Mutants of Downy Mildew Resistance in *Lactuca sativa* (Lettuce)

Patricia A. Okubara, Peter A. Anderson, Oswaldo E. Ochoa and Richard W. Michelmore

Department of Vegetable Crops, University of California, Davis, California 95616

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ABSTRACT

As part of our investigation of disease resistance in lettuce, we generated mutants that have lost resistance to *Bremia lactucae*, the casual fungus of downy mildew. Using a rapid and reliable screen, we identified 16 distinct mutants of *Latuca sativa* that have lost activity of one of four different downy mildew resistance genes (*Dm*). In all mutants, only a single *Dm* specificity was affected. Genetic analysis indicated that the lesions segregated as single, recessive mutations at the *Dm* loci. *Dm3* was inactivated in nine of the mutants. One of five *Dm1* mutants was selected from a population of untreated seeds and therefore carried a spontaneous mutation. All other *Dm1*, *Dm3*, *Dm5/8* and *Dm7* mutants were derived from γ- or fast neutron-irradiated seed. In two separate *Dm1* mutants and in each of the eight *Dm3* mutants analyzed, at least one closely linked molecular marker was absent. Also, high molecular weight genomic DNA fragments that hybridized to a tightly linked molecular marker in wild type were either missing entirely or were truncated in two of the *Dm3* mutants, providing additional evidence that deletions had occurred in these mutants. Absence of mutations at loci epistatic to the *Dm* genes suggested that such loci were either members of multigene families, were critical for plant survival, or encoded components of duplicated pathways for resistance; alternatively, the genes determining downy mildew resistance might be limited to the *Dm* loci.

IN a "gene-for-gene" disease interaction, specificity is determined by a dominant resistance gene in the plant that is matched by a dominant gene for avirulence in the pathogen (FLOR 1971; GABRIEL 1990; KEEN 1990; DE WIT 1992), although additional genes in the plant or pathogen might also influence the interaction (CRUTE 1985). In recent years, much work has been done on the expression and structure of avirulence genes and the function of their products is being elucidated (KEEN 1990; KNOOP *et al.* 1991; TAMAKI *et al.* 1991; DE WIT 1992; BONAS *et al.* 1993). Two resistance genes have recently been isolated from plants. *Hm1*, from corn, codes for a reductase that inactivates a toxin from *Cochliobolus carbonum* and thereby confers resistance to the fungus (JOHAL and BRIGGS 1992). *Pto* from tomato confers specific resistance to the bacterial pathogen *Pseudomonas syringae* pv. *tomato* and has sequence similarity to a protein kinase (MARTIN *et al.* 1993). Many other genes will need to be characterized to determine the diversity of types of resistance genes in plants.

To understand the genetic and molecular basis of disease resistance, we have been studying the interaction between *Lactuca sativa* and the obligate biotrophic fungus, *Bremia lactucae*. The gene-for-gene interaction between *L. sativa* and *B. lactucae* has been genetically well characterized (CRUTE and JOHNSON 1976; JOHNSON *et al.* 1978, FARRARA *et al.* 1987; ILOTT *et al.* 1989). Extensive classical genetic analysis has shown that there are at least 13 dominant, single genes in lettuce for resistance to downy mildew (*Dm* genes) that are matched by specific avirulence genes in *B. lactucae*. Many other sources of resistance in lettuce have been identifed, although these have not been characterized in detail (*e.g.*, FARRARA and MICHELMORE 1987; BONNIER *et al.* 1992).

Mutations have been generated in plants by treatment of seeds with mutagenic chemicals or ionizing radiation. Ethyl methanesulfonate, sodium azide and ionizing radiation have been used to generate mutations in resistance genes from a number of plant species. Detailed mutation studies of *Ml-a12* for resistance to powdery mildew in barley identified a range of altered phenotypes from partial resistance to complete susceptibility as well as mutations at a second suppressor locus (TORP and JORGENSEN 1986; JORGENSEN 1988). Mutational studies of a gene in Arabidopsis for resistance to *P. syringae* identified a single gene, designated *RPS2* (YU *et al.* 1993; KUNKEL *et al.* 1993). Deletion mutations have been useful in the cloning of several animal and plant genes (STRAUS and AUSUBEL 1990; SHIRLEY *et al.* 1992; JOHAL and BRIGGS 1992). Deletions (as well as other chromosomal rearrangements) are preferentially generated by ionizing radiation (*e.g.*, International Atomic Energy Agency, 1977). Therefore, we irradiated lettuce seeds with two kinds of ionizing radiation, γ and fast neutrons.

We generated mutants of downy mildew resistance for several reasons. Genetic analysis of such mutants could lead to the identification of additional loci involved in resistance and establish a hierarchy of gene action in the resistance pathway. Deletion mutants are powerful genetic stocks for identifying new molecular markers close to *Dm* loci. Deletions can be useful for the physical localization of *Dm* genes as part of cloning strategies. Such

TABLE 1

Dm genotypes of lettuce cultivars

Cultivar	*Dm* genes	*B. lactucae* isolates used
Saffier	*1, 7, 16*	CS12+CS9
Diana	*1, 3, 5/8, 7*	C83M47+IM25P11 +CG1+R60
Kordaat × Calmar F_1	*1, 3, 4, 5/8, 7, 13*	C83M47+IM25P11

mutants also provide the opportunity to apply recently developed differential screening techniques for the isolation of sequences from resistance genes or linked loci (LIANG and PARDEE 1992; LISITSYN *et al.* 1993). In this study, we report the identification of mutants at four *Dm* genes and demonstrate deletions at two of these loci.

MATERIALS AND METHODS

Plant material and mutagenesis: Two inbred cultivars and one F_1 progeny were studied (Table 1). Seeds of cv. Saffier were treated with γ-irradiation from Co^{60} at absorbed dosage levels of 40–300 Gy (Crocker Nuclear Laboratory, Davis, California; FRANCIS 1992). Under greenhouse conditions, less than 40% of plants from seeds irradiated at the highest dose survived. Seeds of cv. Diana were irradiated with fast neutrons at dosage levels of 4.5, 6.0 and 7.0 Gy (International Atomic Energy Agency, Vienna, Austria). Germination rates of M_1 seed were 95% at the 4.5- and 6.0-Gy dosage levels and 90% at the 7.5-Gy dosage level. To select for genetically transmissible mutations and to select against large deletions, M_2 progeny were generated for the mutant screen. A large F_1 progeny was generated by crossing cvs. Kordaat and Calmar; this seed received no mutagenic treatment prior to screening for loss of resistance.

Assays for loss of downy mildew resistance: Fungal isolates were propagated on 6-day-old seedlings of cv. Cobham Green (containing no known *Dm* genes) and asexual spores were harvested seven days after inoculation, as described previously (FARRARA *et al.* 1987). For the mutant screen, 15–25 lettuce seeds of each M_2 family were sown on filter paper saturated with mineral nutrient medium in compartmented plastic boxes. Six- to seven-day-old seedlings were inoculated with asexual spores. Loss of *Dm* activity was detected as profuse sporulation of *B. lactucae* occurring 6–10 days after inoculation. Over 1,200 M_2 families of γ-irradiated cv. Saffier were inoculated with CS12 and CS9 to detect loss of *Dm1* or *Dm16* activity, respectively, as indicated in Tables 1 and 2. More than 2,200 M_2 families of fast neutron-treated cv. Diana were inoculated with a pool of four isolates, each diagnostic for one of the four *Dm* genes in Diana (Tables 1 and 2). About 3,000 untreated F_1 progeny of Kordaat × Calmar were inoculated with a mixture of C83M47 (for *Dm1*) and IM25P11 (for *Dm3*). All susceptible seedlings were given multiple treatments of the systemic fungicide Ridomil 2E (Ciba-Geigy Corp.) to kill *B. lactucae* before transfer to soil. Efficiency of rescue was 100%. As a general indicator of the effectiveness of mutagenesis, the segregation of leaf pigment mutants was also noted.

To determine which *Dm* gene was inactivated in each mutant, 15–20 additional M_2 seedlings, or leaf disks taken from susceptible M_2 plants, were inoculated with each of the fungal isolates individually. In both seedling and leaf disk assays, susceptibility was indicated by profuse sporulation and was clearly distinguishable from resistance, in which no sporulation was observed.

Genetic analyses: Mutant M_2 families were scored for the segregation of resistance and susceptibility. To test whether susceptibility to downy mildew segregated in the M_3 generation, 25–40 M_3 seed from two susceptible individuals of each M_2 family were inoculated with appropriate fungal isolates.

To determine whether mutants that were susceptible to the same fungal isolate carried lesions in the same locus or could undergo intergenic complementation, M_2 individuals within the *Dm1* or *Dm3* mutant classes were crossed. In most cases, reciprocal crosses were made. For the allelism test using the spontaneous mutant derived from Kordaat × Calmar, F_2 plants homozygous for the mutant *Dm1* region from Kordaat were identified based on the absence Calmar RAPD markers and the presence of Kordaat markers in the *Dm1–Dm3* region. While selfed individuals could not be distinguished from outcrossed individuals in these allelism tests, crosses between mutants and Cobham Green or wild-type Diana described below indicated the average rate of out-crossing was 53% (ranging from 24 to 100%).

Pollen from several individual M_2 *Dm* mutants were also used in crosses with Cobham Green as the female to determine if loss of resistance was attributable to second-site mutations. F_1 progeny were distinguished from self-fertilized Cobham Green on the basis of their resistance to CG1, conferred by *Dm5/8*. Leaf disks of F_1 individuals were inoculated with single isolates to assay for the lost *Dm* activity.

Analysis for flanking molecular markers: DNA was isolated from lettuce leaves using a modified cetyltrimethylammonium bromide procedure (CTAB; BERNATZKY and TANKSLEY 1986). Mutants were analyzed for the presence or absence of random amplified polymorphic DNA (RAPD; WILLIAMS *et al.* 1990) and restriction fragment length polymorphism (RFLP) markers previously shown to map to the regions of the *Dm* genes (KESSELI *et al.* 1989, 1993; D. LAVELLE, P. ANDERSON and P. OKUBARA, unpublished data). RAPD and sequence characterized amplified region (SCAR) markers were assayed using procedures described previously (PARAN and MICHELMORE 1993).

Pulsed-field gel Southern blot analysis: High molecular weight DNA was isolated from leaf mesophyll protoplasts (CRUCEFIX *et al.* 1987; WING *et al.* 1993). Protoplasts were immobilized in microbeads of agarose, then treated with proteinase K and detergent to remove proteins and lipids. Restriction endonuclease digestion of DNA embedded in microbeads was done according to the specifications of the enzyme manufacturer. Approximately 100 μl of microbeads were used for each enzyme treatment. Digested DNA was fractionated by pulsed-field gel electrophoresis (CHEF DR III, Bio-Rad, Richmond, CA) through 1% agarose at 6 V/cm, 9.8–160-sec switch time, 14°, for 20 hr. Southern blots and hybridization to cloned markers $OPK13_{1700}$, *CL922* and $SCI11_{1200}$ were done according to standard protocols.

RESULTS

Inoculation of M_2 or F_1 lettuce seedlings with pooled isolates of *B. lactucae* allowed for easy and rapid identification of putative *Dm* gene mutants. Approximately 135 M_2 families or 1,000 F_1 seedlings were screened weekly. For all mutants obtained, loss of downy mildew resistance was marked by profuse fungal sporulation six to ten days after inoculation. In all cases, the mutant phenotypes were clearly distinguishable from the resistant phenotype, in which no sporulation was observed. Sporulation on the mutants occurred synchronously with normal compatible reactions; no intermediate

TABLE 2

Virulence phenotypes of isolated of *B. lactucae*

Isolate	Reaction to *Dm* genes														
	1	*3*	*5/8*	*7*	*2*	*4*	*6*	*10*	*11*	*R12*	*13*	*14*	*15*	*16*	*R18*
CS9	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+
CS12	−	−	−	+	+	+	−	−	+	−	−	−	−	+	−
C83M47	−	+	+	+	+	+	+	−	−	+	+	+	−	+	−
IM25P11	+	−	+	+	−	+	+	+	±	+	+	+	+	+	−
CG1	+	+	−	+	−	+	−	+	−	+	+	−	+	−	−
R60	+	+	+	−	+	+	+	+	+	+	+	+	−	−	−

+ = profuse sporulation, − = no sporulation, ± = sparse sporulation.

TABLE 3

***Dm* mutants of lettuce**

Mutant	Gene inactivated	Treatment[a]	Dosage (Gy)[b]	Cultivar
dm1a	*Dm1*	γ	200	Saffier
dm1b	*Dm1*	FN	6.0	Diana
dm1c	*Dm1*	FN	7.5	Diana
dm1d	*Dm1*	FN	7.5	Diana
dm1e	*Dm1*	None		Kordaat × Calmar F_1
dm3a	*Dm3*	FN	4.5	Diana
dm3b	*Dm3*	FN	6.0	Diana
dm3c	*Dm3*	FN	6.0	Diana
dm3d	*Dm3*	FN	6.0	Diana
dm3e	*Dm3*	FN	4.5	Diana
dm3f	*Dm3*	FN	6.0	Diana
dm3g	*Dm3*	FN	6.0	Diana
dm3h	*Dm3*	FN	6.0	Diana
dm3i	*Dm3*	FN	7.5	Diana
dm58a	*Dm5/8*	FN	7.5	Diana
dm7a	*Dm7*	FN	6.0	Diana

[a] γ = γ-irradiation from a Co^{60} source; FN = fast neutron irradiation.
[b] 1 Gy = 100 rad absorbed dosage.

resistant/susceptible phenotypes were observed. The genetic backgrounds of the mutants were verified on the basis of molecular markers, morphological characteristics and their reaction to individual isolates of *B. lactucae.*

Mutations affecting *Dm* gene activity: Sixteen separate mutants showing loss of resistance to downy mildew were obtained from three different experiments (Table 3). Tests with single fungal isolates indicated that the action of only one *Dm* gene was affected in each of the putative *Dm* mutants. Four *Dm1* mutants (dm1a, 1b, 1c, 1d) were selected from M_2 families derived from γ- or fast neutron-irradiated seed. A fifth mutant lacking *Dm1* function was obtained from the screen of 3,000 F_1 individuals that were normally heterozygous for six *Dm* genes. This mutant, designated dm1e, was considered to be spontaneous because no mutagenic treatment had been given prior to screening. A total of nine *Dm3* mutants (dm3a to dm3i), one *Dm5/8* mutant (dm58a) and one *Dm7* mutant (dm7a) were selected from 2,211 M_2 families derived from fast neutron-irradiated seed. No *Dm16* mutants were obtained from the 1,222 γ-irradiated M_2 families.

As an indicator of general mutation rates, M_2 families showing segregation for pale green, yellow or white seedlings were also noted. From the γ-irradiated treatment, ten putative chlorophyll or carotenoid pigment mutants and one *Dm* mutant were detected. The fast neutron treatments resulted in 88 M_2 families deficient in pigmentation and 14 *Dm* mutants. Fertile M_2 *Dm* mutants were obtained at each of the three fast neutron dosage levels, although not all mutants were fully fertile. Frequencies of putative *Dm* and pigmentation mutations in cv. Diana were directly correlated to the level of absorbed dosage of fast neutrons (Table 4), indicating that fast neutron bombardment of lettuce seeds was an effective means of mutagenesis and that the loss of *Dm* activity was related to irradiation. *Dm* gene inactivation was observed in approximately one in every 150 M_2 families, while the frequency of pigmentation mutations was sixfold higher. The relative frequency of mutagenesis of single-copy target genes to pigmentation genes in lettuce was similar to that observed in *Arabidopsis thaliana.* In contrast to populations derived from irradiated seed, only one *Dm* mutant was obtained from approximately 3,000 untreated Kordaat × Calmar F_1 individuals and no chlorotic mutants were detected in this experiment.

Single-locus, recessive nature of *Dm* mutations: Classical genetic analyses indicated that susceptibility in all fast neutron-treated mutants of Diana was a recessive trait segregating at a single locus (Table 5). In the M_2 generation, χ^2 values showed no significant deviation from the 3:1 ratio of resistant to susceptible seedlings expected for single, recessive mutations. A total of 50–80 M_3 seedlings from two different susceptible individuals representing each M_2 family were susceptible to the appropriate fungal isolates, as expected for homozyous recessive mutations. The one apparently resistant dm3a seedling was likely to have been an escape in the screen. The number of dm1c M_2 progeny analyzed was limited by the scarcity of available seed for this family. Due to reduced fertility of dm1c, dm1d and dm3g M_2 individuals, M_3 populations could not be obtained for further analysis.

TABLE 4

Mutation frequency in fast neutron-treated M_2 families

	FN dosage (Gy)			
	4.5	6.0	7.5	Total
No. of families screened	789	916	506	2,211
No. of independent *Dm* mutations	2	8	5	15
Frequency of *Dm* mutations	0.0025	0.0087	0.0099	0.0068
No. of chlorotic mutations	19	37	32	88
Frequency of chlorotic mutations	0.024	0.040	0.063	0.040

TABLE 5

Segregation for resistance in M_2 and M_3 generations of *Dm* mutants

	M_2 generation			M_3 generation[a]	
Mutant	R[b]	S	$\chi^2_{3:1}$	R	S
dm1a				0	67
dm1b	22	5	0.6	0	64
dm1c	9	4	0.2	Not tested	
dm1d	42	7	3.0	Not tested	
dm3a	29	8	0.23	1	72
dm3b	19	8	0.31	0	71
dm3c	30	7	0.62	0	71
dm3d	24	8	0.0	0	76
dm3e	27	5	1.50	0	70
dm3f	27	8	0.08	0	80
dm3g	22	3	2.25	Not tested	
dm3h	24	8	0.0	0	82
dm3i	22	6	0.19	0	74
dm58a	22	7	0.01	0	47
dm7a	31	8	0.41	0	50

[a] Reactions of M_3 progeny from susceptible M_2 plants.
[b] R = number of resistant seedlings; S = number of susceptible seedlings; not tested = M_3 progeny not available for analysis.

TABLE 6

Complementation tests for *Dm1* and *Dm3* mutants

	F_1 progeny[b]	
Mutants crossed[a]	Resistant	Susceptible
dm1a × dm1b	0	43
dm1b × dm1d	0	2[c]
dm1b × dm1e	0	20
dm3c × dm3e	0	13
dm3d × dm3e	0	16
dm3d × dm3f	0	23
dm3e × dm3f	0	13
dm3h × dm3c	0	12
dm3h × dm3d	0	20
dm3h × dm3e	0	18
dm3h × dm3f	0	16
dm3i × dm3c	0	5
dm3i × dm3d	0	9
dm3i × dm3e	0	13
dm3i × dm3f	0	11
dm3i × dm3h	0	28

[a] Average rate of crossing was 53%.
[b] Data from reciprocal crosses were combined.
[c] + indicates susceptibility was obtained on leaf disks of F_1 individuals.

Spontaneous mutants could be detected directly in the F_1 population, as normally all *Dm* genes would be heterozygous. A mutation in the single copy of any *Dm* gene or a heterozygous epistatic locus would result in susceptibility and the F_2 generation would be entirely susceptible. As expected for a mutation at *Dm1*, F_2 and F_3 seedlings of dm1e were susceptible to C83 M47.

Genetic tests for allelism and epistasis: Allelism tests were carried out to determine whether *Dm* function could be restored by crossing two independent mutants, both showing susceptibility to the same fungal isolate (Table 6). Reciprocal crosses were done whenever possible. F_1 progeny from crosses between the *Dm1* mutants were tested for resistance to C83M47 diagnostic for *Dm1*. Lack of resistance in the progeny indicated that the lesion in dm1b was not complemented by crossing to either dm1a, dm1d or dm1e. All F_1 progeny from crosses made between the *Dm3* mutants were susceptible to *B. lactucae* diagnostic for *Dm3*. To date, the allelism tests provide no evidence for intergenic complementation. Therefore, lesions have occurred at the same locus in all *Dm1* mutants tested and at the same locus in the *Dm3* mutants.

To determine whether the loss of *Dm* function could be restored in a different genetic background, several *Dm* mutants were crossed to Cobham Green, a closely related cultivar that contains no known resistance genes. If the mutation had occurred in an epistatic locus rather than in a *Dm* gene, then Cobham Green should provide a wild type epistatic allele in the F_1 progeny. Resistance was not restored by crossing to Cobham Green; leaf disks of F_1 progeny from dm1a, dm7a and four independent *Dm3* mutants (dm3a, dm3c, dm3d and dm3f) were susceptible to the appropriate isolates. The combined data from the epistasis and allelism tests provide evidence for lesions at the *Dm* loci rather than at epistatic loci for all the *Dm1*, *Dm3* and *Dm7* mutants tested; we have no data for *Dm5/8* because this mutant was obtained late in the study and suitable parents were not available.

Evidence for deletions in *Dm* mutants: The five *Dm1* mutants were analyzed for RAPD markers in the *Dm1* region (Table 7, Figure 1). Two fast neutron-irradiated

TABLE 7

Genotypes of *Dm1* mutants

Mutant	Molecular marker			
	OPA07	*OPAB16*	*OPB19*	*OPV02*
Diana	+	+	+	+
dm1a	+	+	+	NT
dm1b	+	+	+	+
dm1c	+	−	−	+
dm1d	+	−	−	+
dm1e	+	+	+	NT

+ = present; − = absent; NT = not tested.

Dm1 mutants, dm1c and dm1d, were lacking the RAPD-based markers $OPAB16_{1700}$ and $OPB19_{1200}$. $OPAB16_{1700}$ cosegregated with *Dm1* in a mapping population of 245 F_2 Calmar × Kordaat individuals. $OPB19_{1200}$ was identified in a screen using the *Dm* mutants; it was not polymorphic between Calmar and Kordaat, and therefore could not be mapped. Both $OPAB16_{1700}$ and $OPB19_{1200}$ were present in dm1a, dm1b and dm1e. Flanking markers, $OPA07_{370}$ and $OPV02_{2030}$, were present in all *Dm1* mutants tested.

Eight putative *Dm3* mutants were analyzed for nine RAPD, one SCAR and two RFLP markers (Table 8). Each *Dm3* mutant lacked at least one marker; dm3f lacked five markers. All markers that were missing in at least one mutant were tightly linked to *Dm3*; all absolutely cosegregated with *Dm3* in a mapping population of 245 F_2 progeny (P. ANDERSON, unpublished data). $OPR14_{860}$ and *CL1795h14* were present in all of the mutants and therefore delineated genetic boundaries of the deletions at approximately 1.8 and 2 cM, respectively.

Additional evidence that *Dm3* mutants harbored deletions was provided by Southern blot analysis of high molecular weight genomic DNA. Hybridization of cloned $OPK13_{1700}$ to DNA from dm3d and dm3f is shown in Figure 2. In dm3d, there was a change in the size of an *FspI* restriction fragment from approximately 1800 kb (in wild-type cv. Diana) to 900 kb (in dm3d). No detectable hybridization of $OPK13_{1700}$ to dm3f was observed. Hybridization of this blot to two other marker probes, *CL922* and $SCI11_{1200}$ (data not shown), indicated that dm3d and dm3f were not detectably different from wild-type Diana in regions flanking *Dm3* (Figure 1). Fragments detected with $OPK13_{1700}$ were either modified or deleted in all the other *Dm3* mutants (except dm3g which could not be analyzed). Detailed analysis of these mutants for physical changes in the *Dm1* and *Dm3* regions is in progress.

Characterization of spontaneous mutant: The single spontaneous mutant, dm1e, had lost the *Dm1* phenotype and did not complement one of the *Dm1* fast neutron mutants, dm1b in allelism tests. All markers linked to *Dm1* were present in dm1e; therefore there was no evidence of a deletion caused by unequal crossing over. The nature of the dm1e mutation will be of particular interest when the structure of the functional *Dm1* allele is known.

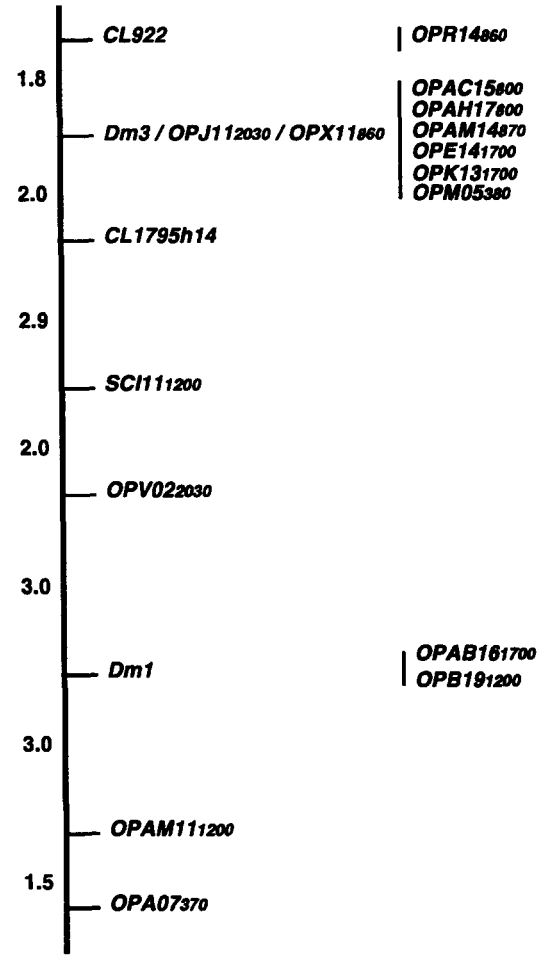

FIGURE 1.—Genetic map of the *Dm1/Dm3* region. RAPD markers are indicated by the prefix OP. SC indicates a SCAR marker amplified with specific 24-base primers. RFLP markers are designated CL. Genetic distances are given in centiMorgans. With the exception of $OPB19_{1200}$ (see text), the markers have been placed by classical genetic mapping (KESSELI *et al.* 1994; D. LAVELLE, P. ANDERSON and P. OKUBARA, unpublished data). The linkage map was constructed in part using Mapmaker, version 2.0 (LANDER *et al.* 1987).

Additional aberrations in mutants: In addition to loss of downy mildew resistance, several mutants exhibited marked differences in fertility and morphology with respect to wild type Diana. Some but not all the dm1b mutants had pale yellow rather than bright yellow flowers, indicating the presence of an independent mutation in flower color. Two other *Dm1* mutants, dm1c and dm1d, showed extreme fasciation, stunting, lack of apical dominance, and severely reduced pollen production; floral organs were often fused, resulting in reduced fertility. These mutants could not be selfed and had to be rescued by outcrossing. All susceptible plants in the two mutant families displayed identical abnormal morphology; therefore, this mutant phenotype was genetically linked to *Dm1*.

TABLE 8

Genotypes of *Dm3* mutants

Mutant	Molecular marker											
	CL922	*OPR14*	*OPJ11*	*OPX11*	*OPAC15*	*OPAH17*	*OPAM14*	*OPE14*	OPM05	*OPK13*	*CL1795*	*SCI11*
Diana	+	+	+	+	+	+	+	+	+	+	+	+
dm3a	+	+	+	+	+	+	−	−	−	+	+	+
dm3b	+	+	+	+	−	−	+	+	+	−	+	+
dm3c	+	+	+	+	+	+	−	+	−	+	+	+
dm3d	+	+	+	+	+	+	+	+	−	+	+	+
dm3e	+	+	+	+	−	−	+	+	−	−	+	+
dm3f	+	+	+	+	−	−	−	+	−	−	+	+
dm3h	+	+	+	+	+	−	+	+	+	−	+	+
dm3i	+	+	+	+	+	−	−	+	−	−	+	+

+ = present; − = absent.

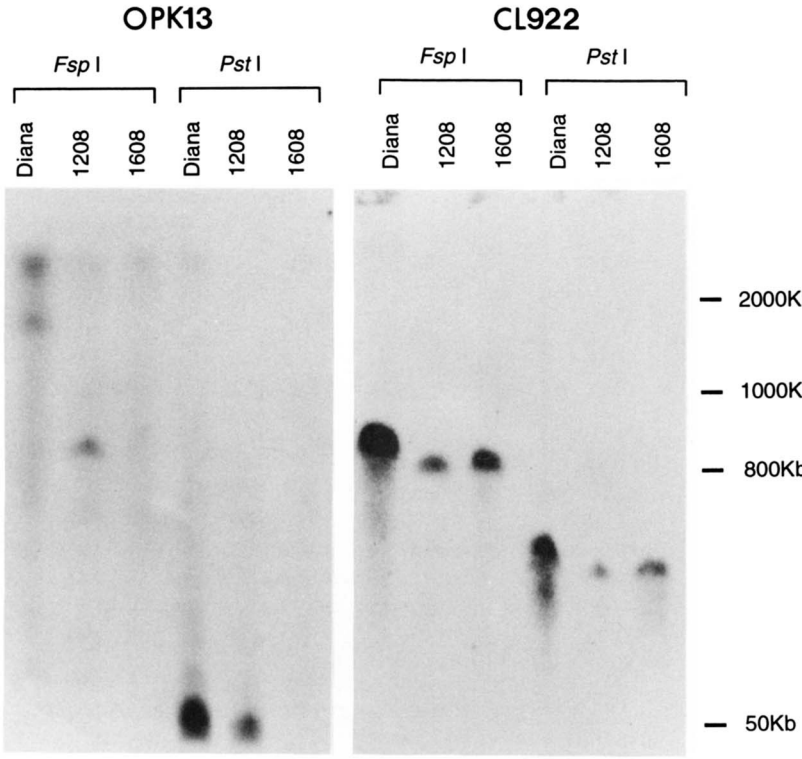

FIGURE 2.—Southern blot analysis of high molecular weight genomic DNA from dm3d and dm3f probed for *OPK13* and *CL922* sequences. DNA was fractionated by pulsed-field gel electrophoresis, transferred to nylon membrane, then probed with a 1200-base pair fragment derived from $OPK13_{1700}$ and then an 800-base pair cDNA clone of *CL922*. The specific activities of the probes were approximately 2×10^9 cpm/μg DNA. The autoradiograph was exposed at −80 ° for 4 days.

In several dm3e mutants, the surfaces of the cotyledons and leaves had a ribbed, crinkled appearance. This abnormal trait was always accompanied by early bolting, but both phenotypes segregated independently of susceptibility to downy mildew. The dm3g family segregated for chlorosis and arrested development as well as for susceptibility. The two phenotypes appeared to be inherited independently as some resistant seedlings were chlorotic; however, in the limited seed available, all six susceptible seedlings were also chlorotic. These mutants could not be rescued as development did not proceed beyond the cotyledon stage. Another family that exhibited the same phenotypes as dm3g, contained seedlings susceptible to the pool of isolates; however, the *Dm* gene lesion(s) were not determined for this family due to the limited number of M_2 seed and the failure of susceptible seedlings to develop beyond the cotyledon stage.

DISCUSSION

A total of 16 independent mutations affecting four different *Dm* gene activities were obtained from several populations of lettuce seeds. Analysis of lettuce seedlings for *Dm* mutations was rapid and unambiguous. Resistance was readily distinguished from susceptibility. In contrast to *RPS2* (YU *et al.* 1993; KUNKEL *et al.* 1993) and *RPP5*, a gene from Arabidopsis for resistance to the fungus *Peronospora parasitica* (PARKER *et al.* 1993), no intermediate resistance phenotypes were observed for the *Dm* genes. The majority of the mutants appeared to be induced by the fast neutron treatment because of the dosage dependence of the mutation rate and because

the mutation frequencies in the irradiated families were higher than those observed in the untreated F_1 population. The possibilities of seed contamination were excluded by analysis of molecular markers, morphological characteristics and their reaction to individual isolates of *B. lactucae.*

Mutations influencing the action of more than one *Dm* gene were not obtained. There are several possibilities that can account for the absence of such mutations. The resistance phenotype could be conferred solely by the *Dm* gene product and therefore such epistatic loci do not exist. Alternatively, if there is more than one step in the pathway determining resistance, as is likely, components in the pathway may be encoded by multigene families, or there may be duplicated pathways. In both cases, lesions in such genes would not have detectable phenotypes in our screen. Another possibility is that an epistatic locus might encode a product which is critical to survival of the plant, so that a mutation would be lethal when homozygous.

We recovered mutations in each of the *Dm* genes at different frequencies. Among the fast neutron-irradiated families, 9 of the 14 *Dm* mutations affected *Dm3* action, while only three *Dm1* and one each of *Dm5/8* and *Dm7* mutations were obtained. The significance of the higher frequency of *Dm3* mutations is unknown. The *Dm3* locus could either be more sensitive to fast neutron irradiation, consist of multiple tightly linked genes (all necessary for the *Dm3* phenotype), or consist of a single gene distributed over a large region of the chromosome. Alternatively, the lower frequencies of deletions at *Dm1*, *Dm5/8* and *Dm7* could be due to the proximity of these *Dm* genes to loci critical for plant growth or development. The absence of severe mutant phenotypes accompanying loss of *Dm3* activity suggested that no important developmental genes were linked to *Dm3*. In contrast, for two mutants at *Dm1*, dm1c and dm1d, loss of resistance was accompanied by similar abnormal developmental phenotypes that resulted in sterility. These were the only confirmed deletion mutants at *Dm1* because they lacked two flanking molecular markers. Therefore, both *Dm1* and gene(s) critical to normal plant development may have been deleted. We obtained too few mutants at *Dm5/8* and *Dm7* to be able to infer any linkage to genes important for viability.

Three types of genetic analysis indicated that loss of function mutations had occurred at individual *Dm* loci. Segregation of resistance in M_2 and M_3 populations indicated that the mutations are recessive and are at single loci. Allelism tests demonstrated that mutations had occurred at one locus in four of the *Dm1* mutants and at a single locus in six of the *Dm3* mutants. In tests for epistatsis, loss of resistance in dm1b, dm7a and four different *Dm3* mutants was not complemented in Cobham Green hybrids and therefore second site mutations were not involved in loss of *Dm* gene function. We have insufficient genetic data for dm58a; further mapping is required to place the lesion relative to *Dm5/8.*

Analyses with molecular markers also indicated that mutations had occurred at *Dm1* or *Dm3*. At least one closely linked molecular marker was missing in two of the *Dm1* mutants and in eight *Dm3* mutants, suggesting that the *Dm* genes had undergone deletions or local rearrangements. Furthermore, high molecular weight genomic DNA fragments that hybridized to cloned RAPD marker $OPK13_{1700}$ in wild-type cv. Diana was either absent or altered in the *Dm3* mutants. Small rearrangments, deletions or both could account for the apparent truncation of *OPK13*-hybridizing sequences. Deletions and rearrangments have been identified in Arabidopsis after fast neutron bombardment (SHIRLEY *et al.* 1992).

While the genetic and marker data indicate that the lesions occurred at a single locus, they do not preclude the possibility that several closely linked genes are involved in the resistance phenotype and that the deletions involved more than one gene. The allelism tests did not provide any evidence of multiple genes. However, the likelihood of intergenic complementation is reduced as the majority of mutants used in the allelism tests were deletions.

The physical proximity of the flanking markers to *Dm3* is unknown. $OPJ11_{2030}$ and $OPX11_{860}$ cosegregate with *Dm3* in genetic studies but are present in all of the mutants. Other markers that also cosegregate with *Dm3* are missing in one or more mutants. However, no marker is missing in all the mutants. Additional genetic recombinants as well as a long-range restriction map of the region are being analyzed to place these markers relative to *Dm3*, prior to initiating a chromosome walk to clone the gene.

We are now using these mutants in several ways to aid in cloning *Dm* genes. A panel of mutants is being screened to identify more markers linked to each of the four *Dm* genes. We are searching for RAPD markers and mRNA sequences (LIANG and PARDEE 1992) that are present in wild-type Diana but are absent in the mutants. We are not, however, pursuing subtractive approaches (STRAUS and AUSUBEL 1990; LISITSYN *et al.* 1993) with these deletion mutants because genetic data and genomic Southern analyses of markers in the *Dm* regions suggest that there is considerable sequence duplication within and between clusters of *Dm* genes (PARAN *et al.* 1992; P. ANDERSON and P. OKUBARA, unpublished results). Our studies are now focused on *Dm3* because this locus has provided the majority of the deletion mutants and has the greatest number of cosegregating markers. A detailed long-range restriction map is being constructed around *Dm3*. The deletion breakpoints in the mutants will help position *Dm3* on the physical and genetic maps.

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