Recessive Resistance to *Plasmopara lactucae-radicis* Maps by Bulked Segregant Analysis to a Cluster of Dominant Disease Resistance Genes in Lettuce

Rick Kesseli,[1,2] Hanneke Witsenboer,[1] Mike Stanghellini,[3] George Vandermark,[3,4] and Richard Michelmore[1]

[1]Department of Vegetable Crops, University of California, Davis 95616 U.S.A.; [2]Biology Department, University of Massachusetts, Boston 02125 U.S.A.; [3]Department of Plant Pathology, University of Arizona, Tucson 85721 U.S.A.; [4]Agritope Inc., Beaverton, OR 97005 U.S.A.

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The recessive gene (*plr*) for resistance in lettuce to the fungus *Plasmopara lactucae-radicis*, was mapped to a cluster of genes for resistance to taxonomically diverse pathogens. Four RAPD (random amplified polymorphic DNA) loci linked to *plr* were identified by bulked segregant analysis of a previously unanalyzed F_2 population segregating for *plr*. Two of these RAPD loci had been previously mapped in a second cross and therefore identified a region of many other loci potentially linked to *plr*. RAPD loci in the region of *plr* were mapped using F_3 families. Informative F_3 families that showed a recombination event in the region were subsequently scored for resistance to *P. lactucae-radicis* to locate *plr* on the genetic map. Ambiguities regarding the precise position of *plr* caused by dominance or misscorings were resolved by scoring individuals of selected F_3 families to define the genotype of the progenitor plants of the F_2 population. The *plr* gene and three other disease resistance genes (downy mildew resistance, *Dm5/8* and *Dm10*, and turnip mosaic virus resistance, *Tu*) are all located on a segment of 12–18 cM. This strategy of quickly identifying linked loci by bulk segregant analysis of the F_2 population followed by precise mapping of the gene with F_3 families proved efficient and accurate. The growing number of disease resistance genes that map into three clusters in lettuce supports the hypothesis that resistance genes are members of multigene families whose members have a common function and origin. The presence of other multigene families associated with these resistance gene clusters suggests that duplications and rearrangements involving these regions may be common.

Additional keyword: Lactuca sativa.

The relative position of specific genes along chromosomes may indicate functional or evolutionary relationships and origins. Genetic studies in several plant species have shown that disease resistance genes are often clustered as multiallelic series at a locus or as multiple linked loci. In flax, 29 genes for resistance to rust (*Melampsora lini*) are located in six clusters (Shepherd and Mayo 1972; Mayo and Shepherd 1980; Islam *et al.* 1989). In maize, resistance genes to rust (*Puccinia sorghi*) are located in five clusters (Hooker and Saxena 1971; Saxena and Hooker 1974). Fine structure maps of the short arm of chromosome 10 indicate that there are tight clusters with many loci within 1 cM of each other (*Rp1* region) and others (*Rp5* and *Rp6*) at slightly greater distances of 2 or 3 cM (Bennetzen *et al.* 1991; Hulbert and Bennetzen 1991). Other examples include resistance to *Clavosporium fulvum* and *Meloidogyne incognita* in tomato (Dickinson *et al.* 1993) and powdery mildew (*Erysiphe graminis* f. sp. *hordei*) in barley (Wise and Ellingboe 1985). In *Lactuca sativa L.* 13 genes for resistance to downy mildew (*Bremia lactucae*) have been mapped to four clusters (Hulbert and Michelmore 1985; Farrara *et al.* 1987). These were located with classical approaches using many crosses each segregating for a few loci. We used molecular markers to develop detailed genetic maps of *L. sativa* and located these clusters of resistance genes to various regions of the genome (Kesseli *et al.* 1990, 1992b). The largest of these contains seven genes for downy mildew resistance (*Dm*; Hulbert and Michelmore 1985) and root aphid resistance (*Ra*; Crute and Dunn 1980) covering approximately 25 cM on linkage group 2 of our most recent map (Kesseli *et al.* 1992b). The second cluster contains *Dm5/8, Dm10* (Hulbert and Michelmore 1985), and resistance to turnip mosaic virus (*Tu*; Zink and Duffus 1973) and covers approximately 20 cM on linkage group 1. The third contains three tightly linked genes or allelic compliments at two or more loci (*Dm4*, *Dm7*, and *Dm11*) on linkage group 4 (Hulbert and Michelmore 1985; and unpublished data). One other downy mildew resistance gene (*Dm13*) has been precisely placed, and a few genes for resistance to other pathogens have been tentatively placed at other unlinked regions of the genome (Kesseli *et al.* 1992a). The close physical relationship of these genes, which bestow resistance to a variety of pathogens, has led to the hypothesis that they may be members of multigene families that have a common origin and be functionally related though encoding different specificities (Michelmore *et al.* 1987; Pryor 1987).

Placement of a gene on the genetic map is a prerequisite for many fundamental studies and applied manipulations (Mich-

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elmore *et al.* 1992). Because most genes of interest do not segregate in any one cross, mapping multiple resistance genes as well as any other morphological, physiological, or developmental traits with respect to each other has been slow. When multiple genes do segregate in one population, epistatic interactions among traits can also confound the scoring of these genes. In addition, scoring multiple disease resistance genes requires the simultaneous handling and screening of multiple pathogens. Locating these genes accurately requires either the construction of specific crosses that cosegregate for multiple genes or the generation of multiple detailed maps each linking different traits to common sets of jointly segregating molecular markers. Both approaches are laborious.

Recently, new classes of molecular markers and advances in mapping strategies have accelerated the production of high density genetic maps and limited the need to construct maps with randomly placed markers. The advent of polymerase chain reaction (PCR) based genetic markers provided thousands of potential markers. Several procedures describe the use of arbitrary primers to amplify specific sequences of the genomic DNA of an individual or genotype (Welsh and McClelland 1990; Williams *et al.* 1990; Caetano-Anollis *et al.* 1991). With 10-base primers, Williams *et al.* (1990) showed that random amplified polymorphic DNAs (RAPDs) segregate as Mendelian markers. In *L. sativa* accessions, which average 8.5 amplified bands per 10-base primer, 170 loci scattered throughout the genome can be screened for polymorphisms with as few as 20 different primers (Kesseli *et al.* 1992a). More than 230 RAPDs have now been mapped in *L. sativa.*

Bulked segregant analysis (BSA) was developed as a rapid method either to saturate previously mapped regions with new markers or to locate efficiently unmapped genes by targeting a specific region and anchoring it to a detailed map (Michelmore *et al.* 1991). BSA can be used to target immediately any gene that segregates in any population. Samples in a segregating population are pooled into different genetic classes determined by the genotype of the target gene or region. The pools differ for markers linked to the target but are randomized for the genotypes of unlinked markers. Hundreds of loci can be screened in a day; only those markers linked to the target will distinguish the pools. Previously, the comparison of near-isogenic lines (NIL) differing for a specific gene was a primary means of targeting markers to a region (Young *et al.* 1988; Paran *et al.* 1991). These NILs are time consuming to make in species for which controlled backcrosses are possible and may be impossible to make in slowly maturing species. BSA allows efficient mapping by targeting specific regions instead of randomly mapping many markers, only a few of which are of interest.

We have continued to test for the clustering of resistance genes to diverse pathogens in *L. sativa.* In this study, we describe the efficient mapping of a new resistance gene to the second largest cluster of resistance genes. Resistance to the oomycete fungus *Plasmopara lactucae-radicis* Stang. & Gilbn., root downy mildew, was recently reported (Stanghellini et al. 1990) and resistance segregated as a single recessive gene, *plr* (Vandemark *et al.* 1992). In contrast, the related fungus *Bremia lactucae*, lettuce downy mildew, predominantly infects leaves, and resistances to the pathotypes segregate as dominant genes. Resistance to *P. lactucae-radicis* is laborious and tedious to score as the production of sporangia must be observed on the roots (Vandemark *et al.* 1992). No NILs existed for this gene, but we had previously constructed an F_2 population involving cultivars that were resistant (Cobham Green) and susceptible (Calmar). By identifying RAPDs with BSA of the F_2 population, we located the approximate position of *plr*. We then constructed, from F_3 families, a detailed genetic map of the region and compared the location of the markers in this map with those for our high-resolution genetic map constructed from another cross (Kesseli et al. 1992b). The mapping strategy was efficient and accurate because we assayed the problematic *plr* gene in only those informative F_3 families that showed recombinants in the region. The *plr* gene was linked to a previously known cluster of resistance genes (Hulbert and Michelmore 1985) that includes two genes for resistance to downy mildew, *Dm5/8* and *Dm10*, and one to turnip mosaic virus, *Tu*.

RESULTS

BSA of the initial F_2 population.

The two bulks, produced by separately pooling the DNA from 25 resistant and 25 susceptible individuals of the F_2 from the cross of cvs. Calmar and Cobham Green, showed identical banding patterns for all but four of the initial 100 primers examined. Each of these four (D8, D19, F12, and F19) showed a band present in the susceptible class that was absent or drastically reduced in the resistant class, indicating (as expected given the construction of the pools) that the dominant alleles of the RAPDs are coupled with the dominant susceptible allele of the resistance gene. Segregation of each of these markers was subsequently scored in the 50 individual samples of the F_2. The four loci identified with these primers were linked to *plr* (χ^2; $P = 0.01$) at recombination distance of 15 ± 13 or less. Although *plr* was clearly linked to these RAPDs, the limited segregation data did not position the locus unambiguously. Two of these RAPDs (*OPd08* and *OPf12*; all band sizes noted in Fig. 1) had been previously located on our detailed map (generated from the Calmar × Kordaat cross) to linkage group 1 (Kesseli *et al.* 1992b). On that map, the two were 4 cM apart , and 27 other markers were within 30 cM of these, a span likely to encompass the position of *plr*. This region included a cluster of three disease resistance genes. One of these, *Dm5/8*, has been precisely mapped in the linkage group. Seeds were not generated from the 50 individuals used to identify the four RAPDs; therefore, this small F_2 was not used in subsequent parts of this study. Instead, we used the 150 F_3 families generated from a larger F_2 population of the same cross.

Identification of informative RAPDs and recombinant F_3 families.

The parental cultivars Calmar and Cobham Green were tested for differences at 15 of the 27 additional loci located in the region of the high-resolution map (Calmar × Kordaat) to which *plr* was preliminarily assigned. Including *OPd08* and *OPf12*, nine RAPDs in both coupled and repulsed phases and downy mildew resistance gene *Dm5/8* clearly segregated in both crosses and could therefore be used as anchors to locate *plr* precisely in the detailed map. Subsequently, the segregation of the nine RAPD markers was scored in the 150 DNA samples generated from pooled individuals of each F_3 family.

The gene *Dm5/8* was scored by inoculation of a minimum of 20 seedlings of each F_3 family. No recombination was detected in this region (between *OPx01* and *OPco8*) for 124 of the families; these families were therefore uninformative for the mapping of *plr* and were not studied further. Twenty-five of the remaining 26 informative F_3 families were subsequently scored for segregation at *plr*. The remaining informative family was not identified as recombinant until late in the study and was therefore not included in the disease screen. We screened 20 plants for each F_3 family so that *plr* could be scored as a codominant gene.

Unlike the scoring for resistance to *B. lactucae*, in which the F_3 families were readily classified as homozygous resistant, segregating, or homozygous susceptible, the *plr* gene was difficult to score, because the sporangia appear on the roots not cotyledons and uniform inoculation was difficult to guarantee (Vandemark *et al.* 1992). Seven of the 25 informative F_3 families from the large F_2 initially appeared misscored on the basis of their flanking RAPD genotypes, and these were subsequently retested. Six had been misscored for *plr*, and one had been misscored for a RAPD. In retrospect, seven individuals from the 50 plants scored in the preliminary screen also appeared misscored. These probably escaped infection, and this explained why we were unable to locate *plr* with that initial F_2 population of 50 plants.

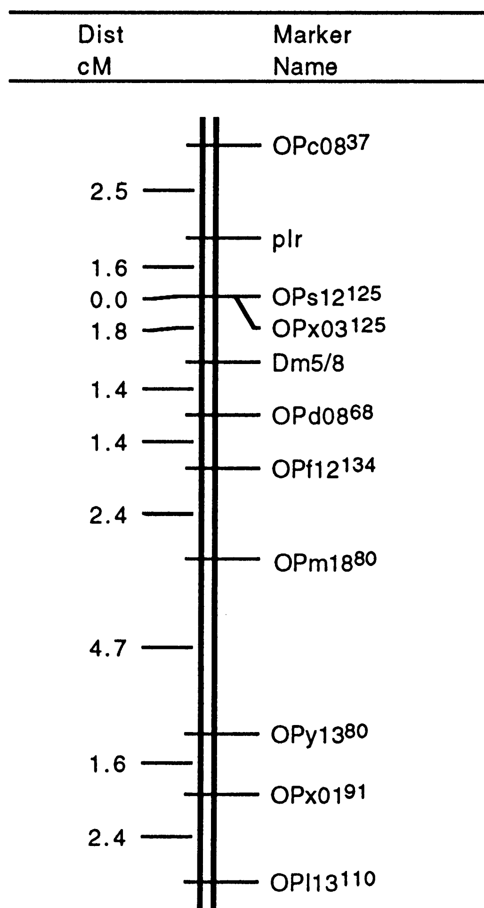

Fig. 1. Genetic map showing the linkage relationships of plr, Dm5/8, and RAPD loci analyzed in the F2 population of a cross between cultivars Calmar and Cobham Green. Superscripts represent the fragment sizes of RAPD loci and can be multiplied by 10 to obtain the size in base pairs.

Precise mapping of *plr*.

The pairwise recombination values and best fit gene orders were determined with MAPMAKER (Lander *et al.* 1987). The *plr* locus was placed with nearly equal likelihood at any location between *Dm5/8* and *OPc08* because of the inability to detect all recombination events for the intervening dominant RAPD loci, *OPs12*, and *OPx03*. These loci were converted to codominant markers for eight F_3 families with recombination in this interval by scoring individuals of the families separately and determining whether the genotypes at locus *OPs12* or *OPx03* in the progenitor F_2 parent had been heterozygous or homozygous dominant. This additional information positioned *plr* between *OPc08* and *OPs12* with odds of greater than 1,000:1 favoring placement; a log of the odds ratio (LOD) score of more than 3.0 separated this order from the next most likely order (Fig. 1). The detailed genetic map of *L. sativa* shows that members of a small multigene family (*CL202e* and *CL390*) also reside in this interval (Kesseli *et al.* 1992b).

The distance between *plr* and *Dm5/8* in the Calmar × Cobham Green cross was 3.4 cM. Based on flanking markers, these genes would be at least 10 cM apart in the high-resolution Calmar × Kordaat cross. This difference may not be significant. The two-point recombinational distances from *Dm5/8* past *plr* to *OPc08*, the nearest flanking marker segregating in both populations are, however, significantly different in the two crosses (t test $P < 0.05$). In the cvs. Calmar × Kordaat F_2 population the recombination value is 0.20 ± 0.06; for Calmar × Cobham Green it is 0.06 ± 0.02.

DISCUSSION

Efficiency of the mapping strategy.

The complexity of the interactions between host and pathogens makes the genetic analysis of disease resistance genes difficult. The similarity of the reaction phenotypes often limits the analysis of multiple resistance genes. Laborious screening procedures can slow analyses. Genetic penetrance may be less than complete. Also, susceptible individuals may die, eliminating a phenotypic class from further study. We used a fast, efficient, and relatively simple five-step approach that is particularly applicable to the mapping of disease resistance genes. First, we identified four RAPDs linked to a target gene (*plr*) with BSA of a small temporary set of individuals from an F_2 population (Calmar × Cobham Green). Second, the simultaneous segregation of two of these RAPDs in a second F_2 population (Calmar × Kordaat) that had previously been used to construct a detailed genetic map with more than 300 markers but did not segregate for *plr*, provided a preliminary position for *plr* on that map and identified 27 additional, potentially linked markers. Third, using pooled individuals from 150 F_3 families of the first cross, we accurately mapped 10 loci in the region of *plr* and identified the families that would be informative for the positioning of *plr*. Limiting the number of families to be screened for resistance to *P. lactucae-radicis* greatly increased our efficiency, since the assay was difficult. Fourth, the 25 informative families were screened for resistance, thus locating the target gene to two intervals

encompassing less than 6 cM. Fifth, we scored individuals of selected F_3 families to resolve ambiguities caused by dominance or scoring errors and precisely located the gene to a 4.1-cM interval.

The efficiency of BSA.

The efficiency of the BSA can be measured by the frequency at which linked markers were identified. Under our reaction conditions, an average of 8.5 ± 2.8 bands are amplified per arbitrary 10-base primer, and approximately 17% of these segregate in crisphead × butterhead F_2 populations (Kesseli *et al.* 1992a). Extrapolating, the 100 primers used in the initial screening should detect roughly 144 polymorphic markers scattered throughout the genome, of which only half could potentially distinguish the bulked resistant and susceptible samples (because of heterozygotes in the susceptible class; see Materials and Methods). We estimated that BSA can usually identify RAPDs up to 30 cM from the target gene (Michelmore *et al.* 1991), which in lettuce represents a window of approximately 3% of the genome (60/2,000 cM; Kesseli *et al.* 1990). Thus, assuming a random distribution of RAPDs in the genome (Kesseli *et al.* 1992a), we expected about 3% of the potential polymorphic loci to be linked to the target gene. About 5% of the loci (4/72) were detected, therefore the efficiency of the method fit or slightly exceeded expectations.

Identification of linked markers.

The separation of the marker identification phase of the study from the precise mapping phase yielded several advantages. Identification involved only the F_2 population, small numbers of individuals, and no seed production. Since the screening for resistance to *P. lactucae-radicis* was laborious and cumbersome, the use of only a small F_2 population, rather than many F_3 families, was particularly beneficial. Although we used 25 plants per pool, fewer than 10 individuals in each pool of the BSA would generally be sufficient to identify linked markers (Michelmore *et al.* 1991). Additionally, as no seed was produced, we did not have to rescue susceptible individuals after the inoculation and the spread of the disease. This was not a consideration for our screen of *plr* but could be a significant advantage for other diseases. It was necessary to save only a small amount of leaf material for DNA extraction (a few grams or less). Indeed, BSA could still be performed even if a debilitating disease destroyed all susceptibles (for recessive resistance this would be 75% of the population). Barring major segregation distortion, the missing class could be mimicked by pooling the parental DNA.

Dominance is tolerated with BSA, since heterozygotes do not have to be excluded from one class. Errors due to poor penetrance of the resistance gene and misscoring of a portion of the individuals (which did occur in our study) could also be tolerated, because occasionally misscored individuals do not prevent the identification of markers by BSA and because maps are constructed from a different set of individuals. These errors are not acceptable in the mapping phase; there must be the opportunity to retest data points.

Because a detailed genetic map already existed in *L. sativa*, linked markers could be identified in two ways. Four markers were identified directly by *de novo* testing of arbitrary primers on the bulks generated from the classes in the Calmar × Cobham Green F_2 that were resistant or susceptible to *P. lactucae-radicis*. Locating two of those four markers on the detailed map constructed from the cross Calmar × Kordaat indirectly identified 27 other potential markers. Many of these potential markers were tested, and eight that jointly segregated in both crosses were eventually used to construct comparative maps for this region.

Precise mapping.

Although using a small temporary F_2 population for identifying linked markers proved most efficient, using the F_2 to map the target gene would have been less accurate and no faster than our approach. Mapping the molecular markers from F_3 families was as fast and easy as mapping from the F_2 for two reasons. The F_2 plants that were used to produce F_3 families can be grown simultaneously with those used in the preliminary disease screen but under optimal conditions. Thus F_3 seed in the mapping population can be produced as markers are being identified by BSA of the temporary population. In addition, leaves are pooled for individuals of each F_3 to produce a single sample representing the F_2 progenitor; no increase in sample number is needed for equivalent information.

The increased accuracy made mapping with the F_3 families superior. For resistance to *P. lactucae-radicis*, we screened only the informative F_3 families, which limited the number of the laborious tests, and we eliminated the problem of dominance at *plr* since the genotypes of the F_2 were inferred from the progeny array. Accurate mapping also required resolving ambiguities caused by dominance at RAPD loci or the inevitable introduction of scoring errors. Dominant markers in coupling provide at most half the information of codominant markers. Dominant markers in repulsion provide almost no information, since most recombination events will be cryptic (Allard 1956). Maintaining the F_3 families allowed us first to quickly score RAPDs as dominant markers in pooled families and then to convert the tightly linked RAPD loci of specific families to codominant markers by scoring individuals of the family. Without the added information obtained by classifying these families as segregating or homozygous dominant, we would not have been able to assign *plr* to a single interval on our map. We also eliminated errors by retesting selected individuals and confirmed the initial misscoring of six families for the *plr* gene and one for a RAPD marker.

Characterization of resistance genes.

Our first attempt to target and locate a new disease resistance gene to a previously unexamined pathogen mapped directly within one of our three known resistance gene clusters. We had shown previously that disease resistance genes are linked in *Lactuca* spp. (Hulbert and Michelmore 1985) and that they are the only class of genes (e.g., RFLP, RAPD, isozyme) that consistently exhibit a clustered distribution (Kesseli *et al.* 1992a). Our finding here further supports this observation and extends it to a wider list of disease resistance genes. This cluster now contains known genes for resistance to two different fungal and one viral pathogen. From earlier analyses, *Dm5/8* and *Dm10* are linked with a recombination frequency between 0.026 and 0.060 (Nakahara and Mich-

elmore, unpublished). Turnip mosaic virus resistance (*Tu*) is also linked to *Dm5/8* (r = 0.12 ± 0.016; Zink and Duffus 1970). The orientation of *Tu* and *Dm10* is presently being determined (H. Witsenboer and M. Fortin, unpublished), but the four resistance genes are likely all on a segment of 12–18 cM. Preliminary data has located an additional downy mildew resistance gene to the linkage group (B. Maisonneuve and R. Michelmore, unpublished).

In addition to the diversity of resistance gene specificities, there is a difference in interallelic interactions for these genes. Most disease resistance genes are dominant, although exceptions are known (e.g., *ml-o*; Jorgensen and Jensen 1976). In lettuce, corky root resistance (*cor*) and lettuce mosaic virus resistance (*mo*) are recessive but have not yet been precisely mapped. Resistance to *Plasmopara* is the first recessive resistance gene to be located in these resistance gene clusters. It has been shown, however, for one resistance gene to *B. lactucae*, that 3:1 ratios can be converted to 1:3 ratios by exposing plants to high levels of inoculum (B. Maisonneuve and R. Michelmore, unpublished). This implies that there are response thresholds for resistance alleles and that the distinction between dominant and recessive disease resistance genes may not be strict and may therefore not reflect fundamental differences in gene action. Variation in the response of *plr* to nonuniform levels of inoculum may have been partly responsible for the difficulties in scoring the gene. Since infected source plants could not be located equidistantly from all individuals in the hydroponic system, a simultaneous and uniformly concentrated inoculation of all individuals was not possible. Resistant individuals exposed to high concentrations of inoculum show a few sporangia. Lightly infected susceptibles yield the same phenotype (Vandemark *et al.* 1992). The genetic analyses of this study identified suspect individuals and allowed for retesting and clarification of this phenomenon.

These genetic data provide further support for the hypothesis that resistance genes may be members of multigene families with common origin and function yet diverged specificity (Michelmore *et al.* 1987; Pryor 1987). The discovery of clusters of resistance genes in several species supports this model. If duplications and rearrangements are prevalent in these regions, other, non-disease-resistance-gene loci in the region may also be affected. The *Ml-a* resistance gene cluster of barley is bracketed by *Hor1* and *Hor2*, two genes that encode seed storage proteins (Jensen *et al.* 1980; Wise and Ellingboe 1985). In *L. sativa,* several multigene families have been detected with our cDNA probes (Kesseli *et al.* 1992b). We have mapped members of those multigene families that segregated in our F_2 populations. For the two largest families, nine and five loci have been mapped, respectively (Paran *et al.* 1992, Kesseli *et al.* 1992b). For the larger family, detected with probe CL1795, four loci map to two of the three disease resistance gene clusters. For the smaller family, CL202, one member maps within the same flanking RAPD markers as *plr*. These data do not show a definitive correlation, since there are other members of these multigene families that do not map to resistance gene clusters, and there are other multigene families that are monomorphic in our populations and therefore are not mapped at all. The data do suggest that genes for resistance to a variety of pathogens may all have a common origin, that duplications and rearrangements in these regions may allow the diversification of their specificity, and that these processes may be affecting many functionally unrelated loci in these regions.

MATERIALS AND METHODS

Plant materials.

Two preexisting F_2 populations between one crisphead cultivar, Calmar (with downy mildew resistance genes *Dm5/8, DmDm7,* and *DmDm13*), and two butterhead cultivars, Kordaat (*Dm1, DmDm3,* and *Dm4*) or Cobham Green (no known resistance genes at the time of the cross) were used. The F_2 population of cvs. Calmar × Kordaat has been used to generate a detailed genetic map for lettuce (Landry *et al.* 1987; Kesseli *et al.* 1990, 1992b). The cross of cvs. Calmar × Cobham Green was created by O. Ochoa (University of California, Davis) as part of our program to generate near-isogenic lines differing for single *Dm* genes. Subsequent to these studies, Cobham Green was shown to carry a single recessive gene for resistance, *plr*, for the root downy mildew pathogen *P. lactucae-radicis* (Vandemark *et al.* 1992). For the initial genetic analysis, a sample of the F_2 population from Calmar × Cobham Green was grown hydroponically at the University of Arizona, inoculated, and scored for resistance as described previously (Vandemark *et al.* 1992). Leaves were harvested from 25 resistant and 25 susceptible individuals for DNA extraction; seeds were not produced from these individuals. For a more detailed analysis, a random F_2 population of 150 individuals was grown, and large F_3 families (1,000+ seeds each) were generated at the University of California, Davis. Pooled DNA samples of 20 or more individuals from each F_3 family were used to regenerate the phenotypes of the RAPD loci in the progenitor F_2 population. More than 20 individuals from each F_3 family were also screened for resistance to *Dm5/8* as described previously (Farrara *et al.* 1987). From these data, all recombinants in the region of *plr* were identified. Because the screening was difficult and laborious, only selected families, those shown to be recombinant in the region, were scored for *plr*. The other F_2 individuals showed no recombination in the region; they were therefore uninformative and not scored for resistance to *P. lactucae-radicis*. In addition, scoring RAPD genotypes for individuals of selected F_3 families increased the information content for the F_2 population by resolving the problems of dominance associated with RAPD loci. The large F_3 families provided enough seed to rescore disease resistance genes and RAPDs as necessary.

DNA extractions and PCR.

The DNA extraction procedure was a modified CTAB protocol (Bernatsky and Tanksley 1986). The DNA was diluted to approximately 4–5 ng/µl in a modified Tris-EDTA buffer with reduced EDTA concentration (0.1 mM), and 20–25 ng was used in each 25-µl PCR reaction. Procedures for RAPDs are similar to Williams *et al.* (1991) and described elsewhere (Paran *et al.* 1991; Michelmore *et al.* 1991). DNA was extracted from leaves of individuals in the F_2 population, pooled F_3, samples, or individuals of selected F_3 families.

Bulked segregant analysis.

Equal quantities of DNA from the 25 resistant and 25 susceptible plants of the initial F_2 population were combined into

two separate pools or bulks. These two bulks were therefore randomized for genotypes of markers not linked to the resistance gene but differed for loci closely linked to the *plr* gene. The procedure has been described in detail elsewhere (Michelmore *et al.* 1991). Since resistance is recessive, the susceptible bulk contains heterozygous and homozygous susceptible genotypes for *plr* and linked loci. This class therefore contains all alleles of the cross, whereas the resistant bulk will be deficient for the alleles that are linked in *trans* with *plr*. Because we lacked a homozygous susceptible class and because we were using dominant markers (RAPDs) for screening, BSA could only identify markers when the amplified product, the band, was in *trans* with *plr*. The two pools were initially screened for polymorphisms with 100 10-base primers (kits A to D and F; Operon Technologies, Alameda, CA).

Genetic mapping.

Primers that detected differences between the bulks were then used to examine the 50 individual samples of the F_2 population to determine the position of the RAPD loci in relation to *plr*. As expected, many loci that segregated in the cvs. Calmar × Cobham Green cross also segregated in the Calmar × Kordaat cross (cvs. Kordaat and Cobham Green are both butterhead types). Segregating loci common to both crosses allowed us to locate *plr* to a position on the detailed map. This identified many other markers that had been previously mapped in the Calmar × Kordaat cross to the region containing *plr*. Many of these could not be detected with the bulked DNA samples, because the amplified RAPD fragments were in *cis* with *plr*. These loci were, however, subsequently scored in the individuals of the Calmar × Cobham Green F_2 population and F_3 families to generate a detailed map of the region in this cross. We used MAPMAKER (Lander *et al.* 1987) to determine orders and calculate multipoint recombinational distances.

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