp1*^{J}, *Rp1*^{F} and *Rp1*^{D}. Recombination in the *Rp1*^{J} and *Rp1*^{G} homozygotes clearly demonstrated that *Rp1* genes are carried on or linked to tandemly duplicated repeats and unequal crossing-over is the predominant mechanism of recombination (Chapter two). Since unequal crossing-over can modify the number of duplications and the position of functional genes in a tandem array, these three *Rp1* genes could be either allelic to each other or one of them might be linked to the other allelic locus. A possible example of such a case was reported previously at *Rp1* (Hulbert and Bennetzen 1991). In a previous attempt to determine the mechanism of instability in *Rp1* homozygotes, two sources of *Rp1*^{A}, GG208R and Golden King, were crossed and the test cross progenies of *Rp1*^{A}/*Rp1*$^{A'}$ homozygotes revealed that the two genes have two different map positions. It was, therefore, speculated that the maize lines carrying these genes

might have a closely linked duplicated sequence which can modify the position of the functional gene in the two different parents (Bennetzen *et al.* 1991). That is, the original single locus with multiple alleles might have been converted to the two closely linked loci as a consequence of changes in number of homologous repeats proximal or distal to the functional gene. Therefore, it is not valid to postulate a gene order for these genes from this data. These results also clearly indicate how the application of a modified *cis-trans* test described for disease resistance genes is not valid when the genes are carried on duplicated sequences which mispair.

Analysis of the individuals that were recombinant individuals between $Rp1^G$ and *Rp5* indicated that $Rp1^G$ maps distally to *Rp5* (Sudupak *et al.* 1992). $Rp1^C$ and *Rp*5 were positioned 1-3 cM distally to the other *Rp1* alleles (Hulbert and Bennetzen 1991), and it was previously suggested that $Rp1^G$ might be allelic to *Rp5* (Bennetzen *et al.* 1990). These results suggest that some *Rp* specificities may have been displaced on the short arm of chromosome 10. Similar observations were made ywith the *Lc* allele of the *R* locus which is located 1.5 cM distally to the *R* locus in maize. The *Lc* allele functions in pigmentation of the leaf base, and it has been shown to retain synaptic homology to components of the *R* locus (Dooner *et al.* 1976). Since we have now shown that both $Rp1^J$ and $Rp1^G$ undergo unequal crossing-over, the instability of $Rp1^G$ and map position of *Rp5* might represent the presence of unstable closely linked loci distal to *Rp1*. If this is the case for the short arm of chromosome 10, it could be possible to detect mispairing between

Rp1 and *Rp1*^{G}, which might generate deletions as large as three cM.

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UNEQUAL RECOMBINATION AND MEIOTIC INSTABILITY OF *Rp1* REGION DISEASE RESISTANCE GENES IN MAIZE

by

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ABSTRACT

The *Rp1* region of maize was originally characterized as a complex locus which conditions resistance to the fungus *Puccinia sorghi*, the causal organism in the common rust disease. Subsequent studies have indicated that more than one *Rp* gene may be present in the *Rp1* region. For instance, we have shown that two presumed alleles of *Rp1*, $Rp1^J$ and $Rp1^G$, map roughly two centiMorgans apart. Some alleles of *Rp1* are meiotically unstable, but the mechanism of instability is not known. We have studied the role of recombination in meiotic instability in maize lines homozygous for either $Rp1^J$ or $Rp1^G$. Test cross progenies derived from a line that was homozygous for $Rp1^J$, but heterozygous at flanking markers, were screened for susceptible individuals. Five susceptible individuals were derived from 9772 progeny. All five had nonparental combinations of flanking markers; three had one combination of recombinant flanking markers while the other two had the opposite pair. In an identical study with $Rp1^G$, twenty susceptible seedlings were detected out of 5874 test cross progeny. Nineteen of these were associated with flanking marker exchange, eleven and eight of each recombinant marker combination. Our results indicate that these disease resistance genes are carried on duplicated fragments which retain synaptic homology, and that unequal exchange is the main mechanism of their meiotic instability.

In second part of this study, closely flanking restriction fragment length polymorphisms (RFLPs) were used to assay recombination in *Rp1* heterozygotes. Susceptible recombinant progenies were obtained from test cross progenies of

several *Rp1* heterozygotes. Recombinants, carrying resistance phenotypes of neither parents or the combined resistance phenotypes of both parents were recovered from test cross progenies of both $Rp1^{J}/Rp1^{F}$ and $Rp1^{D}/Rp1^{J}$ heterozygotes, and all of the recombinants arose on nonparentally marked chromosomes. Both combinations of flanking markers were observed in recombinants derived from the test cross progenies of $Rp1^{J}/Rp1^{F}$ heterozygotes, which suggest that these genes are probably allelic, but mispairing allows them to recombine. As previously observed, most of the *Rp1* genes mapped closely together. $Rp1^{G}$ and *Rp5*, which were positioned 1-3 cM distally to the other *Rp1* genes, mapped approximately 0.2 cM from each other, with $Rp1^{G}$ appearing to be distal to *Rp5*. The close linkage and distal position of $Rp1^{G}$ to *Rp5* suggest that there might be another cluster of *Rp* genes in the *Rp1* area.