Recombination and Spontaneous Mutation at the Major Cluster of Resistance Genes in Lettuce (*Lactuca sativa*)

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ABSTRACT

Two sets of overlapping experiments were conducted to examine recombination and spontaneous mutation events within clusters of resistance genes in lettuce. Multiple generations were screened for recombinants using PCR-based markers flanking *Dm3*. The *Dm3* region is not highly recombinagenic, exhibiting a recombination frequency 18-fold lower than the genome average. Recombinants were identified only rarely within the cluster of *Dm3* homologs and no crossovers within genes were detected. Three populations were screened for spontaneous mutations in downy mildew resistance. Sixteen *Dm* mutants were identified corresponding to spontaneous mutation rates of 10^{-3} to 10^{-4} per generation for *Dm1*, *Dm3*, and *Dm7*. All mutants carried single locus, recessive mutations at the corresponding *Dm* locus. Eleven of the 12 *Dm3* mutations were associated with large chromosome deletions. When recombination could be analyzed, deletion events were associated with exchange of flanking markers, consistent with unequal crossing over; however, although the number of *Dm3* paralogs was changed, no novel chimeric genes were detected. One mutant was the result of a gene conversion event between *Dm3* and a closely related homolog, generating a novel chimeric gene. In two families, spontaneous deletions were correlated with elevated levels of recombination. Therefore, the short-term evolution of the major cluster of resistance genes in lettuce involves several genetic mechanisms including unequal crossing over and gene conversion.

PLANT disease resistance genes are frequently members of multigene families, each member conferring resistance to a specific strain of the pathogen. Classical genetic studies conducted in parallel on the plant host and pathogen have often shown that the interaction follows a "gene-for-gene" principle: for every resistance gene in the host, there is a corresponding avirulence gene in the pathogen (Flor 1956; Crute 1986). Although molecular studies have shown that the gene-for-gene interaction is an oversimplification (Bisgrove *et al.* 1994; Salmeron *et al.* 1994), it remains a useful predictive model for plant-pathogen interactions. Genetic and molecular analyses of resistance genes have increasingly demonstrated that the clustering of disease resistance genes is a common occurrence in plant genomes (Michelmore and Meyers 1998). Some genes, such as the *L* locus in flax, are an allelic series (Islam and Shepherd 1991). More frequently, resistance genes are located in complex, highly duplicated regions with multiple genes that are tandemly arrayed and may encode resistances to diverse pathogens. The *Cf* clusters in tomato, the *M* locus in flax, the *Xa21* locus in rice, and the *Dm* clusters in lettuce all exhibit this organization (reviewed in Michelmore and Meyers 1998).

Approximately 20 disease resistance genes, which confer resistance to an array of bacterial, viral, and fungal pathogens, have been cloned, mostly by transposon tagging or map-based cloning (reviewed in Baker *et al.* 1997; Hammond-Kosack and Jones 1997; Ellis and Jones 1998; Michelmore and Meyers 1998). Nearly all of these genes are predicted to encode proteins involved in signal transduction and thus are implicated in pathogen recognition and elicitation of the resistance response. These proteins and their corresponding genes can be categorized according to the structural motifs that they contain (Baker *et al.* 1997; Hammond-Kosack and Jones 1997). By far the most prevalent group is the class encoding a nucleotide-binding site (NBS) and leucine-rich repeats (LRRs; Meyers *et al.* 1999). This class includes, among others, the *N* gene from tobacco (Whitham *et al.* 1994), the *L6* gene from flax (Lawrence *et al.* 1995), the *I2* gene from tomato (Ori *et al.* 1997), the Arabidopsis genes *RPM1*, *RPS2*, and *RPP5* (Bent *et al.* 1994; Mindrinos *et al.* 1994; Grant *et al.* 1995; Parker *et al.* 1997), as well as the *Dm3* gene from lettuce (Meyers *et al.* 1998a; K. Shen and R. Michelmore, unpublished results).

A variety of genetic events have been observed at resistance gene loci in plants (Ellis *et al.* 1997; Hulbert 1997; Michelmore and Meyers 1998; Ronald 1998).

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Both unequal crossovers and gene conversion have been correlated with meiotic instability and novel resistance specificities at the *Rp1* locus in maize (Sudupak *et al.* 1993; Hu and Hulbert 1994; Richter *et al.* 1995). Unequal intragenic recombination in the LRR-encoding portion of the gene was also implicated in losses of resistance at the *M* locus in flax and the *RPP5* locus in Arabidopsis (Anderson *et al.* 1997; Parker *et al.* 1997). Sequence analyses suggested that recombination or gene conversion had produced chimeric genes in the *Cf-4/9* and *Cf-2/5* clusters in tomato (Parniske *et al.* 1997; Dixon *et al.* 1998; Parniske and Jones 1999), the *Xa21* cluster in rice (Song *et al.* 1997), the *Dm3* cluster in lettuce (Meyers *et al.* 1998a), as well as the Arabidopsis *RPP8* and *RPP5* clusters (McDowell *et al.* 1998; Noel *et al.* 1999). Sequence analysis also provided evidence that transposition was involved in the evolution of the *Xa21* cluster (Song *et al.* 1997). These data led to a model of resistance gene evolution in which unequal crossing over and gene conversion are the major genetic mechanisms leading to the generation of new resistance specificities. Clusters of resistance genes are postulated to be dynamic, unstable arrays of related sequences (Ellis *et al.* 1997; Hulbert 1997).

However, comparative sequence analyses of several loci suggested that resistance genes are evolving slowly and that the structure of some resistance clusters may be fairly stable. Complete sequencing of the resistant and susceptible haplotypes of the *Pto* locus in tomato revealed a conserved structure with obvious orthologous relationships (D. Lavelle and R. Michelmore, unpublished results). At the *Pto*, *Cf*, *Xa21*, and *Dm3* clusters, orthologs are more similar than paralogs and there is little evidence for the sequence homogenization that frequent crossovers between paralogs and gene conversion would produce (Parniske *et al.* 1997; Meyers *et al.* 1998b; D. Lavelle, unpublished data; S.-S. Woo, unpublished data). Comparisons of sequences flanking the *RPM1* locus in Arabidopsis also led to the conclusion that resistance genes are evolving slowly (Stahl *et al.* 1999). In addition, nonsynonymous (K_a) to synonymous (K_s) nucleotide substitution ratios of LRR-encoding portions of genes in the *Cf-4/9*, *Xa21*, *RPP1*, *RPP8*, *RPP5*, and *Dm3* clusters are all >1 (Botella *et al.* 1998; Parniske *et al.* 1997; McDowell *et al.* 1998; Meyers *et al.* 1998b; Michelmore and Meyers 1998; Wang *et al.* 1998; Noel *et al.* 1999). Together these data suggest that diversifying selection acting in concert with random mutation on individual genes may be more important than sequence exchange between paralogs in generating new resistance specificities. This led to an alternative model that resistance genes evolve mainly through divergent selection and a "birth-and-death" process (Michelmore and Meyers 1998), similar to the evolution of vertebrate MHC genes (Nei *et al.* 1997). Genes are continually "born" via duplication events and either are maintained in the genome through evolutionary time or "die" via deletion or mutational events. These two models of resistance gene evolution are not mutually exclusive. The *Cf-4/9*, *Xa21*, *RPP5*, *RPP8*, and *Dm3* loci provide evidence for a variety of genetic events; however, the relative importance of recombination, conversion, transposition, and divergent selection may differ over evolutionary time, for different resistance gene clusters, or for plant species exhibiting different mating systems.

The interaction between lettuce, *Lactuca sativa*, and the obligate biotrophic fungus, *Bremia lactucae*, the causal agent of lettuce downy mildew, has been studied extensively. Classical genetic analyses have demonstrated at least 15 dominant, single genes for resistance to downy mildew (*Dm* genes) located in at least three major clusters in the lettuce genome (Farrara and Michelmore 1987; Bonnier *et al.* 1994). Over 100 additional resistance specificities have been identified (Norwood *et al.* 1981; Farrara and Michelmore 1987; Bonnier *et al.* 1992), but only a few have been genetically characterized (Bonnier *et al.* 1994; Maisonneuve *et al.* 1994). *Dm3*, which is located in the largest cluster, has recently been cloned by a combination of map-based cloning and a candidate gene approach using PCR with degenerate oligonucleotide primers (Meyers *et al.* 1998a,b; Shen *et al.* 1998; K. Shen and R. Michelmore, unpublished results). *Dm3* belongs to a multigene family of resistance gene candidate (*RGC*) sequences, the *RGC2* family, that encodes members of the NBS-LRR class of resistance proteins (reviewed in Baker *et al.* 1997; Hammond-Kosack and Jones 1997; Ellis and Jones 1998; Meyers *et al.* 1999). Family members are interspersed throughout the region surrounding *Dm3* and span at least 3.5 Mb (Meyers *et al.* 1998a). The *RGC2* family is highly duplicated and complex, containing more than 24 family members that share similar molecular markers, as well as 53–96% nucleotide sequence identity. Mutation and transgenic analyses demonstrated that the family member, *RGC2B*, encodes *Dm3* specificity (Meyers *et al.* 1998a; Chin 2000; K. Shen and R. Michelmore, unpublished results).

To investigate the roles of recombination, unequal crossing over, and possibly gene conversion in the evolution of resistance specificities in the *Dm3* cluster, two sets of overlapping experiments were conducted. The first experiment involved the examination of recombination in the *Dm3* region over multiple generations. A large F_2 population was screened to identify individuals with recombination breakpoints near *Dm3*. These individuals were then further examined with molecular markers to compile a profile of recombination breakpoint patterns in the region. The second experiment involved the identification and characterization of naturally occurring, spontaneous mutations in *Dm* resistance from three different populations of lettuce; F_2 recombinants from the first experiment were used to derive one of these three populations. Molecular analyses were

conducted to determine the genetic changes underlying the loss of resistance in these spontaneous mutants.

MATERIALS AND METHODS

Identification of recombinant individuals in the *Dm3* region: Two lettuce cultivars, Kordaat (*Dm1*, *Dm3*, *Dm4*) and Calmar (*Dm7*, *Dm8*, *Dm13*), were crossed and the F_1^{KC} (Kordaat × Calmar) individuals selfed to produce a large F_2 population in which six *Dm* genes were segregating. This was the same cross that was used to produce an intraspecific mapping population analyzed by Kesseli *et al.* (1994). A rapid alkali-treatment method was used to extract DNA suitable for PCR analysis from F_2 seedlings that were 2–3 wk old (Wang *et al.* 1993). Two SCAR (sequence characterized amplified region) markers, *SCV12* and *SCI11*, were used in a multiplexed PCR to screen F_2 individuals for the occurrence of recombination in the *Dm3* region. Data from the intraspecific mapping population (Kesseli *et al.* 1994) indicate that *SCV12* is 3.6 cM from *Dm3* and that *SCI11* is located 4.9 cM from *Dm3*, on the opposite side (Figure 1). Both markers are codominant, allowing the identification of recombinant individuals that had a crossover point within an ~8.5-cM interval surrounding *Dm3*.

Identification of spontaneous mutants in downy mildew resistance: Three populations were screened for spontaneous mutants.

Population 1: F_1^{KC} individuals from a cross of the cultivars Kordaat (*Dm1*, *Dm3*, *Dm4*) × Calmar (*Dm7*, *Dm8*, *Dm13*). This population was screened for losses of *Dm1* and *Dm3* activity. Activity of *Dm4* could not be tested in this population due to the lack of an isolate with the appropriate virulence phenotype. Activities of *Dm7*, *Dm8*, and *Dm13* could not be tested due to unavoidable selfing of the maternal parent (Kordaat).

Population 2: S_2 (selfed twice) families of the lettuce cultivar, Diana (*Dm1*, *Dm3*, *Dm7*, *Dm8*). This population was screened for losses of all four specificities.

Population 3: F_3 recombinant-derived families. This population was derived by crossing Kordaat × Calmar F_2 individuals, which exhibited recombination events between *SCV12* and *SCI11* (identified in the population described in the preceding paragraph) such that the recombinant allele retained *Dm3*.

These crosses produced F_1^R progeny (R for recombinant to distinguish these individuals from the F_1^{KC} population) with a homozygous region immediately surrounding *Dm3*, but heterozygous flanking markers (Figure 2). The region of homozygosity surrounding *Dm3* included only those markers that cosegregated absolutely with *Dm3* in the mapping population (*OPAC15*, *OPAH17*, *OPAM14*, *OPJ11*, *OPM15*, *OPX11*, *MSAT15-34*, *MSATE6*, *SCE14*, *SCK13*, and *SCM05*; Kesseli *et al.* 1994) and was designated the *OPAC15-SCM05* "block" (Figure 1). Eight F_1^R individuals with the appropriate genetic configuration, from three different crosses, were selfed twice to produce the F_3 families that were screened for spontaneous losses of *Dm3* activity. Population 3 was used to analyze the exchange of flanking markers and to determine the association between recombination and spontaneous mutations at *Dm3*.

To screen for spontaneous *Dm* mutants, F_1^{KC} seedlings (population 1) or 20 to 30 S_2 or F_3 seedlings per family (populations 2 and 3) were germinated at 15° in plastic compartmentalized boxes on filter paper saturated with Hewitt's solution (Hewitt 1952). Seven days after germination, seedlings were inoculated with either a single or a pool of *B. lactucae* isolates capable of detecting one to four *Dm* specificities of interest in that population (Table 1). Isolates were maintained and their viru-

TABLE 1

Virulence phenotypes of *B. lactucae* isolates used

Isolate	Origin	*Dm* gene diagnostic for	Reaction to *Dm* genes														
			Dm1	*Dm2*	*Dm3*	*Dm4*	*Dm6*	*Dm7*	*Dm8*	*Dm10*	*Dm11*	*Dm12*	*Dm13*	*Dm14*	*Dm15*	*Dm16*	*Dm18*
C83M47	California	*Dm1*	−	+	+	+	+	+	+	−	−	+	+	+	−	+	−
IM25P11	UK	*Dm3*	+	−	−	+	+	+	+	+	(+)	+	+	+	+	+	−
R60	UK	*Dm7*	+	+	+	+	+	−	+	+	+	+	+	+	−	−	−
CG1	Switzerland	*Dm8*	+	−	+	+	−	+	−	+	−	+	+	−	+	−	−
AM	Australia	*Dm3*	+	−	−	(+)	−	+	+	+	−	+	+	−	−	−	−
IL4	Israel	*Dm3*	+	+	−	+	−	+	+	+	+	+	+	+	+	+	−
C99S744	California	*Dm3*	+	+	−	+	+	+	+	+	+	+	+	+	+	+	−
CG5	Switzerland	*Dm3*	+	+	−	+	−	+	+	+	−	+	+	+	−	(+)	(+)

+, profuse sporulation; −, no sporulation; (+), sparse sporulation.

TABLE 2

Oligonucleotide primers used in PCR and sequencing

Primer	Sequence (5′ to 3′)	Purpose
SCV12T	ACCCCCCACTTGTCCTGCAACTTT	Identification and analysis of recombinants and mutants
SCV12U	ACCCCCCACTACCATATCAATCTC	
SCI11U	ACATGCCGTGTATTACTCAGAGTT	Identification and analysis of recombinants and mutants
SCI11RT	TCTCAAAACAACAGACCAATA	
SCK13F	AAAACCCTAGAACTCATACTTA	Analysis of recombinants and spontaneous mutants
SCK13R	CACGACTTAAATGAAACTA	
SCE14T3	GAGTTATGTCAGTCGTTATT	Analysis of recombinants and spontaneous mutants
SCE14T7	TAAACTCAGACCGTAAACTT	
SCM05U	GGGAACGTGTTAATTAGAGATGTA	Analysis of recombinants and spontaneous mutants
SCM05T	GGGAACGTGTGTGTGTATGGATCA	
MSAT15-3	GTATCACATCCCAAACTCTC	Analysis of recombinants and spontaneous mutants
MSAT15-4	GACAACAAAGTTGAACTGCC[a]	
3EXON4C	AGTGATTGTGAAGAAGGAAGAA	Analysis of recombinants and spontaneous mutants
5MSATE6-1	CCCAAGAAGAATCCTACCA	
RLG2B5-30	GTAAGGAAGATCAGAAGAGACTGTTCACAC	Sequencing of dm3s1977
5-2Bintron2	CCCAAGTTTAGTCATCCCACCTCTTTAT	
2Bintron2-3end	GACCAAGGTTTCTTCCAGGTACCATTTG	Sequencing of dm3s1977
2B.3UTRA-B	CACCAGTCCACCGACTAGCTAACATCTA	
1977CH5	GCTAGAGAGATAGAAATAGTTGGA	Confirmation of dm3s1977 conversion event
1977CO5	GAAGAGCATGATTATTCTCG	
1977CO3	GTTGCACAAACCAATCTC	

Primers for RAPD markers used in analyses (designated by the prefix OP, see text) were supplied by Operon Technologies (Alameda, CA) and their sequences are not included here.

[a] Correction from sequence published in Okubara *et al.* (1997).

lence phenotypes were determined as described previously (Farrara *et al.* 1987). Seedlings were screened for loss of *Dm* resistance, as indicated by profuse sporulation from 6 to 14 days postinoculation. F_1^{KC} and S_2 mutants (populations 1 and 2) were retested using single isolates to determine which *Dm* specificity had been lost. Susceptible seedlings were rescued by treatment with the systemic fungicide, Ridomil 2E (Ciba-Geigy Corp., Greensboro, NC), at 50 ppm. Rescued plants were transferred to soil and grown to maturity in the greenhouse and their selfed seed was collected.

Genetic analyses of recombinants and spontaneous *dm* mutants: Segregation of the mutant originating from population 1 was analyzed in an F_2 population derived by crossing Kordaat to selfed individuals that were homozygous for the mutant allele. Segregation of resistance was scored for the S_2 and F_3 mutant families of populations 2 and 3, respectively. The stability of mutant phenotypes was confirmed by examining progeny of mutants after one or two generations of selfing in all three populations. To determine the number of loci mutated, complementation tests were conducted by intercrossing mutants exhibiting the loss of the same *Dm* specificity. In addition, two representatives of a fast-neutron (FN)-induced panel of *dm3* deletion mutants, dm3r1208 and dm3r1608, one FN *dm7* mutant, dm7r240 (Okubara *et al.* 1994; Anderson *et al.* 1996), as well as a panel of ethyl methanesulfonate (EMS) mutants representing both *dm3* and *dm7* (Chin 2000) were crossed to the spontaneous mutants. Crosses were also made to the closely related cultivars, Cobham Green, which has no known resistance genes, and Diana (*Dm1*, *Dm3*, *Dm7*, *Dm8*), to determine if epistatic or dominant loci had been affected. Reciprocal crosses were made in most cases.

Linkage analyses of the recombinants and spontaneous mutants from population 3 were conducted using formulas from Allard (1956) and the software program LINKAGE-1 (Suiter *et al.* 1983). The Kosambi mapping function was used to convert some recombination frequencies to centimorgans (Kosambi 1944).

Molecular analyses of recombinants and spontaneous mutants: Extraction of DNA for comprehensive marker analyses was conducted using a modified cetyltrimethylammonium bromide protocol (Bernatzky and Tanksley 1986). Flanking molecular markers assayed on recombinants and mutants included randomly amplified polymorphic DNA (RAPDs; Williams *et al.* 1990), SCARs (Paran and Michelmore 1993), and microsatellites (Table 2; Okubara *et al.* 1997; Sicard *et al.* 1999). To search for restriction fragment length polymorphisms (RFLPs), Southern blot analyses were conducted according to standard protocols (Sambrook *et al.* 1989), using a variety of restriction enzymes, including *Hin*dIII, *Acc*I, *Bam*HI, *Bgl*I, *Nco*I, *Sac*I, and *Sca*I (New England Biolabs, Beverly, MA), nylon membrane (Hybond N+, Amersham, Arlington Heights, IL or GeneScreen Plus, New England Nuclear Life Sciences Products, Boston, MA), and ^{32}P-labeled probes made by the random-primer method (MultiPrime, Amersham). Several markers, *e.g.*, *MSAT15-34* and *NBS2B*, represent multicopy sequences that identify multiple *RGC2* family members; individual fragments of such markers are indicated by the marker name followed by a colon and a number, *e.g.*, *MSAT15-34: 1*, which is present in *Dm3*.

Sequencing: Two primer sets, RLG2B5-30/5-2Bintron2 and RGC2Bintron2-3end/2BUTRA-B (Table 2), were used for amplification of *Dm3* sequences using long-range PCR. Two sets of primers were used because the entire gene (~13 kb) could not be amplified in a single reaction. PCR products were

amplified from genomic DNA using the polymerases, BIOXACT (Intermountain Scientific Corp., Kaysville, UT) or DNAZyme (Finnzymes, MJ Research, Watertown, MA), which have 3′ to 5′ proofreading activity, in two-step PCR: an initial denaturation of 94° for 2 min, followed by 15–25 cycles of 92° for 20 sec and 68° for 9 to 11 min, and a final extension of 72° for 6 to 9 min. Amplification products were sequenced directly after gel purification using DEAE cellulose membranes (NA45, Schleicher & Schuell, Keene, NH) or sequenced after subcloning using the TOPO-XL kit (Invitrogen, Carlsbad, CA). All sequencing was performed with a dye terminator cycle sequencing kit and an automated ABI 377 sequencer (Applied Biosystems Inc., Foster City, CA). Analysis of sequence information was performed using the software package, Sequencher 3.0 (Genecodes, Ann Arbor, MI).

RESULTS

Recombination is infrequent within the *Dm3* cluster when it is heterozygous: A total of 167 recombinant individuals were identified from an analysis of ~2220 F_2 seedlings using the codominant SCAR markers, *SCV12* and *SCI11*, that flank *Dm3* (Figures 1 and 2A). Subsequently, 13 additional RAPD, SCAR, and microsatellite markers, which span the length of the *SCV12-SCI11* interval, were assayed on the recombinant individuals to determine the positions of the recombination breakpoints (Figure 1). The block of markers that included *OPAC15*, *OPAH17*, *OPJ11*, *SCE14*, *SCK13*, and *SCM05* cosegregated with *Dm3* in the original mapping population; these markers are interspersed throughout a small region immediately surrounding *Dm3* (MATERIALS AND METHODS; Figure 1; KESSELI *et al.* 1994; MEYERS *et al.* 1998a). The two microsatellite markers, *MSAT15-34* and *MSATE6*, represent sequences within *RGC2B*, the *Dm3* gene (Figure 3), and are also present in several other RGC2 paralogs that are distributed within the *OPAC15-SCM05* block (OKUBARA *et al.* 1997; MEYERS *et al.* 1998a,b; SICARD *et al.* 1999). The molecular marker assays indicated that 163 out of 167 recombination events resolved outside the *OPAC15-SCM05* block.

Only four individuals, C31, C75, C127, and C132, out of the 167 recombinants were identified that had crossover points within the *OPAC15-SCM05* interval producing recombinant *RGC2* haplotypes. The occurrence of these rare meiotic events could be most easily identified using *MSAT15-34*; the recombinants C31, C127, and C132 had nonparental *MSAT15-34* haplotypes. C31 and C127 exhibited a deletion of the fragments 3 and 4 from the parental Kordaat genotype (Figure 4). C132 was missing fragments 1, 3, and 5 (Figure 4). The recombinant C75 was identified by the presence of all the block markers except *SCE14*. The deletion of multiple markers indicated that all four recombinant individuals had nonparental numbers of *RGC2* sequences. All four recombinants were screened for resistance to the isolate IM25P11, which is diagnostic for *Dm3*. Individuals C31, C75, and C127 retained *Dm3* resistance, while individual C132 exhibited a loss of *Dm3* specificity.

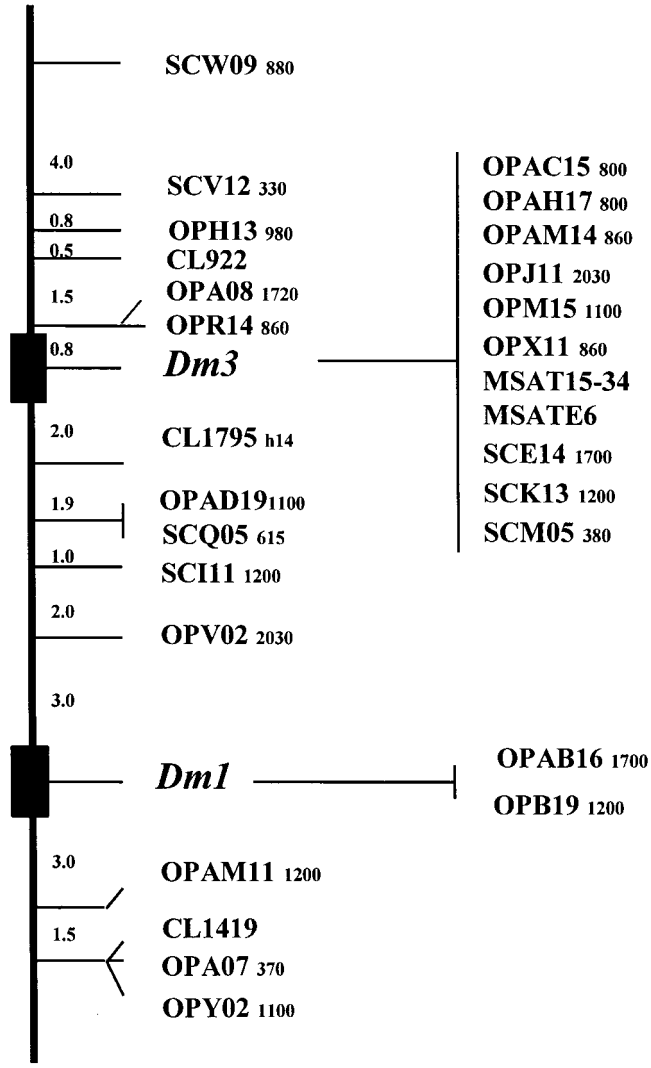

FIGURE 1.—Genetic map of the major cluster of resistance genes in lettuce, the *Dm1/Dm3* cluster. Column 1 represents the map with centimorgans noted between markers. OP markers are RAPDs. SC markers are SCAR markers. CL markers are RFLPs. MSAT markers are microsatellites. Small numerals indicate band sizes. The codominant SCAR markers, *SCV12* and *SCI11*, were used to monitor recombination in a region surrounding *Dm3*. Column 2 represents blocks of markers that cosegregated with *Dm3* or *Dm1* in the mapping population (KESSELI *et al.* 1994).

After selfing to achieve homozygosity of the recombinant chromosome, Southern hybridization analyses were conducted on an array of 18 recombinants representative of the various crossover breakpoints. Several RFLP probes were hybridized to genomic DNA digested with *Hin*dIII, including *NBS2B* (MEYERS *et al.* 1998a; SHEN *et al.* 1998), *AC15* (ANDERSON *et al.* 1996), and *651END* (MEYERS *et al.* 1998a), which represent sequences in the 5′, middle, and 3′ regions of *RGC2* sequences, respectively (Figure 3). All three probes hybridized to multiple fragments (~20 fragments) representing the various *RGC2* family members and thus allowed the monitoring of genetic changes occurring throughout the resistance

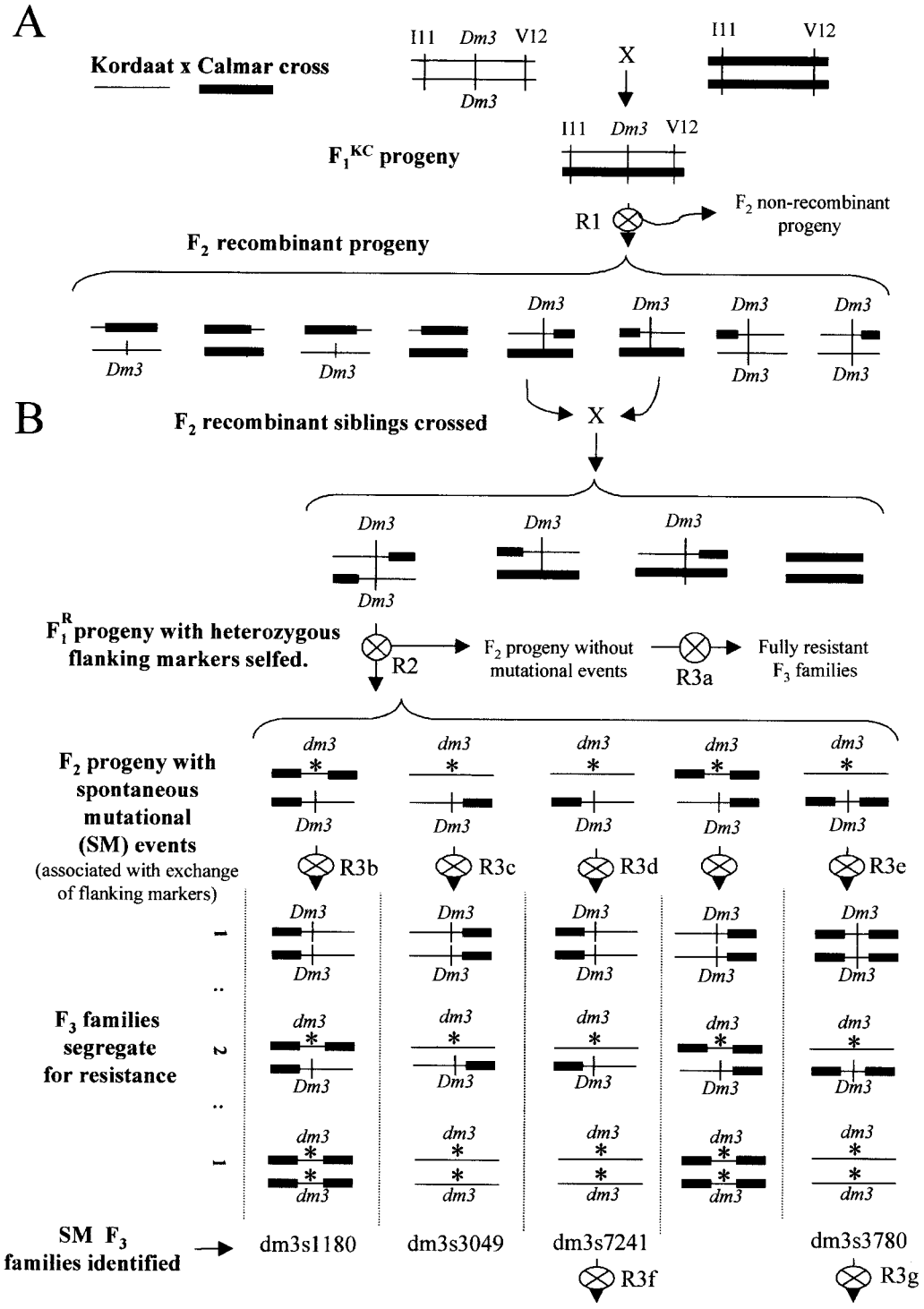

FIGURE 2.—Derivation of F_2 population screened for recombinants and F_3 recombinant families screened for *Dm3* spontaneous mutations. (A) The cultivar Kordaat (*Dm1*, *Dm3*, *Dm4*) was crossed with the cultivar Calmar (*Dm7*, *Dm8*, *Dm13*) to produce a segregating F_2 population. (B) F_2 recombinant siblings were crossed to produce F_1^R individuals (R for recombinant) in which *Dm3* was homozygous but flanking regions were heterozygous. These F_1^R individuals were then selfed twice and the resulting population of F_3 families phenotypically screened for spontaneous mutations in *Dm3* specificity. The SCAR markers, *SCV12* and *SCI11*, were used to monitor recombination in the region over multiple generations. The region of homozygosity surrounding *Dm3* was delimited by the block of markers containing *OPAC15*, *OPAH17*, *OPAM14*, *OPJ11*, *OPM15*, *OPX11*, *SCE14*, *SCK13*, and *SCM05* (Figure 1). Recombination rates (R1, R2, R3a–g) experienced during the various meioses were tabulated in Table 5 (see text).

gene cluster. Each probe detected diagnostic banding patterns for the parents, Kordaat and Calmar. These fragments were coinherited in 14 out of the 18 $F_{2:3}$ progeny as parental haplotypes, with progeny exhibiting either the Kordaat or Calmar haplotype. The four recombinant individuals, C31, C75, C127, and C132, exhibited banding patterns that were either a combination or a subset of the two parental haplotypes (Figure 5), providing further evidence that rare recombination events had occurred within the *RGC2* cluster. The recombination breakpoint data of these four individuals for both the RFLP and PCR-based markers were consistent with the deletion breakpoint map previously generated for the region (Figure 6; ANDERSON *et al.* 1996; MEYERS *et al.* 1998a). Although the crossovers did not apparently generate chimeric *RGC2* genes (see below), the deletion of multiple markers indicates that these events did alter *RGC2* copy number (Figure 5).

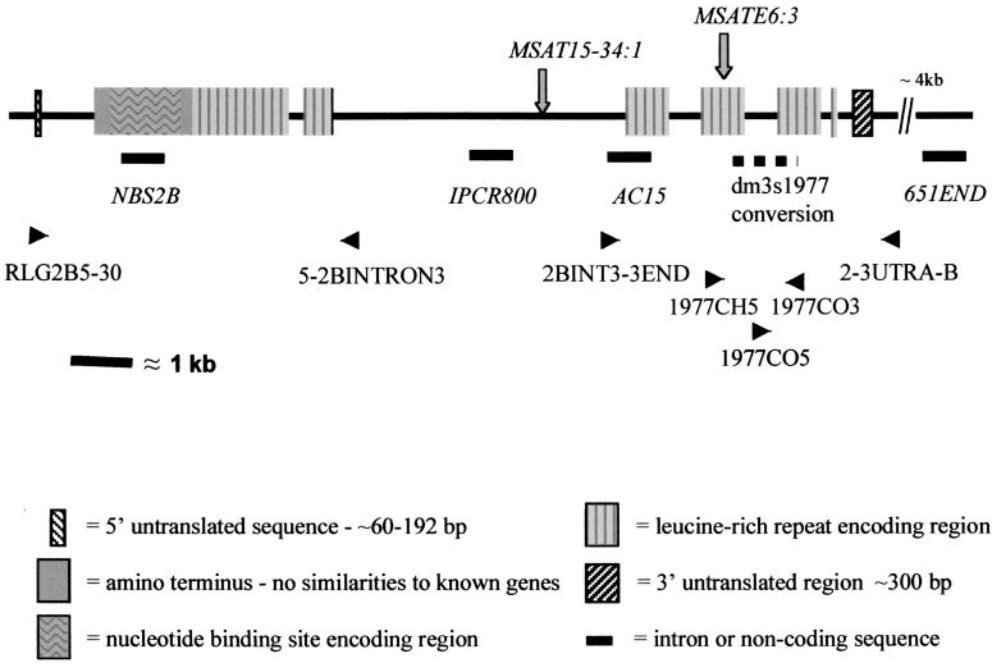

FIGURE 3.—Structure of *Dm3* gene with position of markers, primers, and gene conversion event. Schematic of *Dm3* (*RGC2B*) gene. Location of RFLP probes *NBS2B*, *IPCR800*, *AC15*, and *651END* are indicated as solid bars below the gene. Each of these probes hybridizes to multiple RGC2 family members (Figures 5, 6, and 8). Position of microsatellite markers, *MSAT15-34* and *MSATE6*, and primers used in PCR and sequencing are indicated as arrows. These two microsatellite markers also represent multicopy markers that are present on several RGC2 family members (Figures 4, 6, and 7). The position and length of the converted region in the spontaneous mutant, dm3s1977, is indicated as a dashed bar.

Twelve additional unique restriction endonuclease/probe combinations were used in Southern analyses on the four recombinants with breakpoints within the *RGC2* cluster (data not shown). No novel fragments were detected relative to the parental Kordaat and Calmar haplotypes. Because the majority of *RGC2* homologs resided on unique RFLP fragments, the lack of novel fragments indicated that the recombination events within the cluster did not resolve in or near *RGC2* genes. Although it is possible that recombination between two closely related homologs would not produce changes detectable by a single probe or restriction enzyme digest, no changes were detected by any of the probes or enzymes used. Therefore, there was no evidence for the generation of chimeric *RGC* sequences and the recombination events must have resolved in the noncoding regions between the homologs rather than within the genes.

An estimate of the physical size of the *SCV12-SCI11* interval was unavailable and thus an estimation of the relationship between physical and genetic size for this interval could not be calculated. However, the physical size of the region immediately surrounding *Dm3*, the *OPAC15-SCM05* block, has been estimated to encompass at least 3.5 Mb, based on a partial bacterial artificial chromosome (BAC) contig tiling path, average spacing between *RGC2* family members, and high molecular weight DNA analyses (MEYERS *et al.* 1998a; CHIN 2000). The four recombinants identified from ~2220 screens of F_2 individuals represented a genetic distance of 0.15 cM. Therefore, the ratio of physical to genetic distance was estimated to be 23.3 Mb/cM (3500 kb/0.15 cM), in comparison to an estimated genome-wide average of 1.28 Mb/cM [2500 Mb (ARUMUGANATHAN and EARLE 1991)/1950 cM (KESSELI *et al.* 1994)]. Therefore, the *RGC2* cluster is not highly recombinagenic; in fact, recombination in the *OPAC15-SCM05* interval is 18-fold less in the pairing of Calmar and Kordaat haplotypes, relative to the genome average.

Rates of spontaneous mutation in homozygous *Dm* genes differ: A total of 15 new mutants were identified from the three populations screened for spontaneous losses of *Dm* resistance (Table 3). The F_1^{KC} seedlings of Kordaat (*Dm1*, *Dm3*, *Dm4*) × Calmar (*Dm7*, *Dm8*, *Dm13*) yielded 1 *dm3* mutant out of ~5500 individuals screened (population 1). No spontaneous mutations at *Dm1* were identified in this screen, although 1 mutant had been identified in a previous screen of ~3000 individuals (OKUBARA *et al.* 1994). The S_2 families of Diana (*Dm1*, *Dm3*, *Dm7*, *Dm8*) yielded 7 *dm3* mutant families (including the 2 reported in ANDERSON *et al.* 1996) and 3 *dm7* mutant families out of ~11,000 families screened (population 2). No spontaneous mutations at either *Dm1* or *Dm8* were identified in this screen. The F_3 recombinant families, which were screened only for *dm3* mutations, yielded 4 mutant families out of ~8000 families screened (population 3).

Dm loci differ in their meiotic stability. Both *Dm3* and *Dm7* undergo spontaneous mutations at a rate of $\sim 10^{-4}$ mutations per locus per generation, which is high relative to most other types of genes (DRAKE *et al.* 1998), but similar to rates observed for the *Rp1* locus in maize (BENNETZEN *et al.* 1988; HULBERT 1997). The combined F_1^{KC} data (population 1) indicate that *Dm1* has a similar mutation rate; however, no *Dm1* mutants were observed in the S_2 population (population 2). These spontaneous mutations occurred in meioses involving genotypes that were homozygous for the entire genome (the parents of populations 1 and 2) or for the region immediately surrounding the *Dm3* gene (population 3). No sponta-

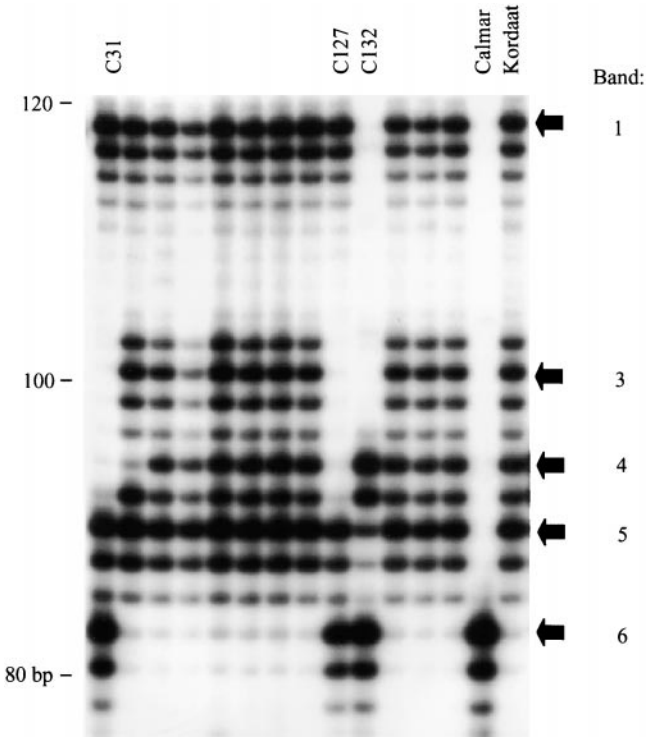

FIGURE 4.—*MSAT 15-34* genotype of selected recombinants in *Dm3* region. MSAT 15-34 is a multicopy microsatellite marker that is represented on several RGC2 family members. Three recombinants were identified with nonparental *MSAT15-34* genotypes or deletions of various copies. C31 and C127 were missing bands 3 and 4 from the Kordaat genotype. C132 was missing bands 1, 3, and 5. C75, which is not shown, exhibited no deletions. Band 2 is present only in the Diana genotype and is not shown here. Bands not indicated with arrows represent "stutter" bands, which are an artifact of the amplification of the repeated array by PCR with *Taq* polymerase.

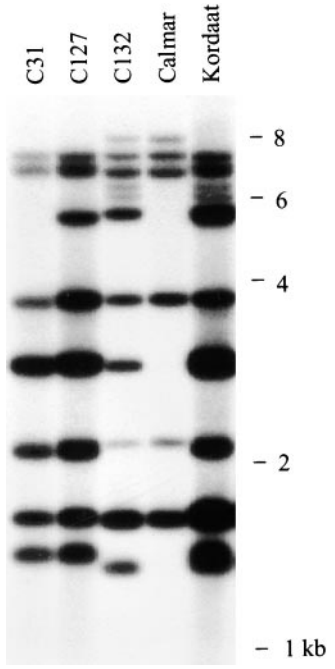

FIGURE 5.—Southern hybridization of *651END* on selected recombinants in the *Dm3* region. The probe *651END* is located ~4 kb downstream of the *Dm3* sequence and detects multiple family members in both parental haplotypes. Each of the recombinants, C31, C127, and C132, displayed a banding pattern that was either a subset or a combination of the two parental banding patterns, Calmar and Kordaat. Deletions of fragments indicate losses of the corresponding *RGC2* family members. No novel fragments were detected.

neous mutations of *Dm8* were found; therefore, this locus appears to be more stable relative to the other *Dm* loci tested.

Mutations are single locus, recessive, and stable: Selfed progeny from all mutants identified from the three populations were retested with the appropriate fungal isolates. The mutant phenotypes for all progeny were confirmed (Table 4). Segregation of resistance was examined to determine the inheritance of the mutant phenotypes. None of the segregation ratios deviated significantly from the expected ratio of 3 resistant:1 susceptible (Table 4). Thus, in all the spontaneous *dm* mutants identified, susceptibility segregated as a recessive, stable trait at a single locus.

To test for allelism, independent spontaneous mutants that exhibited a loss of resistance to the same fungal isolate were intercrossed. Additionally, spontaneous mutants were crossed with a range of FN-induced deletion mutants (OKUBARA *et al.* 1994) and EMS-induced point mutants (CHIN 2000). All crosses between mutants exhibiting a loss of the same resistance specificity produced susceptible progeny, providing no evidence for intergenic complementation within a specificity group. Therefore, lesions had occurred in the same *Dm* locus in both the induced and spontaneous mutant populations.

Additional crosses were made to test for epistasis and dominant mutations. Spontaneous mutants were crossed with Cobham Green, a closely related cultivar with no known *Dm* genes. All F_1 progeny from crosses to Cobham Green were susceptible, indicating that mutations had not occurred in a locus that was epistatic to the *Dm* genes. Mutants were also crossed back to wild-type Diana or Kordaat. All F_1 progeny from these crosses were resistant, confirming that the mutations were not dominant.

Spontaneous mutations at the *Dm7* locus are not associated with detectable deletions: The RAPD markers *OPA01*, *OPK02*, and *OPH14*, which span an ~2-cM interval that includes *Dm7* (KESSELI *et al.* 1994; P. OKUBARA, unpublished results), were assayed in the three *dm7* S_2 mutants from population 2. All markers were present; thus no deletions could be detected at this resolution. The FN-induced *dm7* mutants had also showed no detectable deletions (OKUBARA *et al.* 1994). It is possible that genes required for viability are tightly linked to *Dm7* and therefore large deletions in this region are lethal. Due to the lack of additional tightly linked markers or candidate genes in this region, these mutants were not studied further.

Most spontaneous mutations at the *Dm3* locus exhibit

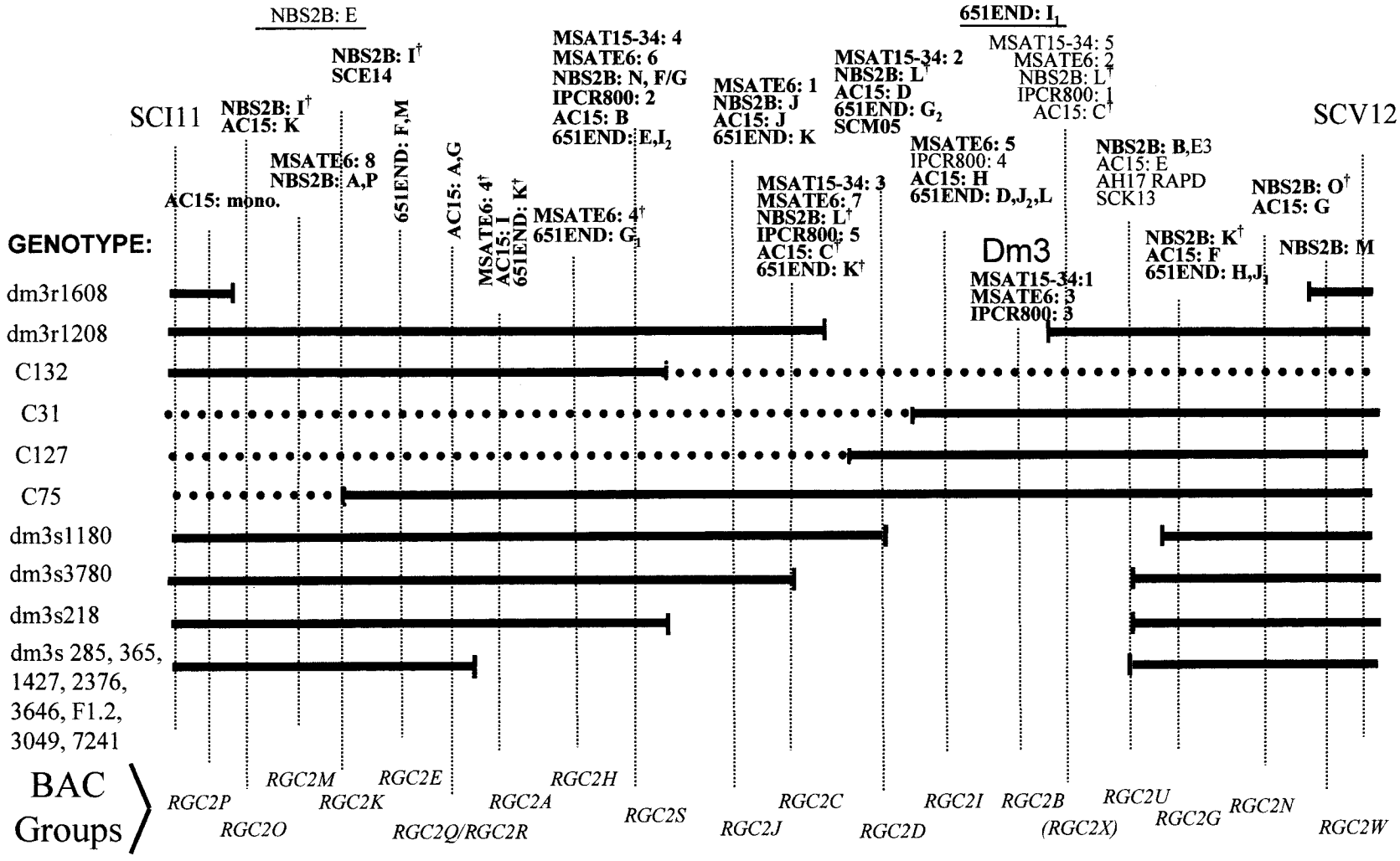

FIGURE 6.—Breakpoint analysis of *Dm3* recombinants and spontaneous mutants. The breakpoints of the Kordaat × Calmar F_2 recombinants and the *dm3* spontaneous deletion mutants were mapped using a combination of PCR-based and RFLP markers. Two FN-deletion mutants are included as references: dm3r1608 and dm3r1208 contained the largest and smallest deletions, respectively (ANDERSON *et al.* 1996; MEYERS *et al.* 1998a). Positions of BAC groups that contain members of the *RGC2* multigene family, of which *Dm3* is a member, are given below. The physical region spanned by the BAC groups is estimated to be at least 3.5 Mb (MEYERS *et al.* 1998a). All markers mapped are from the Kordaat or Diana *Dm3* haplotype and are shown at the top of the figure (see text). OP or SC markers are RAPD and SCAR markers. MSAT markers are microsatellite markers. All other markers are RFLP markers. Markers in boldface type were present on the BACs indicated by the connecting dashed line. Markers in normal typeface mapped to the region but were not detected on a BAC. The position of markers that could not be located precisely is shown above bars at the top. Individual fragments of multicopy markers are denoted by a colon and the letter(s) or number indicating the individual fragment. Identical duplicate markers, detected by their presence on nonoverlapping BACs, are noted by a dagger. The Kordaat alleles in the recombinants, C31, C75, C127, and C132, are noted by solid lines and the inferred Calmar alleles are noted by dotted lines. Data modified and updated from MEYERS *et al.* (1998a).

TABLE 3

Spontaneous mutants identified in lettuce

Population	Pedigree	*Dm* specificities screened	Total no. of screens	No. of mutants identified	Mutant ID no.
1	Kordaat × Calmar F_1	*Dm1*	8,500	1	dm1e (Okubara *et al.* 1994)
		Dm3		1	dm3sF1.2
2	Diana S_2	*Dm1*	11,000	0	
		Dm3		7	dm3s218, dm3s285, dm3s365, dm3s1427, dm3s1977, dm3s2376, dm3s3646
		Dm7		3	dm7s 15, dm7s1188, dm7s2330
		Dm8		0	
3	Kordaat × Calmar F_3 recombinant derivatives	*Dm3*	8,000	4	dm3s1180, dm3s3049, dm3s3780, dm3s7241

deletions: The RAPD markers *OPAC15*, *OPAH17*, and *OPJ11*, as well as the SCAR markers *SCE14*, *SCK13*, and *SCM05* (Table 2; Figure 1), were assayed in the 12 spontaneous *dm3* mutants from the three populations. Eleven mutants exhibited a deletion of the markers *OPAH17*, *SCK13*, and *SCM05*. The microsatellite markers *MSAT15-34* and *MSATE6* (Table 2; Figures 1 and 3) provided greater resolution and identified four types of deletions (Figures 6 and 7). Eight mutants, dm3s285, dm3s365, dm3s1427, dm3s2376, dm3s3646, dm3sF1.2, dm3s3049, and dm3s7241, had the largest deletion and were missing all of the *MSAT15-34* fragments (Figure 7) and all of the *MSATE6* fragments, except *MSATE6*:8 (data not shown). Three of the remaining mutants contained unique deletions (Figures 6 and 7). As with the analysis of the F_2 recombinants (above), the deletion of several molecular markers indicated that mutant haplotypes contained nonparental numbers of RGC2 homologs. Mutant dm3s1977 had no detectable deletions with any of the markers.

Southern hybridizations with sequences derived from *Dm3* were conducted to further characterize the *dm3* spontaneous mutants. Six unique restriction endonuclease/probe combinations were analyzed that were a subset of those used in the analysis of the recombinants. Deletions of several of the ~20 *RGC2* fragments were detected in all of the mutants except for dm3s1977 (Figure 8). The Southern analyses were consistent with the mutant deletion profiles detected by the PCR analyses and the linear order of breakpoints determined from previous studies of fast-neutron mutants and BACs (Figure 6; Anderson *et al.* 1996; Meyers *et al.* 1998a). None of these Southern analyses detected any novel fragments relative to the wild-type haplotype. Thus, although the

TABLE 4

Segregation of resistance in spontaneous *Dm* mutant families

Population		Resistant	Susceptible	$\chi^2_{(3:1)}$ [a]
K × C F_1 individuals	dm3sF1.2[b]	31	9	0.133
Diana S_2 families	dm3s218	39	16	0.491
	dm3s1977	67	27	0.695
	dm3s285	30	10	0.000
	dm3s365	30	11	0.073
	dm3s1427	76	30	0.616
	dm3s2376	30	5	2.143
	dm3s3646	23	4	1.494
	dm7s15	ND	ND	ND
	dm7s1188	27	7	0.274
	dm7s2330	13	2	1.089
K × C F_3 recombinant families	dm3s1180	77	27	0.051
	dm3s3049	48	15	0.048
	dm3s3780	143	33	3.667
	dm3s7241	89	24	0.853

ND, not done.
[a] $P(\chi^2 < 3.841) = 0.95$.
[b] dm3sF1.2 was crossed to Kordaat and the segregation was checked in the F_2 generation.

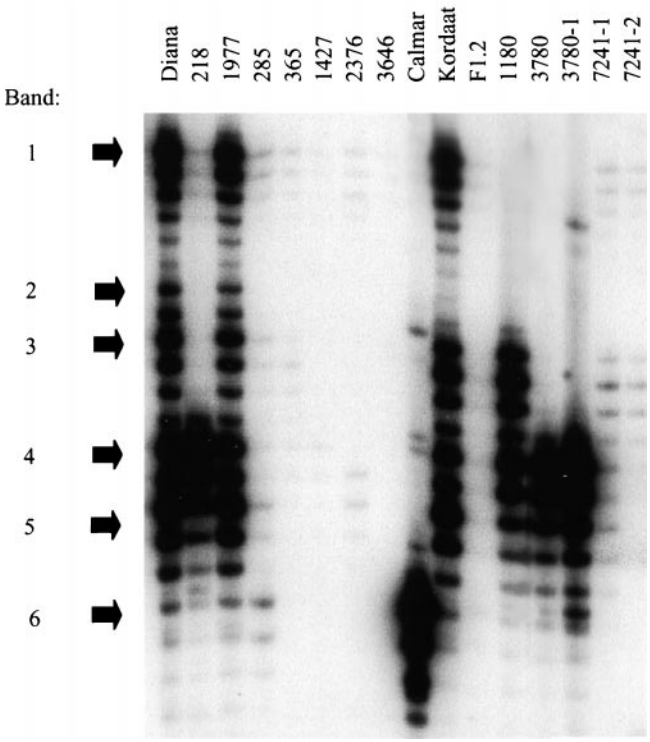

FIGURE 7.—*MSAT 15-34* genotype of spontaneous *dm3* mutants. The *dm3* spontaneous mutants exhibited a variety of *MSAT 15-34* haplotypes, most of which were deletions of various sizes. The wild-type parental controls, Diana, Calmar, and Kordaat are included for comparison. The mutant dm3s3049 is not shown, but exhibited a deletion of all bands.

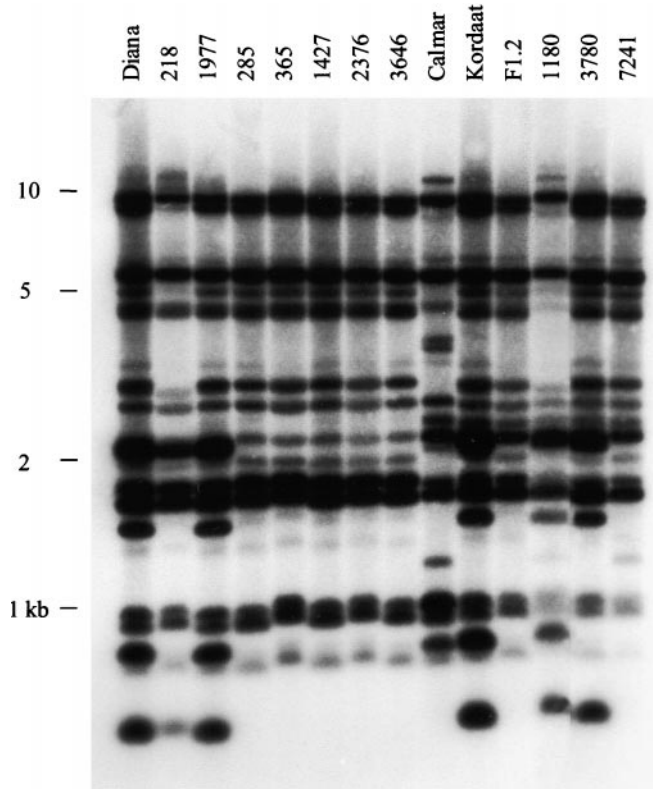

FIGURE 8.—Southern hybridization of *NBS2B* on spontaneous *dm3* mutants. The probe *NBS2B* detects multiple members of the *RGC2* family, even in the cultivar, Calmar, which does not contain any of the *Dm* genes in the major cluster. All of the spontaneous mutants, with the exception of dm3s1977, exhibited a deletion of multiple bands. No mutants exhibited nonparental bands. dm3s3049 is not shown but exhibited a deletion pattern similar to that of dm3s7241.

spontaneous deletion events did change the number of RGC2 homologs, the deletion breakpoints did not occur in or near *RGC2* sequences and the RFLP data provided no evidence for the occurrence of chimeric *RGC2* sequences in these mutants. The deletions detected in these spontaneous mutants can most easily be explained by the occurrence of recombination and unequal crossing over. However, the homozygosity of flanking markers in the eight F_1^{KC} and S_2 mutants from populations 1 and 2 precludes analysis of recombination in these mutants.

Recombination and unequal crossing over are associated with spontaneous mutation events: To determine if recombination could be correlated with spontaneous mutation events, population 3 was derived from F_1^R progeny that were homozygous for the *OPAC15-SCM05* block of markers containing *Dm3*, but heterozygous for markers *SCV12* and *SCI11* that flank this region (Figure 2). Four F_3 mutant families from these F_1^Rs were identified as segregating 3:1 for resistance:susceptibility. The initial marker analysis unexpectedly demonstrated that several of the susceptible F_3 individuals (*dm3 dm3*) were not homozygous for flanking markers *SCV12* and *SCI11*. Therefore, a total of at least 30 resistant and susceptible F_3 individuals from each mutant family were analyzed with *SCV12* and *SCI11* to determine the genotypes of the F_2 progenitors (Figure 9C). Recombination events that had occurred during the F_1^R meioses were detected for the ~8.5-cM interval between *SCV12* and *SCI11* for all four mutant families. The F_3 genotypes were consistent with recombination events in both F_1^R and F_2 meioses and indicated that recombination in the F_1^R meioses was associated with the loss of resistance in all four mutant families. The probability of four spontaneous mutation events in the *SCV12-SCI11* interval being associated with recombination events by chance is $P < 2.6 \times 10^{-6}$ (assuming four independent events with a binomial distribution and a recombination rate of 0.04; Table 5). Therefore the spontaneous losses of *Dm3* resistance apparently resulted from deletions that were associated with unequal crossing over.

Increased recombination frequencies were associated with some spontaneous mutation events: The recombination frequencies in the *SCV12-SCI11* interval were analyzed over four generations during the derivation of the F_3 mutant families in order to determine if (1) homozygosity immediately surrounding *Dm3* affected recombination rates and (2) if the spontaneous mutation events affected recombination rates in the *Dm3* region. The frequency of recombination in the original

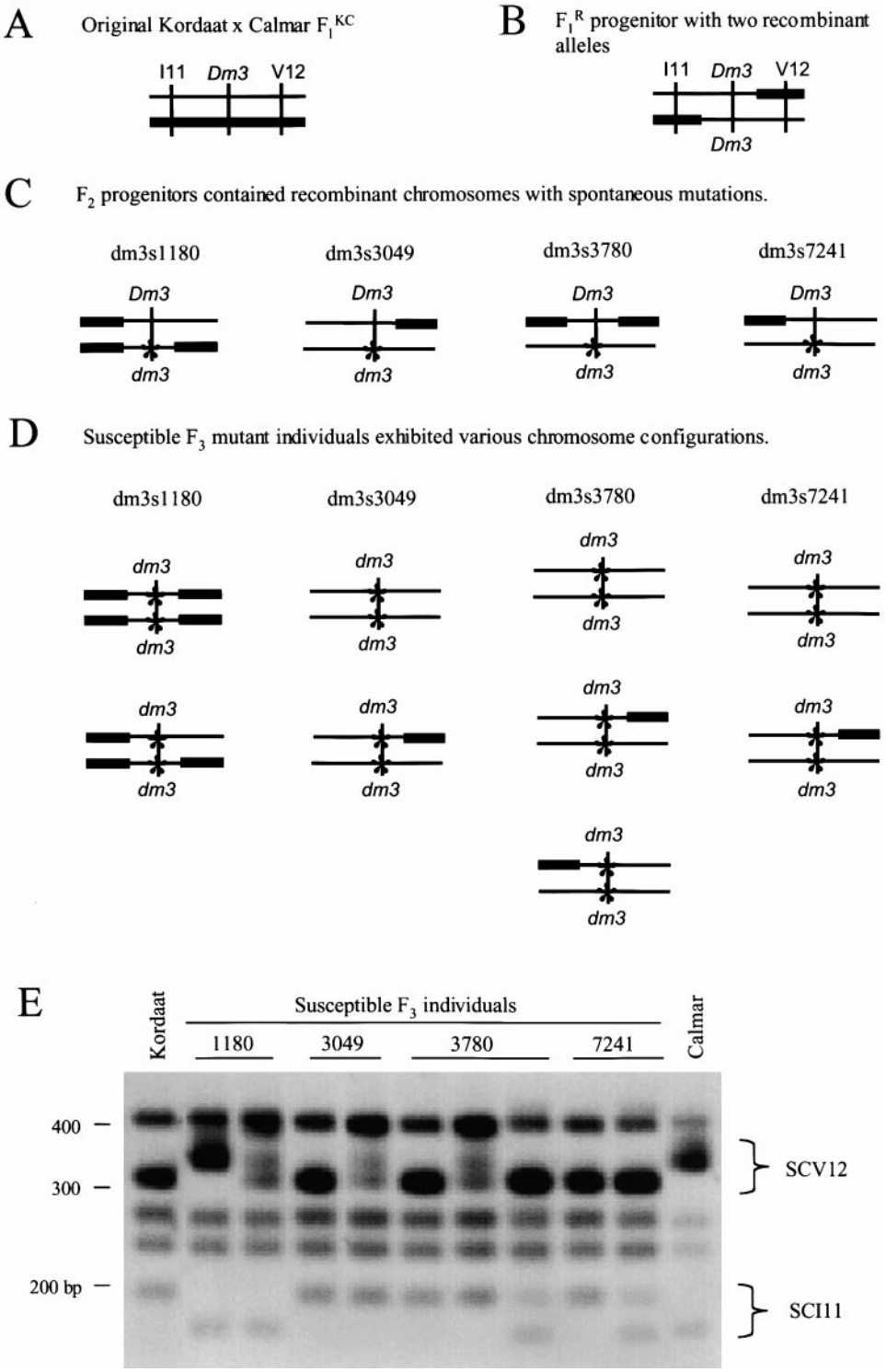

FIGURE 9.—V12/I11 configurations of recombination events in various generations. (A) The *SCV12/SCI11* configuration of the original Kordaat × Calmar F_1^{KC} in which no recombination has occurred. (B) The configuration of the F_1^R progenitors of population 3, resulting from crosses between F_2 siblings with recombination events on opposite sides of *Dm3* (see text and Figure 2). (C) The deduced *SCV12/SCI11* configurations of the F_2 progenitors of the four F_3 mutant families. Recombination events that occurred in the meioses of the F_1^R plants resulted in spontaneous deletions of the *Dm3* gene on that recombinant chromosome. Note dm3s3780, which displayed recombination events in both chromosomes, only one of which resulted in a deletional mutation. (D) The configurations of susceptible F_3 individuals, some of which displayed chromosomes in which additional recombination events occurred during F_2 meioses. (E) A gel of the *SCV12/SCI11* PCR reactions for the four mutant F_3 families. The various possible configurations for each family are shown. Parental genotypes, Kordaat and Calmar, were included for comparison. Braces indicate the polymorphic bands.

F_2 population of over 2000 individuals (R1, Figure 2A; Table 5) was 0.039 ± 0.003 for the interval *SCV12-SCI11*. This rate represents meioses in which the entire interval was heterozygous. The frequency of recombination in 288 "recombinant-derived" F_2 individuals (R2, Figure 2B; Table 5) was slightly higher at 0.044 ± 0.009. This increase was not statistically significant (normal approximation of the binomial, $P \cong 0.49$). A similar recombination rate of 0.040 ± 0.01 was also measured in the nonmutant F_3 families (R3a, Figure 2B; Table 5). Therefore the small region of homozygosity immediately surrounding *Dm3* (0.15 cM and ~3.5 Mb, see above; MEYERS *et al.* 1998a) within the larger heterozygous *SCV12-SCI11* region (3.6 plus 4.9 cM of unknown physical size) did not significantly affect the recombination frequency.

The recombination rates were also determined for

TABLE 5

Recombination frequencies in the *SCV12-SCI11* interval over multiple generations

Meiosis[a]	Population screened[a]	No. of individuals screened	No. of recombinants identified	Recombination frequency[b] (observed)		Standard error (SE)	Recombination frequency[c] (adjusted/total)
R1	Original F_2	2219	167	0.039		0.003	—
R2	Recombinant F_2	288	25	0.044		0.009	—
R3a	Resistant F_3	221	17	0.040		0.010	—
			SCV12-Dm3 Dm3-SCI11[d]				
R3b	dm3s1180[e]	63	2	0.016	—	0.011	0.061
R3c	dm3s3049	83	2	0.012	—	0.008	0.046
R3d	dm3s7241	35	6	—	0.086	0.033	0.120
R3e	dm3s3780	44	12	0.068	0.068	0.027, 0.027	0.14
R3f	dm3s7241-F_4	48	7	—	0.073	0.027	0.102
R3g	dm3s3780-F_4	46	8	0.011	0.065	0.011, 0.026	0.087

[a] See Figure 2 and text.

[b] Estimated recombination frequencies were calculated using the software program, LINKAGE-1 (Suiter *et al.* 1983), and according to Allard (1956).

[c,d] Recombination rates were adjusted for those meioses in which recombination could be monitored for only one side of the *SCV12-SCI11* interval. A total of 26% of the recombinants identified from the R1 meiosis were in the *SCV12-Dm3* interval and 74% were in the *Dm3-SCI11* interval. Therefore, R3b and R3c were adjusted by a factor of 3.8, and R3d and R3f were adjusted by a factor of 1.4.

[e] All F_3 and F_4 individuals screened for meioses R3b–g were susceptible to *B. lactucae* isolates containing *Avr3*.

the four mutant F_3 families that were derived from meioses in which one of the *Dm3* alleles had previously undergone a spontaneous deletion event resulting in hemizygosity at the locus (R3b to R3e, Figure 2B; Table 5). Three of these families, dm3s1180, dm3s3049, and dm3s7241, were homozygous at either *SCV12* or *SCI11* and therefore recombination could be observed only on one side of the interval, using *Dm3* as one of the markers. Consequently the recombination frequencies for the whole interval were inferred by extrapolation using the relative genetic sizes of the *SCV12-Dm3* and *Dm3-SCI11* intervals (Table 5). Two mutant families, dm3s1180 (R3b; Figure 2B) and dm3s3049 (R3c; Figure 2B), exhibited recombination frequencies of 0.061 and 0.046, which were similar to R1, R2, or R3a. The two other families, dm3s7241 (R3d; Figure 2B) and dm3s3780 (R3e; Figure 2B), exhibited significantly elevated recombination frequencies of 0.12 and 0.14, respectively (Table 5; $P \cong 0.0004$ and 0.0002). Recombination in the dm3s3780 family was elevated in both the *SCV12-Dm3* and *Dm3-SCI11* intervals (Table 5). The extrapolation for the *SCV12-SCI11* interval for the dm3s7241 family assumes that the recombination rate is elevated uniformly on both sides of *Dm3*; however, even when only the *Dm3-SCI11* interval is considered, the measured recombination frequency is significantly greater than R1, R2, and R3a for the entire *SCV12-SCI11* interval (Table 5; $P \cong 0.034$). Therefore, the recombinant haplotypes generated by unequal crossing over did not significantly decrease recombination but rather in two families seemed to have stimulated recombination at the *Dm3* locus.

The phenomenon of increased recombination in the dm3s3780 and dm3s7241 families was examined further in selected F_4 progeny. Resistant F_3 individuals that were hemizygous for the deletion of *Dm3* were selfed. Their F_4 progeny were screened for susceptibility to the *Avr3*-expressing isolate, IM25P11 (Table 1). Recombination between *SCV12* and *SCI11* in four F_4 families derived from dm3s3780 was 0.087 (Table 5). Recombination between *SCI11* and *Dm3* in four F_4 families derived from dm3s7241 was 7.3 cM (10.2 cM adjusted for *SCI11-SCV12* interval, Table 5). These rates were lower than those observed in F_3 hemizygotes but still significantly elevated from the other populations. Therefore, the increased recombination found in the dm3s3780 and dm3s7241 F_3 families was a repeatable phenomenon that continued into the next generation.

To determine the position of the crossovers that had occurred in the F_2 meioses, susceptible F_3 individuals were selfed to obtain F_4 progeny that were homozygous for the F_2 recombination product. Six and 12 susceptible F_3 individuals, from the dm3s7241 and dm3s3780 families, respectively, were homozygous for the *Dm3* deletion but heterozygous for either *SCV12* or *SCI11* (Table 5; Figure 9D). F_4 progeny from these 18 F_3 individuals were screened with *SCV12* and *SCI11* to identify those that were homozygous for the F_2 recombination product. Two homozygous individuals from each family were then analyzed with the probe *NBS2B*. For 17 of the 18 F_3 individuals selfed, deletion profiles of these F_4 progeny were identical to the profiles obtained for susceptible F_3 individuals that were homozygous for the F_1^R recombination product (data not shown). Therefore, most of

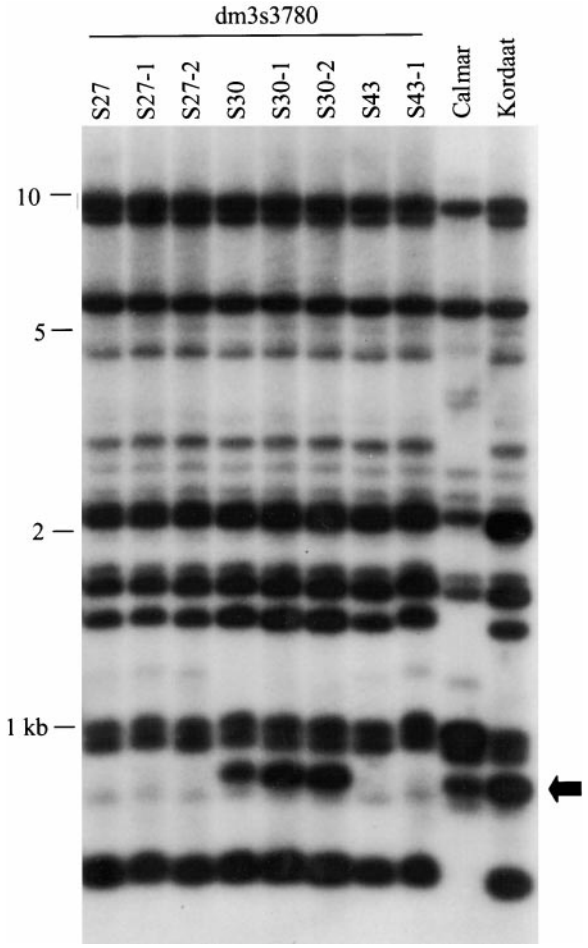

FIGURE 10.—Southern hybridization of NBS2B on F_3 *dm3* mutants and their F_4 progeny. The F_3 *dm3* mutants, S27 and S43, along with their F_4 progeny (indicated with dashes followed by numbers) exhibit the deletion profile found in nearly all of *dm3* spontaneous mutants from the dm3s3780 family, in which the marker NBS2B:L (arrowed; Figure 6) has been deleted. Only the mutant S30 and its progeny exhibit a novel haplotype in which marker NBS2B:L has been recovered through a recombination event in the F_2 progenitor.

the recombination events in the F_2 meioses resolved outside of the region encompassing the cluster of RGC2 sequences, as occurred in the original F_1^{KC} (see above). One of the 18 F_3 individuals selfed contained an additional marker, NSB2B:L (Figure 6); this was confirmed in the next generation because both the F_4 plants from this dm3s3780 individual also had NSB2B:L (Figure 10). Consequently, in this one case, recombination had occurred within the *RGC2* cluster and recombination in the F_2 meiosis of this individual generated a novel haplotype. However, no new restriction fragments were observed with respect to the Kordaat haplotype; therefore, there was again no evidence for the generation of novel recombinant *RGC2* sequences.

One spontaneous loss of resistance resulted from gene conversion: The mutant dm3s1977 contained all the molecular markers screened and therefore exhibited no detectable deletions. The *Dm3* allele in this mutant was sequenced to determine the molecular changes underlying the loss of resistance. The 5′ portion of the gene was sequenced following amplification of a *Dm3*-specific fragment of ~4.2 kb that encompassed the first three exons (Figure 3). Direct sequencing of this fragment from dm3s1977 detected no changes from the wild-type *Dm3* gene in Diana. The remainder of the gene was sequenced following amplification of an ~5.1-kb fragment that encompassed the last four exons (Figure 3). Sequencing of this 3′ fragment detected an ~1.5-kb region that did not match the wild-type *Dm3* allele. The divergent region extended from the middle of the fifth exon (just 3′ of *MSATE6*) to the middle of the sixth exon (Figure 3) and differed from *Dm3* at 120 polymorphic sites (Table 6). The divergent sequence exactly matched the sequence of *RGC2C*, a closely related paralog of *Dm3* (MEYERS *et al.* 1998a,b), and can be explained most parsimoniously as a gene conversion event (as opposed to a closely spaced double recombination event). Precise breakpoints of the conversion event could not be determined because *Dm3* and *RGC2C* share 81.6% overall nucleotide sequence identity; however, the breakpoints could be located to an 107-bp window on the 5′ end of the conversion tract and a 70-bp window on its 3′ end (Table 6). This conversion tract encompassed 38% of the C-terminal LRR (275 amino acids of the 721 amino acids in the C-terminal LRR), altering 9 of the 21 repeats in this region (MEYERS *et al.* 1998b).

To confirm that the gene conversion tract in this mutant was not an experimental artifact, a fragment specific to the gene conversion event was amplified from genomic DNA of dm3s1977 using PCR. Primers were designed to specifically amplify across the breakpoint of the *Dm3* and *RGC2C* sequences. As a control, an overlapping pair of primers was designed that amplified within the converted region from *RGC2C* (Figure 3; Table 2). The control primers, 1977CO5 and 1977CO3, amplified a fragment from the mutant dm3s1977 as well as from the *RGC2C* homolog present in wild-type Diana (Figure 11). However, PCR using the primers 1977CH5 and 1977CO3 only amplified a fragment from the mutant dm3s1977 (Figure 11). This confirmed the gene conversion event in the spontaneous mutant, dm3s1977.

The conversion event in the LRR-encoding region of the *Dm3* gene in dm3s1977 resulted in a loss of the *Dm3* specificity, indicating that this region was necessary for resistance gene function or specificity. This mutant was therefore tested against a variety of Bremia isolates from diverse geographical origins, each expressing the *Avr3* avirulence phenotype (Table 1). The mutant dm3s1977 was susceptible to all 5 isolates, confirming the specificity of this mutation to the *Dm3-Avr3* interaction. Thirty other isolates, which were virulent on wild-type Diana and collectively exhibited 13 different virulence phenotypes, were also tested on mutant dm3s1977; resistance

TABLE 6

Sequence comparison of 3′ LRR-encoding region of the chimeric allele in dm3s1977 with *Dm3* and *RGC2C*[a]

basepair position[b]

genotype	9729 9754 9761 9762 9763 9764 9765 9769 9835 9838 9839 9841 9846 9847 9852 9918 10058 10062 10065 10066 10067 10075 10174 10175 10176 10177 10178 10179 10180 10181 10182 10183 10184 10185 10186 10187 10188 10197 10304 10305 10509 10435 10445 10519 10529 10547 10585 10613 10628 10659 10661 10662 10663 10664 10665
Dm3	T G T T G G A T A G A T T G C C A A A T T G T C T T C T T C T T C T T C T T A G A T T G T A T C G T G T G T G
dm3s1977	T G T T G G A T A G A T T G C C A A A T T G T C T T C T T C T T C T T C T T T C G C C A C G A T A G - - - - -
RGC2C	C A G C A A C C G A T G C A G T C T G G G A - - - - - - - - - - - - - - - - G T C G C C A C G A T A G - - - - -

basepair position

genotype	10666 10667 10668 10669 10670 10671 10678 10713 10722 10723 10724 10725 10726 10727 10728 10732 10734 10735 10736 10737 10738 10739 10740 10741 10742 10743 10744 10745 10746 10747 10748 10749 10750 10751 10763 10764 10765 10771 10823 10862 10872 10873 10900 10904 10918 10943 10980 11111 11210 11213 11214 11274 11289 11420 11431
Dm3	T G G G A G T T C T C G A A C T G A A A C C C T T T T A A T C T T T G A C T T T C T C G G C G T G G G A G A C
dm3s1977	- C G G A C A A C T C A A T C T T G G
RGC2C	- C G G A C A A C T C A A T C T T G G

basepair position

genotype	11435 11443 11515 11518 11522 11530 11531 11532 11533 11574 11591 11593 11596 11597 11598 11599 11600 11601 11602 11607 11608 11609 11611 11619 11620 11624 11625 11630 11643 11680 11681 11685 11712 11719 11727 11732 11733 11735 11736 11737 11742 11744 11803 11804 11805 11809 11810 11812 11882 11892 11893 11898 11925A 11928A 11928B
Dm3	T A T C A A A G G C C T A T C C A A C G T G G A G A T A A G G C G C T A G G A A C T A A G T G G C C G G - - -
dm3s1977	C C A T G T T C A T G G T G G T T T G A C C T T C C A C T C A T A G A C T C T T A A G G A C T C C C G G - - -
RGC2C	C C A T G T T C A T G G T G G T T T G A C C T T C C A C T C A T A G A C T C T T A A G G A C T C A T C A C A T

[a] *Dm3* (*RGC2B*) and *RGC2C* share 81.6% nucleotide sequence identity (Meyers *et al.* 1998a). Only positions in exon 5, intron 5, or exon 6 that are polymorphic between *Dm3* and *RGC2C* are shown.

[b] *Dm3* is used as the reference sequence in numbering base pair positions. Deletions are indicated by dashes. Breakpoints of the conversion event in dm3s1977 are indicated by arrows.

was not observed to any isolate (S. Brown and O. Ochoa, unpublished results). Therefore, there was no evidence for novel specificities encoded by the chimeric allele in dm3s1977. The other spontaneous mutants were not tested against these isolates because they were deletion mutants and did not display novel RFLP patterns indicative of chimeric genes.

DISCUSSION

Although the clustering of resistance genes is common in many plant species, the evolution of these gene clusters remains only partially understood. Comparative genetic and sequence analyses have identified or suggested the involvement of a variety of genetic events, including recombination, unequal crossing over, gene conversion, transposition, and divergent selection (reviewed in Hulbert 1997; Michelmore and Meyers 1998; Ronald 1998). However, the relative importance of each of these events in the immediate and long-term evolution of resistance gene clusters is not known. This article emphasizes genetic analyses of resistance gene clusters and provides further insights into the roles of recombination, unequal crossing over, and gene conversion in the short-term evolution of clusters of resistance genes. Some of our observations are consistent with conclusions from studies of other loci, while some are not.

A variety of factors affect recombination and stability within clusters of resistance genes: The structural and sequence similarities between haplotypes in a particular meiotic pairing, as well as the size and complexity of the region as a whole, will profoundly influence the genetic behavior of multigene families. Sequence and structural identity, as occurs when the locus is homozygous, will favor exact pairing and little structural rearrangement; however, if there are long stretches of duplicated sequences, there will be opportunities for mispairing resulting in further gene duplications and deletions. Structural heterozygosity between haplotypes in a particular pairing prevents precise alignment and, depending on the degree of divergence, will tend to repress recombination in the region and result in novel haplotypes whenever recombination does occur within the cluster. In our experiments, the *Dm3* region was studied in both the heterozygous and homozygous condition.

There is only limited information on the rates of recombination within clusters of resistance genes. When the *SCV12-SCI11* interval encompassing the *Dm3* gene was heterozygous in the intraspecific Kordaat × Calmar F_2 population, crossovers within the *RGC2* cluster were

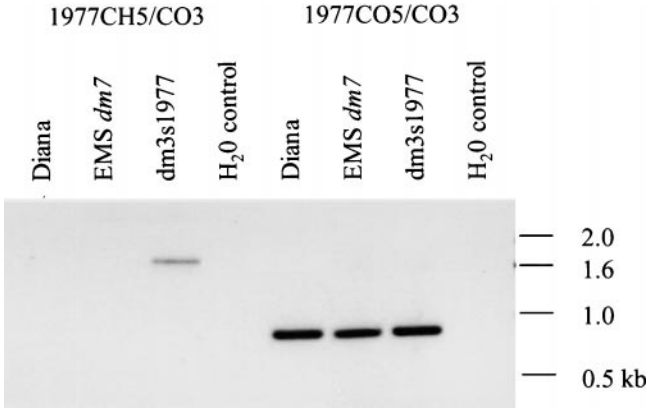

FIGURE 11.—Confirmation of gene conversion event in dm3s1977. Three primers were designed that were diagnostic for the conversion event in the spontaneous mutant dm3s1977 (Table 2; Figure 3). The primers 1977CO5 and 1977CO3 lie within the conversion event and are thus amplified from the family member *RGC2C* in all three genotypes: wild-type Diana, a *dm7* Diana EMS mutant, as well as dm3s1977. The primer 1977CH5, which lies just 5′ of the conversion event, was used in conjunction with 1977CO3; the product of these two primers was a chimeric sequence of *Dm3* and *RGC2C* and was thus amplified only from the mutant, dm3s1977. Genomic DNA was used as template for all samples.

only rarely detected and recombination was suppressed approximately 18-fold from the genome average. The *RGC2* region may be partially hemizygous between Kordaat and Calmar; the RAPD and SCAR markers identified within the *OPAC15-SCM05* block are all linked *in cis* with the *Dm3* Kordaat allele (ANDERSON *et al.* 1996) and the microsatellite markers, *MSAT15-34* and *MSATE6*, as well as Southern hybridizations with *RGC2* sequences, consistently detected fewer fragments in Calmar than Kordaat. Such hemizygosity would be expected to prevent pairing within the cluster. Making the *OPAC15-SCM05* block homozygous (Figure 2) did not result in a detectable increase in recombination (Table 5). Repression of recombination at resistance gene loci is not unexpected as many resistance gene-containing regions have been introgressed from other species and therefore represent divergent haplotypes (CRUTE 1988). There was a 10-fold reduction of recombination in a 240-kb region surrounding the *Mla* resistance gene cluster in barley, relative to regions immediately flanking the cluster (WEI *et al.* 1999). Recombination was also repressed in chromosomal regions of *Lycopersicon esculentum* containing *Mi* (VAN DAELEN *et al.* 1993) and *Tm-2a* (GANAL *et al.* 1989). Recombination rates could also be influenced by chromosome position; however, *Tm-2a* and *Mi* are proximal to the centromere while *RGC2* and *Mla* are telomeric (GANAL *et al.* 1989; VAN DAELEN *et al.* 1993; SHEN *et al.* 1998; WEI *et al.* 1999). None of these data indicate that clusters of resistance genes are highly recombinagenic.

The stability of resistance genes, as evidenced by rare losses in resistance, has been studied when the resistance cluster was both homozygous and heterozygous. The most informative studies are those when the region is homozygous, as will be the case for most meioses in inbreeding species such as lettuce, tomato, and *Arabidopsis thaliana*. The *Dm3* cluster was genetically unstable when homozygous, as evidenced by a high rate of spontaneous mutation (10^{-3} to 10^{-4} mutations per generation, Table 3). This mutation rate was comparable to instability at the *Rp1* cluster in maize (PRYOR 1987; BENNETZEN *et al.* 1988). The instability of the *Dm3* and *Rp1* regions contrasts with the stability of the *Cf-4/9* locus in tomato and the *RPP5* locus in *A. thaliana* when homozygous (PARNISKE *et al.* 1997; NOEL *et al.* 1999). No mutations were identified at the *Cf-9* locus in screens of ~12,000 testcross progeny from homozygous *Cf9* plants. Similarly, no susceptible individuals were identified in screens of ~7500 testcross progeny of *RPP5* homozygous plants. The reasons for such differences in stability between these loci are not clear. The *Dm3*, *Rp1*, *Cf-4/9*, and *RPP5* clusters are all duplicated and complex multigene families. The *Dm3* locus is the largest, both in terms of the number of homologs (24+ homologs *vs.* ~10 homologs at the other loci) and the physical size of the cluster (~3.5 Mb *vs.* ~35 kb and 95 kb for *Cf-4/9* and *RPP5*, respectively; JONES *et al.* 1994; PARNISKE *et al.* 1997; THOMAS *et al.* 1997; MEYERS *et al.* 1998a; COLLINS *et al.* 1999; NOEL *et al.* 1999). The *Cf-4/9* and *RPP5* loci may not be large enough and stretches of sequence affiliations may be distributed such that mispairing does not occur when these loci are homozygous; in contrast, the size and level of duplication at the *Dm3* locus may allow occasional misalignment even when it is homozygous. Thus, the genetic behavior of a resistance gene cluster may change as its structure and level of complexity evolves due to genetic rearrangements. In particular, recent large duplications resulting from unequal crossing over would be expected to stimulate instability in the homozygous condition.

When resistance loci are heterozygous, estimates of stability as evidenced by losses of particular resistance specificities will be heavily dependent on the haplotypes involved, rather than a consequence of the intrinsic properties of the locus. Precise alignment is impossible between divergent haplotypes; recombination within a cluster will always result in nonparental haplotypes, some of which may lack one or more functional resistance genes. Losses of resistance could also result from gene conversion events between divergent haplotypes; however, this would not result in structural rearrangements. When *Dm3* was heterozygous in the Calmar × Kordaat F_2 progeny, all recombinant events within the cluster produced novel haplotypes; three recombinants retained *Dm3* and one did not (Figures 4–6). Similarly, the five susceptible individuals that were identified from screens of 7500 testcross progeny of *Cf-4/Cf-9* heterozygous plants had nonparental haplotypes

at the *Cf-4/9* locus (Parniske *et al.* 1997). Both the frequency of novel haplotypes as well as losses of resistance should be considered in estimating the instability of resistance loci when heterozygous. This will reflect the rates of gene conversion as well as recombination within the cluster that in turn are consequences of the structural and sequence similarities between haplotypes. Furthermore, the mating system of the plant species will determine how often a resistance cluster is heterozygous. In outbreeding species, heterozygosity will be frequent and haplotypes might be expected to evolve constantly. Conversely, in inbreeding species, such as lettuce, heterozygosity will occur rarely; however, occasional outcrossing and heterozygosity may have profound consequences for the evolution of the region.

Losses of resistance were due to unequal crossing over or gene conversion: Eleven of the 12 spontaneous losses of *Dm3* specificity were due to deletions. Interestingly, all of the deletions appeared to terminate in a similar region at one end and 8 of these 11 events had the same large deletion (Figure 6). These 8 events were derived from each of the three populations that were screened for spontaneous mutations in three different years. In the one population in which recombination of flanking markers could be monitored, spontaneous deletion events were associated with recombination, thereby implicating unequal crossing over as the mechanism responsible for all of the deletions. The distribution of deletion profiles may reflect a pattern of sequence duplication within the locus that favors a particular misalignment or resolution of crossover events. The gene density in the region is low and individual *RGC2* genes are separated by an average of 145 kb (Meyers *et al.* 1998a). As with the spontaneous *Cf-9* mutants in tomato (Parniske *et al.* 1997), the breakpoints in novel *RGC2* haplotypes were between rather than within *RGC2* genes. This suggests that the crossovers are not occurring, or at least not being resolved, within genes.

In the one spontaneous mutant that exhibited no deletions, sequencing of the *dm3* allele revealed that gene conversion was probably responsible for the loss of specificity. Sequence exchange between *Dm3* and *RGC2C* necessitates pairing between sequences with 82% nucleotide identity. This alignment of *Dm3* with *RGC2C* would have also paired the neighboring regions containing *RGC2D* and *RGC2I* with those containing *RGC2S* and *RGC2J*; these pairs of *RGC2* sequences shared 82 and 92% nucleotide identity, respectively (Figure 6; Meyers *et al.* 1998a). The regions on either side of the *RGC2D/S* to *Dm3/RGC2C* segments contained more divergent pairs of *RGC2* sequences.

The relationship between rearrangements at the locus and the evolution of new resistance specificities: No chimeric *RGC2* genes were observed as the result of recombination between divergent *Dm3* haplotypes or as the result of unequal crossing over. This contrasts with the analyses conducted on mutants at the *Rp1-D* locus in maize, in which the majority of mutations involved recombination within or close to coding regions of *Rp1-D* homologs (Collins *et al.* 1999). The Southern analyses of the *Dm3* mutants utilized seven different restriction endonucleases with multiple probes and thoroughly sampled the *RGC2* genes as well as the regions immediately adjacent to them. No novel fragments were observed. Recombination and unequal crossing over produced changes in the number of *Dm3* paralogs but did not generate chimeric genes. Therefore, consistent with the birth-and-death model for resistance gene evolution (Michelmore and Meyers 1998), the predominant role of unequal crossing over may be in creating changes in copy number, rather than generating chimeric genes with new resistance specificities. Unequal crossing over may, however, affect the evolution of new specificities by generating gene duplications. Assays for spontaneous losses of resistance can detect only one of the products of unequal crossover events. The reciprocal products that contain the duplicated segments could be templates for divergent selection and thus potentially lead to novel resistance specificities (Michelmore and Meyers 1998).

One spontaneous mutation event generated a chimeric *RGC2* gene, most likely through gene conversion. Comparative sequence analyses of the *Cf-4/Cf-9*, *Xa21*, *L*, *RPP5*, and *RPP8* loci all suggest similar exchanges of sequence information between paralogs, either by recombination or gene conversion (Parniske *et al.* 1997; Song *et al.* 1997; McDowell *et al.* 1998; Ellis *et al.* 1999; Noel *et al.* 1999). Analyses of the *Pto* and *RGC2* loci provided similar evidence (D. Lavelle and R. Michelmore, unpublished results; H. Kuang, E. Nevo and R. Michelmore, unpublished results). However, it is unclear on what timescales these exchanges have occurred. Our data suggest that sequence exchange between paralogs may be rare and further support the predictions of the birth-and-death model for resistance gene evolution (Michelmore and Meyers 1998).

In certain cases, the deleted product of unequal crossing over may also increase evolutionary activity of the locus. Increases in recombination were associated with two spontaneous *Dm3* mutation events in multiple generations. The recombination rates representing meioses in the mutant F_3 families, dm3s3780 and dm3s7241, were significantly increased ~3- and 3.5-fold compared to the recombination rates analyzed over three generations. It is unclear why this increase occurred. The deletion in dm3s7241 was among the largest observed and is probably well over 1 Mb; the deletion in dm3s3780 is at least 500 kb (Meyers *et al.* 1998a; Chin 2000). If recombination is enhanced by the existence of different gene arrangements or novel haplotypes, then evolution of resistance gene clusters may be a punctuated process, with periods of relative stability interspersed with bursts of instability stimulated by unequal crossing over.

Future studies: The majority of studies on resistance gene evolution, including our experiments, have involved the analysis of one or a few haplotypes. The picture emerging from these analyses is complex. However, it is becoming increasingly evident that stability and recombination activity at resistance gene clusters are heavily dependent on several parameters including the size and complexity of the locus, as well as the structural and sequence similarities between the haplotypes in a particular pairing. It is now necessary to extend these analyses to include a greater range of haplotypes and haplotype pairings. We are currently generating progeny from crosses between genotypes with varying levels of diversity; these involve naturally occurring haplotypes or FN-induced deletions of *Dm3* (Okubara *et al.* 1994; Anderson *et al.* 1996). These progeny will be analyzed to determine the effects of haplotype diversity and the presence of deletions on recombination frequencies, instability at the locus, and the generation of chimeric resistance genes.

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