PCR-BASED TEST FOR DIFFERENTIATING VARIETIES OF *Gaeumannomyces graminis*, THE TAKE-ALL PATHOGENS

by

Sansanalak Rachdawong

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APPROVED:

Dr. Erik L. Stromberg, chairman
Dr. Carole L. Cramer
Dr. Elizabeth A. Grabau
Dr. George H. Lacy
Dr. Saghai Maroof
Dr. Orson K. Miller Jr.

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Dr. Erik L. Stromberg, Chairman

Department of Plant Pathology, Physiology and Weed Science

Virginia Polytechnic Institute and State University

ABSTRACT

Take-all is the most devastating root disease of wheat worldwide. The causal agent is *Gaeumannomyces graminis* (Sacc.) Arx & Olivier. Based on morphological characteristics and host ranges, three varieties of *G. graminis* have been recognized. *G. graminis* var. *tritici* Walker (Ggt) is the major causal agent of take-all of wheat and barley and the most economically important take-all pathogen. *G. graminis* var. *avenae* (Turner) Dennis (Gga) attack oats and causes take-all patch of turf grasses while *G. graminis* var. *graminis* (Ggg) is pathogenic on turf grasses but is non-pathogenic on wheat. Conventional diagnosis of take-all pathogens is based on field symptoms such as blackened roots, stunted growth, and white-heads and morphological characteristics such as hyphopodia type, size of perithecia, asci, and ascospores. These procedures are time-consuming, laborious, and often inconclusive. The objective of this study was to develop a rapid, simple, and specific method for differentiation of *G. graminis* varieties using PCR and molecular-based technology. Exploitation of genes associated with pathogenicity of *G. graminis* as markers for the test was proposed. Metabolic activities of *G. graminis* associated with pathogenesis were investigated, namely, the abilities to produce avenacinase and to oxidize manganese. Avenacinase, an avenacin detoxifying enzyme, was associated with Gga pathogenicity for oats but this enzyme is not important in Ggt pathogenicity for wheat. Manganese oxidation was also correlated with Ggt virulence. In this study, avenacinase-like genes were discovered in Ggt and Ggg and manganese oxidation was confirmed for Ggt, Gga, and Ggg. All isolates of Ggt except

isolate ATCC 28230 were manganese oxidizers. Ggg and Gga isolates could oxidize manganese but their precipitation patterns were not as intense or closely correlated with mycelial growth as for Ggt. Pathogenicity assays on oats for Ggt, Gga, and Ggg isolates confirmed that Ggt isolates could not cause disease on oats aside from occasional slight root damage. Root weight was reduced for oat seedlings inoculated with Gga isolates. Comparison of partial sequences of avenacinase-like genes from Ggt and Ggg showed strong homology to that of Gga (94.8% identity to Ggt and 94.6% identity to Ggg). However, the Ggt gene was more closely related to that of Ggg (99.2% identity) than to Gga. DNA restriction endonuclease polymorphisms of the genes supported DNA sequencing information and revealed that there were variations within the genes among Ggt, Gga, and Ggg. Variety-specific electrophoretic patterns were obtained when the genes were digested with *Hae*III. Ggt, Gga, and Ggg upstream (5') variety-specific primers and a downstream (3') universal primer were designed from the avenacinase and avenacinase-like DNA sequences. PCR amplification with Ggt-, Gga-, and Ggg-specific primers generated fragments of 870, 617, and 1,086 bp, respectively. Each 5'-specific primer showed high specificity for its own DNA template in mixed populations of DNA templates. The optimized PCR procedure was sensitive to DNA template concentration as low as 100 pg. Genomic DNA of sixteen Ggt isolates, seven Gga isolates, and five Ggg isolates were tested. Although all Ggt isolates were originally isolated from wheat, seven isolates produced Ggg-specific fragments. This result corresponded well with *Hae*III DNA polymorphisms, pathogenicity assay, and manganese oxidizing ability. All but one Gga isolates produced the variety-specific fragment. Ggt- and Gga- specific products were generated from Gga isolate RB-W. Although Ggg-specific fragments were produced from all Ggg isolates, non-specific products were also observed from isolates that were not from wheat origin suggesting some genetic variations due to host ranges. Additionally, no non-specific amplification was obtained from any closely related fungi such as *Gaeumannomyces cylindrosporus* or *Phialophora* spp. The test developed in this study is the first test capable of identification of Ggt, Gga, and Ggg in a single PCR tube with a basic PCR protocol. The test is rapid and specific. Interpretation of results is simple and conclusive based on differences in size of each variety-specific fragment.

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DEDICATION

I would like to dedicate this dissertation to my father, Dr. Sunt Rachdawong, who passed away in November 25, 1998. I wish you were with us to celebrate my success.

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ABBREVIATIONS

AFLP	amplified fragment length polymorphism
ALPs	avenacinase-like proteins
AV	avenacinase
AVN1	avenacinase gene
BGLs	β-glucosidases
bp	base pair
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphates
Gga	*Gaeumannomyces graminis* var. *avenae*
Ggg	*Gaeumannomyces graminis* var. *graminis*
Ggt	*Gaeumannomyces graminis* var. *tritici*
ITS	internal transcribed spacer
LB	Luria-Bertani broth
L-DOPA	L-β-3,4-dihydroxyphenylalanine
MnP	manganese peroxidases
mtDNA	mitochondrial deoxyribonucleic acid
PCR	polymerase chain reaction
PDA	potato dextrose agar
PDB	potato dextrose broth
RAPD	random amplified polymorphic deoxyribonucleic acid
rDNA	ribosomal deoxyribonucleic acid
RFLP	restriction fragment length polymorphism
R-PDA	rifampicin potato dextrose agar
SM-GGT3	selective medium for *Gaeumannomyces graminis* var. *tritici*
XANES	X-ray absorption near edge structure

CHAPTER 1

LITERATURE REVIEW

1.1 INTRODUCTION

Take-all, historically an Australian disease, was first recognized as early as 1852 by farmers in South Australia (Garrett, 1981). At that time, it was described as a severe seedling blight. The name of take-all was not used until 1870 and is now recognized as a disease of the roots of wheat (*Triticum aestivum* L.) worldwide (Nilsson, 1969). Take-all occurs throughout the temperate areas of the world such as Australia, Europe, Japan, North, and South America where wheat cultivation is intensive (Wiese, 1987). Take-all of wheat is of major economic importance and causes losses in wheat production each year (Garrett, 1981). According to Walker (1981), the causal agent of take-all was first described as *Sphaeria herpotricha* Fr. from grass culm in Sweden. In 1890, Prillieus and Delacroix identified perithecia, ascus and ascospores produced by the wheat take-all fungus and described the fungus as *Ophiobolus graminis* Sacc. However, the wheat take-all fungus was transferred to the new genus *Gaeumannomyces* (Diaporthales) with the type species *G. graminis* (Sacc.) v.Arx & Olivier in 1952 (Walker, 1981). Later the oat take-all and wheat take-all fungi were transferred to *G. graminis* as the varieties *avenae* (Turner) Dennis in 1960 and *tritici* Walker in 1972, respectively.

1.2 TAKE-ALL DISEASE

The first above-ground indication of disease is the appearance of patches of weak plants in young seedlings of the crop. Colonized plants are stunted, produce few tillers and ripen prematurely. The heads of the plant contain sterile, empty spikelets that are bleached earning the name “whiteheads” for this characteristic symptom. The root system of colonized plants is much reduced, blackened, and brittle. A plant can be removed from the soil easily at this stage (Nilsson, 1969). Moisture content and temperature in the soil can be critical factors for disease development. If soil moisture is limited early in the development of the crop, symptoms may be limited to blacken roots. If soil moisture is high throughout the growing season, blackening of the crown and culm base will be obvious. Optimum temperature for development of take-all is between 10-20°C. During a cool and wet growing season, the lower leaf sheath surrounding the culm

base may be speckled by dark, erumpant perithecia. Diseased culms are weakened and may fall non-directionally (Wiese, 1987).

G. graminis is dispersed in infested soil and plant debris. Both hyphae and ascospores serve as inoculum, although ascospores have been considered less important epidemiologically. Ascospores are disseminated in splashing raindrops as well as by wind. Germination of ascospores may play a role by establishing the pathogen through the proximal parts of the roots through root hairs (Weste, 1972). However, three major sources of inoculum are from mycelia in host residues, grassy weeds, and wild plants (Hornby, 1981). Residue from diseased crops has been found the best source of inoculum of *G. graminis* var. *tritici* because the fungus survives as a saprophyte on those residues in soil. Wild plant and grassy weeds serve as the good sources of inoculum and resevoirs in the absence of susceptible crops. Moreover, Ggt spreads in soil better in the presence of living crested wheatgrass (*Agropyron* cristatum (L.) Gaertn.) than in the absence (Hornby, 1981). Most plant-to-plant spread of the pathogen occurs via runner hyphae, brown, thick-walled macrohyphae, advancing through soil and across root bridges (Wiese, 1987). Roots in contact with hyphae are colonized superficially and penetration occurs into the epidermal cells and cortex of the roots via pegs beneath hyphopodia emerging from hyaline hyphae or microhyphae (infection hyphae). Formation of lignitubers, a thickening of the tertiary layer of the cell wall impregnated with lignin or ligneous materials was observed as part of the host response to invading hyphae (Skou, 1981; Weste, 1972). The lignitubers are conical sheaths formed rapidly upon cell wall penetration. Once the microhyphae have penetrated the apices of the lignitubers, plasmolysis of the cells followed by cell collapse occurs very rapidly. At this point, the amount of mycelium in the tissues increases and the cortex is packed with inter- and intra-cellular hyphae. The phloem is destroyed faster than the xylem. However, root death results from occlusion of the xylem vessels that restricts the flow of nutrients and water (Clarkson et al., 1975; Skou, 1981).

1.3 *Gaeumannomyces graminis* (Sacc.) Arx & Olivier, CAUSAL AGENT OF TAKE-ALL.

Gaeumannomyces graminis (Sacc.) Arx & Olivier, a soilborne filamentous fungi belonging to the family *Diaporthaceae* of the *Ascomycetes* (Domsch and Gams, 1972). It was first isolated in Australia in 1962 from stolons of kikuyu grass (*Pennisetum clandestinum* Hochst. ex Chiov.). The order Diaporthales is characterized by "ostiolate perithecia, often with a long neck lined with periphyses, containing clavate, cylindrical to fusiform unitunicate asci with a well-developed refringent apical ring. In several genera, asci become free in the perithecial cavity towards maturity. Asci and ascospores often ooze from the ostiole although, in some species, forcible ejection of ascospores may occur" (Walker, 1972; Walker, 1981). General characteristics of *G. graminis* include brown septate runner hyphae, 4-7 μm wide. Hyphopodia vary from simple, non-lobed to lobed. Perithecia are formed in leaf sheaths and their sizes range from 200-500 x 150-400 μm. They are roughly globose, broadly oval and sometimes flattened. Asci are unitunicate with a distinct apical ring containing eight ascospores, which are faintly yellowish. Length of ascospores varies from 60-130 μm. *G. graminis* grown on potato-dextrose agar gives rise to white mycelia which becomes black in old cultures due to production of melanin-like pigments (Sivasithamparam and Parker, 1981). A characteristic of hyphal growth is the curling back of hyphae on the edge of the colony (Walker, 1972; Wiese, 1987).

Based on morphological characteristics and pathogenicity on host plants, three varieties of *G. graminis* have been recognized. *G. graminis* var. *tritici* Walker (Ggt) is considered the major causative agent of take-all of wheat (*Triticum aestivum* L.), rye (*Secale cereale* L.), and barley (*Hordeum valgare* L.) and is the most important pathogen of this disease. *G. graminis* var. *avenae* (E.M. Turner) Dennis (Gga) attacks oats (*Avena sativa* L.) and causes take-all patch of turf grasses. The variety *graminis* (Ggg) is pathogenic on bermudagrass (*Cyanodon* spp.) and causes crown sheath of rice (*Oryza sativa* L.) but is non-pathogenic on wheat (Henson et al., 1993; Tullis and Adair, 1947). These varieties of *G. graminis* have *Phialophora*-like anamorphs as do other non-pathogenic

Gaeumannomyces and *Phialophora* species found on cereal roots that constitute the *Gaeumannomyces-Phialophora* complex (Deacon, 1973; Deacon, 1974). *Phialophora radicicola* var. *radicicola,* and *P. graminicola* (Deacon) which have been considered as anamorphs of *G. graminis* var. *graminis,* are also non-pathogenic on wheat (Deacon, 1974).

1.3.1 *G. gramins* var. *tritici* Walker (Ggt)

G. graminis var. *tritici* Walker (Ggt), the most important pathogen of the group, produces brown, septate runner hyphae, 4-7 μm wide, which are branched to form hyphopodia-bearing hyphae. Hyphopodia are simple and unlobed, either terminal or intercalary with a minute pore where host penetration occurs (CMI # 383, 1973; Walker, 1972; Walker, 1981). In addition, hyphopodia are often clustered, forming sclerotial patches on culms and leaf sheaths. Black perithecia are 200-400 μm in diameter with a neck 100-400 x 70-100 μm. Ggt has unitunicate and elongated club-shaped asci (80-130 x 10-15 μm). Ascospores are light yellow and slightly curved. Phailides and phialospores are similar to those of Ggg and often formed in culture. Its host range is mainly cereals, particularly wheat. One major characteristic of Ggt is its susceptibility to the antifungal saponin, avenacin, presents in oat roots. Ggt does not produce enzymes that can detoxify this compound, therefore, the fungus is unable to cause disease on oats (Crombie, et al., 1986b; Osbourn et al., 1991).

1.3.2 *G. graminis* (E.M. Turner) Dennis var. *avenae* (Gga)

G. graminis var. *avenae* (Gga) was formerly named *Ophiobolus graminis* (Sacc.) Sacc. var. *avenae* E.M. Turner. The fungus has similar perithecia, asci, and ascospores to those of Ggt but its perithecia are sometimes slightly larger (300-500 x 250-400 μm) than for the other taxa and its asci are slightly longer and wider than those of Ggt. Runner hyphae, hyphopodia, and phialospores are as in Ggt (CMI #382, 1973, Walker, 1981). Variations in ascospore length among different isolates are common. Microhyphae originate from simple hyphopodia or mycelial mats. Dark brown or black perithecia with

curved necks are formed under and protrude through the lower leaf sheath (Smiley et al., 1992). Unlike Ggt, Gga is an oat-attacking species and has the ability to produce a saponin detoxifying enzyme, avenacinase, one of the two major mechanisms of resistance of fungi to saponin (Crombie et al., 1986; Osbourn et al., 1991). This enzyme allows penetration of Gga into host tissues by detoxifying the triterpenoid oat root saponin, avenacin, which is essential for Gga to be pathogenic on oats.

1.3.3 *G. graminis* (Sacc.) Arx & Olivier var. *graminis* (Ggg)

G. graminis var. *graminis* (Ggg) produces black perithecia in lower leaf sheaths (200-400 μm in diameter and 200-400 long x 150-300 μm wide) with a neck very variable in length, usually 100-400 μm long and 70-100 μm wide. Asci and ascospores are similar to those of Ggt and Gga. Ascospores are faint yellow, usually slightly curved and tapering towards the base. A network of hyphae is formed on the surface of rhizomes, leaf sheath and culms. Runner hyphae are brown and septate (4-7μm wide) and hyphopodia are formed from hyaline branching hyphae. Two types of hyphopodia are developed which are brown, lobed and simple, non-lobed terminal, or intercalary with a small clear spot representing the point for host penetration (CMI # 381, 1973; Epstein et al., 1994; Nilsson, 1972). Characteristic hyphopodia readily develop on coleoptiles of cereal seedlings grown on culture plates (Walker, 1972). Phialides are aerial or submerged, usually slightly curved and hyaline and clustered on sides of hyphae or terminal (9-18 x 2-3 μm). Phialospores, often formed in culture, are hyaline, unicellular, and slightly to strongly curved (4-7 x 1-1.5 μm).

1.4 OTHER CLOSELY RELATED FUNGI

1.4.1 *G. cylindrosporus* sp.nov.

G. cylindrosporus sp. nov. was isolated from colonized roots of wheat and barley. Its perithecia are superficial, globose, black, mostly single with a cylindrical neck up to 242 μm long, 48-116 μm wide at the base (Hornby et al., 1977). Asci are clavate, straight or

curved (24 μm long, 65-135 x 9-16 μm) with a tapering but round or slightly truncate apex with a non-amyloid apical ring staining with Congo red. Ascospores are hyaline and cylindrical with septates (37-69 x 3.2- 5.6 μm). Slightly lobed or angular hyphopodia are developed from hyaline hyphae. This fungus can be differentiated from *G. graminis* by small size of ascospores and type of perithecia (Hornby, 1977). In addition, *G. cylindrosporus* grows much slower in culture than *G. graminis*.

1.4.2 *Phialophora radicicola* Cain var. *radicicola and* var. *graminicola*

These fungi produce perithecia, asci, and ascospores, which are structurally similar to those of Ggg. *P. radicicola* var. *radicicola* produces lobed hyphopodia superficially on roots and stems of the host and does not penetrate into the host cells. It grows well on cereal roots but causes disease only on ryegrass (*Lolium* spp.) and has been considered an imperfect stage of Ggg (Deacon, 1974; Walker, 1981). On the other hand, *P. radicicola* var. *graminicola* produces simple and non-lobed hyphopodia and is non-pathogenic on wheat (Deacon, 1973).

1.5 CONTROL OF TAKE-ALL

Reduction of take-all in wheat usually relies on crop rotation to reduce inoculum levels. However, the pathogen survives in soybean double cropping system on the roots and debris of previously colonized crops which becomes the main source of inoculum. Soil infestation levels build up rapidly and resulting in more severe take-all in the next wheat crop. Winter wheat crops are more susceptible and more frequently damaged than spring wheat crops (Huber and McCay-Buis, 1993). The lack of resistant wheat cultivars also restricts the control of take-all. However, there is a potential of generating a genetic resistant wheat via selection of somaclonal variants regenerated from callus culture (Eastwood et al., 1994). Uses of fungicides and microbial suppression also help reduce the severity of take-all (Capper and Campbell, 1986; Maplestone and Campbell, 1989; Wong and Baker, 1984). Use of antagonistic microorganisms isolated from take-all suppressive soils is another way to decrease the severity of take-all. Most of the microorganisms tested have been fluorescent pseudomonads such as *Pseudomonas*

fluorescens (Trevisan) Migula and *P. putida* (Trevisan) Migula (Weller et al., 1988) and bacilli such as *Bacillus pumilus* Meyer and Gottheil (Capper and Campbell, 1986; Wong and Baker, 1984). The mechanisms of suppression by *Pseudomonas* sp. are believed to be the production of antibiotics with broad spectrum against bacteria of fungi or the excretion of siderophores. Siderophores can chelate the limited supply of ferric iron in the rhizosphere, thus, limiting its availability to the pathogen and consequently suppressing its growth (Weller, 1988). However, efficiency of disease suppression by siderophores is greater in neutral or alkaline soils than in acid soils (Ownley et al., 1992). On the other hand, the mechanism of suppression by *Bacillus* sp. is the competitive colonization on the roots. (Maplestone and Campbell, 1989). The phenomenon "take-all decline" has been found in wheat fields after periods of severe damage to wheat crops, as a result of bacterial antagonistic to the take-all pathogens (Wiese, 1987). Take-all decline probably results from the build up of populations of one or more organisms suppressive to the take-all pathogen. In addition, several fungi such as *Phialophora* spp. have demonstrated the potential to be biocontrol agents of take-all (Mathre et al., 1999).

Availability of manganese in soils may play an important role in suppression of take-all. Reduction of disease severity can be achieved by increasing Mn^{2+} supply to plants in situations of Mn^{2+} deficiency. Application of manganese as a seed treatment reduced severity of take-all and increased yields (Huber and McCay-Buis, 1993). The ability of Ggt to oxidize manganese has been studied and correlated with virulence of the fungus (Huber and McCay-Buis, 1993; Pedler et al., 1996). Ggt oxidizes Mn^{2+} to Mn^{3+}/Mn^{4+} making it unavailable in the wheat rhizosphere. Rengel (1997) demonstrated that a decrease in capacity to oxidize manganese of Ggt resulted in a decrease in virulence of the fungus which, in turn, enhanced take-all decline in experimental field plots. In contrast, decreased levels of Mn^{2+} in soils in Eastern Virginia had little or no effect on decreasing take-all severity in the field and greenhouse experiments (Crozier and Stromberg, personal communications). However, whether this mechanism may solely play a significant role in take-all decline is not known. One possibility is that availability of Mn^{2+}, an important enzyme co-factor in the shikimic pathway which is important in

the production of several pathogen response chemicals, may affect disease resistance mechanism in plants resulting in take-all decline (Huber and McCay-Buis, 1993).

1.6 DIAGNOSIS AND IDENTIFICATION OF TAKE-ALL PATHOGENS.

Conventional diagnosis of take-all in wheat is laborious, time-consuming, and inconclusive (Clarkson and Polley, 1981; Mathre, 1992). Diagnosis is usually based on characteristic field symptoms on host plants including patches of weakened young plants, stunting, reduction in tiller numbers, premature ripening, whiteheads, reduced, blackened, and brittle roots. Although these symptoms are the first obvious indications, they are the terminal stages of the disease (Jones and Clifford, 1978; Wiese, 1987). Diagnosis depends on the presence of internal and superficial dark mycelium and runner hyphae on the basal stem when blackened stems are not observed. However, these symptoms are also characteristic of other small grain diseases such as spring dead spot and crown sheath of rice caused by Ggg and *Ophiobolus oryzinus* Sacc., respectively (Juhnke, 1984; Henson et al., 1993; Mathre, 1992). Additionally, other pathogens, insect infestations, or environmental conditions such as nutrient deficiency can also generate similar symptoms (Clarkson and Polley, 1981). Moreover, complexity of the soil microorganisms enhances difficulty in diagnosis of take-all of wheat. For example, the *Gaeumannomyces-Phialophora* complex consists of three varieties of *G. graminis*, its *Phialophora*-like anamorphs and other non-pathogenic *Gaeumannomyces* and *Phialophora* species found on cereal roots. Some isolates of *Phialophora radicicola* are weakly or non-pathogenic on cereals but are pathogenic on grasses (Deacon, 1974; Mathre, 1992). Members of this complex all produce dark ectotropic hyphae on roots. Other fungi such as *Pythium* spp., *Fusarium* spp., *Cochliobolus sativus* (Ito et Kuribayashi) Drechsler ex Dastur, or mycorrhizae also cause root discoloration or blackening of wheat roots (Clarkson and Polley, 1981; Jones and Clifford, 1978).

Traditionally, conclusive diagnosis is achieved by culturing and identifying the fungus from diseased tissues of the host plant, microscopic examination, and pathogenicity assay (Clarkson and Polley, 1981, Mathre, 1992). Isolates are usually obtained from surface-

sterilized tissues plated on potato dextrose agar (PDA) with or without antibiotics. Because Ggt is a slow growing fungus, it is easily overgrown by many opportunistic soil and plant tissue-inhabiting organisms (Asher 1978; Huber, 1981). Positive identification was based on cultural characteristics such as pigmentation, thick runner hyphae and hyphal curling at the colony margins (Hornby, 1969, Nilsson, 1972), type of hyphopodia and ascospore size (Walker, 1972). However, some overlap of these characteristics makes specific identification difficult and inconclusive (Asher, 1980). Additionally, identification based on pathogenicity is sometimes non-specific and time-consuming. For example, some Ggt isolates found in Australia can also attack oats (Bryan et al., 1995; Yeates et al., 1986) and Gga can be pathogenic on wheat as well (Walker, 1972).

Juhnke et al. (1984) developed a selective medium (SM-GGT3) for isolation of Ggt to confirm field diagnosis of take-all. Differentiation of Ggt from other soil fungi was based on formation of melanin pigment in the presence of L-β-3,4-dihydroxyphenylalanine (L-DOPA). It has some advantages over the conventional isolation media in that it allowed Ggt to grow without competing with other fungi and bacteria and was easy to differentiate by pigmentation of the fungus. It was easy and inexpensive to make and had a long shelf life. However, its obvious disadvantages were its inability to inhibit *Rhizopus* and that it could not differentiate Ggt from other varieties *of G. graminis* and *Phialophora* spp. (Juhnke, et al., 1984). In addition, it took weeks to obtain results. In 1994, another semiselective medium for identification of Ggt, R-PDA (rifampicin-PDA) was developed by Duffy and Weller (1994) based on the ability of Ggt to alter the color of rifampicin from orange to purple due to degradation of rifampicin. This reaction can be obtained within 24 hours after culturing procedure and is more effective than SM-GGT3. Although this medium can differentiate Ggt from other soil fungi, it was not useful for differentiation of Ggt from Gga and Ggg.

It is obvious that conventional identification of *G. graminis* is time-consuming and unreliable. Variation in cultural characteristics and pathogenicity of these fungi can lead to inconclusive identification (Asher, 1980). Since take-all of wheat is devastating and difficult to control, there is a need for an accurate and rapid identification for the

pathogen. In the past few years, attention has been paid to development of a rapid and specific identification method for *G. graminis*, especially Ggt, to replace the laborious conventional methods. New DNA technologies such as polymerase chain reaction (PCR) provides a great possibility to accomplish this goal. Recently, several identification methods based on DNA hybridization and PCR technologies have been developed. Reviews of the published work on identification of the varieties of *G. graminis* using DNA-based technology are presented in the following sections.

1.7 MOLECULAR MARKERS FOR IDENTIFICATION OF *G. graminis*

1.7.1 Protein-based markers

Protein markers have been frequently used for identification of plant pathogenic fungi prior to the exploitation of DNA-based techniques. The technique is based on the electrophoretic separation of proteins followed by specific staining of a distinct protein subclass. Allozyme electrophoresis has been successfully applied to many organisms for various purposes such as systematics, population genetics, *etc*. Roy et al. (1982) reported use of aminopeptidase profiles in identification and characterization of Ggg isolated from soybean pod from Ggt and Gga. This assay is completed within hours but it requires a number of substrates to conduct the assay. It may be useful when used in combination with morphological examination and pathogenicity assays. A similar technique has been used to differentiate between morphologically similar fungi and to determine the relationship between Ggt and related taxa such as *Phialophora graminicola* (Deacon) Walker, *P. zeicola* Deacon & Scott, and *Magnaporthe rhizophila* Scott & Deacon (Maas et al., 1990). Analysis is based on comparison of soluble proteins patterns on polyacrylamide gel electrophoresis and spectrophotometric tracings of reference proteins (lysozyme and thyroglobulin) from protein extracts of each fungus.

1.7.2 Restriction fragment length polymorphisms (RFLPs) analyses

DNA probe hybridization and analyses of RFLPs have played a major role in development of modern identification assays for plant pathogenic fungi. Specific probes used for this purpose can be generated from mitochondrial DNA (mtDNA) which has been used extensively with ascomycetes (Grossman and Hudspeth, 1985) or other complementary DNA sequences specific to a particular fungal species. However, RFLP analysis has recently become popular for use in identification and taxonomic studies of plant pathogenic fungi due to its several advantages over a classical DNA probe hybridization (Manicom et al., 1990). For example, RFLP probes are usually locus-specific resulting in a codominant marker behavior, which is easy to screen and can discriminate homo-and hetero-zygotic states in diploid organisms. In addition, RFLP probes usually are species-specific (Weising et al., 1995). In RFLP analyses, genomic DNA is extracted, digested with restriction enzymes, and the resulting fragments are separated by electrophoresis on agarose gels. For observation of smaller fragments, restriction enzymes recognizing a four-base pair sequence and polyacrylamide gel electrophoresis can be used. DNA is transferred from the gel to a membrane and hybridized with a specific labeled probe. DNA fragments which hybridize to the labeled probe are visualized by color development or using X-ray film for either radioactive or chemilluminescent probe DNA (Ward and Gray, 1992).

In fungi, two types of RFLPs have been recognized: RFLP in nuclear DNA and in cytoplasmic DNA, such as mtDNA. RFLP analysis of nuclear DNA typically uses species-specific probes obtained from a cDNA or genomic DNA library of the species of interest or a closely related species (Weising et al., 1995). Ribosomal DNA (rDNA) is also a good source for RFLPs and is easy to detect due to high copy numbers of the DNA sequences. The coding regions (17S, 5.8S and 26S of rRNA) are generally conserved while variability is confined to the noncoding rDNA regions of the intergenic spacer, where polymorphisms in the number of short tandem repeats occur. RFLP analysis of mtDNA in fungi is based on conservation of its gene order due to small genome size in comparison to plant mtDNA (Grossman and Hudspeth, 1985). In addition, its high sequence variability, and high copy numbers make mtDNA a good source for RFLPs in

population studies, especially for the analysis of maternal lineages and population history. A number of diagnostic tests have been accomplished using this technique. For example, differentiation between *Fusarium culmorum* (W.G. Smith) and *F. graminearum* Schwabe, cereal pathogens, using a species-specific probe provides a simple and reliable differentiation method for the species since identification based on morphological traits were highly variable (Koopmann et al., 1994). Differentiation of closely related *Pyrenophora graminea* Ito and Kurib. and *P. teres* Drechs., major seed-borne pathogens of barley, causing different diseases, was also accomplished based on unique RFLP patterns (Husted, 1994).

1.7.3 Polymerase chain reaction (PCR)-based techniques

PCR is a method for *in vitro* amplification of nucleic acid by repeated cycles of high temperature denaturation of double stranded DNA, annealing of oligonucleotide primers to the single stranded template, and DNA polymerase-mediated synthesis (Mullis and Faloona, 1987; Saiki et al., 1988). The specificity of the method is derived from the design of the synthetic oligonucleotide primers. It is so sensitive that a single DNA molecule has been amplified from a complex mixture without using radioactive probe detection. It is also rapid and versatile. A wide range of plant pathogens from various hosts or environmental samples have been detected using PCR (Henson and French, 1993). Applications of PCR to disease diagnosis include nested PCR, multiplex PCR, random amplification of polymorphic DNA (RAPD), and amplified fragment length polymorphisms (AFLP).

Diagnostic PCR is usually based on direct amplification of any DNA or RNA sequence that is specific for a particular organism. For example, mtDNA fragment specific to *G. graminis* facilitated primer design for amplification of the fungal DNA from colonized wheat or bermudagrass (Henson, 1992; Henson et al., 1993). Amplification of the internal transcribed spacer (ITS) regions of ribosomal DNA sequences provides a specific target for molecular detection and phylogenetic studies because ribosomal DNA repeat units contain highly conserved DNA sequences as well as more variable DNA regions.

As a consequence, amplification and sequencing of these fragments can be accomplished simply by using universal primers for fungal nuclear and mitochondrial ribosomal RNA genes. Internal transcribed spacer (ITS) comprises the transcribed region flanking the 5.8S rDNA and has been used to detect variations among related fungal species (White et al., 1990). A number of direct detections of plant pathogens from colonized samples have been achieved via this technique (Foster et al., 1993; Moukhamedov et al., 1994).

Random amplified polymorphic DNA (RAPD) analysis is one of the important applications of PCR because it does not require genome sequence information or radioactive probes. RAPD uses PCR and a set of short, random-sequence oligonucleotide primers (8-10 bases) that produce characteristic profiles of amplified products for each organism (Williams et al., 1990). However, RAPD is not useful for direct detection of plant pathogens in complex environmental samples or organisms that are difficult to culture.

1.8 DEVELOPMENT OF DNA-BASED IDENTIFICATION/DIFFERENTIATION TESTS FOR *G. graminis* VARIETIES.

In *G. graminis*, the first DNA probe for identification purposes was developed by cloning a large fragment of mtDNA (4.3 kb) of Ggt into a multicopy plasmid, pUC 18, generating plasmid pMSU315 (Henson, 1989). This probe showed strong homology to all three varieties of *G. graminis* as well as to other closely related fungi such as *G. cylindrosporus*, *G. incrustans* Landschoot & Jackson, *Phialophora* spp., and *Magnaporthe* spp. Unfortunately, patterns of hybridization for some of these fungi were identical to that of Ggt which makes specific identification difficult (Henson, 1992). However, this probe showed no homology to genomic DNA of plant pathogenic fungi from other genera such as *Rhizoctonia* spp., *Sclerotinia sclerotiorum* (Libert) Korf et Dumont, *Fusarium culmorum*, *etc*. (Henson, 1989; Henson, 1992). This method did not discriminate among the three varieties of *G. graminis*. Nested-PCR has been applied for the detection of Ggt in diseased wheat plants by Schesser et al. (1991). For the first amplification round, two sets of primers were designed from plasmid derived from

pMSU315, which flanked 407 bp and 138 bp fragments. In a second round of amplification, a second set of primers was used to amplify fragments of 287 and 188 bp in size as diagnostic fragments. This procedure was used successfully to detect *G. graminis* from colonized wheat seedlings and was specific to *Gaeumannomyces* spp. Although it does not differentiate among varieties, the procedure may be directly used to detect Ggt, Gga, and Ggg from diseased plants grown in artificially and naturally infested soil (Elliott et al., 1993; Henson et al., 1993). Improvement in specificity has been made in analyses of RFLPs of *G. graminis* and *Phialophora* spp. using pMSU315 probe in combination with PCR (Bateman et al., 1992; Ward and Gray, 1992). They demonstrated that Ggt and Gga generated unique RFLP patterns and were easily distinguished from those of *Philophora* spp. As mentioned previously, polymorphisms in the mtDNA within species is useful for population studies of Ggt from different locations. Different RFLP patterns were observed among isolates of Ggt originating from different host crops as well as between locations (Bateman et al., 1997).

Others have shown that RFLP analysis of rDNA provided a better means for differentiation of *G. graminis* varieties (O'Dell et al., 1992; Tan et al. 1994). They demonstrated that by using universal probes derived from rRNA genes of *Neurospora crassa* Shear et Dodge, they could identify Ggt, Gga, and Ggg specifically based on different sizes of restriction fragments detected. In the method of Tan et al. (1994), isolates of Ggt were divided into three subgroups based on variations in the size of fragments resulting from variations in the rDNA repeats among isolates. Cereal host preference of *G. graminis* isolates can also be predicted from unique RFLPs from the repeated sequences of the coding regions of rRNA (O'Dell et al., 1992). Exploitation of PCR amplification of rDNAs alone or in combination with RFLP analysis of these genes enhanced specificity and sensitivity for differentiation procedures (Bryan et al., 1995; Bryan et al., 1999; Fouly et al., 1997; Goodwin et al, 1995; Ward and Akrofi, 1994). The best target for specific amplification of rDNA are the regions within the internal transcribed spacer (ITS) regions of the non-coding regions of rRNA. Ward and Akrofi (1994) and Fouly et al. (1997) amplified these regions with oligonucleotide primers that are universal for any fungi and generated RFLP patterns of those PCR fragments. They

showed that these patterns were useful in discriminating among the varieties of *G. graminis* as well as identification of fungi in the *Gaeumannomyces-Phialophora* complex. However, some atypical isolates gave intermediate RFLP patterns which made absolute identification difficult to conclude. In 1995, Goodwin et al. cloned and sequenced rRNA genes of Gga and used this information to design a differentiation procedure for turf grass pathogens. A PCR fragment specific to Gga (373 bp) was not present in samples containing Ggt or other turf grass root pathogens. This procedure has proven to be the most simple and specific PCR-based identification test for Gga in the literature. In contrast, Bryan et al. (1995) published DNA sequences for the ITS and 5.8S rDNA of Gga and Ggt and developed a PCR-based identification techniques for these two fungi. Oligonucleotide primers specific to Ggt and Gga were designed based on single nucleotide difference at the 3'-end of each primer and nested PCR was used to produce specific PCR fragments. This technique established several advantages over the others since identification is based on the presence or absence of a single DNA band. Direct detection of the fungus from diseased plants was straightforward and conclusive. This technique is considered one of the best identification methods available to date. However, both of the last two methods still lack an internal control for their PCR reactions to ensure that all negative results obtained are not due to reaction failures (Ward, 1995). Addition of internal control template is very important for a diagnostic test of the pathogens because false negative results can generate serious damage if the test is to be used for field applications.

A few research groups have included RAPD as an identification means for take-all pathogens (Bryan et al., 1999; Fouly et al., 1996; Wetzel III et al., 1996), although results obtained may not be as conclusive as the aforementioned techniques. Since oligonucleotide primers used in this type of PCR are non-specific, more than one PCR profiles are always produced, thus, these procedures are inconclusive for identification purposes. It is agreed that RAPD markers should be applied in conjunction with other identification methods such as pathogenicity assays or morphological examination to produce a reliable assay (Bryan et al., 1999; Fouly et al., 1996; Wetzel III et al., 1996).

Since several groups have established their own methods for identification and differentiation of the varieties of *G. graminis*, there is no universal method available for routine diagnosis of these fungi. However, all of them have both advantages and disadvantages. Comparison of these methods is summarized in Table 1.

Table 1.1. Comparison of current molecular-based methods for identification and differentiation of *G. graminis* varieties.

RFLP-based technique[1]	PCR-based with mtDNA as a template[2]	PCR-based with rDNA as a template[3]
1. Time-consuming, takes about a week to get results	1. Simple and quick. Results can be obtained in a day	1. Simple and quick. Results can be obtained in a day
2. Costly due to restriction enzymes, film, in some cases, radioisotopes used, etc.	2. Less cost. No need for restriction enzymes	2. Less cost. No need for restriction enzymes
3. No definitive RFLP patterns, depending on enzymes used. Results can be varied.	3. If primers are specific, only a single band is produced.	3. If primers are specific, only a single band is produced.
4. Interpretation of results is complicated.	4. Results based on the presence/absence of a single band.	4. Results based on the presence/absence of a single band.
5. Can differentiate *G. graminis* from other closely related fungi but not among the varieties of *G. graminis*	5. Can differentiate *G. graminis* from other closely related fungi but differentiation among varieties of *G. graminis* is non-specific	5. Use of ITS region as a target sequence, provides specific identification among varieties of *G. graminis*
	6. Nested PCR required	6. Nested PCR required
	7. No specific primer for all three varieties available	7. Primers available for Gga and Ggt
6. Direct detection of the fungus from field samples is not possible.	8. Direct detection of the fungus is possible. No need for DNA isolation.	8. Direct detection of the fungus is possible. No need for DNA isolation.
7. Not suitable for routine test	9. Suitable for routine test	9. Suitable for routine test
8. Well-trained personnel required	10. Easy to perform	10. Easy to perform

[1]Ward and Akrofi (1994). [2]Schesser, et al., (1991). [3]Bryan et al. (1995).

1.9 USE OF SPECIFIC MARKERS ASSOCIATED WITH PATHOGENICITY OF *G. graminis* FOR DEVELOPMENT OF AN IDENTIFICATION TEST FOR *G. graminis*.

All of the aforementioned DNA-based identification methods for varieties of *G. graminis* were based on PCR amplification of target regions with high redundancy of the families of DNA in the genome in order to obtain high sensitivity of detection. However, these methods do not provide any link to behavior, pathogenicity, or host range of the take-all pathogens. In addition, variation in the genome of these fungi can affect definitive interpretation of those techniques. To date, little attention has been paid to physiological, biochemical and molecular biological aspects of pathogenesis. Only two metabolic activities, namely, the ability of Ggt to oxidize manganese and the ability of Gga to detoxify a saponin present in oat roots, have been studied as they may be important to and correlate with pathogenicity of *G. graminis*. Based on differential expression of these metabolic activities among the varieties of *G. graminis*, these characteristics may be good candidates as specific markers for development of a specific and informative identification of the take-all pathogens.

1.9.1 Avenacin/avenacinase system

Avenacins, belonging to the saponin family, are antimicrobial substances found in oat roots (Turner, 1953; Turner, 1956). Saponins are glycosylated compounds found in over 100 plant species and can be divided into three major groups, depending on the structure of the aglycone: triterpenoids, steroids, and steroidal glycoalkaloids (Osbourn, 1996). Triterpenoid saponins are found primarily in dicotyledonous plants while steroid saponins occur mainly in monocots. Steroidal glycoalkaloids are found mainly in the members of the family *Solanaceae* such as potato (*Solanum tuberosum* L.) and tomato (*Lycopersicon esculentum* Mill. L.). Saponins exhibit potent antifungal activity and often are found in relatively high amounts in healthy plants, thus, these compounds may play a role in resistance of plants to fungal pathogens (Osbourn, 1996). The major mechanism of toxicity of saponins to fungi is believed to be due to their membraneolytic action.

Saponins can form complexes with the 3β-OH group of plasma membrane sterols leading to membrane disruption and electrolyte leakage and, thus, are potentially toxic to all organisms with these membrane constituents (Weltring, et al., 1997; Wubben et al., 1996).

There are two different families of saponins present in oats, the triterpenoid avenacins and the steroidal avenacosides. The first group contains the mixture of four major compounds namely avenacins A-1, A-2, B-1, and B-2 that are all glycoconjugates containing β-linked glucosyl residues. Avenacin A-1, restricted to the root epidermal cells, is the most abundant and the most fungitoxic among the four substances in oats (Crombie et al, 1986a; Maizel et al., 1963, Osbourn, 1994). The second group consists of avenacosides A and B and are also present at higher concentration in the root epidermis than in the leaves. It has been found that Gga detoxifies avenacin A-1 to a less toxic compound, thus, rendering oats susceptible to disease. On the other hand, avenacin A-1 drastically inhibits growth of Ggt. This results in the mechanism by which resistance of oats to invasion of Ggt is maintained (Crombie, et al., 1986a & b; Osbourn et al., 1991. Turner, 1955). However, not all oat species produce avenacin. For instance, *Avena longiglumis* lacks detectable levels of avenacin and is susceptible to colonization of Ggt (Osbourn et al., 1994b). This finding supports a role of avenacin as a determinant of resistance to fungal pathogen.

Avenacinase, an avenacin detoxifying enzyme, produced by Gga, is a β-glucosyl hydrolase (EC 3.2.1.21) with a single polypeptide chain of 110 kD, pI 4.6, and is highly active towards avenacin. Mode of action of the enzyme is removal of β,1-2-and β,1-4-linked terminal D-glucose molecules from avenacin A-1 to generate both the *mono*- and *bis*-deglucosylated forms of avenacin which are less fungitoxic (Turner, 1961; Crombie et al., 1986a; Osbourn et al., 1991). Similar enzyme action is found in other fungal pathogens that have the ability to detoxify saponins. Saponin detoxifying enzymes were purified from *Septoria lycopersici* Spegazzini, foliar pathogen of tomato (Sandrock, et al., 1995); *Septoria avenae* A.B. Frank, causal agent of speckled blotch of oat (Wubben et al., 1996); *Botryris cinerea* Pers.: Fr., a causal agent of grey mold of tomato (Quidde,

et al., 1998); and *Gibberella pulicaris* (Fries) Saccardo, causal agent of potato dry rot (Weltring et al., 1997). All of these enzymes exhibit similar modes of action to that of avenacinase but their substrate specificity are for specific saponins although their physiochemical properties are very similar (Osbourn et al., 1995; Quidde, et al., 1998; Wubben et al., 1996). For example, avenacosidase purified from *S. avenae* has the same molecular mass as that of avenacinase but it is specific to steroidal saponins but not to triterpenoid saponins in oats (Wubben et al., 1996)

The gene encoding avenacinase (AVN1) from Gga has been isolated and characterized by a reverse genetic approach (Bowyer et al., 1995; Osbourn et al., 1995). Avenacinase produced from Gga was used to raise polyclonal antibody production in rats (*Rattus* sp. L.) which was subsequently used to select positive clones from an expression library of Gga. Northern blot analyses of RNAs from a number of Gga isolates confirmed the consistency in the size (110kD) of the avencinase proteins. Expression of avenacinase was tested in *Neurospora crassa,* which was avenacin sensitive and showed no detectable avenacinase activity. Transformants of *N. crassa* expressing the transformed gene showed increased resistance to avenacin. A single copy of the avenacinase gene was detected from both Southern and northern blot analyses. The full-length gene is about 3 kb and shows some similarity with β-D-glucosidases belonging to the family 3 group of glycosyl hydrolases defined by Henrissat (Henrissat, 1991; Henrissat and Bairoch, 1993; Osbourn, et. al., 1995). The most closely related genes are β_2-tomatinase of *S. lycopersici* (68% similarity and 53% identical amino acids) and the β-glucosidases (BGLs) gene families from *Trichoderma reesei* Simmons (60% similarity and 45% identity) and from *Candida pelliculosa* Redaelli (60% similarity and 38% identity). The BGLs genes play an important role in cellobiose degradation. However, their effects on saponins have not been reported (Osbourn et. al., 1994a). Generation of mutants of Gga via targeted disruption of the avenacinase gene confirmed that the gene plays a role in determining pathogenicity of this fungus since avenacinase-minus mutants were more sensitive to avenacin A-1 than the wild type and unable to cause disease on oats (Bowyer et al., 1995).

So far, little is known about the presence of avenacinase in Ggt and Ggg. Crombie et al. (1986b) and Osbourn et al. (1991) reported that Ggt has the ability to produce an enzyme with similar physicochemical properties as that of avenacinase and can be recognized by avenacinase antisera. However, they showed very weak avenacinase activity after prolonged incubation with the substrate. This results in sensitivity of Ggt to avenacin leading to the inability to cause disease in oats. Osbourn et al. (1994b) also reported the presence of enzyme with weak avenacinase activity in Ggg, *G. incrustans*, *G. cylindrosporus*, and *Phialophora* spp. and named these enzymes as avenacinase-like proteins (ALPs). It has been postulated that these proteins may simply be β-glucosidases required for fungal nutrition. However, they may be important for saponin detoxification during colonization of plants other than oats (Osbourn et al., 1994a). Further investigation by generation of specific mutants of Ggt and Ggg lacking these enzymes needs to be conducted to confirm and determine role(s) of the enzymes in these organisms.

Currently, only the avenacinase gene in Gga has been characterized and found to play an important role in pathogenicity of the fungus on oats (Bowyer et al., 1995). It is likely that this gene is the best candidate for identification purposes. Since Ggt, Gga, and Ggg have the ability to produce avenacinase or avenacinase-like enzymes, the genes encoding this enzyme in all three varieties are presumably closely related although the activity of the enzymes are quite different. Thus, there should be sufficient divergence at the gene level to alter enzyme activity. This could be used as a basis for development of a specific identification for the varieties of *G. graminis*.

1.9.2 Manganese/manganese peroxidase system

Biological oxidation or reduction in soil dynamically alters Mn^{2+} availability to plants. Biological oxidation of divalent manganese yields manganic oxide (Mn_2O_3) as the primary product, which subsequently undergoes spontaneously a dismutation to divalent Mn^{2+} and MnO_2 (Graham et al., 1988; Mann and Quastel, 1946). These kinetics can be used to determine the nature of the microorganisms and the organic matter present in the

soil. Isolation and identification of Mn^{2+} oxidizing microorganisms in soils may be accomplished by using selective media containing manganese sulfate ($MnSO_4$) (Bromfield and Skerman, 1949; Krumbein and Altmann, 1973). The presence of brown-black spot-like deposits of MnO_2 around the colony margin indicates an ability to oxidize Mn^{2+} (Bromfield and Skerman, 1949). In fungi, deposition of MnO_2 in culture medium occurs in the form of a sheath around and along the hyphae (*Cladosporium sp*. and *G. graminis*) and in small masses a short distance from the hyphae (*G. graminis*).

Availability of manganese (Mn^{2+}) in soil has an influence in disease susceptibility of wheat (Graham, 1988; Huber and McCay-Buis, 1993). Graham and Rovira (1984) proposed three mechanisms of Mn^{2+} involvement in disease susceptibility. Firstly, Mn^{2+} affects photosynthesis impacting the amount and composition of root exudates, which, in turn, may affect the rhizosphere microflora including pathogen antagonists. Secondly, Mn^{2+} plays an important role as a cofactor in the shikimic pathway associated with lignification and formation of toxic phenolic compounds, plant compounds which are involved in disease resistance (Brown et al., 1984; Huber and McCay-Buis, 1993). Thirdly, high levels of Mn^{2+} may inhibit some fungal exoenzymes such as pectin-methylesterase and may be toxic to Ggt (Graham et al., 1988; Huber and McCay-Buis, 1993). Marschner et al. (1991) showed that the concentration of Mn^{2+} in soil had a marked effect on fungal growth. High levels of Mn^{2+} strongly decreased growth of Ggt while low Mn^{2+} concentrations slightly increased fungal growth. Oxidation of Mn^{2+} has been associated with virulence where Mn^{2+} is oxidized in advance of the microhyphae (Huber and McCay-Buis, 1993; Pedler et al., 1996). Pathogenic strains of Ggt and Ggg are capable of catalyzing the oxidation of soluble Mn^{2+} to insoluble Mn^{4+} on the rhizoplane and in the soil surrounding the root (Schulze et al., 1995). Because Mn^{4+} is immobilized in phloem, Mn^{2+} supply vital to the plant is ineffective. This hypothesis was supported by direct measurement of Mn^{2+} oxidation states with X-ray absorption near edge structure (XANES) spectroscopy within plant roots. The measurement indicated the presence of Mn^{4+} in black roots colonized by Ggt (Schulze et al., 1995). It was also found that Ggt and Ggg isolates that failed to oxidize Mn^{2+} were non-pathogenic or only weakly virulent. Those that oxidized Mn^{2+} were highly virulent. Ggt and Ggg are Mn^{2+}

oxidizers while no studies have been reported for Gga. Ggt oxidizes Mn^{2+} at a distance from the mycelium and deposits manganese oxide (MnO_2) in cortical cells and root hairs of the plants as well as along the runner hyphae of the fungus (Huber and McCay-Buis, 1993). On the other hand, Mn^{2+} oxidation of Ggg occurs only around the lobed hyphopodia and pathogenesis-related structures. This difference between Ggt and Ggg in Mn^{2+} oxidation could be important in wheat pathogenesis. However, Mn^{2+} oxidation in *G. graminis* in soil can be inhibited by antagonistic microorganisms isolated from take-all suppressive soils such as *Pseudomonas fluorescens* and other Mn^{2+}-reducing bacteria (Marschner et al., 1991). The frequency of Mn^{2+}-reducing bacteria isolated from wheat rhizosphere was higher with Ggg than Ggt. In addition, a decrease in capacity to oxidize manganese of Ggt was correlated with a decrease in virulence on wheat (Rengel, 1997).

Biochemically, Mn^{2+} oxidation in fungi occurs by the catalytic activity of manganese peroxidases (MnP). This enzyme complex catalyzes the oxidation of organic substrates using Mn^{2+}/Mn^{3+} as a mediator (Glenn and Gold, 1985). The MnPs are catalytically distinct from other peroxidases in that Mn^{2+} serves as the reducing substrate resulting in its oxidation to Mn^{3+}. Mn^{3+}, in turn, diffuses away from the enzyme active site and thus serve as a redox mediator in the oxidation of a large number of phenolic substrates (Brown et al., 1990; Glenn and Gold, 1985; Pease et al., 1989). MnP has been widely studied in white rot basidiomycetes such as *Phanerochaete chrysosporium* Burdsall (Godfrey et al., 1990; Mayfield et al., 1994; Orth et al., 1994; Pease et al., 1989; Pribnow et al., 1990), *Phanerochaete sordida* (Karsten) Eriksson et Ryvarden (Pease and Tien, 1992) and *Trametus versicolor* (Linnaeus: Fries) Pilat (Johansson and Nyman, 1995; Jonsson et al., 1994). There are two families of extracellular peroxidases, lignin peroxidases and MnPs, which play a major role in fungal depolymerization of lignin. Lignin peroxidases are responsible only for oxidation of aromatic and nonphenolic substrates (Gold and Alic, 1993). Degradation of wheat straw by the white rot fungi demonstrated that the less lignified tissues were degraded first whereas the xylematic fibers underwent a delayed attack. Spherical black bodies resulting from potassium permanganate ($KMNO_4$) staining were observed in holes or fissures during cell wall degradation indicating the oxidation of Mn^{2+} by the fungi (Barrasa et al., 1995).

MnPs of *P. chrysosporium* and *T. versicolor* have been purified and characterized. MnP is an H_2O_2- and Mn (II)-dependent, heme-containing glycoprotein of 46,000 kDa molecular weight and occurs as a family of isozymes encoded by a small gene family (Godfrey et al, 1990; Pease et al., 1989; Pribnow et al., 1990). At least four different MnP isozymes were detected in the extracellular fluid of nitrogen-limited culture of *P. chrysosporium* (Orth et al., 1994). So far, cDNAs encoding two MnP isozymes of *P. chrysosporium* have been isolated (Pease et al., 1989; Pribnow et al., 1990) whereas one gene has been isolated from *T. versicolor* (Johansson and Nyman, 1995). The gene encoding MnP isozyme 1 (MnP-1) is composed of 2539 bp including 526 bp of 5' flanking sequence and 368 bp 3' to the stop codon (Godfrey et al., 1990). The nucleotide sequence encoding for MnP-2 contains 3297 bp which included 1287 bp of 5' flanking sequence and 490 bp 3' to the stop codon (Mayfield et al., 1994). MnP is regulated by ammonia nitrogen at the level of gene transcription as well as by the presence of Mn^{2+} in the culture medium (Brown et al., 1990). In addition, expression of MnP in nitrogen-limited culture was also up-regulated by heat shock and manganese addition induced transcription in culture medium (Li et al., 1995). Availability of the nucleotide sequences of the MnP genes from these two microorganisms may facilitate isolation of MnP genes from Ggt, Ggg, and Gga using PCR technology and DNA polymorphisms of the genes among the varieties may be useful in developing differential assays.

1.10 DETECTION AND QUANTIFICATION OF FUNGAL PATHOGENS FROM FIELD SAMPLES.

The goal for development of any differential test for plant pathogenic fungi is its application to samples collected from the field. Identification, differentiation and quantification of the pathogen of interest from field samples will be more informative and perhaps useful in prediction of disease severity for subsequent crops and may facilitate disease management programs. Consequently, this may help in improvement of crop yields, possible reduction of pesticide usage, and net economic return to the growers.

1.10.1 Identification and quantification of take-all fungi in soil

Identification and quantification of plant pathogenic fungi in soil is an important tool for studying the biology and ecology of fungi, its epidemiology and population dynamics which, in turn, are essential for development of an effective disease management program. Attempts to isolate the take-all fungus directly from soil were first described by Hornby (1969) using a selective medium containing several antibiotics. However, quantitative studies were not feasible at that time due to the fact that *G. graminis* has little competitive saprophytic ability and is, therefore, present in low numbers in soil. Although it may survive in root fragments, the longevity of survival is very brief (Hornby, 1969). A successful soil bioassay technique was later developed but it is time-consuming and requires large amounts of space in a controlled environment (Herdina et al., 1997; Hornby, 1981).

As mentioned earlier, molecular techniques have played a major role in the development of rapid identification methods for fungal pathogens. Most of those techniques can be applied to soil samples. However, it has been found that isolation of DNA from soil organic matter is a crucial step for success. Soil contains phenolic substances, such as humic acids, that can inhibit *Taq* DNA polymerase activity (Tsai and Olson, 1992). Failure in detection of the presence of *G. graminis* DNA directly from naturally infested soil by PCR-based procedure was observed and some spurious results were attributed to the presence of phenolic substances in the soil (Henson, et al., 1993). Use of DNA-probe hybridization can circumvent this problem and provide a quantification method for plant pathogenic fungi in the soil (Herdina et al., 1996; Whisson et al., 1995). Several DNA isolation procedures have been developed to overcome this problem. Prior to PCR, DNA solutions containing humic acids can be purified by using column chromatography (Whisson, et al., 1995), $CaCl_2$ precipitation with polyvinyl polypyrollidone (PVPP) (Ernst et al., 1996; Johnston and Aust, 1994), skim milk powder as a carrier for nucleic acids (Volossiouk et al., 1995), and solvent extraction and washes of DNA samples (Bahnweg et al., 1998). All of these methods yield PCR-quality DNA ready for

subsequent PCR amplification or DNA hybridization analyses although further optimization of test conditions may be required.

Quantification of *Rhizoctonia solani* J.G. Kühn isolate AG-8 and Ggt in soil has been achieved using slot blot hybridization analyses (Herdina et al., 1996; Whisson et al., 1995). Two DNA probes used for *G. graminis* were derived from its own genomic DNA library fragment and mtDNA fragment (pMSU315), respectively. Probes hybridized to all three varieties of *G. graminis* but different patterns were detected. The amount of Ggt in soil was estimated and approximately 0.3 ng of DNA of Ggt was detected in 1 μg of total extracted DNA (Herdina et al., 1996). By this method, the amount of Ggt mycelia in soil and percentage of root colonization could be estimated. In combination with a model for take-all disease development and effective sampling techniques, this information can be used to predict disease level within a field (Herdina, et al., 1997). To date, none of the PCR-based identification methods for *G. graminis* have been successful in direct detection of the fungus in the soil.

1.10.2 Direct detection of take-all fungi from colonized wheat samples

Direct detection of the fungi from plant tissue samples is more straightforward than from soil due to the presence of fewer *Taq* DNA polymerase inhibitors. However, the type of plant tissue can be an important factor for successful identification. For example, it is more difficult to grind dry or dead tissue than fresh tissue. It would be more useful if identification of the take-all fungus is achieved from dried wheat stubble as it serves as a habitat of the fungus through subsequent growing season. The purity of DNA from old tissue is lower than from green fresh tissue because of the presence of higher levels of phenolic compounds (John, 1992). Most of the PCR-based techniques described earlier showed that they can be used to detect the presence of *G. graminis* in artificially and naturally colonized wheat roots without culturing of the fungus and isolating DNA (Bryan et al., 1995; Henson et al., 1993; Schesser et al., 1991). However, sample preparation is a critical factor for a successful identification process. Boiling is the most favorable method for preparation for PCR-based techniques since the aliquot can be

added directly to a PCR cocktail (Henson et al., 1993). Klimyuk et al. (1993) suggested an alkaline treatment of the sample with boiling for tomato leaves and roots. Bryan et al. (1995) successfully used this method for identification of Ggt from field samples. Internal controls are also important for a PCR-based direct identification assay since false negative results may result in incorrect identification and diagnosis. This is especially true when a sample preparation step is not efficient. Hu et al. (1993) suggested that addition of internal control was useful for detection of the presence of inhibitory substances in samples so that false negative results could be prevented. In addition, a DNA sample could be diluted or re-purified to prevent the artifact. However, none of the aforementioned tests for *G. graminis* has included an internal control for their PCR assays.

In conclusion, significant progress in development of a rapid, simple and specific diagnostic tests for take-all pathogens has been made in the past decade due to advances in molecular techniques. These molecular-based techniques have proved that they can be more reliable and conclusive than conventional diagnostic culturing techniques. However, none of the published methods can completely replace the conventional culturing method and there is a need for further confirmation using conventional methods such as pathogenicity assays. In addition, despite the attention paid to Ggt and Gga, there is, at present, no specific test for Ggg. Since Ggg can be a saprophyte on wheat and is often present in colonized wheat tissue, it is necessary to have a test that clearly differentiates Ggg from Ggt as well as Gga.

The goal of this study is to find a better marker than those previously published to be used as a basis for a simple and specific PCR-based test for identification and differentiation of all three varieties of *G. graminis*. Objectives of this study are to identify or isolate 1) genes encoding the avenacinase-like proteins or 2) genes involved in manganese oxidation from all three varieties of *G. graminis* and identify DNA polymorphisms among those genes. Partial DNA sequencing of the avenacinase genes from all three varieties was obtained and DNA sequence information was used to design PCR primers for identification and differentiation assay. The assay developed will be

used as an identification and differentiation assay for all three varieties of *G. graminis*. This test also has a high potential for field applications of take-all.

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CHAPTER 2

DNA POLYMORPHISMS OF AVENACINASE-LIKE GENES AMONG VARIETIES OF THE TAKE-ALL PATHOGEN, *Gaeumannomyces graminis*

The information presented in this chapter will be submitted to an appropriate journal with additional authors: C.L. Cramer, E.A. Grabau, G.H. Lacy, and E.L. Stromberg

2.1 ABSTRACT

Avenacinase-like genes were identified and partially characterized in four isolates of *Gaeumannomyces graminis* var. *tritici* (Ggt) and one isolate of var. *graminis* (Ggg). cDNAs from Ggt genes were more closely related to the gene from var. graminis (99% identity) than to avenacinase gene from *G. graminis* var. *avenae* (Gga) (96% identity). Comparison of deduced amino acids obtained from the sequences of Ggt also showed greatest similarity to that of Gga (95% identity). Oligonucleotide primers (AV1, AV2 and AV3 were designed from the published avenacinase gene of Gga. Amplification of genomic DNA of all Virginia Ggt isolates with primer AV1, AV2, and AV3 generated 750 and 1,384 bp PCR products similar to those of Gga. While both PCR products were obtained from all Ggg isolates, non-specific amplification with primer AV1 and AV3 were noticeable in some Florida Ggg isolates (FL-19, FL-39 and FL-175). *Alu*I, *Hae*III, and *Msp*I were used to study DNA polymorphisms among these genes. Unique patterns from *Alu*I and *Hae*III digestion were obtained for each variety. Profiles of five out of seven Virginia Ggt isolates were identical to an isolates (M1) from Montana while profiles of two other putative Virginia Ggt isolates (CHe1 and CK1a) and a single isolate from American Type Culture Collection (Ggt ATCC 28230) were identical to the profile of Ggg ATCC 12761. No DNA polymorphisms were obtained using *Msp*I. Pathogenicity assays confirmed that all Ggt isolates could not cause disease in oat seedlings while take-all symptoms were observed from those inoculated with Gga isolates. Root systems of inoculated seedlings were much reduced compared with healthy plants. This confirms that Ggt does not cause disease in oats although they possess avenacinase-like genes

2.2 INTRODUCTION

Take-all is a significant and damaging root disease of cereals and grasses worldwide. It is very important in temperate areas where wheat and grass culture is intensive (Garrett, 1981). Diseased plants usually are stunted, ripen prematurely, white-headed and sterile. *Gaeumannomyces graminis* (Sacc.) Arx & Olivier, a soilborne ascomycete, is the causal agent of take-all of cereal and grasses. Based on pathogenicity of host plants and cultural characteristics, three varieties of *G. graminis* have been recognized (Walker, 1972). *G. graminis* var. *tritici* Walker (Ggt) is the major causative agent of take-all of wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare* L.) and rye (*Secale cereale* L.). *G. graminis* var. *avenae* (E.M. Turner) Dennis (Gga) attacks oats (*Avena sativa* L.) and causes take-all patch of turfgrasses, particularly *Agrostis spp*. (Walker, 1981). *G. graminis* (Sacc.) Arx & Olivier var. *graminis* (Ggg) is pathogenic on turfgrasses (Elliott, 1991; Elliott, et al., 1993) and causes crown sheath rot of rice (*Oryza sativa* L.) (Walker, 1981) but is non-pathogenic or weakly pathogenic on wheat. Ggt is the most economically important take-all pathogen. Traditionally, these three varieties can be morphologically distinguished by the size of perithecia, ascospores, and type of hyphopodia, which has been used as the main distinction characteristic for these fungi. While Ggt and Gga produce simple and non-lobed hyphopodia, Ggg is capable of producing two types of hyphopodia, lobed and simple, non-lobed hyphopodia (Epstein et al., 1994; Nilsson, 1972). Variation in size of the ascospores can also be used to differentiate the three varieties, however, some overlap of this characteristic can make the specific identification difficult and inconclusive. Additionally, identification based on pathogenicity is time-consuming and sometimes non-specific due to loss of virulence during long term storage (Asher, 1980). Besides, some Ggt isolates found in Australia also cause disease on oats (Bryan et al., 1995) and Gga can be pathogenic on wheat as well (Walker, 1972).

A major distinctive characteristic among Ggt, Gga, and Ggg is the ability to produce a saponin detoxifying enzyme named avenacinase for detoxification of avenacin, a preformed inhibitor of fungal growth in oat roots (Crombie, et al., 1986; Osbourn et al.,

1991; Turner, 1953). Avenacins, belonging to the saponin family, are the mixture of four major compounds, namely avenacins A-1, A-2, B-1, and B-2, which are all glycoconjugates containing β-linked glucosyl residues. Avenacin A-1 is the most abundant and the most fungitoxic among the four substances in oats (Crombie et al, 1986a). Avenacinase, a β-glucosyl hydrolase (EC 3.2.1.21), produced by Gga is a single polypeptide chain of 110 kD and is highly active towards avenacin enabling the fungus to be resistant to avenacin and cause the disease in oats. Mode of action of the enzyme is removal of β,1-2 and β,1-4 linked terminal D-glucose molecules from avenacin A-1 to generate both the *mono*- and *bis*-deglucosylated forms of avenacin which are less fungitoxic (Crombie et al., 1986b; Osbourn et al., 1991; Turner, 1961). The gene encoding avenacinase (AVN1) from Gga was isolated and sequenced (Osbourn et al., 1995). The full length gene is about 3 kb and shows some similarity with β-D-glucosidases belonging to the family 3 group of glycosyl hydrolases defined by Henrissat (Henrissat, 1991; Henrissat and Bairoch, 1993; Osbourn, et. al., 1995). The most closely related are the β-glucosidases (BGLs) gene families from *Trichoderma reesei* (60% similarity and 45% identity at the amino acid level) and from *Candida pelliculosa* (60% similarity and 38% identity) which play an important role in cellobiose degradation. However, their effects on saponins have not been reported (Osbourn et. al., 1995).

Ggt and Ggg have the ability to produce enzymes with similar physicochemical properties as that of avenacinase and can be recognized by anti-avenacinase antisera. However, they showed very weak activity on avenacin after prolonged incubation with the substrate. This results in the sensitivity of Ggt to avenacin leading to inability to cause disease in oats. Some Ggt isolates, originally identified based on ascospore length, can infect oats and are resistant to avenacin A-1. These isolates do not produce avenacinase *in vitro* and lack a DNA homology to the AVN1 gene, however, analysis of ribosomal DNA sequences showed that these isolates were more closely related to Gga (Bryan et al., 1995). Ability to produce enzyme with weak avenacinase activity has been observed in *Phialophora* and other related *Gaeumannomyces* species but role of the enzymes in these fungi is not clear (Osbourn, 1994b).

As previously mentioned, conventional methods for identification and differentiation of Ggt, Gga, and Ggg based on morphological characteristics and pathogenicity are time-consuming and inconclusive. Our goal is to use knowledge gained from this study as a basis for development of a rapid and specific diagnostic test for Ggt. Although others have developed rapid techniques for differentiation of *G. graminis* varieties based on amplification of mitochondrial DNA fragments (Elliott, 1993; Henson, 1992; Schesser, et al., 1991), amplification of the internal transcribed spacers (ITS) regions of the ribosomal RNA genes (Bryan, et. al., 1995; Goodwin et al., 1995), RAPDs (Bryan et al., 1999; Fouly et.al., 1996; Wetzel III et al., 1996) and DNA probe hybridization (Henson, 1989; Tan et. al., 1994; Ward and Gray, 1992), identification and classification of the varieties also varied from one to another. This may be due to variation in collection of isolates of each individual research group. Also, some of those methods required restriction enzyme digestion or DNA hybridization with probes which are expensive and time-consuming. We have been exploring different approaches for developing a specific identification technique for *G. graminis* varieties such as using specific genes of interest. Currently, only the avenacinase gene has been found to play an important role in pathogenicity of Gga on oats but not as important in Ggt (Bowyer et al., 1995), it is likely to be the best candidate for our purpose. Since Ggt, Gga, and Ggg have the ability to produce avenacinase or avenacinase-like enzymes, the genes encoding this enzyme in all three varieties must be highly homologous although the activity of the enzymes are quite different. We believe that there should be sufficient divergence at the gene level affecting the enzyme activity. Here, we report the presence of an avenacinase-like gene in Ggt and Ggg isolates and describe DNA polymorphism of the genes from all three varieties. Partial sequencing of the genes identified from Ggt and Ggg isolates revealed that these genes were highly homologous to the avenacinase gene of Gga. Deduced amino acid sequences of the newly identified genes were highly conserved among the three varieties. In addition, pathogenicity assay on oat seedlings was also carried out in order to confirm that all isolates used in this study were not be able to cause disease in oats although they all possessed the avenacinase-like genes.

2.3 MATERIALS AND METHODS

2.3.1 Fungi, plasmid, and media.

All fungal isolates (Table 2.1) were maintained on ¼ x potato dextrose agar (¼ x PDA; 6 g potato dextrose broth and 1.5% w/v agar per liter) at 4°C and no more than three successive transfers were performed to ensure that cultures maintained their pathogenicity. Isolates other than those from American Type Culture Collection (ATCC, Manassas, VA 20110) were purified by single hyphal tip isolation (Appendix A) prior to any DNA work. For long term storage, mycelium plugs were taken from colony margins using a cork borer no. 3 (8-mm dia.) and stored in 15% glycerol at -80°C.

For liquid cultivation of *G. graminis*, three mycelium plugs taken from colony margin using cork borer no. 2 (5-mm dia.) were inoculated in a 50 ml ¼ x potato dextrose broth (¼ x PDB, Sigma, St. Louise, MO 63178) and grown in an orbital incubator (SanyoGallenkamp PLC., Loughborough, UK) for 7 days at 25°C, 120 rpm. Mycelia were then ground in a Waring blender (Dynamics Corporation of America, New Hartford, CT 06057) and transferred to a 1 L flask containing 250 to 300 ml ¼ x PDB. The fungi grown for 7 to 10 days under the condition as above were ready for harvest.

Plasmid pA3G2 containing a 3 kb avenacinase gene was kindly provided by Dr. Ann Osbourn of the Sainsbury Laboratory, John Innes Centre for Plant Science Research, Norwich, UK. For long term storage, plasmids were transformed into *Escherichia coli* (Migula) Castellani and Chalmers strain DH5α using standard methods (Sambrooks et al., 1989) and *E.coli* containing intact plasmid were stored in 50% glycerol at -80°C.

Table 2.1. Isolates of *Gaeumannomyces graminis* used.

Isolate	Host	Source Location	Source/reference
G. graminis var. *tritici*			
CB1	*Triticum aestivum* L.	Bedford Co., VA, USA	J.B. Crozier[1]
CD1	*T. aestivum*	Dinwiddie Co., VA, USA	J.B. Crozier
CH1	*T.aestivum*	Hannover, Co., VA, USA	J.B. Crozier
CHe1	*T. aestivum*	Henrico Co., VA, USA	J.B. Crozier
CK1a	*T. aestivum*	King and Queen Co., VA, USA	J.B. Crozier
CK1b	*T. aestivum.*	King and Queen Co., VA, USA	J.B. Crozier
CS1	*T. aestivum*	Suffork Co., VA, USA	J.B. Crozier
M1	*T. aestivum*	Montana, USA	D. Mathre[2]
ATCC 28230	*T. aestivum*	United Kingdom	ATCC[3]
G. graminis var. *avenae*			
ATCC 15419	*Avena sativa* L.	United Kingdom	ATCC
FR-W	*Agrostis stolonifera* L.	France	H.C. Wetzel III[4]
WW-W	*A. stolonifera*	Ohio, USA	H.C. Wetzel III
G. graminis var. *graminis*			
ATCC 12761	*T. aestivum*	United Kingdom	ATCC
FL-19	*Cyanodon dactylon* (L.) Pers.	Florida, USA	M. Elliott[5]
FL-39	*Stenotaphrum secundataum* (Walt.) Kuntze	Florida, USA	M. Elliott
FL-175	*Oryza sativa* L.	Florida, USA	M. Elliott
2033	*Glycine max* (L.) Merr.	Florida, USA	M. Elliott

[1]Mr. James B. Crozier, Department of Plant Pathology, Physiology and Weed Science, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061-0331

[2]Dr. Don E. Mathre, Department of Plant Sciences, Montana State University-Bozeman, Bozeman, MT 59717-3150.

[3]American Type Culture Collection (ATCC), 10801 University Boulevard, Manassas, VA 20110-2209

[4]Mr. Henry C. Wetzel III, Department of Plant Pathology, Kansas State University, Manhattan, KS66506-5502.

[5]Dr. Monica L. Elliott, Fort Lauderdale Research and Education Center, University of Florida, Ft. Lauderdale, FL 33314-7799.

2.3.2 Pathogenicity assays.

Two winter oat (*Avena sativa* L. cultivar 'Rodger') seeds were planted in 150 g Kempsville sandy loam coastal plain soil from a freshly plowed field at the Eastern Virginia Agricultural Research and Extension Center, Warsaw, Virginia. Prior to the experiment, the soil was amended with monobasic calcium phosphate (25 μg P/g soil), potassium chloride (25 μg K/g soil) and calcium carbonate (15 g/30 kg soil) in a plastic planting cone, 4 x 21 cm (Ray Leach Containers®, Portland, OR). Two mycelium plugs (cork borer no. 3) of a specific *G. graminis* isolate were placed in contact with the oat seeds and the seeds were then covered with 1.5 cm of soil. At the time of planting, approximately 30 ml of ammonium nitrate solution was added to each cone such that 30 μg N/g soil at final concentration was achieved. A few days after germination, plants were thinned to one plant per Conetainer®. Plants were grown in the greenhouse for 6 weeks under artificial lights (1000 Watts, lamp type M47) on a 12 hour cycle (Sylvania Lighting Equipment, Fall River MD 02724). Light intensity was approximately 143.2 μmol M^{-2} S^{-1} PAR (945 ft-c) under lower lighting conditions and 193.8 μmol M^{-2} S^{-1} PAR (1279 ft-c) under higher lighting conditions.

After 6 weeks, plants were removed intact from containers and the soil was rinsed from the roots. Roots were rated on the basis of degree of necrosis, *i.e.*, no, low, moderate, and severe necrosis. Plant height, fresh shoots and root weights were measured at the time of harvest. Dry weights of shoots were also obtained after oven drying at 60°C for 3 days. Each experiment was set up in a completely randomized block design with 6 replicates. Negative controls were also added to each experiment. The experiment was repeated twice.

2.3.3 Preparation of fungal genomic DNA.

After 7 to10 days of cultivation in ¼ x potato dextrose broth (¼ x PDB) at 25°C, 120 rpm, the mycelia were collected on 4 layers of 46 x 91 cm cheesecloth wipes (Fisher Scientific, Pittsburgh, PA 15238) and transferred onto a sterile Whatman® no. 1 filter

paper (Whatman International Ltd., Maidstone, UK). Wet mycelia were freeze-dried (FreezeMobile 6, The Virtis Company Inc., Gardiner, NY 12525) at 200 μτ overnight. Dried mycelia were collected on the next day and kept in a desiccator until used for DNA isolation.

Two procedures were used for preparation of *G. graminis* genomic DNA. The procedure modified by Picknett et al. (1987) was used for large scale preparation in which approximately 2-5 g of dried mycelia were ground in liquid nitrogen using sterile mortar and pestle. Mycelial powder was resuspended in 10 ml of extraction buffer (25 mM Tris-HCl [pH 8], 25 mM EDTA, 50mM NaCl, 1% SDS) and incubated in an ice-water slurry (0°C) for 1 hour. This was followed by addition of 5 M NaCl to a final concentration of 1 M and incubation in ice-water slurry for 1 hour. The mixture was centrifuged for 30 min (2,500 x g, 4°C). The supernatant, following separation from cell debris, was treated with proteinase K (20 mg/ml) to obtain a final concentration of 50 μg/ml at 37°C for 20 min. The supernatant was extracted with an equal vol. of phenol:chloroform:isoamyl alcohol (25:24:1), centrifuged for 15 min (2,500 x g, room temp.), treated with RNase A (50 μg/ml) at 37°C for 3hours and extracted again with phenol:chloroform:isoamyl alcohol. If needed, additional phenol:chloroform extractions were performed until a clear aqueous phase was obtained. A clear aqueous phase was extracted once with an equal vol. of chloroform:isoamyl alcohol (24:1) and centrifuged to separate the phases for 15 min. (14,000 x g, room temp.). DNA was precipitated by adding 0.03 vol of 3 M sodium acetate and 2.5 vol of cold ethanol (-20°C) and incubating at -20°C overnight. At the end of this period, the mixture was centrifuged for 30 min. (14,000 x g, 4°C), and the pellet was washed twice with 70% ethanol and air-dried. DNA was dissolved in either 1x TE (10mM Tris-HCl [pH8] and 1mM EDTA) or deionized water and stored at 4°C until used.

Fungal DNA used as templates for PCR were prepared according to the protocol modified by Lee and Taylor (1990). Approximately 1 g of dry mycelia was powdered in liquid nitrogen and 10 ml of lysis buffer (50 mM Tris-HCl [pH, 7.2], 50mM EDTA, 3% v/v SDS, 1% v/v β-mercaptoethanol) was added immediately to the powder. The mixture

was stirred with a 5 ml pipet tip and vortexed vigorously until it became homogeneous. The mixture was incubated for 1-2 hr at 65°C, extracted with phenol:chloroform until the aqueous phase was clear, incubated with RNase A (10 mg/ml) to obtain a final concentration of 50 μg/ml at 37°C for 3 hr, re-extracted once with phenol:chloroform, and the DNA was precipitated with 0.03 vol. of 3 M sodium acetate and 0.6 vol. of 99% isopropanol at 4°C for at least 30 min. The DNA pellet was subsequently obtained by centrifugation for 20 min (14,000 x g, 25°C), rinsed with 70% ethanol, dried, and re-dissolved in either 1 x TE or deionized water. DNA solutions were stored at 4°C.

2.3.4 Plasmid propagation and transformation.

Plasmid pA3G2 was transformed into competent *E. coli* DH5α. Transformation was conducted as described in the standard protocol (Sambrook et al., 1989) with slight modification. Two to three hundred nanograms of pA3G2 were mixed with 300 μl of competent *E. coli* DH5α, the mixture was incubated on ice for 40 minutes, and the cells were heat-shocked in a 42°C water bath for 3 min before immediate transfer to an ice-water slurry (0°C) for 2 min. Two ml of Luria-Bertani (LB) broth (Difco Laborotories, Detroit, MI, 48232-7058) was added to the mixture and incubated for 2 hours at 37°C to allow the cells to recover and express antibiotic resistance (Amp^r) encoded by the plasmid. Cells were plated on LB agar plates containing 50 μg/ml ampicillin and incubated at 37°C for at least 16 hours. Colonies that were able to grow in the presence of ampicillin were transferred onto a second plate for further identification. Plasmid DNA was prepared as described in Appendix A.

To ensure that pA3G2 contained the expected avenacinase insert, double digestion with *Apa*I and *Bgl*II was carried out for 1 hour at 37°C followed by a second digestion overnight at the same incubation temperature or with *Sst*I for 1 hour at 37°C. Digested samples were electrophoresed on 0.8% agarose gel, stained with ethidium bromide (0.5 μg/ml final concentration and visualized under UV light at 302 nm (Sambrooks et al., 1989).

2.3.5 PCR and PCR conditions

PCR amplification of putative avenacinase genes from Ggt and Ggg isolates was achieved using 3 primers flanking partial coding sequences of the gene. As shown in Fig. 2.1, the only 5' primer, AV1, was 5'-AGATGTTGCGCTCAAGTGCTT-3'. The 3' primers were AV2 and AV3 and their sequences were 5'-CTGCTCATTGCCGATGAAGTG-3' and 5'-TGCTCATGGTGGTTCCTGGC-3', respectively. All primers were synthesized by Integrated DNA Technologies Inc. (Coralville, IA 52241). For PCR conditions, unless otherwise specified, each 50 µl reaction volume contained 50 pmol of each of a 5' and 3' primer, 2.5 U of *Taq* DNA polymerase (Perkin Elmer AmpliTaq® DNA polymerase, PE Biosystems, Foster City, CA 94404), 200 µm of each deoxynucleotide triphosphates, 5 µl of 10 x reaction buffer, 4.5 mM $MgCl_2$ and 50 ng of fungal DNA. For reactions with plasmid DNA as templates, 5-10 ng of template was used. Polymerase chain reactions were performed in a thermal cycler (Robocycler® Gradient 40 Temperature Cycler, Stratagene, La Jolla, CA 92037) programmed for an initial denaturation of 3 min at 95°C, followed by 35 cycles of denaturation at 94°C for 45 sec, annealing at 59°C for 1 min, and extension at 72°C for 2:30 min. At the end of this incubation, additional incubation for 7 min at 72°C was always included to ensure complete extension of PCR products. PCR products were separated by electrophoresis (Small Horizontal Gel System, FB MSU-1, Fisher Biotech, Pittsburgh, PA, 15238) at 75 V for 1.5 hours in a 1.5% agarose gel (Ultrapure Agarose Electrophoresis Grade, Life Technologies, Inc., Gaithersburg, MD 20877) in 0.5xTBE buffer (45 mM Tris-borate and 1mM EDTA). Gels were stained with ethidium bromide (0.5 µg/ml final concentration) for 15 min followed by destaining in deionized water, if necessary, and photographed with a Polaroid camera (with Polaroid film type 667 or 55) under UV light at 302 nm with a Wratten 22A filter.

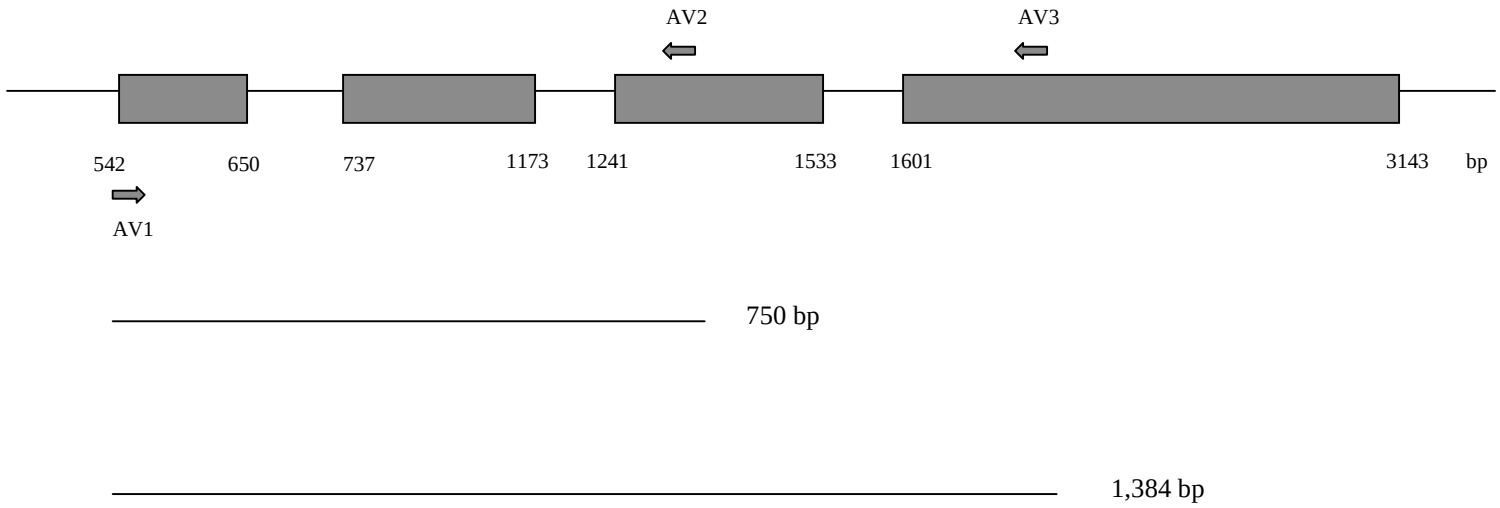


Fig. 2.1 Locations of PCR primers used in this study on the avenacinase gene of *Gaeumannomyces graminis* var. *avenae* (Gga) (Genbank accession number U35463). The filled boxes represent the coding regions (exons) and the lines connecting the boxes represent the non-coding regions (introns) of the gene. The arrows represent the approximate locations of the primers on the gene. PCR amplification with AV1 + AV2 and AV1 + AV3 produce products of approximately 750 and 1,380 bp, respectively.

Optimization of PCR components such as $MgCl_2$ (1.5-9mM), dNTPs (50-200μM) and amount of templates were always performed for each pair of primers as well as optimization of annealing temperature and time (50-65°C).

2.3.6 Southern hybridization and analysis of genomic DNA

Ten micrograms of fungal genomic DNA was digested at 37°C with *Hin*dIII, *Eco*RI, and *Kpn*I for at least 16 hours and, electrophoresed on 0.8% agarose gels, stained and photographed as described previously. Genomic DNA was then transferred to a nylon membrane (MagnaGraph Nylon Transfer Membrane, Micron Separations Inc., Westborough, MA 01581) using a standard method for Southern blot (Sambrook et al., 1989). Hybridization was carried out at 68°C in Church's buffer (1% BSA, 1mM EDTA pH 8, 0.5 M $NaHPO_4$ pH 7.2 and 7% SDS) overnight with DIG-labeled 750 bp amplified fragment from pA3G2 as a probe. Probe was prepared and quantified according to the manufacturer's protocol (Digoxigenin-11-dUTP, Roche Molecular Biochemicals, Indianapolis, IN 46250). Membranes were washed four times for 15 min each in 1 x SSC (15mM sodium citrate and 150mM NaCl) and 0.5% SDS at 68°C and rinsed in wash buffer (1mM EDTA and 40mM $NaHPO_4$) for a few minutes at room temperature. Detection was performed according to the manufacturer's protocol (DIG High Prime DNA Labeling and Detection Starter Kit II, Roche Molecular Biochemicals, Indianapolis, IN 46250) with slight modification such that CDP-Star™ (Roche Molecular Biochemicals, Indianapolis, IN 46250) was used as a substrate.

For analysis of PCR products, hybridization was carried out at 42°C overnight. Membranes were washed three times in 0.5x SSC, 0.4% SDS at 55°C, and twice in 2xSSC at room temperature. A 750 bp amplified fragment from pA3G2 with AV1 and AV2 was used as a probe. DNA probe labeling and detection were carried out as described by the manufacturer's protocol (ECL™ Direct Nucleic Acid Labeling and Detection Systems, Amersham Pharmacia, Biotech Inc., Piscataway, NJ 08855).

2.3.7 DNA polymorphism of avenacinase gene.

To remove unincorporated dNTPs, PCR products generated from AV1 and AV2 (750 bp) and from AV1 and AV3 (1,388 bp) were precipitated from the reaction mixtures by using 7.5 M ammonium sulfate (Sigma, St. Louis, MO 63178) to obtain a final concentration of 2.5 M and adding two volumes of 95% cold ethanol (-20°C). The mixture was incubated for at least 30 min at –20°C and centrifuged for 15 min at 13,000 x g. DNA pellet was rinsed with 100% ethanol and centrifuged for another 2 min at 13,000 x g. The resulting pellet was air-dried, and dissolved in an appropriate volume of sterile deionized water.
Short PCR fragments (750 bp) were digested separately with *Alu*I, *Hae* III, and *Msp*I and the 1,388 bp fragments were digested separately with *Alu*I and *Hae*III for 1 hour at 37°C Digestion with *Taq*I was carried out separately for 1 hour at 65°C. The DNA fragments were mixed with 6x gel loading buffer and separated by a vertical gel electrophoresis (Model V16, Bethesda Research Laboratories, Gaithersburg, MD 20877) at 70 V for 8 hours in a 10% polyacrylamide gel (acrylamide:bis acrylamide, 29:1), stained with ethidium bromide (0.5μg/ml) for 30 min, destained in deionized water for 15 min, and photographed with a Polaroid camera (Polaroid film type 55) under UV light at 302 nm using a Wratten 22A filter.

2.3.8 DNA sequencing.

The amplified products using the AV1 and AV3 primers were cloned and sequenced. In case of multiple bands obtained from PCR, the expected fragments were excised from the gel, purified (Gel Extraction Kit, QIAGEN Inc., Valencia, CA 91355) and resuspended in 30 μl of Tris-HCl, pH8. Otherwise, fresh PCR products were used directly for cloning. Cloning and transformation of PCR products were carried out according to the manufacturer's protocol (TOPO™ TA Cloning®, Invitrogen Corporation, Carlsbad, CA 92008) with slight modifications such that 35-75 μl of each transformation mixture were spread onto selective plates. White or light blue colonies appeared on selective plates (positive clones) were transferred to a second plate for further isolation of plasmid using the procedure described earlier. Restriction analysis of the plasmid with *Eco*RI was

carried out for 1 h at 37°C and visualized on 0.8% agarose gel electrophoresis. In some cases, PCR analysis of positive clones was carried out. Each 25 µl reaction contained 10xPCR buffer, 4.5 mM $MgCl_2$, 200 µM dNTPs, 50 pmol of each primer and 2.5 U of *Taq* DNA polymerase (Qiagen Inc., Valencia, CA 91355). Positive colonies were picked directly from plates and individually resuspended in the PCR cocktail. PCR condition and detection of positive clones were performed as previously described. Plasmids of the size expected with the insert were purified (Qiaquick Gel Extraction Kit, QIAGEN, Inc., Valencia, CA 91355) and sequenced using a dye-terminator cycle sequencing reaction based on manufacturer's protocol (BigDye™ Terminator Cycle Sequencing, PE Applied Biosystem, Foster City, CA 94404). Sequencing was performed with an ABI Prism DNA sequencer either model 377 or 373A Stretch (PE Applied Biosystem, Foster City, CA 94404). Two internal primers used in sequencing were 5'-CGCACCAGTCCGACACGACG-3' and 5'-CCTGCACCAAGCACTTCATC-3'. Sequence data were analyzed with the aid of the Lasergene Sequence Analysis Software (DNAStar Inc., Madison, WI 53715) and on-line basic BLAST search (BLAST 2.0) provided by National Center for Biotechnology Information (URL address: http://www.ncbi.nlm.nih.gov/BLAST).

2.4 RESULTS

2.4.1 Pathogenicity assay on oat seedlings.

Oat seedlings inoculated with Gga ATCC 15419 and Gga WW-W showed some root necrosis. Some root browning was also observed from plants inoculated with all Ggt isolates (Table 2.2). As shown in Fig. 2.2, average of root weights of seedlings inoculated with Gga was only half of that of seedlings inoculated with either Ggt or Ggg confirming that Gga is more virulent than Ggt or Ggg on oats. Analyses of variance by Duncan's MRT method for plant height, shoot weight, and root weight did not indicate significant differences at $p \leq 0.05$ (Table2). No other characteristic symptoms were observed and all plants inoculated with Ggt and Ggg isolates showed no stunting and reduced growth.

Table 2.2 Pathogenicity ratings of *Gaeumannomyces graminis* varieties on oat seedlings[1]. The experiment was repeated twice and data shown were from experiments 1 and 2.

Isolate	Virulence rating[2]		Plant height (cm)		Shoot weight (g)				Root weight (g)	
					Fresh weight		Dry weight			
	1	2	1	2	1	2	1	2	1	2
Gga ATCC 15419	+	+++	49.4c[3]	51.3a	3.33a	4.08a	0.49a	0.58ab	0.31ab	0.67bc
Gga WW-W	++	++	51.1bc	52.8a	2.96ab	3.14a	0.41ab	0.50ab	0.33ab	0.43c
Ggt M1	+	-	52.3abc	52.3a	3.32a	4.13a	0.45ab	0.57ab	0.62a	0.85ab
Ggt ATCC 28230	+	+	49.3 c	51.8a	3.26a	4.04a	0.47a	0.58ab	0.55ab	1.15a
Ggt CB1	+	+	53.0ab	51.3a	3.25a	3.48a	0.47a	0.49ab	0.61a	0.43c
Ggt CH1	+	+	50.6bc	53.4a	3.08ab	3.46a	0.44ab	0.49ab	0.55ab	0.59bc
Ggt CS1	+	-	53.3ab	50.7a	2.98ab	3.19a	0.42ab	0.46b	0.39ab	0.52bc
Ggg ATCC 12761	-	-	55.3a	54.3a	3.56a	3.85a	0.48a	0.54ab	0.52ab	0.88ab
Non-treated + agar plug[4]	-	-	50.9bc	52.0a	2.99ab	3.98a	0.43ab	0.61a	0.44ab	0.71bc
Non-treated - agar plug[5]	-	-	49.3c	51.1a	2.49b	3.54a	0.36b	0.52ab	0.27b	0.66bc

[1] Winter oat seeds cultivar "Rodgers" were planted 1.5 cm deep in Kempsville sandy loam coastal plain soil in a plastic planting cone, inoculated with mycelium plugs and amended with NH_4NO_3 solution. Plants were grown in the greenhouse for 6 weeks under artificial lights on a 12 hour cycle.

[2] Virulence ratings: (-) = no symptoms, + = some browning of roots, ++ = one to several root lesions, +++ = extensive root necrosis and browning of leaf sheaths, ++++ = same as 3 and leaves wilting, +++++ = plant dead.

[3] Means followed by the same letter do not significantly differ (Duncan's MRT, $p \leq 0.05$).

[4] Non-treated + agar plug = oat seeds were planted with sterile ¼ x PDA plugs (5-mm dia.).

[5] Non-treated –agar plug = only oat seeds were planted.

a b c d

Fig. 2.2. Comparison of the oat root systems among seedlings inoculated with *Gaeumannomyces graminis* varieties. Winter oat seeds cultivar "Rodgers", were planted 1.5 cm deep in Kempsville sandy loam coastal plain soil in a plastic planting cone, inoculated with mycelium plugs and amended with NH_4NO_3 solution. Plants were grown in the greenhouse for 6 weeks under artificial lights on a 12 hour cycle. (a) *G. graminis* var. *avenae* ATCC 15419, oat pathogen. (b) *G. graminis* var. *avenae* WW-W, turf grass pathogen. (c) *G. graminis* var. *tritici* M1, non-oat pathogen. (d) *G. graminis* var. *graminis* ATCC 12761, non-oat pathogen. This picture was taken 6

2.4.2 Identification of avenacinase-like gene in Ggt and Ggg.

Using primer pairs AV1 and AV2 or AV1 and AV3, DNA amplification from genomic DNAs from all isolates of Gga, Ggg and Ggt tested yielded, respectively, 750 or 1,384 bp fragments (Fig. 2.3). In Fig. 2.4a, all Ggg isolates amplified with AV1 and AV2 produced the same size of fragments and the intensity of all the bands was strong (Fig. 2.4a). However, when DNAs were amplified with AV1 and AV3, only Ggg 2033 produced a single band of 1,384 bp (Fig. 2.4, lane 11). All other isolates produced a band of 1,384 bp and other non-specific bands. Two PCR fragments of 1,384 and approximately 500 bp were generated from FL-19 and FL-39 (Fig. 2.4, lane 8 and 9) while FL-175 produced fragments of 1,384, 500 and 250 bp (Fig. 2.4, lane 10). In addition, yield of final PCR products from all Florida isolates except FL-175 was poor compared with ATCC 12761 (Fig. 2.4). All PCR experiments were repeated at least twice with appropriate controls (e.g., no template control). Southern blot analysis also confirmed that all bands were hybridized strongly to the 2.8 kb *Apa*I-*Bgl*II fragment of the avenacinase gene (data not shown).

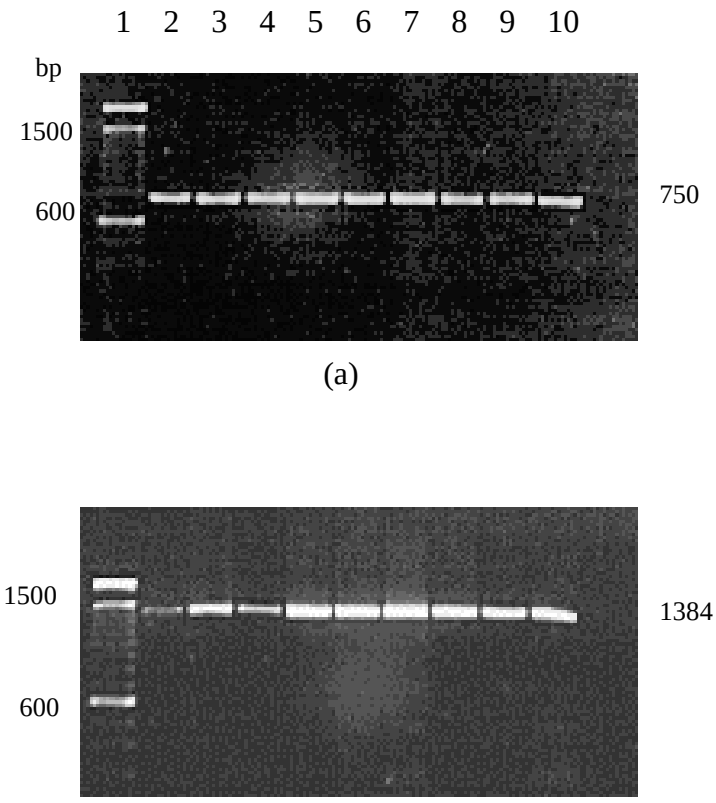


Fig. 2.3. Amplification of avenacinase or avenacinase-like gene fragments from *Gaeumannomyces graminis* var. *avenae* (Gga), var. *tritici* (Ggt) and var. *graminis* (Ggg). Electrophoresis and ethidium bromide staining indicated that PCR amplification with primers AV1 and AV2 (panel a) and AV1 and AV3 (panel b) produced 750 and 1,384 bp products, respectively. For both panels, lane 1 contains 100 bp DNA ladder for molecular weight standards. Genomic DNA used as templates for amplification were as follows: lane 2, Gga ATCC 15419; lane 3, Ggg ATCC 12761; lanes 4-10 are Ggt isolates M1; ATCC 28230; CB1; CD1; CHe1; CK1a, and CK1b, respectively. PCR products were electrophoresed on 1.8% agarose gel, stained with ethidium bromide, visualized and photographed with 302 nm UV light using a Wratten 22A filter.

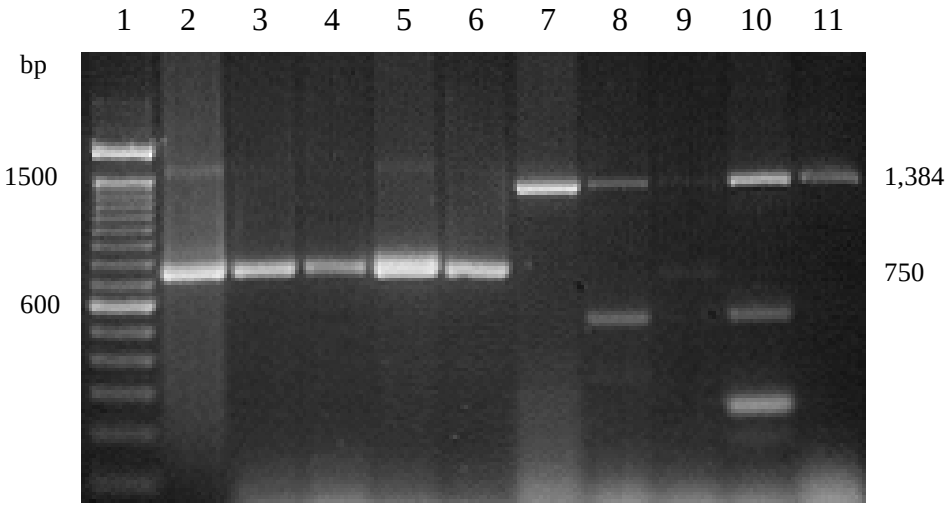


Fig. 2.4. Amplification of genomic DNAs from *Gaeumannomyces graminis* var. *graminis* (Ggg) isolates with primers AV1 and AV2 (lanes 2-6) and with primers AV1 and AV3 (lanes 7-11). PCR products were electrophoresed on 1.8% agarose gel, stained with ethidium bromide, visualized and photographed with 302 nm UV light using a Wratten 22A filter. Lane 1 contains 100 bp DNA ladder for molecular weight

2.4.3 DNA polymorphism of avenacinase-like genes of Ggt, Gga, and Ggg.

Restriction endonuclease digestion of the 750 and 1,384 bp fragments by *Alu*I and *Hae*III revealed unique polymorphism patterns for Ggt, Gga, and Ggg. Digestion of the 750 bp fragment with *Msp*I yielded common patterns (Figs. 2.5 and 2.6). Although *Alu*I profiles of 750 bp fragments were distinguishable among the three varieties, profiles from 1,384 bp fragments were quite similar (lane 5-7 in Figs. 2.5 and 2.6). Based on these results, other Ggt isolates were not digested with these two enzymes. DNA polymorphisms of *Hae*III digestion indicated that there were some differences at the nucleotide sequence level among three varieties of *G. graminis*. From sequence information, there were 17, 17, and 18 restriction sites on the 1,384 bp fragments of Ggt, Gga, and Ggg, respectively, however, only 6 to 7 bands were visible on 10% polyacrylamide gel (Table 2.3). *Hae*III profiles of both 750 and 1,384 bp fragments exhibited unique profile for each variety (Figs. 2.5 and 2.6). Interestingly, profiles of Ggt isolates M1, CB1, CD1, CH1, and CK1b were identical. Profiles of Ggt isolates ATCC 28230, CHe1, and CK1a were matched with those of Ggg ATCC 12761 (Fig. 2.6, lane 9-16). *Hae*III profiles of the 750 bp fragments also strongly supported this results (Fig. 2.6, lane 8-15). Although isolate ATCC 28230 has been designated as Ggt from the culture collection, I have observed unusual colony morphology and suspect that this isolate was mis-identified and is actually Ggg. None of Ggt isolates used in this study showed profiles identical to Gga.

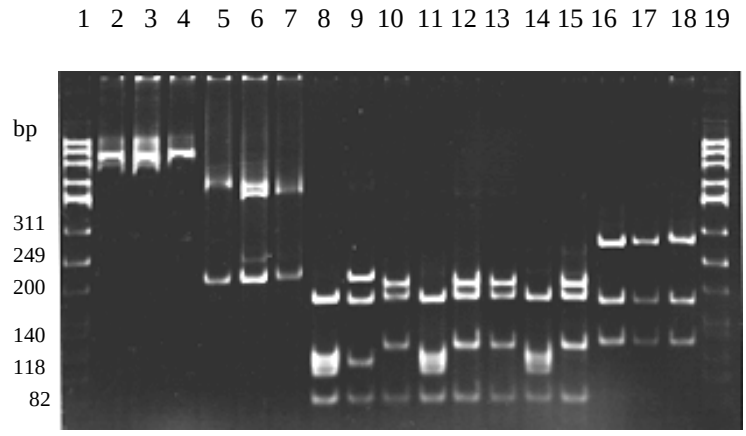


Fig. 2.5. Restriction endonuclease profiles of the 750 bp PCR product generated from primers AV1 and AV2 among isolates of *Gaeumannomyces graminis* var. *avenae* (Gga), var. *graminis* (Ggg), and var. *tritici* (Ggt). Digested samples were electrophoresed on 10% polyacrylamide gel, stained with ethidium bromide, visualized and photographed under UV light at 302 nm using a Wratten 22A filter. Lanes 1 and 19 contain ϕX174 DNA digested with *Hinf*I for molecular weight standards. Lanes 2-4 contain undigested genomic DNA of Gga ATCC 15419, Ggg ATCC 12761 and Ggt M1. Lanes 5-7 and 16-18 contain DNA of Gga ATCC 15419, Ggg ATCC 12761, and Ggt M1 digested with *Alu*I and *Msp*I, respectively. Lanes 8-15 contain DNA of Ggg ATCC 12761, Gga ATCC 15419 and isolates of Ggt M1, ATCC 28230, CB1, CD1, CH1, CHe1 and Ck1b, respectively, digested with *Hae*III.

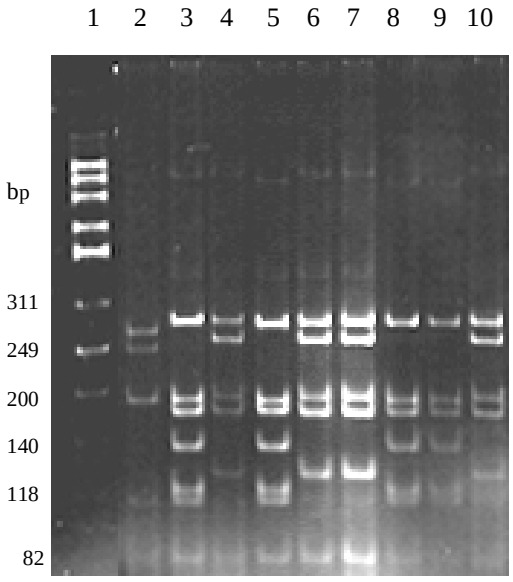


Fig. 2.6. Restriction endonuclease profiles of the 1,384 bp PCR product generated from primers AV1 and AV3 among isolates of *Gaeumannomyces graminis* var. *avenae* (Gga), var. *graminis* (Ggg), and var. *tritici* (Ggt). Digested samples were electrophoresed on 10% polyacrylamide gel, stained with ethidium bromide, visualized and photographed under UV light at 302 nm using a Wratten 22A filter. Lane 1 contains ϕX174 DNA digested with *Hinf*I for molecular weight standards. Lanes 2-9 contain genomic DNA of Gga ATCC 15419, Ggg ATCC 12761 and isolates of Ggt M1, ATCC 28230, CB1, CD1, CHe1, CK1a and CK1b, respectively, digested with *Hae*III.

Table 2.3 Patterns of DNA polymorphisms of PCR products from primers AV1, AV2, and AV3.

Enzyme	Varieties/isolates	Estimated visible band size (bp)	
		AV1 + AV2	AV1 + AV3
*Alu*I	Ggt	( ), 485, 230	651, 423, 230
	Gga	523, ( ), 227	686, 423, 227
	Ggg and Ggt ATCC 28230	512, ( ), 229	651, 423, 229
Hae III	Ggt	216, 195, 136, ( ), ( ), 87	286, 264, 195, ( ), 185, ( ), 136, ( ), ( ), 87
	Gga	231, 192, ( ), 118, ( ), 87	276, 254, 196, 192, ( ), ( ), ( ), ( ), 118, 87
	Ggg and Ggt ATCC 28230	( ), 190, 123, 118, 106, 87	286, ( ), ( ), 190, 185, 153, ( ), 123, 118, 87

( ) = no band of similar size present.

2.4.4 DNA sequencing and analysis of avenacinase-like genes of Ggt and Ggg isolates

Both strands of the 1,384 bp fragments from Ggt M1 and Ggg ATCC 12761 were sequenced. The fragment from Ggt M1 was 1,388 bp long and Ggg ATCC 12761 was 1,387 bp long (Fig. 2.7). Both sequences contained three introns, which corresponded to the published nucleotide sequence of Gga. Simulation of *Hae*III restriction sites on the nucleotide sequences with DNAstar™ software also corresponded with our previous results on DNA polymorphism of the gene. Comparison of the deduced amino acid sequences among Ggt, Ggg, and Gga showed that the avenacinase-like sequences of Ggt and Ggg were more closely related to each other (99.2% identity) than to Gga (94.8% to Ggt and 94.6% to Ggg) (Fig. 2.8). Blast search results also indicated that both genes were highly homologous to the avenacinase gene of Gga (95% identity). The first base of the sequences properly aligned with base 540 of the avenacinase gene confirming correct location of the 5' primer. In addition, these genes showed little homology with other genes in the family 3 of glucosyl hydrolases such as β-glucosidase genes from *Aspergillus kawachii* (Genbank accession number AB003470) and GTPase activation proteins of *Cochliobolus heterostrophus* Drechsler (Genbank accession number AF029913) (data not shown).

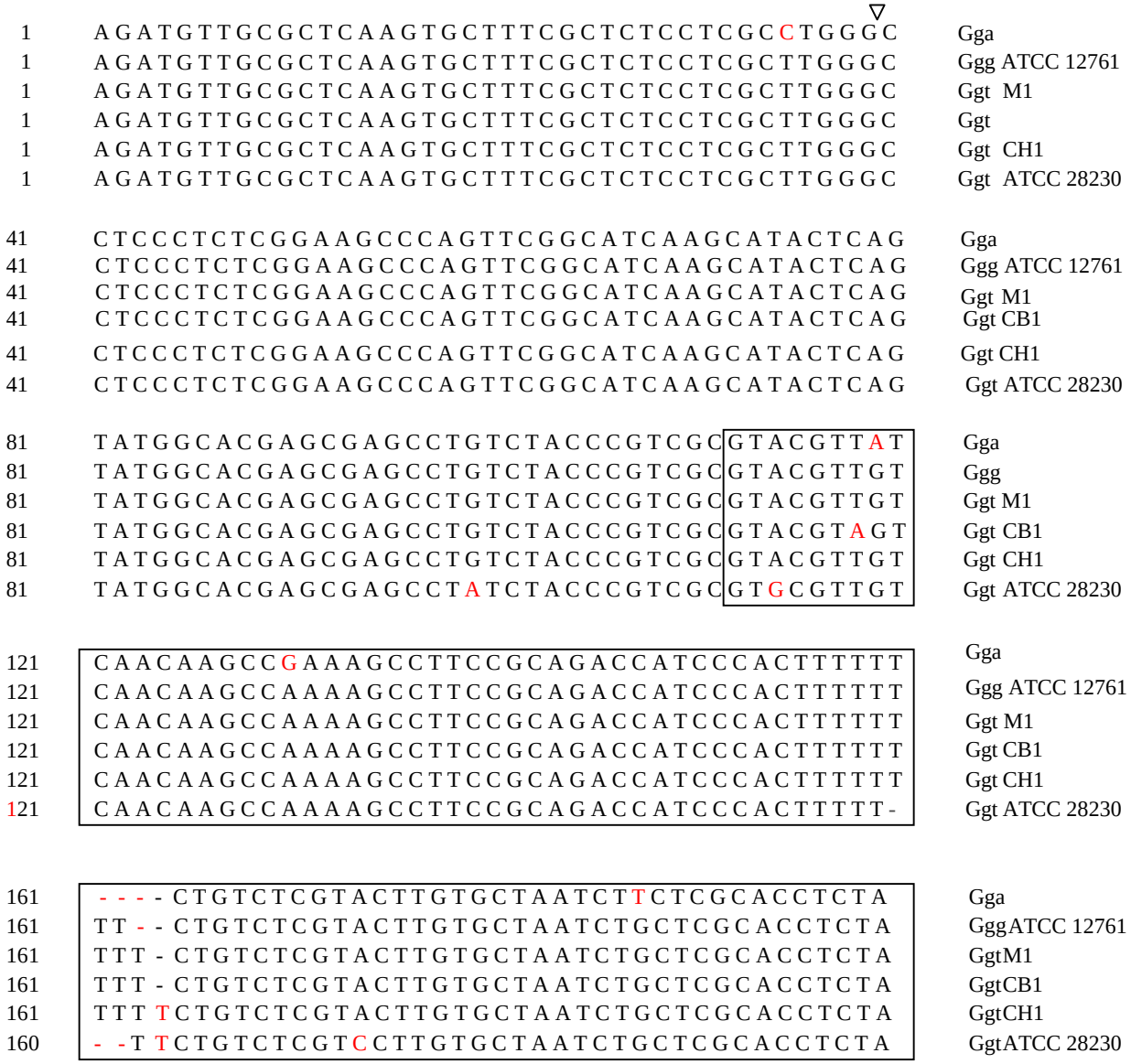
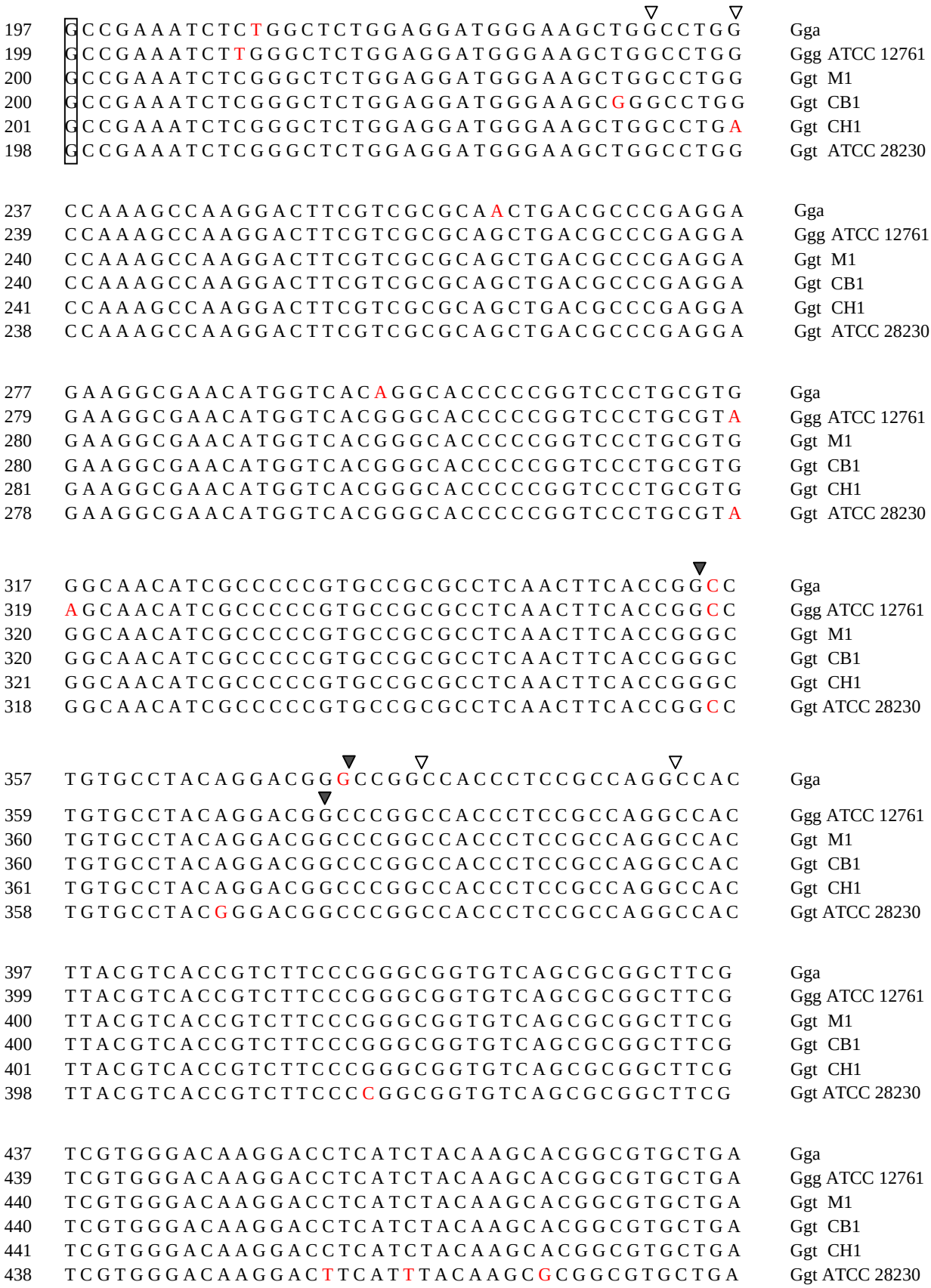


Fig. 2.7. Nucleotide sequence alignments of parts of the avenacinase gene from Gga and the avenacinase-like genes from Ggg and Ggt isolates generated from PCR amplification with primers AV1 and AV3. Alignments were performed using Clustal method with Weighted residue weight table. Published sequence of Gga (Genbank accession number U35463) was used as the reference sequence. Nucleotides differing from the consensus sequence are indicated in red and intron regions are boxed. Common *Hae*III sites among Gga, Ggg, and Ggt are indicated by opened triangles. *Hae*III sites presented in one or two varieties are indicated by filled triangles.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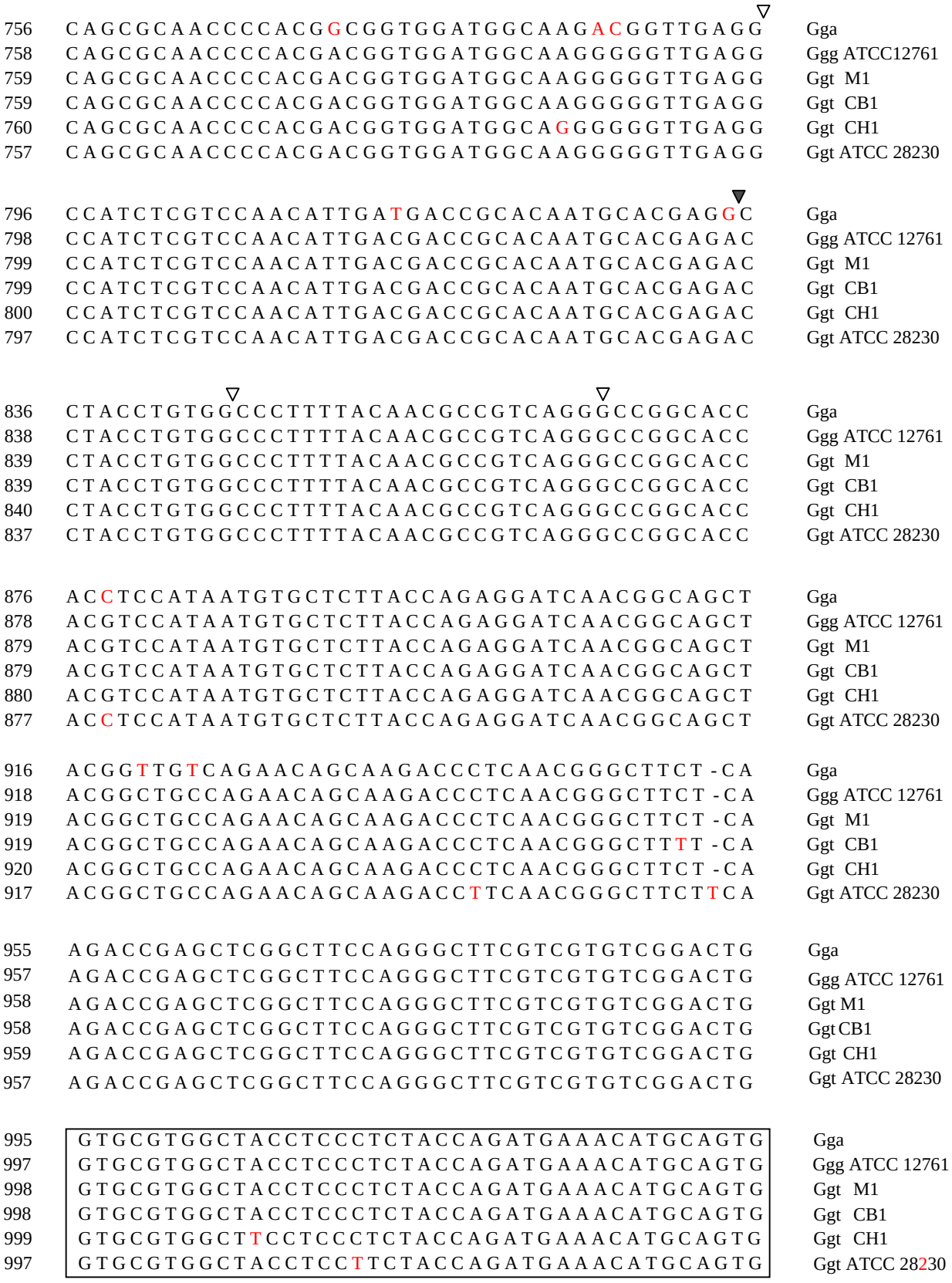
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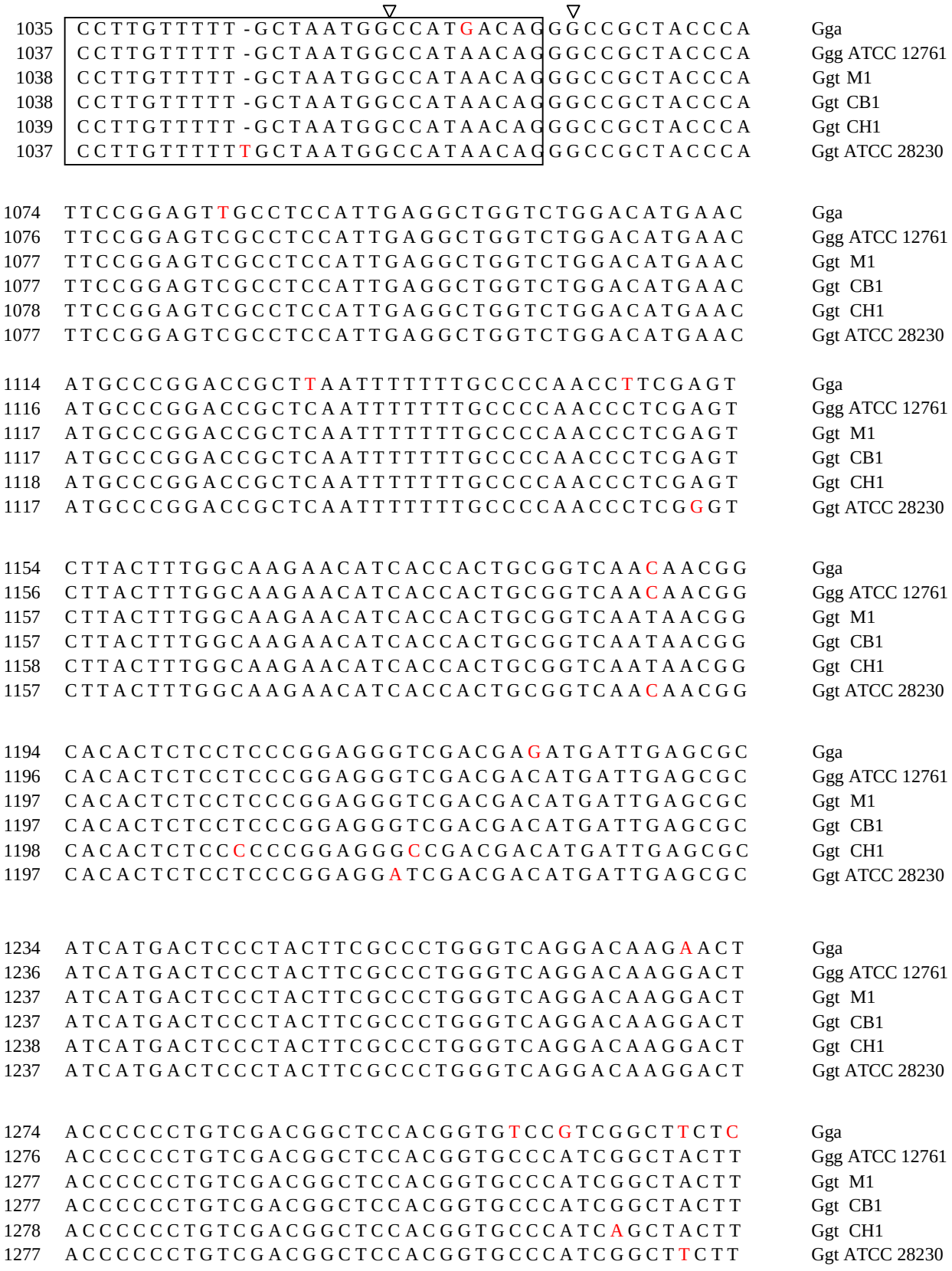
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1157 CTTACTTTGGCAAGAACATCACCACTGCGGTCAATAACGG Ggt CB1
1158 CTTACTTTGGCAAGAACATCACCACTGCGGTCAATAACGG Ggt CH1
1157 CTTACTTTGGCAAGAACATCACCACTGCGGTCAACAACGG Ggt ATCC 28230
1194 CACACTCTCCTCCCGGAGGGTCGACGAGATGATTGAGCGC Gga
1196 CACACTCTCCTCCCGGAGGGTCGACGACATGATTGAGCGC Ggg ATCC 12761
1197 CACACTCTCCTCCCGGAGGGTCGACGACATGATTGAGCGC Ggt M1
1197 CACACTCTCCTCCCGGAGGGTCGACGACATGATTGAGCGC Ggt CB1
1198 CACACTCTCCCCCCGGAGGGCCGACGACATGATTGAGCGC Ggt CH1
1197 CACACTCTCCTCCCGGAGGATCGACGACATGATTGAGCGC Ggt ATCC 28230
1234 ATCATGACTCCCTACTTCGCCCTGGGTCAGGACAAGAACT Gga
1236 ATCATGACTCCCTACTTCGCCCTGGGTCAGGACAAGGACT Ggg ATCC 12761
1237 ATCATGACTCCCTACTTCGCCCTGGGTCAGGACAAGGACT Ggt M1
1237 ATCATGACTCCCTACTTCGCCCTGGGTCAGGACAAGGACT Ggt CB1
1238 ATCATGACTCCCTACTTCGCCCTGGGTCAGGACAAGGACT Ggt CH1
1237 ATCATGACTCCCTACTTCGCCCTGGGTCAGGACAAGGACT Ggt ATCC 28230
1274 ACCCCCCTGTCGACGGCTCCACGGTGTCCGTCGGCTTCTC Gga
1276 ACCCCCCTGTCGACGGCTCCACGGTGCCCATCGGCTACTT Ggg ATCC 12761
1277 ACCCCCCTGTCGACGGCTCCACGGTGCCCATCGGCTACTT Ggt M1
1277 ACCCCCCTGTCGACGGCTCCACGGTGCCCATCGGCTACTT Ggt CB1
1278 ACCCCCCTGTCGACGGCTCCACGGTGCCCATCAGCTACTT Ggt CH1
1277 ACCCCCCTGTCGACGGCTCCACGGTGCCCATCGGCTTCTT Ggt ATCC 28230

*Eco*RI

1314	G C A G C C C G G C T T C T G G A G C C A C G A A T T C C C C C T C G G C C C C			Gga
1316	G C A G C C C G A C G C C T G G A A C C A C G A A T T C C C C C T C G G C C C C			Ggg ATCC 12761
1317	G C A G C C C G A C G C C T G G A A C C A C G A A T T C C C C C T C G G C C C C			Ggt M1
1317	G C A G C C C G A C G C C T G G A A C C A C G A A T T C C C C C T C G G C C C C			Ggt CB1
1318	G C A G C C C G A C G C C T G G A A C C A C G A A T T C C C C C T C G G C C C C			Ggt CH1
1317	G C A G C C C G A C G T C T G G A G C C A C G A A T T C C C C C T C G G C C C C			Ggt ATCC 28230

1354	A C G G T C G A C G T G C G C A G G A A C C A C C A T G A G C A	1385	Gga
1356	A C G G T C G A C G T G C G C A G G A A C C A C C A T G A G C A	1387	Ggg ATCC 12761
1357	A C G G T C G A C G T G C G C A G G A A C C A C C A T G A G C A	1388	Ggt M1
1357	A C G G T C G A C G T G C G C A G G A A C C A C C A T G A G C A	1389	Ggt CB1
1358	A C G G T C G A C G T G C G C A G G A A C C A C C A T G A G C A	1390	Ggt CH1
1357	A C G G T C G A C G T G C G C A G G A A C C A C C A T G A G C A	1388	Ggt ATCC 28230

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1    MLRSSAFALLAWASLSEAQFGIKHTQYGTSEPVYPSPEIS   Ggt M1
1    MLRSSAFALLAWASLSEAQFGIKHTQYGTSEPVYPSPEIS   Ggt CB1
1    MLRSSAFALLAWASLSEAQFGIKHTQYGTSEPVYPSPEIS   Ggt CH1
1    MLRSSAFALLAWASLSEAQFGIKHTQYGTSEPVYPSPEIL   Ggg ATCC 12761
1    MLRSSAFALLAWASLSEAQFGIKHTQYGTSEPVYPSPEIS   Gga

41   GSGGWEAGLAKAKDFVAQLTPEEKANMVTGTPGPCVGNIA   Ggt M1
41   GSGGWEAGLAKAKDFVAQLTPEEKANMVTGTPGPCVGNIA   Ggt CB1
41   GSGGWEAGLTKAKDFVAQLTPEEKANMVTGTPGPCVGNIA   Ggt CH1
41   GSGGWEAGLAKAKDFVAQLTPEEKANMVTGTPGPCVSNIA   Ggg ATCC 12761
41   GSGGWEAGLAKAKDFVAQLTPEEKANMVTGTPGPCVGNIA   Gga

81   PVPRLNFTGLCLQDGPATLRQATYVTVFPGGVSAASSWDK   Ggt M1
81   PVPRLNFTGLCLQDGPATLRQATYVTVFPGGVSAASSWDK   Ggt CB1
81   PVPRLNFTGLCLQDGPATLRQATYVTVFPGGVSAASSWDK
81   PVPRLNFTGLCLQDGPATLRQATYVTVFPGGVSAASSWDK   Ggg ATCC 12761
81   PVPRLNFTGLCLQDGPATLRQATYVTVFPGGVSAASSWDK   Gga

121  DLIYKHGVLMAREFRDKGSHIILGPVIGPLGRSPYAGRNW   Ggt M1
121  DLIYKHGVLMAREFRDKGSHIILGPVIGPLGRSPYAGRNW   Ggt CB1
121  DLIYKHGVLMAREFRDKGSHIILGPVIGPLGRSPYAGRNW   Ggt CH1
121  DLIYKHGVLMAREFRDKGSHIILGPVIGPLGRSPYAGRNW   Ggg ATCC 12761
121  DLIYKHGVLMAEEFRDKGSHVILGPVIGPRGRSPYAGRNW   Gga

161  EGFSPDSYLAGVLAEQTVKGMQSVGVQTCTKHYIGNEQEE   Ggt M1
161  EGFSPDSYLAGVLAEQTVKGMQSVGMQTCTKHYIGNEQEE   Ggt CB1
161  EGFSPDSYLAGVLAEQTVKGMQSVGVQTCTKHYIGNEREE   Ggt CH1
161  EGFSPDSYLAGVLAEQTVKGMQSVGVQTCTKHFIGNEQEE   Ggg ATCC 12761
161  EGFSPTSYLAGVMAEQTVKGMQSVGVQACTKHFIGNEQEE   Gga

201  QRNPTTVDGKGVEAISSNIDDRTMHETYLWPFYNAVRAGT   Ggt M1
201  QRNPTTVDGKGVEAISSNIDDRTMHETYLWPFYNAVRAGT   Ggt CB1
201  QRNPTTVDGRGVEAISSNIDDRTMHETYLWPFYNAVRAGT   Ggt CH1
201  QRNPTTVDGKGVEAISSNIDDRTMHETYLWPFYNAVRAGT   Ggg ATCC 12761
201  QRNPTAVDGKTVEAISSNIDDRTMHEAYLWPFYNAVRAGT   Gga

241  TSIMCSYQRINGSYGCQNSKTLNGLLKTELGFQGFVVSDW   Ggt M1
241  TSIMCSYQRINGSYGCQNSKTLNGLFKTELGFQGFVVSDW   Ggt CB1
241  TSIMCSYQRINGSYGCQNSKTLNGLLKTELGFQGFVVSDW   Ggt CH1
241  TSIMCSYQRINGSYGCQNSKTLNGLLKTELGFQGFVVSDW   Ggg ATCC 12761
241  TSIMCSYQRINGSYGCQNSKTLNGLLKTELGFQGFVVSDW   Gga
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Fig. 2.8. Alignment of amino acid sequences deduced from nucleotide sequences of the avenacinase gene from Gga and the avenacinase-like genes from Ggg and Ggt using Clustal method with PAM250 residue weight table. Deduced amino acid sequences from nucleotide sequence of the avenacinase gene of Gga (Genbank accession number U35463) was used as the reference sequence. Amino acids differing from the consensus are indicated in red.

281	A A T H S G V A S I E A G L D M N M P G P L N F F A P T L E S Y F G K N I T T A	Ggt M1
281	A A T H S G V A S I E A G L D M N M P G P L N F F A P T L E S Y F G K N I T T A	Ggt CB1
281	A A T H S G V A S I E A G L D M N M P G P L N F F A P T L E S Y F G K N I T T A	Ggt CH1
281	A A T H S G V A S I E A G L D M N M P G P L N F F A P T L E S Y F G K N I T T A	Ggg ATCC 12761
281	A A T H S G V A S I E A G L D M N M P G P L N F F A P T F E S Y F G K N I T T A	Gga

321	V N N G T L S S R R V D D M I E R I M T P Y F A L G Q D K D Y P P V D G S T V P	Ggt M1
321	V N N G T L S S R R V D D M I E R I M T P Y F A L G Q D K D Y P P V D G S T V P	Ggt CB1
321	V N N G T L S P R R A D D M I E R I M T P Y F A L G Q D K D Y P P V D G S T V P	Ggt CH1
321	V N N G T L S S R R V D D M I E R I M T P Y F A L G Q D K D Y P P V D G S T V P	Ggg ATCC 12761
321	V N N G T L S S R R V D E M I E R I M T P Y F A L G Q D K N Y P P V D G S T V S	Gga

361	I G Y L Q P D A W N H E F P L G P T V D V R R N H H E	Ggt M1
361	I G Y L Q P D A W N H E F P L G P T V D V R R N H H E	Ggt CB1
361	I S Y L Q P D A W N H E F P L G P T V D V R R N H H E	Ggt CH1
361	I G Y L Q P D A W N H E F P L G P T V D V R R N H H E	Ggg ATCC 12761
361	V G F S Q P G F W S H E F P L G P T V D V R R N H H E	Gga

2.4.5 Genomic Southern hybridization analysis.

Southern hybridization analysis of genomic DNA of Gga, Ggg, and Ggt was carried out with three restriction endonucleases. DNA sequences of the Gga avenacinase gene and the Ggt and Ggg avenacinase-like genes indicated that there was a single internal *Eco*RI recognition site within the Gga, Ggg, and Ggg genes located approximately on the same position (Fig. 2.7). There is no internal recognition site for *Hind*III and *Kpn*I in all of them. High stringency hybridization (68°C) of the avenacinase gene probe to genomic DNA of Ggt, Gga, and Ggg digested with *Hind*III and *Kpn*I showed that avenacinase may be present as a single copy gene (Fig. 2.9). *Eco*RI-digested Gga DNA generated two fragments of approximately 6 and 0.8 kb corresponding to a single restriction site but a single and intense band of approximately 5 kb was detected in Ggt. Since the intensity of the Ggt band was very strong, it could possibly be a doublet of similar sized fragments. One strong band of the same size as that of Ggt and two faint bands of high molecular weights were observed in *Eco*RI-digested Ggg DNA. DNA polymorphisms among three varieties were observed when genomic DNA of all three varieties was digested with *Eco*RI and *Hind*III. In addition, no DNA polymorphisms were detected for any sample digested with *Kpn*I.

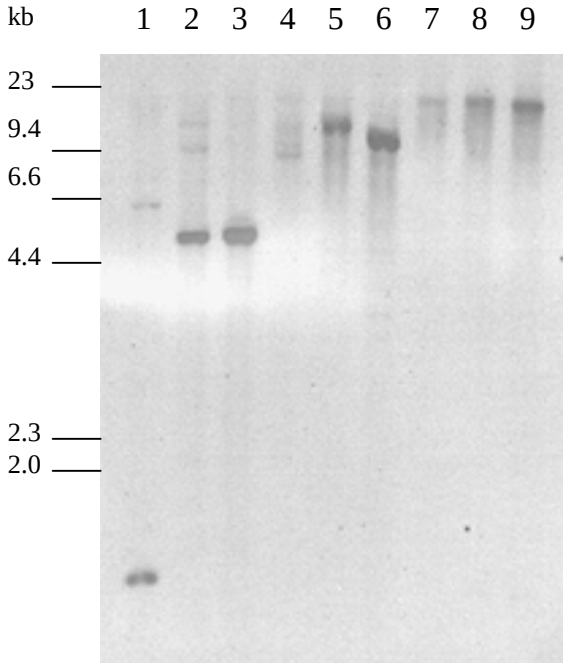

Fig. 2.9. Southern hybridization of genomic DNA of *Gaeumannomyces graminis* var. *avenae* (Gga), var. *graminis* (Ggg), and var. *tritici* (Ggt) digested with *Eco*RI, *Hind*III, and *Kpn*I, respectively. Digested DNA samples were electrophoresed on an 0.8% agarose gel in 0.5 x TBE, transferred on to a nylon membrane and hybridized with a 750 bp DIG-labeled avenacinase probe at 68°C overnight. Chemilluminescent detection was carried out with anti-DIG-alkaline phophatase conjugate, visualized with CDPstar™ as a substrate and exposed to an X-ray film for 45 min. Lanes 1-3 contain DNA of Gga, Ggg, and Ggt, respectively, digested with *Eco*RI. Lanes 4-6 contain DNA of Gga, Ggg, and Ggt, respectively, digested with *Hind*III. Lanes 7-9 contain DNA of Gga, Ggg, and Ggt, respectively, digested with *Kpn*I.

2.5 DISCUSSION

Mechanism of resistance of oats to Ggt colonization has been well established by Turner (1953). Avenacin present in oat roots plays a major role in disease resistance. Maizel et al. (1963) reported that only a small amount of avenacin completely inhibited growth of Ggt but did not significantly affect the growth of other fungi. Several groups have reported that Ggt had very weak avenacinase activity in culture rendering the fungus susceptible to avenacin A-1 in oat roots. In contrast, Gga has the ability to produce avenacinase, the avenacin detoxifying enzyme enabling the fungus to be pathogenic on oats (Crombie et al, 1986b; Osbourn et al, 1991; Turner, 1961). Our oat pathogenicity assay confirmed that Gga isolates caused take-all symptoms on oats as indicated by root lesions and significant reduction in root weight and size. However, virulence was not as severe as expected. Only two seedlings inoculated with Gga isolate WW-W were killed prior to harvest. The top of the oats appeared as healthy as those inoculated with Ggt and Ggg. This may be due to sub-optimum conditions for disease development in the greenhouse. Although slight root rotting occurred in some seedlings inoculated with Ggt, the average of root weight was twice as much as in Gga-inoculated seedlings. No root symptoms were observed on oats inoculated with Ggg.

Although Ggt and Ggg isolates did not cause significant reduction of oat root systems in our greenhouse assay, they possess the avenacinase-like genes. Deduced amino acid sequence alignment showed that the genes are highly homologous to avenacinase gene of Gga (95% identity). This is the first report on partial DNA sequences of avenacinase-like genes in Ggt and Ggg isolates although Osbourn et al. (1994) briefly described the presence of avenacinase-like proteins (ALPs) produced by Ggt and Ggg. All isolates of Ggt and Ggg produced identical PCR products to those of Gga when genomic DNA was amplified with primers AV1, AV2, and AV3. We confirmed their identity using Southern blot analyses, electrophoretic profiles from restriction enzyme digestions, and DNA sequencing of the 1,384 bp PCR products of four Ggt isolates and one Ggg isolate. DNA alignment clearly showed that the genes isolated from those Ggt and Ggg isolates are nearly identical to the Gga avenacinase gene with less than 50 bases difference within

the 1.4 kb fragment. According to Bowyer et al. (1995), the gene encoding for avenacinase in Gga is 2.8 kb in length and is a single copy gene present in the genome. We have shown that the avenacinase-like genes of Ggt and Ggg were present as single copies as well. In contrast to the strong morphological similarity of Ggt to Gga, based on DNA sequences alignment of 1,384 bp products, Ggt was more closely related to Ggg (99.2% identity and 98% identity at amino acid level) than Gga. Differences in the amino acid sequence, especially towards the 3'end, among Gga, Ggg, and Ggt may suggest differences in enzyme activities among these three varieties. This leads to a speculation on co-evolution of these fungi. The ancestor of Gga, Ggg, and Ggt might have an avenacinase-like gene. In the co-evolution of Ggt and Ggg with their hosts, there was no selective pressure on avenacinase-like genes since their hosts lacked avenacin. However, in the co-evolution of Gga, the fungus appears to have evolved avenacinase from avenacinase-like gene template for successful pathogenesis and survival. Avenacinase-like proteins may simply be β-glucosidases required for fungal nutrition or may play a role in detoxification of saponins during pathogenesis in other plants (Osbourn et al., 1994). However, our avenacinase-like genes showed little similarity to β-glucosidase genes (data not shown). Isolation of the full-length genes and generation of mutants of Ggt and Ggg may be necessary in order to confirm the identity or to study the function of these genes.

It is worth noting that other plant pathogenic fungi also possess detoxification mechanisms similar to that of *G. graminis* rendering them pathogenic on particular hosts. *Septoria lycopersici* Spegazzini, foliar pathogen of tomato, and *Botrytis cinerea* Pers.:Fr., causal agent of grey mold of fruits, vegetables, and flowers, produced enzymes that can detoxify α-tomatine, a saponin found in tomatoes and other members of the family *Solanaceae* (Quidde et al., 1998; Sandrock, et al., 1995). The physicochemical properties and mode of action of β_2-tomatinase produced by *S. lycopersici* are very similar to those of avenacinase. However, substrate specificity of β_2-tomatinase was restricted to α-tomatine reflecting the specificity of the fungi towards their original hosts (Osbourn et al., 1995; Quidde et al., 1998). In confirmation, an isolate of *B. cinerea* lacking the ability to detoxify α-tomatine was more sensitive to this compound than α-tomatine-

degrading isolates and did not cause disease on tomatoes (Quidde et al., 1998). In addition, isolates of *Septoria avenae* A.B. Frank, a causal agent of speckled blotch of oat, have the ability to detoxify oat leaf saponins while isolates that are pathogenic on wheat can not detoxify these substances (Wubben et al., 1996). These studies support the hypothesis that the ability to detoxify saponin compounds in host plants may be a pathogenicity factor of the fungal pathogens. Moreover, these results suggested that the substrate specificity of an individual enzyme may play a role in determining of the host ranges of these fungi.

DNA polymorphisms that were observed from *Hae*III digestion of avenacinase or avenacinase-like genes can be used to differentiate Ggt, Gga, and Ggg. Unique profiles were obtained from each of the varieties, which corresponded to slight variations in nucleotide sequences described earlier. Our study revealed that three isolates (CHe1, CK1a, and ATCC 28230) that were isolated from diseased wheat tissue and identified as Ggt actually have *Hae*III profiles suggesting that they should be classified as Ggg. This is consistent with pathogenicity assay carried out in our laboratory where these two isolates were avirulent on wheat (Crozier, 1999).

Based on our results, variations of avenacinase-like genes at the nucleotide level can be used for development of a specific identification method for Ggt. We can differentiate Ggt from Gga or Ggg by using *Hae*III profiles of the gene. DNA polymorphisms or restriction fragment length polymorphisms (RFLPs) have been widely used for identification of plant pathogenic fungi, however, previous methods have involved RFLP of multicopy genes such as ribosomal DNA and mitochondrial DNA genes. For ribosomal DNA, probes were generally obtained from the internal transcribed spacer regions of the gene. Tan et al. (1994) and Ward and Gray (1992) reported that this method allowed identification of *G. graminis* varieties as well as discrimination of isolates within varieties. Others have exploited redundancy of rDNA to show RFLPs for each varieties (O'dell et al., 1992) or establishing sets of RFLP profiles for each varieties (Bryan et al., 1999). The use of RFLPs of mitochondrial DNA for differentiation of *G. graminis* varieties and *Phialophora* spp. has been somewhat successful but it is not as

definitive as the use of ribosomal DNA (Henson, 1992). Although use of ribosomal DNA is believed to provide high sensitivity for identification, they are not perfect. Use of a specific gene such as avenacinase gene may provide a better means for differentiation of *G. graminis* varieties. Currently, *Hae*III profile can be used as a tool for differentiation of *G. graminis* varieties. However, our goal is to develop a better means for result interpretation. With sequence information for avenacinase-like genes from all three varieties, it is possible to design specific primers for each of the varieties and use a PCR-based strategy to create a simple and specific identification test for the take-all fungus.

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