Theor Appl Genet (1994) 89: 96–104

B. Maisonneuve · Y. Bellec · P. Anderson
R. W. Michelmore

Rapid mapping of two genes for resistance to downy mildew from *Lactuca serriola* to existing clusters of resistance genes

Received: 4 January 1994 / Accepted: 12 January 1994

Abstract Two resistances to downy mildew derived from *Lactuca serriola* were characterized genetically and mapped using molecular markers. Classical genetic analysis suggested monogenic inheritance; however, the presence of multiple, tightly-linked genes in each case could not be eliminated. Therefore, they were designated resistance factors R17 and R18. Analysis with molecular markers known to be linked to clusters of resistance genes quickly revealed linkage of R18 to the major cluster of resistance genes and provided six linked markers, three RAPD (Random Amplified Polymorphic DNA) markers and three codominant SCAR (Sequence Characterized Amplified Region) markers. The mapping of R17 required the screening of arbitrary RAPD markers using bulked segregant analysis; this provided five linked markers, three of which segregated in the basic mapping population. This demonstrated loose linkage to a second cluster of resistance genes and provided additional linked markers. Two RAPD markers linked to R17 were converted into SCARs. The identification of reliable PCR-based markers flanking each gene will aid in selection and in combining these resistance genes with others.

Key words RAPDs · SCAR · Lettuce · Downy mildew *Lactuca serriola*

Communicated by H. F. Linskens

B. Maisonneuve · P. Anderson · R. W. Michelmore (✉)
Department of Vegetable Crops,
University of California,
Davis, CA 95616, USA

B. Maisonneuve · Y. Bellec
Institut National de la Recherche Agronomique,
Station de Génétique et d'Amélioration des Plantes,
Route de Saint Cyr,
78026 Versailles Cedex, France

Introduction

Lettuce downy mildew, caused by the oomycete fungus *Bremia lactucae*, is an important disease wherever lettuce is grown (Crute 1992b). The genetics of resistance in the host and of avirulence in the pathogen have been studied extensively (Crute and Johnson 1976; Johnson et al. 1978; Farrara et al. 1987; Ilott et al. 1989). To-date, 13 single dominant genes (*Dm*) for resistance to *B. lactucae* have been described that are matched by single dominant avirulence genes (*Avr*) in *B. lactucae*. The 13 *Dm* genes map to four clusters in the lettuce genome (Farrara et al. 1987). Many others sources of resistance exist in wild germplasm (e.g., Farrara and Michelmore 1987; Bonnier et al. 1992); however, their genetic bases have not been determined.

Breeding of cultivars containing new *Dm* genes has not provided long term durable resistance. There is extensive variation in virulence phenotype between different isolates of *B. lactucae*. The pathogen undergoes sexual reproduction in several parts of the world resulting in the generation of new variants, as well as having a prolific asexual cycle resulting in rapid spread of virulent phenotypes (Michelmore 1981). However, there are no clear alternatives to the use of *Dm* genes. The fungicide, metalaxyl, provided effective control until insensitive strains developed (Crute 1987; Leroux et al. 1988; Schettini et al. 1991); now this fungicide provides little protection unless used as part of an integrated strategy that combines *Dm* genes and metalaxyl treatments (Crute 1992a). Field resistance has been identified in several cultivars (Eenink and Dejong 1982; Norwood et al. 1985); however, its genetic basis and screening requirements are complex, making it difficult to manipulate in breeding programs. Also, it is not clear whether this form of resistance may also exhibit isolate specificity and whether it would be durable. Therefore, control of downy mildew currently relies on the continual identification and introduction of new *Dm* genes. Consequently, it is important to maximize the efficiency with which *Dm* genes can be introgressed from wild germplasm into commercially-acceptable genotypes.

Molecular markers provide several opportunities for studying the inheritance of *Dm* genes and for manipulating them in breeding programs. Molecular markers can be used to map new *Dm* genes quickly. Map position indicates which resistance genes are unlinked and therefore can be combined easily. It also indicates whether there is a danger of eliminating one resistance gene while introgressing another when resistance genes are linked. Molecular markers can be used to identify recombinant individuals with new combinations of *Dm* genes linked in *cis*; such combinations can then be manipulated as a single Mendelian unit. Also, molecular markers can be used to monitor the introduction of multiple resistance genes; this is particularly advantageous if the individual genes are otherwise only detectable with exotic isolates of *B. lactucae* or confer resistance to every known isolate.

In this paper we describe the rapid mapping of two new genes for resistance to *B. lactucae* from *L. serriola* using bulked segregant analysis (Michelmore et al. 1991) and PCR-based markers.

Materials and methods

Plant materials

One set of F_2 and derived F_3 families was used to analyze each source of resistance. Both analyses involved crosses between butterhead types. The resistance from *L. serriola* LS102 was analyzed in a population derived from cv Girelle×YYD. Girelle is a butterhead cultivar from INRA (Institut National de la Recherche Agronomique, Versailles) that contains *Dm2*, *Dm3* and *Dm4*. YYD is a breeding line generated at INRA to introgress the new resistance from LS102; it is an F_5 selection from BC_3 (*ms4 Ms5* Girelle×an interspecific F_1 between LS102 and an iceberg breeding line).

The other resistance was analyzed in a population derived from cvs Cobham Green×Mariska. Cobham Green is a butterhead cultivar without any known genes for resistance to downy mildew. Mariska is a butterhead cultivar from Nunhems with a new resistance from *L. serriola* (T. Wolters, Nunhems, Haelen, The Netherlands, personal communication); it is a selfed line derived from a complex pedigree involving four butterhead cultivars and *L. serriola* LS17.

Tests for resistance to *B. lactucae*

The isolates of *B. lactucae* used in this study were selected on the basis of their virulence phenotype from the collections maintained at UC Davis and INRA (Table 1). The virulence phenotypes of all isolates were checked prior to use by inoculation onto differential sets of resistant cultivars (Farrara et al. 1987). At Davis, isolates were rescued from storage at −80 °C by culture on seedlings of cv Cobham Green. Inoculations and incubation were made as described previously (Hulbert and Michelmore 1985). For analysis of F_2 populations, seedlings were grown individually in vermiculite in Magenta boxes. At Versailles, isolates were initially cultured on seedlings of a cultivar selected as susceptible to the isolate being cultured (e.g., GL659 for KT2; R4T57 for NL1; Valverde for R60, NL2 and NL13; Capitan for NL16; Mariska for 49/83). Suspensions were prepared by shaking spore-bearing cotyledons in distilled water and directly spraying seedlings in the growth chamber at 16/12 °C day/night temperatures with 16 h per day. At both locations, seedlings were scored 7 and 14 days after inoculation for the presence or absence of asexual sporulation and the intensity of necrosis.

The genetics of resistance in YYD was first investigated at Versailles using crosses to a susceptible cultivar (cv Hilde) and to lines carrying known resistance genes for allelism tests. A single F_2 Girelle×YYD population was analyzed at Davis using isolate CS9. Seedlings were inoculated 6 or 14 days after sowing. Susceptible plants were rescued by treating twice with 1 ml of Ridomil (50 ppm); the first treatment was at the first day of emergence of conidiophores and the second 8 days later. One-hundred-and-thirty-three F_2 plants were transferred to the greenhouse and grown to seed. Thirty to fifty seedlings of each F_3 line were reinoculated to confirm the resistance genotype and to determine the zygosity of the progenitor resistant F_2 plants.

The segregation of resistance in the F_2 Cobham Green×Mariska population was determined at Versailles using isolates NL1, Tv, KT2, or R60 applied 7 days after sowing. Eighty-five of the plants that were tested with isolate R60 (59 resistant and 26 susceptible) were rescued by treatment with Ridomil and grown to seed in the greenhouse. F_3 families were tested with isolates CS9 and R60.

DNA extraction

DNA was extracted, using a modified CTAB procedure (Bernatzky and Tanksley 1986) from young fresh leaves by homogenizing with a polytron in 20 ml of extraction buffer (Tris 0.1 M, sorbitol 0.35 M, and Na_2EDTA 5 mM). Approximately 2 g of leaves from each of the 133 F_2 individuals from Girelle×YYD and of pools of 16–24 seedlings (1–2 leaves each) from the 85 F_3 families from Cobham Green×Mariska were used. The DNA was diluted to approximately 4-5 ng/µl in a TE buffer modified with reduced EDTA concentration (0.1 mM) prior to use as a substrate for PCR.

Molecular markers

Two types of PCR-based genetic markers were used in this study, RAPD (Williams et al. 1990) and SCAR markers (Paran and Michelmore 1993). For RAPDs, the protocol was similar to Williams et al. (1990) with minor modifications (Paran et al. 1991). RAPD primers were supplied by Operon Technologies (Alameda, Calif.). The amplification products were resolved by electrophoresis in a 2% agarose gel in TBE buffer and stained with ethidium bromide. If potentially-informative fragments could not be adequately resolved in 2% agarose, a 1% synergel (Diversified Biotech, Newton Centre, Mass.) plus 0.6% agarose gel with TAE buffer was used.

RAPD markers linked to resistance genes were identified by bulked segregant analysis (Michelmore et al. 1991). Only homozygous plants were used in the bulks, therefore markers both in *cis* and in *trans* to the resistance gene could be identified. For Girelle×YYD, the bulks were made by pooling young leaves of nine susceptible and 15 resistant F_2 plants prior to DNA extraction. It was possible to distinguish the homozygous resistant plants as they had a more complete resistant reaction than the heterozygotes (see below). For Cobham Green×Mariska, the bulks were made by combining an equal quantity of DNA from 19 susceptible and 13 resistant non-segregating F_3 families. Initial tests were made using RAPD markers known to be linked to one of the four clusters of *Dm* genes. If no polymorphic markers were identified, random primers were then used (Operon kits O to Y).

When a new RAPD marker was found linked to resistance, SCARs were made as described previously (Paran and Michelmore 1993). If the SCAR primers amplified a band of the same size from both parental genotypes, the amplification products were screened for restriction site polymorphisms using endonucleases with 4 bp recognition sites.

Mapping

When a RAPD band was detected as polymorphic between the DNA bulks, linkage was confirmed using DNA from the parents and segregating F_2 individuals. Segregation data were analyzed using MAPMAKER (Lander et al. 1987) with a LOD threshold of 3.0 for linkage. If a marker had not yet been located on the lettuce map and was polymorphic between Calmar and Kordaat, it was mapped using our basic mapping population of 66 F_2 individuals from Calmar×Kordaat (Landry et al. 1987; Kesseli et al. 1994).

Table 1 Virulence phenotypes of isolates of *B. lactucae* used in this study and their reaction to new sources of resistance

a, tests at Davis

		Isolates of *B. lactucae*													
	Dm gene	CG1	CG5	CS9	CS12	JPN	NL1	NL6	NL16	SF3	Tv	IL4	IM25R7	C83M47	C82P24
Differential varieties															
Cobham Green		+[a]	+	+	+	+	+	+	+	+	+	+	+	+	+
Lednicky	1	+	+	+	–	+	+	+	+	+	+	+	+	–	–
UCDM2	2	–	+	+	+	+	+	+	+	+	+	+	–	+	+
Dandie	3	+	–	+	–	–	–	–	+	+	+	–	–	+	+
R4T57	4	+	+	+	+	*	+	+	+	–	+	+	+	+	–
Valverde	5/8	–	+	+	–	+	–	+	+	+	+	+	+	+	+
Sabine	6	–	–	+	–	–	–	–	+	*	+	–	+	+	+
LSE57/15	7	+	+	+	+	+	*	*	+	+	+	+	+	+	+
UCDM10	10	+	+	+	–	+	+	+	+	*	+	+	+	–	+
Capitan	11	–	–	+	+	+	–	+	+	+	–	+	+	–	+
Pennlake	13	+	+	+	–	+	+	+	+	+	+	+	+	+	+
UCDM14	14 (+13?)	–	+	+	–	+	+	+	–	+	+	+	+	+	+
PIVT1309	15	+	–	+	*	+	*	–	–	+	–	+	+	–	–
LSE18	16	+	+	–	+	–	+	+	+	–	+	+	+	+	–
Mesa659 (= GL659)	7+13	+	+	+	–	+	*	–	+	+	+	+	+	+	+
Saffier	1+7+16	*	+	–	–	–	–	–	+	–	+	+	+	–	–
Kinemontepas	10+13+16	–	*	–	–	–	–	–	+	–	+	+	+	–	–
Girelle	2+3+4	–	–	+	–	–	–	–	+	–	+	–	–	+	–
New sources															
LS102	R17	–	–	–	–	–	–	–	*	–	–	–	–	–	–
YYD	R17	–	–	–	–	–	–	–	*	–	–	–	–	–	–
Mariska	R18	–	*	–	–	–	–	–	–	–	–	–	–	*	–

b, tests at Versailles[b]

		Isolates of *B. lactucae*															
	Dm gene	KT2	49/83	42/83	239/78	C19:D	R60	R74/89	NL2	NL4	NL5	NL13	NL14	NL16	B	M	Q	S
Differential varieties																		
Cobham Green		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Lednicky	1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
UCDM2	2	+	+	+	+	+	+	+	+	+	–	–	+	+	+	+	+	+
Dandie	3	+	+	+	+	+	+	+	+	–	+	+	+	+	+	+	+	+
R4T57	4	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+	+	+
Valverde	5/8	+	+	+	+	+	+	+	+	+	–	+	+	+	+	–	–	+
Sabine	6	–	+	–	–	+	+	+	+	–	–	–	+	+	+	+	+	+
UCDM10	10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Capitan	11	+	+	+	+	–	+	+	–	–	–	+	+	+	–	+	–	–
Pennlake	13	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
UCDM14	14 (+13?)	+	+	+	+	+	+	+	nt[c]	nt	nt	+	+	–	nt	+	nt	+
LSE18	16	–	*	+	nt	+	*	+	nt	nt	nt	–	–	+	nt	–	nt	nt
GL659	7+13	+	+	+	+	+	–	+	–	+	+	–	–	+	–	–	+	+
Saffier	1+7+16	–	–	–	–	+	–	+	–	–	–	–	–	+	–	–	–	–
Kinemontepas	10+13+16	–	–	–	–	+	–	+	–	–	–	–	–	+	–	–	–	–
Girelle	2+3+4	+	+	+	+	+	+	+	+	–	–	–	+	+	+	+	+	+
New sources																		
LS102	R17	–	–	nt	nt	–	nt	–	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt
YYD	R17	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Mariska	R18	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–

[a] +, compatible reaction (sporulation); –, incompatible reaction (no sporulation); *, intermediate reaction (sparse and late sporulation)
[b] Tests at Versailles with Tv and NL1 provided the same virulence phenotypes as shown for the tests at Davis
[c] nt, not tested

Results

Genetics of resistance to *B. lactucae* in YYD

YYD and its progenitor LS102 were resistant to all of the isolates tested (38 for YYD and 18 for LS102); only late and sparse sporulation occurred with isolate NL16 in some tests. The results with 30 of these isolates with distinct virulence phenotypes are presented in Table 1. These results suggested that YYD and LS102 contained at least one new resistance gene or a combination of several previously-characterized resistance genes.

Tests of the F_2 YYD×Hilde population with each of 11 isolates that sporulated on cv Girelle (the recurrent parent used to generate YYD from LS102) gave results consistent with a 3:1 ratio except with isolate KT2 (Table 2). The segregation observed with KT2 showed a significant excess of resistant individuals but was still closer to a monogenic rather than a digenic ratio. Therefore, a single new locus for resistance seems to have been introgressed into YYD by backcrossing from LS102; we designated this locus R17.

To investigate the linkage of R17 to other *Dm* genes, the segregation of resistance was tested in the F_2 YYD×Hilde population using each of four isolates (NL1, NL4, NL5, NL13) that were also avirulent against one or more of the *Dm* genes in cv Girelle (*Dm2*, *Dm3*, *Dm4*); this resulted in segregation ratios consistent with two (15:1) or three (63:1) independent dominant genes (Table 2). Therefore, the three *Dm* genes from Girelle had been retained in YYD. Tests of F_2 YYD×GL659 with isolate R60 (396:24 resistant to susceptible) and F_2 YYD×Capitan with isolate Tv (399:28 resistant to susceptible) gave results consistent with a 15:1 ratio (χ^2=0.21 and χ^2=0.07, respectively). Therefore, R17 must be unlinked to the two clusters of resistance genes that contain *Dm2*, *Dm3*, *Dm4*, *Dm7* and *Dm11* (Farrara et al. 1987).

Segregation of resistance in F_2 and F_3 populations from Girelle×YYD after inoculation with isolate CS9 were consistent with a single dominant gene. When very young seedlings (6 days after sowing, only small cotyledons and no leaf visible) were inoculated with a large number of spores (2×10^5 conidia/ml), half of the plants (37 out of 75) showed delayed and sparse sporulation and/or extensive necrosis. This resulted in a ratio of 1:2:1 for resistant: sparsely sporulating and/or necrotic:susceptible plants (15:37:23; χ^2=1.72). In a second experiment, inoculation of older plants (14 days after sowing, cotyledons and first leaf visible) resulted in a ratio of 3:1 resistant to susceptible (54:19; χ^2=0.12) with very little variation in reaction within the resistant class. Testing F_3 families with isolate CS9 confirmed that the intermediate reaction of the young seedlings reflected heterozygosity at R17. Overall, we identified 35 homozygous resistant, 68 heterozygous, and 44 homozygous susceptible F_2 plants ($\chi^2_{1:2:1}$=1.92).

Table 2 Segregation of resistance in F_2 populations between YYD or Mariska and a susceptible cultivar inoculated with a variety of isolates of *B. lactucae*

Isolate	F_2 YYD × Hilde				F_2 Cobham Green × Mariska	
	No. of seedlings	χ^2			No. of seedlings	χ^2
	Res:susc.	3:1	15:1	63:1	Res:susc.	3:1
KT2	221:59	6.76*	65.24**		334:117	0.21
49/83	125:43	0.03	*			
42/83	86:27	0.07	*			
239/78	200:55	1.60	*			
C19:D	76:23	0.16	*			
Tv	142:40	0.89			191:52	1.68
R60	471:247	0.49	*		371:111	1.00
NL2	71:32	2.02	*			
B	108:26	2.24				
M	103:34	0.00	*			
Q	191:71	0.62	*			
S	82:30	0.19	*			
NL1	124:3	*	3.28	0.53	296:108	0.65
NL4	98:8	*	0.30	*		
NL5	101:7	*	0.01	*		
NL13	104:1		*	0.25		

* Significantly different from hypothesis ($P< 0.05$)
** Significantly different from hypothesis ($P< 0.01$)

Identification of markers linked to resistance in YYD (R17)

The initial study of 11 RAPD markers that had been previously mapped to the four clusters of *Dm* genes, identified no polymorphism between the bulks; therefore, arbitrary RAPD primers were tested. Fourteen out of two-hundred-and-thirty primers (Kits O to Y; Operon Technologies, Alameda, Calif.) revealed potential polymorphisms between the bulks. Primers OPS02, OPT08 (two bands), OPR13, OPX04, and OPO03 identified six markers (Fig. 1) whose linkage to R17 was confirmed by segregation analysis. Screening of Calmar vs Kordaat with these RAPD markers gave a useful polymorphism with OPS02 and OPO03; analysis of the basic mapping population placed $OPS02_{1470}$ and $OPO03_{1900}$ on the genetic map, loosely linked to the *Dm5/8* cluster. Therefore, additional markers known to be linked to this cluster of resistance genes were analyzed on the YYD×Girelle population. Only one ($OPG02_{1100}$) out of twenty-one RAPD markers that were linked to the *Dm5/8* cluster in the analysis of Calmar×Kordaat was polymorphic between Girelle and YYD. Four SCARs previously developed for the *Dm5/8* cluster identified no polymorphism between Girelle and YYD (band present in both genotypes for SCF12, SCU16 and SCX03; no band in either genotype for SCD08). $OPT08_{1100}$ was also polymorphic between Calmar and Kordaat but the band was too faint to score reliably in their progeny.

RAPD fragments $OPS02_{1470}$ and $OPT08_{490}$ were cloned and sequenced. Two new SCARs were made using primers which included the 14 bases internal to the RAPD primer sequence in addition to the sequence of the RAPD primers (Table 3). After PCR with 60°C annealing tem-

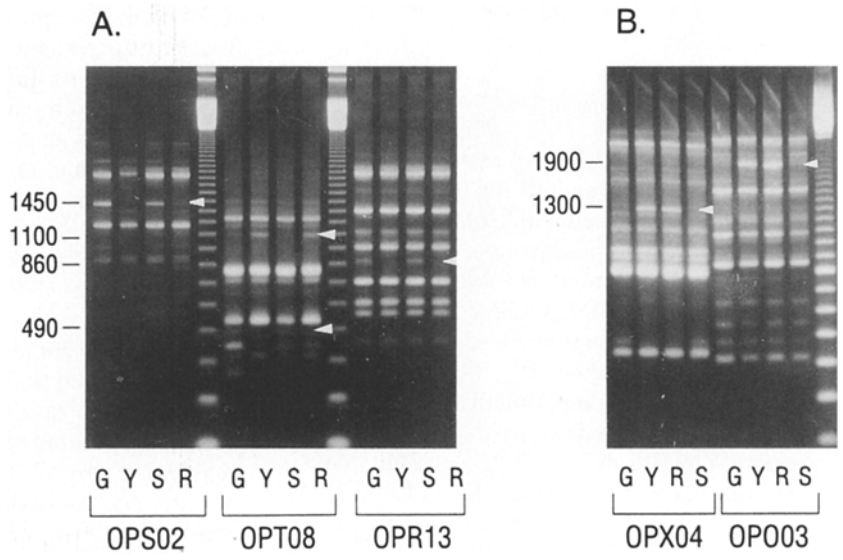

Fig. 1 A, B RAPD markers linked to R17 for resistance to downy mildew. Amplification with five primers (OPS02, OPT08, OPR13, OPX04, OPO03) of genomic DNA from the susceptible cv Girelle (*G*), the resistant breeding line YYD (*Y*), and two bulks of F_2 plants generated from Girelle×YYD (*R*, homozygous resistant; *S*, homozygous susceptible). *Lanes 5 and 10* are 123-bp molecular-weight ladders, the *last lane* is a 100-bp molecular-weight ladder. Polymorphic fragments are *arrowed* and their sizes shown in bp

Table 3 Sequence of 24-mer oligonucleotide primers for the SCAR loci derived from RAPD markers linked to R17 and the polymorphism detected

Locus	Primer[a]	Sequence[b]	Polymorphism in Girelle×YYD[c]
SCS02	$OPS02A_{1470}$	CCTCTGACTGTTGTAGAAGCCCTG	Co-dominant after cutting with *Hae*III
	$OPS02B_{1470}$	CCTCTGACTGCCTCATATAGACGA	
SCT08	$OPT08A_{490}$	AACGGCGACAACTTGGACGACACT	Co-dominant after cutting with *Alu*I+*Mse*I
	$OPT082B_{490}$	AACGGCGACAAGATGATTTGGAGT	

[a] The third letter and the fourth and fifth numbers refer to the kit and primer number (Operon Technologies) used to identify the progenitor RAPD marker. The subscript number refers to the size in bp of the amplified RAPD product from cv Girelle
[b] The underlined sequences represent the sequences of the progenitor RAPD primers
[c] An annealing temperature of 60 °C was used for SCS02 and 65 °C for SCT08

peratures, each of these two SCARs amplified the same-sized band from both YYD and Girelle that was the same size as their progenitor RAPD bands. Cutting with restriction enzymes, *Hae*III for $SCS02_{1470}$ and *Alu*I+*Mse*I for $SCT08_{490}$ (annealing temperature 65 °C) revealed codominant alleles (Fig. 2). The inheritance of both SCARs was determined in the F_2 Girelle×YYD population, but only *SCT08* segregated in the F_2 Calmar×Kordaat population.

The segregation of four RAPD markers ($OPS02_{1470}$, $OPG02_{1100}$, $OPX04_{1300}$, $OPO03_{1900}$) and two SCARs (*SCS02* and *SCT08*) were determined for 133 F_2 individuals of Girelle×YYD. The recombination values and best-fit gene orders were determined using MAPMAKER (Fig. 3a). The markers identified by bulked segregant analysis were in a genetic window of 36 cM with R17 close to the middle. The closest markers were $OPX04_{1300}$ at 3.5 cM and *SCT08* at 6.2 cM from R17. Analysis of 57 F_2 individuals of Calmar×Kordaat with the four polymorphic markers indicated a different best order ($OPS02_{1470}$, $OPG02_{1100}$, $OPO03_{1900}$, *SCT08*). Analysis of the Girelle×YYD data revealed that this order was 10^6 less likely; while the two gene orders were not significantly different for the Calmar×Kordaat data. Therefore, the gene order computed from the more extensive Girelle×YYD data is probably correct. The genetic distance between $OPG02_{1100}$ and $OPO03_{1900}$ was approximately the same in both crosses.

To estimate the size of the genomic segment introgressed with R17, *L. serriola* LS102, YYD, and Girelle were compared for 26 RAPD and SCAR markers that mapped to the region. Twelve markers from $OPH04_{720}$ to $OPA05_{690}$ distinguished *L. serriola* LS102 from Girelle. Of these, YYD had the genotype of Girelle for $OPL13_{1100}$ to $OPN07_{1230}$ and for $OPB08_{1480}$. YYD had the *L. serriola* genotype for $OPS02_{1450}$ to $OPO03_{1900}$. Therefore, the cross-over points appear to have occurred between $OPN07_{1230}$ and $OPS02_{1450}$ and between $OPO03_{1900}$ and $OPB08_{1480}$, indicating that 36 to 75 cM was introgressed during the three backcrosses used to generate YYD.

Genetics of resistance in cv Mariska to *B. lactucae*

Segregation ratios of the F_2 Cobham Green×Mariska population for resistance to each of four isolates (NL1, Tv, KT2 and R60) were consistent with a single dominant locus (R18) for resistance (Table 2). Twenty-five F_3 lines from F_2 plants that were susceptible to R60 were homozy-

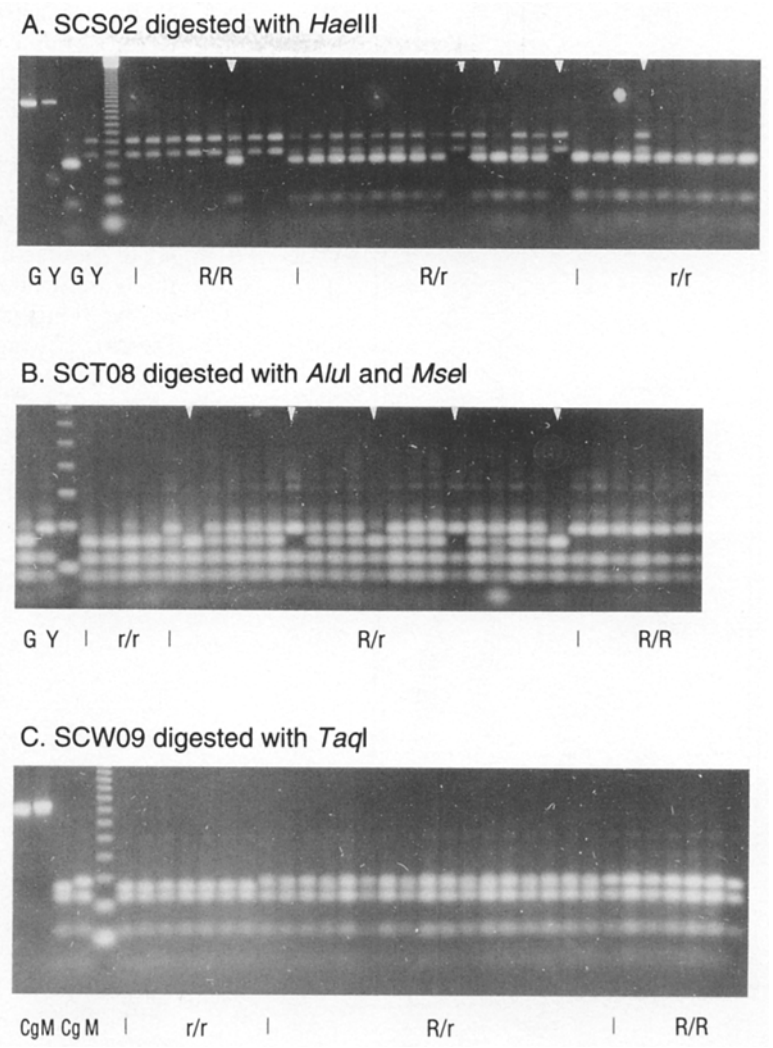

Fig. 2 A–C Amplification with SCAR primers of genomic DNA from two segregating populations. The 123-bp molecular-weight ladder, in either *lane 3* (**B**) or *lane 5* (**A** and **C**), separates the parental and progeny samples. **A** Segregation of *SCS02* in F_2 Girelle (*G*)×YYD (*Y*); *lanes 1 and 2* are uncut amplification products of -c_2 parental DNA; all other lanes have been digested with *Hae*III. **B** Segregation of *SCT08* in F_2 Girelle (*G*)×YYD (*Y*); amplification products were digested with *Alu*I and *Mse*I. **C** Segregation of *SCW09* in F_3 families from Cobham Green (*Cg*)×Mariska (*M*); *lanes 1 and 2* are uncut amplification products of parental DNA; all other lanes have been digested with *Taq*I. *R*, resistant allele; *r*, susceptible allele. Recombinants between the SCAR markers and the R factors are *arrowed*

gous susceptible to R60 and to CS9. Twenty-one F_3 families from the 58 F_2 plants that were resistant to R60 were homozygous resistant to R60 and CS9 and the remaining 37 families segregated for resistance to both isolates. In only two cases did the F_3 results not correlate with the F_2 phenotype. Experimental error may have caused one inconsistency (segregating F_3 from susceptible F_2). In the other family, the result was not clear: all of 147 F_3 seedlings were resistant to R60 but 11 out of 82 F_3 seedlings showed sparse sporulation and necrosis after inoculation with CS9. For analysis in MAPMAKER this family was scored as not homozygous susceptible.

Identification of markers linked to resistance in Mariska (R18)

The initial study of RAPD markers that had been previously mapped to the *Dm1/Dm3* cluster quickly identified polymorphism between the bulks of 13 homozygous resistant families and 19 homozygous susceptible families. Therefore, only markers linked to this cluster were analyzed further; four out of twenty RAPD primers tested detected polymorphism ($OPA08_{1720}$, $OPW09_{980}$, $OPY02_{1100}$, $OPV02_{1970}$) (Fig. 4). Also, three out of the six SCARs from this cluster (Paran and Michelmore 1993) were polymorphic (*SCI11*, *SCV12* and *SCW09*); codominance was revealed only after cutting the amplified fragment with restriction enzymes for two of these SCARs (*Mbo*I for *SCI11*, *Taq*I for *SCW09*) (Figs. 2 and 4).

The segregation of three RAPD markers ($OPY02_{1100}$, $OPA08_{1720}$, $OPW09_{980}$) and three SCARs (*SCW09*, *SCI11* and *SCV12*) was determined with DNA from 85 F_3 families from Cobham Green×Mariska. RFLP analysis of recombinant F_3 Cobham Green×Mariska families with probe CL922 was consistent with results using RAPD and SCAR primers; *CL922* cosegregated with $OPA08_{1720}$. The recombination values and best-fit gene orders were determined using MAPMAKER (Fig. 3b). The closest markers were *SCW09* at 0.6 cM and *SCI11* at 3.1 cM from R18. Analysis of F_2 Calmar×Kordaat with these markers gave a slightly different order. Comparison of the relative LOD scores for the two gene orders on both data sets indicated that the two gene orders were significantly different in

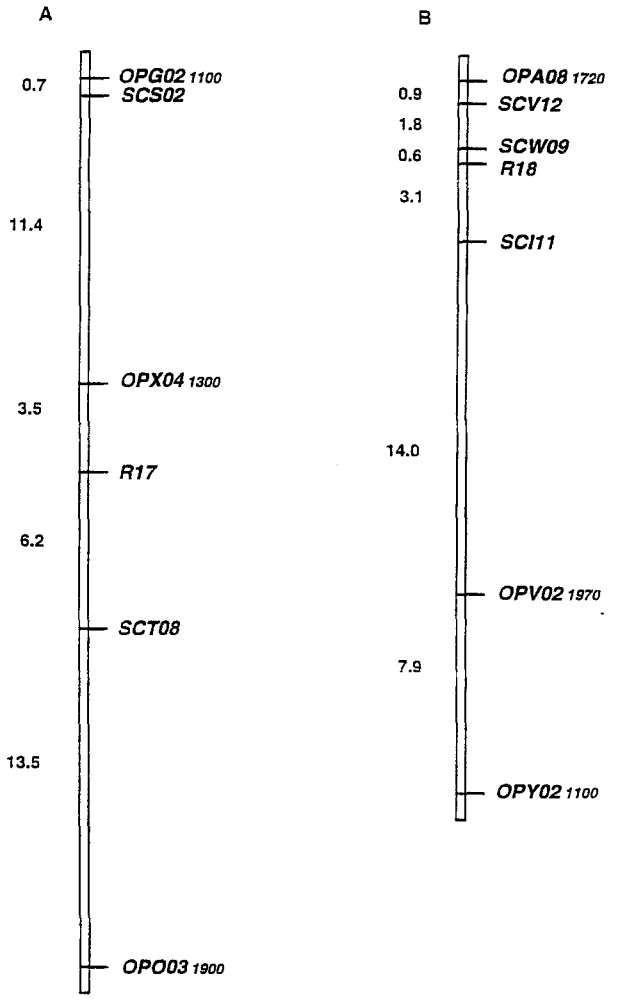

Fig. 3 Genetic map of the regions around R17 (**A**) and R18 (**B**). Genetic distances are shown in cM to the left. The map around R17 was generated from the analysis of Girelle×YYD and that around R18 from the analysis of Cobham Green×Mariska

each case (difference in LOD scores=6 for Cobham Green×Mariska and 3 for Calmar×Kordaat); whether this reflects real differences in gene order, or is an experimental artifact, requires further study.

To investigate the size of the genomic segment introgressed with R18, *L. serriola* LS17, Mariska and the four butterhead cultivars in the pedigree of Mariska were analyzed for 20 RAPD and SCAR markers in the region. For six of these markers, LS17 was identical to one or more of the butterhead cultivars and they were therefore uninformative. Only three markers, $OPH13_{980}$, $OPD18_{580}$, and $OPA20_{480}$, were the same in Mariska and the butterhead cultivars. All other informative markers were the same between LS17 and Mariska; therefore these markers defined the minimum limits of the introgressed region. There were no PCR-based markers in the 120 cM interval between $OPA07_{370}$ and $OPD18_{580}$, so that the position of the cross-over point cannot be determined accurately. Between 29 and 120 cM has been introgressed from *L. serriola* into Mariska.

Discussion

Our results indicate that the resistances in YYD and Mariska to *B. lactucae* are determined by single dominant loci. The resistance in YYD was monogenic with isolates 49/83 and Tv is, therefore, a new resistance locus. The resistance in Mariska was also monogenic with isolates R60 and Tv, therefore, is again a new resistance locus. As these resistances map to different genomic positions, they must be different loci. We have designated these loci R17 and R18 respectively. The R17 designation has been used once before and *Dm17* has not been assigned (Farrara et al. 1987); however, the previous use of R17 was restricted to our laboratory and has never been in common use. Therefore,

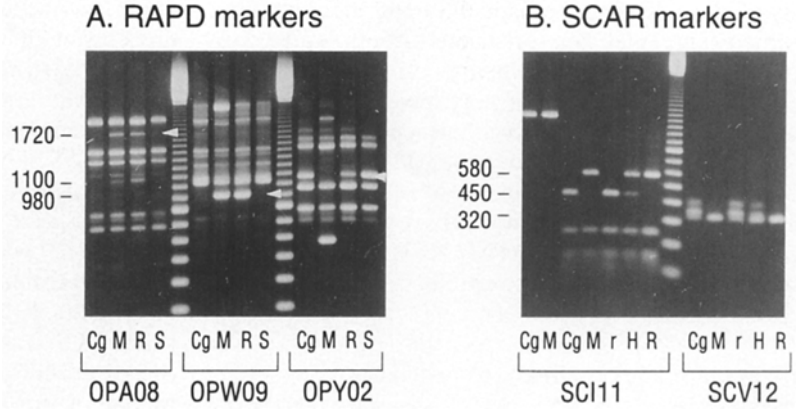

Fig. 4 A, B PCR-based markers linked to R18 for resistance to downy mildew. **A** Amplification with three primers (OPA08, OPW09, OPY02) of genomic DNA from susceptible cv Cobham Green (*Cg*), resistant cv Mariska (*M*) and two bulks of F_3 families generated from Cobham Green×Mariska (*R*, homozygous resistant; *S*, homozygous susceptible). Polymorphic fragments are *arrowed* and their sizes shown in bp. **B** Amplification with two SCARs (SCI11: uncut in *lanes 1 and 2*, or cut with *Mbo*I; SCV12: uncut) of genomic DNA from cv Cobham Green (*Cg*), cv Mariska (*M*) and three individuals F_3 families (*r*, homozygous susceptible; *H*, heterozygous; *R*, homozygous resistant). The molecular-weight marker is the 100-bp ladder (*lanes A5, A10 and B8*)

we are reusing this designation to minimize the numbers employed. R18 has been commonly used for the resistance in Mariska. Our data for R18 confirm the recent classical genetic analysis of Bonnier et al. (1994).

We have termed the newly-discovered resistances as resistance factors (R#) rather than assigning them a formal *Dm* gene designation because we have yet to demonstrate that these resistances are determined by single genes rather than multiple tightly-linked genes. Resistance genes tend to be clustered in the genome (Farrara et al. 1987; Kesseli et al. 1993) and a monogenic segregation ratio is consistent with either a single gene or a cluster of linked genes. Our data unambiguously place R18 in a 3.7 cM interval between *SCW09* and *SCI11*. Therefore, if R18 is two genes, they must be tightly linked.

One method to determine the numbers of genes determining specificity is to study the genetics of virulence in the pathogen (Farrara et al. 1987). As avirulence genes tend to be unlinked, segregation of the corresponding avirulence genes will tend to reveal the number of resistance genes determining specificity in a particular line. However, this approach is experimentally laborious and requires at least one isolate that is virulent on the new resistance; this is often not the case for newly-discovered resistances that have been selected to provide resistance against all known isolates. For R17, we observed only sparse and late sporulation in some tests with one isolate, NL16; but the irregularity of this reaction obstructs segregation analysis of the progeny between NL16 and a fully-avirulent isolate. For R18, segregation analysis of avirulence in *B. lactucae* will also be difficult because the virulence of isolate 49/83 on R18 seems unstable. Continual multiplication on Mariska is necessary to maintain profuse sporulation on Mariska; after several multiplications of 49/83 on a variety without R18 the sporulation on Mariska becomes sparse. As other isolates that are fully virulent on Mariska become available, such studies will be possible.

An alternative, although slightly less rigorous, approach to determine gene number relies on detailed recombination analysis of the region containing the resistance. If many recombinants are analyzed and the resistance cannot be broken into multiple resistance genes, then the resistance can be considered to be a single gene or else to involve extremely tightly-linked genes, and the use of the *Dm* designation is probably warranted. However, this approach requires tightly-linked flanking markers and the identification and characterization of numerous recombinants with a number of isolates. In an attempt to dissect R18, we have started to analyze the reaction of recombinants between markers SCW09 and SCI11 to many isolates. Therefore, *Dm17* or *Dm18* will only be assigned after further genetic analysis of either many recombinants within these regions or progenies from crosses between fully-virulent and avirulent isolates of *B. lactucae*.

Bulk segregant analysis (BSA) was an efficient method to map new resistance genes and identify new markers. For the resistance in YYD, the initial screening of the two bulks of homozygous plants with 230 random primers identified five linked markers; three of these markers (*OPS02*, *OPX04*, *OPO03*) could be mapped within a window of 17 to 20 cM either side of R17; the two other linked markers (*OPT08* and *OPR13*) were difficult to score reliably. These results are consistent with the sensitivity of the BSA determined by artificial mixtures of two DNA samples (Michelmore et al. 1991). It was important to identify the homozygous resistant plants because four markers (*OPG02*, *OPR13*, *OPS02*, $OPT08_{490}$) were in repulsion with R17; if a bulk of susceptible plants had been compared to a bulk of all resistant F_2 plants, these markers would not have been identified. The partial resistance observed with high inoculum levels and young seedlings distinguished heterozygous and homozygous resistant F_2 plants, allowing only homozygous plants to be bulked immediately without waiting for the production and testing of F_3 families.

BSA can very quickly locate genes that map to an existing cluster of genes. R18 was known to be linked to six markers after screening the two bulks with only twenty RAPD primers and six SCARs. Therefore, it is realistic to map some new resistance genes over a period of only a few weeks. The time-consuming step is the generation of the segregating population and the screening for resistance. During such analyzes, it is necessary to screen the parental lines to determine which markers are informative. Only 12 out of 30 RAPD or SCAR markers surrounding the *Dm1/Dm3* region from the Calmar×Kordaat map were polymorphic between Cobham Green and Mariska. Similarly, only four out of twenty-three markers in the *Dm5/8* region were polymorphic between Girelle and YYD.

Using RAPD markers to map a gene requires the scoring of only those bands that are experimentally reproducible and easy to identify. Several potential RAPD polymorphisms were identified between the bulks that were not subsequently useful. This was not a major problem because of the large number of potential markers that we could screen. The conversion of RAPD markers into SCARs increased the reliability of the markers. $OPT08_{490}$ was difficult to score but *SCT08* was easy to analyze in both the F_2 Girelle×YYD and Calmar×Kordaat populations. An additional advantage of SCAR markers is the possibility of codominance. After trying eight endonucleases, it was possible to distinguish alternate alleles for four out of five SCARs used in this study. The polymorphisms for SCARs with Cobham Green and Mariska differed from those identified for Calmar and Kordaat (Paran and Michelmore 1993); in the case of *SCI11*, *Rsa*I detected polymorphism between Calmar and Kordaat while *Mbo*I was used for Cobham Green and Mariska. Polymorphism could not be detected for *SCW09* between Calmar and Kordaat. Because half of our loci were codominant, due to the scoring of resistance in F_3 families and the use of SCARs, our data were more informative than if based on RAPD markers alone.

Our results provide further evidence for the clustering of resistance genes in *Lactuca* spp. R17 is in the same cluster as *Dm5/8* and *Dm10* as well as genes for resistance to two other pathogens (*plr* and *Tu* Kesseli et al. 1993; Robins et al. 1994); R18 is in a cluster with seven other *Dm* genes (*Dm1*, *Dm2*, *Dm3*, *Dm6*, *Dm14*, *Dm15*, *Dm16*) as well as resistance to root aphid (*Ra*). As both R17 and R18

were recently independently derived from wild species, the data suggest that the clustering of resistance genes is not an artifact of plant breeding introgressing blocks of resistance genes. Detailed genetic maps were established for the both areas of the lettuce genome containing these new resistances. Both R17 and R18 occupy unique positions relative to linked markers indicating that they are not alleles of any of the *Dm* genes that have been analyzed in detail so far.

The generation of SCARs on both sides of these new resistances provides the opportunity to use them as indirect markers in breeding programs. Although it is easy to screen for resistance using isolates of *B. lactucae*, it will be possible with markers to combine R17 with other *Dm* genes; this was not previously possible as there is no isolate that is virulent on R17 and therefore allows detection of other *Dm* genes. Also, it will be possible to use markers to identify recombinants that carry the new resistances in *cis* with known resistance genes. Resistance can then be manipulated as a single Mendelian unit. Identification of such recombinant individuals would be very difficult without markers that identify each chromosome.

Acknowledgments We wish to acknowledge H. Bannerot who started the program at INRA using resistance from *L. serriola*. We are grateful to B. Deffontaine and F. Favre for technical assistance with the downy mildew tests made at INRA, O. Ochoa for taking care of the plants at Davis, and R. Kesseli for help with the genetic analysis. We gratefully acknowledge the financial support to INRA provided previously by Societe Vilmorin for the generation of the YYD line. We thank Operon Technologies (Alameda, Calif.) for supplying the oligonucleotide primers. The studies at UC Davis were supported by USDA grant 91-37300-6364.

References

Bernatzky R, Tanksley SD (1986) Genetics of actin-related sequences in tomato. Theor Appl Genet 72:314–321

Bonnier FJK, Reinink K, Groenwold R (1992) New sources of major gene resistance in *Lactuca* to *Bremia lactucae*. Euphytica 61:203–211

Bonnier FJK, Reinink K, Groenwold R (1994) Genetic analysis of *Lactuca* accessions with new major gene resistance to lettuce downy mildew. Phytopathology 84:462–468

Crute IR (1987) The occurrence, characteristics, distribution, genetics, and control of a metalaxyl-resistant pathotype of *Bremia lactucae* in United Kingdom. Plant Disease 71:763–767

Crute IR (1992a) The role of resistance breeding in the integrated control of downy mildew (*Bremia lactucae*) in protected lettuce. Euphytica 63:95–102

Crute IR (1992b) Downy mildew of lettuce. In: Chaube HS, Kumar J, Mukhopadhyay AN, Singh US (eds) Plant diseases of international importance. Prentice Hall, Englewood Cliffs, New Jersey, pp 165–185

Crute IR, Johnson AG (1976) The genetic relationship between races of *Bremia lactucae* and cultivars of *Lactuca sativa*. Ann Appl Biol 83:125–137

Eenink AH, Dejong CJ (1982) Partial resistance in lettuce to downy mildew (*Bremia lactucae*). 3. Correspondence between resistance levels of cotyledons and leaf discs and resistance of adult plants. Euphytica 31: 761–769

Farrara BF, Michelmore RW (1987) Identification of new sources of resistance to downy mildew in *Lactuca* spp. HortScience 22:647–649

Farrara BF, Ilott TW, Michelmore RW (1987) Genetic analysis of factors for resistance to downy mildew (*Bremia lactucae*) in species of lettuce (*Lactuca sativa* and *L. serriola*). Plant Pathol 36:499–514

Hulbert SH, Michelmore RW (1985) Linkage analysis of genes for resistance to downy mildew (*Bremia lactucae*) in lettuce (*Lactuca sativa*). Theor Appl Genet 70:520–528

Ilott TW, Hulbert SH, Michelmore RW (1989) Genetic analysis of gene-for-gene interaction between lettuce (*Lactuca sativa*) and *Bremia lactucae*. Phytopathology 79:888–897

Johnson AG, Laxton SA, Crute IR, Gordon PL, Norwood JM (1978) Further work on the genetics of race-specific resistance in lettuce (*Lactuca sativa* L.) to downy mildew (*Bremia lactucae*). Ann Appl Biol 89:257–264

Kesseli RV, Witsenboer H, Vandemark GJ, Stangellini ME, Michelmore RW (1993) Recessive resistance to *Plasmopara lactucae-radicis* maps by bulked segregant analysis to a cluster of dominant resistance genes in lettuce. Mol Pl Microbe Interact 6:722–728

Kesseli RV, Paran I, Michelmore RW (1994) Analysis of a detailed genetic linkage map of *Lactuca sativa* constructed from RFLP and RAPD markers. Genetics 136:1435–1446

Lander ES, Green P, Abrahamson J, Barlow A, Daly MJ, Lincoln SE, Newburg L (1987) MAPMAKER: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. Genomics1:174–181

Landry BS, Kesseli RV, Farrara B, Michelmore RW (1987) A genetic map of lettuce (*Lactuca sativa* L.) with restriction fragment length polymorphism, isozyme, disease resistance, and morphological markers. Genetics 116:331–337

Leroux P, Maisonneuve B, Bellec Y (1988) Detection en France de souches de *Bremia lactucae*, agent du mildiou de la laitue, resistantes au metalaxyl et a l'oxadixyl. Pépiniéristes, Horticulteur, Maraîchers, Revue Horticole 292:37–40

Michelmore RW (1981) Sexual and asexual sporulation in the downy mildews. In: Spencer DM (ed) The downy mildews, pp 165-181

Michelmore RW, Paran I, Kesseli RV (1991) Identification of markers linked to disease-resistance genes by bulked segregant analysis: a rapid method to detect markers in specific genomic regions by using segregating populations. Proc Natl Acad Sci USA 88:9828–9832

Norwood JM, Johnson AG, O'Brien MJ, Crute IR (1985) The inheritance of field resistance to lettuce downy mildew (*Bremia lactucae*) in the cross Avoncrisp×Iceberg. Plant Breed 94:259–262

Paran I, Michelmore RW (1993) Development of reliable PCR-based markers linked to downy mildew resistance genes in lettuce. Theor Appl Genet 85:985–993

Paran I, Kesseli RV, Michelmore RW (1991) Identification of restriction fragment length polymorphism and random amplified polymorphic DNA markers linked to downy mildew resistance genes in lettuce using near-isogenic lines. Genome 34:1021–1027

Robins MA, Witsenboer H, Michelmore RW, Laliberte J-F, Fortin MG (1994) Mapping of turnip mosaic virus resistance in lettuce. Theor Appl Genet (in press)

Schettini TM, Legg EJ, Michelmore RW (1991) Insensitivity to metalaxyl in California populations of *Bremia lactucae* and resistance of California lettuce cultivars to downy mildew. Phytopathology 81:64–69

Williams JGK, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res 18:6531–6535