Differentiation and Detection of Sugar Beet Fungal Pathogens Using PCR Amplification of Actin Coding Sequences and the ITS Region of the rRNA Gene

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ABSTRACT

Weiland, J. J., and Sundsbak, J. L. 2000. Differentiation and detection of sugar beet fungal pathogens using PCR amplification of actin coding sequences and the ITS region of the rRNA gene. *Plant Dis.* 84:475-482.

The DNA sequences of the actin genes of several fungi were compared and highly conserved regions in the coding sequence were identified. Deoxyoligonucleotide primers were synthesized based on conserved sequence blocks in the 5' and 3' ends of the open reading frame encoding the actin protein. In addition, primers (internal transcribed spacer [ITS] regions 1 and 4) based on conserved regions of the ribosomal RNA (rRNA) genes of fungi were synthesized. Use of the primers in the polymerase chain reaction (PCR) resulted in the amplification of DNA products from the genomes of sugar beet fungal pathogens of a size consistent with the amplification of the actin gene and rRNA gene sequences, respectively, in these fungi. With one primer pair (5FWDACT and MIDREVACT) directed to the actin gene, the major products amplified from the DNA of *Aphanomyces cochlioides*, *Pythium ultimum*, *Cercospora beticola*, *Phoma betae*, *Fusarium oxysporum*, and *Rhizoctonia solani* were of the sizes of 0.9, 0.9, 1.1, 1.1, 1.2 and 1.7 kilobase pairs (kbp), respectively, whereas no product was generated from the DNA of sugar beet (*Beta vulgaris* L.). Restriction endonuclease digestion of products amplified using 5FWDACT and MIDREVACT permitted the differentiation of *A. cochlioides* from *A. euteiches*. Use of ITS1 and ITS4 in PCR reactions employing the same template DNAs and reaction conditions yielded single products of 0.7, 0.8, 0.5, 0.5, 0.6, and 0.7 kbp, respectively, as well as a 0.7-kbp product from DNA of sugar beet. The data indicate that actin and rRNA gene sequences are appropriate targets for the development of PCR-based strategies for distinguishing sugar beet fungal pathogens at the genus level. The presence of *A. cochlioides* DNA in extracts of diseased sugar beet seedlings was detected using PCR with primers 5FWDACT and MIDREVACT.

The yields of sugar beet can be compromised severely by diseases caused by fungal pathogens (4). This can result from poor stand establishment due to seedling pre- and post-emergence damping off, caused principally by *Pythium* spp., *Aphanomyces cochlioides*, and *Rhizoctonia solani*. Additionally, adult plant root rots, caused by *A. cochlioides* and *R. solani*, and leaf spot, caused by *Cercospora beticola*, can reduce sugar beet sucrose accumulation and tonnage (4). Seed treatments show effectiveness in the control of *Pythium* damping off and black-root seedling disease caused by *A. cochlioides* (2). Leaf-

spot disease continues to be controlled though foliar fungicide application, although resistance of the fungus to the benzimidazole fungicides and tolerance to triphenyltin hydroxide has been documented (3,26). No chemical means currently are implemented for controlling adult plant root rot in sugar beet, and plant resistance to root-rot pathogens has been difficult to incorporate into high-yielding varieties (4).

The accurate identification of pathogens that cause crop disease is critical to the implementation of successful disease-management strategies. Fungi infecting sugar beet often are readily identified by the asexual or sexual spores that they produce. Conidiophores and conidia of *C. beticola* can be seen within lesions on infected leaves using light microscopy (4,26), although lesions devoid of these structures might be confused with leaf spot caused by *Phoma betae* and *Ramularia beticola* (25). Fungal pathogens causing damping off and root rot of sugar beet are not so easily distinguished, however. Detection methods usually require culture of the diseased tissue to permit growth of the disease-causing organism, followed by identification of the organism by microscopy. This method can lead to the misidentification of a fungus

due to lack of a distinguishing asexual or sexual spore type and can be confounded by the presence of saprophytic organisms that may outgrow the pathogen in culture.

Interests in our laboratory include the development of more sensitive and rapid methods for the detection and differentiation of sugar beet fungal pathogens, as well as the functional analysis of sugar beet pathogenesis by fungi. To this end, we are engaged in experiments aimed at cloning and characterizing the actin genes from the fungi *C. beticola*, *A. cochlioides*, and *Rhizoctonia solani*. The gene for actin is conserved throughout the eukaryota; therefore, it has received attention as a "molecular clock" gene for studies examining organism phylogeny (16). Typically, actin is a highly expressed gene, making the promoter of this gene valuable for the expression of heterologous genes in gene fusions (13). The rRNA genes of organisms also have been used in phylogenetic studies, although they have been less useful for the expression of foreign genes (14). Examination of polymorphisms in amplified DNA of the internal transcribed spacer (ITS) region of rRNA genes in the present study provides a benchmark against which diversity in the actin gene can be compared (24). Genes encoding rRNA have been used extensively in polymerase chain reaction (PCR) tests designed to differentiate fungal phytopathogens (5,6,8-10,12,18,20-22).

The current study was undertaken to determine whether common fungal pathogens of sugar beet could be distinguished by PCR using primers directed to the coding region of the actin gene and the ITS region of the rRNA genes. The ability to detect *A. cochlioides* in infected sugar beet seedlings also was investigated. Information generated from the study would be useful in the development of PCR-based disease diagnostics and in the application of DNA sequence data to fungal phylogenetics.

MATERIALS AND METHODS

Fungal isolates and primer design. Fungal isolates were maintained on potato dextrose agar (PDA), except for isolates of *A. cochlioides* and *A. euteiches*, which were maintained on cornmeal agar plates and stored in oatmeal broth (Table 1). Other fungi used in the study were stored on sterile barley grain at -80°C to minimize genotype changes that might occur

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Accepted for publication 5 January 2000.

Publication no. D-2000-0218-02R

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from excessive isolate transfer (19). Growing cultures were incubated in the dark at 22°C. Pathogenicity of fungal isolates, except for *A. euteiches*, on sugar beet was confirmed using standard inoculation procedures (19); pathogenicity of *A. euteiches* isolates was confirmed on pea (*Pisum sativum* cv. Wando).

Nucleotide sequences of the actin genes of *Saccharomyces cerevisiae*, *Phytophthora infestans*, *Trichoderma reesei*, *Aspergillus nidulans*, and *Puccinia graminis* were obtained from the Genbank sequence database. Positioning of conserved amino-acid sequences encoded by actin genes (16) onto the gene sequences from these fungi permitted the identification of nucleotide sequences conserved amongst these genes (Fig. 1). Consensus sequences for each of four regions in the actin structural gene were generated and primers were designed based upon these sequences. Primers 5FWDact and MIDFWDact were designed to be homologous to the sequence blocks near the 5' end of the coding strand of the gene, whereas primers MIDREVact and 3REVact were designed to be complementary to the sequence blocks near the 3' end of the coding strand of the gene (Fig. 1). Primers ITS1 (5'TCCG-TAGGTGAACCTGCGG3') and ITS4 (5'TCCTCCGCTTATTGATATGC3') are directed to conserved regions in rRNA genes of fungi (Fig. 1B; 24).

DNA preparation and PCR. Fungal mycelium was grown to near confluency on PDA or corn meal agar plates. Sterile

potato dextrose broth (PDB) was poured onto the mycelial mat and the mycelium was scraped from the agar surface using a sterile razor blade. The suspension was transferred to a sterile Petri dish and incubated at 22°C in the dark for 20 h. Mycelia were captured by filtration of the suspension through Miracloth (Calbiochem, La Jolla, CA). After several rinses with distilled, sterile water, mycelia were transferred to a mortar containing quartz sand (-50+70 mesh, Sigma-Aldrich, St. Louis). Preparation of fungal DNA was according to Weiland (23). Extraction buffer (0.1 M TRIS-HCl, pH 8.0, 20 mM Na₂EDTA, 0.5 M NaCl, and 1% sodium dodecylsulfate) was added to the mortar and the mixture was homogenized. The homogenate was extracted with buffer saturated phenol:chloroform:isoamyl alcohol (25:24:1) and the aqueous phase was precipitated with isopropanol. The precipitate was collected by centrifugation and the pellet was washed with 95% ethanol and dried in vacuo. The pellet was resuspended in 10 mM TRIS-HCl, pH 8.0, 1.0 mM Na₂EDTA (TE), including ribonuclease A at 20 µg/ml, and was incubated for 30 min at 37°C. After a second extraction with phenol:chloroform:isoamyl alcohol (25:24:1), the DNA was precipitated from the aqueous phase using ethanol and ammonium acetate and the precipitate again was collected by centrifugation. The final DNA pellet was washed with 95% ethanol, dried, and dissolved in 0.1 ml of TE.

Fungal DNA preparations for use in PCR were adjusted to a concentration of 1.0 µg/ml and oligonucleotide primers were diluted to 10 µg/ml. PCR reactions, optimized according to the recommendations of Innis and Gelfand (7), were performed in a volume of 50 µl and included 10 ng of fungal DNA, 50 ng of each forward and reverse primer, 10 mM TRIS-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 1.5 mM MgCl₂, 0.1 mM each of d(G,A,T,C)TP, and 2 units of *Taq* DNA polymerase (Promega Corp., Madison, WI). Samples were incubated in a thermocycler (Model PTC100V, MJ Research, Watertown, MA) for 40 cycles with cycling parameters of 94°C for 1 min, 45 to 55°C for 1 min, and 72°C for 2 min. Reactions were terminated by the addition of loading buffer (10× = 0.01% bromophenol blue, 50% glycerol, 0.5 M TRIS, pH 8.5, 0.5 M boric acid, and 10 mM EDTA), except for cases where samples were digested with restriction endonucleases prior to electrophoresis. A total of 10% of each PCR reaction was loaded onto a 1% agarose gel containing TRIS-borate-EDTA (TBE) electrophoresis buffer (1) and electrophoresed at 10V/cm.

Amplified products were digested with restriction endonucleases by combining 10 µl of each PCR reaction, 2 units of each of the enzymes *AluI*, *HaeIII*, and *MspI* (American Allied Biochemical, Aurora, CO), and 2 µl of restriction enzyme buffer (10 mM TRIS-HCl, pH 7.8, 10 mM MgCl₂, 50 mM NaCl, and 1 mM β-mercaptoethanol), and adjusting the solution to 20 µl. The digests were incubated for 2 h at 37°C, terminated by the addition of 2.3 µl of 10× loading buffer, and the products were separated on a 6% polyacrylamide gel using 1× TBE electrophoresis buffer (1). Gels were photographed using an AlphaImaging Chemimager 4000 gel documentation system.

Plant inoculation and disease diagnosis. Sugar beet seed of cv. Ultramono was seeded in 6-inch pots of peat-perlite (Sunshine Mix #1) at a rate of 5 seeds/pot. At 7 days post-planting, an agar plug containing a fresh culture of *Aphanomyces cochlioides* was placed just below the soil surface and next to the seedlings. Seedlings were watered twice daily with tap water after the day of inoculation. By 4 to 7 days post-inoculation, symptoms of seedling black root were observed.

Extracts from diseased and uninoculated (healthy) seedlings were prepared based on a modification of the procedure by Saghai-Marouf et al. (17) by surface sterilizing the excised seedling hypocotyl and boiling for 10 min in CTAB extraction buffer (100 mM TRIS-HCl, pH 8.0, 2% [wt/vol] cetyltrimethylammonium bromide, 1.4 M NaCl, 20 mM EDTA, and 1% [wt/vol] soluble polyvinylpyrrolidone) at a rate of 1 ml of buffer/4 excised hypocotyls. The solution was extracted once with an equal

Table 1. Isolates of fungal pathogens used in the development of a polymerase chain reaction differentiation assay

Fungal pathogen	Isolate	Bait plant	Region	Source
<i>Aphanomyces cochlioides</i>	17-1z	Sugar beet	Minnesota	C. Windels
	19-1z	Sugar beet	Minnesota	C. Windels
	20-1z	Sugar beet	Minnesota	C. Windels
	Russ 2A	Sugar beet	Michigan	J. Halloin
	Bera 3A	Sugar beet	Michigan	J. Halloin
<i>A. euteiches</i>	Bob 24	Sugar beet	Michigan	J. Halloin
	174	Pea	Minnesota	C. Windels
	467	Pea	Minnesota	C. Windels
	MM35	Pea	Minnesota	C. Windels
<i>Cercospora beticola</i>	98-23	Sugar beet	North Dakota	W. Bugbee
	898A	Sugar beet	Minnesota	J. Weiland
	898B	Sugar beet	Minnesota	J. Weiland
	898C	Sugar beet	Minnesota	J. Weiland
<i>Fusarium oxysporum</i>	898A	Sugar beet	Minnesota	J. Weiland
	898B	Sugar beet	Minnesota	J. Weiland
	898C	Sugar beet	Minnesota	J. Weiland
	898C	Sugar beet	Minnesota	J. Weiland
<i>Phoma betae</i>	11A11	Sugar beet	Minnesota	W. Bugbee
	3A15	Sugar beet	Minnesota	W. Bugbee
	10A13	Sugar beet	Minnesota	W. Bugbee
	1098A	Sugar beet	Montana	J. Weiland
	1098B	Sugar beet	Montana	J. Weiland
<i>Pythium ultimum</i>	1098C	Sugar beet	Montana	J. Weiland
	898A	(Soil)	Minnesota	J. Weiland
	898B	(Soil)	Minnesota	J. Weiland
	898C	(Soil)	Minnesota	J. Weiland
	"heterothallic"	Potato	North Dakota	B. Salas
<i>Rhizoctonia solani</i> AG2-2	153-7	Potato	North Dakota	B. Salas
	2A13	Sugar beet	Minnesota	W. Bugbee
	R9	Sugar beet	Colorado	E. Ruppel
	898A	Sugar beet	Minnesota	J. Weiland
	898B	Sugar beet	Minnesota	J. Weiland

A

5' end of actin coding sequence:

	"5FWDACT"
	5' GT ATG TGC AAG GCC GGT TT 3' 19-mer
P.inf	TCC GGT ATG TGC AAG GCC GGT TTC
A.nid	TCG GGT ATG TGC AAG GCC GGT TTC
T.rees	TCT GGT ATG TGC AAG GCC GGT TTC
S.cerv	TCT GGT ATG TGT AAA GCC GGT TTT
P.gram	TCG GGT ATG TGC AAA GCC GGT TTC

	"MIDFWDACT"
	5' TGG CAC CAC ACC TTC TAC AA 3' 20-mer
P.inf	ATC TGG CAC CAC ACC TTC TAC AAC GAG CTG CGT
A.nid	ATT TGG CAC CAC ACA TTC TAC AAC GAG CTT CGT
T.rees	ATC TGG CAC CAC ACC TTC TAC AAT GAG CTG CGT
S.cerv	ATC TGG CAT CAT ACC TTC TAC AAC GAA TTG AGA
P.gram	ATC TGG CAT CAC ACC TTC TAC AAC GAG CTC CGA

3' end of actin coding sequence:

P.infe	AAG TAC TCG GTC TGG ATC GGT GGT TCC ATC
A.nidu	AAA TAC TCC GTC TGG ATC GGT GGT TCC ATC
T.rees	AAG TAC TCT GTC TGG ATC GGT GGT TCC ATT
S.cerv	AAG TAC TCC GTC TGG ATT GGT GGT TCT ATC
P.gram	AAA TAC TCC GTA TGG ATC GGT GGT TCT ATC
23-mer	3' ATG AGG CAG ACC TAG CCA CCA AG 5'
	"MIDREVACT"

P.inf	TCG ATC GTG CAC CGC AAG TGC TTC TAA G
A.nid	TCG ATC GTC CAC CGC AAG TGC TTC TAA G
T.rees	AGT ATT GTC CAC CAC AAG TGC TTC TAA G
S.cerv	TCT ATC GTT CAC CAC AAG TGT TTC TAA T
P.gram	TCG ATC GTC CAT CGT AAA TGT TTC TAA G
19-mer	3' GTG GCG TTC ACG AAG ATT C 5'
	"3REVACT"

B

Fig. 1. (A) Alignment of actin gene sequences from selected filamentous fungi and design of primers for the polymerase chain reaction. Deduced protein sequences from gene sequences were visually aligned according to highly conserved regions of actin across eukaryotes. The DNA sequences in these regions then were aligned and a consensus sequence, derived from this alignment, was used as the basis for primer design (bold type). Primer designation is presented above and below the primer sequence. **(B)** Diagrammatic representation of location of primers on a generic actin gene and rRNA gene. Most fungal actin genes contain introns which are not shown here (16). Regions of the primary rRNA removed by splicing within the internal transcribed spacer regions are depicted as lines; shaded boxes represent sequences that become the mature rRNAs (nls = nuclear large subunit, nss = nuclear small subunit).

volume of chloroform:isoamyl alcohol (24:1) and was precipitated with 0.6 vol. of isopropanol. The final nucleic acid pellet was washed with 95% ethanol, dried, and resuspended in 50 µl of TE. The preparation (1 µl) was used as template in the PCR reaction using the conditions described above and the primers 5FWDACT and MIDREVACT.

RESULTS AND DISCUSSION

Amplification of actin gene sequences.

Initial experiments performed with primers directed to the actin gene indicated that an annealing temperature of 55°C during PCR permitted the amplification of DNA products from genomic DNA from most of the fungi examined without the amplification of products from sugar beet genomic DNA. A 50°C annealing temperature was necessary for the consistent generation of amplified DNA by PCR from the genomic DNA

of *Rhizoctonia solani*, although DNA products were amplified from genomic DNA of sugar beet in some of the experimental replications at this annealing temperature. The PCR conditions for the differentiation of fungi from pure culture using actin-based primers, therefore, employed a 50°C annealing temperature, whereas a 55°C annealing temperature was used in the detection of *A. cochlidioides* in diseased seedlings. Of the actin-based primers tested, primers 5FWDACT and MIDREVACT gave the most consistent amplification products in these tests; only results using this pair of actin-based primers are presented.

A single DNA product was amplified from the genomic DNA of *Pythium ultimum*, *C. beticola*, and *Phoma betae* when using primers 5FWDACT and MIDREVACT (Fig. 2A). The amplification of *Fusarium oxysporum* DNA generated a

major product at 1.2 kilobase pairs (kbp), with weakly amplified products at 0.7 and 0.6 kbp. Whereas only one weakly amplified product (0.4 kbp) was generated from *A. cochlidioides* DNA in addition to the major 0.9-kbp product, weak products of sizes 0.2, 0.5, 0.8, and 1.6 kbp were amplified from two *R. solani* (898A and 898B) isolates and products of sizes 0.4, 0.7, and 1.6 kbp were amplified from a third *R. solani* (2A13) isolate. From the examination of several cloned actin genes, the coding region for actin appears to be minimally around 1.0 kbp (16). The faster-migrating products of the PCR reactions, therefore, could be the result of mispriming by the actin gene-based primers or of correct priming on actin pseudogene or other sequences. Nevertheless, the application of PCR using 5FWDACT and MIDREVACT to the fungi examined here resulted in the generation of DNA products of differing

Fig. 2. (A) Amplification of DNA from the genomes of representative sugar beet fungal pathogens with actin gene-specific primers and (B) analysis of restriction enzyme digestion products. Oligonucleotide primers 5FWDACT and MIDREVACT were used in all reactions. Reactions were performed in duplicate and a sample of the products was analyzed by agarose gel electrophoresis (A). In A, the 1-kilobase-pair (kbp) ladder (Life Technologies, Gaithersburg, MD) was co-electrophoresed as a size standard (Marker). Abbreviations for lane labeling are F.o. (*Fusarium oxysporum*), R.s. (*Rhizoctonia solani*), P.u. (*Pythium ultimum*), A.c. (*Aphanomyces cochlidioides*), C.b. (*Cercospora beticola*), and P.b. (*Phoma betae*) with isolate designators included directly above each lane. In B, a subset of the products shown in A were subjected to digestion with a restriction endonuclease cocktail containing *AluI*, *HaeIII*, and *MspI* prior to electrophoresis on a 6% polyacrylamide gel. The 100-bp ladder was co-electrophoresed as a size standard (Marker).

molecular size, enabling these pathogens to be distinguished. Sequence analysis of the 1.6-kb product generated from *R. solani* DNA should yield information permitting the design of primers for the optimal amplification of the actin gene in this organism. Sequence comparison analysis of DNA amplified in our laboratory from *A. cochlioides* and the fungal entomopathogen *Metarhizium anisoplae* using the primers 5FWDACT and MIDREVACT indicates that the DNA amplified in this study is, indeed, that coding for the actin protein (not shown).

For most of the PCR tests described above, products amplified with primer pair 5FWDACT and MIDREVACT differed in their migration after electrophoresis between the organisms tested (Fig. 2A). The major product amplified from the genomic DNA of *C. beticola*, however, was of a similar size to that amplified from the genomic DNA of *P. betae*. Likewise, the PCR product generated from the genomic

DNA of *Pythium ultimum* was of a similar size to that generated from *A. cochlioides* DNA. Sugar beet leaf spot might be misdiagnosed as being caused by *C. beticola* in instances where *Phoma betae* is the causal agent. Likewise, seedling damping off caused by *A. cochlioides* might be misdiagnosed as Pythium damping off. Additional sequence differences in the actin genes of the respective pathogen pairs need to be revealed, therefore, if the PCR assay is to be sufficiently discriminating.

The digestion of the 1.2-kbp actin gene products common to *C. beticola* and *P. betae* with a mixture of the restriction endonucleases *Alu1*, *Msp1*, and *HaeIII* yielded polymorphisms that permitted the differentiation of these two pathogens based on the isolates examined (Fig. 2B). Electrophoretic patterns generated by restriction digest of amplified products were consistent between the isolates of the fungal genus under examination. A total of seven and eight restriction-enzyme prod-

ucts generated from the digestion of the 1.2-kbp product characterizing *C. beticola* and *P. betae*, respectively, were resolved by native polyacrylamide gel electrophoresis. Five of the digestion products from each of the pathogens had a similar migration in the gel. Two of the digestion products originating from the amplification of *C. beticola* actin gene sequences were absent from that of *P. betae*, whereas three of the digestion products originating from the amplification of *P. betae* actin gene sequences were absent from that of *C. beticola*.

The digestion with *Alu1*, *Msp1*, and *HaeIII* of the 0.9-kbp product common to the amplified actin gene sequences of *A. cochlioides* and *Pythium ultimum* yielded distinguishing DNA banding patterns to a greater degree than that in the comparison of *C. beticola* and *Phoma betae*. Digestion of the 0.9-kbp product yielded eight and six bands resolved by electrophoresis for *Pythium ultimum* and *A. cochlioides*, re-

Fig. 3. (A) Amplification of DNA from the genomes of several sugar beet fungal pathogens with rRNA gene-specific primers and (B) analysis of restriction enzyme digestion products. Oligonucleotide primers ITS 1 and 4 were used in the amplification. Reactions were performed in duplicate and a sample of the products was analyzed by agarose gel electrophoresis (A). Abbreviations for lane labeling are F.o. (*Fusarium oxysporum*), R.s. (*Rhizoctonia solani*), P.u. (*Pythium ultimum*), A.c. (*Aphanomyces cochlioides*), C.b. (*Cercospora beticola*), P.b. (*Phoma betae*), and B.v. for sugar beet (*Beta vulgaris* L.) DNA, with isolate designators included directly above each lane. In B, a subset of the products shown in A were digested with a restriction endonuclease mixture of *Alu1*, *HaeIII*, and *Msp1* prior to electrophoresis on a 6% polyacrylamide gel. For both A and B, the 100-base-pair ladder was co-electrophoresed as a size standard (Marker).

spectively. Only one of the bands generated (approximately 200 bp) appeared common to the two pathogens. Sequence polymorphisms in the actin gene within *P. ultimum* are suggested by the different bands observed for isolate 898B as compared to isolates 898A and 898C after restriction-enzyme digest of the PCR products (Fig. 2B). The additional products may represent amplification of a distinct haplotype of the actin gene in this isolate of *P. ultimum*. Nevertheless, the actin gene appears to possess sufficient sequence divergence between fungi of different genera for the development of PCR-based methods aimed at differentiating sugar beet fungal pathogens.

Amplification of rRNA gene sequences. Much focus in molecular phylogenetics has been placed on sequences of the ITS region of rRNA genes (14,24). Use of the primers ITS1 and ITS4 directed to the ITS region of the rRNA gene by PCR generated amplification products of 0.5 to 0.8 kbp for the sugar beet fungal pathogens examined in this study. Only the 50°C annealing temperature was used, which yielded abundant, discrete amplification products for all fungi tested. Approximate sizes of the products amplified from the ITS region were 0.5 kbp for *C. beticola*, 0.7 kbp for *A. cochlioides*, 0.5 kbp for *F. oxysporum*, 0.7 kbp for *R. solani*, 0.8 kbp for *P. ultimum*, 0.6 kbp for *Phoma betae*, and 0.7 kbp for sugar beet (*Beta vulgaris*) (Fig. 3A). Product sizes are consistent with those previously reported for the ITS re-

gions in other *Fusarium* spp. (18), as well as for *Pythium ultimum* (8) and *R. solani* (10). Based on the study by Ward et al. (22), amplification of the ITS region of *Polymyxa betae*, the fungal vector of *Beet necrotic yellow vein virus* (BNYVV; causal agent of beet rhizomania), would yield a product of approximately 0.5 kbp, which would be distinguishable from the ITS products generated from many of the fungi analyzed here. The 0.7-kbp product amplified from sugar beet DNA (Fig. 3A) agrees with the data presented by Ward et al. (22) using a similar primer pair.

As with products amplified from the actin gene using primers 5FWDCT and MIDREVACT, some of the products amplified from the rRNA gene ITS region of two different fungi were of a similar size (Fig. 3A). Digestion of these products with the endonuclease mixture of *AluI*, *HaeIII*, and *MspI* revealed the presence of nucleotide differences in these products. As above, patterns generated by restriction digest of amplified products were consistent between the isolates of the fungal genus being examined (*not shown*). Thus, the analysis of restriction fragment length polymorphisms of the products amplified by PCR using primers ITS1 and ITS4 give an additional level of resolution in the differentiation of sugar beet fungal pathogens. It should be noted that the ITS primers used in the present test would be of limited use in the detection of fungi within sugar beet, because a product of a size similar to that observed for the fungi tested

was amplified from uninfected sugar beet. Nevertheless, the amplification of DNA from this region followed by digestion with restriction endonucleases remains useful for the discrimination of pure isolates of the fungi investigated here and elsewhere (24).

Identification of *P. betae* isolated from field samples of diseased sugar beet. In October of 1998, diseased mature sugar beets were received from growers in the Yellowstone River valley of Montana. Petioles of the leaves had a blackened appearance and many of the older leaves had senesced. Surface-sterilized petiole sections were placed onto PDA. After 2 days, the hyphal tips of the fungus growing out of the petiole were transferred to PDB and to a fresh plate of PDA. At 3 days post-plating, DNA was prepared from the liquid culture of the fungus and PCR assays, using the primer pairs 5FWDCT-MIDREVACT and ITS1-ITS4 in separate reactions, were performed. By the evening of the same day, electrophoresis of the PCR products (*not shown*) combined with the disease symptoms suggested that the isolated organism was *Phoma betae*. At 4 days after plating of the petiole sections, restriction-enzyme digestion of the PCR products yielded patterns supporting the probability that the isolated fungus was *P. betae* (Fig. 4). At 10 days after the plating of the fungus on PDA, pycnidia were observed under the mycelia. Spore discharge from isolated pycnidia was characteristic of that described for *P. betae* (25). The example illustrates the usefulness of the information obtained using the PCR assay in generating rapid information leading to the identification of organisms associated with, or causing, a disease. The data presented further indicate the existence of sequence polymorphism at the actin locus within *P. betae* (Fig. 4).

Differentiation of *A. cochlioides* from *A. euteiches* and detection of *A. cochlioides* in diseased sugar beet seedlings. The pathogen *A. euteiches* can be found in association with legumes in regions where sugar beet is grown. A host-range bioassay is the principle means by which *A. cochlioides* and *A. euteiches* currently are differentiated (15), although random amplified polymorphic DNA analysis has permitted the separation of these organisms based on DNA fingerprinting as well (11). *A. euteiches* and *A. cochlioides* can induce low levels of disease in the laboratory in sugar beet and pea, respectively (11); therefore, it would be informative to determine to what extent these organisms are found in association with the respective non-host plant. In order to determine if amplification of the actin gene would provide a DNA product with sufficient divergence to permit the differentiation of these two pathogens, isolates of these two pathogens were subjected to the PCR assay. The results demonstrate that amplification of

Fig. 4. Native polyacrylamide gel (6%) of restriction endonuclease-digested products of amplified actin gene sequences from a fungus isolated from diseased sugar beet. Three isolates (1098A, 1098B, and 1098C), each from a separate, diseased sugar beet plant, were cultured in potato dextrose broth. Total DNA prepared from fungal mycelia was used as template in polymerase chain reactions that included primers 5FWDCT and MIDREVACT. The amplified product from each isolate of approximately 1.1 kilobase pairs (kbp) (*not shown*) was digested with a mixture of the restriction enzymes *AluI*, *HaeIII*, and *MspI*. Digestion products were fractionated by electrophoresis and the gel was photographed. The 100-bp ladder was co-electrophoresed as a size standard (Marker). Compare the DNA banding pattern to that generated from *Phoma betae* DNA in Figure 2B. Note the slightly faster migration of the approximately 0.1-kb product originating from isolate 1098B as compared to those originating from isolates 1098C and 1098A.

actin gene sequences by itself is insufficient for the differentiation of these species (Fig. 5, top). Restriction-enzyme digestion of the amplified products, however, clearly shows that *A. cochlioides* and *A. euteiches* can be differentiated based on DNA sequence differences in the actin gene (Fig. 5, lower panel).

An anticipated use of the PCR assay is in the rapid detection of *A. cochlioides*

within diseased sugar beet seedlings. To test the ability of the PCR assay to detect the presence of *A. cochlioides* DNA in an extract prepared from diseased seedlings, 1-week-old seedlings of sugar beet cv. Ultramono were either inoculated with *A. cochlioides* or left uninoculated. At 7 days post-inoculation, approximately 50% of the seedlings that had been inoculated with *A. cochlioides* began to show symptoms of

seedling black-root disease. No disease symptoms were observed on uninoculated seedlings. Although the method described by Henson et al. (6) was tested for the ability to yield DNA products by PCR from diseased sugar beet seedlings, results in our laboratory indicated that a contaminant was co-extracted with the nucleic acids that inhibited PCR amplification of DNA (*data not shown*). For this reason, the

Fig. 5. Differentiation of *Aphanomyces cochlioides* from *A. euteiches* based on actin gene sequence differences. In the top panel, products amplified from the genomic DNA of *A. cochlioides* and *A. euteiches* with primers 5FWDACT and MIDREVACT were separated on a 1% agarose gel. The 1-kilobase-pair (kbp)-ladder was co-electrophoresed as a size standard (Marker). In the lower panel, these same products were digested with a mixture of the restriction endonucleases *AluI*, *HaeIII*, and *MspI* before electrophoresis on a 6% native polyacrylamide gel. For the *A. cochlioides* isolates, only the digest of the products from isolate Russ 2A is shown. Compare the digestion pattern of Russ 2A and the *A. cochlioides* isolates in Figure 2 to that of the *A. euteiches* isolates in this panel. For both panels, the 1-kbp ladder was co-electrophoresed as a size standard (Marker).

method described by Saghai-Maroo et al. (17) was adapted to the procedure for fungal pathogen detection in diseased sugar beet. The presence of polyvinylpyrrolidone and cetyltrimethylammonium bromide during DNA extraction has been proposed to facilitate the removal of compounds that might inhibit DNA modifying enzymes (17).

Extracts were prepared from healthy and diseased seedlings from three independent experiments and the samples were subjected to the PCR assay using the primers 5FWDCT and MIDREVACT. The results of a typical experiment, in which the assay was applied to extracts from diseased tissue, clearly show amplification of DNA of a size consistent with the amplification of *A. cochlioides* actin DNA (Fig. 6). Restriction-enzyme digest of these products supported the contention that the amplified products contained *A. cochlioides* actin sequences (not shown). Using the annealing temperature of 55°C, no amplified products from healthy, uninoculated tissue were observed.

The data suggest that the primer sequences comprising 5FWDCT and MIDREVACT are useful for distinguishing fungal pathogen from host sugar beet tissue and for detecting *A. cochlioides* in diseased sugar beet seedlings. It is likely that diseased, field-grown sugar beet may often harbor fungal opportunists that confound that ability to diagnose that causal agent of disease; therefore, it is cautioned that the techniques described here at the present state of development be used to complement standard disease diagnosis. Nevertheless, the positive identification of a disease-causing organism in the diseased tissue is an important element of diagnosis

and can be facilitated by the PCR technology outlined. Future work will be directed at the design of specific primers, based on the sequence of the cloned actin gene-specific PCR products, for the detection of *A. cochlioides* in diseased, field-grown sugar beet, in non-diseased weed hosts, and in the soil.

ACKNOWLEDGMENT

We thank G. Nielsen for providing expert technical assistance. This work was supported in part by Beet Sugar Development Foundation Project no. 620 to JJW.

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Fig. 6. Detection of *Aphanomyces cochlioides* in diseased sugar beet seedlings using polymerase chain reaction (PCR) with primers 5FWDCT and MIDREVACT. Extracts were prepared from healthy and diseased seedlings 7 days post-inoculation. Each sample extract was prepared from 4 to 5 seedlings. Tests for the presence of *A. cochlioides* based on the amplification of the 0.9-kilobase-pair (kbp) actin-gene PCR product were performed in duplicate. The 1-kbp ladder was co-electrophoresed as a size standard (Marker).