Development of Ustilago hordei-specific DNA probes
by Karla Hedman Dugan

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Plant Pathology
Montana State University

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Since the mechanisms causing resistance are poorly understood, U. hordei-specific DNA probes should be useful in analyzing this plant-pathogen interaction. A U. hordei genomic DNA library was constructed, and potentially useful probes were selected by dot blot and Southern hybridization techniques. The probes were pMTSU-1 through pMTSU-13. Probes were tested for specificity to U. hordei. copy number, and lack of hybridization to barley genomic DNA. Seven probes which did not hybridize to barley DNA and showed different banding patterns when hybridized to restriction enzyme-digested genomic U. hordei DNA were further analyzed. One probe appeared to be a low copy number sequence while the others contained multiple copy, tandem repeat or dispersed repeat sequences. Hybridization with these probes to U. hordei races 1A through 14A identified polymorphisms between the fourteen races. Evidence for probe specificity was determined by hybridization with bacterial DNA and DNA from various unrelated and related fungi. Detection of the pathogen in plant tissue was observed with hybridization of pMTSU-11 to DNA extracted from stem tissue of two inoculated barley cultivars. After separation of chromosome-sized U. hordei DNA by pulsed-field-gel-electrophoresis, hybridization patterns indicated that one chromosomal band of race 14 does not contain the pMTSU-11 sequence.

This project describes the development of U. hordei probes which identify polymorphisms between the fourteen U. hordei races, are species-specific, and can detect the pathogen in the plant. These probes' will be useful for future analysis of the U. hordei infection process and mechanisms of host resistance.

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ABSTRACT

Ustilago hordei is the causative agent of barley covered smut. The primary method of control has been through fungicide seed treatment even though a number of host resistance genes have been identified. This resistance appears to be monogenic, resulting in a gene-for-gene relationship with *U. hordei* avirulence genes. Since the mechanisms causing resistance are poorly understood, *U. hordei*-specific DNA probes should be useful in analyzing this plant-pathogen interaction. A *U. hordei* genomic DNA library was constructed, and potentially useful probes were selected by dot blot and Southern hybridization techniques. The probes were pMTSU-1 through pMTSU-13. Probes were tested for specificity to *U. hordei*, copy number, and lack of hybridization to barley genomic DNA. Seven probes which did not hybridize to barley DNA and showed different banding patterns when hybridized to restriction enzyme-digested genomic *U. hordei* DNA were further analyzed. One probe appeared to be a low copy number sequence while the others contained multiple copy, tandem repeat or dispersed repeat sequences. Hybridization with these probes to *U. hordei* races 1A through 14A identified polymorphisms between the fourteen races. Evidence for probe specificity was determined by hybridization with bacterial DNA and DNA from various unrelated and related fungi. Detection of the pathogen in plant tissue was observed with hybridization of pMTSU-11 to DNA extracted from stem tissue of two inoculated barley cultivars. After separation of chromosome-sized *U. hordei* DNA by pulsed-field-gel-electrophoresis, hybridization patterns indicated that one chromosomal band of race 14 does not contain the pMTSU-11 sequence.

This project describes the development of *U. hordei* probes which identify polymorphisms between the fourteen *U. hordei* races, are species-specific, and can detect the pathogen in the plant. These probes will be useful for future analysis of the *U. hordei* infection process and mechanisms of host resistance.

INTRODUCTION

Smut diseases infect more than 75 species of angiosperms worldwide. These basidiomycetes, in the order Ustilaginales, are responsible for significant economic losses in cereal crops. Although barley is not a major commodity crop, smut diseases are responsible for decreased barley yields. Barley smuts vary in prevalence, symptoms, infection, and severity. However, the presence of the pathogen does not become evident until plant maturity when the grain kernel is replaced by the "smutty" teliospores of the pathogen. In the case of barley covered smut, caused by Ustilago hordei, the teliospores are covered with a thin membrane. Knowledge of barley genetics in relation to U. hordei and the similarities of the barley smuts make this a valid area of research.

The disease cycle begins at harvest when the dark teliospores are released into the air. The teliospores drop to the ground where they overwinter and survive in soil, plant debris, and seed. Harvested seed also becomes inoculated with teliospores. Under proper conditions, teliospores germinate to form haploid sporidia which are nonpathogenic. Since U. hordei is heterothallic with a bipolar mating type, haploid sporidia from each mating type

(A and a) must be present for formation of the pathogenic dikaryotic mycelium. The dikaryotic mycelium invades the seedling, advances through the meristem, penetrates ovarian tissue, undergoes nuclear fusion at plant maturity, and finally forms the diploid teliospores in the plant spike which provide the inoculum for the next disease cycle.

Control of U. hordei has been through the use of fungicides such as Vitavax, Baytan, Thiram, and Vitaflo. Recommendations for chemical and fungicide application can affect the development of fungicide resistant mutants. When more is known about the pathogen, fungicide applications may be decreased or at least better targeted for that specific pathogen. Resistant or partially resistant varieties such as Vantage, Hannchen, and Excelsior have provided fairly good control of barley smuts in the past. However, the threat of new races and the need for new cultivars continues. In order to develop new cultivars a better understanding of the host-pathogen interaction is needed.

U. hordei-specific DNA probes were developed for detection of the pathogen in plants. Use of such probes would greatly shorten the lengthy process of waiting for plant maturity to determine infection, provide a means to detect the pathogen in the seed for a disease-free seed program, monitor the movement of the pathogen throughout

the plant, and provide a mechanism to understand the interactions involved in resistance and avirulence.

LITERATURE REVIEW

The Pathogen: Ustilago hordei

Historical and Economic Significance

Smut diseases are distributed worldwide and have been associated with significant cereal crop losses. Incidence of smut diseases were first reported in the 1700's (23). The major damage is associated with yield reduction; however, increased production costs, reduced grain value and damaged equipment are other results of smut infections (23, 61). Smut diseases have been greatly reduced through chemical seed treatments but are still problematic in areas where seed treatment is not economically feasible.

Historical data regarding the incidence and monetary losses from barley smuts is not readily available. Estimated losses from 1918 to 1935 in the United States were 2.25 million bushels annually (87). Much of the research on barley smuts during this century has been done in Canada. Losses from Ustilago nuda (true loose smut), Ustilago nigra (semiloose smut), and Ustilago hordei (covered smut) in Manitoba and Eastern Saskatchewan from 1972-1974 ranged from 0.6% to 0.9% (92). The Canadian prairie provinces yield decreases ranged from 0.7%-1.4%

from 1978-1982. These percentages represent monetary losses from $5.8 million in 1979 to $17.6 million in 1981 (94). Between 1983-1988, barley smuts caused an annual yield loss of 0.7% to 1.6% or $8.8 to $13.2 million losses. Field analysis showed one Manitoba field (1987) and one Alberta field (1988) to have the highest infection due to U. hordei. The continued presence of U. hordei in Canada in 1989 and 1990 indicates the significance of this barley pathogen.

Farming practices in the Middle East increase the probability of smut infections. In areas of the Punjab, Haryana, and Himachal Pradesh, India, barley covered smut causes average annual losses of 2% to 5%. For susceptible varieties, that percentage may increase to 20% to 40% when environmental conditions are conducive to disease development (6, 7). Western Rajasthan has also reported average losses of 2% to 5% with epidemic proportions reaching 30% (2). The International Center for Agricultural Research in Dry Areas (ICARDA) has performed disease surveys throughout Syria from 1984 to 1987 and determined that disease severity varied among fields and years; however, barley covered smut was present in a large proportion of fields on a yearly basis (101).

Taxonomy

Fungi are classified by division (-mycota), subdivision (-mycotina), class (-mycete), order (-ales), family (-aceae), genus, and species. Smuts are true fungi (Eumycota) in the subdivision Basidiomycotina based on their characteristic spore-bearing basidium. Hemibasidiomycetes (Teliomycetes) have simple, septate mycelia, thick-walled teliospores, and a promycelium which develops into sporidia. This class includes both the smut and rust fungi. Rusts and smuts differ in host range, sporidial development and culturability. The smuts are in the order Ustilaginales, which develop an indefinite number of sporidia from the promycelium, are parasitic on Angiosperms and are readily cultured (104). Ustilaginales is divided into Ustilaginaceae and Tilletiaceae. The Ustilaginaceae have a transversely septate promycelium that bears lateral basidiospores (4). Further classification of the smut fungi into genus and species has been wrought with controversy and difficulty. Host range, mode of infection, and spore morphology are only some factors considered when determining genus and species of the smut fungi (4, 23).

The Ustilago genus is described as having a dark and powdery spore mass with the sorus limited by a host membrane (3). Species differentiation is based on sori, spore characteristics, symptomatology, and the host family (23). Based on these priorities, three important barley

smuts have been identified. Ustilago nigra (semiloose or black loose smut), Ustilago nuda (true loose or brown loose smut), and Ustilago hordei (covered smut) are recognized today (61). Each of these pathogens have races designated by their virulence on differential cultivars.

Pathogen Description

Barley covered smut is discernible from other barley smuts by the characteristic membrane enclosing the smut sorus (61). The teliospores which comprise the smut sorus of U. hordei are smooth surfaced, globose to subglobose, and light olive-brown to brown in color. These resting spores are 5-8 μm in diameter (61) and represent the sexual stage of the fungus. The process of U. hordei teliospore germination from pregermination to septation of the promycelium and nuclear condition in germinating teliospores has been investigated (48, 76, 77). Under appropriate conditions, teliospores germinate to produce a long tube-like metabasidium. The diploid nucleus of the teliospore undergoes meiosis (reduction division) which is followed by formation of a single septum forming a two-celled metabasidium each having a haploid nucleus. These daughter nuclei divide and two more septa are formed resulting in a four-celled promycelium (48, 77). Each of the four cells continuously produces haploid, uninucleate sporidia. These sporidia may detach and produce additional

sporidia in a yeast-like budding fashion (104). The yeast-like phase of U. hordei is culturable on artificial media; however, the host plant is required for completion of the sexual stage (95).

The haploid sporidia represent the non-pathogenic phase of U. hordei. Since U. hordei is a heterothallic basidiomycete with a bipolar mating type, each haploid sporidium contains one-half of the genetic information originally found in the diploid teliospore. These haploid sporidia form delicate hyaline hyphae (3) which require fusion with another haploid cell of the opposite mating type for parasitism. When haploid mycelia of opposite mating types (A and a) fuse, a pathogenic dikaryotic mycelium is formed (104). Formation of dikaryotic mycelium is possible on Vogel's Minimal Medium + 1% charcoal (59); however, the continuous growth of the diploid phase in culture has not yet been documented for U. hordei (54, 65). In the host plant the dikaryotic mycelial (n + n) nuclei fuse (karyogamy) to form the diploid nucleus (2n) that will be contained in the resting spore (teliospore).

The Disease Cycle

Barley covered smut is considered a single-cycle disease in that it undergoes only one infection cycle per growing season (61). The disease cycle begins with teliospores, which have replaced the grain kernels, being

released from infected barley heads during the previous harvesting process. These spores overwinter in soil, plant debris, and on seed. A seedling infecting smut, U. hordei teliospores germinate at the same time as the seedling. If conditions are optimal, 20-24°C with a moderately high soil moisture level, teliospores lodged under the hull of the seed are better able to parasitize the host (53, 61, 96). The mycelium proliferates in the embryonic region, invading the growing point, node initials, and internodal regions as the growth of the tiller begins (53). Colonization of the meristematic tissues and advancement through the coleoptile allows for the development of more infected tillers (53, 61). Both intercellular and intracellular hyphae are present until plant flowering (53). Upon penetration of ovarian tissue and reduction in plant nutrients, the hyphal segments thicken, break up and begin rounding to form the diploid teliospores which replace the grain kernel (53, 61).

Barley heads containing the dark, membrane enclosed teliospores tend to emerge later than healthy heads. Often these infected heads will be trapped in the flag leaf and fail to emerge completely. Stunted heads also occur in the presence of U. hordei infections (61). Some races of U. hordei result in teliospore development in leaves (27). To date, detection of U. hordei infection is not possible until plant maturity and observance of smutted heads. The

teliospores maintain viability for up to 15 years and readily provide inoculum for the next disease cycle (104).

Control

Control of smut diseases through the use of chemical and heat seed treatments began over three centuries ago. By 1897, Bolley had replaced copper sulphate treatments for wheat bunt with formaldehyde for the control of all seed-borne, seedling-infecting cereal smuts at a reduced cost (23). Unfortunately, formaldehyde treatments were harmful to human and seed health and had application problems. Organic mercury compounds introduced in the early 1900's proved to be effective but hazardous and expensive while hot water or steam treatments remained questionable and effective for only some smut diseases (23).

Effective control of covered smut can be obtained through a variety of protective and systemic seed treatments; however, no one method is effective for all smuts or bunts. Wallace (1969) showed that the chemical seed treatment BEI 16 controlled smut of oats and barley but not bunt (103). Mills (1972), using a number of seed treatments, showed control of oat and barley smut. This treatment either failed to control bunt or controlled barley smut and bunt but did not control oat smut (67). Vitaflo 280 and 18 gave significantly reduced levels of bunt and oat and barley smut in studies performed by Mills

in 1975 (68). A variety of fungicide seed treatments have proven to be effective (Vitavax, Baytan, Pano-ram, Thiram, Vitaflo, Nusan) while others have shown phytotoxic characteristics (e.g., Imazalil and Serinal) (5, 39, 49, 61, 72, 74, 81). Combinations of these treatments (i.e. Baytan and Lindane or Baytan and Thiram) often enhances disease control (49).

Barley covered smut can also be controlled by the use of resistant cultivars. Because fungicide seed treatment is available and relatively inexpensive in developed countries, little emphasis has been placed on the development of resistant cultivars. Early selection and breeding of resistant barley cultivars was limited by artificial inoculation techniques. In 1937, Tapke used a spore-suspension method to identify eight physiologic races of U. hordei (87). Eight differential varieties, Excelsior, Hannchen, Himalaya, Lion, Nepal, Trebi, Pannier, and Odessa were used to identify thirteen physiological races of U. hordei in 1945 (88). These cultivars should provide adequate information for the development of new resistant cultivars. Several new barley cultivars resistant to races 5, 7, and 8 (reported by Tapke in 1937) and races 14 and 15 (reported by Shrivastava and Srivastava in 1974) have been identified (83, 84, 88). Resistant cultivars are described as having major gene resistance which may be conditioned by a single dominant factor or by

two to four independent factors with minor genes involved in partial resistance (61). Environmental factors and variations in morphological characteristics of the physiological races also influence the infection process (13, 61, 71, 88). Clearly, the interaction between barley resistance genes and U. hordei virulence genes is not understood.

Genetics of Ustilago hordei

Mutation Studies

The prospects of performing genetic studies are enhanced by Ustilago hordei's culturability and mutative qualities. The teliospores of U. hordei remain viable for at least 23 years providing a lasting source of material for research (95). Teliospores germinate on a variety of media which are suitable for ordered tetrad analysis, selection of mating type, and culturing of the haploid, yeast-like phase of U. hordei. The asexual stage produces daughter cells by budding. These budding, haploid cells can be cultured in large quantities and are easily mutated as shown by Hood in 1968 (44). Using ultraviolet (UV) light, Hood studied mutation and lethality of synchronous populations of U. hordei. Three hundred and twenty three mutants were developed with twenty-three of these not being identifiable by specific nutritional requirements. A delay in colony development during different stages of sporidial

development due to chemical effect on cell growth and repair processes was noted (44). A 90-99.5% sporidial kill was determined by growth on complete media. After plating the surviving sporidia on complete and minimal media, 0.35% were found to be auxotrophic mutants (44). In 1972, Thomas was able to increase the frequency of U. hordei auxotrophic mutants to 1.9% by combining UV irradiation and inositol starvation. This technique resulted in ten different amino acid, one purine, and five vitamin mutants (91).

U. hordei mutants have provided valuable markers for numerous genetic studies. Through genetic complementation of U. hordei arginine-requiring mutants, Dinoor and Person improved determination of mating type, genetic loci, and segregating mutant gene(s). Instead of mixing cultures, test strains were replica-plated onto plates of complete media which contained a lawn of one or the other wild-type or mating-type strains. This technique enhanced detection of specific metabolites, heterokaryon formation, and back mutations (17). Using biochemically marked strains, U. hordei parasexual and somatic recombination events have been described which provided evidence of diploid formation (54, 65). Spontaneous fungicide mutants often have a low survival rate due to competition with the wild-type strain or absence of the fungicide. In order to determine more reliably whether resistance is genetic or nongenetic, mutagen-treated sporidia are necessary for genetic studies

of fungicide resistance (36). Fungicide tolerance is determined by plating mutated sporidia on gradients of various fungicides. Dominance, fitness, and stability traits of carboxin and benomyl resistance have been reported (8). Furthermore, polygenic involvement has been noted for chloroneb and thiabendazole resistance (9, 35). The use of mutagenized strains of U. hordei has provided valuable information regarding competition between or among tolerant strains, the physiological basis of tolerance, significance of the sexual stage, and information regarding linkage analysis (9, 10, 36, 37). This information plays an important role in recommendations for future fungicide treatment strategies.

Virulence

Virulence is defined as the degree of pathogenicity of a given pathogen (1). In 1937, Tapke described virulence or percent infection in terms of barley cultivar resistance to U. hordei infection (0-5% infection is resistant, 6-35% infection is tolerant, and 36-100% infection is susceptible). This classification can be used for determining physiological races and cultivar value; however, it does not account for pathogen presence without teliospore development and is inadequate for identifying resistance and avirulence gene interactions. In 1965, tetrad analysis was used to determine that a single

recessive allele for virulence caused 5% infection on two barley cultivars indicating the need for clarification between avirulence (zero reaction) and virulence (any infection) (96).

Genetic control of virulence for the Hordeum vulgare-U. hordei system has been investigated. Sporidia from three parental teliospores were selfed or crossed, and used to inoculate five barley cultivars (Excelsior, Vantage, Hannchen, Trebi, and Lion). From the infection results, Sidhu and Person (85) were able to identify two recessive virulence genes, Uhv-1 (effective on Hannchen and Vantage) and Uhv-2 (effective on Excelsior) (85). Further analysis determined dominant resistance genes present in the host cultivars in a gene-for-gene interaction with U. hordei virulence genes as described by Flor in 1954 (25). In this interaction, host resistance occurs when any resistance allele is not met by its corresponding pathogen virulence allele. Susceptibility requires that all resistance alleles be confronted by their corresponding virulence alleles (86). Ebba and Person discovered that modifier genes, environmental conditions, and the presence of duplicate and recessive genes at two different loci (v_4 and v_5 determining virulence on Keystone and Himalaya, respectively) complicate this gene-for-gene relationship (18). A single recessive gene (v_6) (unlinked to v_1 or v_2) is responsible for virulence on Lion and Plush, v_3 (linked

to v_2) confers virulence on Nepal and Pannier, and both v_1 and v_6 are necessary for virulence on Vantage indicating that some cultivars have more than one resistance gene (93). Crosses of U. aegilopsidis x U. hordei and U. nigra x U. aegilopsidis showed that U. aegilopsidis alleles necessary to overcome barley resistance alleles were absent in U. aegilopsidis (95).

Polygenes which modify the effects of virulence and evidence for reversal of dominance have been described (12, 73). Environmental factors and morphological characteristics of spores also affect virulence. How these factors influence changes in U. hordei populations is not clear (12).

Although the gene-for-gene theory provides a model for understanding the specificity between host cultivar resistance and pathogen virulence, it does not completely describe the host-parasite interaction. Interpretation of the host-parasite interaction has been over simplified by unbalanced information regarding host and parasite genetics (14). The genetics of both the host and the parasite need to be simultaneously investigated to fully understand the interaction between the plant and the pathogen. Hott et al. (45) used classical genetics to determine a gene-for-gene interaction between lettuce (Lactuca sativa) and Bremia lactaucae but noted that this method does not provide information regarding biochemical mechanisms

involved in resistance (46). New advances in DNA technology combined with genetic analyses may help clarify the complex relationship between host resistance and pathogen avirulence genes (14, 15). Both pathogen and host genetics need to be studied to fully understand these factors (12, 33).

Aggressiveness

Aggressiveness is the variation in the degree of infection of susceptible cultivars by a virulent pathogen. Genetic aspects of this variation have been investigated for developing horizontal cultivar resistance and understanding disease incidence.

Using biometrical genetic analysis, Emara (1972) detected a range of aggressiveness from 1.7% to 41.9% among combinations of the 13 U. hordei physiological races (19). Both environmental factors and nuclear genes accounted for this variability in aggressiveness. Genetic control was largely due to additive genetic effects with dominance and epistasis affecting a small portion (96). By investigating the expression of the recessive virulence allele Uhv-1 on the barley cultivar Vantage, it was determined that environmental factors (35%), additive genetic effects (43.9%) and non-additive factors (21.3%) control variability in aggressiveness. A continuous character, aggressiveness is controlled by polygenes which are mainly

additive in effect. Crossing results indicated alleles for higher levels of aggressiveness are dominant and heterosis appears to be present (21). A similar study by Emara and Freake (1981) of $\underline{v}_1$ on Hannchen with different environmental conditions showed variations due to genetic causes (28%), macroenvironment (31%) and microenvironment (14%) (20, 96).

Odessa (universally susceptible) was used to test aggressiveness from progeny of three dikaryons varying in origin and cultural history. Homozygous genes affecting aggressiveness were observed in race 10, while mating of unrelated sporidia produced a heterozygous dikaryon with highly variable progeny. Both additive and dominant gene effects were observed. Evidence of aggressiveness being linked to mating type was also detected. A mating-type segregants of E3a and I4A crosses displayed a higher incidence of aggressiveness than did progeny with the a allele (11). Since aggressiveness affects the severity of epidemics on compatible hosts, pathogen fitness, and the evolution of new races, understanding this aspect of the disease process is important (11).

Pope and Wehrhahn (1991) used barley cultivars Trebi (partially resistant) and Odessa for aggressiveness analyses and found environmental factors contributed to disease incidence in both cultivars. Both cultivars displayed significant variations between A sporidia and

microenvironments indicating linkage of the mating type locus to environmental aggressiveness factors. Variations on Trebi and Odessa were also attributed to additive and nonadditive gene effects. The additive genetic variance was higher on Trebi than Odessa while nonadditive variance effects were lower on Trebi. Interactions within and/or among aggressiveness polygenes and polygenes with virulence genes were hypothesized (75).

U. hordei races display variation in aggressiveness among and within races. The 13 physiological races of U. hordei were observed for variation of peduncle compaction and extent of sorus formation in heads, leaves, and nodes on barley cultivars Hannchen, Nepal, and Odessa. Gaudet and Kiesling (1991) found races 1, 7, 8, and 12 to be heterozygous for genes controlling the above traits as well as plant dwarfing. Highest variability occurred on races 7 and 12 while the lowest variability was in races 1 and 8. These data are indicative of U. hordei's ability to genetically induce morphological changes in the barley host. These changes may play a significant role in maintaining a balance between host and plant survival (27).

Molecular Genetics

Molecular genetic analyses of fungi can be greatly enhanced by DNA-mediated transformation. Transformation of fungi has been reviewed by Fincham (24). The first

description of transformation of U. hordei was described by Holden (41). Frequencies between 10 and 50 resistant colonies per microgram of DNA were reported. This system utilized the U. maydis heat shock gene (hsp 70) promoter fused to a bacterial gene conferring resistance to hygromycin B. Hygromycin B resistance was transferred to U. hordei and was shown to remain stable through many mitotic divisions. Transformation provides the option of using selectable markers for elucidation of U. hordei virulence genes (41).

Isozyme variation has been found to be useful for taxonomy, population genetics, and evolution studies. Pathogens with the same or similar host ranges and species within a genus have been differentiated using this technique. Hellman and Christ (1991) used starch gel electrophoresis to study isozyme variation of 55 North Dakota U. hordei isolates with different virulence genotypes and 8 Ethiopian U. hordei isolates with unknown genotypes. Nine enzymes were examined. Six enzymes had a single allele common to all isolates, two enzymes had two alleles, and one enzyme had three alleles. Differentiation of isolates by isozyme analysis was not detected. Races that were genetically closely related by isozyme analysis were genetically distant by virulence analysis. Thus, isozyme analysis indicated that virulence genes and isozyme genes are not linked. The scarcity of isozyme polymorphisms

for U. hordei were considered to be due to inbreeding, common origin, and lack of necessity for isozyme variation for this organism. These data are consistent with other research where virulence genes showed no correlation with isozyme polymorphisms (34).

A promising aspect of molecular genetics involves pulsed-field-gel-electrophoresis (PFGE) technologies for the separation of large molecular weight or chromosome-sized DNA. Several systems such as orthogonal-field-alternating-gel electrophoresis (OFAGE), transverse-alternating-field electrophoresis (TAFE), and contour-clamped-homogenous-electrophoretic-field (CHEF) have been used for fungal karyotyping and gene identification. Using OFAGE, Kinscherf and Leong estimated 20 chromosome-sized DNA's (300 - 4000kb) in U. maydis (52). The TAFE system has been used for isolate differentiation of Leptosphaeria maculans and karyotyping of Schizophyllum commune (45, 90). Karyotyping of Neurospora crassa, Phytophthora megasperma, Aspergillus niger, Colletotrichum gloeosporioides, Penicillium janthinellum, and Curvularia lunata by CHEF analyses represents a diverse group of fungi where chromosome size have been estimated (16, 47, 50, 60, 69, 70).

Once chromosome separation has occurred, other techniques can be utilized for identification of gene location, linkage data and mapping strategies. A

Magnaporthe grisea repeat (MGR) sequence was detected using cloning, Southern hybridization, CHEF, and classical genetic analyses (32). The A and B mating-type loci of Schizophyllum commune (45), location of the nuclear gene coding for the glyceraldehyde-3-phosphate dehydrogenase (gpd) gene of Curvularia lunata (70), and chromosomal locations of rDNA for Penicillium janthinellum strains (50) have been identified by combining PFGE and Southern hybridization analyses. McClusky and Mills (1990) were able to identify chromosome length polymorphisms for 14 races of U. hordei using CHEF analysis. Fifteen to nineteen chromosome-sized bands were detected ranging in size from 170 to 3150 kilobases. Using U. hordei probes, linkage groups and chromosome-length polymorphisms were identified. Race differentiation by total soluble protein analysis proved to be ineffective. This preliminary data provides incentive to pursue PFGE analysis for determining gene location on specific chromosomes and mapping strategies (62, 66). A rapid method for PFGE chromosomal DNA preparation has been described for U. hordei, Tilletia spp., and Saccharomyces cerevisiae as well as other fungi (63).

Cloning specific genes, especially pathogen virulence genes, would greatly enhance understanding the plant-pathogen interaction. Previously cloned avirulence genes, their significance, and the prospects of cloning plant

disease resistance genes has been discussed by Keen (51). One avirulence gene, avr9 of the fungal pathogen Cladosporium fulvum, has been identified (100). Isolation and characterization of the avr9 gene, along with its regulatory elements, needs to be investigated further (100). The cloning of U. maydis genes has also been described, which increases the possibility of cloning U. hordei avirulence genes (26, 42, 55, 56).

DNA Probes

The use of DNA probes has become a widely used tool for species identification, RFLP analyses, gene location, and detection of pathogens in plant tissue and soil. cDNA libraries, lambda libraries, and plasmid libraries have been sources of DNA probes. Plasmid libraries are prepared by digestion of genomic DNA, ligation of DNA fragments to the appropriate vector, and selection of transformed colonies. Dot blot or slot blot hybridization techniques provide a fairly rapid method of determining whether single, high, or low copy number sequences of genomic DNA are present in the plasmid inserts (58). For species-specific probes, use of high copy number sequences which do not hybridize to plant tissue or unrelated DNA sequences is often preferred (28).

Cloned DNA probes have been used for identification of Phytophthora parasitica (28, 29). Phytophthora is an

Oomycete with more than 40 species causing root, foot and crown rots and blights of foliage and fruits of numerous herbaceous and woody plants (80). Chromosomal DNA digested with HindIII and EcoRI was ligated to pUC8 and used to transform Escherichia coli. Plasmids which hybridized to P. parasitica but not other Phytophthora species, healthy tomato roots, or Pythium species were selected for probes. The species-specific probes were found to have a high copy number sequence which provided better detection signals and increased sensitivity for soil and plant assays (28, 29).

Peronosclerospora sacchari is another Oomycete which causes downy mildew of maize and sugarcane. This obligate pathogen is difficult to detect under conventional isolation methods. The recombinant plasmid pCLY83 was used to develop a probe which provided rapid, accurate, and sensitive detection of seedlots infected with Peronosclerospora. All Peronosclerospora spp. were determined to have a repetitive sequence homologous to the probe which enhances detection of low amounts of the pathogen in host tissues. RFLP analysis indicated that P. philippinensis and P. sacchari may be the same organism (105).

A mitochondrial DNA probe was developed for isolate identification of two Phythium spp. A mitochondrial DNA fragment of P. oligandrum was subcloned into pBluescript. The resulting probe was not highly species and isolate

specific. Investigation into DNA sequence analysis may enhance probe specificity (58).

Septoria tritici, a Deuteromycete, (teleomorph Mycosphaerella graminicola) causes Septoria tritici leaf blotch on wheat. Isozyme and virulence studies have not been successful in identifying genetic variation within populations. McDonald and Martinez selected eight DNA probes for RFLP analysis of 93 isolates of S. tritici sampled from a single wheat field (64). Genetic variability was determined to be on a local scale and sufficient for rapid selection of isolates resistant to fungicides or virulence on resistant cultivars.

Leptosphaeria korrae is an ascomycete which causes spring dead rot of bermudagrass. The symptoms of the disease are similar to other soil-borne fungi causing patch-type symptoms on turf grasses which makes isolation and identification difficult. Two clones, pLK66 and pLK88, were developed for detection of L. korrae. pLK88 proved to be suitable for diagnosis, and ecological and epidemiological studies of patch diseases (97).

Gaeumannomyces graminis is a soilborne ascomycete which colonizes the roots and crown tissue of many members of the Gramineae plant family. Take-all disease of wheat (G. graminis var. tritici) is diagnosed by symptoms which may also be caused by other microorganisms or environmental conditions. A G. graminis var. tritici mitochondrial DNA

probe was developed for identification of Gaeumannomyces spp. which was quite specific and useful for identification of strains and transformants (38). Through the use of polymerase chain reaction (PCR), a target sequence of the mtDNA probe amplified. This method allowed for the detection of G. graminis in infected wheat seedlings (78).

The use of PCR, random amplified primer DNA (RAPD), and RFLPs in conjunction with cloned DNA probes will enrich the capabilities of detecting pathogens within plant tissue and soil, alleviate lengthy isolation and identification procedures, and provide valuable information regarding pathogen genetics.

MATERIALS AND METHODS

Ustilago hordei Strains

U. hordei strain I4 (mating-type A, ATCC No. 34037) was supplied by Dr. Barbara Christ, Pennsylvania State University, and strain E3 (mating-type a, ATCC No. 34038) was provided by Dr. David Pope, University of Georgia. These strains were used for identification of the mating type of isolated sporidia from race 8 and race 14 teliospores, which were provided by Dr. Pederson, University of Illinois. Strains 8A and 14a were from randomly isolated sporidial cultures from race 8 and race 14 respectively.

U. hordei races 1 through 7 and races 9 through 13 were kindly provided by Dr. Mills, Oregon State University. The mating type of randomly isolated sporidia from each of the respective races was identified using strains 8A and 14a.

Selection of Auxotrophic Mutants of U. hordei

Auxotrophic mutants provide excellent selectable markers for genetic analyses. In preparation for mutagenesis, race 8A and race 14a were grown to log phase in liquid Holliday's Complete Medium (HCM) (43) at room

temperature on a VWR orbital shaker (275 rpm). The cells were washed in 1 ml SCS buffer (1.2 M KCl, 20 mM Na citrate pH 5.8) to remove polysaccharides, pelleted in a Sorval-RC5B centrifuge at 5,000 rpm for 10 min, washed in 0.1 M potassium phosphate buffer (pH 6.5), pelleted as above and resuspended in SCS by vortexing. Cell concentrations were determined using a hemacytometer and adjusted to a final concentration of $6x10^7$ cells/ml in 170 μl 0.1 M phosphate buffer (pH 6.5) containing 5 μl of the chemical mutagen ethyl-methanesulfonate (EMS). Cultures were gently agitated on a VWR orbital shaker in the dark at 22°C for 1 h. The reaction was stopped by adding 10 μl of treated cells to 400 μl 5% sodium thiosulfate. Controls consisted of cells treated as above without EMS. Exposure to EMS required for a 60% sporidial kill was determined by removing treated cells at 0, 10, 20, 30, 45, 60, and 120 min. Cells were diluted in sterile distilled water and plated on HCM agar (100-500 colonies per plate). After three days incubation at 21°C, colonies were replica plated onto Vogel's Minimal Medium (VMM) (102) and HCM agar plates. Colonies varying in morphology or growth were patched onto HCM agar and incubated as above. A HCM agar master plate with selected auxotrophic mutants was replica-plated onto VMM supplemented with various amino acids, nucleotides, and vitamins for determination of specific auxotrophic requirements (82).

Selection of Mating Type

Teliospores from race 1 through race 14 were added to 1 ml sterile distilled water, vortexed, and 100 µl was plated onto HCM agar. These plates were incubated for two days at 21°C. Single colonies were streaked for isolation on HCM agar and tested for mating type and purity. Randomly isolated colonies were suspended in 1 ml sterile distilled water. A 10 µl drop of each suspension was pipetted onto VMM + 1% charcoal, allowed to air dry, and combined with 10 µl suspensions of race 8A or race 14a. A positive mycelial reaction with a single wild-type strain after overnight incubation at 21°C indicated the mating type (A or a) (59). Controls consisted of 8A alone, 14a alone, and a combination of the two wild types.

Extraction of DNA from U. hordei.

The procedure for extraction of U. hordei DNA was modified from two methods described for U. maydis DNA extraction (79, 98). Sporidia were grown in 30 ml HCM and washed as previously described for EMS mutagenesis. After washing, the pellets were suspended in 1 ml SCS plus 20 mg Novozyme 234 and incubated for 1.5 hours with gentle rocking at 21°C. A 90-95% protoplast formation was determined microscopically. The protoplasts were washed in 5 ml SCS, centrifuged (3,000xg, 10 min), washed in 5 ml

0.1 M EDTA (pH 8.0) with 1.2 M KCl, and centrifuged as above. The cells were incubated at 37°C for 1 h in 2 ml of lysis buffer (0.05 mM Tris, pH 8.0, 0.1 mM EDTA, 1% SDS). After centrifugation (10,000xg, 5 min), the supernatant was transferred to 1.5 ml microfuge tubes. Proteins were removed by mixing the solutions first with an equal volume of phenol-chloroform (1:1) and then with chloroform-isoamyl alcohol (1:24). Following each extraction, the phases were separated by centrifugation at 14,000xg for 10 min. The final top layer was removed and combined with 2.5 volumes of cold 95% ethanol, incubated for 1 h at -20°C, pelleted by centrifugation at 14,000xg for 10 min, and air dried at room temperature for 1 h. The DNA pellet was resuspended in TE (10 mM Tris, pH 8.0, 1 mM EDTA) buffer. DNA concentration was determined by comparing fluorescence of ethidium bromide stained fungal DNA to HindIII-digested lambda DNA standards following electrophoresis in a 0.7% agarose gel.

Restriction Enzyme Digestion of Fungal DNA

U. hordei genomic DNA was partially digested with SauIIIA (New England BioLabs, Inc.) restriction endonuclease as recommended by the manufacturer. The partially digested DNA was fractionated by sodium chloride gradient (22). The size of DNA in each fraction was determined by electrophoresis in 0.7% agarose. Fractions

with DNA from 400 to 2,000 base pairs (bp) were used for ligation to pBluescript (Stratagene).

DNA from races 1 through 14 were prepared as described previously and digested with EcoRI, BamHI, and HindII (New England Biolabs, Inc.). Digests were performed by incubating an appropriate volume of DNA with the enzyme, buffer, water, and incubating at 37°C overnight (57). Equal volumes of digested DNA were electrophoresed in 0.7% agarose (Seakem) at 42 volts for 8 hours with HindIII-digested lambda DNA as standards.

Construction of Genomic Library

Preparation of Competent E. coli.

Preparation of competent Escherichia coli (E. coli) MV1193 cells was modified from the procedure described by Maniatis et.al. (57). E. coli MV1193 cells were grown overnight at 37°C on LM agar (1% tryptone, 0.5% yeast extract, 1% NaCl, 10 mM $MgSO_4 \cdot H_2O$). Single colonies were incubated in 50 ml SOB media (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 1% $MgSO^4 \cdot 7H_2O$) in a 500 ml flask at 37°C for 2.5 h at 275 rpm. Cells were placed on ice for 10 min and centrifuged at 3,000 rpm for 10 min (Sorval RC-5B). The pellet was suspended in 0.3 volume (16.6 ml) FSB (10 mM K acetate, 100 mM KCl, 45 mM $MnCl_2 \cdot 4H_2O$, 10 mM $CaCl_2$, 10% glycerol, 3 mM $CaCL_2 \cdot H_2O$, pH 6.4), vortexed, placed on ice for 10 min, and pelleted

as above. The pellet was suspended in 4 ml FSB and placed on ice for 10 min. A 3.5% concentration of dimethyl sulfoxide (DMSO) was added and the suspension was placed on ice for 5 min. An additional 3.5% DMSO was mixed into the suspension and 200 µl aliquots were transferred to cold microfuge tubes and stored at -70°C.

Transformation of Competent MV1193 Cells

Competent MV1193 cells were thawed on ice, 1 µl of the pBluescript was added, and the suspension was placed on ice for 30 min. The cells were then incubated for 3 min at 42°C and placed on ice for one to 2 min. 800 µl of SOC (99 ml SOB plus 1 ml 2 M glucose) was added, followed by incubation at 37°C for 1 h with shaking at 250 rpm. The cells were diluted with SOB, spread on a plate of LB agar with 50 mg/ml ampicillin (LB-amp), and incubated overnight at 37°C. The control consisted of performing the protocol with competent MV1193 cells without adding plasmid. Transformants were selected by growth on LB-amp at 37°C overnight (57).

DNA Mini-Preps

A mini-preparation of plasmid DNA which used lysis by boiling was adapted from Maniatis (56). Recovery of plasmid DNA from E. coli was performed by inoculating isolated MV1193 colonies, which had been grown on LB-amp,

into 3 ml LB broth containing 20 μg/ml ampicillin. The cultures were incubated overnight at 37°C with shaking at 275 rpm. 1.5 ml of the growing cultures was transferred to microfuge tubes and centrifuged at 10,000xg for 1 min. The supernatant was discarded, the pellet resuspended in 350 μl STET (8% sucrose, 0.5% Triton X100, 250 mM EDTA, 1 M TRIS, pH8.0), and 25 μl of fresh 10 mg/ml lysozyme (Sigma) was added. The suspension was placed in a boiling water bath for 45 s and centrifuged at 14,000xg for 10 min. The cells were placed on ice, the supernatant transferred to a new microfuge tube and the DNA precipitated with an equal volume of isopropyl alcohol at -20°C for 90 min. After centrifugation (14,000xg, 10 min), the supernatant was removed and the pellet was dried under vacuum for 15 min. The pellet was resuspended in 50 μl TE. The plasmid DNA was cut with BamHI (New England Biolabs, Inc.) in 10 μl volumes containing 5 μl plasmid DNA, 1 μl enzyme, 1 μl 10X buffer, and 3 μl distilled water. The enzyme digestions were incubated at 37°C overnight. DNA digestions and concentrations were determined by electrophoresis of the samples in 0.7% agarose with uncut pBluescript and HindIII-digested lambda DNA as standards.

Ligation Procedure

pBluescript was phosphatase treated by combining 70 μl BamHI-cut plasmid DNA, 20 μl distilled water, 10 μl 10X

phosphatase buffer, 1 µl calf intestine alkaline phosphatase (Sigma) and incubating at 37°C for 30 min. The phosphatase reaction was stopped by adding 1 µl 0.5 M EDTA and incubating at 65°C for 1 h. Sized SauIIIA-cut U. hordei DNA (10 µl) was combined with 35 µl phosphatase treated plasmid DNA and phenol/chloroform and chloroform extracted. A 1/10 volume of potassium acetate was added and the DNA was precipitated using 2.5 volumes of cold 95% ethanol at -20°C for 1 h. The pellet was dried and resuspended in a ligation mix consisting of 1 µl 10X ligation buffer, 0.5 µl T4 DNA ligase (New England Biolabs, Inc.), 1 µl 10 mM ATP, and 7.5 µl sterile distilled water. The mixture was incubated at 16°C for 2 h. After incubation, 1 µl of the ligation mixture was used to transform competent MV1193 cells as described previously. Transformants were selected for insertion of fungal DNA by plating on LB-amp agar which had been overlaid with 5-Bromo-4chloro-3indolyl-ß-D-galactoside (X-gal, 0.8 mg/ml) and isopropylthio-ß-D-galactoside (IPTG, 0.8 mg/ml) and incubated overnight at 37°C. White colonies were transferred to LB-amp agar plates, DNA was extracted by the mini-prep method, and analyzed by agarose gel electrophoresis. Plasmids which were larger than the 3.0kb pBluescript DNA were chosen for dot blot hybridization analysis.

Dot Blot and Southern Hybridizations

Dot blot hybridization was performed according to manufacturer's instructions (Bethesda Research Laboratories). Either 1 μl, 2 μl, or 4 μl of plasmid DNA was added to 80 μl 2 M NaOH in 120 μl distilled water and gently vortexed. The Zetaprobe nylon membrane (BioRad) was cut to fit the dot blot apparatus, moistened in distilled water, and washed in 1 M NH_4 acetate. The membrane was placed on the dot blot apparatus and a vacuum applied for 30 seconds. The DNA was neutralized by the addition of 200 μl 2 M NH_4 acetate to each sample. The samples were loaded into the 96 well dot blot apparatus and a vacuum applied until the liquid was removed from the wells. The membrane was removed from the apparatus and vacuum dried at 80°C for 2 h. Controls consisted of plasmid DNA without insert and no DNA.

Southern hybridization was performed with high stringency conditions. Prehybridization buffer consisted of 50% formamide, 4X SSPE, 1% SDS, 0.5% Blotto, and 0.5 mg/ml of carrier DNA (Biorad). Carrier DNA was prepared by boiling a 1 ml aliquot of 10 mg/ml salmon sperm DNA for 5 min and cooling on ice. The nylon membrane was incubated in the prehybridization buffer at 42°C for 30 min.

Nick translation of U. hordei genomic DNA was performed according to manufacturer's instructions

(Boehringer-Mannheim). Solutions consisted of 3 μl nucleotide mix, 2 μl 10X buffer, 10 μl distilled water, 1 μl U. hordei genomic DNA, 2 μl enzyme, and 2 μl ^{32}P-dCTP with a specific activity of 10.0 mCi/ml. The solution was incubated for 2 h at 16°C. ^{32}P labeled DNA was recovered by running through a Sephadex GS-50 column. The labeled DNA was combined with the prehybridization mixture in which the membrane was immersed. Hybridization occurred overnight at 42°C. The membrane was washed briefly in 2X SSC, followed by 15 min washes at 42°C in 2X SSC/0.1% SDS, 0.5X SSC/0.1% SDS, and 0.1X SSC/0.1% SDS. The membrane was covered with plastic wrap and used to expose an X-ray film for two days. The autoradiogram was developed and the hybridization reaction observed for relative copy number of the insert DNA.

This same labeling method was used for Southern hybridization of selected probes to various bacterial and fungal DNAs. Rhizobium, Sclerotinia, Cochliobolus, Sordaria, Alternaria, Aspergillus, Gaeumannomyces, and Trichoderma DNAs were provided by Dr. Henson, Montana State University (MSU). Candida and Neurospora DNAs were provided by Dr. Cutler, MSU. Pyrenophora DNA was provided by Dr. Raboy, MSU. Teliospores for DNA extraction of Tilletia caries and T. foetida were supplied by Dr. Mathre, MSU. Pulsed-field-gel-electrophoresis (PFGE) separated U. hordei chromosomes, U. hordei restriction enzyme

digested DNA, and DNA isolated from inoculated plant tissue were also examined by Southern hybridization. In later experiments, the hybridization mixture was changed to 50% formamide, 0.25 M $NaPO_4$ (pH 7.2), 0.25 M NaCl, 7% SDS, and 0.01 M EDTA.

Plant Preparations

Plant Inoculations

Race 14 and race 12 were selected for inoculating the differential barley cultivars described by Tapke (88). One to two inoculating loops of sporidia were placed into 1 ml sterile distilled water and vortexed. Husks were removed from seeds of Lion, Himalaya, Jet, Hannchen, Excelsior, Nepal, Trebi, Pannier, and Odessa. The seeds were surface sterilized in undiluted Clorox bleach for 5 min, washed three times in sterile distilled water and dried for 30 min in a laminar flow hood. Fifteen seeds from each cultivar were placed in a suspension of race 14A x 14a sporidial cells and vortexed. The vacuum inoculation technique described by Tapke and Bever (89) was used. A vacuum was applied for three 10 min intervals, the supernatant discarded, and the seeds dried prior to planting. Five replications of three seeds per 6" pot were grown in the MSU Plant Growth Center. Odessa seeds were infected with a suspension of race 12A x 12a sporidia as described above. This race has been reported to develop teliospores in

leaves as well as heads (27). Infection was noted after a three month growth period.

Plant DNA Extractions

Stems and leaves from inoculated barley plants were cut into 2.5 cm sections from the head to the base of the plant, frozen in liquid nitrogen, and stored at -70°C. DNA extraction was as described by Goodwin et al. (29). A 2.5 cm leaf or stem section was placed into a 1.5 ml microfuge tube and crushed with a sterile spatula. A 500 μl aliquot of barley extraction buffer (100 mM Tris, 50 mM EDTA, 100 mM NaCl, 1% SDS) was added to each tissue sample and vortexed. The samples were incubated at 68°C for 20 min and centrifuged (14,000xg, 10 min). The supernatant was removed and boiled for 5 min, cooled, and 200 μl 2M NH_4 acetate was added. The entire amount was loaded into a well of the dot blot apparatus as described previously.

CHEF Analysis

DNA Extraction Without Protoplasting

Race 1 through race 14 U. hordei sporidial cultures were grown as previously described. After washing twice in SCS, the pellets were suspended in SCS and warmed in a 50°C water bath. A 2.5% low melting temperature (LMT) agarose (SeaPlaque) solution was prepared and placed in a 50°C

water bath. The LMT agarose and cell suspension were combined for a final concentration of $1X10^9$ cells/ml. The suspension was transferred to a precooled casting plug and allowed to set. The plugs were transferred to a petri dish and overlayed with lysis buffer (0.45 M EDTA, 10%SDS, 50 mg/ml Proteinase K) which had been warmed to 50°C. The plugs were incubated overnight at 50°C in a sealed container. The lysis buffer was removed, the plugs were washed with 0.5 M EDTA, and stored in 0.5 M EDTA at 4°C.

Gel and Buffer Preparation

A 0.5XTBE running buffer (45 mM Tris-borate, 1 mM EDTA) (57) was prepared and 100 ml of the buffer was removed for preparation of the gel. The remaining 0.5XTBE buffer was placed at 4°C. A 1% agarose gel was prepared by adding 1 g of SeaGem agarose to 100 ml 0.5X TBE buffer. The agarose solution was microwaved for 5 min, cooled prior to pouring into the casting apparatus containing a comb with the desired number of wells (Biorad), and the gel was allowed to set for 1 h. A 2 mm section of each plug was inserted into the CHEF gel wells and overlayed with 1% LMT agarose.

Run Conditions

A CHEF-DRII (Biorad) system was used for separation of chromosome-sized DNA. The cold 0.5XTBE buffer was added to the CHEF chamber and allowed to equilibrate to 14°C before

loading the gel. The gel was loaded into the chamber and again the temperature was allowed to equilibrate to 14°C. The run consisted of a ramp from 55 s to 120 s during a 50 h period at 150 volts.

Staining and Photography

The gel was stained by gentle agitation in 5 µl of 10 mg/ml ethidium bromide in 100 ml 0.5X TBE for 1 h. The gel was destained in 100 ml distilled water from 1 h to overnight. Results were photographed on a UV transilluminator using Polaroid 665 film.

RESULTS

Selection of U. hordei Auxotrophic Mutants

Following EMS mutagenesis, several auxotrophic mutants were identified. Mutants with changes in colony morphology were also developed. A 60% sporidial kill was obtained by incubation of race 8A and 14a sporidial colonies in EMS for 60 min.

Both 14a and 8A EMS-treated sporidia produced morphology mutants. Nonmutated U. hordei sporidia grow as smooth, yeast-like colonies on HCM agar. On HCM agar, the morphology mutant colonies were wrinkled, mycelial, or doughnut shaped. Isolated colonies were stored in glycerol at -70°C.

Race 8A auxotrophs requiring lysine, isoleucine, arginine, leucine/adenine, or pyrimidine were isolated. Race 14a methionine, nicotinic acid, valine, proline, cysteine, histidine, adenine, and arginine auxotrophic mutants were also isolated. The reversion of the auxotrophic mutation was determined by plating $1x10^7$ cells/plate on 10 plates of VMM agar and VMM agar with the required amino acid. Reversion rates were rated high ($\geq 1x10^{-4}$ revertants), medium ($\leq 3x10^{-5}$), or low ($\leq 1x10^{-8}$). High reversion rates were observed for 8A pyrimidine,

14a methionine, 14a proline, and 14a histidine. Medium reversions were noted for 8A isoleucine, 8A leucine/adenine, 14a nicotinic acid, 14a cysteine, and 14a adenine. 8A lysine, 8A arginine, 14a valine, and 14a arginine showed low reversion rates with 14a arginine and 14a valine having no revertants in the 10^8 cells plated on VMM agar. All auxotrophic mutants were stored in glycerol at -70°C.

Construction of a U. hordei Genomic Library

Competent E. coli MV1193 cells which had been transformed with a library of pBluescript containing sized U. hordei DNA were recovered on LB agar with 50 mg/ml ampicillin overlayed with X-gal and IPTG. This selection media provides rapid detection of white colonies which indicates that the lacZ gene of pBluescript had been disrupted by the insertion of the U. hordei DNA fragment. In order to confirm that the plasmid contained a U. hordei DNA fragment, DNA was extracted from selected colonies by the mini-prep method and separated by agarose gel electrophoresis (Figure 1). Migration patterns compared to HindIII-digested lambda DNA (Lane 1) and pBluescript DNA (Lane 12) showed plasmids larger than unmodified pBluescript (Lanes 8, 9, and 11) and plasmids without apparent inserts (Lanes 2-7 and 10). From over five hundred DNA mini-preps, twenty-six recombinant plasmids were selected in the above fashion.