Marker-Based Cloning of the Region Containing the *UhAvr1* Avirulence Gene From the Basidiomycete Barley Pathogen *Ustilago hordei*

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ABSTRACT

Race-cultivar specialization during the interaction of the basidiomycete smut pathogen *Ustilago hordei* with its barley host was described in the 1940s. Subsequent genetic analyses revealed the presence of dominant avirulence genes in the pathogen that conform to the gene-for-gene theory. This pathosystem therefore presents an opportunity for the molecular genetic characterization of fungal genes controlling avirulence. We performed a cross between *U. hordei* strains to obtain 54 progeny segregating for three dominant avirulence genes on three differential barley cultivars. Bulked segregant analysis was used to identify RAPD and AFLP markers tightly linked to the avirulence gene *UhAvr1*. The *UhAvr1* gene is located in an area containing repetitive DNA and this region is undetectable in cosmid libraries prepared from the avirulent parental strain. PCR and hybridization probes developed from the linked markers were therefore used to identify cosmid clones from the virulent (*Uhavr1*) parent. By walking on *Uhavr1*-linked cosmid clones, a nonrepetitive, nearby probe was found that recognized five overlapping BAC clones spanning 170 kb from the *UhAvr1* parent. A contig of the clones in the *UhAvr1* region was constructed and selected probes were used for RFLP analysis of the segregating population. This approach genetically defined an ~80-kb region that carries the *UhAvr1* gene and provided cloned sequences for subsequent genetic analysis. *UhAvr1* represents the first avirulence gene cloned from a basidiomycete plant pathogen.

GENETIC studies of the rust pathogen *Melampsora lini* and its host, flax (Flor 1942), and of the smut *Ustilago tritici* and its host, wheat (Oort 1944), demonstrated that genes in both the host and the pathogen are involved in conditioning the compatibility (susceptibility) and incompatibility (resistance) of plant-pathogen interactions. The gene-for-gene hypothesis developed from this work predicted that incompatibility will occur if a host carries at least one dominant resistance allele that allows recognition of at least one dominant allele in the pathogen. Conversely, when the pathogen or the host harbor homozygous recessive alleles, no recognition ensues and the plant becomes diseased. These genes have been called avirulence or *Avr* genes because they render the pathogen unable to cause disease symptoms. Isolates of a pathogenic species can carry different complements of avirulence genes, making up a larger variety of so-called races. Similarly, a host species may harbor different combinations of resistance genes that recognize its cognate avirulence gene, making up a collection of differential cultivars. In many pathosystems this race-cultivar surveillance mechanism is genetically superimposed on the basic potential of the particular pathogen species to infect the host species. That is, recognition of a single *Avr* gene (product) by a host plant harboring the cognate resistance gene (product) is epistatic over this basic compatibility and is sufficient to completely prevent disease (Flor 1971; Keen 1990; de Wit 1992; Staskawicz *et al.* 1995; Knogge 1996; Van der Biezen and Jones 1998; Dangl and Jones 2001; Van't Slot and Knogge 2002).

Much research has focused on isolating and understanding the molecular workings of *Avr* genes because they (a) represent single, often dominant genes and are therefore easy to track genetically; (b) restrict host range; and (c) are recognizable factors of pathogens that allow host plants to trigger defense mechanisms. These mechanisms are often correlated with a conspicuous, necrotic resistance reaction termed the hypersensitive response (HR), which is thought to localize infection by killing off surrounding plant tissue (Hammond Kosack and Jones 1996; Heath 2000; Lam *et al.* 2001). Resistance reactions and initial responses to compatible interactions produce many other molecular events in

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both host and pathogen, often through induced gene expression. In plants these include membrane changes and depolarization, production of reactive oxygen species, and production of PR proteins (Hammond Kosack and Jones 1996; Dangl and Jones 2001).

Many *Avr* genes have been isolated from bacterial phytopathogens using classical bacterial genetic techniques such as transforming a virulent receptor strain with a genomic library made from an avirulent strain and selecting for *Avr*-containing clones using the visual HR response (reviewed in van den Ackerveken and Bonas 1997; Collmer 1998). Far fewer fungal *Avr* genes have been isolated due to larger genomes involved and inefficient transformation procedures (reviewed in Lauge and de Wit 1998; Knogge 2002). However, several *Avr* genes were isolated using reverse genetic approaches because it was shown that their products elicited defense reactions in a race-cultivar or nonhost interaction, such as *Avr9* (van Kan *et al.* 1991), *Avr4* (Joosten *et al.* 1994), *Ecp1*, *Ecp2* (van den Ackerveken *et al.* 1993), *Ecp3*, and *Ecp4* (Lauge *et al.* 2000) of *Cladosporium fulvum* and *Nip1* of *Rhynchosporium secalis* (Rohe *et al.* 1995). In contrast, *PWL2*, *AVR-Pita* (formerly called *Avr2-YAMO*), and *avrCO39* of *Magnaporthe grisea* have been cloned by map-based approaches (Sweigard *et al.* 1995; Farman and Leong 1998; Orbach *et al.* 2000; Farman *et al.* 2002). Several more fungal *Avr* genes have been mapped on short genetic intervals in *M. grisea* (Dioh *et al.* 2000), *Leptosphaeria maculans* (Attard *et al.* 2002), and *Blumeria graminis* (Pedersen *et al.* 2002). The only basidiomycete phytopathogen in which *Avr* genes have been provisionally mapped is the wheat stem rust, *Puccinia graminearum* f.sp. *tritici* (Zambino *et al.* 2000), although the existence of many *Avr* genes has been described in several cereal rusts. In addition, an oomycete *Avr* gene, *Inf1*, has been isolated (Kamoun *et al.* 1998) and several more from *Phytophthora infestans* (van der Lee *et al.* 2001; Whisson *et al.* 2001), *Phytophthora sojae* (Gijzen *et al.* 1996; MacGregor *et al.* 2002), and *Peronospora parasitica* (Rehmany *et al.* 2003) have been mapped.

The gene-for-gene hypothesis reflects a purely genetic concept and the molecular mechanisms underlying the recognition of an *Avr* gene product by its cognate resistance gene product are still largely unknown despite the isolation of several fungal and many bacterial avirulence genes and many plant resistance genes. It is thought that avirulence gene products encode or produce (through enzymatic functions) elicitors that are recognized by the product of a specific plant resistance gene, possibly as described in ligand-receptor interaction models or by a complex of proteins, which consists of at least the resistance gene product and another effector protein as proposed in the "guard hypothesis" (reviewed in Ji *et al.* 1998; Holt *et al.* 2000, 2003; Staskawicz *et al.* 2001; Van der Hoorn *et al.* 2002; Axtell and Staskawicz 2003; Mackey *et al.* 2003). Also, a molecular understanding of the function of *Avr* genes in the disease process, apart from eliciting a defense response, is still unclear although mounting evidence attributes virulence and fitness functions to these genes. Apparently, plants have co-opted *Avr* gene products as triggers for active defense (Collmer 1998; Knogge 1998; White *et al.* 2000; Van't Slot and Knogge 2002; Wolpert *et al.* 2002). Hints of the potential function of some avirulence genes have recently emerged. NIP1 (Rohe *et al.* 1995) and ECP2 (Lauge *et al.* 1997) appear to act as virulence factors that enable the fungus to kill host cells and obtain nutrients. AVR-Pita, a metalloprotease with an N-terminal secretory signal and pro-protein sequences, possibly releases an elicitor (Jia *et al.* 2000), and AvrD from the bacterial pathogen *Pseudomonas syringae* is involved in the biosynthesis or release of an elicitor (Keen *et al.* 1994).

The smuts from the genus Ustilago are basidiomycete plant pathogens that cause diseases world-wide, leading to substantial crop losses in many countries including Canada (Thomas 1989; Menzies *et al.* 1996). The *Ustilago hordei*/barley pathosystem is a model for smuts that infect members of the Gramineae, such as cereal crops and forage grasses. Infection occurs at the seedling stage and the fungus grows within the meristematic tissue during development of the plant without showing symptoms. At flowering, the fungus undergoes massive sporulation in the floral tissue and replaces the seeds with smut sori (Hu *et al.* 2002). Teliospores from infected plants overwinter in the soil or on grain and reinitiate infection after germination, meiosis, and mating. Germinated, single haploid cells are amenable to molecular techniques but cells need to mate with compatible partners to produce dikaryotic infectious mycelia. Therefore, sex and pathogenicity go hand-in-hand because fusion brings together avirulence alleles that determine compatibility or incompatibility. However, susceptibility is indistinguishable at the macroscopic level from resistance until heading of the plant when kernels of susceptible plants, which are replaced with the black sooty teliospores of the fungus, become visible. Little is known about how the infection process is blocked in incompatible or resistant interactions. The presence of avirulence gene *UhAvr1* triggers a very localized, microscopic HR-like reaction in cultivars harboring the cognate resistance gene *Ruh1* (Hu *et al.* 2003).

Fourteen races of *U. hordei* have been described (Tapke 1945) and subsequent genetic studies have identified six avirulence genes and six corresponding resistance genes in barley cultivars (Sidhu and Person 1972; Ebba and Person 1975; Thomas 1976). Three of the avirulence genes, *UhAvr1*, *UhAvr2*, and *UhAvr6*, have been demonstrated to act consistently in a stable genetic manner, although *UhAvr2* is influenced by environmental variability. We decided on a PCR marker-based approach to isolate these *Avr* genes because no race-specific elicitors had been described to allow a biochemical

approach. In addition, the complexity of the *U. hordei* genome is $\sim 2 \times 10^7$ bp and integrative transformation for *U. hordei* is inefficient. Consequently, it would not be feasible to screen for avirulence activity by transformation of libraries. Moreover, no visible HR is observed in an incompatible interaction. A compatible (disease) interaction is detected by scoring plants at heading (after 2 months) and an incompatible (resistant) interaction is scored as zero plants infected out of a significant number of plants inoculated compared to a positive (diseased) control. Here we describe the generation of populations segregating for the three avirulence genes as well as the mapping and cloning of the region containing the *UhAvr1* gene, which conditions resistance on the cultivar "Hannchen" harboring resistance gene *Ruh1*.

MATERIALS AND METHODS

Strains and plasmids: Ustilago isolates are listed in Table 1. Strains were received as teliospore populations on smutted ears and were germinated on potato dextrose agar (PDA, Difco Laboratories, Detroit) for random basidiospore isolation. Strains were grown on PDA or in potato dextrose broth (PDB) at 22° and stored on PDA slants at 4° until used. To establish the mating type, candidate strains were mixed with strains of known mating type on complete medium plates amended with 1% charcoal (Holliday 1974), incubated at room temperature for 48 hr, and the presence or absence of infection hyphae was verified using a compound microscope. Mating type was verified by restriction fragment length polymorphism (RFLP) analysis on DNA blots (Bakkeren *et al.* 1992; Bakkeren and Kronstad 1993). *Escherichia coli* DH5α (Invitrogen Canada, Burlington, ON) was used for DNA manipulations, and *E. coli* DH10B/r was used for propagation of bacterial artificial chromosome (BAC) clones, the BAC vectors pBeloBACII (Shizuya *et al.* 1992) and pEcBAC1 (Frijters *et al.* 1997), and related clones and constructs. pUsBAC5 is a 9.6-kb Ustilago-specific integrative BAC vector and is derived from pEcBAC1. To construct this vector, the 3.1-kb *Hin*dIII fragment from pJW42 containing the Ustilago-specific hygromycinB expression cassette (Wang *et al.* 1988) was cloned into the unique *Hin*dIII site of pEcBAC1 after which the *Hin*dIII site nearest to the *Bam*HI site was destroyed. Finally, the 0.3- and 0.4-kb *Sac*I fragments were deleted to create unique *Sac*I, *Not*I, and *Hin*dIII sites. Similarly, cosmid vector pGBcos1 is a Ustilago-specific, integrative, phleomycin-resistant derivative of vector pJW42 that also allows GUS expression (G. Bakkeren, unpublished results).

DNA procedures: DNA manipulations were performed as described (Sambrook *et al.* 1989). *U. hordei* genomic DNA was isolated by a glass bead/high salt/phenol:chloroform extraction technique as described (Bakkeren *et al.* 1992). Bulked pools consisted of genomic DNA preparations from eight progeny with a final DNA concentration of 12.5 ng/μl. DNA fragments from BAC inserts were subcloned into pUsBAC5 using the method of Korch (1987) to partially fill in 5′ extensions of restriction sites to generate compatible ends; *e.g.*, we cloned *Xma*I fragments into a *Not*I site and *Xba*I fragments into a *Hin*dIII site of pEcBAC1. High-copy plasmids and BAC clones and vectors were isolated and purified with a QIAGEN-100 plasmid kit according to the manufacturer's protocol (QIAGEN, Mississauga, ON). Restriction enzyme mapping and fingerprinting of BAC clones were performed by field inversion gel electrophoresis (Bio-Rad Laboratories Canada, Mississauga, ON). PCR products were excised from the gel, purified with the Geneclean kit (Bio 101, La Jolla, CA), and cloned directly into the *Eco*RV site of pBluescriptIIK/S (Stratagene, La Jolla, CA). The DNA sequence of randomly amplified polymorphic DNA (RAPD) markers was obtained using a T7 sequencing kit (Invitrogen Canada) and M13 universal and reverse primers.

RAPD and SCAR analysis: RAPD primers (nos. 1–890) were purchased from the Nucleic Acid-Protein Service Unit at the University of British Columbia. RAPD analysis (Williams *et al.* 1990) was performed on a Perkin-Elmer 9600 thermocycler (Applied Biosystems, Foster City, CA). Successful RAPD primers were 359, 5′-AGGCAGACCT-3′, and 743, 5′-CCACCCA CAC-3′, which were included in the design of sequence characterized amplified region (SCAR) primers R359N, 5′-AGG CAGACCTAACCAAATCTTAAA-3′; R359P, 5′-AGGCAGACC TCTGTGGACACTCCG-3′; 743R, 5′-CCACCCACACGAGACAG CCAAAGA-3′; and 743F, 5′-CCACCCACACCAGACCCTGGT GTC-3′. Specific "junction" primers (359-2.0) were JCT2A, 5′-AACTAGTTATTGTCAATG-3′; JCT2B, 5′-AGGCCCATGA CAATAAC-3′. PCR conditions for each 25-μl reaction were as follows: 2.0 mM $MgCl_2$; 100 μM each of dGTP, dATP, dTTP, and dCTP; 15 ng of a single RAPD primer; 0.5 units AmpliTaq polymerase (Applied Biosystems); 25 ng template genomic DNA; and 1× PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 0.001% gelatin). Components for all reactions were assembled in a Labgard laminar flow biological safety cabinet (NuAire, Plymouth, MN) using aerosol resistant tips (Continental Laboratory Products, Burlington, MA). The following amplification profile was used for RAPD reactions with primers 1–800: initial denaturation at 94° for 30 sec; 40 cycles of 12 sec at 94°, 60 sec at 36°, 60-sec ramp to 72°, and 65 sec at 72°; and a final 5-min extension at 72°. A higher annealing temperature of 42° was used for the longer primers 801–890 (16–18 bases in length). Reaction products were subjected to electrophoresis in 1.4% agarose gels in 0.5× TBE (45 mM Tris-Borate, 1 mM EDTA, pH 8.0). PCR conditions for SCAR analysis were as for RAPD reactions but with 1.5 mM $MgCl_2$, 0.1 mM of each primer, and 30 cycles. Annealing temperature was 65° (or 50° for primers JCT2A and JCT2B), and the 2-min extension was at 72°.

AFLP analysis: Amplified fragment length polymorphism (AFLP) analysis (Vos *et al.* 1995) was performed as described previously (Bakkeren *et al.* 2000). For template preparations, combinations of the "six-cutter" restriction enzymes, *Bam*HI, *Bgl*II, or *Pst*I, and the "four-cutter" restriction enzymes, *Mse*I or *Taq*I, were employed and corresponding adapters were ligated. Primer sequences were as follows: BamPa (5′-GGACT GCGTACGATCCa; core in uppercase letters, specifying nucleotides in lower case); BglPaa (5′-GGACTGCGTACGATCTaa); PstPga (5′-GACTGCGTACATGCAGga), -gt, -gg, -ag; MsePat (5′-GATGAGTCCTGAGTAAat), -gaa, -aca, -aat, -aag, -aac, -agc, -ggc, -gcg, -aata, -aagc; TaqPca (5′-TGAGTCCTGACCGAca), -acc, -aca. Oligonucleotide primers were synthesized on a Beckman Oligo 1000M DNA synthesizer. The six-cutter primers were labeled with [γ-^{32}P]dATP (6000 Ci/mmol, New England Nuclear, Boston) using a standard kinase procedure (Vos *et al.* 1995). For analysis, 4 μl of the AFLP products (mixed 1:1 with formamide/dye and heated at 94° for 3 min) were separated on 4.5% polyacrylamide (the ratio of acrylamide:bis was 20:1), 7.5 M urea, 0.5× TBE gels at constant power (110 W, 50 V/cm) in 1× TBE buffer. Gels were dried and exposed to Kodak X-OMAT AR X-ray film.

Construction of libraries: A cosmid library of partially digested *Sau*3A genomic DNA fragments from virulent parental strain Uh362 in pGBcos1 was constructed as described (Kronstad and Leong 1989). The library yielded ~500,000 CFUs

after packaging with Gigapack Gold II extract (Stratagene, Cedar Creek, TX) and transfection of *E. coli* strain DH5α. A BAC library was constructed from genomic DNA from avirulent parental strain Uh364. Approximately 2×10^8 cells were trapped in low-melting-point agarose blocks of 100 μl, converted into protoplasts, and subsequently digested with protease K as described (Lee *et al.* 1999). The trapped high-molecular-weight DNA was partially digested with *Hin*dIII, run out in a 1% low-melting-point agarose gel in a contour-clamped homogeneous electric field (CHEF) apparatus (LKB, Pharmacia) in 1× TAE buffer (40 mM Tris.acetate, 1 mM EDTA, pH 8.0) for 16 hr at 120 V, using 70-sec pulses at 11°. Size-selected DNA of between 200 and 300 kb was excised, equilibrated in 0.1 M EDTA, and sent to Genome Systems (St. Louis) for library construction in vector pBeloBACII and transformation of *E. coli* DH10B/r. The library, containing 2200 clones (~10 genome equivalents), was spotted on high-density nylon membranes for hybridization analysis. A second BAC library from the same avirulent parent, Uh364, was constructed in our laboratory in vector pUsBAC5, by cloning size-selected, partially *Hin*dIII-digested genomic DNA fragments.

DNA blot hybridization analysis: DNA from agarose gels was blotted onto nylon membranes (Biodyne B, Pall, Mississauga, ON) by capillary transfer in 20× SSC (0.3 M sodium citrate, 3.0 M sodium chloride, pH 7.0) or by alkaline transfer with 0.4 N NaOH onto Hybond N+ (Amersham Biosciences, Piscataway, NJ). DNA fragments used as probes consisted of restriction fragments or PCR products and were gel purified using the QIAEX II kit (QIAGEN). Labeling and detection were done using the enhanced chemiluminescence random prime labeling and chemiluminescent detection system, a random primer kit (Amersham Pharmacia Biotech or Roche Diagnostics Canada, Laval, Quebec) with [α^{32}P]dCTP (New England Nuclear), or deoxygenin (DIG)-labeling with DIG-dUTP through random priming or by PCR and detection with CDP-STAR (Roche Diagnostics Canada). All procedures were performed as detailed in the manufacturer's manual. Hybridization was carried out overnight in 0.5 M Na-phosphate, pH 7.0, 7% SDS-buffer, at 65° and filters were washed at 65° in 0.1× SSC, 0.1% SDS. Autoradiography was performed with Kodak X-OMAT AR X-ray film.

Pathogenicity tests: Seeds of each of the four cultivars, Hannchen (*Ruh1*), Excelsior (*Ruh2*), Plush (*Ruh6*), and Odessa (*ruh1 ruh2 ruh6*), a universally susceptible cultivar, were inoculated with parental crosses and progeny from backcrosses. At Winnipeg, Manitoba, seeds were soaked for 1 hr in a 0.12% formaldehyde solution, washed thoroughly in water, and then air dried. Equal amounts of liquid culture of strains of opposite mating type were mixed in complete medium (Holliday 1974) and grown for an additional 24 hr. This inoculum mixture was then placed in a vial with the pretreated seeds and introduced under the seed hull for 20 min at 20 lb vacuum. The remaining inoculum was poured off and seeds were air dried for 3 days at room temperature. Sixty inoculated seeds per testcross were planted and grown in the greenhouse, and 100 each in the field. In the greenhouse test the number of plants showing smutted ears (on any of the tillers) after 3 months was recorded; in the field the percentage of smutted tillers was determined among 100–200 tillers sampled. At Corvallis, Oregon, seeds were dehulled by hand, surface-sterilized, and germinated in sterile distilled water on filter paper at room temperature. After 24 hr, sporidia that had been growing on PDA plates for 48 hr were scraped from the plates and approximately equal proportions of two compatible sporidial lines were physically mixed with a sterile paintbrush. The resulting paste was then painted on the germinated and emerging coleoptile. The inoculated seedlings were further incubated at room temperature for 48 hr, transplanted to pots containing an autoclaved soil mixture of sand, peat moss, and loam (1:1:1 v/v/v), and grown under greenhouse conditions as described by Hu *et al.* (2002). Fifteen inoculated seedlings were seeded, three seeds per pot, and disease was scored after 3 months as the percentage of infected tillers. Discrepancies were retested for verification using the latter method at the University of British Columbia, Vancouver.

RESULTS

Construction of a family segregating for three avirulence genes: Diploid teliospore collections of *U. hordei* homozygous for three avirulence genes served as starting material for the construction of families. Fifty-four progeny originating from the cross Uh362 (*MAT-2 avr1 avr2 avr6*) with Uh364 (*MAT-1 Avr1 Avr2 Avr6*) were evaluated on the differential cultivars Hannchen (*Ruh1*), Excelsior (*Ruh2*), Plush (*Ruh6*), and Odessa (*ruh1 ruh2 ruh6*; Figure 1, Table 1). Traditional disease severity ratings consider a reaction between a *U. hordei* race and a barley cultivar "resistant" when 0–5% of the plants show disease symptoms, that is, at least one smutted spike. When 5–35% of the plants become infected, the reaction is called "intermediate," and levels >36% are rated as "fully susceptible" (Sidhu and Person 1972; Thomas *et al.* 1987). In this rating, the first category represents "field resistance," which is not appropriate for geneticists studying gene-for-gene interactions. In our studies, the *U. hordei* parental isolates carrying *UhAvr1* or *UhAvr6* have never produced teliospores on cultivars Hannchen or Plush, respectively, whereas on these cultivars inoculations with parents carrying the respective recessive alleles have resulted in up to 70% infection on Hannchen and up to 100% on Plush under greenhouse conditions. Pathogenicity tests were performed independently in Corvallis, Oregon, and in Winnipeg, Manitoba (see materials and methods). We scored the interactions as incompatible when no plants showed any smutted tillers during greenhouse and field tests at both sites, when the same combination resulted in at least 12% infection on the universal susceptible cultivar Odessa. Infections on Hannchen varied from 3 to 89% and from 3 to 100% on Plush, but comparison of the results from the three tests at both sites allowed for confident scoring of a given progeny as being virulent. For example, progeny 20 gave 4% infected plants in the greenhouse but 25% in the field in Winnipeg and 62% in the Corvallis greenhouse. Eleven of the progeny were found to give inconsistent results between the test sites. The crosses with these strains were repeated at the University of British Columbia and six of these progeny were again found to give inconsistent results and were not included in selections for the pools.

The *UhAvr1* and *Uhavr1* alleles segregated in a ratio of 25 to 29, as expected for a single locus. The *UhAvr6/Uhavr6* pair gave a similar ratio of 28 to 25. *UhAvr1* and *UhAvr6* segregated independently from the mating-type locus, *MAT* (Figure 1, Table 2).

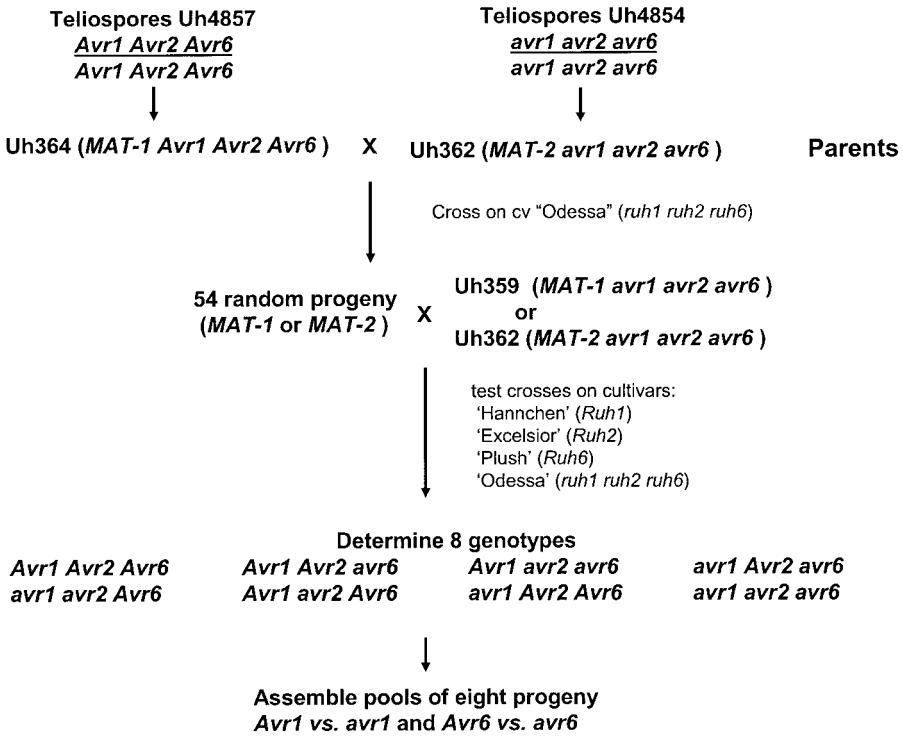

FIGURE 1.—Flow chart of the construction of a *U. hordei* mapping population to detect segregation of avirulence genes. Starting material consisted of diploid teliospore collections homozygous for three avirulence genes, *UhAvr1*, *UhAvr2*, and *UhAvr6*, conditioning incompatibility on barley cultivars Hannchen (harboring resistance gene *Ruh1*), Excelsior (*Ruh2*), and Plush (*Ruh6*), respectively. Upon germinating these teliospores, random haploid basidiospores were obtained and their mating types determined. This yielded parental strains Uh362 (*MAT-2 avr1 avr2 avr6*) and Uh364 (*MAT-1 Avr1 Avr2 Avr6*), a cross of which was initiated on the universal susceptible cultivar Odessa (*ruh1 ruh2 ruh6*). The strains are described in Table 1 and the resulting genotypes of the progeny are presented in Table 2. Note that avirulence genes *UhAvr1* and *UhAvr6* segregate independently and are not linked to the mating-type locus.

In contrast, the *UhAvr2* gene proved difficult to score in our crosses because an unexpectedly small number of the progeny appeared to contain the virulent *Uhavr2* allele; this series has been tested only in Winnipeg and resulted in infection rates generally <8% in the greenhouse and <12% in the field. Similar results have been found previously and the action of the *UhAvr2* gene on Excelsior (*Ruh2*) is thought to be modified by environmental factors (P. THOMAS, unpublished results).

Pools for bulked segregant analysis: The identification of markers linked to a locus of interest is facilitated by bulked segregant analysis (MICHELMORE *et al.* 1991). In our study, two sets of pools with eight progeny each were generated for two of the avirulence alleles (*UhAvr1*, *Uhavr1*, *UhAvr6*, or *Uhavr6*); a primary set was employed for the initial identification of RAPD primers yielding candidate markers linked to the desired allele and a secondary set allowed rescreening of another pool composed of different progeny. Progeny within the pools were distributed evenly with respect to *MAT* and the other *Avr* gene (*UhAvr1* or *UhAvr6*) segregating in the cross (Table 2).

Identification of RAPD markers linked to the *Avr1/avr1* locus: RAPD analysis (WILLIAMS *et al.* 1990) was initially used and 890 RAPD primers were screened by PCR with the bulked genomic DNAs to identify differences in amplification profiles (data not shown). The various V6 and v6 pools generated potential polymorphisms but these failed to show linkage to either allele upon rechecking with a number of individual progeny. RAPD analysis with the V1 and v1 pools identified two primers, 359 and 743, which amplified markers exhib-

TABLE 1

Strains and isolates used

Name	Genotype	Comment
Uh4854[a]	*avr1/avr1 avr2/avr2 avr6/avr6 MAT-1/MAT-2*	*U. hordei* diploid teliospore isolate
Uh4857[a]	*Avr1/Avr1 Avr2/Avr2 Avr6/Avr6 MAT-1/MAT-2*	*U. hordei* diploid teliospore isolate
Uh359	*avr1 avr2 avr6 MAT-1*	Random progeny no. 4 from Uh4854
Uh362	*avr1 avr2 avr6 MAT-2*	Random progeny no. 10 from Uh4854
Uh364	*Avr1 Avr2 Avr6 MAT-1*	Random progeny no. 4 from Uh4857
Uh365	*Avr1 Avr2 Avr6 MAT-2*	Random progeny no. 5 from Uh4857
Um001	*a2b2*	*U. maydis* isolate 518[b]
Um002	*a1b1*	*U. maydis* isolate 521[b]

[a] THOMAS (1976); THOMAS *et al.* (1987); collections from Winnipeg, Manitoba, Canada.
[b] KRONSTAD and LEONG (1989).

TABLE 2

Scoring of progeny from a cross of Uh362 and Uh364 for mating type (*MAT-1* or *MAT-2*) and avirulence (*UhAvr1*, *UhAvr6*) or virulence (*Uhavr1*, *Uhavr6*) phenotypes

Genotype	Progeny no.	Total
MAT-1 Avr1 Avr6	17,[a,b,c] 24,[a] 30,[d] 34,[d] 35,[a,b,c] 44[e], 45[e]	7
MAT-1 Avr1 avr6	5,[f,g] 15,[a,h] 42,[d] 43,[a,h] 49[f,g]	5
MAT-1 avr1 Avr6	18,[c,i,j] 21,[i,j] 22,[e,k] 23,[e,k] 26, 28, 37,[b] 51[b,c]	8
MAT-1 avr1 avr6	1,[j] 12,[j] 13,[g,k] 32,[h,i,k] 33,[f,g] 50,[f] 52[h,i]	7
MAT-2 Avr1 Avr6	9,[a,b,c] 14,[a,b,c] 16,[e,d] 25,[e,d] 53	5
MAT-2 Avr1 avr6	10,[a,h] 38,[d,f,g] 39[d,f,g]	3
MAT-2 avr1 Avr6	6,[b,c,d,j] 8,[i,j] 31,[e] 40,[e,k] 47[b,c,k]	5
MAT-2 avr1 avr6	2,[i,j] 3,[i,j] 4,[h,k] 7,[h,k] 20,[h] 27,[g] 29,[f,g] 46[f]	8

Composition of primary pools for RAPD analysis: [a]V1-1 pool; [j]v1-1 pool; [c]V6-1 pool; [h]v6-1 pool; secondary pools: [c]V6-2 pool; [d]V1-2 pool; [k]v1-2 pool; [g]v6-2 pool. Composition of pools for AFLP analysis: [a]V1 pool; [b]V6 pool; [i]v1 pool; [f]v6 pool. Progeny 11, 19, 36, 41, 48, and 54 gave low levels of infection. *UhAvr2* could not be scored reliably in this cross.

iting linkage. The first of these, primer 359, amplified a 2.0-kb marker exclusively in the v1 pools (marker 359-2.0) and a 1.55-kb marker was strongly amplified only in the V1 pools (marker 359-1.55; Figure 2A). Primer 743 generated a 1.0-kb product (743-1.0) in pools bulked for *Uhavr1* but not for *UhAvr1* (data not shown). Amplifications with primer 359 and DNA from individual progeny confirmed the linkage of markers 359-2.0 and 359-1.55 with their respective alleles. The markers segregated 1:1 among the progeny and all progeny contained either one or the other, but not both markers, suggesting that they were allelic. Of the 53 progeny, 52 exhibited cosegregation between marker 359-1.55 and the *UhAvr1* allele and between marker 359-2.0 and the *Uhavr1* allele. The remaining isolate (no. 12) was scored as *Uhavr1* but amplified the 1.55-kb product presumably due to a recombination event; this would translate into a map distance of 2 cM. Marker 743-1.0 was amplified in 26 of the 28 *Uhavr1* progeny and was not amplified from 2 progeny scored as *Uhavr1* (nos. 12 and 40), placing it distal to 359-2.0 with respect to *Uhavr1* and 3.8 cM from this avirulence allele. Marker 743-1.0 was not amplified in any of the individual progeny classified as *UhAvr1*.

Characterization of RAPD markers: Restriction enzyme analyses of the three cloned markers indicated that 359-2.0 and 359-1.55 shared nearly identical sites and possibly differed by a 450-bp region constituting an imperfect duplication responsible for the size polymorphism. This was confirmed by sequence analysis that

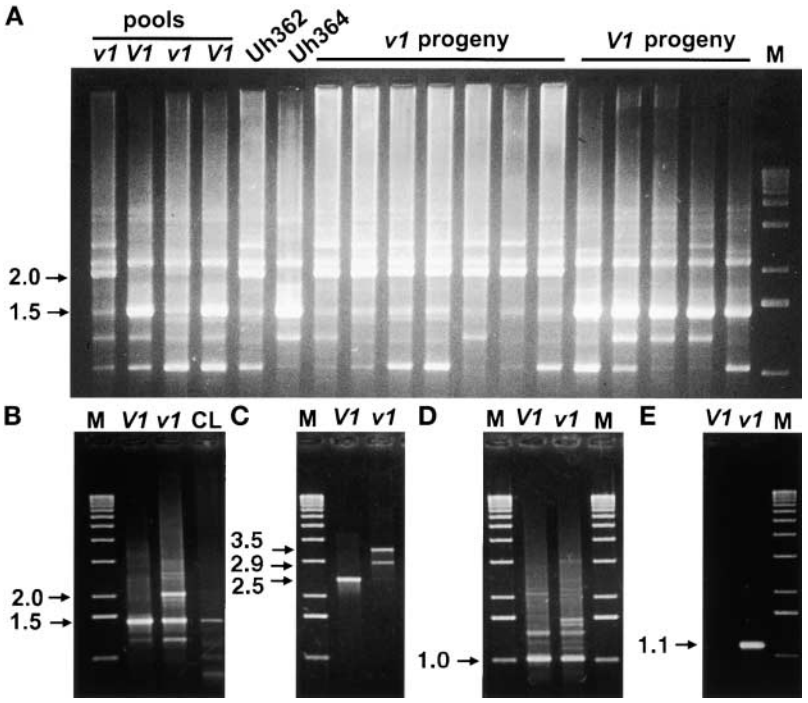

FIGURE 2.—Identification of RAPD and SCAR markers linked to the *UhAvr1/Uhavr1* alleles. (A) Ethidium-bromide-stained agarose gel of the amplification results for RAPD primer 359 revealing linkage of a 2.0-kb PCR product to the *Uhavr1* allele and a 1.55-kb product to *UhAvr1* (arrows). The RAPD products are shown in the pools, the two parents (Uh362, *Uhavr1*, and Uh364, *UhAvr1*) and a subset of individual progeny. (B–E) Ethidium-bromide-stained agarose gels showing amplification with SCAR and junction primers on genomic DNAs from the parental strains and, in B, pooled *UhAvr1* cosmid library DNA (CL) as template. (B) PCR reactions with SCAR primers R359N and R359P. (C) PCR reactions with SCAR primers R359N and 743R. (D) PCR reactions with SCAR primers 743R and 743F. (E) PCR reactions with SCAR primer R359N and junction primer JCT2B. M, the 1-kb marker lane. Sizes are indicated in kilobase pairs.

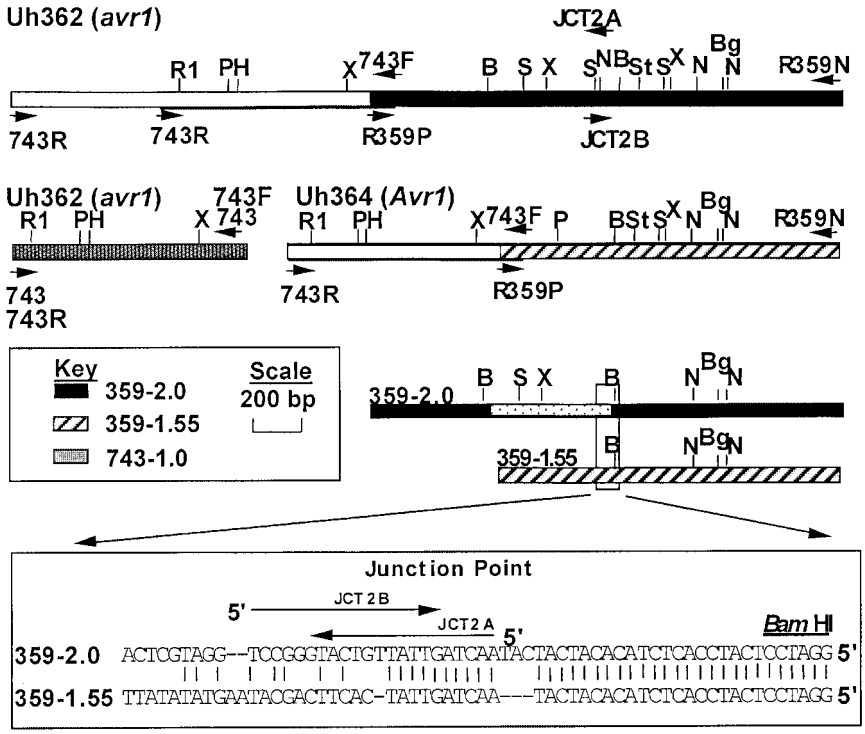

FIGURE 3.—Restriction enzyme map and molecular analysis of the polymorphic RAPD markers. The solid box represents marker 359-2.0, a 2.0-kb PCR product generated with RAPD primer 359 and linked to the *Uhavr1* allele. The hatched box represents marker 359-1.55, a 1.55-kb PCR product generated with RAPD primer 359 and linked to the *UhAvr1* allele. The shaded box represents marker 743-1.0, a 1.0-kb PCR product generated with RAPD primer 743 and linked to the *Uhavr1* allele. Note the extra 450 bp of DNA present in marker 359-2.0 but not in marker 359-1.55 (marked as a dotted box). The enlarged boxed area shows the DNA sequences flanking the point of divergence between markers 359-2.0 and 359-1.55, which allowed for the design of the 359-2.0-specific junction primers, JCT2A and JCT2B. R359N, R359P, 743R, and 743F are SCAR primers whose positions and orientations are also shown. Note that primers 743R and 743F have primer binding sites on DNA physically attached to both marker 359-2.0 and marker 359-1.55 as well as on the original 743 RAPD fragment, which was mapped between 10 and 20 kb or 2 cM away. In addition, 743R primes from two locations on the sequence associated with marker 359-2.0. Restriction enzyme sites: B, *Bam*HI; Bg, *Bgl*II; H, *Hin*dIII; N, *Nde*I; P, *Pst*I; R1, *Eco*RI; S, *Sal*I; St, *Stu*I; X, *Xho*I.

revealed that the 359-2.0 and 359-1.55 RAPD products showed 92 and 96% sequence identity for 250-bp segments compared at their respective ends. These data confirmed that the two markers represent allelic forms of a related sequence. Surprisingly, complete sequence identity over a 100-bp region was also apparent between markers 743-1.0 and 359-2.0. The original RAPD marker 743-1.0 must be a related sequence copy that is separated from 359-2.0 as indicated by the measured genetic distance of 1.8 cM (Figure 3) and a physical distance of ~15 kb (Figure 5) between the two markers. No significant matches between the markers and known sequences in public databases were found and short tandem repeats were not detected in any of the sequences. The DNA sequence provided the information necessary to design SCAR primers with which to generate a more robust and simple PCR profile (PARAN and MICHELMORE 1993). In addition, a region of sequence divergence between markers 359-2.0 and 359-1.55 seemed suitable for designing so-called junction primers for discriminating between alleles (Figure 3). Subsequent SCAR analyses did indeed reveal a simpler PCR profile and yielded several single PCR products linked to either the *UhAvr1* or the *Uhavr1* allele (Figure 2, B–E). However, when SCAR primers 743R and 743F were tested, the original RAPD polymorphism was lost and additional fragments showed up due to the presence of primer binding sites on the DNA stretch physically attached to the 1.55 and 2.0 markers (Figure 2D and Figure 3).

The suspected repetitive nature of the RAPD markers was confirmed by their hybridization to DNA blots containing digested genomic DNA. This analysis revealed intense hybridization in a smeared pattern that was characteristic of repetitive sequences, although single bands were distinctly visible (data not shown). To determine the distribution of these repetitive elements within the *U. hordei* genome and to determine whether the RAPD marker probes might locate the *UhAvr1* allele on one particular chromosome, hybridization studies were carried out with intact chromosomes separated by pulsed-field gel electrophoresis. A strong signal resulted from every chromosome, indicating that this repeated DNA unit is dispersed throughout the genome (Figure 4, A and B). No hybridization to the DNA of the corn pathogen *U. maydis* was detected.

Identification of an AFLP marker linked to the *Avr1/avr1* locus: Literature describing the use of RAPD products for molecular mapping has shown that amplified products frequently contain repetitive elements (see, for example, ARNAU *et al.* 1994). Our study corroborates these findings. Because this severely limits the utility of the markers for chromosome walking, we subjected our bulked pools to AFLP analysis (VOS *et al.* 1995) to search for alternative molecular markers that might be unique. After screening >100 primer pair combinations using different restriction enzyme pairs (see MATERIALS AND METHODS; BAKKEREN *et al.* 2000), a *Bam*HI/*Mse*I combination was found that successfully generated a polymorphism that was tightly linked to the *Uhavr1* allele at a distance of ~2 cM (Figure 5). Unfortunately, the cloned

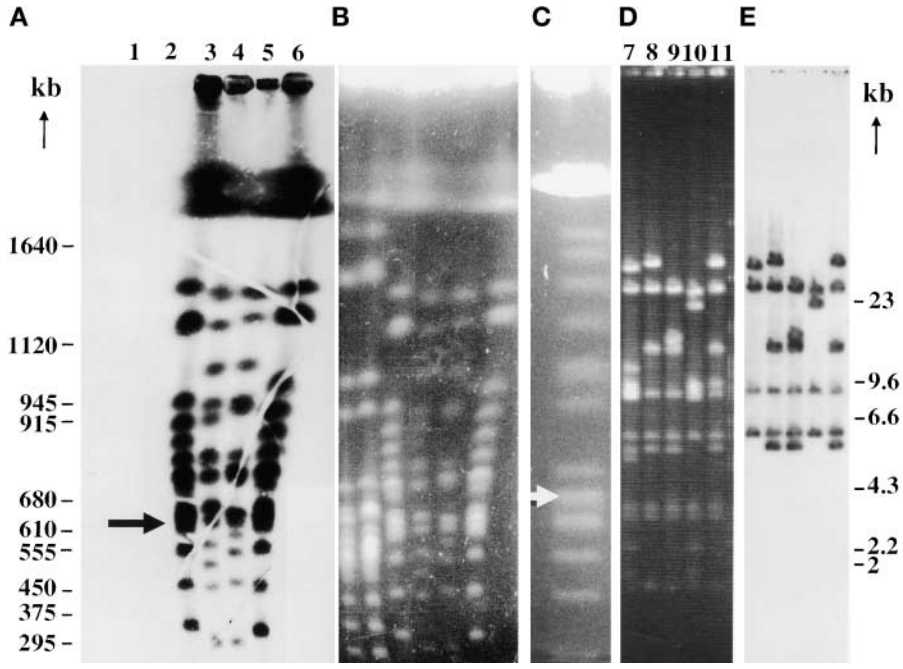

FIGURE 4.—Repetitive nature of the RAPD markers identified with primer 359 and their distribution over the genome and BAC clones. (A) Hybridization of cloned RAPD marker 359-1.55 to a DNA blot of separated chromosomes of *U. maydis* and *U. hordei*, the ethidium-bromide-stained agarose gel of which is shown in B. CHEF gel of embedded chromosomes (see MATERIALS AND METHODS); electrophoresis conditions were: 1% (w/v) agarose gel in 0.5× TBE buffer run at 12° and 180 V for 22 hr with a 70-sec pulse and for 18 additional hr with a 120-sec pulse. Lane designations: 1, Um002; 2, Um001; 3, Uh364; 4, Uh362; 5, Uh359; and 6, Uh365 (see Table 1). No hybridization was detected for lanes 1 and 2, indicating that related sequences are not present in *U. maydis*. The size markers in kilobases refer to A and B and represent *Saccharomyces cerevisiae* chromosome pulsed-field gel size standards (New England Biolabs, Beverly, MA). (C) Ethidium-bromide-stained gel of chromosomes from strain Uh364. Electrophoresis conditions were: 1% (w/v) agarose gel in 1× TBE buffer run at 11° and 135 V for 65 hr with a 125-sec pulse. The arrow denotes the bands that hybridize with the probe RFLP1, indicating the chromosome on which *UhAvr1* is located (data not shown). The probe location relative to the other markers is given in Figure 5. (D) Ethidium-bromide-stained gel of BAC clones pBAC1-J3 (lane 7), pBAC1-G21 (lane 8), pBAC1-M18 (lane 9), pBAC1-P7 (lane 10), and pBAC3-A2 (lane 11) digested with *Kpn*1. (E) Hybridization of cloned RAPD marker 359 to a DNA blot of the agarose gel in D. Size markers in kilobases on the right refer to D and E.

marker, designated AFLP1, did not hybridize to genomic DNA from the avirulent parent due to a deletion of homologous sequences in that strain (data not shown). However, the marker was useful for further genetic characterization at the locus and for library screening. Despite the use of >50 primer combinations, no AFLP markers have been identified in the various V6 and v6 pools to date.

Library screening: To initiate a genomic walk toward the *UhAvr1* allele, a 250-bp *Pst*I fragment of marker 359-1.55 that had previously given a less repetitive hybridization signal on genomic DNA was used to screen a cosmid library from the avirulent parent Uh364 (in pGBcos1). A screen of 2496 clones from the library (harboring approximately five genome equivalents) yielded 154 positive clones that were subsequently analyzed by direct PCR using SCAR primers R359N and R359P to detect a 1.55-kb product (see Figure 2B). Surprisingly, the majority of these cosmids yielded amplification products, although few were of the expected 1.55-kb size. Screening with SCAR primers for positive clones eliminated all but 13 cosmids as possible candidates. Further analyses revealed that none of these represented the original RAPD marker 359-1.55.

In an alternative approach to obtain representative clones from the region, we decided to screen a cosmid library (in pGBcos1) from the virulent parent Uh362 with SCAR primers R359N and JCT2B. These primers consistently generated a specific PCR fragment (Figure 2E). A single cosmid, 359RAPDv1, was identified by screening pools of decreasing complexity (starting from 20,000 CFUs harboring ~20 genome equivalents). The cloned AFLP1 marker was used as a probe to screen 50,000 CFUs from the same library to yield cosmid clone AFLPv1 (Figure 5B). Restriction enzyme mapping and hybridization analyses revealed the location of the respective markers on these cosmid clones and identified a single-copy probe represented by a 1.7-kb *Hin*dIII fragment located ~12 kb proximal to the AFLP1 marker. Importantly, this fragment hybridized to genomic DNA from the avirulent parent. A 400-bp *Bam*HI-*Hin*dIII subfragment of this 1.7-kb *Hin*dIII fragment, designated RFLP1, was used in an RFLP analysis of the population and no recombinants were detected (Figure 5C). However, this probe failed to identify positive clones in the cosmid library from the avirulent parent.

Our analysis indicated that *UhAvr1* sits in a region harboring repetitive DNA and that sequences from this area appear to be underrepresented or absent from the cosmid library. It is possible that *E. coli* does not tolerate clones with multiple copies of the repetitive elements identified by the RAPD markers. To circumvent this problem, we constructed a BAC library from the avirulent parent Uh364 in the commonly used vector pBeloBACII (SHIZUYA *et al.* 1992). The 1.7-kb *Hin*dIII fragment was used as a probe to screen 2200 BAC clones

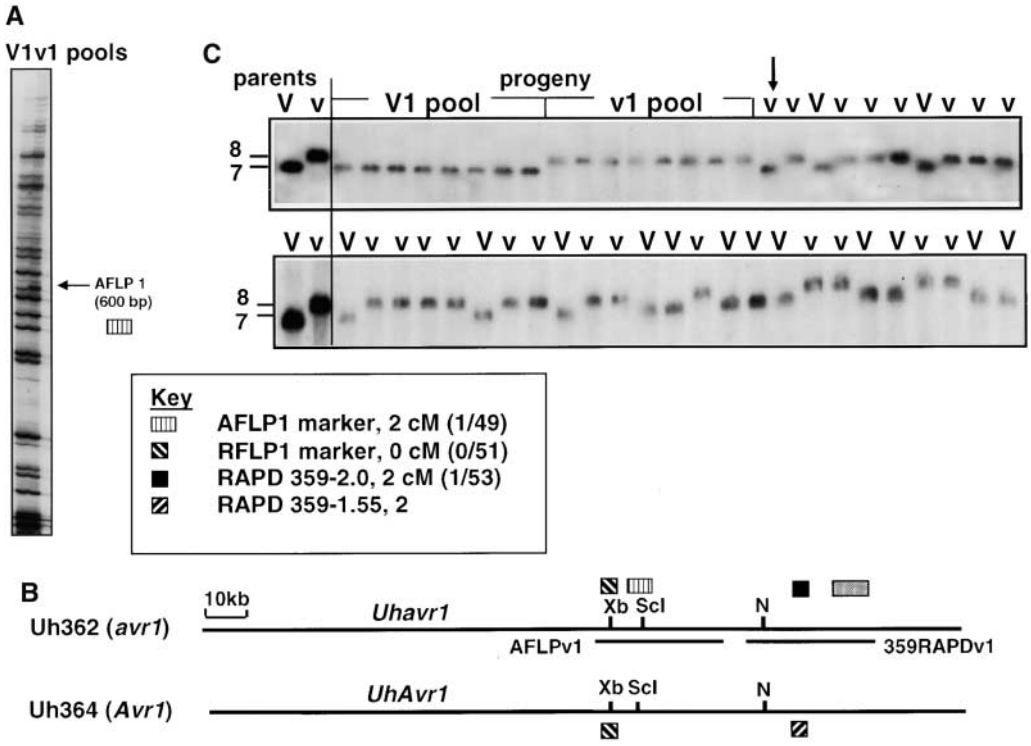

FIGURE 5.—Identification of the AFLP1 marker and its map position relative to the RAPD markers. (A) Autoradiograph of a PAGE analysis of ^{32}P-labeled AFLP fragments generated with primer combinations BamPa + MsePggc. These primers were used with *Bam*HI/*Mse*I-digested templates from bulked pools of DNA from eight segregants harboring alleles *UhAvr1* (V1 pool) and *Uhavr1* (v1 pool; see Table 2 and MATERIALS AND METHODS). Note that the dominant polymorphic AFLP1 marker is linked to the *Uhavr1* allele but the cloned fragment from this marker did not hybridize to DNA of the avirulent parent. (B) Maps of the allelic genomic regions harboring the *UhAvr1/Uhavr1* locus. The positions of the RAPD, AFLP, and RFLP markers as determined by restriction mapping of the cosmids AFLPv1 and 359RAPDv1 are indicated. The cosmids each contain 35-kb inserts. The genetic distances in centimorgans were measured in the population by RAPD, AFLP, and RFLP analysis; the recombinant progeny were no. 12 for the RAPD 359 and AFLP1 markers, and nos. 12 and 40 for RAPD marker 743. (C) An example of an RFLP analysis with a DNA blot of *Sac*I/*Xba*1-digested genomic DNA from the two parents and a subset of their progeny. The blot was hybridized with a 400-bp *Bam*HI/*Hin*dIII fragment, designated RFLP1, located ~12 kb proximal to the AFLP1 marker. The sizes on the left are in kilobases. The arrow indicates a potential recombinant (progeny 1) that was excluded from the population after it became clear that its genotype had been scored incorrectly. Restriction enzyme sites: N, *Not*I; ScI, *Sac*I; Xb, *Xba*I (note that not all *Sac*I and *Xba*I sites are indicated).

harboring ~10 genome equivalents. Eight positive clones were recovered and restriction mapping with rare-cutting enzymes generated a set of 5 tiled BAC clones spanning ~170 kb. Comparative mapping and PCR analysis with the RAPD and SCAR primers placed these markers and the cosmids on a physical map of the region (Figure 6). The mapping efforts were considerably hampered by the fact that probes from various regions often contained repetitive sequences present at several locations (Figure 4, D and E; data not shown). In addition, spot sequencing in, for example, subclone 5-12 (Figure 6), revealed repetitive sequences similar to those in RAPD359-1.55 and -2.0 that also matched sequences in other BAC clones spanning the mating-type region *MAT-1* (Q. JIANG, G. BAKKEREN and J. KRONSTAD, unpublished results).

Delimiting the region containing the *Avr1/avr1* locus: We next sought to discover a recombination breakpoint to more closely delimit the *UhAvr1* locus on the particular region of cloned DNA defined by our BAC clone tiling set. Subclones of the region were obtained in the Ustilago-specific transformation vector pUsBAC5 (see MATERIALS AND METHODS) to allow for the isolation of probes and future testing of potential avirulence activity. These subclones were mapped on the BAC clones and smaller fragments were isolated to serve as probes in RFLP analyses. The genomes of the two parents are apparently quite similar in this region and it proved difficult to discover RFLPs. For example, 25 restriction enzymes, 17 recognizing 6-bp sites and 8 recognizing 4-bp sites, were routinely tested on DNA from the two parents. One additional probe revealing an RFLP, RFLP2, was located 15 kb proximal to RFLP1 but did not reveal recombinants (Figure 6). A probe at the end of BAC clone pBAC1-J3 however, revealed five new recombinants in the population and this probe defined the marker RFLP3 that sits 65 kb from RFLP2 (Figure 6). Because pBeloBACII cannot be used to transform *U. hordei*, an additional BAC library was constructed in pUsBAC5, resulting in the isolation of clones pUsBAC1-P19 and pUsBAC1-E6 using probe RFLP1 (Figure 6). However, initial experiments to introduce complete BAC clones and test for avirulence activity were inconclusive. Taken together, the genetic data position the *UhAvr1* locus within an 80-kb interval between AFLP1 and RFLP3; this region is contained on our BAC clones pBAC1-J3, pBAC1-P7, and pUsBAC1-P19 as shown in Figure 6.

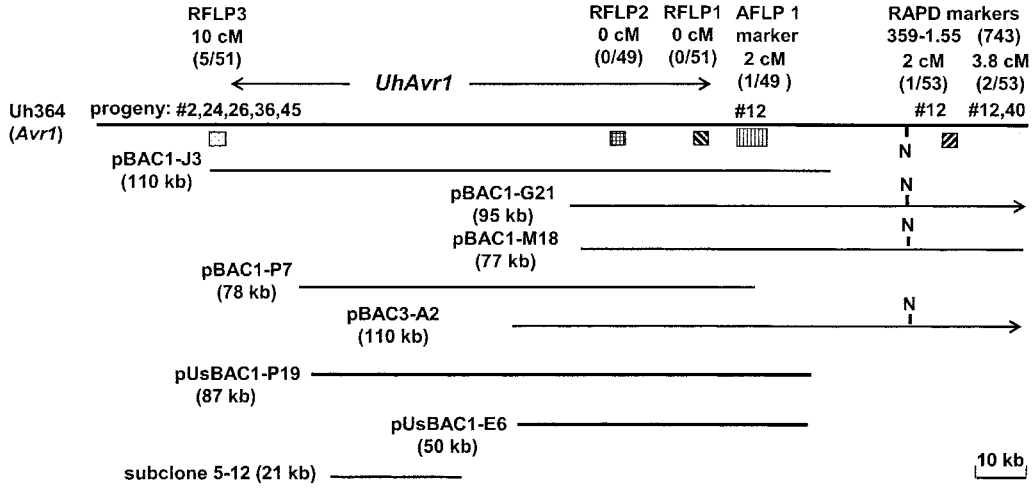

FIGURE 6.—Physical and genetic map of the *UhAvr1*-containing region. The long bold line at the top represents the genomic region of the avirulent parent Uh364. The RFLP1 probe was used to recover eight overlapping BAC clones. Of these, five tiled BAC clones are depicted as solid lines and their ID number and insert sizes (in parentheses) are shown. Several fragments spanning the 80-kb region to the left of the initial RFLP1 probe were tested by RFLP analysis to find a recombination breakpoint (see legend to Figure 5). BAC clones pUsBAC1-P19 and pUsBAC1-E6 were isolated from a different BAC library in the Ustilago-specific vector pUsBAC5 (see MATERIALS AND METHODS). The positions of the markers on the map are shown at the top along with the ID numbers of the recombinant progeny detected by each marker. Note also that the RFLP2 marker did not detect any recombinants in this population. N, *Not*I restriction enzyme site as reference.

DISCUSSION

In this study, a population of *U. hordei* isolates was generated in which we confirmed the segregation of two dominant avirulence genes, *UhAvr1* and *UhAvr6*, as the first step toward the isolation of these genes. Two RAPD markers and one AFLP marker were identified tightly linked to the *UhAvr1* gene. This analysis demonstrated the feasibility of combining RAPD and AFLP marker analysis with the bulked segregant technique for tagging specific genes in *U. hordei* and allowed us to identify several BAC clones carrying the *UhAvr1* locus. Additionally, one of the BAC libraries that we generated was employed to construct a physical map of the *U. hordei* genome (Q. JIANG, G. BAKKEREN and J. KRONSTAD, unpublished results).

The combination of bulked segregant and RAPD or AFLP analyses provides a means to quickly saturate a region of interest with molecular markers. In our study, pools bulked for *UhAvr1* and *UhAvr6* were screened with 890 available RAPD primers, allowing for an extensive survey of the *U. hordei* genome. Not all primers resulted in the synthesis of RAPD products from *U. hordei* DNA (Table 3). Presumably, there is a lack of inwardly oriented annealing sites close enough for amplification for these primers. Alternatively, and additionally, sequences that are complementary to these RAPD primers simply may not be present in the *U. hordei* genome. Interestingly, almost all of the primers that failed to give products had a guanine and cytosine base (G + C) composition of 50% or less. The short length of RAPD primers may require a higher G + C content for amplification. Each of our 667 successful PCR amplifications yielded an average of six products so that our RAPD primer screening produced ~4000 RAPD loci. These loci are assumed to be randomly dispersed throughout the genome and only three cosegregated with the *Avr1/avr1* locus. Assuming that each RAPD locus is able to detect base-pair differences at either of the primer binding sites, as well as DNA rearrangements between each site, ~72 kb of the *U. hordei* genome was directly scanned by primer annealing.

The large number of AFLP primer combinations that had to be tested corroborated the results from the RAPD analyses. Even though preliminary screens with several RAPD primers revealed the presence of sufficient polymorphisms between the two parents, very few were discovered with either the RAPD or AFLP techniques once the *UhAvr1* and *UhAvr6* loci were targeted in the respective pools. The two parents are related and lack substantial polymorphisms because they are derived from teliospore populations originating from southern Manitoba. They have some common ancestry due to three back-

TABLE 3

Results of RAPD analysis with avirulence gene pools

Primer set[a]	A (%)	B (%)	C (%)	D (*N*)
1	34	8	10	2
2	39	12	15	3
3	24	18	10	3
4	11	17	10	2
5	21	16	21	2
6	32	15	15	1
7	24	10	7	4
8	14	17	17	2
9	22	11	13	4
1–9	25	14	13	23

A, primers giving no amplification; B, primers rechecked against secondary V1-1 and v1-1 pools; C, primers rechecked against secondary V6-1 and v6-1 pools; D, primers checked against individual progeny.

[a] Each set contained 100 10-mer primers.

crosses performed to obtain homozygous teliospores (P. Thomas and F. Matheson, unpublished results). Ideally, one would want to employ new populations using isolates that are genetically more diverse to generate polymorphic markers. Also, for the smut fungi, it should be possible to produce interspecific crosses between different species such as *U. hordei* and *U. nigra* that share a common host and are interfertile. It has been proposed that both of these barley smuts should be merged into a single species and it has recently been shown that many of the small grain-infecting smut fungi are closely related (Bakkeren *et al.* 2000; Menzies *et al.* 2003). The absence of polymorphisms in certain regions of the genome may explain why no RAPD markers linked to the *UhAvr6* locus were identified. AFLP analysis of the V6 and v6 pools was also unsuccessful and it may be necessary to use pools that are bulked for fewer segregants to screen for less tightly linked markers. In addition, it is clear that very limited differences could account for avirulence/virulence differences between alleles. For example, at least one report describes a single base-pair change as the key difference between an active and an inactive allele of the avirulence gene *Avr4* of the tomato mold *C. fulvum* (Joosten *et al.* 1994).

Even though the RAPD markers that we identified in the screen with *UhAvr1* harbored repetitive DNA with copies dispersed over the genome, including several areas of the BAC clones spanning the *UhAvr1* locus, the RAPD primers themselves and the SCAR primers derived from one of them were tightly linked to the *Uhavr1* allele. Single base-pair changes at the primer binding sites may be responsible for this result. Indeed, SCAR primers 743R and 743F derived from RAPD primer 743 still produced the 1.0-kb product but it was no longer linked to *Uhavr1*. Recent use of RAPD analysis for molecular mapping has shown that amplified products frequently contain repetitive elements. The proportion of RAPDs composed of highly repeated DNA does appear to vary according to the source of the template DNA and the specific site being targeted. For example, molecular analysis of 49 RAPDs amplified from the fungal tomato pathogen *C. fulvum* revealed that all but one of the products represented repetitive DNA (Arnau *et al.* 1994). RAPD analysis of tomato to target the *jointless* gene revealed that 50% of the markers were repetitive (Wing *et al.* 1994). This apparent bias toward the amplification of repetitive DNA represents an unforeseen and serious limitation of the RAPD technique in marker-based cloning. The genome organization and complexity of *U. hordei* has not been studied to determine the proportion of repetitive DNA in the genome. However, sequence analysis of the 500-kb *MAT-1* region indicates a high density of repetitive sequences, including copies of sequences related to 359-2.0 (Lee *et al.* 1999; J. Kronstad, unpublished results). Other basidiomycete genomes appear to contain a substantial level of repetitive DNA. For example, reassociation kinetic studies for the genome of the wheat stem rust *P. graminis*, a fungus in the hemi-basidiomycete class with *U. hordei*, measured a repetitive DNA content of 30% (Backlund and Szabo 1993). Further, the genomes of *Coprinus lagopus* and *Schizophyllum commune*, two higher basidiomycetes, were reported to possess 10–20% repetitive DNA (Dutta 1974; Ullrich *et al.* 1980).

E. coli cells carrying either of the two cosmid clones harboring the RAPD and AFLP markers exhibited weak growth, suggesting that the presence of the *U. hordei* DNA is somehow detrimental to cell growth. In addition, we were unsuccessful in using the RFLP1 marker to identify positive clones in an extensive screen of the cosmid library of the avirulent parent Uh364. It was necessary to resort to BAC libraries harboring large genomic DNA stretches in low copy number in *E. coli* to identify clones carrying the *UhAvr1* region. Moreover, subcloning of various smaller fragments from this region was unsuccessful in standard multi-copy vectors, and again we had to modify a BAC vector for the construction of these subclones. It is unclear whether the repetitive nature of the region is responsible or whether any of the encoded gene products, serendipitously produced in *E. coli*, are detrimental for growth. Future sequence analysis might clarify the nature of this problem.

The next step in our analysis will involve the subcloning of the *UhAvr1* region and the functional analysis of subclones for their ability to convert a virulent dikaryon to avirulence on the cultivar Hannchen. Preliminary experiments with the BAC clone pUsBAC1-P19 indicated the possible presence of avirulence activity, as expected, whereas clone pUsBAC1-E6 did not result in any significant reduction in disease. However, the stable transformation of *U. hordei* with BAC clones harboring large inserts has proven to be problematic (G. Bakkeren, unpublished results). No significant difference in transformation efficiency or stability of resulting transformants was found between using uncut (supercoiled) or linearized constructs, and hygromycinB-resistant transformants were seen to revert to sensitivity upon prolonged culture under nonselective conditions. Transformation of *P. infestans* with BAC clones appears to be efficient and to yield stable inserts (Randall and Judelson 1999). For *U. hordei*, a more directed gene knock-out strategy in the avirulent parent seems more suitable. Toward this goal, a shotgun sequencing approach has been initiated and the BAC clone inserts from the *UhAvr1* region are being used to screen cDNA libraries for potential genes located in the area. We are also mapping the region harboring *UhAvr1* more precisely to help in identifying target genes for disruption.

In summary, this study reports the localization of *UhAvr1* in *U. hordei* and the identification of BAC clones carrying the gene. Our experience with this gene suggests that the region harbors repetitive DNA sequences and that many DNA fragments from this region are difficult to propagate in *E. coli*. This may reflect an

association of the avirulence gene with an unusual part of the genome. However, our analysis of the repetitive RAPD markers suggests that some of these sequences have a widespread distribution and are therefore an important feature of the *U. hordei* genome. Despite the difficulties presented by the repetitive sequences, we were able to clone the region harboring *UhAvr1* and this represents the first isolation of an avirulence gene from a basidiomycete pathogen.

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