

IDENTIFICATION AND CHARACTERIZATION OF RAPD MARKERS
LINKED TO THE VI AVIRULENCE GENE OF *USTILAGO HORDEI*

by

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ABSTRACT

The basidiomycetous fungus *Ustilago hordei* is the causal agent of covered smut of barley and oats. The resistance or susceptibility of the barley host to this phytopathogen is determined by the resistance genes in the plant and the corresponding avirulence genes in the pathogen. A map-based cloning strategy was adopted for the isolation of the avirulence genes, *VI* and *V6*, in *U. hordei*. Randomly amplified polymorphic DNA (RAPD) analysis of bulked segregant pools for *VI*, *vI*, *V6* and *v6* was performed to obtain molecular markers linked to these alleles. Polymerase chain reaction (PCR) amplifications with 890 available RAPD primers identified 2 markers cosegregating closely with the *vI* allele and one marker tightly linked to the *VI* allele, but no markers linked to either *V6* or *v6* were obtained. One marker, designated 743-1.0, mapped 5.5 cM from *vI* while two allelic markers, 359-1.55 and 359-2.0, were 3.7 cM from the *VI* and *vI* alleles. Hybridization studies of the identified markers determined that all three RAPD products were part of a repetitive DNA element which was present on all chromosomes of the *U. hordei* genome. Nucleotide sequence information from the markers was obtained and used to design sequence characterized amplified region (SCAR) primers. These primers were useful for the further characterization of the markers and for the screening of genomic libraries using PCR. A cosmid library of parental strain Uh 4857-4 (*VI*, *V2*, *V6*, *MAT-1*) was constructed for the purpose of a chromosome walk toward the *VI* allele. A screen of 2496 clones of the cosmid library by a combined approach of hybridization and direct PCR with the SCAR primers did not result in the isolation of cosmids carrying marker 359-1.55. Successful PCR amplification of a 1.55 kb SCAR product from pooled cosmid clones, however, suggests that this cosmid clone is present within the library. The results of the work described here have subsequently led to the isolation of two overlapping cosmids carrying marker 359-2.0 from another cosmid library. These cosmid clones will be invaluable tools for the isolation of the *VI* avirulence gene.

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1. Introduction

1.1 Literature Review

1.1.1 The Gene-for-Gene Hypothesis

The ability of a phytopathogen to cause disease on a set of plant species defines its host range. The pathogen is said to be compatible with these host species. Yet even within this range, resistance or incompatibility can occur. That is, in certain plant-pathogen interactions, another system is superimposed upon basic pathogenicity to determine resistance or susceptibility. In the last century, this system has been of significant economic importance because plant breeders have incorporated resistance genes into agricultural crops. The mechanics of resistance and susceptibility is also of scientific interest in the investigation of the nature of disease and interactions between organisms.

Studies of the rust pathogen *Melampsora lini* on its host, flax, demonstrated that genes in both the host and the pathogen were involved in compatible and incompatible disease reactions (Flor, 1942). This gene-for-gene hypothesis proposed that a resistance gene in the host and a corresponding avirulence gene in the pathogen conditioned resistance or susceptibility. In its simplest form, the hypothesis predicts that incompatibility will occur if a host carries a dominant resistance allele that allows recognition of a dominant avirulence allele in the pathogen (Table 1). Since the proposal of this model, gene-for-gene interactions have been demonstrated by classical genetics for phytopathogens as diverse as bacteria, fungi, viruses, insects and nematodes (Day, 1974; McHale *et al.*, 1989; Sidhu, 1986).

Table 1. Expected compatibility between genotypes in a single locus, gene-for-gene interaction.

	Plant Host Genotype	
Pathogen genotype	<i>R</i> -	<i>r</i>
<i>V</i> -	Incompatible	Compatible
<i>vv</i>	Compatible	Compatible

An incompatible interaction results in resistance and a compatible interaction leads to disease. *V* represents a dominant avirulence locus and *v* indicates a recessive virulence allele. *R* indicates a dominant locus conditioning resistance; *r* denotes the recessive allele of the *R* locus.

How the gene-for-gene system of resistance operates at the molecular level, however, has only recently begun to be elucidated. The most widely accepted mode by which gene-for-gene complementarity operates has followed along the lines of a straightforward ligand-receptor model. Resistance is postulated to be initiated by host recognition of the pathogen, mediated via the binding of the putative avirulence gene product by the putative resistance gene-encoded receptor. Host recognition results in the activation of plant defense responses which serve to limit growth of the pathogen and, thereby, prevent disease. Most notable of the defense reactions of many plant species is the hypersensitive response, a rapid necrosis of plant cells in the vicinity of the invading pathogen.

The utilization of the hypersensitive response as a simple assay for avirulence, along with the application of the tools of molecular biology, has allowed for the cloning of several avirulence genes and, more recently, of resistance genes (reviewed in: Keen, 1992; de Wit, 1992; Dangl 1995). For the most part, DNA sequencing of cloned avirulence genes has provided few clues to their function in the pathogen or to why these genes are retained in light of their seemingly detrimental effect on the pathogen. In contrast, the cloning and characterization of a handful of resistance genes has revealed a novel class of gene products sharing a number of motifs. These shared structural features implicate the encoded products as possible receptors at the front end of a signalling pathway (Mindrinos *et al.*, 1994; Bent *et al.*, 1994; Whitham *et al.*, 1994; Jones *et al.*; 1994). The isolation of both genetic components of the system, resistance genes and avirulence genes, has confirmed the validity of the gene-for-gene hypothesis and has begun to provide a molecular understanding of plant-pathogen interactions.

1.1.2 Gene-for-Gene Interactions in the Life Cycle of *Ustilago hordei*

The interaction of the basidiomycetous fungus *Ustilago hordei*, causal agent of covered smut of barley and oats, with one of its hosts, *Hordeum vulgare*, conforms to the gene-for-gene hypothesis and provides a model system for study. As with most fungal pathogens exhibiting gene-for-gene interactions, *U. hordei* is a biotrophic pathogen highly specialized on a narrow range of plant hosts (Ellingboe, 1976; Heath, 1981; Keen, 1982). Several features of the life cycle of this fungal pathogen facilitate the study and isolation of avirulence genes (Figure 1).

U. hordei exhibits dimorphic growth in that it has both a sporidial haploid phase and, after mating, a filamentous dikaryotic phase that grows obligately in the plant host.

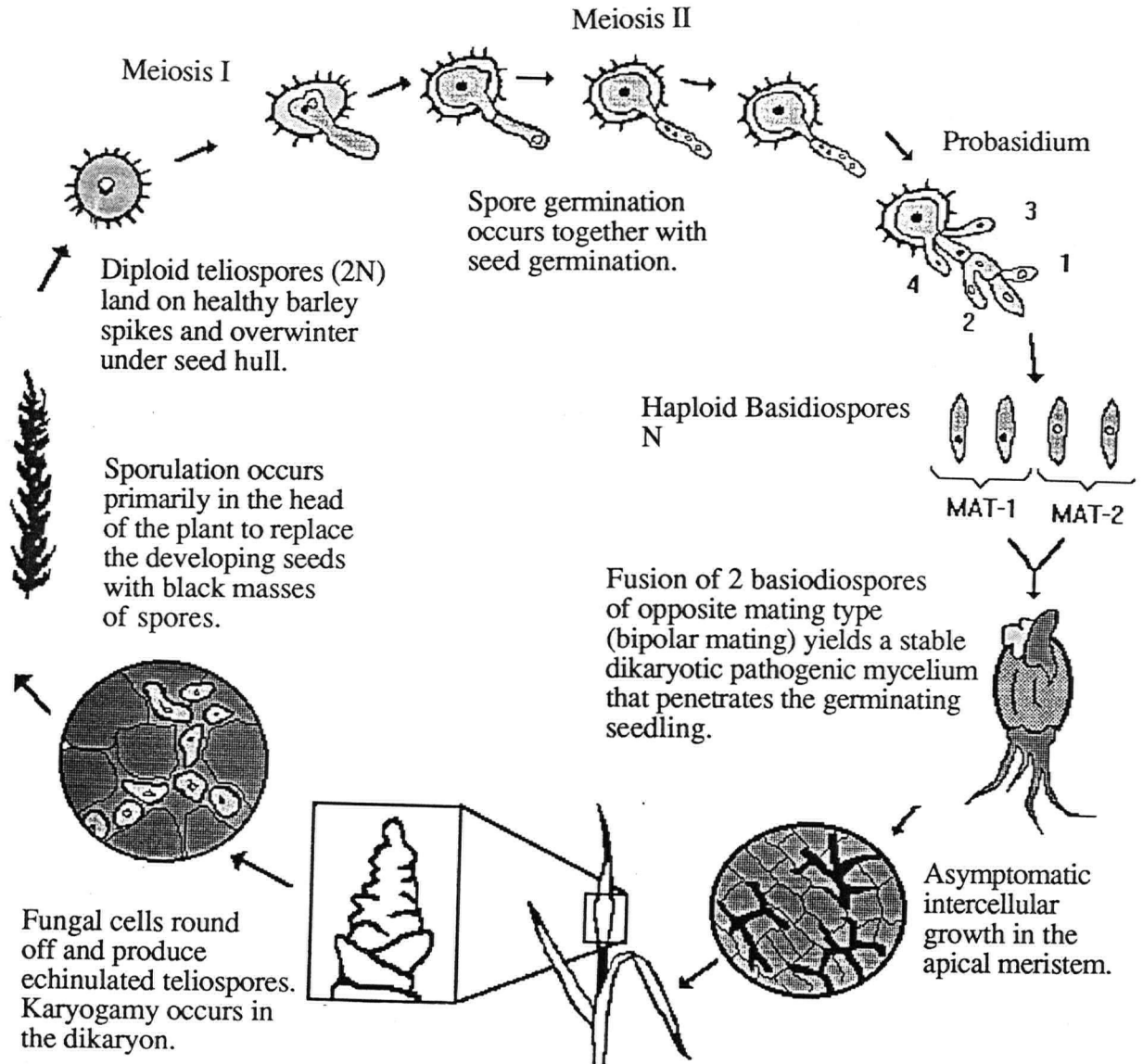


Figure 1. Life cycle of *Ustilago hordei*, causal agent of covered smut of barley and oats. This figure was adapted from figures 11-98 and 11-100 in Agrios (1988).

While attempts to replicate *in planta* nutrient requirements have not successfully resulted in stable growth of the dikaryon *in vitro*, the initial stages of the life cycle proceed readily in the laboratory. Diploid teliospores germinate on standard mycological media to produce a probasidium in which meiosis occurs to give rise to four haploid basidiospores. Separation of the meiotic products by micromanipulation allows for the analysis of gene segregation among the progeny. The resulting haploids grow by budding as yeast-like cells that are easily cultured and are amenable to molecular techniques. The adaptation of such tools as DNA transformation, gene disruption techniques, selectable markers and autonomous replicating vectors from protocols developed for the corn smut pathogen *Ustilago maydis* (Bakkeren and Kronstad, 1993; Wang *et al.*, 1988; Banks and Taylor, 1988; Tsukuda *et al.*, 1988; Kronstad *et al.*, 1989; Fotheringham and Holloman, 1989) has greatly facilitated the genetic studies of *U. hordei*.

Transition to the infectious dikaryon occurs by the fusion of sporidial cells of opposite mating type. Cell fusion also serves to bring together the avirulence alleles which determine compatibility or incompatibility to the host. Regardless of which reaction is to occur, the dikaryon appears to be able to penetrate the coleoptile of the germinating barley seedling. How susceptible and resistant reactions diverge at this point, however, is difficult to determine due to the absence of detectable symptoms in the former case and a lack of a visible host defense response in the latter.

During compatible interactions, growth of the infectious hyphae continues asymptotically into the growing point of the plant where the fungus resides until differentiation of the meristem to the floral tissue. Macroscopically, susceptibility is indistinguishable from resistance until heading of the plant; at this point, barley kernels are replaced with the black sooty teliospores of the fungus. Dissemination begins when the

plant-derived membrane that covers the sori (spore cluster) is broken, most often by threshing, and spores are carried (primarily by the wind) to healthy plants. Germination of spores that have overwintered under the seed hull completes the infection cycle.

Not much is known about how the infection process is halted in incompatible interactions. Separate histological studies by Kiesling and Chatterjee observed some anatomical changes in tissues of resistant barley varieties after pathogen penetration (Kiesling, 1952; Chatterjee, 1956). Most notable of these was the formation of a sheath in epidermal cells around the penetrating hyphae. The formation of the barrier does not appear to be solely responsible for resistance, however, since this host response was also present at low frequencies during infection of susceptible varieties. Both studies also observed that a common expression of incompatibility was simply the failure of the hyphae to grow further into the host.

These observations were made for interactions involving only two avirulence genes in total and may only, therefore, be specific for those genes. As a result, the possibility remains that different avirulence genes may elicit defense responses at different points in the sequence of infection. In the case of all known avirulence genes in *U. hordei*, though, incompatibility does not result in a detectable hypersensitive response. Growth of the dikaryon in a number of incompatible reactions is currently being investigated through the use of a β -glucuronidase (GUS) -reporter construct in the pathogen (G. Bakkeren, unpublished results).

Six avirulence genes in *U. hordei*, and their complementary resistance genes in barley cultivars, have been identified by classical genetic studies (Tapke, 1945; Sidhu and Person, 1971a; Sidhu and Person, 1972b; Ebba and Person, 1975; Thomas, 1976). Much of this work was performed at the University of British Columbia by Dr. C. Person and his

colleagues. Of the 6 avirulence genes, 4 do not conform exactly to the simple gene-for-gene model and are, therefore, less suited for study. *V2* controls resistance on cultivar Excelsior, which carries the *R2* resistance allele. The action of *V2*, however, was found to be modified by differing conditions of environment and genetic background. Studies performed in British Columbia and California indicated that the interaction of *V2* with *R2* does not behave consistently between the two locations. Linked to *V2* is the avirulence gene *V3* which determines resistance on cultivars Nepal and Pannier. Very little work has been performed with this gene due, in part, to the difficulty in detecting segregation away from *V2*. *V4* and *V5*, existing as duplicate genes at unlinked loci, do not act in the traditional single gene model; these genes are involved in resistance on the cultivars Keystone and Himalaya.

The two remaining avirulence genes, *V1* and *V6*, have been demonstrated to act consistently in a stable genetic manner that is relatively unaffected by environment variability. *V1* conditions resistance on cultivar Hannchen (*R1*) and *V6* on cultivar Plush (*R6*). Therefore, these two independently inherited genes are prime candidates for the isolation of avirulence genes from *U. hordei* and are the focus of this study.

1.1.3 Strategies for the Cloning of Avirulence Genes

1.1.3.1 Shotgun and Reverse Genetic Approaches

Several approaches have been taken towards the cloning of avirulence genes. In bacterial systems, cloning has relied largely on shotgun approaches involving the transformation of portions of the genome from an avirulent strain into a virulent one using the hypersensitive response as a quick assay. The absence of such a response and the considerably larger fungal genome would make such a strategy laborious and time-

consuming for *U. hordei*. Since not all plants inoculated with a compatible strain result in disease, a large number of replicates would be necessary; all of which would require two to three months for plant maturation before scoring.

Three of the four fungal avirulence genes cloned to date, *avr9* and *avr4* of *Cladosporium fulvum* and *Nip1* of *Rhynchosporium secalis*, were isolated by reverse genetics (Van Kan *et al.*, 1991; Joosten *et al.*, 1994, Hermann *et al.*, 1994). In these cases, researchers were able to isolate the polypeptide products of the avirulence genes. From the N-terminal protein sequences, degenerate oligonucleotides were designed for gene isolation. For these avirulence genes, the reverse genetic cloning strategy was greatly facilitated by the observations that the mature products were secreted and were able to elicit strong biological reactions in the plant. The presence of signal peptide sequences in all of the fungal avirulence genes characterized so far represents their only common feature. No other distinctive motifs are shared between these fungal elicitors or with any other known gene product. Whether or not any of the *U. hordei* avirulence gene products are secreted is not yet known.

What is known is that the putative elicitors in *U. hordei* do not possess a detectable activity comparable to the hypersensitive response triggered by the *C. fulvum* *avr4* and *avr9* gene products or the stimulation of the plant plasmalemma H⁺-ATPase by the *Nip1* necrosis inducing peptide. The difficulty in isolating the products of the avirulence genes of *U. hordei* does not favor the adoption of a reverse genetics cloning approach. Such a strategy, however, was adopted by P. Thomas in an attempt to isolate the putative V6 gene product by comparison of two-dimensional iso-electric focussing gels from avirulent and virulent strains (Thomas *et al.*, 1987). A candidate polypeptide identified by the

comparison of four strains was subsequently found to be encoded by a gene unlinked to V6.

1.1.3.2 RAPD markers and Map-based Cloning

Map-based cloning provides an alternative strategy particularly well suited for situations where genome size precludes shotgun cloning and where the nature of the gene product is not known. This approach has been pursued to a successful end in the cloning of avirulence genes in the rice blast fungus, *Magnaporthe grisea*, and of several plant disease resistance genes (Valent *et al.*, 1994, Martin *et al.*, 1993; Bent *et al.*, 1994; Mindrinos *et al.*, 1994). Until recently, the labor required to identify markers linked to the desired gene by traditional means, such as restriction fragment length polymorphisms (RFLPs), had limited the map-based approach. The development of a polymerase chain reaction (PCR) based technique, however, has provided a rapid means to obtain randomly amplified polymorphic DNAs (RAPDs) that function as classical genetic markers (Williams *et al.*, 1990).

RAPD analysis utilizes ten base oligonucleotide primers to generate a distinctive PCR amplification profile specific for a genomic DNA template. Being of short sequence, the single primer used per reaction anneals to many locations within the genome, but products are generated only when two inwardly oriented primer binding sites are in close enough proximity for amplification. Thus, the utility and power of RAPD analysis lies in the ability of each of the individual primers to scan multiple sites dispersed throughout the genome in a quick and relatively easy to perform reaction.

Differences in sequence between template DNAs are detectable as differences in the amplification pattern when RAPD products are resolved on agarose or, for greater

sensitivity, polyacrylamide gels. Polymorphisms are scored most frequently as the presence or absence of a band, but may also be manifested as allelic RAPD markers of different size. The basis of these polymorphisms is either nucleotide changes at the primer annealing sites, in the former case, or rearrangements and deletions between these sites, in the latter. The specificity of primer annealing confers sensitivity of the RAPD assay to even single base pair changes.

More detailed analysis of polymorphic markers can be achieved by the conversion of RAPD products to sequence characterized amplified region (SCAR) markers (Paran and Michelmore, 1993). The DNA sequence of the ends of cloned RAPD markers can be used to design 24mer primers consisting of the original 10 nucleotides of the RAPD primer and 14 additional bases downstream. The greater specificity of these longer SCAR primers, compared with the RAPD primer, allows for their use in the physical characterization of the linked region and in screening of genomic libraries using PCR.

A map-based cloning approach can be further facilitated using bulked segregant analysis (Michelmore *et al.*, 1991), a pooling technique compatible with the RAPD assay. Bulk segregant analysis provides the advantages of screening near-isogenic lines but requires only the segregation of the gene of interest in a defined cross. By generating pools of progeny sharing only the phenotype conferred by the gene to be isolated, the specific genomic region of interest can be studied in an otherwise randomized genetic background.

1.2 Research Outline

The combination of bulked segregants and RAPD analysis to obtain linked markers constitutes the first step in a cloning strategy for the *V1* and *V6* avirulence genes. To achieve this first step, the initial goal was to identify markers by screening RAPD primers

against pools of DNA bulked for the *VI*, *vI*, *V6* or *v6* alleles. The degree of linkage of candidate markers could then be measured by screening with the primer and each of the individual progeny. Tightly linked markers would then serve as starting points for a chromosome walk in a cosmid library constructed from the avirulent parental strain. The first screen of the cosmid library could be carried out either by hybridization of the cloned RAPD marker or by screening using PCR and the SCAR primers. Successful cloning would be confirmed by the conversion of a virulent strain to an avirulent strain after transformation with candidate cosmid clones obtained from the chromosome walk.

Several preliminary steps have been made towards this end. An initial cross of haploid strains Uh 4854-10 (*vI*, *v6*, *MAT-2*) and Uh 4857-4 (*VI*, *V6*, *MAT-1*), in which both avirulence genes were segregating, was performed by G. Bakkeren. The two parental lines are related but are not isogenic, with Uh 4854-10 being derived from earlier crosses to strain Uh 4857-4. This was confirmed by the screening of RAPD primers 101-200 against the two parental lines to determine the degree of relatedness. Twenty-seven single band differences were evident with twenty-seven of the primers, indicating that although the strains are very similar, sufficient variability exists for the identification of polymorphic markers (J. Kronstad, unpublished results).

Fifty-four progeny resulting from the cross between parental strains Uh 4854-10 and Uh 4857-4 were tested for avirulence. Inoculations of differential cultivars Hannchen (*R1*), Plush (*R6*) and the universal suscept Odessa (*r1*, *r6*) were performed independently by D. Mills at Oregon State University in Corvallis, Oregon and by P. Thomas at the Agriculture Canada Research Station in Winnipeg, Manitoba. Discrepancies between the two evaluations were re-checked at the University of British Columbia by G. Bakkeren to

ensure the accuracy of the scoring. Despite this re-checking, some uncertainty remained in the classification of six of the progeny (Table 2).

The preliminary work, together with the results described in this study, provide a foundation for the molecular dissection of gene-for-gene interactions between *U. hordei* and its barley host. An understanding of this system should provide insight into the evolution of the concurrent attempts of pathogens to avoid recognition and of the host to improve pathogen detection. Elucidation of this gene-for-gene system will also provide information on the nature of pathogenicity, the role of avirulence genes in the determination of host range and the pathway of activation for the plant defense response. The isolation of components that determine gene-for-gene interactions will also be of significant practical application in the development of biotechnological means to combat a pathogen, such as *U. hordei*, that is responsible for considerable crop losses (Thomas, 1989).

1.3 Specific Objectives

The specific objectives of this work are as follows:

1. To identify RAPD markers linked to the *V1* or the *V6* gene.
2. To characterize linked RAPD markers for use in map-based cloning of avirulence genes.
3. To construct a cosmid library from the avirulent parental strain.
4. To screen the cosmid library for the isolation of clones carrying the linked RAPD marker.

Table 2. Results of avirulence gene scoring of 54 progeny for *VI* and *V6* on barley cultivars Plush, Hannchen and Odessa.

Progeny	Barley Cultivar									GENOTYPE	Mating Type
	Plush r1 R6			Hannchen R1 r6			Odessa r1 r6				
	DG	PT	GB	DG	PT	GB	PT	GB			
1	S	S		R	S		S		v1 v6	1	
2	S	S		S	S		S		v1 v6	2	
3	S	S		S	S		S		v1 v6	2	
4	S	S		S	S		S		v1 v6	2	
5	S	S		?	R	R	S	S	V1 v6	1	
6	R	R		S	S		S		v1 V6	2	
7	S	S		R	S		S		v1 v6	2	
8	R	R		S	S		S		v1 V6	2	
9	R	R		R	R		S		V1 V6	2	
10	S	S		R	R		S		V1 v6	2	
11	?	R		?	R		S		V1 V6	1	
12	S	S		R	S		S		v1 v6	1	
13	S	S		S	S		S		v1 v6	1	
14	R	R		R	R		S		V1 V6	2	
15	S	S		R	R		S		V1 v6	1	
16	R	R		R	R		S		V1 V6	2	
17	R	R		R	R		S		V1 V6	1	
18	R	R		S	S		S		v1 V6	1	
19	?	R		?	?	R	S	S	V1 V6	1	
20	S	S		S	S		S		v1 v6	2	
21	R	R		S	S		S		v1 V6	1	
22	R	R		S	S		S		v1 V6	1	
23	R	R		S	S		S		v1 V6	1	
24	R	R		R	R		S		V1 V6	1	
25	R	R		R	R		S		V1 V6	2	
26	?	R		S	S		S		v1 V6	1	
27	S	S		S	S		S		v1 v6	2	
28	R	R		S	S		S		v1 V6	1	
29	S	S		R	S		S		v1 v6	2	
30	R	?	R	R	R		S	S	V1 V6	1	
31	R	R		S	S		S		v1 V6	2	
32	S	S		R	S		S		v1 v6	1	
33	S	S		S	S		S		v1 v6	1	
34	R	R		R	R		S		V1 V6	1	
35	R	R		R	R		S		V1 V6	1	
36	?	?	R	S	S		S	S	v1 V6	1	
37	?	R	R	S	S		S	S	v1 V6	1	
38	S	S		R	?	R	S	S	V1 v6	2	
39	S	S		?	R	R	S	S	V1 v6	2	
40	R	R		S	S		S		v1 V6	2	
41	?	S		?	?	R	S	S	V1 v6	1	
42	S	S		R	?	R	S	S	V1 v6	1	
43	S	S		R	R		S		V1 v6	1	
44	R	R		R	R		S		V1 V6	1	
45	R	R		R	R		S		V1 V6	1	
46	S			S					v1 v6	2	
47	R	R		S	S		S		v1 V6	2	
48	?	?		?	R	R	S	S	V1 ?	1	
49	S	S		?	R	R	S	S	V1 v6	1	
50	R	S		S	S		S		v1 v6	1	
51	R	R		S	S		S		v1 V6	1	
52	S	S		S	S		S		v1 v6	1	
53	R	R		R	R		S		V1 V6	2	
54	S			R					V1 v6	2	

Tests were carried out by D. Gaudet/D. Mills (DG) in Corvallis, Oregon, P. Thomas (P.T.) in Winnipeg and G. Bakkeren in Vancouver, British Columbia. The methods for plant inoculation and disease scoring were performed as described previously (Thomas, 1987). R, resistant; S, susceptible; ?, indeterminate.

2. Materials and Methods

2.1 Strains

U. hordei strains were obtained from Percy Thomas and have been described previously (Thomas, 1976; Thomas, 1987). The strains are of the following genotype: strain Uh 4857-4 (*V1, V2, V6, MAT-1*), strain Uh 4857-5 (*V1, V2, V6, MAT-2*), strain Uh 4854-10 (*v1, v2, v6, MAT-2*) and strain Uh 4854-4 (*v1, v2, v6, MAT-1*). *Escherichia coli* strain DH5 α (*F⁻, endA1, hsdR17(rk⁻, mk⁻), supE44, thi⁻, recA1, gyrA96, relA1, ϕ 80lacZM15*) from Bethesda Research Laboratories (BRL) was used for DNA manipulations.

2.2 DNA Procedures

DNA manipulation protocols were performed as described by Sambrook *et al.* (1989). Small-scale, alkaline lysis plasmid preparations were carried out by the '10 minute' method (Zhou *et al.*, 1990). Restriction and DNA modifying enzymes were obtained from BRL, Boehringer Mannheim, New England Biolabs and Pharmacia.

2.3 Generation of Pools for Bulk Segregant Analysis

Small scale preparations of *U. hordei* genomic DNA were isolated by a glass bead/phenol:chloroform extraction technique adapted from a protocol for the isolation of DNA from *Saccharomyces cerevisiae* (Elder *et al.*, 1983). Briefly, 5 mL cultures were grown in potato dextrose broth (Difco) for 48 hours at 23^o C with agitation until an OD₆₀₀ of 2.0 was reached. Cells were lysed by vigorous vortexing in the presence of phenol:chloroform:isoamyl alcohol (24:24:1), acid washed glass beads and lysis buffer (0.5 M NaCl, 0.2 M Tris-HCl pH 7.5, 0.01 M EDTA pH 8.0, 1% SDS). Two further phenol/chloroform/isoamyl alcohol and one chloroform/isoamyl alcohol extraction were performed before isopropanol precipitation of the DNA. Preparations were resuspended

in a final volume of 50 μ L in TE (10mM Tris-HCl, 1mM EDTA pH 8.0) buffer. The DNA concentration was estimated by agarose gel electrophoresis and comparison to a λ *Hind* III standard. Bulked pools consisted of genomic DNA preparations from eight progeny to yield a total DNA concentration of 12.5 ng/ μ L. All DNA dilutions were made in sterile deionized water.

2.4 RAPD Analysis

All reactions were performed using the Perkin-Elmer PCR System 9600 and MicroAmp tubes (Perkin Elmer). PCR conditions for each 25 μ L reaction were as follows: 2.0 mM MgCl₂; 100 μ M each of dGTP, dATP, dTTP and dCTP; 15 ng of a single RAPD primer; 0.5 units AmpliTaq polymerase (Perkin-Elmer); 25 ng template genomic DNA and 1X PCR buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 0.001% gelatin). RAPD primer sets 1-8 were purchased from the Nucleic Acid - Protein Service Unit at the University of British Columbia (U.B.C.). Primers #1-800 were received in 10 μ g dry quantities. Each primer set was resuspended in 100 μ L sterile deionized water. Further dilutions of 1 in 20 were made for a working concentration of 5 ng/ μ L. Resuspended primer stocks were stored at -20^o C while dilutions were kept at 4^o C. Primer set 9 (#801-890), based on microsatellite repetitive DNA sequences, was received as a gift from Dr. John Carlson (U.B.C.). Components for all reactions were assembled in a Labgard laminar flow biological safety cabinet (Nuair) and aerosol resistant tips (Continental Laboratory Products) were employed for all transfer of PCR reagents.

The following amplification profile was used for RAPD reactions with primer sets 1 through 8: initial denaturation at 94^o C for 30 seconds; 40 cycles of 94^o C/ 12 seconds, 36^o C/ 60 seconds, 60 second ramp to 72^o C, 72^o C/ 65 seconds; five minute final extension at 72^o C and an indefinite hold at 4^o C. A higher annealing temperature of 42^o C was used for the longer primers of set 9 (16-18 bases in length). Reaction products were electrophoresed through 1.4% agarose gels in 0.5X TBE (45 mM Tris-Borate, 1 mM

EDTA pH 8.0) buffer and detected on an UV transilluminator after ethidium bromide staining.

2.5 Cloning and Sequence Analysis of RAPD products

PCR products were directly cloned by a T-tailed vector method (Holton and Graham, 1991). RAPD products amplified from genomic DNA preparations of the *U. hordei* parental strains were resolved by agarose gel electrophoresis and the band of interest was isolated by excision from the gel followed by purification with the 'GeneClean' kit (Biol. 101 Inc.). To prevent digestion of the deoxyadenosine residue at the 3' end of the duplex PCR products (added by the terminal transferase activity of Taq polymerase), RAPD products were used immediately in ligation reactions or were stored at -20° C until further use.

Incubation of *Eco* RV digested pBluescript II K/S (Stratagene, La Jolla CA.) with 100 µM ddTTP and terminal deoxynucleotide transferase for 1 hour at 37° C was sufficient for tailing of the vector. Ligations were carried out overnight at 12° C. Cloning of the correct RAPD product was confirmed by comparison of the restriction patterns of the cloned product and the 'GeneClean' preparations of the DNA marker fragments isolated from the RAPD reactions. Medium scale plasmid preparations of the cloned products were made using the Wizard Maxipreps DNA Purification System (Promega) for double strand sequencing and for use as hybridization probes. Sequencing of the RAPD markers (T7 Sequencing Kit, Pharmacia) was performed using the dideoxy chain termination method (Sanger *et al.*, 1977) and the M13 universal and reverse primers.

2.6 Southern Analysis

DNA from agarose gels was blotted onto nylon membranes (Biodyne B, Pall Inc.) by capillary transfer in 20X SSC (0.3 M Sodium Citrate, 3.0 M Sodium Chloride pH 7.0).

The DNA was fixed to the membrane by baking for 15 minutes at 80° C in a vacuum oven (Fisher Scientific). Probe labelling and blot development were done using the ECL random prime labelling and chemiluminescent detection system (Amersham) as detailed in the manufacturer's manual. A hybridization temperature of 65° C was used for all overnight incubations. Stringency was maximized with washes at 65° C in 0.1X SSC, 0.1% SDS. Autoradiography was performed with Kodak X-OMAT AR and Island Scientific X-ray film.

2.7 Primer Design and Analysis

Four 24mer SCAR primers were designed based upon the sequence obtained from each of the two ends of RAPD products 359-1.55 and 743-1.0. The primers are composed of the 24 bases at each end of the markers and, therefore, contain the sequence of the original RAPD primer at the 5' end plus an additional 14 bases internal to the RAPD products. Sequence information was obtained from the central *Bam* HI fragment of marker 359-2.0 for the synthesis of 17mer primers JCT2A and JCT2B. The locations of the primers are marked in Figure 4 and their respective sequences shown in Table 3. All primers were synthesized by the Nucleic Acid - Protein Service Unit at U.B.C.

PCR conditions for SCAR analysis were identical to those outlined for RAPD reactions but with 1.5 mM MgCl₂ and 0.1 μM concentrations for each primer. Longer primers allowed for increased annealing temperature and a decreased number of cycles. The amplification program parameters consisted of a 30 second initial denaturation at 94° C followed by 30 cycles of 94° C/ 1 minute, 65° C/ 1 minute, 72 ° C / 2 minute and a final extension of 5 minutes at 72° C. Conditions for primers JCT2A and JCT2B were as described above except that a reduced annealing temperature of 50° C was employed to accomodate a lower primer length and GC content. Template concentration was maintained at 1 ng/ μl for both genomic DNA and cosmid library DNA prepared by the alkaline lysis method (Sambrook *et al.*, 1989).

Table 3. Nucleotide sequence of primers.

Primer Designation	Sequence
RAPD primer 359	5' AGGCAGACCT 3'
R359N	5' AGGCAGACCTAACCAAATCTTAAA 3'
R359P	5' AGGCAGACCTCTGTGGACACTCCG 3'
RAPD primer 743	5' CCACCCACAC 3'
743R	5' CCACCCACACGAGACAGCCAAAGA 3'
743F	5' CCACCCACACCAGACCCTGGTGTC 3'
JCT2A	5' AACTAGTTATTGTCAATG 3'
JCT2B	5' AGGCCCATGACAATAAC 3'

2.8 Cosmid Library Construction

High molecular weight *U. hordei* genomic DNA was prepared by the lysis of protoplasts. Briefly, a 400 mL culture of strain Uh 4857-4 (*VI, V6, MAT-1*) was grown in YEPS medium (1% (w/v) yeast extract, 2% bacto-peptone, 2% sucrose) to an OD₆₀₀ of approximately 1.0. Cells were collected by centrifugation and protoplasts were obtained by treatment with Novozyme 234 (Sigma) according to the protocol of Wang *et al.* (1988). Protoplast suspensions were lysed by the addition of 3% sodium N-lauroyl sarcosinate in 0.5 M Tris-HCl pH 9.0 and 0.2 M EDTA and incubation at 65° C for 3 hours.

Nucleic acids were separated from cell debris by centrifugation of the lysate on a 10-50% sucrose step gradient. The gradient was centrifuged in a Beckman SW27 rotor for 3 hours at 26,000 RPM and 20° C. The bottom 6 mL of the 30 mL gradient was removed and dialyzed against 2 L of TE buffer pH 8.0 for 48 hours with 3 buffer changes. The DNA preparation was concentrated to 1 mL by laying the dialysis tubing (MWCO 10,000, Spectra-por CE membrane, Spectrum Inc.) on solid sucrose for 2 hours. For sucrose removal, the concentrated sample was re-dialyzed against TE pH 8.0 for another 48 hours. The size and concentration of the DNA in the preparation was

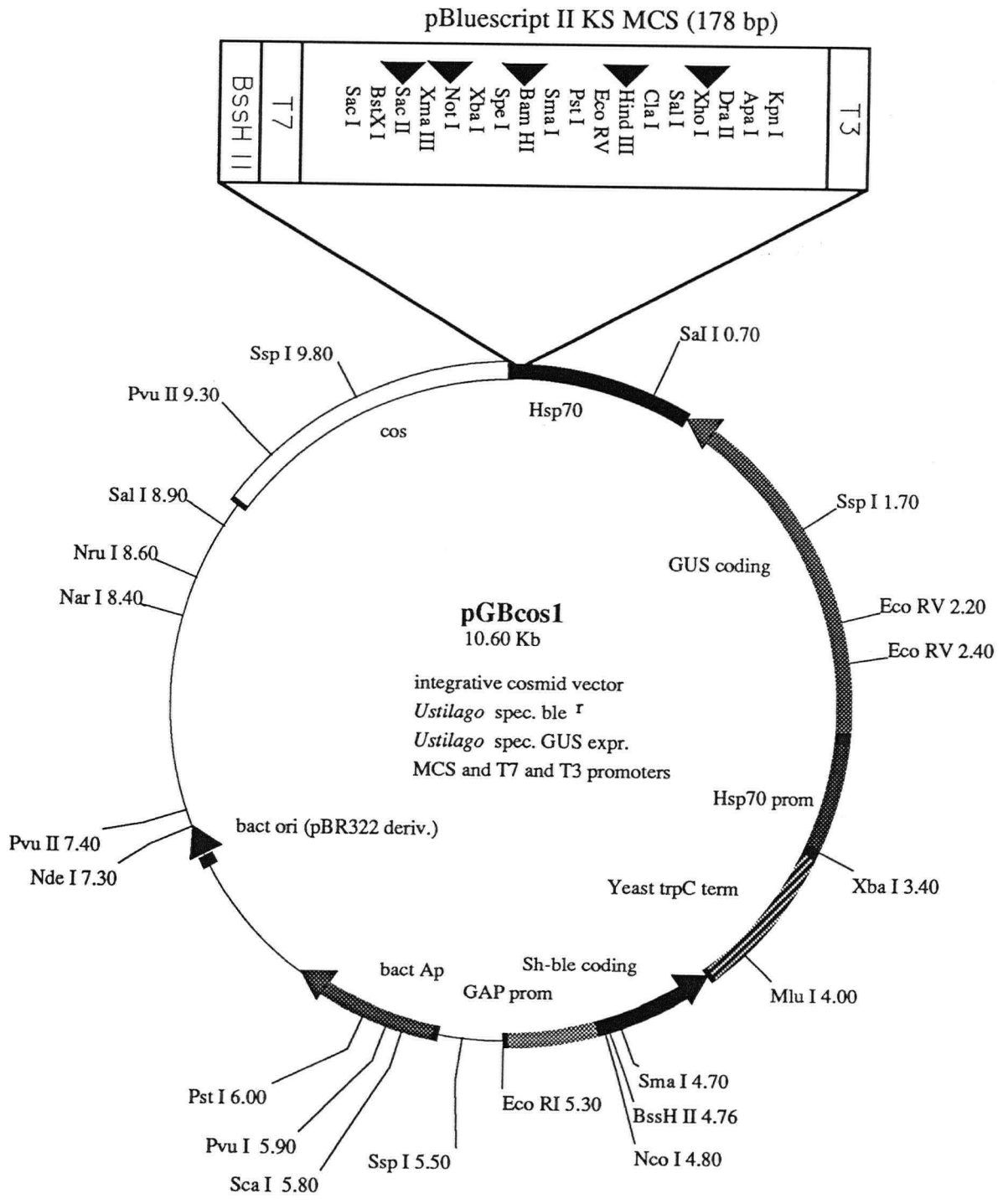
determined by agarose gel electrophoresis in a 0.3% agarose gel in 0.5X TBE buffer using intact λ DNA as a standard for comparison.

DNA fragments of 35-40 kb were obtained by *Sau* 3A partial digestion as outlined by Sambrook *et al.* (1989). Pilot reactions established digestion conditions of 0.01 U *Sau* 3A/ μ g DNA for 20-30 minutes. Large-scale digests of 20, 25 and 30 minutes were size fractionated on a 5-20% linear NaCl gradient for 4.5 hours at 37,000 RPM and 22° C in a Beckman SW41 rotor. The gradient was separated into 250 μ L fractions and a 20 μ L aliquot of each was checked for size and DNA content on a 0.3% TBE gel. Fractions of the gradient carrying insert sizes of 35-40 kb were pooled and concentrated for ligation into the *Bam* HI site of cosmid vector pGBcos1. This vector was constructed by G. Bakkeren and is diagrammed in Figure 2. The ligation products were packaged into λ phage using the Gigapack Gold II extract (Stratagene) and transfection of *E. coli* strain DH5 α yielded approximately 500,000 colony forming units.

2.9 Cosmid Library Screening

Individual cosmid clones were randomly seeded into the wells of 26 96-well microtitre dishes containing 100 μ L Luria Broth (Sambrook *et al.* 1989) with 50 μ g/mL ampicillin and 1% glucose. These dishes were incubated overnight at 37° C with slow agitation. Cultures from each dish were transferred using a sterilized 48 tine prong to two 150 x 15 mm LB agar petri dishes containing 50 μ g/ mL ampicillin, one with and one without a nylon filter (Biodyne B, Pall Inc). The transferred cells were allowed to form colonies by overnight incubation at 37° C. Filters carrying colonies were processed for colony lysis and DNA binding as described by Sambrook *et al.* (1989). Petri dishes carrying colonies were kept at 4° C for use as master plates. The original cultures in microtitre dishes were stored as -70° C stocks with the addition of sterile glycerol to a final concentration of 15%.

Figure 2. Map of cosmid vector pGBcos1.



Notes:

1) Parent plasmid : pGB666 (Sph I/ Hind III), Klenow treated

2) ▼ = unique sites

The protocol for hybridization screening of the cosmid library was as described for southern analysis. Individual cosmid clones were screened by direct PCR with the SCAR primers using a method outlined by Joshi *et al.* (1991). Intact bacterial cells restreaked from the master plates were transferred to PCR reaction tubes. Colonies were touched with sterile 1-200 μ l plastic pipette tips and the transferred cells were resuspended in a 20 μ l volume containing all PCR components except for Taq polymerase. A 10 minute lysis step at 99.9 $^{\circ}$ C, followed by cooling to room temperature, was carried out before the addition of the polymerase. Reaction conditions and the amplification profile subsequent to the lysis step were identical to those previously described for PCR reactions with the SCAR primers.

The cosmid library was amplified after the initial screening. Transfected clones were plated on 6 150x15 mm LB/ampicillin plates and the colonies removed using a sterile spreader after the addition of 3 mL LB broth to each plate. An additional wash with 3 mL of LB broth was carried out before transfer to a series of 15 mL tubes. Sterile glycerol was added to 18% and ampicillin to 50 μ g/ mL prior to the storage of the library at -70 $^{\circ}$ C. The titre of the amplified library was measured to be 1.0×10^7 clones/ μ L. Alkaline lysis preparations (Sambrook *et al.*, 1989) were made directly from aliquots of the amplified library for screening using PCR and the SCAR primers. PCR conditions for screening were as described in the Primer Design and Analysis section.

3. Results

3.1 RAPD Analysis of Bulk Segregants

3.1.1 Design of Bulk Pools

Two sets of pools were generated for each of the avirulence alleles (*V1*, *v1*, *V6* or *v6*); a primary set for the initial identification of RAPD primers yielding candidate markers linked to the desired allele and a secondary set to allow re-screening on another pool comprised of different progeny. Experiences in other laboratories with the reproducibility of RAPD reactions (Penner *et al.*, 1993; Ellsworth *et al.*, 1993) suggested that the use of two pools for each trait would be helpful in ensuring that differences in RAPD amplification profiles reflected true polymorphisms and not artifactual variation.

Two considerations were involved in the selection of the individual progeny in each pool. Since the correct scoring of the progeny for avirulence was crucial, individuals whose classification was in doubt were not included in any of the pools. To accommodate this exclusion and the design of two different pools for each avirulence allele, a pool size of eight progeny was used. Secondly, an effort was made to ensure that the pools were bulked only for the desired avirulence allele and nothing else. As a result, the progeny within the pools were distributed evenly with respect to mating type and the other avirulence gene (*V1* or *V6*) segregating in the cross. A pool of DNA from eight progeny is also of sufficient size such that the likelihood of obtaining polymorphic markers unlinked to the desired avirulence gene is fairly small. The composition of the eight pools that were constructed is shown in Table 4.

Table 4. Composition of pools employed for bulked segregant analysis.

Pool	Progeny in pool of type <i>V6</i> , <i>MAT-1</i>	Progeny in pool of type <i>v6</i> <i>MAT-1</i>	Progeny in pool of type <i>V6</i> , <i>MAT-2</i>	Progeny in pool of type <i>v6</i> , <i>MAT-2</i>
<i>VI</i>	17, 24, 35	15, 43	9, 14	10
<i>vI</i>	18, 21	1, 12	6, 8	2, 3
<i>VI-1</i>	30, 34	6, 42	16, 25	38, 39
<i>vI-1</i>	22, 23	13, 32	40, 47	4, 7
	Progeny in pool of type <i>V6</i> , <i>MAT-1</i>	Progeny in pool of type <i>v6</i> , <i>MAT-1</i>	Progeny in pool of type <i>V6</i> , <i>MAT-2</i>	Progeny in pool of type <i>v6</i> , <i>MAT-2</i>
<i>V6</i>	44, 45	22, 23	16, 25	31, 40
<i>v6</i>	15, 43	32, 52	10	4, 7, 20
<i>V6-1</i>	35, 17	51, 18	9, 14	6, 47
<i>v6-1</i>	5, 49	33, 13	38, 39	29, 27

The numbers in this table are used to designate each of the individual progeny and are the same as those employed in Table 2. Individuals in each pool are classified according to their genotype in each column to indicate an even distribution within the bulks.

3.1.2 RAPD Primer Screening

RAPD primers were used in polymerase chain reactions with the bulked genomic DNAs to identify differences in their amplification profiles. Careful comparisons of the RAPD reaction patterns between the *V6* and the *v6* pools and between the *VI* and *vI* pools were made to detect polymorphic bands. Bands present in one allelic pool but not the other constituted a candidate linked marker which was investigated further.

Not all primers resulted in the synthesis of RAPD products from *U. hordei* DNA (Table 5). Presumably, there is a lack of inwardly oriented annealing sites close enough for amplification for these primers. Alternatively, and additionally, there simply may not be sequences present in the *U. hordei* genome complementary to these RAPD primers. It is interesting to note that almost all primers which failed to give products had a guanosine and cytosine base (GC) composition of 50% or less. The short length of RAPD primers may require a higher primer GC content for amplification.

Table 5. Results of RAPD analysis with avirulence gene pools.

Primer Set	% of primers giving no amplification	% of primers re-checked against secondary V1-1 and v1-1 pools	% of primers re-checked against secondary V6-1 and v6-1 pools	No. of primers checked against individual progeny
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

The number of RAPD bands in successful reactions varied from one to as many as 20 with an average of six per primer. The amplified bands varied in intensity from barely detectable by ethidium bromide staining to a yield of as much as 300 ng and in size from 0.2 to 4 kb. Despite the standardization of reaction conditions, the amplification profiles for a few of the primers were not entirely reproducible on repeated runs. Variability was evident largely with the weakly amplified bands while the stronger ones were consistently amplified.

The *V6* and *v6* pools were screened with 890 RAPD primers. Of these, 13% were re-checked against the secondary *V6-1* and *v6-1* bulk pools (Table 5). Primers continuing to display polymorphisms were screened against a number of individual progeny for confirmation of linkage. The majority of candidate markers, however, consisted of the weakly amplified bands whose variability reflected their less reproducible nature, and not true polymorphisms. When reactions were performed with DNA from individual progeny, no RAPD markers linked to *v6* or *V6* were identified.

RAPD analysis of the *V1* and *v1* pools identified two primers, 359 and 743, which amplified markers exhibiting linkage to the desired gene. The first of these, primer 359, amplified a 2.0 kb marker, designated 359-2.0, exclusively in the *v1* pools and a 1.55 kb marker, designated 359-1.55, was strongly amplified only in the *V1* pools (Figure 3). Amplifications with primer 359

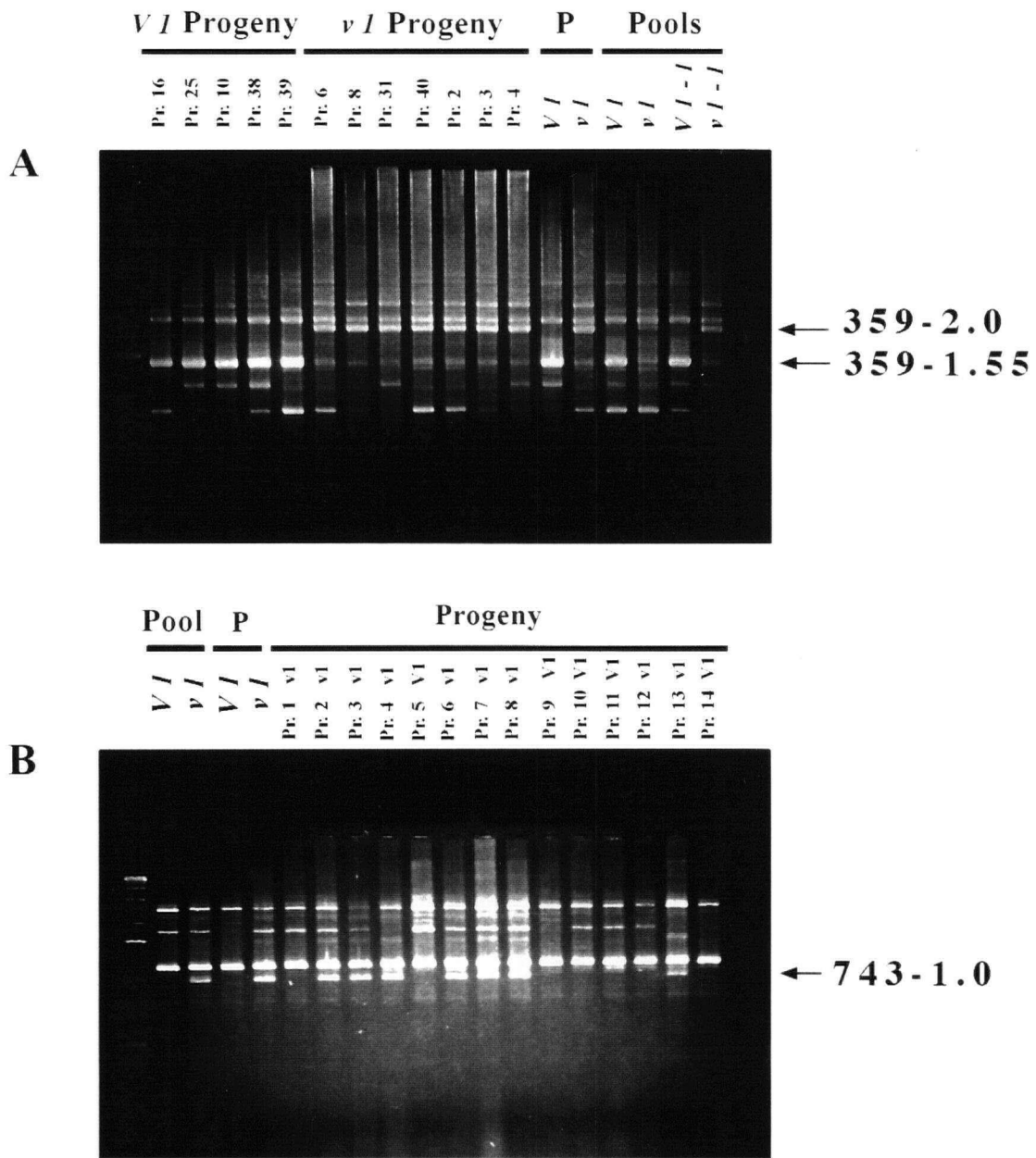


Figure 3. RAPD reactions on template DNA from progeny, parents and bulked segregant pools. P: *U. hordei* parental strains Uh 4857-4 (*V I*) and Uh 48545-10 (*v I*). Pr.: progeny strains as listed in Table 2.

(A) Amplifications with RAPD primer 359.

(B) Amplifications with RAPD primer 743.

and DNA from individual progeny confirmed the linkage of these two markers with their respective alleles. The markers segregate 1:1 amongst the progeny and are never both present or both absent in the same individual. The lack of recombinants between these markers suggest that they are allelic. Of the 54 progeny, 52 exhibited co-segregation; marker 359-1.55 with *VI* and marker 359-2.0 with *vI*. The remaining two, progeny 1 and progeny 12, were scored as *vI* but amplified the 1.55 kb product due to a recombination event between the allele and the marker. Two recombinants among 54 progeny translates to a map distance of 3.7 cM.

Amplification of DNA from the pools with primer 743 identified a 1.0 kb product (743-1.0) in pools bulked for *vI* but not for *VI* (Figure 3). This marker was amplified in 26 of the 29 *vI* progeny, not being present in the *vI*-scored progeny numbers 1, 12 and 40. Marker 743-1.0 was not amplified in any of the progeny classified as *VI*. The frequency of recombination between the 743-1.0 and *vI* indicated a genetic distance of 5.5 cM. Relative to *vI*, this marker is distal to 359-2.0 with a 1.8 cM distance between the two RAPD markers (Figure 4).

3.2 Characterization of RAPD Markers

3.2.1 Restriction Site Mapping and Hybridization Studies of the Linked Markers

The cloning of the three markers (359-1.55, 359-2.0 and 743-1.0) allowed their characterization. Digestion with restriction enzymes and analysis of the resulting DNA fragments on agarose gels indicated that 359-2.0 and 359-1.55 share nearly identical sites (Figure 4). With the exception of an extra *Pst* I site in 359-1.55 and an additional 450 bp near the middle of 359-2.0, the two markers have the same restriction pattern. Interestingly, the 450 bp in 359-2.0 has similar sites to an adjacent region, raising the possibility that an imperfect duplication may be responsible for the size polymorphism between markers 359-2.0 and 359-1.55.

All three markers were labeled and used as hybridization probes to determine their utility for a chromosome walk toward the *VI* gene. DNA from the parental strains Uh 4857-4 and

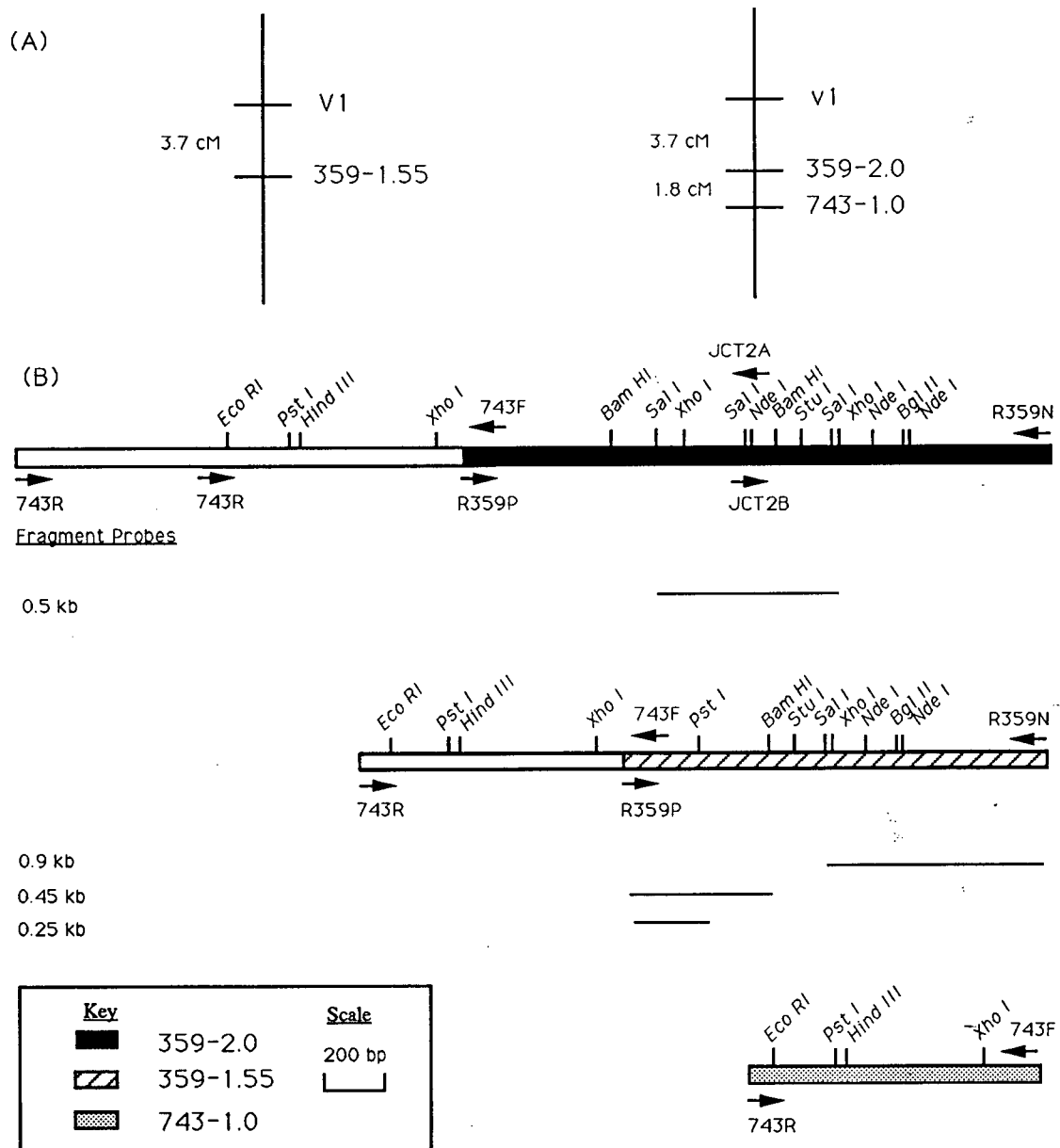


Figure 4. Maps of linked markers.

(A) Genetic maps of the *VI* and *vI* regions. cM distances between sites are marked to the left of the vertical line.

(B) Restriction maps of markers 359-2.0, 359-1.55, 743-1.0 and their flanking regions. Fragments used as hybridization probes are illustrated below each map.

→ designates the location from which PCR primers were designed. The length of the primer icon is not to scale.

Uh 4854-10 were each digested to completion with thirteen different restriction enzymes and were used to prepare a genomic DNA blot. Hybridization with 359-2.0 as a labeled probe indicated the presence of highly repetitive DNA within the marker (Figure 5). While single bands are distinctly visible, the strong smearing pattern of hybridization is characteristic of a repetitive element.

Similar results were obtained when marker 743-1.0 was hybridized to the genomic DNA blot (data not shown). Interestingly, the multiple banding pattern obtained with this marker as a probe is nearly identical to that of the 359-2.0 probe, suggesting that the two markers may be part of the same repeat unit. Fragments of 359-2.0 and 359-1.55, marked in Figure 4, were also used as probes to determine if any segment of the markers was comprised of unique DNA. Upon hybridization, however, all four of the probes gave hybridization patterns consistent with repetitive DNA (data not shown). A 250 bp *Pst* I fragment did, however, appear to be somewhat less repetitive, hybridizing more strongly to the single bands within the smear (Figure 6).

To determine the distribution of this repetitive element within the *U. hordei* genome and to see whether the RAPD probe could locate the *VI* allele on one particular chromosome, hybridization studies were carried out with intact chromosomes separated by pulsed-field (agarose) gel electrophoresis (PFGE). Use of marker 359-1.55 as a probe resulted in a strong signal from every chromosome indicating that this repeated DNA unit is dispersed throughout the genome. No hybridization to the DNA of the corn pathogen *U. maydis* was detected (Figure 7).

3.2.2 Nucleotide Sequence and SCAR Analysis

Nucleotide sequence information was obtained for portions of the three cloned RAPD products. The 359-2.0 and 359-1.55 RAPD products showed 92% (250 bp at the left end as diagrammed in Figure 4) and 96% (250 bp at the right end) sequence identity at their ends. These data confirmed that the two markers represent nearly identical allelic forms of the repetitive element. Surprisingly, complete sequence identity over a 100 bp region was also apparent with markers 743-1.0 and 359-2.0. This result places 743-1.0 or a copy of it adjacent to 359-2.0 with

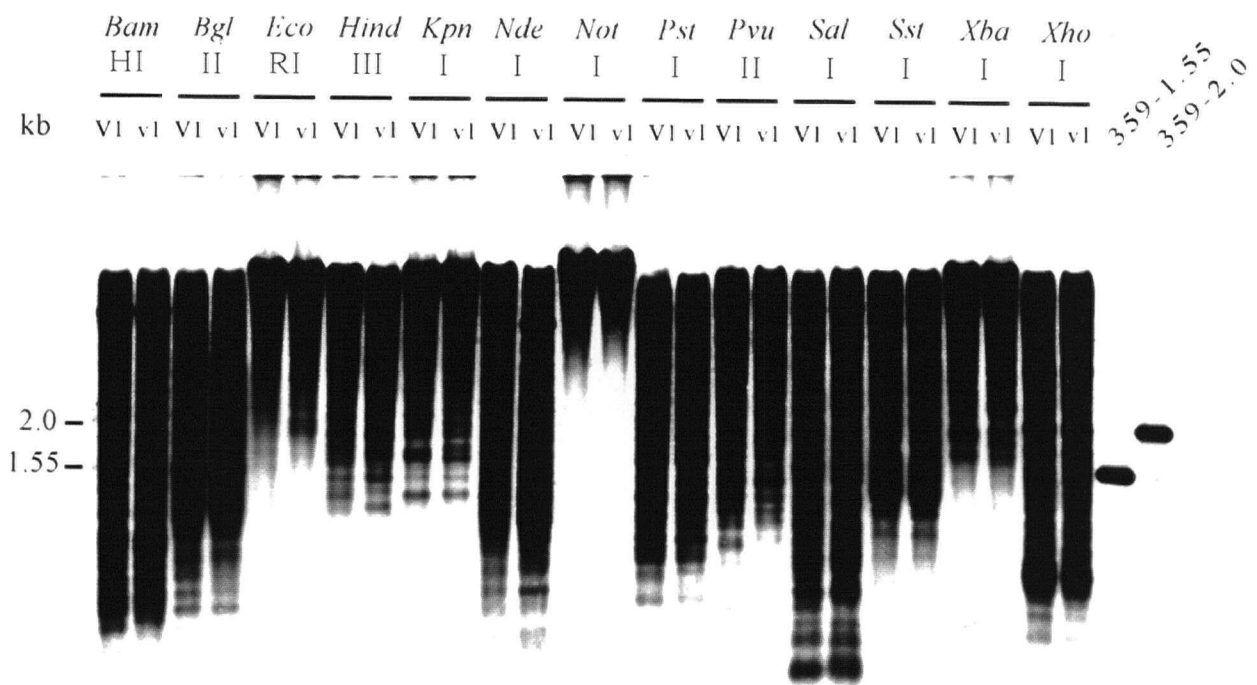


Figure 5. Southern blot of *U. hordei* genomic DNA hybridized with the cloned 359-2.0 probe. For all pairs of lanes, the first lane is DNA from parental strain Uh 4857-4 (VI) and the second from Uh 4854-10 (vI). Genomic DNA was digested to completion with the restriction enzyme labelled above. Intact RAPD markers 359-1.55 and 359-2.0 were included on the gel as positive controls.

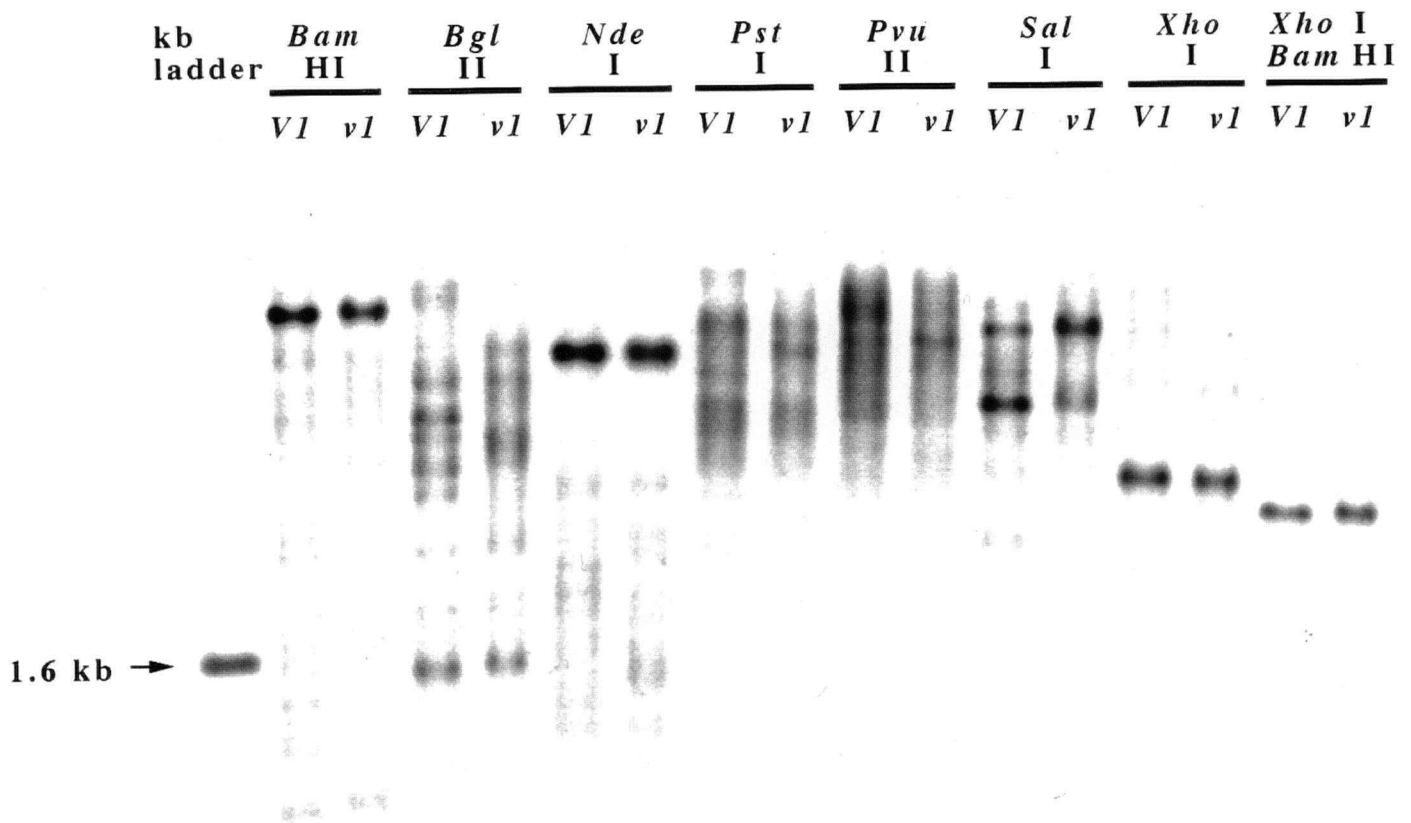


Figure 6. Genomic DNA blot of *U. hordei* DNA hybridized with the 0.25 kb *Pst* I fragment of the cloned 359-1.55 marker. For all pairs of lanes, the first is DNA from parental strain Uh 4857-4 (*VI*) and the second from Uh 4854-10 (*vI*). Genomic DNA was digested to completion with the restriction enzyme labelled above. A hybridization signal from the 1.6 kb band of the kilobase ladder marked to the side is due to the presence of a small amount of the pBluescript vector in the probe.

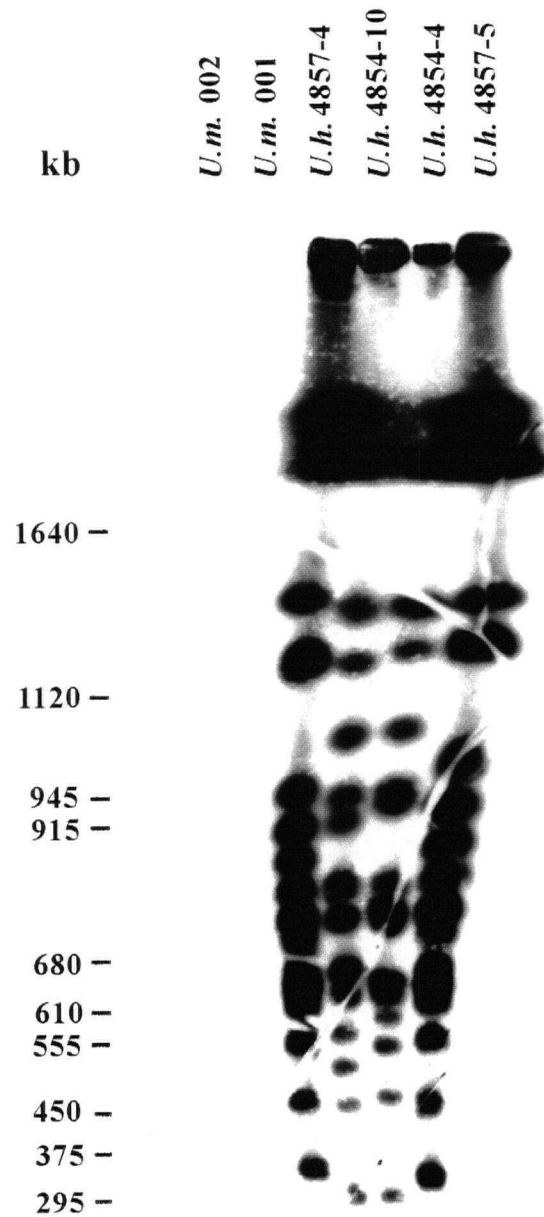


Figure 7. Hybridization of the RAPD marker 359-1.55 to a DNA blot of separated chromosomes. Chromosome separation on a contour-clamped homogenous electric field (CHEF) PFGE gel was performed by G. Bakkeren. Chromosomes were resolved through a 1.0 % agarose gel in 0.5X TBE buffer at 12° C for 40 hours. Electrophoresis was conducted in a Bromma LKB apparatus programmed for 2 steps: 22 hours at 180 volts with a 70 second pulse and 18 hours at 180 volts with a 120 second pulse. *U. m.* 001, 002: laboratory strains of *U. maydis*. *U. h.* 4857-4, 4854-10, 4854-4 and 5857-5: *U. hordei* strains of avirulence phenotype *V1*, *v1*, *v1* and *V1*, respectively. The size markers denoted on the left are from the *Saccharomyces cerevisiae* chromosome PFG size standards (New England Biolabs).

an overlap of 100 bp. Most likely, it is a copy that is adjacent to 359-2.0, given the measured 1.8 cM genetic distance between the two markers. Evaluation of the sequence information from the three markers provided few clues to the nature of the repetitive DNA. For example, a search of the nucleotide sequence database using the BLAST program did not produce any significant matches to known sequences. The presence of short tandem repeats was not detectable in any of the sequences.

SCAR primers, of greater length compared to the RAPD primers, were designed from the sequence information to allow analysis of the region under more stringent PCR conditions. Primers based on the sequence of the ends of marker 743-1.0 did not maintain the polymorphism seen with RAPD primer 743. Even at a stringent annealing temperature of 65° C, the 1.0 kb marker was apparent in both parental strains (Figure 8). This result does not negate the validity of 743-1.0 as a marker linked to *vI*. Rather, the inability of these SCAR primers to detect the polymorphism likely reflects the difference in using the longer SCAR primers as opposed to the 10mer RAPD primer. While the 24 base SCAR primers provide greater specificity, their sensitivity to base mismatches is diminished especially toward the 5' end of the primer. That these longer PCR primers were able to amplify the product in the *VI* parent suggests that the basis for the 743-1.0 polymorphism lies in the original base sequence for the RAPD primer binding site. The greater length of the SCAR primers would allow annealing to the site in the *VI* parent despite the base mismatches which prevented the RAPD primer from annealing.

Amplification with SCAR primers (R359N, R359P), designed from the ends of marker 359-1.55, yielded the same co-segregation pattern, but eliminated all but one of the additional bands seen with the RAPD primer. With these SCAR primers, however, amplification of a 1.55 kb product does occur in the *vI* parent and progeny although not to the same level as for *VI* (Figure 8). This result indicates that a 1.55 kb product is present in *vI* individuals that is only amplified with the longer SCAR primers and not the original RAPD primer.

In contrast, the 2.0 kb SCAR product obtained from conversion of marker 359-2.0 showed tight linkage to *vI* and gave no amplification in *VI* individuals. This observation suggests that

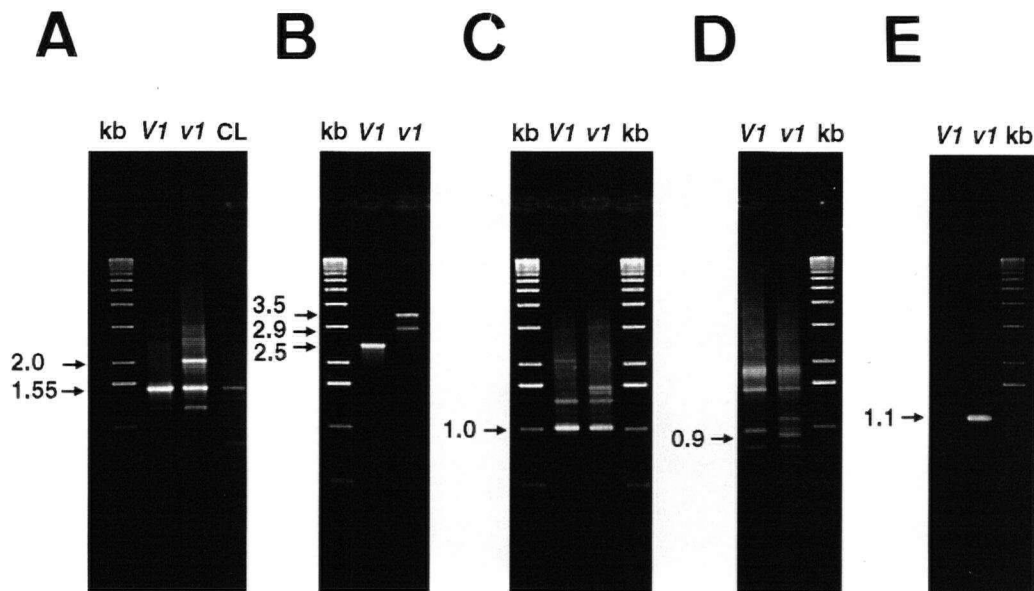


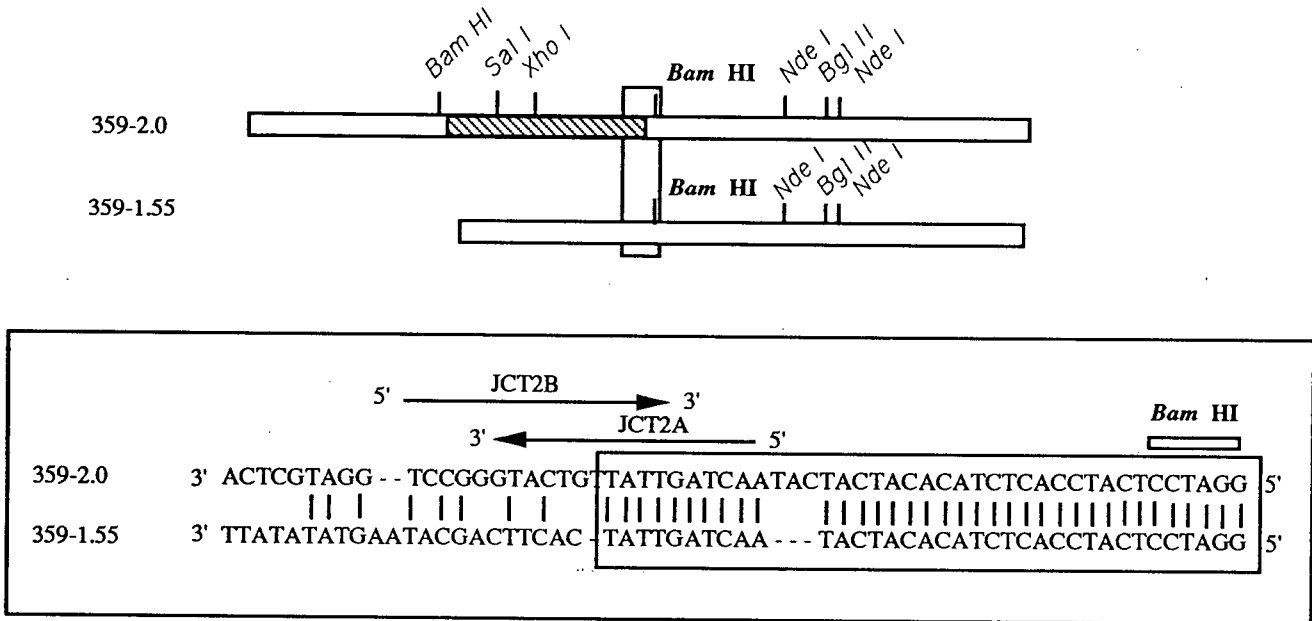
Figure 8. Amplification with SCAR and junction primers on parental genomic DNA and pooled cosmid library DNA. The numbers marked to the side of each panel is the size in kilobases (kb) of the PCR product of interest. kb: kb ladder. *V1*: genomic DNA from parental strain Uh 4857-4 used as template for amplification. *v1*: genomic DNA from parental strain Uh 4854-10 as template. CL: DNA preparation of the *V1* cosmid library as template.

- (A) PCR reactions with SCAR primers R359N and R359P.
- (B) PCR reactions with SCAR primers R359N and 743R.
- (C) PCR reactions with SCAR primers 743R and 743F.
- (D) PCR reactions with SCAR primer R359P and junction primer JCT 2A.
- (E) PCR reactions with SCAR primer R359N and junction primer JCT2B.

359-2.0 may be a unique unit due to the presence of the extra 450 bp of DNA in this marker compared with the 359-1.55 marker. If this were the case, the region of DNA spanning the 450 bp may have greater specificity for use as a short hybridization probe and for the design of specific PCR primers. Sequence information was obtained for 359-2.0 and 359-1.55 at one end of the predicted junction point of the 450 bp repeat (Figure 9). Upon comparison, a point of sequence divergence was identified which could constitute a unique junction of repetitive DNA. Two seventeen nucleotide primers, JCT2A and JCT2B, (Figure 4) were synthesized to span the point of divergence in marker 359-2.0 ; these primers are of opposite orientations.

The specificity of the JCT2A and JCT2B primers was checked in conjunction with SCAR primers R359N and R359P on PCR amplification of genomic DNA. As expected, a 1.1 kb product was present with primers R359N and JCT2B specifically in the *vI* but not the *VI* parent (Figure 8). Similarly, a 0.9 kb PCR product was amplified in only the *vI* parent with primers JCT2A and R359P. Primers with specificity to this *vI*-linked junction point have proven useful for the isolation of specific cosmids from a library prepared with DNA from a strain carrying *vI* (G. Bakkeren, unpublished results).

A combination of the two sets of SCAR primers was utilized to determine the physical localization of the markers relative to each other. If a copy of 743-1.0 is adjacent to both 359-1.55 and 359-2.0, as suggested by sequence analysis, SCAR primers located at the end of each should amplify products composed of both markers. Primers R359N and 743R amplified co-dominant markers; a 2.5 kb product co-segregating with *VI* in the same manner as 359-1.55 and two products, 2.9 kb and 3.5 kb in size, linked to *vI* and mapping to the same location as 359-2.0 (Figure 8). Restriction mapping of the 2.5 kb and the 2.9 kb products confirmed that a copy of 743-1.0 is positioned next to the 359 markers as diagrammed in Figure 4. The 3.5 kb SCAR product appears to contain the 2.9 kb product plus an additional 600 bp due to a second annealing site for primer 743R. Characterization of the markers by SCAR analysis indicates that all are part of the same repetitive DNA unit which is at least 3.5 kb in size in the *vI* parent.



Junction Point

Figure 9. Restriction map location and DNA sequence of the junction point of divergence between markers 359-2.0 and 359-1.55. The extra 450 bp of DNA present in marker 359-2.0 but not in 359-1.55 is marked as a hatched box. Only a portion of the sequence flanking the junction point is shown. The *Bam* HI site marked in bold face on the restriction map corresponds to that on the sequence. The locations and orientations of the 359-2.0 specific primers JCT2A and JCT2B are marked.

3.3 Screening of the Cosmid Library

3.3.1 Cosmid Library Size and Diversity

The identification of a marker mapping only 3.7 cM from the *VI* avirulence allele (359-1.55) prompted an attempt to begin a chromosome walk toward that allele. A cosmid library was constructed for the isolation, first, of clones carrying the genomic amplification sites of marker 359-1.55 and, then, of the subsequent overlapping cosmids leading to the *VI* gene. DNA fragments (35-40 kb) of parental strain Uh 4857-4 (*VI*, *V2*, *V6*, *MAT-1*), from which the cloned 359-1.55 marker was obtained, were ligated to cosmid vector pGBcos1. For the purpose of a chromosome walk, care was taken to prevent tandem ligation which could result in non-contiguous fragments. The appropriate size range of the partially digested DNA fragments was selected after centrifugation (as described in the Materials and Methods).

The cosmid vector used for this library was designed especially for the purpose of isolating *U. hordei* genes by chromosome walking. Flanking the multiple cloning site are T3 and T7 promoters for the preparation of RNA probes in the next steps of the walk. pGBcos1 also carries several *Ustilago* DNA sequences including a phleomycin resistance gene for selection and a *GUS*-reporter gene to track the progress of infection in the host plant.

Prior to the screening of the cosmid library, its size and diversity were assayed. The number of colony forming units obtained was approximately 500,000. Assuming the insert size to be 40 kb, this number of clones represents about one thousand times the *U. hordei* genome (*U. hordei* has a genome complexity of approximately 2×10^7 bp). The diversity of the library was confirmed by the unique restriction patterns obtained from *Xba*I/*Hind* III double digestion of 30 random clones. As a positive control to determine if a specific *U. hordei* sequence could be detected within the library, PCR amplification was performed with two sets of primers to the previously cloned and characterized mating type genes (Bakkeren and Kronstad, 1993). DNA of a pool of approximately 1.0×10^8 clones yielded the expected products (data not shown).

3.3.2 Hybridization and PCR Screening of the Cosmid Library

A 250 bp *Pst* I fragment of marker 359-1.55 which had previously appeared to give a less repetitive hybridization signal on genomic DNA (Figure 6) was used as a probe on the cosmid library. 2496 clones of the library, equivalent to approximately five times the genome, were screened with the probe. A positive hybridization signal was evident with 154 of the clones. This large number of positives (6% of the screened library) was consistent with the repetitive nature of the probe. While screening the cosmids by hybridization did narrow the field of candidates, a secondary process was required to identify the cosmid clones carrying the correct region identified by the RAPD marker.

All of the 154 clones were analyzed by direct PCR using SCAR primers R359N and R359P. This screen provided a quick means to identify the cosmid bearing marker 359-1.55 by the ability to amplify a 1.55 kb product. Surprisingly, the majority of these *E. coli* cosmid clones yielded amplification products when used as templates, although few were of the expected 1.55 kb size. Screening with SCAR primers for positive clones eliminated all but thirteen cosmids as possible candidates. Only one of these clones, 3E#2, strongly amplified the band while the remainder yielded a weak product of corresponding size.

To determine whether these remaining 13 cosmids carried the linked 359-1.55 marker, *Bam* HI/ *Bgl* II digests were performed on the DNA of each cosmid. The fragments were separated by electrophoresis and blotted for hybridization analysis. Restriction mapping of 359-1.55 had determined that such a double digest would release a 0.45 kb internal fragment. Therefore, use of the *Bam* HI/ *Bgl* II fragment from marker 359-1.55 as a probe on the blot should result in a positive signal from a cosmid fragment 0.45 kb in size if the correct cosmid were present.

Restriction enzyme digests of the cosmid clones revealed that, of the thirteen clones, three comprised one set of overlapping cosmids, two constituted a different set while the remainder did not overlap with these or with each other. Although hybridization with the 359-1.55 derived *Bam* HI/ *Bgl* II probe resulted in a signal from 11 of the candidate cosmids, none were of the correct 0.45 kb size (Figure 10). Interestingly, the probe hybridized to fragments between 400 and 600

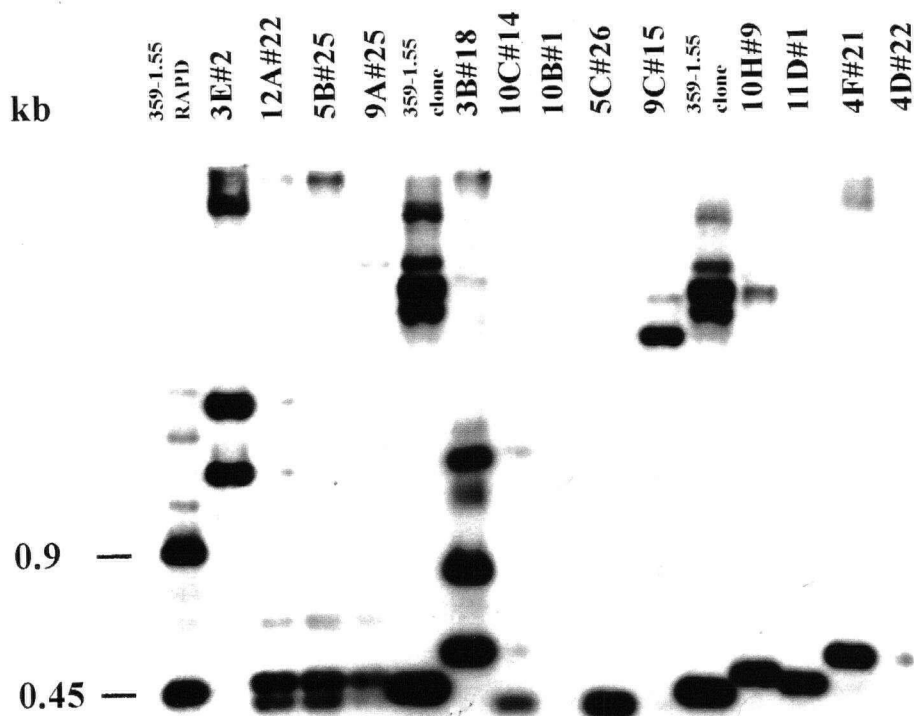


Figure 10. Hybridization analysis of candidate cosmid clones with a 0.45 kb *Bam* HI/ *Bgl* II probe derived from marker 359-1.55. Cosmid and marker DNA were digested with *Bam* HI and *Bgl* II. The 0.9 kb signal from the 359-1.55 RAPD is due to only partial digestion of the marker. 359-1.55 RAPD product: marker isolated from RAPD reaction using the gene-clean extraction kit (Biol. 101 Inc.). 359-1.55 clone: marker cloned in pBluescript II.

bp in eight of the cosmids. The size similarity of the signal bands suggests that although none of the cosmids carry the marker, these copies of the repetitive unit may be fairly similar but not identical with respect to restriction sites.

PCR reactions with the R359N and R359P primers were performed on DNA preparations of pooled cosmid clones of the amplified library to determine if the presence of marker 359-1.55 could be detected within the library. Cosmid DNA preparations (performed as described in Sambrook et al., 1989) were made directly from glycerol stocks of the amplified library. A SCAR product of the expected 1.55 kb size was amplified from cosmid DNA prepared from 10 μ l of the stored library (representing approximately 1.0×10^8 clones) as shown in Figure 8. SCAR products amplified from the genomic DNA of the *VI* parental strain and from the cosmid library of that parental strain were purified using the Gene-clean kit (Biol. 101 Inc.) and digested with several restriction enzymes. Comparison of the fragment patterns indicated that these two products share the same restriction sites and suggests that the SCAR product amplified from the cosmid library is marker 359-1.55 (Figure 11).

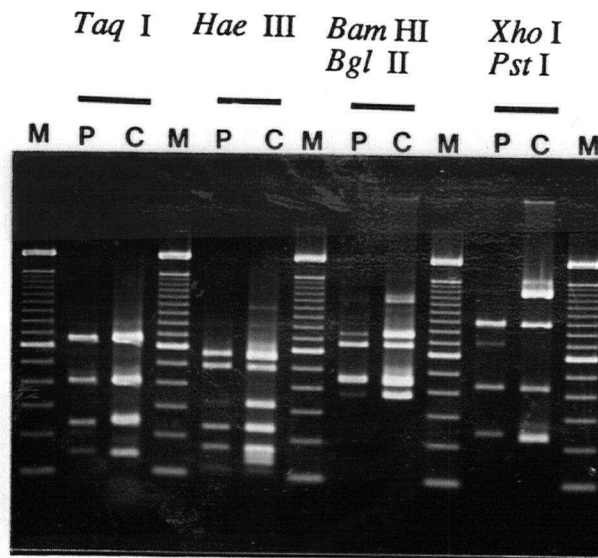


Figure 11. Restriction enzyme digests of PCR products amplified with SCAR primers R359N and R359P from the genomic DNA of the *VI* parental strain and from a DNA preparation of the *VI* cosmid library. Several of the PCR products have not been digested to completion resulting in a few partially cut bands. M: 100 bp marker. P: SCAR product amplified from strain UH 4857-4 digested with the restriction enzyme labelled above. C: SCAR product amplified from the *VI* cosmid library digested with the restriction enzyme labelled above.

4. Discussion

4.1 Evaluation of the RAPD screen of Bulked Segregant Pools

The combination of bulked segregant and RAPD analysis provides a means to quickly saturate a region of interest with molecular markers. In this study, pools bulked for *U. hordei* avirulence genes, *VI* and *V6*, were screened with 890 available RAPD primers. Analysis with this number of primers represents an extensive survey of the *U. hordei* genome. With an average of six products per successful reaction, primer screening produced a total of approximately 4000 RAPD loci randomly interspersed throughout the genome. Each RAPD locus is able to detect both base pair differences at primer binding sites and DNA rearrangements between sites. In the former case, alone, the number of bases of *U. hordei* DNA directly scanned by primer annealing can be calculated to be a total of 72 kb (Appendix IA). Of the 4000 RAPD loci, three exhibited co-segregation with the *VI* gene, but none were determined to be linked to the *V6* avirulence gene.

Application of RAPD analysis in conjunction with bulked segregant pools has been successful in identifying markers linked to desired genes in a number of crop plants including lettuce, barley, tomato, oats, bean and apple (Michelmore *et al.*, 1993; Barua *et al.*, 1993; Wing *et al.*, 1994; Penner *et al.*, 1993; Haley *et al.*, 1993; Koller *et al.*, 1994). For these studies, the number of primers required for marker isolation varied from as few as 100, for the identification of three markers linked to downy mildew resistance in lettuce, to as many as 800, for two tightly linked markers to the *jointless* gene of tomato. Overall, the frequency of linked marker identification is slightly greater than the results obtained for the *V1* avirulence gene (three markers from 667 successful RAPD primer amplifications). However, given the greater plant genome size and complexity, a higher number of linked markers might have been expected from a fungal genome.

The relatively lower efficiency of marker isolation may be due to the use of closely related parental strains as starting material for the bulked segregant analysis. Although the parents are not isogenic, they are genetically similar enough such that the variability amongst the progeny was

low. The subsequent pooling of these progeny for avirulence resulted in a tight focus upon the target region. As a result, markers mapping further away from the *V1* or the *V6* genes may not have been detectable. For the purpose of isolating markers for a chromosome walk, however, markers only loosely linked to the avirulence genes would have been less useful. The narrow window of linkage is consistent with the observation that all markers identified were within a measured interval of 5.5 cM from the *V1* avirulence gene. In contrast, the three RAPD markers identified by Michelmore *et al.* (1991) for the *Dm5/8* resistance genes were located within a 12 cM genetic distance.

The low number of linked markers identified may also reflect the difficulty in distinguishing actual polymorphisms from the artifactual variation of a few weakly amplified bands. RAPD products that were dismissed as being unusable due to their low reproducibility may have been overlooked as linked markers. Separation of the RAPD products on PAGE gels and detection by silver staining would have given greater resolution and sensitivity for these weakly amplified bands. The ability to obtain the highly reproducible and strongly amplified 359-2.0, 359-1.55 and 743-1.0 markers, however, suggests that the time and effort required in pursuing these more variable RAPD bands would not have been productive.

While the two factors described above may also partially account for the lack of *V6* linked-markers, the result also provides some clues to the nature of the *V6* target region. The success of the bulked segregant technique relies upon the presence of a detectable level of genetic divergence between the parental strains within the area of interest (Michelmore *et al.*, 1991). The inability to identify any markers linked to *V6* suggests that the region at and surrounding this avirulence gene is fairly conserved between the two parents. High sequence conservation has been documented where alleles of avirulence genes differ by as little as a single base pair change. For example, a point mutation resulting in a substitution of a tyrosine for a cysteine residue is sufficient to distinguish virulence from avirulence in *avr4* of the fungal pathogen *C. fulvum* (Joosten *et al.*, 1994). Alternatively, significant genetic divergence may exist around the *V6* locus that was simply not detected with the RAPD primers. The use of another means to identify linked markers other

than by RAPD analysis may meet with greater success. The bulked segregant pools are currently being analyzed by D. Mills and his colleagues to identify amplified fragment length polymorphisms (Zabeau *et al.*, 1994) linked to *V6*.

4.2 The Utility of Markers 359-1.55, 359-2.0 and 743-1.0 for Map-based Cloning

PCR amplifications from RAPD primers 359 and 743 yielded products mapping 3.7 cM and 5.4 cM from the alleles of the *VI* avirulence gene, respectively. Markers 359-1.55, 359-2.0 and 743-1.0 were each extensively characterized. The properties of these markers both facilitate their use in a map-based cloning strategy and limit the ease of their application.

The tight linkage of the identified RAPD markers to the target gene greatly reduces the number of steps required in a chromosome walk. The kilobase distance between the markers and the *VI* and *vI* alleles cannot be calculated exactly since the correlation between genetic and physical distance has not been examined in this phytopathogen. In the closely related corn smut fungus, *U. maydis*, a centimorgan unit has been measured to be equivalent to 0.8-2.4 kb based upon the distance between the *pan1* gene and the *al* locus (Froeliger and Leong, 1991). Assuming this conversion to be valid for the *U. hordei* genome, the *VI* avirulence gene is likely to be located on the same cosmid of the genomic library as the template for the 359-1.55 RAPD marker. Centimorgan to kilobase ratios are not uniform across the entire genome, however, since recombinational suppression in such areas as the *U. hordei* mating type loci does occur (Bakkeren and Kronstad, 1994). If the *VI* gene resides in such a suppressed region, the physical distance to the marker could be greater, necessitating additional steps in the chromosome walk. Even under this unlikely scenario, the identified markers are of sufficient proximity for walking toward the *VI* gene.

The repetitive nature of the markers, however, complicates the first step in a chromosome walk. The observation that all three markers linked to *VI* are part of the same highly repeated unit may reflect the prevalence of this form of DNA in the *U. hordei* genome but is most likely an unfortunate characteristic of the RAPD technique. The genome organization and complexity of *U.*

hordei has not been studied to determine the proportion of repetitive DNA in the genome. An indication of the likely value, however, can be estimated based upon the results of reassociation kinetic studies of the genomes of other basidiomycetes. Characterization of the genome of *Puccinia graminis*, a cereal rust pathogen of the same hemibasidiomycete class as *U. hordei*, measured a repetitive DNA content of 30% (Backlund and Szabo, 1993). 10-20% of the genomes of *Coprinus lagopus* (Dutta *et al.*, 1974) and *Schizophyllum commune* (Ullrich *et al.*, 1980), two higher basidiomycetes with which *U. hordei* shares some characteristics, were comprised of the repetitive class of DNA. These measured values suggest that a similar proportion of the *U. hordei* genome consists of repetitive DNA.

Recent use of RAPD products 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. Molecular analysis of 49 RAPDs amplified from the imperfect fungus *C. fulvum* revealed that all but one of the products represented repeat DNA (Arnau *et al.*, 1994). A considerably lower occurrence of one in four RAPD products was obtained by Martin *et al.* (1991) with markers to the *Pto* resistance gene in tomato. Identification of RAPDs to another locus in tomato, the *jointless* gene, by Wing *et al.* yielded yet another frequency of 50%. This apparent bias toward the amplification of repetitive DNA products represents an unforeseen and serious limitation of the RAPD technique in map-based cloning.

Despite their repetitive nature, however, RAPD markers 743-1.0, 359-1.55 and 359-2.0 do originate from single, unique loci as evidenced by their co-segregation pattern with the alleles of the *VI* gene. The tight linkage to a specific locus of genetic markers comprised of repetitive DNA distributed throughout the genome may seem difficult to reconcile, but can be easily explained. A variation in one copy of the highly repeated unit can give rise to a unique polymorphism that is detectable by RAPD analysis. In the case of marker 743-1.0, it is likely that a base-pair change at a RAPD primer annealing site located in a *vI*-linked repeat unit resulted in amplification of a co-segregating 1 kb product. Markers 359-1.55 and 359-2.0 are allelic due to a suspected imperfect

duplication in 359-2.0. The unique character of this event was confirmed by the specific amplification of products from the junction primers designed from the borders of the additional 450 bp of DNA. In these ways, identifiable single RAPD loci can arise from regions of repeated DNA. While their repetitive nature precludes a simple chromosome walk by hybridization, these linked markers represent unique RAPD primer amplification sites that can be exploited in the cloning strategy for the *VI* gene.

4.3 The Screen for Marker 359-1.55 in the *VI* Cosmid Library

The screening of the equivalent of five times the *U. hordei* genome (2496 clones) did not yield the desired cosmid clone. Theoretically, the probability of isolating a particular unique sequence from this number of clones of a *U. hordei* cosmid library is just above 99% (Appendix IB). In light of this, a likely explanation for why the clone was not among those analyzed is that cosmids bearing RAPD marker 359-1.55 may be represented infrequently within the library.

A possible scenario accounting for the under-representation of this sequence is if the marker were located close to the telomere of the chromosome. Proximity to the ends of a chromosome decreases the probability of obtaining 35-40 kb sized fragments carrying the marker. In this case, the closer the marker is to the telomere, the fewer the number of *Sau* 3A restriction sites are present to generate the restriction fragments of sufficient size. Difficulties in the cloning of the *avr2-yamo* avirulence gene of the fungal pathogen *M. grisea* were due to this situation (Valent *et al.*, 1994). The effects of the chromosomal location of this gene manifested itself in the instability of the avirulence phenotype due to frequent spontaneous mutation events. The instability in avirulence which supports this explanation has not been evident with the *VI* gene, however.

A more plausible explanation may be that the *U. hordei* DNA fragment carrying the 359-1.55 marker amplification site is somehow toxic to the *E. coli* host strain. In the screen of 2496 clones, an effort was made to randomly select clones irrespective of colony size. Despite this effort, it is possible that some of the small, slow-growing colonies, which would have included the

desired clones, may have been overlooked. The results of the screening of another cosmid library (described below) for the allelic marker 359-2.0 are consistent with this hypothesis.

To compensate for this possible under-representation, a screen of a much larger number of cosmid clones would be required. Screening of many more clones using the same two-step process (hybridization and direct PCR screening), however, would be extremely labour-intensive due to the large proportion of positive clones identified by the first step of hybridization with a repetitive DNA probe. The use of DNA preparations from pools of the amplified library as templates for PCR reactions with SCAR primers permitted the analysis of a considerably larger number of clones. A 1.55 kb amplification product was observed with primer R359N and R359P on a pool of approximately 1.0×10^8 clones. Characterization of this product by restriction site analysis showed that, unlike the 13 cosmid candidates identified in the first screen, the fragment pattern matches that of marker 359-1.55. This observation suggests that, despite the possible under-representation of cosmid clones bearing the amplification site of marker 359-1.55, this marker is present within the cosmid library and can be isolated eventually.

4.4 Conclusions and Future Research

For the most part, the specific objectives of the research outlined in the introduction were achieved. Several markers tightly linked to the *VI* gene were identified and characterized. These results provide useful markers for the cloning of this gene as well as information on the feasibility of the RAPD and bulked segregant techniques for tagging specific genes in the basidiomycete *U. hordei*. The cosmid library constructed from parental strain Uh 4857-4 should be of use for the eventual isolation of avirulence as well as other genes from this organism. While the initial attempt to isolate cosmids carrying linked marker 359-1.55 in 2496 clones of the library was not successful, the results of this work provide the tools for the cloning of the *VI* gene by an alternate method.

In fact, further work on the avirulence gene project has led to the isolation of two overlapping cosmid clones carrying the amplification site of marker 359-2.0 (G. Bakkeren and J.

Kronstad, unpublished results). Specifically, a cosmid library of the other parental strain, Uh 4854-10, was constructed and screened with the *vI*-specific junction primer (JCT2B) and the SCAR primers (R359N and R359P) developed in this study (Figure 9). The two cosmid clones both exhibited weak growth suggesting that the *U. hordei* DNA insert carried in *E. coli* is somehow detrimental to cell growth. With the two cosmids in hand, unique genomic sequences have been identified from the inserts for use as hybridization probes. Upon confirming the linkage of the sequences to *vI* and their presence in the *VI* parental strain, the unique sequences can then be used in a chromosome walk toward the *VI* avirulence allele. This more round-about approach using the molecular markers, the primers and the cosmid library described in this work should result in the cloning of the *VI* avirulence and *vI* virulence alleles in the very near future.

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APPENDIX I. Calculations

A. Diagnostics of RAPD Primer Annealing in Successful Amplifications

The number of bases in the template DNA scanned by the annealing of a primer during amplification of a RAPD product has been determined to be 18 bases (Williams *et al.*, 1993). Experiments performed by changing a single base at a time in the primer determined that all bases of the 10mer except for the nucleotide at the 5' end resulted in a completely different RAPD amplification profile. Therefore, primer annealing appears to be dependent upon 9 of the 10 bases. Each successful amplification requires annealing of the primer at two inwardly oriented sites, making each RAPD band diagnostic for 18 bases (2 primer sites x 18 bases). The amplification of approximately 4000 RAPD bands from the RAPD primer screening of the bulked segregant pools is scans a total of 4000 bands x 18 bases/band = 72000 bases or 72 kb.

B. Probability of a Particular Unique Sequence being in a Genomic Library

The likelihood of having a given DNA sequence in the cosmid library can be calculated from the following equation (Clarke and Carbon, 1976):

$$N = \frac{\ln(1-P)}{\ln(1-f)}$$

where: P=probability
f=fractional proportion of the genome in a single recombinant
N=necessary number of recombinants

For the *U. hordei* VI cosmid library, given an insert size of 40 kb and an *U. hordei* genome size of 2.0×10^4 kb, the probability of having the sequence present among 2496 clones is:

$$2496 = \frac{\ln(1-P)}{\ln(1-[40 \text{ kb}/20000 \text{ kb]})}$$

Solving for P, the probability is 99.3%.