A polymerase chain reaction for the detection of the take-all fungus, Gaeumannomyces graminis, in infected wheat seedlings
by Kurt Robert Schesser

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Microbiology
Montana State University
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Abstract:
Gaeumannomvces araminis var. tritici. a filamentous ascomycete, causes the wheat and barley disease take-all. Symptoms of take-all include blackened roots, sterile heads, and eventual death of the plant, all of which can be caused by other plant pathogens or environmental conditions. Currently diagnosis of G. araminis var. tritici as the etiologic agent of take-all involves culturing the fungus on a highly selective medium which is variable in its ability to recover the fungus from infected tissue. In this project I used the polymerase chain reaction (PCR) to detect the presence of G. araminis var. tritici DNA in infected wheat seedlings.

PCR is a method of DNA amplification which is both highly specific and extremely sensitive. The specificity arises from the use of two oligonucleotide primers which are made complementary to sequences of the DNA fragment of interest. Sensitivity is a result of the amplification process which proceeds exponentially. Here I describe the use of PCR to amplify a G. araminis-specific DNA fragment which was present in wheat seedlings inoculated with the fungus. The fragment was not produced in uninfected seedlings or seedlings infected with other phytopathogenic fungi. This method of detection has potential use in soil assays for the take-all fungus which would facilitate disease forecasting.

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ABSTRACT

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INTRODUCTION

Gaeumannomyces graminis is a soilborne, filamentous ascomycete of worldwide distribution in the north and south temperate zones (22). In the field the fungus is associated almost exclusively with root and crown tissue of many members of the Gramineae plant family. Colonization of roots and crowns of susceptible plants by G. graminis can lead to either a pathogenic or nonpathogenic condition for the plant, depending on both the variety of the fungus and the species of the host plant (73). G. graminis var. tritici is the etiologic agent of take-all disease of wheat and barley, which economically is the most important root disease for these cereals worldwide. It is characterized by penetration of the root cortical layers and colonization of the stele by the pathogen which leads to blackened roots, stunted growth, sterile heads, and eventual death of the plant. G. graminis var. avenae causes take-all in oats as well as in wheat and barley. G. graminis var. graminis, which causes crown sheath rot disease of rice and spring dead spot of bermuda, is considered a nonpathogenic parasite of wheat and barley since it does not extend into the stele or cause symptoms characteristic of take-all. This project concerns the take-all fungus, G. graminis var. tritici, and its detection in infected wheat seedlings.

Take-All and its Control

The disease take-all was first recognized in South Australia in 1852 and the first recorded assignment of G. graminis var. tritici as the etiologic agent of the disease was made in France in 1890 (22). In 1913 take-all was first recognized in England and by the late 1940's it had become a significant yield-limiting disease in England as well as the rest of Europe, Australia, and the United States. This dramatic increase in take-all's destructiveness was for the most part due to a change in farming practices, as a shift towards monoculturing and away from rotational farming techniques had occurred (30). The reason monoculturing has increased the relative success of G. graminis var. tritici can best be understood in terms of its disease cycle.

Disease cycle

The pathology of take-all can be divided into three somewhat overlapping phases; parasitism and infection, spread of the fungus through a stand of wheat or barley within a growing season, and saprophytic survival of the fungus providing inoculum for the next growing season (31). Under laboratory conditions 2-3 germ tubes emerge from each ascospore. These pigmented germ tubes, or runner hyphae, give rise to infection hyphae anywhere along the length of the former in response to the presence of susceptible plant roots. Infection hyphae penetrate the epidermal or root

hair cells by forming an appressorium-like swelling termed
an infection peg (77). It is believed that this penetration
is mostly an enzymatic process since cell wall material is
observed to dissolve just ahead of the infection peg (65).
In response to this insult the host plant thickens its
secondary cell wall with lignin or ligneous material,
forming stuctures called lignitubers in an effort to contain
the invading fungus. Infection hyphae eventually are able
to penetrate the lignitubers which subsequently leads to the
cell's death. When the infection hyphae reach the
epidermis, blockage of the xylem occurs both by hyphae and
lignitubers which results in the breakdown of stelar tissue
and extensive host cell death. The fungus grows freely in
the dead tissue. How far the fungus is able to proceed
through the cell layers of the host determines the extent of
pathogenicity of the disease. Weakly pathogenic isolates of
G. graminis var. tritici are more often found in the
cortical layer while more pathogenic strains are isolated
from the stele (14). The overall effects of infection on
the plant include blackened roots, stunted growth, and
whiteheads (65).

How the spread of G. graminis var. tritici occurs
within the soil is not well understood due to the almost
complete lack of a reliable soil isolation method (discussed
below). Samual and Garrett (56) demonstrated that moistened
perithecia, when present in whiteheads, eject ascospores at

a rate of several hundred per min from a single diseased wheat culm. This observation agreed with epidemiological data which suggested that production of whiteheads is influenced by precipitation and wind. These dispersed ascospores survived 3-4 days in dry conditions and about a week in wet conditions. For longer term survival G. graminis mycelia appears to remain associated with wheat residue by which inoculum for the next growing season is provided. Soil within planted wheat and barley rows are more infectous than the soil between rows suggesting that the fungus survives better as a parasite than as a saprophyte (29). Brown and Hornby (7) showed that G. graminis var. tritici grew very little, if at all, in soil without wheat seedlings being present and other studies demonstrated a strong relationship between pathogenicity of the fungus and its survival on wheat stubble (11). These studies suggest how monoculturing increases the severity of take-all by increasing the amount of plant residue harboring the fungus. The fungus survives poorly in soil conditions which favor microbial growth, such as highly aerated and moist soils, due to its low competitive saprophytic ability (21).

Disease control

Presently the only completely effective control strategy for take-all involves crop rotation of susceptible

hosts away from the infested fields for a period of one or two years. The success of this control measure is due to the poor saprophytic survival of the fungus, as outlined above, and the activity of both self-inhibitory strains of G. graminis var. tritici and other fungi and bacteria. The microbiological basis of the latter was demonstated by showing that the suppressive properties of inhibitory soils were lost upon heat treatment or irradiation (62,64). Romanos, et al.,(52) described a factor released by certain cultures of G. graminis var. tritici grown at pH 3.5-4.5 which was both self-inhibitory for the take-all fungus and inhibitory for the growth of other fungi. Highly pathogenic strains, when grown at pH 4, produced very little of this inhibitory compound while weaker pathogenic strains produced relatively more and as a result grew slower (45). The pH dependency of the production of this compound suggests the basis for work which showed that field treatments which decreased the soil pH reduced the severity of the disease and increased the pathogen-suppressiveness of the soil (12,24,63). G. graminis var. graminis, a nonpathogenic parasite of cereals, to some extent confers cross-protection to host plants against take-all attack (79). The grassland fungus, Phialophora radicicola, has an infection habit almost indistinguishable to G. graminis var. tritici infection, covering the roots with dark, branching hyphae, but penetrating mostly senescent or dead cells and thus

causing little lignituber formation or vascular discoloration (28,60,66). P. radicicola is considered an avirulent parasite of wheat which provides some protection against take-all when cross-inoculated with G. graminis var. tritici (60). P. radicicola was once believed to be the main microbial agent responsible for the phenomenon of take-all decline (T.A.D.) (30) which is currently believed to be caused by fluorescent pseudomonads (76). T.A.D. follows the maximum development of the disease in soils which have grown cereals continuously for 7-8 years. The bacterium responsible for T.A.D., Pseudomonas fluorescens, produces a phenazine antibiotic which is active in vitro against the take-all fungus and other root rotting fungal pathogens (69). Weller et al. (76) were able to show that isolates of P. fluorescens from roots grown in suppressive soils were more inhibitory for G. graminis var. tritici growth in vitro than those isolated from roots growing in noninhibitory soils. Further work showed that the presence of antibiotic-producing strains of P. fluorescens could be correlated with the lack of take-all disease symptoms displayed by field grown plants (70). Treatment of wheat seeds with antibiotic producing stains of P. fluorescens resulted in plants with fewer disease symptoms when challenged with G. graminis var. tritici in both greenhouse and field tests (75). Yields were increased 147% in fumigated soil and 27% in natural soil over nontreated seeds.

Take-all diagnosis

The traditional approach of diagnosis of plant diseases

has been based on identification of the pathogen either by

its isolation or by symptoms displayed by the host (10,32).

G. graminis var. tritici is difficult to isolate from

diseased tissue and causes disease symptoms similar to those

caused by other fungal plant pathogens or environmental

conditions. Traditionally, take-all has been diagnosed when

plants display blackened roots, stunted growth, prematurely

ripened heads (whiteheads), and ears containing shriveled

grain (15). Whiteheads, which may not occur on all G.

graminis infected plants, may be caused by poor soil

conditions, insects, or other microbial pathogens. Black

roots may be caused by Cochliobolus sativus or mycorrhizae

and stem base blackening by Fusarium spp. or Leptosphaeria

narmair (30). As previoulsy discussed, Phialophora

radicicola covers roots with dark, branching hyphae which

penetrate the plant cell wall inducing the plant to form

lignitubers to a limited extent (60). Therefore, to confirm

a field diagnosis, isolation of the fungus is required.

Pathogen isolation and disease forecasting

Direct isolation of G. graminis var. tritici from

infected plants is difficult due to its slow growth rate

relative to other soil and plant tissue microorganisms (16).

In addition, the fungus in culture does not normally produce

characteristic asexual or sexual spores which aid in identification. One isolation technique takes advantage of G. graminis penetration of host tissue and involves the surface sterilization of a small section of infected root tissue followed by plating on potato dextrose agar (PDA) containing streptomycin (2). Juhnke, et al., (33) developed a medium which is able to isolate the fungus directly from plant material. The medium includes a variety of antibiotics and synthetic antimicrobics specific for other soil and plant microorganisms. Also included in this medium is L-beta-3,4-dihydroxyphenylalanine (L-DOPA) which G. graminis uses as a substrate for production of a melanin pigment. On such a highly selective medium the growth of the fungus is relatively slower than on a nonselective medium and there is variability in recovering viable G. graminis from infected plant tissue (Henson, unpub. observations).

Establishing that G. graminis is the causative agent of take-all-like symptoms is important for crop-loss appraisal and forecasting of the disease. The forecasting of take-all is very difficult due to the fact that an epidemic is a process that involves several growing seasons and can be discontinuous in time (30). Additionally very little is known about the biology of the saprophytic phase of the fungus (discussed above) and our ability to detect G. graminis in soil either directly or using host reponse

assays is very limited (discussed below). Environmental
factors such as the past weather have been somewhat
successful in the USSR and East Germany for disease
prediction (30). Take-all epidemics appear to be associated
with warm dry periods in winter, probably because competing
microorganisms are somewhat inhibited under these
conditions.

Molecular Diagnosis

Recently some of the techniques of molecular biology
have been used in detecting plant pathogens in diseased
plants. These methods involve the detection of pathogen-
specific molecules, either unique antigens or DNA sequences,
in standardized assays. The detection of pathogen-specific
antigens or DNA sequences is not dependent on an observable
host reponse or the development of disease symptoms.
Antigens specific for a given phytopathogen are detected
immunologically with either polyclonal or monoclonal
antibodies (PAbs and MAbs, respectively). Both viral and
bacterial plant pathogens have been detected directly in
infected tissue using enzyme-linked immunosorbant assays
(ELISA's). A MAb raised against citrus tristeza virus was
able to differentiate viral pathovars (48). Using ELISA's
and immunogold siver stain dot-immunobinding assays
Xanthomonas campestris can be detected in infected geranium
plants with a PAb although there was some degree of cross-

reactivity with other X. campestris pathovars and

phytopathogenic bacteria and fungi (1).

DNA Probes

The detection of pathogen-specific DNA sequences

potentially has a greater degree of specificity, and when

used in conjunction with the polymerase chain reaction,

sensitivity. Deoxyribonucleic acid (DNA) serves as the

genetic material and exists in two complementary strands

hydrogen-bonded together. Under appropriate conditions such

as high temperature or low salt concentrations the two

complementary strands denature and exist as single-stranded

molecules. When the temperature is lowered or the salt

concentration raised, each DNA single-stranded molecule will

rehybridize with its complementary strand. Cloned or

chemically synthesized DNA may hybridize to genomic DNA if

the two sequences are complementary and if the conditions

are appropriate. If DNA is extracted from diseased plant

tissue and immobilized on a solid support such as

nitrocellulose filter paper, then the blotted DNA can be

probed with radioactively-labeled cloned DNA which will

hybridize to strands with sufficient homology. After

washing off unhybridized DNA, the blot is exposed to X-ray

film and any radioactive DNA bound to the filter paper can

be detected. Cloned DNA derived from a pathogen with little

homology to DNA sequences found in the host plant or other

microorganisms can be used diagnostically for plant disease detection. DNA probes have been developed for the detection of bacterial, fungal, nematodal, and mycoplasma-like plant pathogens. Thompson, et al. (71), cloned a 5 kilobase (kb) probe from the bacterium <u>Clavibacter</u> <u>michiganense</u> subsp. <u>michiganense</u> which did not hybridize, under the conditions tested, to DNA from a closely related avirulent <u>C</u>. <u>michiganense</u> strain. The virulent strain was detected directly in sap of infected tomato plants. The degree of hybridization can also be used to distinguish between closely related pathogens. A DNA probe directed against <u>Pseudomonas</u> <u>syringae</u> pv. tomato could differentiate, based on the strength of hybridization, this pathogen from <u>P</u>. syringae pv. <u>syringae</u> (18). An internal probe which hybridized to both strains equally well was used to differentiate between weakly hybridizing <u>P</u>. syringae pv. <u>syringae</u> and small amounts of <u>P</u>. <u>syringae</u> pv. <u>tomato</u> DNA. The detection limit of this assay was 2 ng of purified <u>P</u>. <u>syringae</u> pv. <u>tomato</u> DNA which corresponds to the <u>in situ</u> DNA released from 10^5 bacterial cells. Besal et al., (5) was able to detect single nematode cysts with a mitochrondial-derived probe when cyst homogenates were applied directly to nitrocellulose filters. The direct detection of pathogens using DNA probes make it possible to detect microbes which are difficult or impossible to culture <u>in vitro</u> such as mycoplasma-like organisms (MLO's). In this case the

development of the probe is difficult since purified DNA from the pathogen is unobtainable. Kirkpatrick et al., (34) cloned random fragments of DNA derived from infected tissue and was able to isolate a fragment which hybridized only to DNA from infected tissue and not to DNA from healthy tissues.

DNA probes have been used successfully for detection of fungal plant pathogens and for epidemiological studies. Probes for the latter purpose are able to genetically separate closely related populations. Restriction fragment length polymorphic (RFLP's) probes hybridize to fragments of different sizes generated after the fungal DNA is digested with a restriction enzyme and subjected to electrophoresis through an agarose gel. Hybridization patterns can be compared between closely related fungi and in some cases a specific pattern can be correlated to some other characteristic of the pathogen such as host range. The molecular basis for the differential hybridization pattern is probably due to a single base change which either creates or abolishes a restriction enzyme site. Manicom, et al., (41) cloned a 3.4 kb probe from Fusarium oxysporium which produced hybridization patterns that correlated to vegetative compatibility groups (VCG's) and therefore could be used to assign Fusarium forma specialis. Currently the VCG assignment of a Fusarium isolate is determined by pathogenicity tests on a number of possible hosts requiring

a considerable amount of time and labor.

A number of DNA probes have been developed for use in the diagnosis of fungal plant pathogens. A probe directed against a very highly repeated DNA sequence (approximately 4% of the genome) of Phoma tracheiphila, a fungal pathogen of lemon, was able to detect the pathogen directly in infected seedlings (50). The detection of lower-copy pathogen-specific DNA directly in infected tissue may require amplification of the target DNA sequence. A probe specific for Phytophthora citrophthora, which causes a citrus root disease, could not detect the pathogen-specific DNA sequence in hybridization assays with total DNA extracted from infected roots (25). The probe could detect P. citrophthora after the infected root was plated on a semiselective medium which allowed a limited amount of growth of the fungus.

Recently a 4.3 kb DNA fragment was cloned from the mitochondrial genome of G. graminis var. tritici (26). Although this probe specifically hybridized to purified Gaeumannomyces DNA, it was unable to detect fungal DNA in diseased tissue by direct hybridization of the probe with DNA extracted from infected wheat seedlings. In order to detect G. graminis infection it was necessary to subculture the fungus from infected seedlings and extract DNA from the subculture for hybridization with the probe. Thus, amplification of the target sequence for the detection of

the pathogen directly in infected tissue can be accomplished either by culturing diseased tissue on a medium selective for the pathogen (25,26) or as described here by using the polymerase chain reaction (PCR).

The Polymerase Chain Reaction

PCR involves the denaturing of the target DNA followed by hybridization of two oligonucleotide primers which are homologous to opposite strand sequences that flank the DNA to be amplified. Primed template DNA is then extended with DNA polymerase and deoxynucleotides to produce two copies of the target DNA. Completed strands of one primer extension become a template for the other primer. Repetition of denaturation, primer annealing, and primer extension result in exponential amplification of the fragment of interest which can be visualized on a stained agarose gel. The specificity of PCR is a function of the primers, which are usually 20-30 base pairs in length, and the annealing temperature, which usually ranges from 37 C to 65 C. Pairs of chemically synthesized primers that hybridize to many regions of template DNA will result in a large number of PCR products. A low annealing temperature will allow the primers to hybridize to sequences with less homology which likewise will result in a number of nonspecific PCR products.

The theoretical basis of primer directed DNA

amplification was first proposed a number of years ago (35) and only recently shown to be possible. The first published research using PCR involved using a pair of primers which flanked the single base polymorphic site which causes Sickle Cell Anemia (54). The single base change alters a restriction site and therefore its presence can be detected by the hybridization pattern of the amplified DNA in southern blots. The use of PCR increased the level of sensitivity by two orders of magnitude by reducing the amount of starting DNA required for the detection of the base change. The specificity of PCR increased dramatically with the use of the thermostable DNA polymerase of Thermus aquaticus (Taq) (53). Previously the Escherichia coli Klenow fragment polymerase I was used and had to be added fresh after each denaturing step and had a maximum activity at 37 C. The Taq polymerase can withstand extended and repeated incubations at 95 C and has maximum activity at 72 C. In addition to simplifying the procedure and increasing the specificity of the products, the use of Taq polymerase also was reported to increase both the yield of PCR products and the maximum length product which could be obtained, 2-3 kb (53).

PCR for diagnosis

In a relatively short time PCR has become a widely used tool in molecular biology research and the basic

amplification process has given rise to a large array of
related techniques, some of which are outlined in Table 1.

Table 1. Related PCR techniques.

Technique	Purpose	Ref.
Inverse PCR	Chromosome crawling	46
Competitive nucleotide priming	Mutation analysis	23
MOPAC	cDNA amplification	37
Anchored PCR	cDNA amplification	39
RACE	cDNA synthesis	20
Asymmetric PCR	Single-strand synthesis	55
Multiplex PCR	Genetic screening	9

In the area of diagnostics of infectous and genetic diseases
the technology has spawned a growth industry. Plant and
animal pathogens which were before too few in number to
detect in the infected host have become assessable in situ.
This detection is not dependent on isolating or culturing
the pathogen and requires only a minute amount of DNA to be
present in the diseased tissue or sample. PCR has been used
successfully for detecting a wide range of microbial
pathogens in a variety of sample types (Table 2).

Table 2. Detection of pathogens using PCR.

Microbe	Sample	Reference
Viral		
Pseudorabies	Cell culture	4
HTLV-1	Spinal fluid	3
HIV-1	Blood	47
Hepatitis B	Serum	40
Cytomegalovirus	Urine	17
Bacterial		
Shigella flexneri	Lettuce	36
Protozoan		
Trypanosoma brucei	Blood	44
Fungal		
Phoma tracheiphila	Lignified tissue	51

To date there has been only one published account of using PCR for the detection of a phytopathogen in infected plant material. This involved the amplification of the highly repeated sequence of Phoma tracheiphila discussed above (51). Although the probe containing a portion of the repeated sequence could detect the fungus in the soft tissue of infected lemon trees it performed poorly with lignified branch samples. The use of PCR allowed for the direct detection of the pathogen in the woody tissue as well as the infected soft tissues without hybridizations and radioactivity. Fungal DNA has been amplified for phylogenetic studies in which PCR was used to facilitate sequencing of a portion of the 16S-like rDNA gene of a

variety of fungal species (78). Also PCR has been
successfully used to amplify DNA from dried fungal herbarium
specimens which had been stored for 1-50 years under a
variety of conditions (8). Here I describe the use of the
polymerase chain reaction for the amplification of G.
graminis-specific DNA. This method was successful in
detecting G. graminis var. tritici DNA in infected wheat
seedlings directly without subculturing the fungus. The PCR
products were visible on stained agarose gels and they were
shown to be G. graminis-specific by southern blot analysis.
The method described here has potential use for diagnosis of
diseases caused by Gaeumannomyces and the detection of the
fungus in soil which would aid in take-all forecasting.

MATERIALS AND METHODS

Fungi, DNA Isolation, and Plant Infections

Fungi used in this study are listed in Table 3.
Cultures were grown on L medium (43) at 24 C and DNA was
isolated as previously described (26). DNA from infected
and uninfected control plants (six seedlings, approximately
0.6 g/DNA extract) was isolated by a fungal DNA protocol
(38) except that whole roots were used as starting material.
Ponderosa spring wheat seeds were surface sterilized in a
solution of bleach (10%) and ethanol (10%) for 20 min,
washed with distilled water, and placed in 200 ml volume
glass jars containing 25 ml 1.2% agar. Seeds were
inoculated at the time of planting with a single agar plug
taken from a fungal culture with a pasteur pipet. Seedlings
usually displayed disease symptoms within two weeks of
planting and DNA extracted either three weeks or one month
after planting.

Table 3. Fungi used in this study.

Strain	Source
Cochliobolus sativus	D. Mathre
Fusarium culmorum	D. Mathre
Gaeumannomyces graminis	
var. avenae	S. Wong via D. Mathre
var. graminis	C. Rothrock via D. Mathre
var. tritici	D. Mathre
Rhizoctonia	D. Mathre
Trichoderma	D. Mathre

Restriction Mapping and Subcloning

Restriction enzymes were obtained from Stratagene (La Jolla, CA) and USB (Cleveland, OH) and were used according to the directions of the manufacturers. For large scale plasmid preparations the alkali/SDS isolation method was used (6) followed by Sephacryl S-1000 chromatography (Pharmacia, Uppsala) using a high salt column buffer (10 mM Tris [pH 8], 1 mM EDTA, 1 M NaCl). The DNA concentration of the collected fractions was determined by measuring the absorbance at 260 nm. The second A260 peak fractions were pooled and precipitated with one volume isopropanol and resuspended in TE buffer (10 mM Tris [pH 8], 1 mM EDTA) at a concentration of 70-1185 ug/ml. Plasmid minipreps for transformant screenings also utilized alkali\SDS starting

with 2 ml overnight bacterial cultures.

Restriction mapping of pMSU315, a pUC18 plasmid with a 4.3 kb Gaeumannomyces-specific insert (26), was done by digesting 2 ug of the plasmid with a variety of restriction enzymes in separate 20 ul reactions. Digests were analyzed by electrophoresis using 0.75% agarose gels with a running voltage of 4.4 V/cm. A lambda HindIII digest was used as a molecular weight marker and restriction fragment sizes were calculated with the GEL computer program written by Fristensky and based on the method of Schaffer and Sederoff (59).

Subtraction subcloning was accomplished by digesting 20 ug of pMSU315 with restriction enzymes in separate reactions that cut within the 4.3 kb insert as well as the multiple cloning site. The plasmid was then religated and approximately 1 ug used to transform Escherichia coli strain DH-alpha (Bethesda Research Laboratories) (13). Transformants were screened and those with plasmids of the appropriate size (i.e. pMSU315 minus the fragment which was removed by the restriction enzyme digestion as predicted by the restriction map) were cultured for large-scale plasmid preparations. Restriction enzymes used for deletion plasmid constructions were EcoRI, HindIII, PstI, SacI, and XbaI, and their corresponding plasmids were named pMSU542, pMSU543, pMSU544, pMSU541, and pMSU540, respectively.

Sequencing

DNA sequencing was performed using the Sanger chain-termination method (58). The plasmids constructed above, plus the parent plasmid pMSU315, were prepared for sequencing by the alkaline-denaturation method (72) which involved denaturing the plasmid in 0.2 M NaOH, 0.2 mM EDTA for 5 min at room temperature. The solution was then neutralized with 0.1 volume of 3 M sodium acetate (pH 5) and the DNA was precipitated with 2 volumes 95% ethanol. After incubation on ice for 10 min, a 5 min spin in a microfuge was used to pellet the DNA. The supernatant was decanted and the DNA pellet was washed with 160 ul cold 70% ethanol and allowed to dry. The DNA pellet was resuspended in 7 ul of distilled water. Sequencing was performed using a Sequenase version 2.0 kit (USB, Cleveland) following the instructions of the manufacturer. Labeling reactions were done at 18 C for 10 min and the termination reactions were performed at 40 C for 5 min. Three oligonucleotide sequencing primers were used; M13 -20 forward, M13 Reverse (Stratagene), and KS2R (described below). Electrophoresis was done on a BRL model SO sequencing apparatus with 6% polyacrylamide gels as described by Mann (42,57). The gels were fixed in 5% acetic acid, 5% methanol overnight, bonded to 3MM Whatman paper, and vacuum dried at 65 C for one hour. XO-MAT AR film (Kodak) was exposed 4 days and processed with GBX developer and fixer (Kodak) according to the

manufacturer's instructions. Sequencing ladders were read manually and sequence comparison was made by searching GENEMBL and GENEBANK (19).

PCR

Oligodeoxyribonucleotide primers were chosen from the pMSU540 and pMSU541 sequencing data. Primers KS1F and KS2R flanked a 407 bp fragment containing nested primer sequences KS4F and KS5R which encompassed a 138 bp fragment (see Table 4 for sequences). Primers were prepared on an ABI 381A DNA synthesizer (Applied Biosystems, Foster City, CA) followed by cleavage of the trityl group. The primers were resuspended in 150 ul distilled water and the concentration was determined by reading the A260 (1 A260 unit equals 20 ug/ml of single-stranded oligonucleotides).

Master PCR reaction mixes were prepared in 1.5 ml microcentrifuge tubes placed on ice. The mixtures contained distilled water, primers (100 pmol each), all four nucleotides (0.125 mM each) (USB), Taq polymerase (3

Table 4. Sequences of oligodeoxyribonucleic PCR primers used in this study.

Primer	Sequence
KS1F	5'-TACGGCTGTACCGCATGATCTACTA-3'
KS2R	5'-ATGAGGCCAGAGGTCCCGTCAAAA-3'
KS4F	5'-CTGTCGGCAAGCGAAGAAGTAC-3'
KS5R	5'-GTAAGTCTACGCGTAGTTGGC-3'

units/100 ul) (Perkin-Elmer-Cetus, Norwalk, CT), and PCR

buffer (50 mM KCl, 10 mM Tris[pH 8.3], 15 mM MgCl$_2$, and 0.1%

gelatin, final concentration). The mixtures were gently

mixed and 100 ul were aliquoted into 0.5 ml microcentrifuge

tubes containing the template DNA. Two drops of light

mineral oil (Sigma, St. Louis) were added to the reaction

tubes which were kept on ice until placed in the thermal

cycler. Template DNA, in 1 ul volumes, used per reaction

was 1 ng pMSU315, 0.2 ug purified total fungal, or 0.02-0.1

ug from infected or uninfected plants. PCR reactions,

unless otherwise specified, were started with the outside

primers, KS1F and KS2R, and after 20 cycles (1 min at 92 C,

2 min at 42 C, and 4 min at 72 C) reaction products were

diluted 50-fold, and 1 ul was amplified another 30 cycles (1

min at 92 C, 2 min at 52 C, and 4 min at 72 C) with the

inside primers, KS4F and KS4R. Automated PCR was performed

with a Perkin-Elmer-Cetus thermal cycler (Norwalk, CT) and

manual temperature cycling with three heat blocks (55).

Product Analyses

PCR reaction samples (20 ul per lane) were run on a 2%

NuSieve (FMC, Rockland, ME), 1% agarose gel at 4.4 V/cm for

3.5 h. A BstNI digest of pBR322 purchased from New England

Biolabs (Beverly, MA) was used as a molecular weight

standard, yielding fragments of 1857, 1060, 929, 383, and

121 base pairs. Gels were stained with ethidium bromide and

photographed with type 57 high speed film (Polaroid). PCR products were transferred directly without depurination to a nylon membrane (Zeta-Probe, Bio-Rad, Richmond, CA) by alkaline capillary transfer (49). Nick translations were done with a kit purchased from Bethesda Research Laboratories (Gaithersburg, MD). Overnight, 65 C hybridizations were performed with labeled pMSU315 (10^8 cpm) (67). Membranes were washed according to the instructions of the manufacturer (Bio-Rad) and X-ray film (X-Omat AR, Kodak) was exposed for 8 h before development.

RESULTS

Restriction Mapping And Subcloning

Plasmid pMSU315, which contains a 4.3 kb fragment

cloned from G. graminis var. tritici, was mapped with a

variety of restriction endonucleases which have sites within

the pUC18 multiple cloning site. This simplified the

analysis by making it possible to quickly assign the

polarity of the restriction sites. For example, digesting

pMSU315 with SacI, which has a restriction site in the

multiple cloning site 3' of the insert, generated fragments

of approximately 5.2 kb and 2.0 kb. Since pUC18 is 2.8 kb

in length, the SacI site within the insert must be located

2.0 kb from the multiple cloning restriction site (Fig. 1).

When restriction enzyme digestion resulted in more than two

fragments or in two closely sized fragments it was necessary

to digest the plasmid with two restriction enzymes

simultaneously. For example, KpnI digestion generated a 3.8

kb and a 3.2 kb fragment, and it was impossible to

distinguish the fragments since either one could contain the

vector. Performing a KpnI, SacI double digest generated

fragments of 3.2 kb, 2.0 kb, and 1.9 kb, which indicated

that the 3.2 kb fragment generated in the KpnI digestion

contained the vector and about 500 base pairs of the insert.

The KpnI site is therefore located 3.8 kb 5' of the right

multiple cloning site. Other pMSU315 restriction sites are
diagrammed in Fig. 1.

Fig. 1   Partial restriction map of pMSU315. Numbers in
          parenthesis refer to distance, in kilobase pairs,
          of the restriction site from the lefthand border
          of the insert. Multiple cloning restriction sites
          are shown in bold. Double bar portion is
          sequenced.
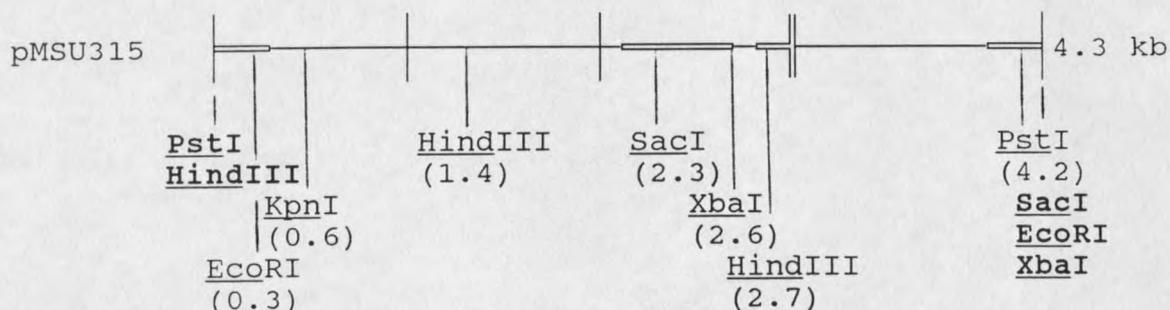
In order to obtain DNA sequence data of regions within
the cloned fragment with one set of primers, subtraction
subcloning was performed. This involved digesting pMSU315
with a restriction enzyme that cuts within the insert as
well as in the multiple cloning site. The cut plasmid is
then religated and used to transform E. coli. Religation of
the plasmid minus the cut out fragment is a first-order
reaction which is favored over the second-order reaction of
pMSU315 reforming intact after digestion (55). For example,
pMSU315 digested with SacI generates 5.2 kb and a 2.0 kb
fragments and when plasmids were recovered from ampicillin-
resistant bacteria transformed with the religated DNA, they
were of the predicted size of 5.2 kb. The newly constructed

plasmid, in this case pMSU541, could be used in sequencing reactions using a universal primer (below) which hybridizes to the flanking region of the right multiple cloning site and is extended 250-350 base pairs into the insert during the sequencing reactions. Therefore, the sequencing of pMSU541 generates approximately 330 base pairs of sequencing data in a region, as predicted from the restriction map, 2.0 kb 5' of the right multiple cloning site of the parent plasmid, pMSU315. In addition to <u>Sac</u>I, <u>Pst</u>I, <u>Eco</u>RI, <u>Hind</u>III, and <u>Xba</u>I, were used to generate the derivative plasmids pMSU544, pMSU542, pMSU543, and pMSU540, respectively.

<u>Sequencing And Primer Selection</u>

The target DNA fragment must be partially sequenced before selection and subsequent synthesis of primers for amplification of PCR. The plasmids, pMSU540, pMSU541, pMSU544, pMSU543, and pMSU542, were sequenced with either the M13 forward or reverse oligonucleotide primer depending on where the corresponding restriction site used to construct the derivitive plasmid appears. The parent plasmid, pMSU315, was sequenced with both the forward and reverse primers to obtain sequence information at both ends of the cloned fragment. Plasmid pMSU544, when sequenced with the forward primer, yielded 48 bp of insert sequence which defined the 3' end of the cloned fragment (Fig. 1).

Priming pMSU315 with the forward oligonucleotide resulted in a sequence ladder which corresponded to 265 bp at the 5' end of the cloned fragment. When the reverse primer was used, pMSU315 yielded 232 bp of sequence information 5' of the PstI restriction site. Plasmid pMSU543 primed with the forward oligonucleotide generated 221 bp of sequence located approximately 2.7 kb downstream of the 5' end of the insert. For unexplained reasons, sequencing pMSU542 with the reverse primer did not result in a readable sequencing ladder.

Plasmids pMSU540 and pMSU541 were partially sequenced with the reverse primer. As already mentioned, 330 bp of sequence, located approximately 2.0 kb 5' of the right multiple cloning site, was obtained when pMSU541 was sequenced. Plasmid pMSU540 generated 246 bp, including the SacI site near the 3' end, of sequence information located approximately 1.7 kb upstream of the right muliple cloning site (Fig. 2). Restriction mapping predicted the XbaI and SacI sites to be approximately 300 bp apart which closely agrees with the sequencing data which suggests that these sites are about 275 bp apart. Ten unsequenced bases, as was determined later by sequencing with the primer KS2R (below), separated the 3' of the pMSU540 and the 5' end of the pMSU541 sequencing data. The beginning of the pMSU540 sequence to the end of the pMSU541 sequence encompassed a 587 bp region which was judged to be appropriate for the synthesis of PCR oligonucleotide primers.