Gaeumannomyces graminis vars. *avenae, graminis,* and *tritici* Identified Using PCR Amplification of Avenacinase-like Genes

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ABSTRACT

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Identifying take-all pathogens, *Gaeumannomyces graminis* varieties *avenae* (*Gga*), *graminis* (*Ggg*), and *tritici* (*Ggt*), is difficult. Rapid identification is important for development of disease thresholds. We developed a single-tube, polymerase chain reaction (PCR) method differentiating among *Gga*, *Ggg*, and *Ggt*. Nucleotide base sequence analyses of avenacinase-like genes from *Gga*, *Ggg*, and *Ggt* isolates provided the basis for designing variety-specific primers. Sequences from *Ggg* and *Ggt* were highly related (99% identity), but *Gga* sequences were <95% identical to *Ggg* and *Ggt* sequences. Three 5′ primers specific for *Gga*, *Ggt*, and *Ggg* and a single 3′ common primer allowed amplification of variety-specific fragments of 617, 870, and 1,086 bp, respectively. Each 5′ primer was specific in mixed populations of primers and templates. No PCR products were amplified from related fungi including *Gaeumannomyces cylindrosporus* and *Phialophora* spp. We surveyed 16 putative *Ggt* isolates using our assay; nine produced *Ggt*-specific fragments and seven produced *Ggg*-specific fragments. Five *Gga* isolates produced *Gga*-specific fragments. However, *Gga*- and *Ggt*-specific fragments were observed from a sixth *Gga* isolate, RB-W, which indicates a mixed culture or a heterokaryon. Our single-tube, PCR method rapidly differentiates among the important take-all pathogens commonly encountered together in cereal fields.

Additional keywords: avenacin, *Avenae sativa,* grasses, oats, phylogenetic relatedness, *Triticum aestivum,* wheat

Gaeumannomyces graminis (Sacc.) Arx & Olivier, a soilborne ascomycete, is the causal agent of take-all of cereal and grasses worldwide. The fungus colonizes the roots of susceptible plants, producing symptoms including stunted shoot growth, white-heads, reduced and blackened roots, and premature grain ripening. Based on pathogenicity assays and cultural characteristics, three varieties of *G. graminis* have been recognized (33). *G. graminis* var. *tritici* Walker (*Ggt*) causes take-all of wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare* L.), rye (*Secale cereale* L.), and, occasionally, oats (*Avenae sativa* L.) (4,38), and is the most economically important take-all pathogen (6,20). Other varieties of *G. graminis* may also colonize wheat roots and commonly occur in wheat fields on weeds. *G. graminis* var. *avenae* (E.M. Turner) Dennis (*Gga*) is a pathogen of oats and causes take-all patch of turfgrasses (34), and may affect wheat (33). *G. graminis* var. *graminis* (*Ggg*) is pathogenic on cereals and grasses (12,33), causes crown sheath rot of rice (*Oryza sativa* L.), and is weakly virulent on wheat (34). To complicate the situation, other species of *Gaeumannomyces* and morphologically similar members of the genus *Phialophora* (*Gaeumannomyces–Phialophora* complex) are capable of colonizing and causing root rot on cereals and grasses. For example, *G. cylindrosporus* and *G. incrustans* cause root rots when inoculated on wheat, barley, and turfgrasses but are not considered to be pathogens in field situations (21,24).

For predicting disease loss thresholds, epidemiology studies, and estimating the effectiveness of disease control strategies, a need exists for reliable identification of the three varieties of *G. graminis* among the *Gaeumannomyces–Phialophora* complex and other soil and rhizosphere organisms. Conventional identification methods for distinguishing *G. graminis* varieties are laborious, time-consuming, and often inconclusive. Identification is based on a combination of the characteristic disease symptoms, perithecia and ascospore morphological characters and size, hyphopodia type, and pathogenicity assays (20). While *Ggt* and *Gga* produce simple and nonlobed hyphopodia, *Ggg* and *Phialophora* spp. produce hyphopodia that are lobed or nonlobed (9,13,26). Variation in size of the ascospores can also be used to differentiate among the three varieties; however, overlapping size ranges makes specific identification difficult or inconclusive. Pathogenicity assays are time-consuming and sometimes difficult to interpret because the organism in question may lose virulence during long-term storage (1) or may only colonize or even be weakly virulent on "nonhost" or "host" plants (12,21,24,33,34).

Selective media have made isolation of *G. graminis* more effective, but they are not useful for identifying the most important varieties, *Gga, Ggg*, and *Ggt* (23). Further, during isolation, these slow-growing fungi are easily overgrown by other organisms.

Molecular techniques have played a significant role in the recent development of specific and rapid identification tests for *G. graminis*, especially *Ggt*. Previous work focused on the use of restriction fragment length polymorphisms (RFLPs) (2,17,32,36), amplification of mitochondrial DNA fragments (11,18,31), amplification of specific sequences within the internal transcribed spacer (ITS) region of ribosomal DNA (rDNA; 4,16,27), or randomly amplified polymorphic DNA (RAPD) profiles (5,15,37). Recently, polymerase chain reaction (PCR) primers have been developed for a fragment of the 18S rDNA gene and allow amplification of distinct *Gga* and *Ggt* products (14). However, no *Ggg* products were amplified using these primers.

To develop a rapid and definitive identification method for differentiation among *G. graminis* varieties *Gga*, *Ggg*, and *Ggt,* we proposed to utilize nucleotide base differences in avenacinase-like genes to design variety-specific PCR primers. Avenacinase-like genes are of special interest because at least one gene is involved in a specific host–pathogen interaction (7). We reasoned that genes important to the selectivity of plant–fungus interactions would require conservation of specific sequences. Avenacinase produced by *Gga* detoxifies avenacin A-1, the most abundant fungitoxic substance present in oat roots (7). The ability to detoxify avenacin A-1 is a pathogenicity factor for *Gga* on oats

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(7,29). *Ggt* and *Ggg* also have the ability to produce avenacinase-like proteins, but they do not affect avenacin A-1 (7,29,30), contributing to the inability of these fungi to cause disease on oats. Our objectives were to isolate and sequence avenacinase-like genes from *Gga, Ggg,* and *Ggt,* to determine if there was adequate divergence at the nucleic acid level to allow variety differentiation, and using simple PCR protocols, to amplify variety-specific bands.

MATERIALS AND METHODS

Fungal isolates, maintenance, and cultivation. All fungi used in this study are listed in Table 1. *Gaeumannomyces* spp. and *Phialospora* spp. isolates were cultured at 25°C and stored at 4°C on dilute (0.25×) potato dextrose agar (PDA; Sigma, St. Louis, MO). For long-term storage, mycelium plugs were taken from the colony margin using a cork borer (no. 3; 8 mm diameter) and stored at –80°C in 15% glycerol. Isolates other than those from the American Type Culture Collection (ATCC, Manassas, VA) were purified by single hyphal tip isolation prior to DNA work.

For fungal cultivation for DNA isolation, three mycelial plugs were taken from the colony margin using a cork borer (no. 2; 5 mm diameter) and inoculated into 50 ml of 0.25× potato dextrose broth (PDB; Sigma) in a 250-ml flask and grown in an orbital incubator for 7 days at 25°C, 120 rpm. Resulting mycelia were chopped in a blender and transferred to a 1-liter flask containing 250 to 300 ml of 0.25× PDB, and incubation was continued as previously described for 7 to 10 days.

Preparation of fungal DNA. Mycelia were harvested from PDB on four layers of cheesecloth and transferred onto sterile Whatman No. 1 filter paper. Wet mycelia were lyophilized overnight, collected, and stored in a desiccator at room temperature until used. Fungal DNA was prepared according to the protocol of Lee and Taylor (25). Approximately 1 g of dry mycelia was ground in liquid nitrogen, immediately mixed with 10 ml of lysis buffer (50 mM Tris-HCl, pH 7.2; 50 mM EDTA; 3% wt/vol sodium dodecyl sulfate (SDS); and 1% vol/vol β-mercaptoethanol), stirred with a 5-ml pipette tip, vortexed vigorously until homogeneous, incubated for 1 to 2 h at 65°C, extracted with phenol:chloroform (1:1 vol/vol) until the aqueous phase was clear, incubated with RNase A (final concentration of 50 µg/ml) at 37°C for 3 h, re-extracted once with phenol:chloroform, and 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 was pelleted by centrifugation for 20 min (14,000 × *g*, 25°C), rinsed with 70% ethanol, dried, and redissolved in Tris-EDTA (TE) or deionized water. DNA solutions were stored at 4°C.

Cloning avenacinase-like genes. About 1.4 kb of the 5′ ends of avenacinase-like genes from *Ggt* and *Ggg* isolates were amplified by PCR using oligonucleotide primers AV1 and AV3 (see Figure 1 for annealing sites and primer sequences) synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). PCR reactions were performed in 50-µl reaction volumes containing 50 pmol of each primer, 2.5 U of *Taq* DNA polymerase (Perkin Elmer AmpliTaq, PE Biosystems, Foster City, CA), 200 µm of each deoxynucleotide triphosphate (dNTPs), 5 µl of 10× reaction buffer, 4.5 mM $MgCl_2$, and either 50 ng of fungal DNA or 5 to 10 ng of plasmid DNA as template. Amplifications were performed in a thermal cycler (Robocycler Gradient 40 Temperature Cycler, Stratagene, La Jolla, CA) programmed for an initial denaturation of 3 min at 95°C, followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 59°C for 1 min, and extension at 72°C for 2.5 min. At the end of these cycles, an additional incubation for 7 min at 72°C ensured complete extension. PCR products were separated in 0.7% agarose dissolved in 0.5× TBE buffer (45 mM Tris-borate, 1 mM EDTA, pH 8.0) by electrophoresis, stained with ethidium bromide, and photographed (Polaroid Corp., Cambridge, MA; film types 667 or 55) under UV light at 304 nm with a Wratten 22A filter. The amplified avenacinase-like gene fragments were excised, purified (Gel Extraction Kit, QIAGEN Inc., Valencia, CA), and cloned into plasmid pCR 2.1-TOPO (TOPO TA Cloning kit, Invitrogen Corporation, Carlsbad, CA). Recombinant plasmids consistent with the size expected for inserted avenacinase-like gene fragments were confirmed by *Eco*RI restriction endonuclease digestion, PCR, and sequence analyses. Two additional internal primers (5′ seq and 3′ seq; see annealing sites in Figure 1) were designed and used for sequencing.

DNA sequencing. Clones containing plasmids with the PCR fragments inserted were purified (Qiaquick Gel Extraction

Table 1. Fungal isolates used in this study

Isolate	Host	Source (location)	Source (reference)
Gaeumannomyces graminis var. *tritici*			
CB1	*Triticum aestivum* L.	Bedford Co., VA, USA	(8)
CD1	*T. aestivum*	Dinwiddie Co., VA, USA	(8)
CE1	*T. aestivum*	Essex Co., VA, USA	(8)
CH1	*T. aestivum*	Hanover Co., VA, USA	(8)
CHe1	*T. aestivum*	Henrico Co., VA, USA	(8)
CK1a	*T. aestivum*	King and Queen Co., VA, USA	(8)
CK1b	*T. aestivum*	King and Queen Co., VA, USA	(8)
CS1	*T. aestivum*	Suffolk Co., VA, USA	(8)
M1	*T. aestivum*	Montana, USA	D. Mathre[a]
ATCC 28230	*T. aestivum*	United Kingdom	ATCC[b]
3053	*T. aestivum*	Washington, USA	M. Elliott[c]
3055	*T. aestivum*	Oregon, USA	M. Elliott
3056	*T. aestivum*	Indiana, USA	M. Elliott
3060	*T. aestivum*	Idaho, USA	M. Elliott
3066	*T. aestivum*	Montana, USA	M. Elliott
G. graminis var. *avenae*			
ATCC 15419	*Avena sativa* L.	United Kingdom	ATCC
PG-W	*Agrostis stolonifera* L.	Pinegrove, Canada	H.C. Wetzel III[d]
FR-W	*A. stolonifera*	France	H.C. Wetzel III
RB-W	*A. stolonifera*	Delaware, USA	H.C. Wetzel III
RI-W	*A. stolonifera*	Rhode Island, USA	H.C. Wetzel III
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
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
Gaeumannomyces cylindrosporus			
ATCC 64420	*Poa pratensis* L.	Rhode Island, USA	M. Elliott
Phialophora radicicola ATCC 64414	*P. pratensis*	Rhode Island, USA	M. Elliott
Phialophora sp.	Unknown	Unknown	M. Elliott
Cercospora zeae-maydis FOO1	*Zea mays* L.	Wythe Co., VA, USA	E.L. Stromberg

[a] Don E. Mathre, Department of Plant Sciences, Montana State University-Bozeman, Bozeman 59717-3150.
[b] American Type Culture Collection, 10801 University Boulevard, Manassas 20110-2209.
[c] Monica L. Elliott, Fort Lauderdale Research and Education Center, University of Florida, Ft. Lauderdale 33314-7799.
[d] Henry C. Wetzel III, Department of Plant Pathology, Kansas State University, Manhattan 66506-5502.

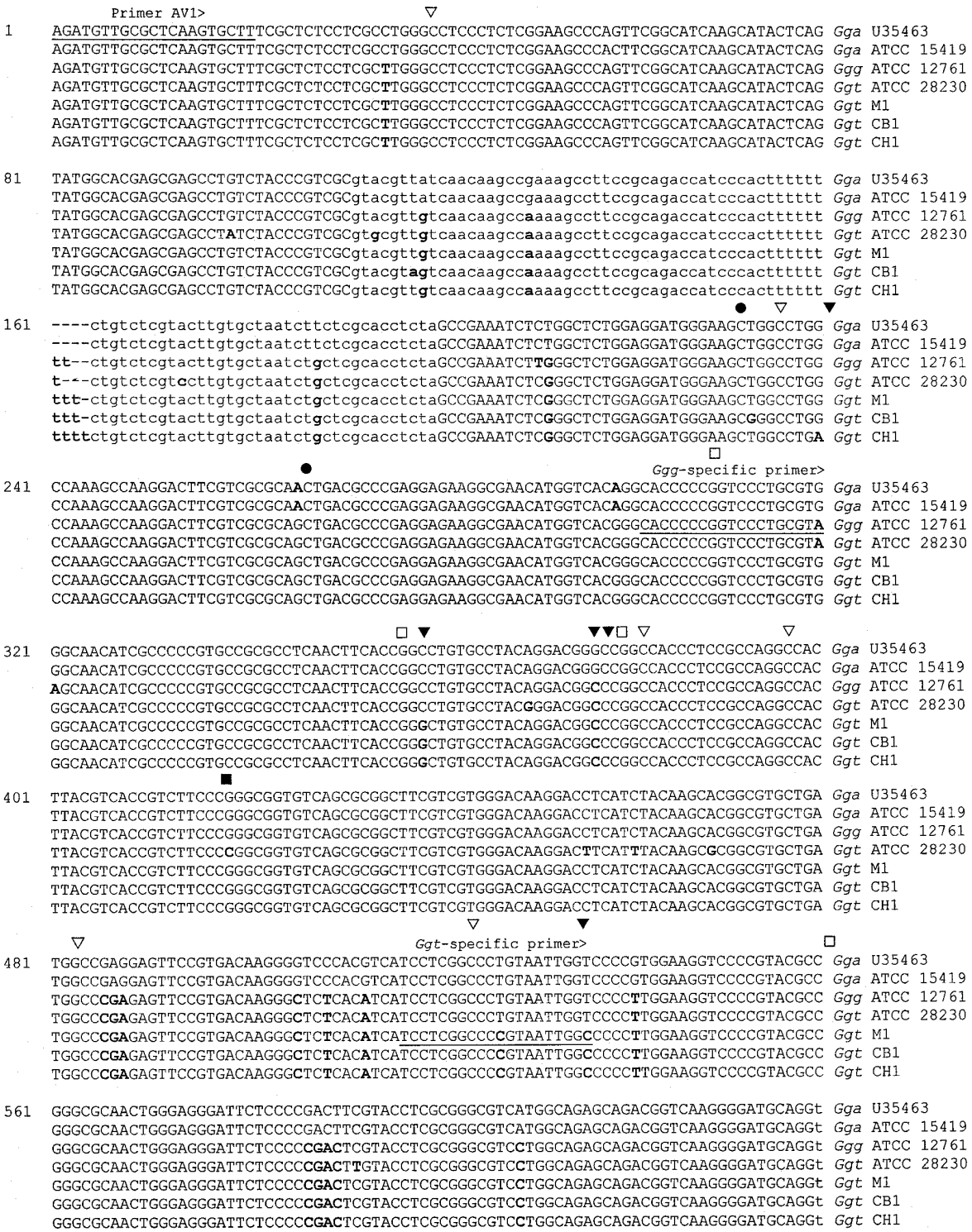

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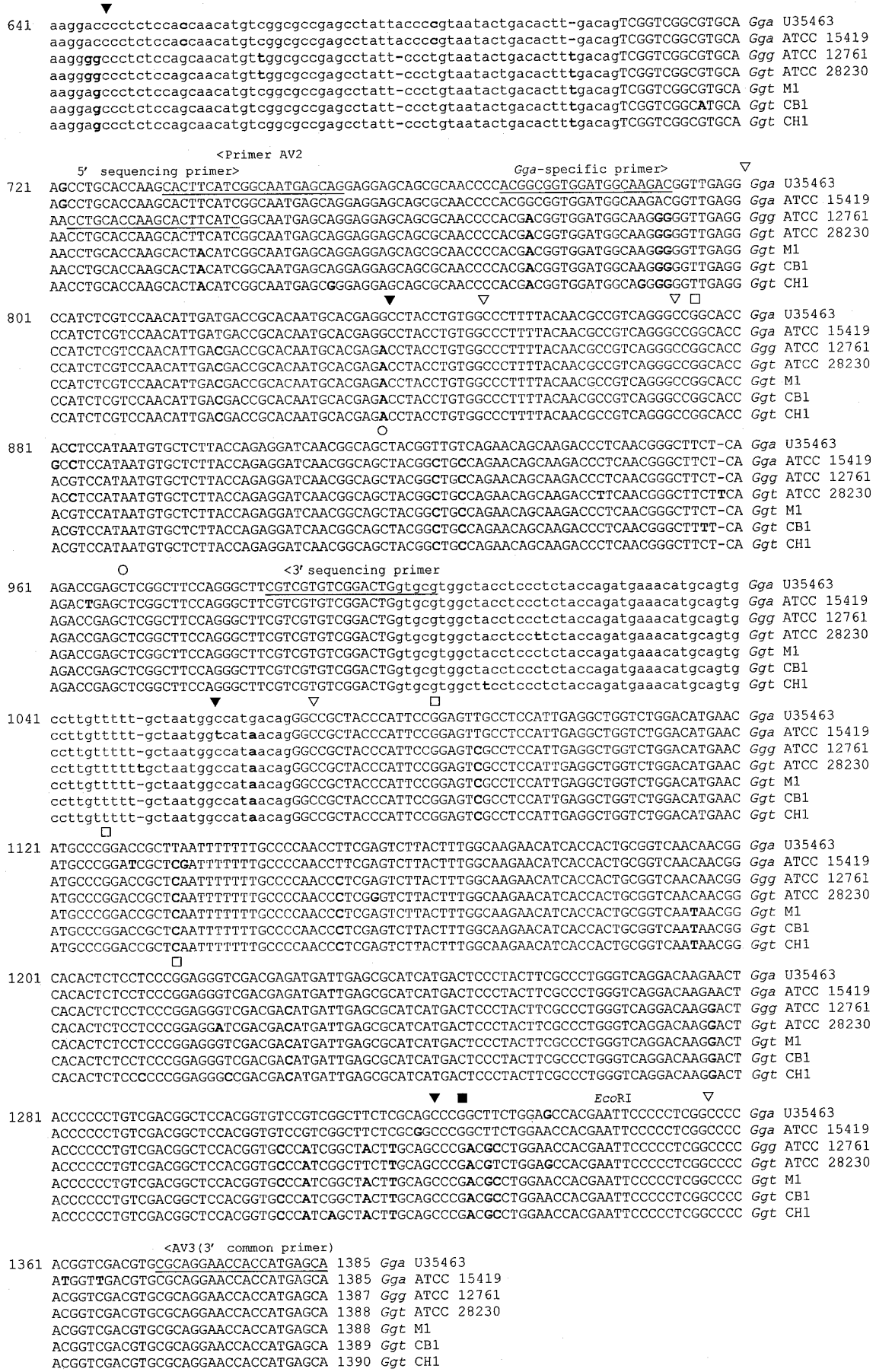
Fig. 1. Aligned partial avenacinase and avenacinase-like nucleotide base sequences. The upstream (5′) sequence (1 to 1,385 bp) of the 3,143 bp of the complete *Gaeumannomyces graminis* var. *avenae* (*Gga*) avenacinase gene (GenBank accession U35463 from Osborne et al. [28]) is compared with those for the avenacinase gene from *Gga* isolate ATCC 15419 (GenBank accession AF365953) and avenacinase-like genes from *G. graminis* var. *graminis* (*Ggg*; ATCC 12761 [AF365954]) and var. *tritici* (*Ggt*; ATCC 28230 [AF365956], M1 [AF365958], CB1 [AF365956], and CH1 [AF365957]). Nucleotides differing from the U35463 sequence are indicated in bold print. Nucleotides in intron regions are shown in lowercase. Sequences were generated by polymerase chain reaction (PCR) amplification using primers AV1 and AV3. Annealing sites for primers (AV1, AV2, AV3, *Gga*-specific, *Ggg*-specific, *Ggt*-specific, and internal primers 5′ and 3′ used for sequencing) are indicated by underlining. Consensus restriction endonuclease sites in all *Gga, Ggg*, and *Ggt* sequences are indicated with open symbols, nonconsensus sites are indicated with filled symbols: *Alu*I (○,●; 5′-AGCT-3′), *Hae*III (▽,▼; 5′-GGCC-3′), and *Msp*I (□,■; 5′-CCGG-3′). The single *Eco*RI restriction site is indicated. Alignments were performed using the CLUSTAL method with Weighted residue weight table. Hyphens were used to maximize the alignment.

Fig. 1. (*continued from preceding page*)



Kit, QIAGEN, Inc., Valencia, CA) and sequenced using a dye-terminator cycle sequencing reaction based on the manufacturer's protocol (BigDye Terminator Cycle Sequencing, PE Applied Biosystem, Foster City, CA). Sequencing was performed with an ABI Prism DNA Sequencer model 373A Stretch (PE Applied Biosystem). Sequence data were analyzed with the aid of Lasergene Sequence Analysis Software (DNASTar Inc., Madison, WI) and on-line basic BLAST search (BLAST 2.0) provided by the National Center for Biotechnology Information.

PCR of *Gga*-, *Ggg*-, and *Ggt*-specific fragments. DNA was amplified using three 5′ primers, one specific for each variety of *G. graminis*, and a single 3′ common primer. Annealing locations and nucleotide sequences for these primers on the avenacinase-like genes are shown in Figure 1. Primers used were synthesized by GIBCO BRL Custom Primers (Life Technologies Inc., Gaithersburg, MD). PCR reaction conditions differed from the procedures used to amplify avenacinase-like genes: a 50-µl reaction mixture contained 25 pmol of each of the three variety-specific primers, 75 pmol of 3′ common primer, 2.5 U of *Taq* DNA polymerase (Qiagen Inc., Valencia, CA), 100 µM of each dNTP, reaction buffer, 3 mM $MgCl_2$, and 50 ng of fungal DNA. PCR was performed in a thermal cycler (Mastercycler Gradient, Eppendorf Scientific Inc., Westbury, NY) programmed for an initial denaturation of 3 min at 95°C, followed by 30 cycles, each consisting of denaturation at 94°C for 45 s, primer annealing at 68°C for 45 s, and extension at 72°C for 2 min. At the completion of these cycles, an additional incubation for 10 min at 72°C ensured complete extension of PCR products. PCR products were separated by gel electrophoresis in 1.8% agarose. Negative controls with no DNA template were included in all PCR experiments. To control for false-positives, some samples were amplified with only a single, variety-specific primer (50 pmol).

Verification of primer specificity in PCR assays was carried out as described above with some modifications. If more than one template was added, the ratio of all templates was kept constant at 1:1 or 1:1:1 with 50 ng of each DNA. To demonstrate the sensitivity of a variety-specific primer, PCR was carried out with a dilution series of DNA template specific to each primer. The templates were diluted in sterile water, and the concentrations of DNA used ranged from 200 ng to 1 pg. PCR cycling parameters were as described above in this section. To confirm that each amplified variety-specific fragment had the expected DNA sequence, PCR products were cloned and sequenced.

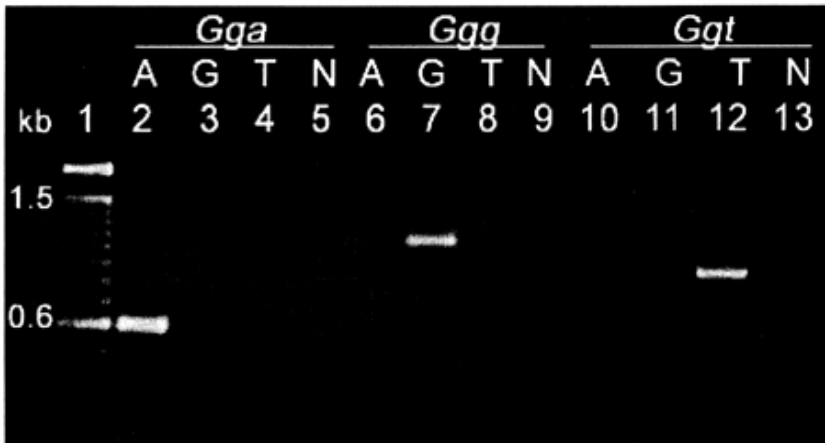


Fig. 2. Specificity of primers for templates. Variety-specific primers for *Gaeumannomyces graminis* var. *avenae* (*Gga*; lanes 2 to 5), var. *graminis* (*Ggg*; lanes 6 to 9), and var. *tritici* (*Ggt*; lanes 10 to 13) were tested against individual genomic DNA templates. Lanes 2, 6, and 10 contain *Gga* (ATCC 14519) template DNA (A); lanes 3, 7, and 11 contain *Ggg* (ATCC 12761) template DNA (G); lanes 4, 8, and 12 contain *Ggt* (M1) template DNA (T); and lanes 5, 9, and 13 contain no DNA (N). Lane 1 contains a 100-bp DNA ladder as a molecular weight standard. Ethidium bromide-stained variety-specific polymerase chain reaction (PCR) products (*Gga,* 617 bp; *Ggg,* 1,086 bp; and *Ggt,* 870 bp) were visualized by fluorescence with ultraviolet light.

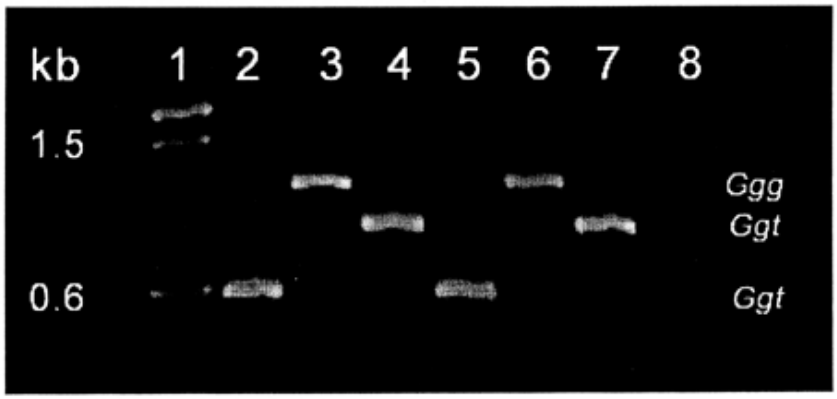


Fig. 3. Variety-specificity with mixed primers. Single template DNAs from *Gaeumannomyces graminis* var. *tritici* (*Ggt*), var. *avenae* (*Gga*), and var. *graminis* (*Ggg*) primed with single and mixed variety-specific primers were used to amplify variety-specific polymerase chain reaction (PCR) products (*Gga,* 617 bp; *Ggg,* 1,086 bp; and *Ggt,* 870 bp). Lane 1 contains a 100-bp DNA ladder as a molecular weight standard. Lane 2 contains *Gga* (ATCC 14519) DNA amplified with *Gga*-specific primer. Lane 3 contains *Ggg* (ATCC 12761) DNA amplified with *Ggg*-specific primer. Lane 4 contains *Ggt* (M1) DNA amplified with *Ggt*-specific primer. Lanes 5 to 7 contain, respectively, *Gga*, *Ggg*, and *Ggt* template DNAs. Lane 8 contains no DNA. Lanes 5 to 8 were amplified using a mixture of all three variety-specific primers. Ethidium bromide-stained PCR products were visualized by fluorescence with ultraviolet light.

RESULTS

Cloning avenacinase-like genes. The presence of single copies of avenacinase-like genes was confirmed in *Gga* and *Ggt* isolates by DNA:DNA hybridization (results not shown) using fungal genomic DNAs digested with *Eco*RI, *Hin*dIII, or *Kpn*I and probed with the 750-bp fragment, amplified using AV1 and AV2, from the authentic *Gga* avenacinase gene cloned in plasmid pA3G2 (3,28) using a kit (DIG Nucleic Acid Detection Kit, Roche Biochemicals, Indianapolis, IN). This plasmid was provided by Ann Osbourn, Sainsbury Laboratory, John Innes Centre for Plant Science Research, Norwich, UK.

Sequences of avenacinase-like genes. The published sequence for the *Gga* avenacinase gene (GenBank accession U35463) was compared with the sequences of the 1.4-kb fragments obtained from *Gga* isolate ATCC 15419 (GenBank accession no. AF365953), *Ggg* isolate ATCC 12761 (AF365954), and *Ggt* isolates M1 (AF365958), CB1 (AF365956), CH1 (AF365957), and ATCC 28230 (AF365955) (Fig. 1). These fragments varied from 1,387 to 1,390 bp. All sequences contained three introns, corresponding to the *Gga* sequence. Interestingly, the sequence for *Ggt* isolate ATCC 28230 most closely resembles the sequence for *Ggg* isolate ATCC 12761. Comparison of the deduced amino acid sequences 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*) (data not shown). The avenacinase and avenacinase-like genes showed little homology with other genes in family 3 of glucosyl hydrolases such as β-glucosidase genes from *Aspergillus kawachii* (GenBank AB003470) and GTPase activation proteins of *Cochliobolus heterostrophus* (GenBank AF029913) (data not shown).

Design, specificity, and sensitivity of *Ggt*-, *Gga*-, and *Ggg*-specific primers. Three PCR primers were designed to be variety-specific from DNA sequences of the avenacinase-like genes of *Ggt* and *Ggg* and the avenacinase gene of *Gga* (Fig. 1). Variety specificity was established by designing these three primers, nearer the upstream end of the genes, with single or double base differences at the 3′ end of each primer. A primer common to all three sequences was used for the downstream end of the PCR product. With this strategy, PCR products amplified from *Ggt*-, *Gga*-, and *Ggg*-specific primers and the 3′ common primer can be distinguished by size: 870, 617, and 1,086 bp, respectively.

Variety specificity of the 5′ primers was tested several ways. Using single templates and either one or all three specific 5′ primers, the expected single amplification products were observed (Figs. 2 and 3). When more than one specific primer was added into a PCR reaction, the amplification efficiency of the expected products was as high as for reactions with single specific primers (Fig. 3; compare intensity of ethidium bromide fluorescence in lanes 2 to 4 with lanes 5 to 7), suggesting that primers were not limiting. In addition, nonspecific product amplification was not observed in any of these experiments. However, less efficient amplification of longer PCR products, i.e., *Ggg*- and *Ggt*-specific fragments, was observed in reactions containing all three templates and all three variety-specific primers, possibly due to competition for dNTP substrates (data not shown). The experiments shown in Figures 2 and 3 were repeated with similar results.

Sensitivity was based on titration of template until amplification no longer produced ethidium bromide–stained variety-specific products visible as UV fluorescent bands in agarose gels (results not shown). The lowest concentration of DNA templates that generated products with *Ggg*- and *Ggt*-specific primers was 100 pg. The *Gga*-specific primer amplified from 50 pg of template DNA.

PCR-based test for differentiating *G. graminis* varieties. All isolates of *G. graminis* and related fungi tested in this study are listed in Table 1. When DNAs of 15 putative *Ggt* isolates were amplified with only the *Ggt*-specific primer, PCR products were obtained from CB1, CD1, CE1, CH1, CK1b, CS1, M1, 3053, and 3055. No *Ggt*-specific fragments were generated from isolates ATCC 28230, CHe1, CK1a, 3056, 3060, and 3066 (panel A of Figures 4 and 5). The atypical *Ggt* isolates that failed to generate *Ggt*-specific fragments were subjected to diagnostic PCR with all three variety-specific primers in a single reaction; they all produced abundant *Ggg*-specific PCR fragments

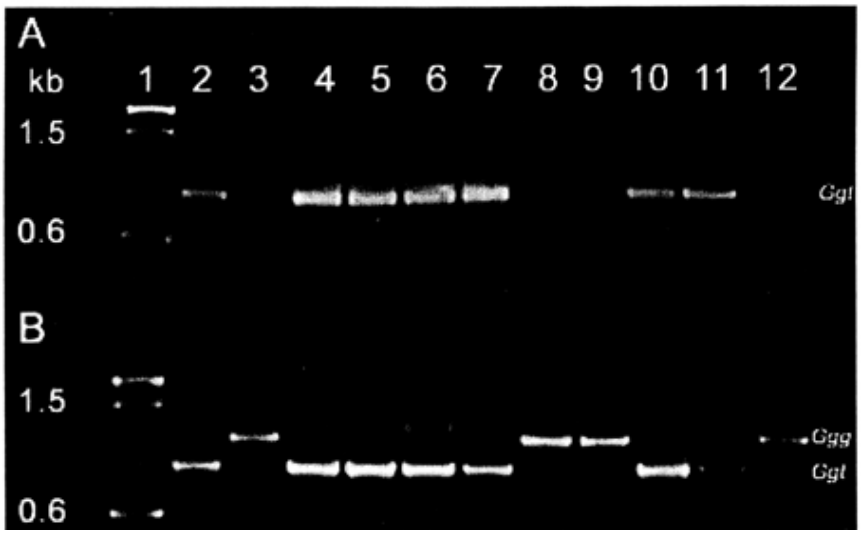


Fig. 4. Survey of *Gaeumannomyces graminis* var. *tritici* (*Ggt*) isolates. *Ggt* isolates from wheat were amplified with **A,** the *Ggt*-variety specific primer, and **B,** a mixture of all three variety-specific primers. Ethidium bromide-stained polymerase chain reaction (PCR) products (*G. graminis* vars. *avenae* [*Gga*], 617 bp; *graminis* [*Ggg*], 1,086 bp; and *Ggt,* 870 bp) were visualized by fluorescence with ultraviolet light. Lane 1 contains a 100-bp DNA ladder as a molecular weight standard. For both panels, lanes 2 to 11 contain DNAs from *Ggt* isolates M1, ATCC 28230, CB1, CD1, CE1, CH1, CHe1, CK1a, CK1b, and CS1, respectively. Lane 12, present only in **B,** contains DNA from *Ggg* ATCC 12761 as a control. The artifact above the major PCR product in **B,** lane 6, was not observed in two other amplifications with this DNA template.

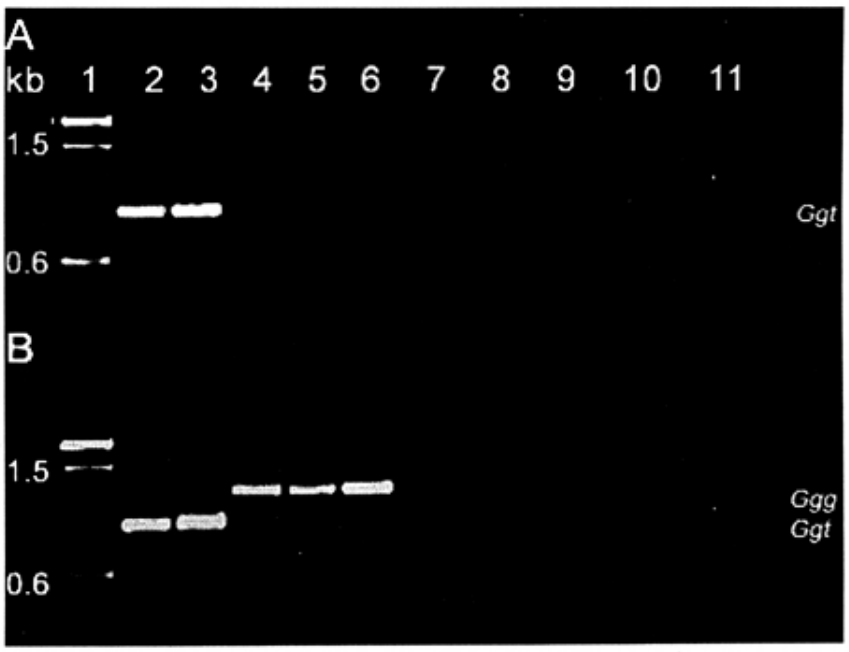


Fig. 5. Survey of isolates of the *Gaeumannomyces-Phialophora* complex. DNA from *Gaeumannomyces graminis* var. *tritici* (*Ggt*) isolates and other fungi were amplified using only the *Ggt*-specific primer (panel A) and all three variety-specific primers (*G. graminis* vars. *avenae, Gga*; *graminis, Ggg*; and *Ggt*) (panel B). Lane 1 contains a 100-bp DNA ladder as a molecular weight standard. For both panels, lanes 2 to 6 contain DNAs from *Ggt* isolates 3053, 3055, 3056, 3060, and 3066, respectively. Lanes 7 to 9 contain DNA from, respectively, *Gaeumannomyces cylindrosporus* ATCC 64420, *Phialophora radicicola* ATCC 64414, and *Phialophora* sp. Lane 10 contains DNA from *Cercospora zeae-maydis* F001, a fungus not related to the *Gaeumannomyces-Phialophora* complex. Lane 11 contains no DNA. Ethidium bromide-stained, variety-specific PCR products for *Ggg,* 1,086 bp, and *Ggt,* 870 bp, were visualized by fluorescence with ultraviolet light.

(panel B of Figures 4 and 5). These six isolates also produced *Ggg*-specific fragments when amplified with only *Ggg*-specific primer (data not shown). The experiments shown in Figures 4 and 5 were repeated with similar results.

To further characterize these atypical *Ggt* isolates, we studied restriction endonuclease digestion profiles of PCR products from the avenacinase-like genes. The predicted restriction endonuclease digestion sites of the partial avenacinase-like gene (1.4 kb) from one atypical *Ggt* isolate ATCC 28230 were identical to *Ggg* isolate ATCC 12761 (Fig. 1). Partial avenacinase-like gene PCR products (1.4 kb) from three of six atypical *Ggt* isolates, ATCC 28230, CK1a, and CHe1, were digested with the restriction endonucleases *Alu*I, *Msp*I, and *Hae*III and separated and visualized as described above (see Figure 1 for restriction sites). Comparison with *Ggt* isolates CD1, CB1, and M1 indicated that the atypical *Ggt* isolates, ATCC 28230, CK1a, and CHe1, had restriction fragment profiles identical with those of *Ggg* wheat isolate ATCC 12761 (results not presented). Results in Figure 5 (panels A and B) confirmed that our primers were specific for amplification only to take-all pathogens of *G. graminis*. No PCR products were detected from DNA of *G. cylindrosporus, P. radicicola* (considered an anamorph of *Ggg*), *Phialophora* sp., or *C. zeae-maydis* (DNA supplied by VKS).

Similar PCR-based differentiation tests were achieved with all of the *Gga* and *Ggg* isolates listed in Table 1. All of the *Gga* isolates produced *Gga*-specific fragments when amplified with *Gga*-specific primer alone (Fig. 6, panel A). Unexpectedly, when all three primers were added to each sample, one isolate, RB-W, produced two fragments of 870 and 617 bp, specific for *Ggt* and *Gga*, respectively (Fig. 6, panel B). This experiment was repeated with similar results for isolate RB-W. *Ggg* isolates from other plant species (Table 1) all amplified the 1,086-bp band but less efficiently than ATCC 12761 isolated from wheat. *Ggg* isolate, FL-19, which was isolated from bermudagrass (*Cyanodon dactylon* L.), generated a PCR product of 300 bp that was not related by nucleotide sequence to any of the variety-specific fragments (data not shown). More *Ggg* isolates from wheat and other host plants should be tested before the significance of nontypical bands can be addressed. DNA sequences of *Ggt-, Gga-,* and *Ggg*-specific PCR fragments demonstrated that both *Ggt*- and *Ggg*-specific fragments were 100% identical to their avenacinase-like genes. However, the *Gga*-specific fragment showed slight divergence (10 bp) from the published sequence of the avenacinase gene. This is not unexpected since *Gga* isolate ATCC 15419 used in this study was not the same isolate sequenced by Osbourn et al. (30).

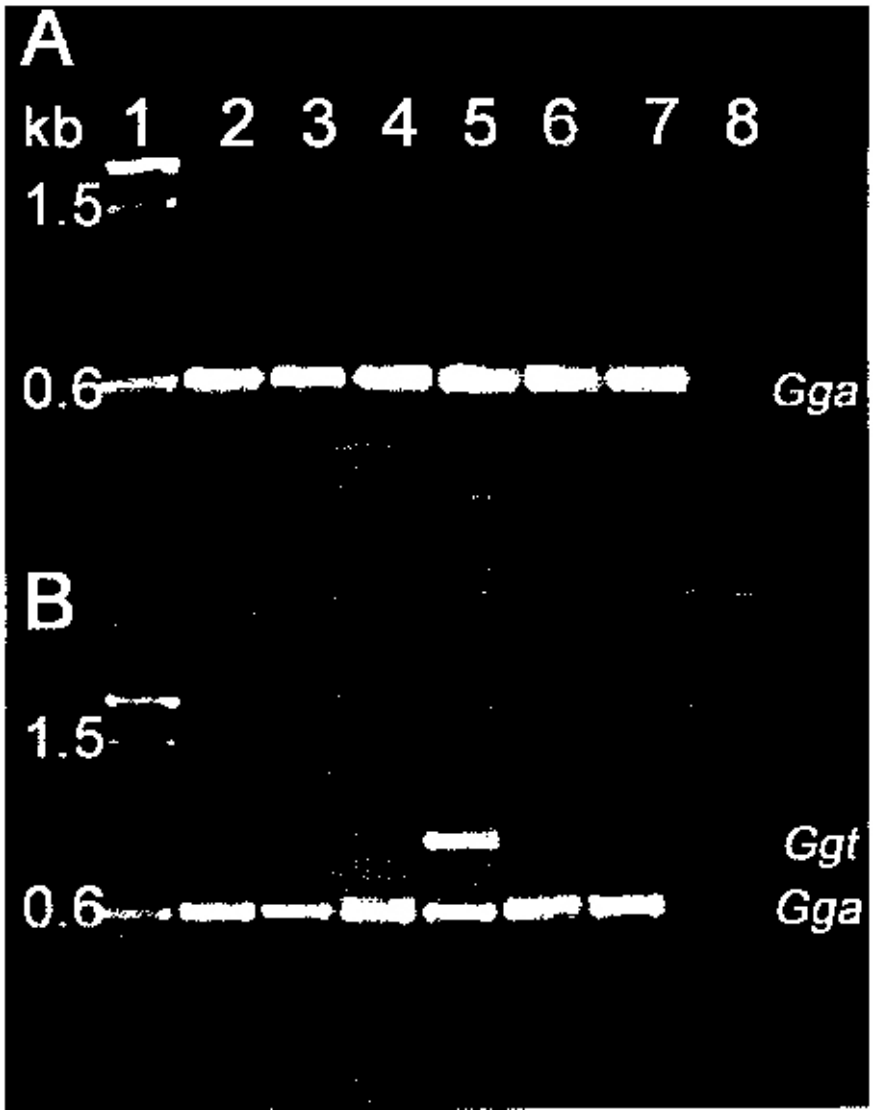


Fig. 6. Survey of *Gaeumannomyces graminis* var. *avenae* (*Gga*) isolates. *Gga* isolates with **A,** only the *Gga*-specific primer, and **B,** all three variety-specific primers. Ethidium bromide-stained variety-specific polymerase chain reaction (PCR) products (*Gga,* 617 bp and *G. graminis* var. *tritici* [*Ggt*], 870 bp) were visualized by fluorescence with ultraviolet light. Lane 1 contains 100-bp DNA ladder as a molecular weight standard. For both panels, lanes 2 to 8 contain DNA from *Gga* isolates ATCC 15419, FR-W, PG-W, RB-W, RI-W, WW-W, and no DNA, respectively.

DISCUSSION

We have identified large fragments (1.4 kb) of genes for *Ggt* and *Ggg* that are very similar to *Gga* avenacinase with less than 50 nucleotide base differences (95% identity). This is the first report of partial DNA sequences for avenacinase-like genes in *Ggt* and *Ggg* isolates, although Osbourn et al. (30) briefly described the presence of avenacinase-like proteins produced by *Ggt* and *Ggg*. In addition, BLAST search results strongly indicated that avenacinase-like gene fragments closely resembled the *Gga* avenacinase gene in GenBank. We also show that the avenacinase-like genes of *Ggt* and *Ggg* are present as single copies as Bowyer et al. (3) found for avenacinase in *Gga.* Based on DNA sequence alignment products, *Ggt* isolates are more closely related to *Ggg* (99.2% identity) than Gga (94.6%). We speculate that the ancestor of *Gga, Ggg,* and *Ggt* might have had an avenacinase-like gene. In the coevolution of *Ggt* and *Ggg* with their hosts, there was presumably no selective pressure on avenacinase-like genes since their hosts lacked avenacin. However, in the coevolution of *Gga* with oats, the fungus evolved avenacinase from an avenacinase-like gene template for successful pathogenesis and survival. Avenacinase-like proteins may simply be β-glucosidases required for fungal nutrition, or they may play a role in detoxification of saponins during pathogenesis in other plants (3). 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.

PCR technology offers a number of advantages over conventional methods for differentiation of take-all–causing varieties of *G. graminis*. Several groups have developed PCR-based methods for differentiation of varieties of *G. graminis*; however, most were based on amplification of the conserved regions of the ITS or 18S rDNA with either specific or random primers (4,14–16,37). In contrast, we demonstrate a PCR differentiation method based on a single gene target that is simple and specific for three varieties of take-all pathogens: *G. graminis* vars. *avenae*, *graminis*, and *tritici*.

Specificity of the PCR identification of *Ggt, Gga,* and *Ggg* is based on nucleotide sequence differences among the genes encoding avenacinase or avenacinase-like proteins. Three upstream primers, annealing in the 5′ half of the genes, provide variety specificity by incorporating one to two mismatched bases at their 3′ end which do not anneal to the sequences of the nontarget varieties. A single common downstream primer, closer to the 3′ end of the genes, was paired with each of the variety-specific primers. Bryan et al. (4) successfully used similar primer design to amplify *Gga*- and *Ggt*-specific products from the ITS regions of rDNA.

An important feature of our differential identification test is that all three variety-specific primers may be used in a single PCR reaction. In samples that amplify multiple bands, retesting with individual primer sets will eliminate the possibility of false-positive results. It should be noted that, based on PCR products from avenacinase-like genes, some fungi isolated from wheat tissues showing characteristic symptoms of take-all may be classified as *Ggg*, a common saprophyte or a weak pathogen on wheat. It is difficult to differentiate between *Ggt* and *Ggg* based on cultural appearance, especially since both of them grow on selective medium for *Ggt*. Use of ascospore size, perithecial characters, and hyphopodia shape to differentiate among these varieties is difficult because overlaps occur among all these traits (20,35,38). Although mycological characters (ascospore, perithecia, and hyphopodia) were not part of this work, in a separate project in our laboratories, several of the strains used in this study were compared directly (8). Perithecial, ascospore, and hyphopodial type (nonlobed in all cases) differences among putative wheat *Ggt* isolates, ATTC 28230, CB1, CD1, CK1a, CHe1, and M1, were not great enough to suggest identification as *Ggg* rather than *Ggt*. However, in pathogenicity studies, *Ggt* isolates CD1, CB1, and M1, producing 870-bp PCR fragments, caused moderate to severe damage on wheat while *Ggt* isolates, ATCC 28230, CK1a, and CHe1, producing 1,086-bp PCR fragments, caused mild damage (8). Our results confirm what others have stated or implied; it is difficult to differentiate between *Ggt* and *Ggg* (20,33–35,38). Although our survey, including 16 putative *Ggt* isolates and five putative *Ggg* isolates, is small for taxonomic purposes, it does suggest that identification difficulties may be a component in the variation of virulence among *Ggt* isolates.

Other closely related fungi such as *G. cylindrosporus*, *Phialophora* sp., and *P. radicicola* failed to amplify with any of the three variety-specific primers demonstrating primer specificity. This is one of the most important criteria for a test since these fungi are present together in the soil as a *Gaeumannomyces–Phialophora* complex (9,10). The *Gaeumannomyces–Phialophora* complex also includes other morphologically similar fungi that are nonpathogenic on cereal roots (34,35). *P. radicicola* produces lobed hyphopodia, while *Phialophora* spp. may produce either lobed or simple hyphopodia which cannot be distinguished from those of *Ggg*. Further confirmation with more isolates of *Ggt, Gga, Ggg, Phialophora* spp., and other closely related fungi such as *G. incrustans* and *Magnaporthe* spp. should be conducted to verify primer specificity.

The *Gga*- and *Ggt*-specific PCR products recovered from diverse isolates indicated a great deal of uniformity among *Gga* populations, except for *Gga* isolate RB-W. Using all three variety-specific primers, *Ggt*- and *Gga*-specific products were observed from isolate RB-W. When individual variety-specific primers were used with this isolate, expected PCR products were recovered with both *Gga*- and *Ggt*-specific primers. Two possible explanations exist. First, isolate RB-W may not be a pure culture, although it was cultured from a hyphal tip. Second, isolate RB-W may be a heterokaryon produced by anastomosis among *G. graminis* varieties. Further purification of the culture and sequencing of the products may determine the correct hypothesis.

The detection level for our diagnostic test was 100 pg for *Ggt* and *Ggg*, and 50 pg for *Gga*. In mixed DNA populations, PCR detection sensitivity decreased to 1 ng of total DNA of each variety (data not shown). These levels are consistent with sensitivity for direct detection of the fungi from diseased plant tissues and soil samples (19). Those authors were able to detect *Ggt* directly from diseased wheat roots and infested soil samples using slot-blot hybridization with a mitochondrial DNA probe. They were able to detect DNA levels as low as 100 and 30 pg in root and soil samples, respectively. In contrast, Hu et al. (22) showed that, with autoradiography, DNA of *Verticillium albo-atrum* (Rienke & Berthold) from inoculated alfalfa (*Medicago sativa* L.) could be detected by amplification of the ITS regions down to 10 pg.

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