Theor Appl Genet (1997) 95:474–478

L. A. Achenbach · J. A. Patrick · L. E. Gray

Genetic homogeneity among isolates of *Fusarium solani* that cause soybean sudden death syndrome

Received: 22 November 1996 / Accepted: 4 April 1997

Abstract *Fusarium solani* f. sp. *phaseoli* is the etiological agent of soybean sudden death syndrome (SDS). This form species includes both members that cause SDS and those that do not. Despite the extensive use of SDS isolates in soybean plant breeding studies, no information regarding genetic relatedness of isolates is available. Sequencing of the D2 region of the large-subunit (28S) ribosomal DNA of 19 isolates of *F. solani* f. sp. *phaseoli*, both SDS and non-SDS isolates, resulted in identical sequences and thus indicated a very low level of genetic variation within the form species. Sequencing of the ITS region resulted in low-level intra-individual as well as intra-specific variation. Random amplified polymorphic DNA (RAPD) analysis was used for a genome-wide estimate of genetic variation and was able to resolve only two amplitypes of the SDS isolates. Thus, SDS isolates from throughout the U.S. comprise an almost clonal population with an extremely low level of genetic variation among individuals.

Key words Ribosomal DNA · RAPD · *Fusarium solani* · Genetic variation · Fungi

Introduction

Fusarium solani (Mart.) Appel & Wollenw. emend. Snyd. & Hans., a common soilborne fungus, is the etiological agent of soybean sudden death syndrome (SDS) (Roy et al. 1989; Rupe 1989). Some characteristic symptoms of SDS are extensive root rot and interveinal necrosis of the leaves of infected soybean plants. *F. solani* isolates that cause SDS (SDS isolates) are commonly identified by morphological characteristics of the macroconidia and the absence of microconidia (Rupe 1989). However, greenhouse pathogenicity tests and/or molecular diagnostic analysis is required to differentiate the isolates that cause SDS from closely related isolates that do not cause the disease (Achenbach et al. 1996).

Because soybean plant breeders depend on stable, virulent SDS isolates to aid in the identification of SDS-resistant soybean cultivars, many SDS isolates have been collected and studied in those states in which SDS is a problem. Despite the extensive greenhouse work that has been done, races of SDS isolates have not been identified todate and little is known about the genotypic diversity of these pathogenic fungi. Information regarding genetic variation is important as the development of resistant soybean cultivars depends on an accurate assessment of the extent of genotypic differences among fungal SDS isolates between and within specific geographic regions.

Ribosomal RNA (rRNA) sequence analysis has been well-documented as a means of determining phylogenetic relationships in all of the major organismal domains. Variable sequences within the mature small-subunit (SSU) and large-subunit (LSU) rRNA genes have been found to be appropriate for assessing subgeneric relationships in many eukaryotes. One of these variable regions, the D2 region of the LSU, has been used to determine phylogenetic relationships in a number of pathogenic fungal genera (Logrieco et al. 1995). The ITS region of the rDNA operon, which is more variable than the D2 region, has proven useful in distinguishing relationships at the species level (Kusaba and Tsuge 1995).

Random amplified polymorphic DNA (RAPD) analysis is a polymerase chain reaction (PCR) technique

Communicated by G. Wenzel

L. A. Achenbach (✉) · J. A. Patrick
Department of Microbiology, Southern Illinois University, Carbondale, IL 62901, USA

L. E. Gray
USDA/ARS and Department of Plant Pathology, University of Illinois, Urbana, IL 61801, USA

that utilizes short, arbitrary oligonucleotides to generate amplification products that can be used as genetic markers (Williams et al. 1991). Because amplifications are performed on total genomic DNA, RAPD analysis can be used to assess genetic variation within the entire genome rather than variation within a single genetic region (e.g. rDNA). This characteristic of RAPD analysis has been exploited by researchers investigating genetic relatedness in a number of fungal species, often to the extent that race differences can be identified via RAPD markers (Crowhurst et al. 1991; Assigbetse et al. 1994). In the present paper, we assess the genetic variation among *F. solani* SDS isolates by sequencing of the D2 and ITS regions of the rDNA and by RAPD pattern analysis.

Materials and methods

Fungal cultures and genomic DNA isolation

The fungal isolates used in this study and their geographic origins are shown in Table 1. Each fungal culture was started from a single spore and was maintained on slants of Bilay's medium at 19°C (Booth 1971). The slant culture was used each time a liquid culture was started. Agar plugs were asceptically transferred to 50-ml aliquots of Potato Dextrose broth (Difco Laboratories) in 250-ml Erlenmeyer flasks. Cultures were grown at 28°C with shaking for 1–2 weeks. Mycelium was collected by vacuum filtration and DNA extractions were performed as described by Nickrent (1994).

D2 and ITS amplification, cloning, sequencing and analysis

Primer sequences for the D2 and ITS regions were D2F: 5′-GATGAAAAGAA-CTTTGAAAAGAGAG-3′, D2R: 5′-CTCCTTGGTCCGTGTTTCAAGACGG-3′, ITS5F: 5′-GGAAGTAAAAGTCGTAACAAGG-3′, ITS4R: 5′-TCCTCCGCTTATTGATATGC-3′. Amplification reactions were performed in 50-µl volumes containing 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 1.2 mM $MgCl_2$, 0.2 mM of each dATP, dCTP, dGTP and dTTP, 125 ng of primer, 1 unit of *Taq* polymerase, and approximately 10 ng of genomic DNA. The samples were overlaid with 30 µl of mineral oil. Amplifications were performed in a Perkin-Elmer-Cetus DNA Thermal Cycler programmed for the following parameters: 94°C for 3 min, followed by 35 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 2 min with a 2-s autoextension. Amplification products were gel-purified using a DEAE membrane and either cloned into vector pCRII (Invitrogen) or sequenced directly as previously described (Nickrent 1994). In addition to the two primers used for amplification, sequencing of the ITS employed two internal primers: ITS2R:

Table 1 Origins of the fungal isolates used in this study

Species	Isolate	Origin	SDS
Plectosphaerella cucumerina	22819(NRRL)[a]	Indiana	–
Nectria haematococca	22820(NRRL)	Indiana	–
Fusarium oxysporum f. sp. *phaseoli*	FO	–	–
Fusarium solani f. sp. *pisi*	T8	–	–
Fusarium solani	D1	Wisconsin	–
	D5	Wisconsin	–
	S1177	Australia	–
	B4-F2-13	Iowa	–
	IA-3	Iowa	–
	W8-2	Washington	–
	91-11-2A	North Dakota	–
	91-115-3A	North Dakota	–
Fusarium solani f. sp. *phaseoli*	Mont	Illinois	+
	RW-1	Illinois	+
	VR-309	Illinois	+
	St1-1	Illinois	+
	St90-1	Illinois	+
	Cora-2	Illinois	+
	IL isolates (40)	Illinois	+
	17-1	Arkansas	+
	171	Arkansas	+
	269	Arkansas	+
	Mo1792	Missouri	+
	FSA-1	Mississippi	+
	KS	Kansas	+
	Wf1-19-3	Iowa	+
	22823(NRRL)	Indiana	+
	22825(NRRL)	Indiana	+
	Boyer isolates (30)	Illinois	+
	Ice isolates (30)	Illinois	+
	18006(ATCC)[b]	California	–
	38135(ATCC)	Washington	–
	42361(ATCC)	–	–

[a] NRRL numbers correspond to cultures from the USDA/ARS NCAUR, Peoria Ill. and were originally designated by Abney et al. (1993) and cross-referenced in O'Donnell and Gray (1995)
[b] ATCC isolates are from the American Type Culture Collection, Rockville, Md.

Table 2 Primers used for RAPD analysis in this study

Code	Sequence
RC09[a]	GATAACGCAC
OPA-01[b]	CAGGCCCTTC
OPA-19	CAAACGTCGG
OPB-05	TGCGCCCTTC
OPG-06	GTGCCTAACC
OPL-12	GGGCGGTACT
OPN-13	AGCGTCACTC

[a] Referenced in Crowhurst et al. (1991)
[b] OP primers were purchased from Operon Technologies (Alameda, Calif.)

5′-GCTGCGTTCTTCATCGATGC-3′ and ITS3F: 5′-GCATCGATGAAGAACGCAGC-3′. Sequence alignments were done manually using the UNIX-based Genetic Data Environment (GDE) sequence analysis program (S. Smith, University of Illinois) on a SUN SparcStation (alignments available on request).

RAPD primers and amplification conditions

RAPD primers used in this study are listed in Table 2. All primers were diluted to 20 ng/ µl. Amplification reactions were performed in 25-µl volumes containing 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 1.2 mM $MgCl_2$, 0.2 mM of each dATP, dCTP, dGTP and dTTP, 20 ng of primer, 0.5 units of *Taq* polymerase, and approximately 100 ng of genomic DNA. The samples were overlaid with 30 µl of mineral oil. Amplifications were performed in a Perkin-Elmer-Cetus DNA Thermal Cycler programmed for the following parameters: 94°C for 3 min, followed by 45 cycles of 94°C for 1 min, 36°C for 1 min, 72°C for 2 min, and a final incubation at 72°C for 10 min. Amplification products (6 µl of a 25-µl reaction) were electrophoresed in 0.7% agarose gels with TBE running buffer, stained with ethidium bromide and either scanned into a computer imaging file or photographed (Sambrook et al. 1989). Computer scanning produced images with better resolution and, thus, all conclusions were based on analysis of the scanned images.

Results

Amplification of fungal genomic DNAs using D2-specific primers produced a single amplification product of approximately 250 bp in all fungal isolates tested. Sequencing resulted in 211 base pairs (bp) which comprise the entire D2 region of the SDS isolates and close relatives. Examination of D2 sequences reported from other *Fusarium* species revealed sequences longer than 211 bp for the D2 region. However, folding of the D2 sequence from the SDS isolates into a secondary structure conformation following the model of Gutell et al. (1993) for *Cryptococcus neoformans* confirmed that the 211 bp comprised the entire D2 region as first described by Michot et al. (1984) for mouse nuclear LSU. (Secondary structure information of the *F. solani* D2 region is available on request.)

Sequences of the D2 region of the large-subunit (28S) ribosomal DNA were generated from 31 fungal isolates including 16 SDS isolates, three *F. solani* f. sp. *phaseoli* isolates that do not cause SDS, *F. oxysporum*, *Nectria haematococca* MPVI, *F. solani* f. sp. *pisi* , eight *F. solani* isolates and *Plectosphaerella cucumerina*. There was 100% sequence identity among the 16 SDS and three non-SDS *F. solani* f. sp. *phaseoli* isolates. There was also 100% sequence identity between isolates 22820, T8, D1, D5, 91-11-2A and 91-115-3A. Thus, the D2 region contains insufficient sequence variation to distinguish isolates within a single form species. However since there was 94.3% sequence identity between the *F. solani* f. sp. *phaseoli* clade and *F. solani* f. sp. *pisi*, and less than 90% sequence similarity between these *F. solani* sequences and the *F. oxysporum* sequence, the D2 region is suitable for distinguishing isolates at the species level in the *F. solani* complex.

Amplification of the ITS region from 12 SDS isolates (Mont, St90-1, St1-1, VR, Mo1792, 269, 171, 22825, KS, Wf1-19-3 and Boyer isolates B4-9 and B5-9) resulted in products of 595 bp. However, direct sequencing of the amplification products was not reliable as faint minor bands could be detected on the sequencing gels. Therefore, cloning of the PCR products was performed and two clones from each isolate were sequenced.

ITS sequences generated from clones from the same organism were not identical. Intra-individual variation at the level of approximately 0.2–1.3% was detected. This variation was in the form of base substitutions only; no length variants were detected. Variation between individuals was from 0 to 2.0%, only slightly higher than that displayed within the same individual. Thus, the ITS polymorphisms alone cannot be used to distinguish among the SDS isolates.

Because the base-substitution sites were apparently random and because the intra-individual and inter-individual variation was approximately the same, the question arose as to whether the intra-individual variation could be explained by misincorporation by *Taq* polymerase, which has an error rate of 2×10^{-4} errors/bp (Saiki et al. 1988). To address this question, the D2 region of one of the SDS isolates, Mont, was amplified and cloned and four of the clones were sequenced. All four clones had exactly the same D2 sequence, demonstrating that *Taq* polymerase infidelity could not account for the variation seen among ITS clones from the same individual.

Approximately 60 RAPD primers were screened for their ability to show pattern variation among the SDS isolates. A total of 55 SDS isolates were examined from a number of states in the central USA (Table 1) plus 30 SDS isolates each from two field plots (Ice and Boyer) in Illinois. Amplification with most of the primers resulted in identical banding patterns among the SDS isolates. However, seven of the primers (Table 2) showed differences in the RAPD patterns among the pathogenic isolates. Of the 35 total amplification products generated from these seven primers from the SDS isolates, 19 were informative (i.e. polymorphic among the SDS isolates). Amplification of non-SDS members

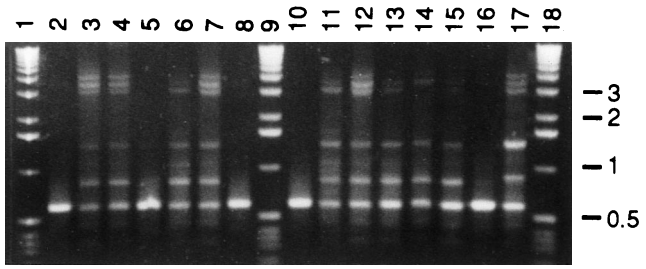

Fig. 1 RAPD pattern obtained by amplification of DNA from fungal SDS isolates with primer OPG-06. *Lanes 1, 9, 18*: 1-kb marker, *2*: Mont, *3*: RW-1, *4*: VR-309, *5*: St90-1, *6*: St1-1, *7*: Cora-2, *8*: 269, *10*: 17-1, *11*: Mo1792, *12*: KS, *13*: 22823, *14*: 22825, *15*: FSA-1, *16*: 171, *17*: Wf1-19-3. The fragment size in kilobase pairs is indicated on the right

of the *F. solani* f. sp. *phaseoli* complex was also performed. Many bands from each RAPD pattern were shared among all of the SDS isolates and several bands were shared among both the SDS and non-SDS isolates, highlighting the relatively low level of genetic variation among this group of fungi.

Analysis of RAPD patterns from this select group of seven primers identified two discrete amplification profiles, or amplitypes, among the SDS isolates. Each primer produced distinctive amplitype markers. For example, amplification with primer OPG-06 resulted in a single marker band (OPG06-630) for isolates Mont, St90-1, 269, 17-1 and 171 while all other isolate profiles contained marker band OPG06-630 in addition to several other bands (Fig. 1). All of the SDS isolates were consistently associated with only one amplitype with all seven primers. One of the amplitypes was represented by SDS isolates Mont, St90-1, 17-1, 269 and 171 while the other amplitype comprised the remaining SDS isolates included in this study.

Discussion

An assessment of genetic relationships within a group of organisms often begins with an estimate of rDNA variation. Because there are regions of high conservation and regions of extreme variability in the rDNA operon, this region of the genome can be used to assess relationships between both distantly- and closely-related organisms. Since our interest was in assessing the genetic variation within the SDS isolates, we focused on the variable D2 and ITS regions of rDNA to determine if sequences in these regions were variable enough to distinguish among SDS isolates from a wide geographic area.

D2 sequences were generated from 31 fungal isolates including 16 SDS isolates. Insufficient variation was found in the D2 region to identify subgroups of the fungus and, in fact, D2 sequences of the known SDS isolates were identical. D2 sequences of the SDS isolates were also identical to sequences obtained from three *F. solani* f. sp. *phaseoli* isolates that do not cause SDS.

ITS sequencing also generated insufficient variation to distinguish among the SDS isolates. However, a low level of intra-individual variation (0.2–1.3%) was detected in the ITS region. This finding has implications for researchers wishing to design species-specific ITS primers in that direct sequencing of ITS amplification products would most likely not detect base-substitution variants.

RAPD analysis gives a much broader picture of genetic variation than is possible with rDNA sequence analysis since RAPD data is based on the entire genome of an organism. We therefore performed RAPD analyses in an effort to estimate the genetic variability among the pathogenic isolates that cause soybean SDS. We analyzed a large number of SDS isolates (55) from diverse geographic regions (Arkansas, Illinois, Indiana, Iowa, Kansas, Mississippi and Missouri; see Table 1). In addition, we examined two fields in Illinois in detail (30 isolates each from the Boyer and Ice field sites) to ascertain genetic relatedness of fungal SDS populations.

Using the RAPD approach, we were able to identify two amplitypes within the SDS isolates. These amplitypes are consistent within the confines of the analysis; that is, every primer that showed differences among the SDS isolates always separated the isolates into the two amplitypes described here and none of the primers were polymorphic within the amplitypes. One amplitype (type I) was composed solely of isolates from Arkansas and central Illinois. The other amplitype (type II) was represented by the remainder of the SDS isolates tested, including those from Illinois, Iowa, Kansas, Missouri and Mississippi.

The broad geographic region represented by isolates in the type-II amplitype is of particular note. Although some geographic areas were represented by a single isolate, these results argue against genetic variation based on geographic isolation. Because we failed to identify hybrids of the two amplitypes, there may exist some genetic barrier between the amplitypes. Greenhouse assays previously performed by our group and others (J. Rupe, G. Hartman and X. B. Yang, personal communication) have failed to define pathogenicity differences based on different soybean host cultivars, indicating that the amplitypes described in this paper do not appear to represent pathogenic races of the fungus. In addition, virulence tests among the pathogenic isolates are unable to differentiate between the amplitypes. Therefore, the biological significance of the amplitype genetic differences is unknown at this time.

Isolates St90-1 and St1-1 were obtained from the same field plot on consecutive years. The placement of isolate St90-1 in the type-I amplitype and of isolate

St1-1 in type II is evidence for the existence of different amplitypes of this pathogenic fungus in the same field. However, based on our relatively limited sampling, this situation is rare.

Considering the fact that all SDS isolates that were collected from the Boyer and Ice field plots at the same time were all classified as type II may indicate the propensity of a single clonal type to dominate a given population. In addition, the absence of genetic variation among Arkansas isolates collected several years apart indicates that the SDS population has maintained a nearly monomorphic state since its appearance in this region. Whether or not the existence of one amplitype (type I) in a field one year and the existence of the other amplitype (type II) in the same field the next year is indicative of a mutation event followed by selection within the fungal population remains speculative.

Despite the sequence identity within the SDS isolates, RAPD analysis revealed some genetic variation in this fungal group with the identification of two amplitypes of SDS isolates. However, the large number of RAPD primers that were screened to find the few that revealed differences, taken together with the sequence identity of the D2 region, highlights the low level of genetic variation among SDS isolates. These results may indicate the introduction of one or two genetically similar SDS isolates into the U.S.

There has been some debate regarding the existence of races within SDS isolates. Although extensive pathogenicity assays on a number of soybean cultivars would need to be performed in order to unambiguously answer the race question, the data presented here indicates that, because of the relatively narrow genetic base within SDS isolates, races of the pathogen may not exist in the U.S. This finding directly impacts on soybean breeding efforts in that a given SDS-resistant soybean variety would be likely to be equally resistant to most, if not all, fungal SDS isolates.

Acknowledgements The authors thank Dr. C. Y. Yoon for SDS isolates from the Ice and Boyer fields, L. L. Lange (USDA/ARS Agricultural Research Technician) for technical assistance with the greenhouse assays, and all of the researchers who generously provided the fungal isolates used in this study. This work was supported by grants from the North Central Soybean Research Program and the Illinois Soybean Program Operating Board to L.A. and L.G. Mention of a trademark or proprietary product is solely to identify materials used and does not constitute a guarantee or warranty of the product by the USDA and does not imply its approval to the exclusion of other products that may also be suitable.

References

Abney TS, Richards TL, Roy KW (1993) *Fusarium solani* from ascospores of *Nectria haematococca* causes sudden death syndrome of soybean. Mycologia 85:801–806

Achenbach LA, Patrick J, Gray L (1996) Use of RAPD markers as a diagnostic tool for the identification of *Fusarium solani* isolates that cause soybean sudden death syndrome. Plant Dis 80:1228–1232

Assigbetse KB, Fernandez D, Dubois MP, Geiger J-P (1994) Differentiation of *Fusarium oxysporum* f. sp. *vasinfectum* races on cotton by random amplified polymorphic DNA (RAPD) analysis. Phytopathology 84:622–626

Booth C (1971) Methods of isolation, culture and stimulation of sporulation. In: The genus Fusarium. Commonwealth Mycological Institute, Kew England, pp 19–23

Crowhurst RN, Hawthorne BT, Rikkerink EHA, Templeton MD (1991) Differentiation of *Fusarium solani* f. sp. *cucurbitae* races 1 and 2 by random amplification of polymorphic DNA. Curr Genet 20:391–396

Gutell RR, Gray MW, Schnare MN (1993) A compilation of large subunit (23S and 23S-like) ribosomal RNA structures. Nucleic Acids Res 21:3055–3074

Kusaba M, Tsuge T (1995) Phylogeny of *Alternaria* fungi known to produce host-specific toxins on the basis of variation in interal transcribed spacers of ribosomal DNA. Curr Genet 28:491–498

Logrieco A, Peterson SW, Bottalico A (1995) Phylogenetic relationships within *Fusarium sambucinum* Fuckel *sensu lato*, determined from ribosomal RNA sequences. Mycopathologia 129:153–158

Michot B, Hassouna N, Bachellerie JP (1984) Secondary structure of mouse 28S rRNA and a general model for the folding of the large RNA in eukaryotes. Nucleic Acids Res 12:4259–4279

Nickrent DL (1994) From field to film: rapid sequencing methods for field-collected plant species. BioTechniques 16:470–475

O'Donnell K, Gray LE (1995) Phylogenetic relationships of the soybean sudden death syndrome pathogen *Fusarium solani* f. sp. *phaseoli* inferred from rDNA sequence data and PCR primers for its identification. Mol Plant-Microbe Interact 8:709–716

Roy KW, Lawrence GW, Hodges HH, McLean KS, Killebrew JF (1989) Sudden death syndrome of soybean: *Fusarium solani* as incitant and relation of *Heterodera glycines* to disease severity. Phytopathology 79:191–197

Rupe JC (1989) Frequency and pathogenicity of *Fusarium solani* recovered from soybeans with sudden death syndrome. Plant Dis 73:581–584

Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, Mullis KB, Erlich HA (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239:487–491

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning, a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York

Williams PH, Kubelik AR, Livak KJ, Rafolski JA, Tingey SV (1991) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res 18:6531–6535