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A genetic map of the lettuce downy mildew pathogen, *Bremia lactucae*, constructed from molecular markers and avirulence genes

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Abstract

The genetic map of *Bremia lactucae* was expanded utilizing 97 F_1 progeny derived from a cross between Finnish and Californian isolates (SF5 × C82P24). Genetic maps were constructed for each parent utilizing 7 avirulence genes, 83 RFLP markers, and 347 AFLP markers, and a consensus map was constructed from the complete data set. The framework map for SF5 contained 24 linkage groups distributed over 835 cM; the map for C82P24 contained 21 linkage groups distributed over 606 cM. The consensus map contained 12 linkage groups with markers from both parents and 24 parent-specific groups. Six avirulence genes mapped to different linkage groups; four were located at the ends of linkage groups. The closest linkages between molecular markers and avirulence genes were 3 cM to *Avr4* and 1 cM to *Avr7*. Mating type seemed to be determined by a single locus, where the heterozygote determined the B_2 type and the homozygous recessive genotype determined the B_1 type.

Keywords: *Bremia lactucae*; Oomycete; Plant pathogen; Downy mildew; Linkage map; Mating type; Avirulence gene; AFLP; RFLP

1. Introduction

Oomycete phytopathogens, which include the biotrophic downy mildews and many species of *Phytophthora* and *Pythium*, are responsible of significant losses of food crops and ornamental plants world-wide. The genetic and molecular bases of interactions between plant and Oomycete pathogens remain largely unknown. Although these organisms exhibit a filamentous growth, biochemical, and phylogenetic analyses have demonstrated that oomycetes are distinct from the main fungal groups and are more closely related to golden-brown and heterokont algae in the kingdom Protista (Kamoun, 2001; Sogin and Silberman, 1998). Because of their distinct origin, oomycete plant pathogens may have distinct molecular mechanisms for interacting with plant hosts than the true fungi (Kamoun, 2001). However, oomycetes and fungal plant pathogens exhibit similar modes of parasitism that suggest convergent evolution (Tyler, 2001). Therefore, comparing the molecular genetics of oomycete and fungal plant pathogens could identify common functions in plant parasitism.

Classical genetic analyses have shown resistance to oomycetes can be mediated by gene-for-gene interactions in which the plant carrying a particular resistance gene (R gene) is resistant to a pathogen expressing a matching avirulence (*Avr*) gene (Flor, 1956; Tyler, 2001). Within the hemi-biotrophic *Phytophthora* spp., seven single dominant avirulence loci corresponded to seven R genes in the interaction between *Phytophthora sojae* and soybean (Cregan et al., 1999; MacGregor et al., 2002; Tyler et al., 1995; Whisson et al., 1994, 1995) and 12 single dominant avirulence loci corresponded to 11 R genes in the interaction between *Phytophthora*

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infestans and potato (Al-Kherb et al., 1995; Judelson, 1996a). Within the obligately biotrophic downy mildew, *Bremia lactucae*, over 14 *Avr* loci have been identified as detailed below.

Despite their economic importance, few genetic maps have been made for oomycete species. This is in part due to the difficulties in experimental manipulation such as the construction of controlled crosses and germination of the sexual spore as well as the biotrophic mode of nutrition of the downy mildews. A genetic map with 21 major and 7 minor linkage groups was constructed from *P. sojae* and comprised 35 RFLP, 236 RAPD, 105 AFLP, and 10 avirulence genes (May et al., 2002; Whisson et al., 1995). A genetic map for *P. infestans* comprising of 17 linkage groups has been developed based on 183 AFLP markers (van der Lee et al., 1997). Ten avirulence genes have been mapped in *P. sojae* and six mapped in *P. infestans* (May et al., 2002; van der Lee et al., 2001). In contrast to most avirulence genes mapped in true fungi, three pairs of *Avr* genes in *P. sojae* and three *Avr* genes in *P. infestans* were clustered. Bulked segregant analysis (Michelmore et al., 1991) has been used to identify additional molecular markers linked to avirulence genes in *P. sojae* and *P. infestans* (MacGregor et al., 2002; van der Lee et al., 2001) and allowed the identification of 10 AFLP markers defining a genetic mapping interval and physical contig spanning an avirulence locus in the downy mildew, *Peronospora parasitica* (Rehmany et al., 2003). No genome-wide genetic map for a downy mildew has been reported except for the preliminary genetic map of *B. lactucae* (Hulbert et al., 1988).

Recently, significant progress has been achieved on the genetic and molecular basis of resistance of plants to oomycete pathogens. Several resistance genes (R genes) against downy mildews *P. parasitica* (several *RPP* genes) and *B. lactucae* (*Dm3*) and against *P. infestans* (*R1*) have been cloned (Ballvora et al., 2002; Bittner-Eddy et al., 2000; Botella et al., 1998; McDowell et al., 1998; Meyers et al., 1998; Parker et al., 1997; Shen et al., 2002). They are members of clustered multigene families and encode receptor-like proteins that contain a nucleotide binding site and leucine rich repeats. Three subclasses of R genes that are effective against oomycetes are known and can be distinguished by their N-terminal regions. The *RPP1* and *RPP5* genes in *Arabidopsis* have homology to the toll-interleukin receptor (TIR) domain; the *RPP8* and *RPP13* genes in *Arabidopsis* and the *R1* gene in potato has a N-terminal, coiled-coil motif; while in lettuce the *Dm3* gene has little obvious N-terminal homology. There is also a growing understanding of the signaling pathways and downstream plant genes and proteins that are involved in resistance (Dangl and Jones, 2001; Glazebrook, 2001). In contrast to the data from the plant host, our knowledge of genome structure and of avirulence gene function and evolution in oomycetes remains poor. Several map-based projects are underway to clone avirulence genes from oomycetes (reviewed in MacGregor et al., 2002; Tyler, 2001). The only report of cloning avirulence genes is the isolation of two genes required for resistance determined by the *Rps1b* gene in soybean that have been identified in *P. sojae* (Tyler, 2001).

Bremia lactucae is the causal agent of lettuce downy mildew. It is an obligate biotroph that can only be cultured on living lettuce plants. The existence of both asexual and sexual reproductive cycles has allowed several genetic studies. *B. lactucae* is diploid for the majority of its lifecycle and is predominantly heterothallic (Michelmore and Ingram, 1981a, 1982; Michelmore and Sansome, 1982). Mating type seems to be determined by two alleles at a single locus with one compatibility type (B_1) being conferred by a homozygous recessive condition and the other (B_2) by a heterozygous condition. Simultaneous genetic studies on resistance in lettuce and virulence in *B. lactucae* revealed 13 dominant genes for resistance (*Dm* genes) matched by 14 avirulence genes (*Avr* gene), avirulence to *Dm11* being determined by two independent genes (Farrara et al., 1987; Ilott et al., 1989; Michelmore et al., 1984; Norwood et al., 1983; Norwood and Crute, 1984). Many additional sources of resistance are known but have yet to be characterized genetically in parallel with pathogen virulence (Bonnier et al., 1992, 1994). Avirulence genes were all inherited independently except two, *Avr5/8* and *Avr10*, which seemed to be linked (Michelmore et al., 1984). Cytological analysis of *B. lactucae* resolved at least 7 or 8 chromosome pairs at meiosis (Michelmore and Sansome, 1982). Pulse field gel electrophoresis revealed a minimum of 7 chromosomes between 3 and at least 8 Mb and a set of linear polymorphic molecules between 300 kb and 1.6 Mb (Francis and Michelmore, 1993). The physical genome size was estimated to be 50 Mb with reassociation kinetics, genomic reconstruction, and CHEF gel electrophoresis (Francis et al., 1990; Francis and Michelmore, 1993). With Feulgen absorbance cytophotometry, the estimated genome size varied from 70 to 144 Mb depending on the isolate of *B. lactucae* (Voglmayr and Greilhuber, 1998). Of the nuclear DNA, 65% is comprised of repeated sequences and low-copy sequences are interspersed with repeated sequences (Francis et al., 1990). RFLP analysis revealed high levels of heterozygosity and significant polymorphism between isolates from around the world (Hulbert and Michelmore, 1988).

A preliminary genetic linkage map of *B. lactucae* was constructed to define the genomic organization of avirulence genes and identify molecular markers linked to these genes (Hulbert et al., 1988). This investigation involved segregation analysis of 53 RFLP loci, 8 *Avr* loci, and the mating type locus in a total of 70 F_1 individuals from two crosses (38 progeny from isolates

SF5 × C82P24; 32 progeny from SF5 × IMOS6b). The resulting map consisted of 13 small linkage groups, including 35 RFLP loci and one *Avr* gene. Construction of a more detailed genetic map was hindered by the ambiguous phase of the alleles in the parents and an insufficient number of markers due to the marker technology available at that time. The aim of the current study was to develop a more comprehensive genetic map of *B. lactucae* predominantly using PCR-based markers to facilitate the cloning of *Avr* genes and gain a better understanding of genome organization and variability. The more heterozygous of the two crosses that had been previously used for mapping (SF5 from Finland × C82P24 from California) was expanded more than twofold by the addition of 59 individuals to give a total of 97 F_1 progeny to facilitate the identification of the phase of the alleles in each parent and improve the detection of linkage. Additional RFLP markers were also identified from several distinct DNA libraries to increase the efficiency of identification of informative probes. This provided a total of 83 RFLP markers and 347 AFLP markers to construct a genetic map including six *Avr* genes and the mating type locus.

2. Materials and methods

2.1. Isolates of B. lactucae, mating and virulence phenotyping

A preliminary map had been developed from segregation analysis of markers that included 38 F_1 individuals of the cross between SF5 × C82P24 (Hulbert et al., 1988). These parental isolates from distinct geographical areas had been selected to maximize the number of segregating avirulence loci and marker polymorphisms (Hulbert et al., 1988). Isolate SF5 is a Finnish isolate of the B_1 mating type (Osara and Crute, 1981). Isolate C82P24 is a Californian isolate of the B_2 mating type (Ilott et al., 1987). Maintenance and storage of isolates, determination of virulence phenotype and mating type of progeny were as described previously (Hulbert et al., 1988; Ilott et al., 1987; Michelmore and Ingram, 1980; Michelmore and Crute, 1982; Michelmore et al., 1984).

In the current study, the number of F_1 progeny from this cross was expanded to a total of 97 isolates. The parental isolates, SF5 and C82P24 were rescued from storage at −80 °C, cultured on cv. Cobham Green that expresses no known *Dm* genes, then inoculated onto the differential series of resistance cultivars to confirm their virulence phenotype. These isolates were then co-inoculated onto cv. Cobham Green to generate the sexual oospore. Oospores were allowed to mature for several weeks in decaying plant tissue and macerated to produce a fine suspension. Isolation of sexual progeny from crosses was achieved as described previously (Michelmore and Ingram, 1981b) by growing lettuce seedlings cv. Cobham Green in a dilute suspension of oospores in GA-7 boxes (Magenta, Chicago, IL). The rate of oospore germination was determined empirically by assessing the number of infected seedlings resulting from a serial dilution of the oospore suspension; the suspension of oospores was then diluted so that on average there was one infected seedling per GA-7 box. Each infected seedling was considered to have arisen from a single oospore and represent an individual sexual progeny. Progeny isolates were propagated asexually on seedlings of the susceptible lettuce cultivar Cobham Green. Virulence phenotypes were characterized by the ability to sporulate on a differential set of 24 resistant lettuce cultivars selected to include all characterized *Dm* genes (*Dm1*, *Dm2*, *Dm3*, *Dm4*, *Dm5/8*, *Dm6*, *Dm7*, *Dm10*, *Dm11*, *Dm12*, *Dm13*, *Dm14*, *Dm15*, *Dm16*, and *R18*); each cultivar contained one or two known *Dm* genes. Each isolate was tested on at least 20 seedlings of each cultivar that represent independent tests. Mating type was identified by pairing the isolate on susceptible lettuce cotyledons with known isolates of either mating type B_1 or B_2.

2.2. DNA isolation

For RFLP analysis, DNA was isolated using a procedure modified from Hulbert et al., 1988. Nuclei were obtained as described in Hulbert et al. (1988). Nuclei were lysed in a solution of 1% *N*-laurylsarcosine and ribonuclease A, incubated at 60 °C for 30 min to 1 h, followed by 30 min with 2 U/ml proteinase K. The insoluble carbohydrates were pelleted by centrifugation at 10,000*g* for 15 min and the lysate extracted with phenol/chloroform. The DNA was precipitated in ethanol, spooled out on a glass micro-pipet, and resuspended in TE buffer (10 mM Tris–Cl and 1 mM EDTA, pH 8.0).

For AFLP analysis, a more rapid DNA extraction was utilized. The cotyledons exhibiting profuse sporulation (between 7 and 9 days after inoculation) were placed in cold ddH_2O and agitated gently to remove the conidia from the cotyledons. The suspension of conidia was filtered through a 35 μm sieve (CellMicroSieve, BioDesign, NY) and kept on ice. After centrifugation for 5 min at 1000*g* to pellet the conidia, the supernatant was removed and conidia resuspended in 5 ml cold ddH_2O. After a second centrifugation for 5 min at 2400 rpm 1000*g*, the supernatant was removed and the pellet resuspended in 250 μl of 2× CTAB extraction buffer (2% hexadecyltrimethyl ammonium bromide, 100 mM Tris, pH 8.0, 20 mM EDTA, pH 8.0, 1.4 M NaCl, 1% polyvinylpyrolidone M_r 40,000). The solution was transferred to a microfuge tube containing approximately 200 μl of 425–600 μm glass beads (SIGMA, St Louis, MN) that had been previously treated with Rainex (Unelko, Scottsdale, USA). The mix of beads

and conidia were shaken at maximum speed (Tomy microtube-mixer MT-360, Tomy Seiko, Japan) for 15 min to break open the conidia. The resulting suspension of nuclei was transferred to a fresh microfuge tube. The beads were washed with an additional 150 µl of 2× CTAB buffer that was added to the suspension already transferred. Nuclei were lysed by incubation for 30 min at 65 °C. DNA was purified using a phenol/chloroform extraction followed by an ethanol precipitation. The same rapid DNA extraction process was conducted on seedlings that had not been inoculated with *B. lactucae* to obtain DNA from contaminating microorganisms as a control. DNA was also extracted from the cultivar Cobham Green using a modified CTAB procedure to obtain control DNA from the plant cultivar on which the *B. lactucae* had been cultured (Bernatsky and Tanskley, 1986).

2.3. RFLP probes

Three genomic libraries were used as sources of RFLP probes: (1) Genomic DNA of *B. lactucae* was digested with *Mbo*I, *Bam*H1, or *Eco*RI and cloned into the appropriate cloning site of pUC13 (Hulbert et al., 1988). (2) *Pst*I-digested genomic DNA of *B. lactucae* was size-selected for 0.5–1.5 kb fragments on a sucrose gradient (Sambrook et al., 1989) and ligated into pUC19. (3) A small chromosome-specific library was created from a 4.2 Mb fragment resolved by pulsed field gel electrophoresis (PFGE) (Francis and Michelmore, 1993). The band was cut from an agarose gel, digested with *Bam*HI and ligated into the *Bam*HI–*Sal*I sites of pUC19. High copy number genomic clones in each genomic DNA library were identified by reverse Southern hybridization to ^{32}P labeled total genomic DNA (Landry et al., 1987) and discarded.

RFLP probes were also generated from two cDNA libraries: (1) a library derived from poly(A) RNA of conidia of *B. lactucae* (Hulbert et al., 1988). (2) A library derived from poly(A) enriched RNA isolates from germinating conidia of *B. lactucae* (Judelson and Michelmore, 1989). Poly(A) RNA of germlings was chosen because of increased transcription relative to the limited synthesis in conidia. Both the conidial and germling cDNA clones required prescreening to eliminate highly abundant mRNAs that comprise ≈20% of the library (Hulbert et al., 1988). Abundant messages in the germling library had been characterized as pHAM families (Judelson and Michelmore, 1989), representatives of which were combined and used to eliminate most redundant clones from the germling library.

Restriction digests, electrophoresis, blotting, nick-translation, and hybridization to radiolabelled probe were done as described by Hulbert et al. (1988). In order to identify those combinations of probes and enzymes that detected RFLPs, DNA of parental isolates and DNA from eight random progeny were digested with each of the seven restriction enzymes (*Bam*HI, *Dra*I, *Eco*RI, *Hin*dIII, *Pst*I, and *Sst*I).

2.4. AFLP analysis

AFLP protocol was adapted from that described by Vos et al. (1995). A total of 250 ng of DNA was digested with 5 U *Eco*RI, 5 U *Mse*I in 50 µl restriction–ligation buffer (10 nM Tris–HAc, pH 7.5, 10 mM MgAc, 50 mM KAc, 5 mM DTT, and 50 ng/µl BSA) at 37 °C for 3 h. Following restriction, 50 pmol *Mse*I oligonucleotide adapter, 5 pmol of *Eco*RI oligonucleotide adapter, 0.2 mM ATP, 5 U of *Eco*RI, 5 U *Mse*I, and 1 U T4 DNA-ligase in 10 µl restriction-ligase buffer were added to the template DNA and incubated at 37 °C for 3 h. Five microliter of the template DNA was mixed with 30 ng of primers complementary to the *Eco*RI and *Mse*I adapters (E + 0 and M + 0 primers), 0.2 mM dNTPs, and 1 U *Taq* DNA polymerase in 15 µl of 1× AFLP buffer (10 mM Tris–HCl pH 8.3, 1.5 mM $MgCl_2$, and 50 mM KCl). PCR amplification consisted of 30 cycles of 30 s at 94 °C, 30 s at 60 °C, and 1 min at 72 °C. These first-round amplification products were diluted 8× and 5 µl of the diluted template DNA was used for the second amplification. The second amplification was conducted as described by van der Lee et al. (1997) using primers with two selective 3′ nucleotides (E + 2 and M + 2 primers; Table 2). In order to confirm the reliability of the markers, the pattern obtained with two selective bases on the *Eco*RI primer (E + 2) and two selective bases on the *Mse*I primer (M + 2) was compared with the pattern obtained using the four E + 2/M + 3 primer combinations from independent restriction/ligation and amplification procedures. As expected the fragments obtained with E + 2/M + 2 primer combination represented the sum of the fragments obtained using the four M + 3 primers.

2.5. Pulse field gel electrophoresis

High molecular weight DNA was prepared for pulse field gel electrophoresis from conidia of the two parents SF5 and C82P24 as described in Francis and Michelmore (1993). The molecular karyotype was resolved by clamped homogeneous electric field (CHEF) electrophoresis (Orbach et al., 1988). The electrophoresis was conducted through 0.8% Nusieve GTC agarose (FMC Bioproducts, Rockland, MA, USA) in 0.5× TBE running buffer. Initial Southern blots were generated following electrophoresis of the high molecular weight DNA for 6 days as described in Francis and Michelmore (1993). This resulted in a pattern of four bands: the largest fragments remained in the well, the 7 and 6 Mb fragments were not resolved from each other, and the 4.0 and 3.5 Mb fragments also co-migrated (Francis and

Michelmore, 1993). Subsequent blots were generated by electrophoresis over an 8-day period. The conditions were altered to include longer pulse times early in the course of the electrophoresis to allow optimal conditions for resolution of larger size molecules. Under these conditions, five bands were resolved, excluding the 10 Mb band and separation of the 4.0 and 3.5 Mb bands (Francis and Michelmore, 1993). Size markers consisted of two lines of *Saccharomyces cerevisiae* (PYB and YPH149 with synthetic chromosomes of 90 kb–2.2 Mb) and *Schizosaccharomyces pombe* strain SP2 (3.7, 4.7, and 5.7 Mb). DNA was transferred to Hybond-N membrane (Amersham Biosciences, Piscataway, USA). The DNA was immobilized by incubation at 80 °C for 30 min. Blots were pre-hybridized and hybridized in a $NaPO_4$-based solution containing 7.0% SDS with a sample of the same RFLP probes that used for genetic analysis.

2.6. Data analysis and map construction

For RFLP markers, each fragment was considered as an allele and five types of segregating patterns were observed: ab × aa (or aa × ab), ab × ab, ab × ac, ab × cc (or cc × ab), and ab × cd. Each RFLP marker was designated by the library of origin of the probe and the name of the probe as well as the restriction enzyme used to identify the polymorphism. The first letter of each RFLP marker indicates whether the probe was a genomic clone (G), a size-selected *Pst*I-digested genomic clone (Gp), a cDNA clone from conidia (C), a cDNA clone from germlings (Cg), or a chromosome-specific clone (K). The restriction enzyme was indicated by a single letter code (http://bremiadb.ucdavis.edu).

AFLP markers exhibited two segregation patterns. When an amplification product was found in only one parent and segregated as a presence/absence polymorphism in the progeny, the parental genotypes were assumed to be $Aa \times aa$ or $aa \times Aa$. When an amplification product was found in both parents and segregated in the progeny, the parental genotypes were assumed to be $Aa \times Aa$ (Fig. 1). Each AFLP marker was designated by the heterozygous parent, the AFLP primer combinations and a number: the marker was designated as 's' when isolate SF5 was heterozygous and C82P24 was homozygous null and 'p' when isolate C82P24 was heterozygous and SF5 when homozygous null; there was no prefix when both isolates were heterozygous. The name of the AFLP primer combination was given by xxyy, xx being the selective bases on the 3′ end of the *Eco*RI oligonucleotide primer and the yy being the selective bases on the 3′ end of the *Mse*I oligonucleotide primer.

All autoradiograms were scored visually by two independent readers. Goodness of fit of the observed-to-expected allelic ratios was analyzed using a χ^2 test. Separate genetic linkage maps were constructed for each of the parental isolates SF5 and C82P24 using a pseudo-testcross mapping strategy (Grattapaglia and Sederoff, 1994). AFLP markers that were heterozygous in both parents could not be included in this analysis. Consequently, for the map of SF5, AFLP markers with genetic model $Aa \times aa$ and RFLP markers with genetic model $ab \times aa$ and $ab \times cc$ were used. For the map of C82P24, AFLP markers with genetic model $aa \times Aa$ and RFLP markers with genetic model $aa \times ab$ and $cc \times ab$ were used. RFLP markers with genetic model $ab \times ab$, $ab \times ac$, and $ab \times cd$ were used in both parental maps.

Parent-specific maps were constructed using the software program MAPMAKER version 3.0 for PC (Lander et al., 1987). Because the phase of the alleles was unknown, the data set was duplicated and recoded to allow the detection of linkage of markers in repulsion phase. An initial LOD score of 5.0 and maximum $\theta = 0.25$ were set as linkage thresholds for grouping markers. The LOD threshold was lowered to 3 to merge linkage groups. Markers in each linkage group were ordered using the "order" command, with interval support LOD >3. The resulting linkage order was checked using the "ripple" command. Map distances in centi-Morgans were calculated using Kosambi's mapping function. Initial maps were constructed using markers that did not deviate significantly from expected Mendelian ratios at the $p = 0.05$ level. Markers that exhibited segregation distortion were added to the linkage maps subsequently.

A consensus map was constructed using all of the markers used to generate the parental map as well as AFLP markers that were heterozygous in both parents. The datasets were analyzed using the software program JoinMap, Version 2.0 (Stam, 1993). Grouping of markers was identified at a LOD score of 5.0. Within each linkage group, the linear order of markers as determined by JoinMap was compared with that obtained using MAPMAKER. When ordering was different on the maps, markers on the consensus map were listed beside the framework map. As before, the map was initially constructed using markers that did not significantly depart from Mendelian ratios at $p = 0.05$. Molecular markers exhibiting distorted segregation ratios were added subsequently.

3. Results

Fifty-nine additional F_1 progeny were generated from the cross SF5 × C82P24. A total of 97 progeny were analyzed for 7 avirulence genes, mating type, 83 RFLP and 347 AFLP markers. Segregation data and images of the markers can be found at http://bremiadb.ucdavis.edu.

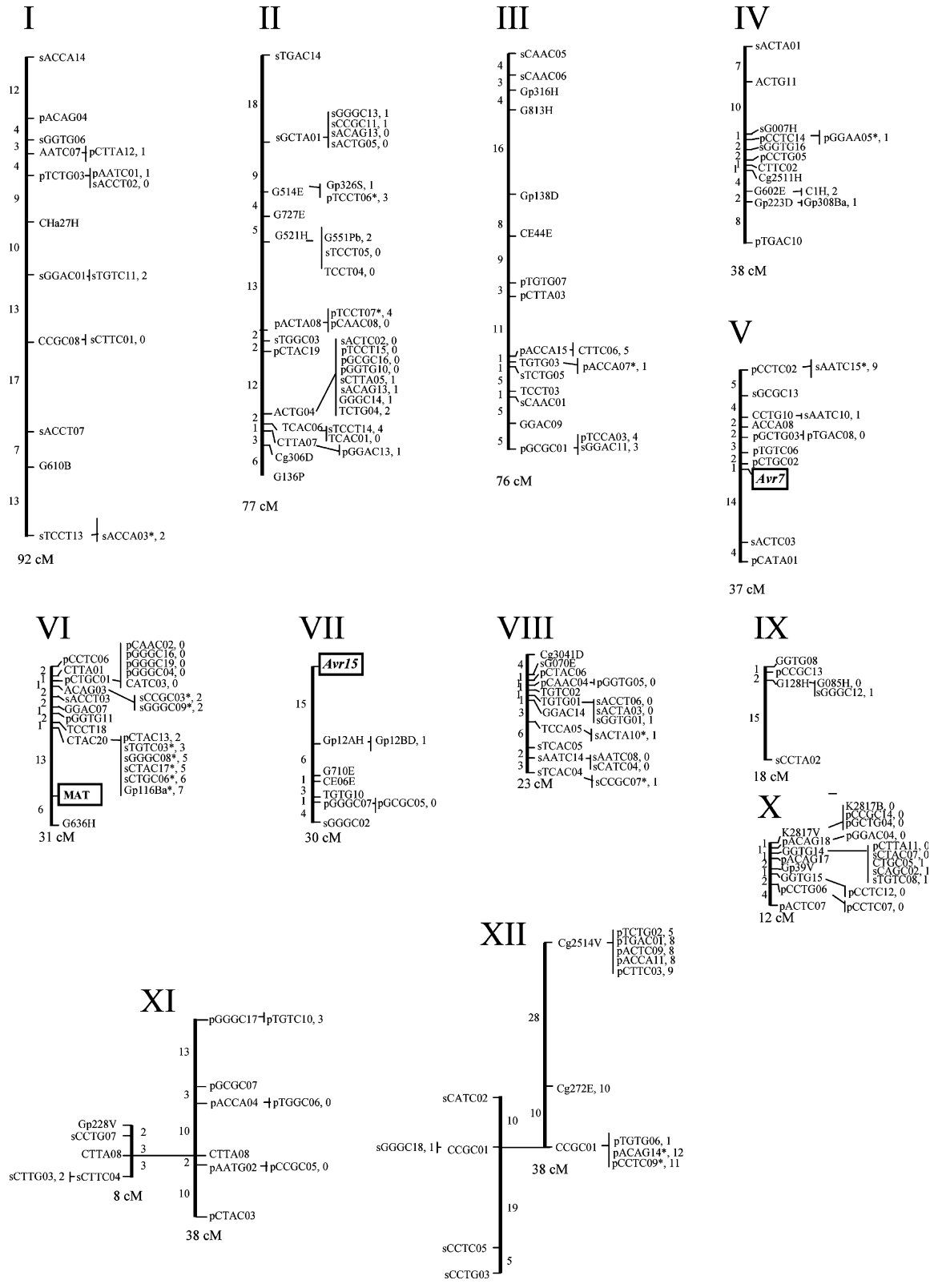

Fig. 1. Consensus linkage map of *B. lactucae*. Linkage relationships of the 347 markers shown was established using a LOD threshold of 5. Linkage groups I–XII represent the 12 merged linkage groups, P1–11 indicate the groups specific to P24, S1–13 describe the groups specific to SF5. Framework markers are indicated beside the vertical line. Markers exhibiting segregation distortion (indicated by *) or close linkage with a framework marker are shown to the right. Genetic distances are in cM. Distances between framework markers are shown to the left of the line. Distances between a framework marker and the other markers are shown after the marker name. Mating type and avirulence genes are highlighted in boxes.

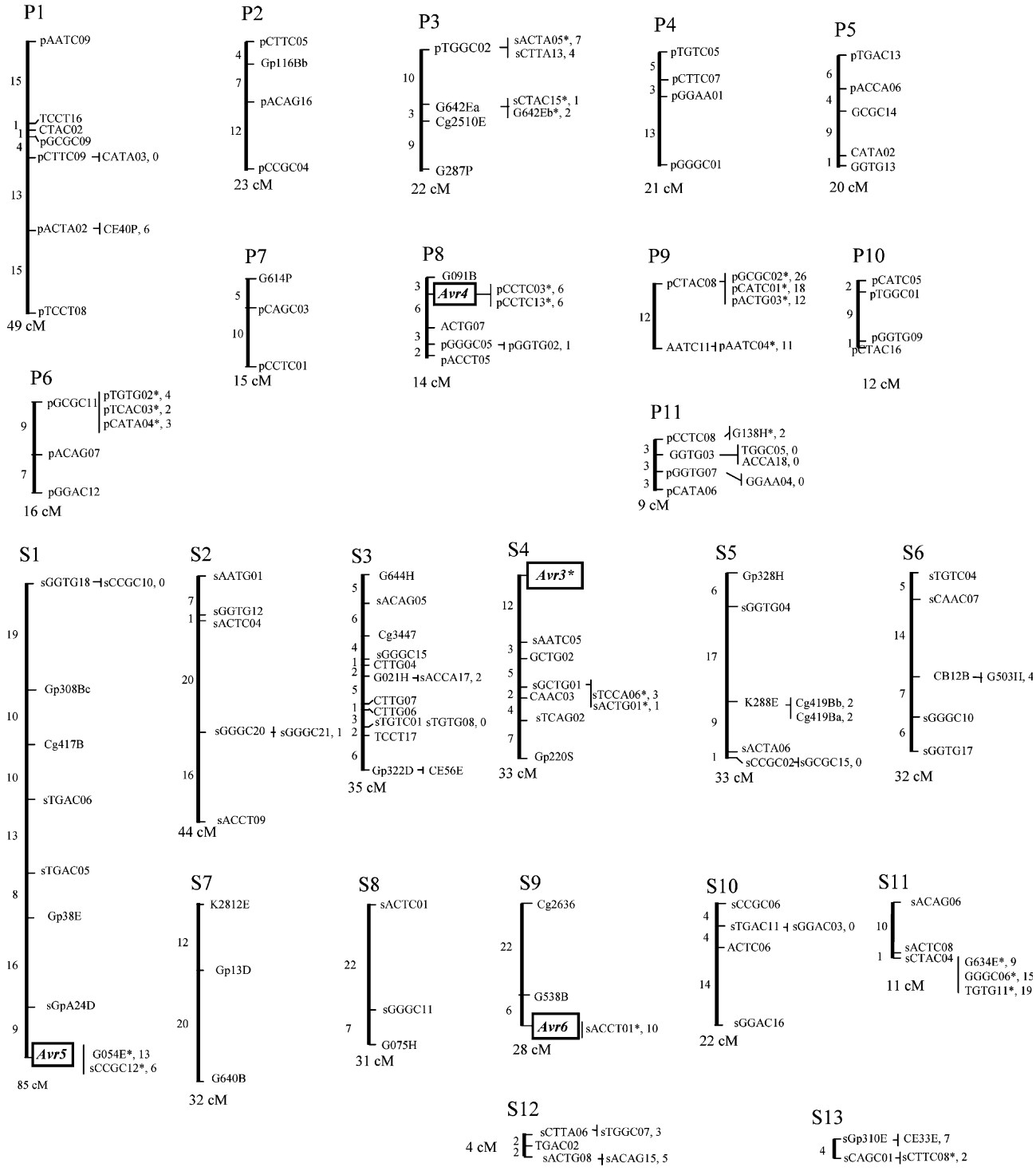

Fig. 1. (*continued*)

3.1. Segregation of avirulence genes and mating type

The progeny exhibited good vigor and sporulated 6–8 days after inoculation of susceptible lettuce seedlings. The two parents were virulent on cultivars expressing *Dm2*, *Dm10*, *Dm12*, *Dm13*, *Dm14* and avirulent on cultivars expressing *Dm1*, *Dm16*, and *R18*. The two parents differed in virulence on lettuce cultivars expressing each of seven *Dm* genes. SF5 was avirulent on cultivars carrying *Dm3*, *Dm5/8*, *Dm6*, *Dm7*, and *Dm11*, while C82P24 was avirulent on cultivars carrying *Dm4* and *Dm15*. No segregation was observed for avirulence

when the parents were monomorphic providing no evidence for heterozygosity for determinants of avirulence at such loci. With the exception of *Avr11* and *Avr3*, all of the polymorphic avirulence loci as well as the mating type loci segregated in a 1:1 ratio expected for a single dominant gene (Table 1). The segregation of *Avr11* was inconsistent with segregation of two alleles at a single locus (Table 1). The observed segregation ratio of 3:1, avirulence to virulence, was consistent with avirulence to *Dm11* being determined by two segregating loci, a dominant allele at either locus resulting in avirulence; this was similar to previous segregation data for *Avr11* observed with other isolates (Michelmore et al., 1984). Segregation of *Avr3* also deviated significantly from the expected ratio 1:1 and fit a 1:3, avirulence to virulence ratio (Table 1). A similar excess of progeny virulent on *Dm3* have been observed previously (Ilott et al., 1989). This could reflect heterozygosity at a locus that inhibited avirulence to *Dm3* in C82P24; alternatively, this could reflect severe segregation distortion, genome instability contributing to loss of avirulence or could be related to heterokaryotic or hyperploid isolates (Hulbert and Michelmore, 1988; Ilott et al., 1989).

3.2. Identification and segregation of molecular markers

Reverse hybridization of *B. lactucae* genomic DNA to the library of size-fractionated *Pst*I fragments indicated that 56% of the clones contained little or no repetitive DNA. This was similar to frequencies previously obtained with *Eco*RI, *Bam*H1, and *Hin*dIII-digested genomic DNA; therefore, there was no indication that the frequency of clones containing repetitive sequences was reduced relative to libraries constructed using methylation insensitive enzymes (Hulbert et al., 1988). Twenty-three RFLP polymorphic markers were identified using the *Pst*I library as a source of probes, bringing the total number of RFLP markers revealed by genomic probes to 58.

A cDNA library was generated from poly(A) RNA isolated from germinated conidia (Judelson and Michelmore, 1989). After the removal of several families of the highly abundant mRNAs (pHAM) (Judelson and Michelmore, 1989), there was no indication of extensive additional redundancy in this library. This was in contrast to the original cDNA library of *B. lactuace* that was constructed from mRNA extracted from ungerminated conidia; this had exhibited a low level of complexity as indicated by frequent identification of redundant sequences (Hulbert et al., 1988). Eleven new RFLP markers were detected using cDNA clones in addition to the 10 cDNA markers obtained previously (Hulbert et al., 1988).

Finally, a small library of clones was generated from the 4.2 Mb band of the electrophoretic karyotype of *B. lactucae* (Francis and Michelmore, 1993). While a chromosome-specific library should allow the rapid development of a saturated map of individual chromosomes, the low efficiency of cloning such fragments permitted the identification of only three chromosome-specifc low-copy number probes. These probes did identify four polymorphic loci.

A total of 36 new RFLP probes were developed in addition to the 40 RFLP probes utilized to construct the previous map of the SF5 × C82P24 cross. This provided 40 new RFLP markers to make a total of 83 RFLP markers. Of these, 46 segregated in the SF5 while only 18 segregated in the C82P24. Among the 19 RFLP markers heterozygous in both parents, 11 were highly informative markers with three or four different alleles.

A total of 347 segregating AFLP markers were identified using 37 AFLP primer combinations (Table 2). The number of polymorphic markers per primer combination ranged from 1 to 21 with an average of 9 (Table 2). There was no evidence of a relationship between the composition of the selective nucleotides and the number of polymorphic fragments obtained. The number of segregating AFLP markers inherited from each parent was slightly higher in SF5; of the 347 AFLP markers, 137 were heterozygous in the parent SF5 and 127 were heterozygous in the parent C82P24. The other 83 AFLP markers segregated in both parents.

Segregation distortion was observed for both RFLP and AFLP loci. Segregation ratios for nine (10%) of the RFLP markers departed from Mendelian expectation at $p = 0.05$. Of these, eight were detected with genomic

Table 1
Segregation of avirulence genes among F_1 progeny from the cross SF5 × C82P24

Avr	SF5[a]	C82P24	F_1 ratio (A:V)	χ^2	*P* value
Avr15	V	A	42:49	0.54 (1:1)	0.46
Avr4	V	A	54:37	3.18 (1:1)	0.07
Avr3	A	V	25:67	19.17 (1:1)	1.19×10^{-5}
				0.23 (1:3)	0.63
Avr5	A	V	51:40	1.33 (1:1)	0.25
Avr6	A	V	40:51	1.33 (1:1)	0.25
Avr7	A	V	46:45	1.33 (1:1)	0.92
Avr11	A	V	64:27	15.04 (1:1)	1.05×10^{-4}
				1.06 (3:1)	0.3

[a] V, virulent; A, avirulent.

Table 2
Number of segregating and distorted AFLP markers for each primer combination

3′ selective nucleotides on AFLP primers		No. of segregating markers	No. of distorted markers
*Eco*R1	*Mse*1		
AA	TC	14	3
AA	TG	3	0
AC	AG	17	2
AC	CA	17	5
AC	CT	9	1
AC	TA	7	2
AC	TC	9	0
AC	TG	9	3
CA	AC	8	0
CA	GC	3	0
CA	TA	6	2
CA	TC	6	1
CC	GC	14	3
CC	TA	2	0
CC	TC	14	4
CC	TG	10	2
CT	AC	17	4
CT	GC	6	1
CT	TA	12	4
CT	TC	10	1
CT	TG	5	1
GC	GC	11	2
GC	TA	1	0
GC	TG	5	0
GG	AA	4	1
GG	AC	12	0
GG	GC	21	4
GG	TG	18	0
TC	AC	6	1
TC	AG	3	0
TC	CA	5	1
TC	CT	14	3
TC	TG	4	0
TG	AC	12	0
TG	GC	7	0
TG	TC	9	1
TG	TG	12	4
Total		342	56

probes and one with a cDNA probe. Only one of the distorted markers was heterozygous in both parents, one was heterozygous only in C82P24, and seven were heterozygous only in SF5. Segregation distortion resulted from an excess of the allele present in SF5 for seven markers. Segregation of 18% of AFLP markers did not fit Mendelian ratios at $p = 0.05$. As for RFLP markers, the number of AFLP markers exhibiting segregation distortion was higher among markers heterozygous in SF5; 31 markers heterozygous in SF5 deviated significantly from the expected ratio, while 18 markers heterozygous in C82P24, and seven loci segregating in both parents departed from expected ratios. Distortion at AFLP loci was again usually due to an excess of the presence of the SF5 allele.

3.3. Parental maps

The SF5 parental map was constructed using four *Avr* loci (*Avr3*, *Avr5*, *Avr6*, and *Avr7*), the mating type locus, and 202 molecular markers: 137 AFLP markers, 46 RFLP markers that segregated in SF5, and 19 RFLP markers that segregated in both parents. Twenty-five linkage groups consisting of 172 molecular markers and four *Avr* loci were formed at thresholds of $\theta = 0.25$ and LOD = 5. *Avr*11 as well as 30 molecular markers were unlinked, 11 of which exhibited segregation distortion. A framework map spanning 835 cM was constructed using 109 of the 172 markers linked at LOD = 5. There were two major linkage groups (82 and 87 cM), one group of 53 cM, and 21 groups of sizes ranging from 44 to 7 cM. Sixteen of the 31 markers that exhibited segregation distortion were located in four linkage groups; all had an excess of the allele for the presence of the fragment.

The C82P24 parental map was constructed using two *Avr* loci (*Avr4* and *Avr15*), the mating type locus, and 164 molecular markers: 127 AFLP markers, 18 RFLP markers that segregated in C82P24, and 19 RFLP markers that segregated in both parents. Twenty-one linkage groups were formed at a threshold of $\theta = 0.25$ and LOD = 5 including 133 molecular markers, 2 *Avr* loci, and the mating type locus. There were 31 molecular markers that either remained unlinked or were paired at this threshold. In C82P24, 96 markers were placed on a framework map spanning 606 cM. There were one major linkage group (80 cM), two groups of 50 and 55 cM, and 15 groups ranging from 43 to 5 cM.

3.4. Consensus map

A consensus map was obtained by integrating the segregation data of all 430 molecular markers, seven *Avr* loci, and the mating type locus. This consensus map comprised of 6 *Avr* loci the mating type locus, and 340 markers in groups of three or more with 90 remaining unlinked or only in pairs. Eighteen percent of the molecular markers with Mendelian segregation ratios were unlinked, while 32% of the markers exhibiting segregation distortion were unlinked. The linkages and gene orders identified using JOINMAP were similar to those previously generated for the parent-specific maps using MAPMAKER. Twelve linkage groups merged markers located on the two parental maps; 13 linkage groups were specific to SF5; 11 linkage groups were specific to C82P24. Among the 12 merged linkage groups, there were three major linkage groups (76–92 cM), and seven linkage groups ranging from 38 to 12 cM. Two pairs of merged linkage groups were each joined by a single marker; therefore the relative orientation of the markers and consequently the sizes of these groups were unknown. Most of the large parent-specific linkage groups

merged. However, one of the largest SF5-specific linkage groups of 85 cM did not; the remaining SF5-specific groups were less than 44 cM. Among the unmerged C82P24-specific linkage groups, there was one linkage group of 49 cM, all the others were less than 23 cM.

Among the 340 molecular markers in the consensus map, 210 molecular markers as well as the 6 *Avr* loci, and the mating type locus were ordered on the framework map. The remaining 130 molecular markers were placed beside the framework map for one of two reasons (Fig. 1). Sixty-five markers either cosegregated or were within 1 cM from a framework map marker and therefore could not be unambiguously ordered; many of these markers were in large clusters. The average spacing of markers was highly variable from one linkage group to another ranging from 0.6 to 11 cM. The largest cluster of cosegregating loci contained six markers. Thirty-nine markers exhibited segregation distortion and were also placed beside the framework map (Fig. 1). Seventeen of these markers were located within eight of the linkage groups. The remaining 22 mapped at the ends of linkage groups; however, this may have been an artifact of the way mapping programs treat loci that cannot be readily ordered rather than reflecting their true position.

The six avirulence phenotypes that segregated as single genes were located in different linkage groups (Fig. 1). The avirulence phenotype (*Avr*11) that segregated as two loci was not mapped. The mating type locus, *Avr7* and *Avr15* were located in merged linkage groups of the consensus map. *Avr4* was located on a C82P24-specific linkage group and *Avr3*, *Avr5*, and *Avr6* were located on SF5-specific linkage groups. Although markers linked to each of the avirulence genes and to the mating type locus were identified, no marker absolutely cosegregated with any of these phenotypic loci. The mating type locus as well as *Avr4* and *Avr7* were flanked by molecular markers. The mating type locus was flanked by an RFLP marker (*G636H*) and an AFLP marker (*CTAC20*) at distances of 6 and 13 cM away, respectively. The closest marker to an avirulence gene was the AFLP marker, *pCTGC02* located 1 cM away from *Avr7*. *Avr3*, *Avr5*, *Avr6*, and *Avr15* were located at the ends of linkage groups and were linked to molecular markers at greater genetic distances. The four *Avr* loci that were located on parent-specific linkage groups were either distorted (*Avr3*) or linked to distorted molecular markers, while the two *Avr* loci *Avr7* and *Avr15* that mapped on merged linkage groups were not (Fig. 1).

3.5. Identification of chromosomes carrying RFLP markers and avirulence genes

Both cytogenetic and pulsed field gel electrophoresis analysis had been previously performed to determine the number and the size of chromosomes of *B. lactucae* (Francis and Michelmore, 1993; Michelmore and Sansome, 1982). Four RFLP probes had been previously hybridized to the electrophoretic karyotype and had allowed the identification of the 3.5 Mb chromosome as carrying *Avr6* (Francis and Michelmore, 1993).

In order to assign more loci to chromosomes, additional RFLP probes were hybridized to Southern blots of the PFGE karyotype of *B. lactucae*. A total of 25 probes were successfully hybridized; of these, 23 were mapped and represented 16 of the linkage groups in the consensus map and two were unlinked. Of the 23 mapped markers, two mapped markers hybridized to the 4.0 fragment, six probes hybridized to the pairs of 6/7 Mb or 3.0/3.5 Mb fragments that were unresolved in the initial round of hybridizations (see Section 2); two other mapped markers were assigned to the 6 or 7 Mb fragments that were resolved in the later hybridizations (Fig. 2). Hybridization of one of the unlinked markers to the resolved chromosomal fragments assigned it to the 4.5 Mb fragment. Four probes, G727E, Gp138D, Cg306, and CHa27H, hybridized to all the chromosome-sized bands. Ten additional probes hybridized predominantly to high molecular weight DNA that had not migrated from the well and there was little or no signal associated with smaller molecules; however, the specificity of such hybridization was treated with caution and assignment of these probes to the large chromosome(s) was regarded as provisional.

Two of the 12 merged linkage groups and seven parent-specific linkage groups could be assigned to chromosomal fragments (Fig. 2). One minor merged linkage group was assigned to the 6 Mb band and an-

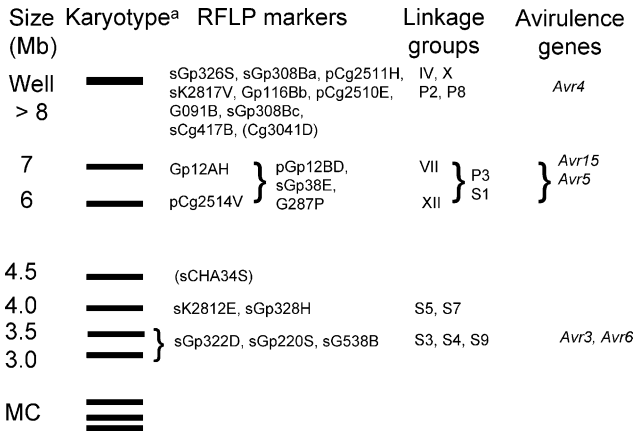

Fig. 2. Distribution of RFLP markers on the electrophoretic karyotype of *B. lactucae*. Ten of the mapped RFLP probes hybridized to fragments in the 3.0–7 Mb allowing these markers to be assigned to chromosomes. Nine mapped probes hybridized to high molecular weight DNA that did not migrate from the well; assignment of these markers to large chromosome(s) over 8 Mb is regarded as provisional. Four probes (see text) hybridized to all chromosomes. Probes for two markers that were unlinked also hybridized; these are shown in parentheses. [a]Karyotype as determined by Francis and Michelmore (1993). MC = Minichromosome.'}' indicate fragments that could not be resolved (see Section 2).

other containing *Avr15* to the 7 Mb band. The major SF5-specific linkage group S1 and a minor C82P24 linkage group was assigned to the 6/7 Mb pair of fragments. In addition, three minor SF5-specific linkage groups, S5 and S7 could be assigned to the 4.0 Mb fragment suggesting these two linkage groups should also be merged. Three minor SF5-specific linkage groups S3, S4, and S9 could be assigned to the unresolved 3.0/3.5 Mb pair of fragments. In addition, two minor merged linkage groups and two parent-specific groups contained RFLP markers that hybridized to the unresolved high molecular weight DNA.

Linkage to RFLP markers allowed avirulence loci to be assigned to chromosomal fragments. *Avr15* was located to the 7 Mb chromosome, *Avr5* to the 6 or 7 Mb chromosome pair and *Avr3* to the 3.0 or 3.5 Mb chromosomes. *Avr4* was linked to RFLP markers that hybridized to the well. The mating type locus and *Avr7* could not be assigned to any chromosome-sized molecule.

4. Discussion

The number of F_1 progeny was doubled from our previous analysis and the number of markers increased sevenfold to develop a more detailed map of *B. lactucae*. This resulted in a genetic map comprised of 340 molecular markers, 6 *Avr* genes and the mating type locus distributed over 12 merged linkage groups plus 24 parent-specific linkage groups. This study did not saturate the genetic map and 90 (21%) markers remained unlinked. It is difficult to estimate the total number of chromosomal groups and the genome size represented by our data because of the possible redundancy between the parent-specific linkage groups. The parent-specific maps had 24 and 21 linkage groups spanning 835 and 606 cM for SF5 and C82P24 respectively. This analysis was more extensive than previous studies of *P. infestans* and comparable in scope to those of *P. sojae*. The map for *P. infestans* based on 73 F_1 progeny consisted of seven RFLP markers, 183 AFLP markers, and the mating type locus distributed over 10 merged linkage groups and 7 parent-specific linkage groups spanning 827 cM (van der Lee et al., 1997). The map for *P. sojae* based on four F_2 progeny each of 53 individuals consisted of 35 RFLP loci, 236 RAPD markers, 105 AFLP markers, and 10 avirulence genes as distributed over 21 major and 7 minor linkage groups spanning 1640 cM (May et al., 2002; Whisson et al., 1995). The number of linkage groups in *B. lactucae* was close to those found in *P. infestans* and *P. sojae*.

The number of linkage groups was considerably higher than the estimated number of chromosomes. This could be due to an underestimation of the number of chromosomes as well as a lack of saturation of the map. Cytogenetic studies identified seven or eight chromosomes in *B. lactucae*; however, chromosomes in *B. lactucae* are too small for clear visualization under the light microscope (Michelmore and Sansome, 1982). PFGE resolved a minimum of seven chromosomes between 3 and 8 Mb and the large band greater than 8 Mb that might contain unresolved chromosomes (Francis and Michelmore, 1993). In the present study, none of the three major merged linkage groups could be assigned to any of the fragments resolved by PGFE; this suggests these linkage groups correspond to chromosomes that were unresolved by PGFE either because of their high molecular weight or due to DNA degradation during high molecular weight DNA extraction. Together the genetic and physical data suggest that there may be at least 10 chromosomes in *B. lactucae*.

The total genome size calculated for each of the two parent-specific maps was 835 and 606 cM. The nuclear genome size of *B. lactucae* was estimated to be between 48 and 53 Mb using PGFE and reconstruction and reassociation kinetics, respectively (Francis and Michelmore, 1993; Francis et al., 1990). Therefore, on average there is 1 cM per ~70 kb; however, there are likely to big large differences in recombination frequency across the genome. This value is comparable to that for *P. sojae* (38–56 kb/cM) but considerably less than the average for *P. infestans* (~200 kb/cM; MacGregor et al., 2002; van der Lee et al., 1997).

Additional markers or analysis of other mapping populations are needed to merge the currently parent-specific groups and assimilate the unlinked markers. The lack of linkage or large gaps between molecular markers may have resulted from high frequencies of recombination or from a lack of polymorphic markers (Kesseli et al., 1994). The high number of parent-specific linkage groups probably reflects regions with little or no polymorphism within each parental isolate. Although the two parents came from widely separated geographical origins, the analysis is based on meiosis within the parental isolates; therefore there is the possibility of identity by descent for regions of the genome when mating has occurred between sympatric isolates, even if the organism is heterothallic. If this has been the case, increasing the number of markers alone will not result in merging of the parent-specific groups. The SF5 parental isolate had a higher level of heterozygosity than C82P24 isolate; this would have contributed to the more extensive map generated for SF5. Molecular markers were not evenly distributed within and between linkage groups. There were several distinct clusters of markers. These could have been due to reduced recombination, chromosomal structural hybridity, or unequal detection of markers. Distribution of AFLP markers may have been biased towards relatively AT rich regions because *Eco*RI (G/AATTC) and *Mse*I (T/TAA) were used for restriction of the genome (Rouppe van der Voort et al.,

1997). The current map of *B. lactucae* was not sufficiently saturated for the distribution of markers to be indicative of centromeric regions.

The genetic and molecular basis of mating type in oomycetes is unclear. Unlike the situation in Ascomycete and Basidiomycete fungi where mating type is expressed in a haploid life stage, oomycetes are diploid in the vegetative stage; therefore, in heterothallic oomycetes one of the two mating types is potentially conditioned by a heterozygous locus (Brasier, 1992). The mating type locus has not been cloned from any oomycete. Several models have been proposed for determination of mating type in oomycetes. One model based on cytological observations associated the mating type locus with chromosomal structural hybridity (Michelmore and Sansome, 1982; Sansome, 1980); however, the current genetic data provided no support for this in *B. lactucae* because a large cluster of markers that would be expected due the repression of recombination associated with a reciprocal translocation was not observed with the mating type locus. A second model was proposed for *B. lactucae* where segregation of the mating types generally fits a 1:1 ratio; mating type is determined by a dominant allele at a single locus, where the heterozygote determines one mating type and the recessive homozygote determines the other (Michelmore and Sansome, 1982). The current genetic data are consistent with this model. The mating type locus could be mapped based on meioses of the B_2 parental isolate (C82P24) but not on those of the B_1 parental isolate (SF5) suggesting that the B_2 mating type was determined by the heterozygous genotype while the homozygote recessive genotype determined the B_1 mating type. This is consistent with the proposed genetic basis of secondary homothallism (Michelmore and Sansome, 1982). However, the situation may be more complex; six of the seven markers linked to mating type but segregating only in SF5 exhibited segregation distortion with an excess of heterozygotes, suggesting heterozygosity was favored in the mating type region in the SF5 parent. In another model that has been proposed to explain pattern of non-Mendelian segregation at the mating type locus in *P. infestans*, both mating types are heterozygous, but only one of the two alleles in each genotype determines the mating type; the other two alleles are proposed to be neutral or null in function (Judelson et al., 1995; Judelson, 1996b). Heterozygotes for the two functional alleles and heterozygotes of the two neutral/null alleles are prevented by a system of balanced lethality. Additional markers tightly linked to mating type or the cloning of the mating type locus are required to test this hypothesis in *B. lactucae*.

Map positions were identified for six avirulence loci and four were assigned to chromosomal fragments. All six mapped to different linkage groups. There was no evidence of clustering of avirulence genes. This is consistent with classical segregation data that considered linkage between 12 avirulence loci in *B. lactucae* (Ilott et al., 1989). This is in contrast, however, to *P. sojae* and *P. infestans* in which three pairs and a triplet of avirulence genes have been identified, respectively (Gijzen et al., 1996; May et al., 2002; van der Lee et al., 2001; Whisson et al., 1995). Our genetic data provides no evidence for pathogenicity islands that are increasingly being identified in bacteria (Alfano et al., 2000; Guttman et al., 2002; Jackson et al., 1999). No RFLP markers, and consequently none of the avirulence genes, were assigned to the small polymorphic 300 kb–1.6 Mb fragments following PFGE. Therefore there was no evidence that these small variable molecules are involved in variation of specificity of *B. lactucae*. However, four avirulence loci were located at the ends of linkage groups in *B. lactucae*. In *P. infestans*, five out of six avirulence genes mapped at the ends of linkage groups (van der Lee et al., 2001). Such terminal positions have also been reported for avirulence genes in several true fungi such as *Puccinia graminis* (Zambino et al., 2000) and *Magnaporthe grisea* (Dioh et al., 2000). In *M. grisea*, four out of eight *Avr* genes are close to the telomere (Dioh et al., 2000). Subtelomeric regions are subject to frequent rearrangements and gene silencing in yeast (Sandmeier et al., 2002). Losses in avirulence in *M. grisea* are associated with deletions (Mandel et al., 1997). Telomeric locations of *Avr* genes would be consistent with the high instability of the avirulence phenotype in *B. lactucae* (Freitas-Junior et al., 2000). Linkage of three *Avr* genes with distorted markers in the *B. lactucae* genetic map may also be indicative of other mechanisms of instability of *Avr* genes such as high frequencies of mitotic gene conversion observed in *P. sojae* (Chamnanpunt et al., 2001).

Close linkages were not identified between avirulence genes and molecular markers. The closest marker was 1 cM from *Avr7* and only loose linkages were identified for the majority of *Avr* genes. Whether this represents a dearth of polymorphic low-copy sequences or high rates of recombination close to avirulence genes is unknown. The genome-wide average relationship between genetic and physical distance of 70 kb/cM is favorable for map-based cloning. However, more markers or an estimation of the relationship between genetic and physical distance around the avirulence gene are required before map-based cloning of avirulence genes from *B. lactucae* is initiated. We have attempted bulked segregant analysis (BSA; Michelmore et al., 1991) to identify markers closely linked to several avirulence genes; however, this was unsuccessful (D. Zungri and R. Michelmore, unpublished). BSA has been used successfully to target markers to some avirulence genes but not others in *P. infestans* (van der Lee et al., 2001). Map-based cloning of *Avr* genes is in progress in several oomycetes (MacGregor et al., 2002; Randall and Judelson, 1999;

Rehmany et al., 2003; van der Lee et al., 2001; Whisson et al., 2001). Alternative approaches such as the identification of candidate genes and mapping relative to avirulence phenotypes are also underway (Kamoun et al., 2002; May et al., 2002; van der Biezen et al., 2000). We are currently characterizing ~8000 ESTs enriched for sequences from a subtracted library of *B. lactucae* made using healthy and infected material to provide candidates for further genetic analysis.

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