A Genetic Map of Lettuce (*Lactuca sativa* L.) With Restriction Fragment Length Polymorphism, Isozyme, Disease Resistance and Morphological Markers

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ABSTRACT

A detailed linkage map of lettuce was constructed using 53 genetic markers including 41 restriction fragment length polymorphism (RFLP) loci, five downy mildew resistance genes, four isozyme loci and three morphological markers. The genetic markers were distributed into nine linkage groups and cover 404 cM which may be 25–30% of the lettuce genome. The majority (31 of 34) of the RFLP probes detected single segregating loci, although seven of these may have been homologous to further monomorphic loci. When several loci were detected by a single probe, the loci were generally linked, suggesting tandem duplications. One probe, however, detected loci in three linkage groups suggesting translocations. The five downy mildew resistance genes (*Dm1*, *Dm3*, *Dm4*, *Dm5/8* and *Dm13*), segregating in the Calmar × Kordaat cross, represented each of the four resistance gene linkage groups. *Dm5/8* is flanked by two cDNA loci, each located 10 cM away. These flanking markers will be used to study the source of variation in downy mildew genes and are also part our strategy to clone resistance genes.

DETAILED genetic linkage maps are fundamental tools for studies on selection, identification and organization of plant genomes (TANKSLEY 1983; BECKMANN and SOLLER 1986; LANDRY and MICHELMORE 1987). Traditionally, a large number of segregating populations were required to develop a linkage map as only a limited number of loci segregated in each cross. Genetic markers were mainly morphological and more recently biochemical (isozyme) variants. In some extensively studied species, tester lines have been constructed from wide crosses to carry many markers (*e.g.*, TANKSLEY, MEDINA-FILHO and RICK 1982). For a few plant species, mapping has also been facilitated by using aneuploids [wheat, SEARS (1954)], chromosome substitution lines [tomato, RICK (1975)] or A/B translocations stocks [maize, ROMAN and ULLSTRUP (1951)]. The lack of genetic markers and the difficulties in developing tester lines has hindered the construction of linkage maps in many important plant species. Detailed genetic maps exist only for a few crop plants (KING 1975; O'BRIEN 1984; VLAMING *et al.* 1984; WEEDEN 1985). A new class of genetic markers, restriction fragment length polymorphisms (RFLPs), provides the opportunity to develop detailed genetic maps from a limited number of crosses. RFLPs are being used in maize and tomato to saturate their already extensive genetic maps (HELENTJARIS *et al.* 1986; BERNATZKY and TANKSLEY 1986b). RFLP analysis employs cloned sequences to probe specific regions of the genome for variations at the DNA level. These variations are seen as changes in the length of DNA fragments produced by digestion with restriction endonucleases. The segregation of many polymorphic RFLP markers can often be followed in a single cross. The different types and sources of probes as well as the different techniques to detect polymorphisms and the potential applications of RFLP analyses for plant improvement have been discussed previously (LANDRY and MICHELMORE 1987).

As lettuce lacks a linkage map, RFLP analysis of a single cross was used as the most efficient method to generate a detailed map. Our interest lies primarily in obtaining genetic markers which flank genes for resistance to lettuce downy mildew caused by *Bremia lactucae* (HULBERT and MICHELMORE 1985). These markers will be used to study sources of variation in downy mildew genes. Flanking genetic markers are also part our strategy to clone resistance genes as they will be used to confirm putative insertional inactivation by cloned transposable elements. The construction of a linkage map using RFLPs has three phases: (1) development of probes (LANDRY and MICHELMORE 1985), (2) choice of parental lines and identification of polymorphic probes (LANDRY 1987) and (3) segregation analysis. The segregation analysis of RFLP markers is facilitated by: (1) generally, the codominance of alleles, (2) the frequent polymorphism, (3) the minimal pleiotropic effect of individual markers

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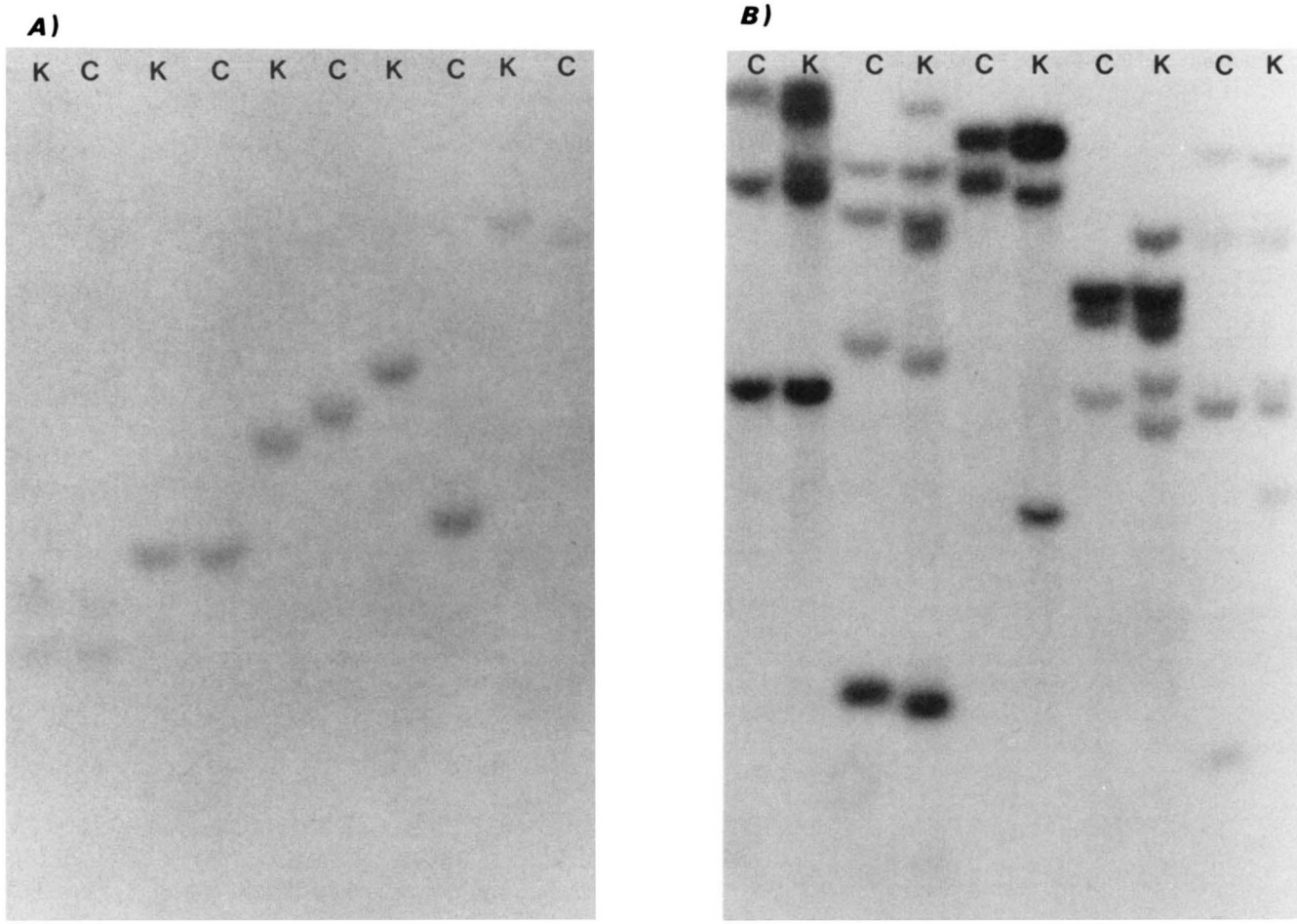

FIGURE 1.—Hybridization patterns of two types of cDNA probes detected by autoradiography. Southern blots of genomic DNA of lettuce cultivars Calmar (C) and Kordaat (K), digested as pairs with *Bam*HI, *Eco*RI, *Eco*RV, *Hin*dIII and *Msp*I were probed with: (A) cDNA probe, CL673, homologous to a single locus and (B) cDNA probe, CL573, homologous to multiple loci.

and (4) the absence or limited influence of the environment (BOTSTEIN *et al.* 1980; BECKMAN and SOLLER 1983).

MATERIALS AND METHODS

Isolation of DNA, restriction endonuclease digestions and Southern blotting: Procedures for the preparation of lettuce genomic DNA, digestions by restriction endonucleases, electrophoresis, Southern blotting, isolation of cloned lettuce inserts for ^{32}P-labeling by nick-translation and hybridizations were performed as described earlier (LANDRY and MICHELMORE 1985; LANDRY 1987).

Probe sources and nomenclature: Two sources of probes were used. Procedures for the isolation and characterization of single/low copy genomic DNA (LANDRY and MICHELMORE 1985) and cDNA (LANDRY 1987) probes have been described. Further polymorphic probes were identified using five restriction endonucleases, *Bam*HI, *Eco*RI, *Eco*RV, *Hin*dIII (Bethesda Research Laboratories) and *Msp*I (Pharmacia Laboratories) to digest genomic DNA from the parental lines, cvs. Calmar and Kordaat. Replicated Southern blots of these digests were hybridized with inserts from lettuce cDNA clones (Figure 1). Segregating loci are listed in Table 1. Loci detected by cDNA probes were identified as, for example, *CL162*. Similarly, the locus detected by a genomic DNA probe was identified as *GL358*. When a single probe detected several loci, individual loci were designated by the same number but distinguished by a lower case letter (*e.g.*, *CL573a*, *CL573b*); the alphabetical order corresponded to the decreasing size of the segregating fragment for each locus (*i.e.*, $a > b > c > d$) in the restriction endonuclease digest used for mapping the loci. Downy mildew resistance, isozyme and morphological loci were identified as described previously (ROBINSON, MCCREIGHT and RYDER 1983; KESSELI and MICHELMORE 1986; FARRARA and MICHELMORE 1987). Loci *Cts* and *Ctc* were new morphological markers and corresponded to the shape (round *vs.* oblong) and the color of the cotyledons (dark *vs.* light green), respectively (dominant phenotype given first).

Lettuce cross and segregation analysis: Cultivars Calmar and Kordaat were obtained from the UCD lettuce germ plasm collection and crossed with Calmar as the female parent. This cross was chosen for three reasons: (1) the cross would segregate for five Downy Mildew resistance genes located in four different linkage groups (HULBERT and MICHELMORE 1985; FARRARA and MICHELMORE 1987), (2) a relatively high number of isozyme loci (five) differentiate these two accessions (KESSELI and MICHELMORE 1986), and (3) these are cultivars of a single species. This is an intra specific cross which should minimize the genetic distortion and error encountered by other workers who have used wide crosses to establish genetic maps (HELENTJARIS *et al.* 1986). F_1 plants were self-pollinated to generate the F_2 population. A single F_2 population (66 plants) was used

TABLE 1

Segregations and chi-square goodness-of-fit tests for 41 RFLP, four isozyme, three morphological and five Downy Mildew resistance genes in a F_2 population derived from a single cross between Calmar and Kordaat

Locus name	Homozygous Calmar allele	Heterozygous	Homozygous Kordaat allele	χ^{2a}
CL162	14	28	14	0.00
CL201	12	33	11	1.82
CL202a	15	24	17	1.29
CL202b	10	32	14	1.71
CL202c	14	27	15	0.11
CL202d	13	24	19	2.43
CL205	13	34	9	3.14
CL206	12	22	22	6.14*
CL207	16	28	12	0.57
CL220	12	31	13	0.68
CL222	6	38	12	8.43*
CL242	8	41	16	6.41*
CL250	15	24	17	0.53
CL257	6	43	15	10.09**
CL258	10	39	16	3.71
CL284	9	40	16	4.97
CL502	9	30	17	2.57
CL505	15	32	18	0.29
CL514	10	40	15	4.23
CL516	7	26	7	3.60
CL517	14	31	20	1.25
CL527	14	36	15	0.78
CL531	9	43	13	7.28*
CL537	12	47	5	15.59***
CL545	7	43	15	8.75*
CL573a	13	39	13	2.60
CL573b	12	40	13	3.49
CL592	10	43	12	6.91*
CL608	16	33	16	0.02
CL613	— 45 ——		20	1.15
CL623	— 44 ——		21	1.85
CL635	12	39	14	2.72
CL643	10	40	15	4.23
CL658	19	31	15	0.63
CL662a	16	23	17	1.82
CL662b	15	24	17	1.29
CL662c	12	26	18	1.57
CL662d	12	25	19	2.39
CL670	8	37	20	5.67
CL673	22	29	14	2.72
GL358	— 43 ——		13	0.10
Adh3	16	26	16	0.62
Cts	21	23	17	4.21
Ctc	21	29	11	3.43
Dia3	18	23	21	4.42
Dm1	15	26	14	0.20
Dm3	18	20	22	7.20*
Dm4	14	28	14	0.00
Dm8	17	25	17	1.37
Dm13	9	15	5	1.14
Est6	13	18	15	2.35
Est8	14	26	9	1.20
w	— 49 ——		14	0.26

[a] Chi-square values greater than this would be expected by chance at probably (P): * $0.01 < P < 0.05$; ** $0.001 < P < 0.01$; *** $P < 0.001$. Chi-squares tested to 1:2:1 or 3:1 ratios.

because of the high genetic information per individual (ALLARD 1956) and the difficulty in producing backcross populations in lettuce. Genomic DNA was isolated from the mature leaves of F_2 plants grown in a greenhouse. Lettuce cannot be propagated vegetatively *in vivo*; therefore, F_3 populations from each F_2 plant were scored for isozyme and downy mildew resistance genes (a minimum of eight plants for each assay) according to previously described techniques (KESSELI and MICHELMORE 1986; HULBERT and MICHELMORE 1985). Morphological characters were also scored in the F_3 families.

Chi-square goodness-of-fit values for segregation and independent assortment ratios of all pairs of polymorphic loci and the maximum likelihood estimates of recombination frequency between linked loci were calculated on an IBM AT personal computer with the LINKAGE-1 program (SUITER, WENDEL and CASE 1983). This program was convenient for RFLP analyses as an unlimited number of individuals and loci (both dominant and codominant) could be analyzed; additional loci could be added and missing data were tolerated. Recombination values and their standard errors were obtained for all possible pairs of loci. The linkage map was deduced as the best fit to these values.

RESULTS

Several patterns of segregation were observed (Figure 2). Twenty-four single segregating loci displayed one or a few bands (Figure 2, A and B). Three loci (*GL358*, *CL613* and *CL623*) segregated for null alleles (*i.e.*, presence *vs.* absence of detectable bands). Two clones were homologous to four segregating loci (*CL202a,b,c* and *d* Figure 2C and *CL662a,b,c* and *d*); one clone was homologous to two loci (*CL573a* and *b*). Therefore, the majority (31 of 34) of these probes detected single segregating loci. Seven clones however segregated for single loci and also showed one or more additional monomorphic bands which may have corresponded to one or more additional loci. The F_2 segregation of most loci fitted the expected Mendelian ratios; significant deviations were, however, detected for eight RFLP loci and one downy mildew resistance gene (Table 1). These deviations were not biased towards alleles of one parent.

All but four loci were distributed into nine linkage groups (Figure 3); the linked markers delimit 404 cM of the lettuce genome. When several loci were detected by a single probe, the individual loci were generally linked (*e.g.*, *CL662a* and *b* and *CL573a* and *b*), suggesting tandem duplications. The five loci homologous to clone *CL202*, however, were found on linkage groups 2, 3 and 6 suggesting translocations. It is probably coincidental that the number of linkage groups identified in this study equaled the haploid chromosome number of *Lactuca sativa* ($n = 9$) since several of the shorter linkage groups may belong to the same chromosome.

For 66 plants, the standard errors on recombination frequencies (0.01 to 0.50) ranged from 0.009 to 0.092. With two codominant markers, recombination frequencies larger than 0.38 were not significantly

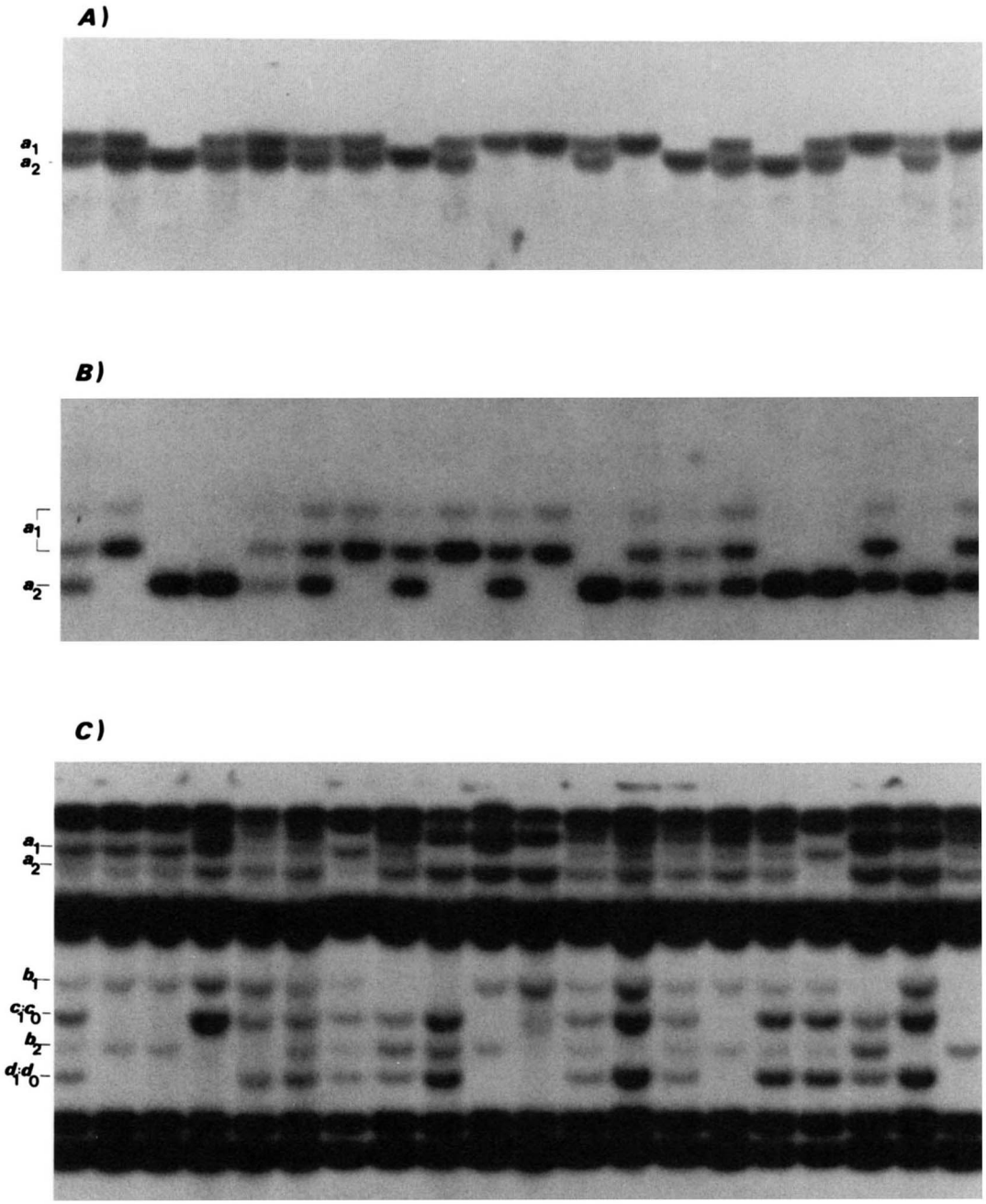

FIGURE 2.—Autoradiographs of RFLP segregation. Three patterns of RFLP segregation observed in Southern blots from the F_2 population of Calmar × Kordaat. The origin and negative pole are at the top for each. (A) Segregation at a single locus, *CL162*, with single banded alleles; (B) segregation at a single locus, *CL206*, with a single and a double banded allele; and (C) segregation at multiple loci detected by probe CL202; alleles of each locus are indicated in the left margin.

different from independent assortment; the significant recombination frequency decreased to 0.36, when one dominant marker and one codominant marker were analyzed, and to 0.35, when analyzing two dominant markers in coupling [calculated from ALLARD (1956)]. For some loci, less than 66 plants were analyzed because certain individuals could not be scored unambiguously, further decreasing the significant recombination frequencies. Most loci showed multiple point linkages to other members of the linkage groups. For four loci, linkages could not be confirmed by multiple point analyses due to the lack of nearby loci. These unconfirmed linkages are denoted by dashed lines in our map and need verification (Figure 3). Linkage values are only estimates. Since the standard error of recombination is a function of the sample size and the recombination value, more accurate values will be obtained as we add markers to our map and as these markers are used in other crosses.

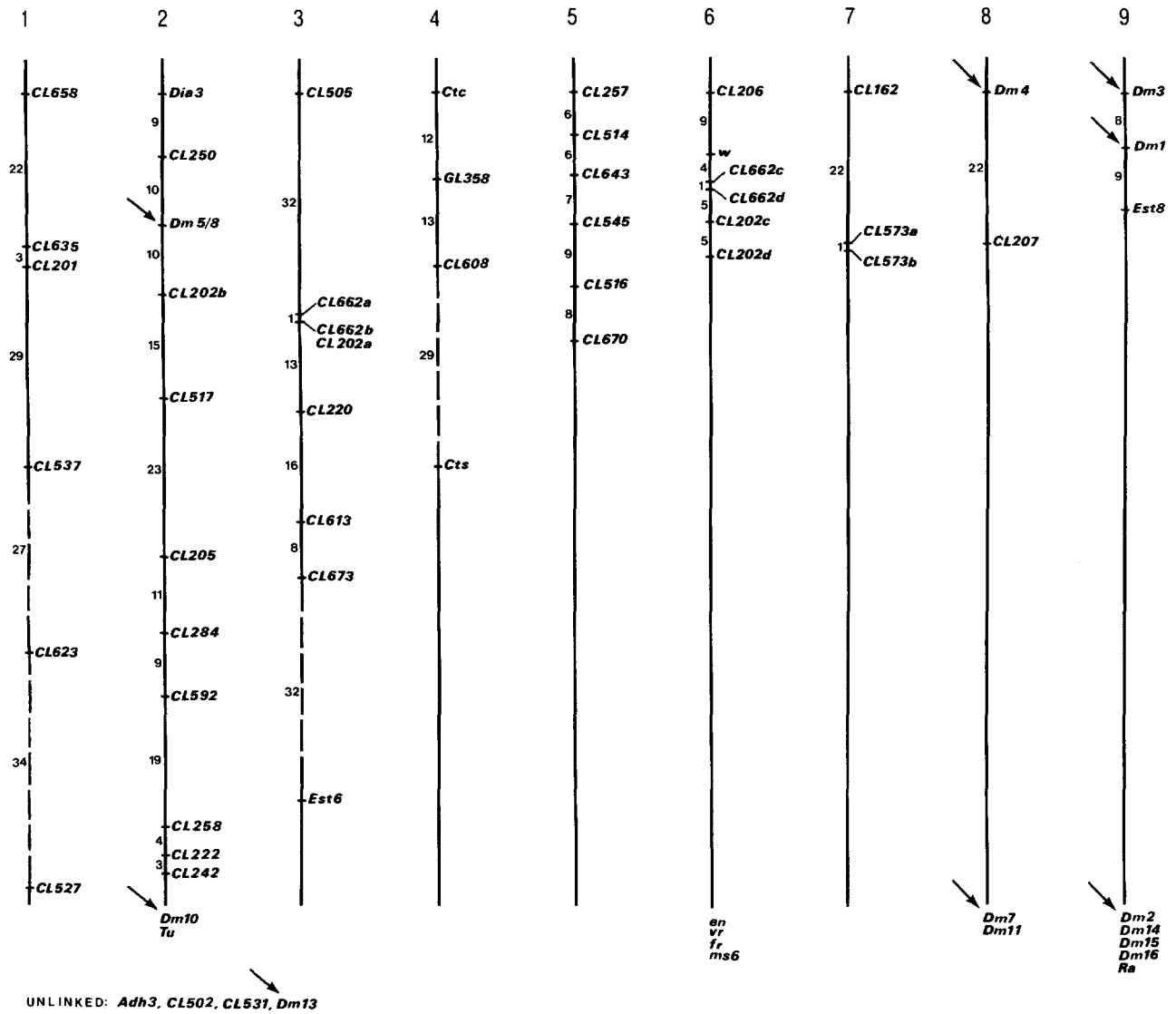

FIGURE 3.—Linkage map of lettuce. The nine linkage groups are listed at the top. Loci are listed on the right. Recombination frequencies are listed on the left; these are estimates with standard errors ranging from 0.01 to 0.09 (see text). Linkage which could not be confirmed by multipoint analysis are shown as dashed lines and are subject to confirmation. Genes for resistance to downy mildew (Dm) are arrowed. *Adh3*, *CL502*, *CL531* and *Dm13* are unlinked. Markers listed at the bottom of some linkage groups have been shown to be linked to one of the segregating markers but their positions are unknown (ZINK and DUFFUS 1973; RYDER 1975, 1983; HULBERT and MICHELMORE 1985; FARRARA and MICHELMORE 1987).

DISCUSSION

We selected the cross, Calmar × Kordaat, as the source of the segregating population to construct a genetic linkage map of lettuce because of the adequate frequency of RFLPs and isozyme markers (KESSELI and MICHELMORE 1986; LANDRY *et al.* 1987) and the segregation of loci representing all four linkage groups of downy mildew resistance genes (FARRARA and MICHELMORE 1987). cDNA probes were used as the main source of probes for mapping as they detected 2.5 times more polymorphism than random genomic DNA probes enriched for single/low copy sequences (LANDRY *et al.* 1987). We also selected the restriction endonucleases *Bam*HI, *Eco*RI, *Eco*RV and *Hin*dIII as being efficient in displaying polymorphisms and strong hybridization signals. In our initial studies, *Msp*I did not display frequent polymorphisms; we continued to use it because of its frequent application and high efficiency in studies in humans (BARKER and WHITE 1983). However, polymorphism was detected solely in digests with *Msp*I for only 4 out of 350 additional lettuce cDNA clones. Our results parallel observations in maize and tomato where *Msp*I did not display more polymorphism than other enzymes (HELENTJARIS *et al.* 1985). Therefore, only *Bam*HI, *Eco*RI, *Eco*RV and *Hin*dIII will be used for future RFLP analyses of lettuce.

The distances between markers represent the best fit of all overlapping pairs of loci (Figure 3). In all three-point analyses the overlapping recombination

frequencies were within the standard errors of the individual recombination frequencies. Distances are given in recombination frequencies but will be transformed using a mapping function (KOSAMBI 1944), once the number of markers approaches saturation and when most map distances can be confirmed by three-point analysis. The 49 linked markers delimited 404 cM which is 25–30% of the lettuce genome if its size is similar to maize and tomato. In maize, 112 markers covered 651 cM (HELENTJARIS, WEBER and WRIGHT 1986). In tomato, 112 markers covered 760 cM (BERNATZKY and TANKSLEY 1986b) and 104 markers covered 506 cM (HELENTJARIS *et al.* 1986). The largest lettuce linkage group covered 115 cM and may represent the majority of a chromosome since the largest chromosomes in tomato and maize are *ca.* 130 cM (BERNATZKY and TANKSLEY 1986b; HELENTJARIS *et al.* 1986).

Individual members of two of the three multigene families, *CL662a* and *b* and *CL573a* and *b*, were tightly clustered with each other. This may reflect either functional clustering or recent tandem duplication events. One pair of duplicated loci, *CL662a* and *b*, on linkage group 3 seems to have been translocated to or from linkage group 6, *CL662c* and *d*. This translocation, however, must have been smaller than 10 cM since it is flanked by *w* and *CL202c*. In pea, clustering for members of gene families has been found for the small subunit of ribulose-1,5-bisphosphate carboxylase (*rbcS*) and chlorophyll *a/b* binding protein (*cab*) gene families (POLANS, WEEDEN and THOMPSON 1985). In tomato, however, a dispersed pattern characterized the distribution of most members of multiple copy cDNA clones (BERNATZKY and TANKSLEY 1986a,b).

The genotypes of RFLP loci can usually be determined directly; however, there was dominance for loci with null alleles. Consequently, the hemizygote was combined with the homozygous hybridizing class. If hybridization bands for other loci were present, however, heterozygotes for null alleles could be scored (*e.g.*, *CL202c* and *d*, Figure 2C), since the other bands provided a reference for hybridization intensity.

The five downy mildew resistance genes segregating in the Calmar × Kordaat cross represented each of the four resistance gene linkage groups (HULBERT and MICHELMORE 1985; FARRARA and MICHELMORE 1987). *Dm5/8* is linked by two cDNA loci, *CL250* and *CL202b*, each located 10 ± 3.1 cM away. Other crosses located *Dm10 ca.* 4 cM from *Dm5/8* and, therefore, *DM10* is *ca.* 6 cM from either *CL250* or *CL202b* (B. FARRARA and R. W. MICHELMORE, unpublished data). When RFLPs linked to the other downy mildew genes are detected, we will test whether regions of the genome containing resistance genes are more variable than other regions across a diverse group of 20 accessions within *Lactuca* spp.

A saturated linkage map will allow us to test whether transcribed loci are clustered by comparing the distribution of regions homologous to cDNA probes with those homologous to random genomic DNA probes. If cDNA probes are clustered, they may be more useful than random genomic DNA sequences for tagging genes for disease and physiological stress resistance.

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