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*sti*35, a Stress-Responsive Gene in *Fusarium* spp.†

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A stress-induced mRNA was identified in the phytopathogenic fungus *Fusarium oxysporum* f. sp. *cucumerinum*. Treatment of the fungus with ethanol resulted in the induction of a major mRNA species encoding a protein of approximate M_r 37,000. A full-length cDNA clone of the induced message was obtained. RNA blot analysis indicated that the mRNA was induced by various other stresses, including treatment with copper(II) chloride and heat (37°C). However, it was not greatly induced by treatment with phaseollinisoflavan, an antifungal isoflavonoid produced by *Phaseolus vulgaris* (French bean). In contrast, phaseollinisoflavan induced the homologous mRNA in the related bean pathogen *Fusarium solani* f. sp. *phaseoli*. A genomic clone of the *F. solani* f. sp. *phaseoli* gene was obtained, and both this and the cDNA clone from *F. oxysporum* f. sp. *cucumerinum* were sequenced. The latter indicated an open reading frame of 320 codons encoding a 34,556-dalton polypeptide. The corresponding reading frame in *F. solani* f. sp. *phaseoli* was 324 codons, 89% identical to the *F. oxysporum* f. sp. *cucumerinum* sequence, and was interrupted by a short intron. The gene was designated *sti*35 (stress-inducible mRNA). Although computer homology searches were negative, the cloned gene was observed to cross-hybridize to DNAs of other filamentous fungi, *Saccharomyces cerevisiae*, and soybean. Thus, *sti*35 appears to be a common gene among a variety of eucaryotes.

Stress-responsive genes have been identified in eucaryotes and bacteria, commonly on the basis of their inducibility by heat treatment. In eucaryotes, several classes of heat shock and related proteins are known, including the hsp70, hsp60, and hsp82 families, and a heterogeneous set of smaller (<30,000 daltons [Da]) heat shock proteins (19). Considerable study has been made of their expression, inducibility by stress, associated regulatory sequences, and localization of the protein products. Recent work has indicated that several of these proteins serve important or essential roles in cellular function. For example, proteins of the hsp70 family may be involved in protein translocation into the endoplasmic reticulum. Two 70-kDa heat shock-related proteins of *Saccharomyces cerevisiae* (yeast) have been purified and shown to stimulate translocation of in vitro-synthesized prepro-α-factor into isolated microsomes (5). Yeast hsp60 is required for assembly of multisubunit proteins in mitochondria (3). Another yeast stress-induced gene, *sti*1, was shown to be necessary for growth at elevated temperatures (23).

We are investigating the biochemical interactions of plants and phytopathogenic fungi. Plant tissues can constitute stressful environments for most fungi, replete as these tissues may be with various physical and chemical barriers. Among the barriers to infection are the phytoalexins, plant secondary compounds which exhibit antibiotic activity. The phytoalexin biosynthetic pathways are induced by infection and by various abiotic stresses (33). Although some fungi are capable of enzymatically detoxifying the phytoalexins characteristic of their particular hosts, even these organisms exhibit some sensitivity to the compounds (32). Thus, it is likely that both host and pathogen are subjected to stresses in the infection court. The mechanisms by which they respond to, avoid, and ameliorate the effects of those stresses may be important in the survival of both plant and microbe and in the progress of disease.

We have begun a systematic investigation to identify genes in *Fusarium* species that are induced by phytoalexins and other stresses. It is expected that phytoalexins may induce a variety of genes, some of which are determinants of pathogenicity. An example is the gene encoding pisatin demethylase in *Nectria haematococca* (the perfect stage of *Fusarium solani* f. sp. *pisi*) (40). This enzyme catalyzes the enzymatic detoxification of pisatin, the major isoflavonoid compound produced by the plant host *Pisum sativum* (pea) in response to infection. The product of this reaction is much less fungitoxic than is the substrate, so the enzyme is thought to ameliorate one of the plant defenses. The related pathogen *F. solani* f. sp. *phaseoli*, one of the subjects of this report, is known to enzymatically detoxify four phytoalexins of its host, *Phaseolus vulgaris* (French bean) (33). It is also possible that some of the genes induced by phytoalexins are not involved in phytoalexin detoxification but are involved in other aspects of pathogenicity. Still other phytoalexin-responsive genes may not be determinants of pathogenicity per se. Rather, their induction may be part of a response that is initiated by a number of stresses, including exposure to the antibiotic products of their plant hosts.

This report describes stress-responsive genes identified in two phytopathogenic *Fusarium* species, *F. solani* f. sp. *phaseoli* and *Fusarium oxysporum* f. sp. *cucumerinum*. The closely related genes were designated *sti*35 because they encoded stress-inducible mRNAs for 35-kDa polypeptides. Homologous sequences were not present in sequence data bases. However, cross-hybridizing sequences were observed in a number of fungi and plants. Thus, *sti*35 appears to be a common eucaryotic gene and may serve a basic biological function.

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MATERIALS AND METHODS

Biological materials. *F. oxysporum* Schl. f. sp. *cucumerinum* Owen, isolate B1-GK, was a gift from J. Kuc' (University of Kentucky). *F. solani* (Mart.) Sacc. f. sp. *phaseoli* (Burk.) Snyd. and Hans. (ATCC 60860), a pathogen of *P. vulgaris* (French bean), has been used for studies on phytoalexin metabolism for several years (34, 42). *F. solani* isolate X was described previously (34, 6). *Nectria haematococca* T9 (the perfect form of *F. solani* f. sp. *pisi*) was from H. D. VanEtten (Cornell University). *Emericella* (*Aspergillus*) *nidulans* UCD-1 was from W. E. Timberlake (University of Georgia). DNAs from *Acremonium coenophialum* (a fungal endophytic symbiont of the grass *Festuca arundinacea*), *S. cerevisiae*, and *Glycine max* (soybean) were from K. L. Mogen, R. C. Dickson, and D. F. Hildebrand, respectively (University of Kentucky). Media and maintenance and growth conditions of the fungi were as described previously (6, 27).

Plasmids and bacteriophage. The plasmid vector pIBI76 and the phage vector M13um20 were from International Biotechnologies, Inc. (New Haven, Conn.). Plasmid Bluescript KS(−) was from Stratagene Cloning Systems (La Jolla, Calif.). The cosmid vector pKBY2 (41) was from K.-M. Weltring (Cornell University). The pKAES plasmids were developed in this work. pKAES010 contains the full-length *sti*35 cDNA from *F. oxysporum* f. sp. *cucumerinum*; pKAES018 is a cosmid clone containing the *F. solani* f. sp. *phaseoli sti*35 gene; pKAES052 is a pIBI76 subclone of the 5.5 kilobase-pair (kb) *Kpn*I fragment containing *F. solani* f. sp. *phaseoli sti*35. Plasmid KAES066 contains an 859-base-pair (bp) fragment from pKAES052, which includes the *F. solani* f. sp. *phaseoli sti*35 sequence from nucleotide 251 (*Bam*HI) of the reading frame to nucleotide 1080 (*Eco*RI), 60 bases 3′ to the presumed translation termination codon (see Fig. 4). This fragment is ligated into pBluescript KS(−).

Chemicals. The phytoalexin phaseollinisoflavan was from the laboratory stocks. Enzymes were obtained from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.) or New England BioLabs, Inc. (Beverly, Mass.). Agarose HGT was from FMC Bioproducts (Rockland, Maine). Other electrophoresis chemicals and supplies were from Bio-Rad Laboratories (Richmond, Calif.) or Sigma Chemical Co. (St. Louis, Mo.). Other chemicals were from Aldrich Chemical Co. (Milwaukee, Wis.) or Sigma.

RNA isolation. Total RNA was isolated by a modification of the methods of Chirgwin et al. (4) and Turpen and Griffith (38). Mycelia were flash-frozen in liquid nitrogen in a mortar on dry ice and ground to a fine powder. The powder was wetted with homogenization buffer containing 4.5 M guanidine isothiocyanate, 50 mM EDTA (pH 8.0), 100 mM 2-mercaptoethanol, 25 mM sodium citrate (pH 7.0), and 2% sodium lauroyl sarcosine. The homogenate was then thawed at room temperature with stirring. Debris was removed by centrifugation at 8,000 × *g* for 10 min at 4°C. Thereafter, 0.2 g of CsCl was dissolved in each 1 ml of supernatant; then the homogenate was layered onto a cushion of 5.7 M CsCl in 50 mM EDTA (pH 7.3) and centrifuged at 150,000 × *g* for 16 h at 20°C. Each pellet was dissolved in 10 mM Tris hydrochloride (pH 7.4)–2 mM EDTA–1% sodium dodecyl sulfate (SDS), extracted with phenol-chloroform (1:1), and precipitated with 2.5 volumes of ethanol. Poly(A)$^+$ RNA was prepared by the method of Aviv and Leder (2).

cDNA cloning and screening. Poly(A)$^+$ RNAs isolated from ethanol-treated *F. oxysporum* f. sp. *cucumerinum* mycelia were used for cDNA synthesis by the method of Gubler and Hoffman (10). After second-strand synthesis, oligo-d(G) tails were added with terminal deoxynucleotide transferase. The tailed cDNA was annealed to *Kpn*I-cleaved pIBI76, tailed with oligo-d(C). *Escherichia coli* TB-1 was transformed, and transformants were screened by standard differential colony hybridization (21).

Radioactively labeled cDNA probes were prepared from mRNA, using avian myeloblastosis virus reverse transcriptase (Life Sciences, Inc., St. Petersburg, Fla.). The reaction was performed in 50 mM KCl–100 mM Tris hydrochloride (pH 8.3)–10 mM $MgCl_2$–10 mM dithiothreitol–2 mM sodium pyrophosphate–0.2 mM dATP–0.2 mM dTTP–0.2 mM dGTP–50 μCi of [α-^{32}P]dCTP (>3,000 Ci/mmol; ICN Radiochemicals, Irvine, Calif.), with random hexamer nucleotides (Pharmacia, Inc., Piscataway, N.J.) used as primers. After 40 min at 37°C, the reaction was stopped by phenol-chloroform (1:1) extraction, and then the RNA-DNA hybrids were precipitated with ethanol. The nucleic acids were redissolved in water, made 0.3 N in NaOH, and incubated for 40 min at 37°C to hydrolyze RNA. Labeled DNA was boiled for 2 min and used to probe colony lifts.

Genomic DNA isolation and cloning. Isolation of genomic DNA from *F. oxysporum* f. sp. *cucumerinum* was done by the method of Turgeon et al. (37), with modifications. Briefly, 5 g of fresh mycelia was frozen with liquid nitrogen in a mortar on dry ice, ground to fine powder, and suspended in 150 mM EDTA–50 mM Tris hydrochloride (pH 8.0)–1% sodium lauroyl sarcosine–proteinase K (2 mg/ml). After centrifugation at 2,000 × *g* for 10 min at 4°C, the supernatant was incubated at 37°C for 20 min and then extracted successively with phenol, phenol-chloroform-isoamyl alcohol (25:24:1), and chloroform-isoamyl alcohol (24:1). The aqueous phase was centrifuged once at 25,000 × *g* for 20 min at 4°C to remove the bulk of polysaccharides. One volume of isopropanol was added to the supernatant, and the nucleic acids were pelleted at 8,000 × *g* for 10 min at 4°C. The pellet was redissolved in TE buffer (10 mM Tris hydrochloride [pH 7.5], 1 mM EDTA) and banded in a CsCl-ethidium bromide buoyant density gradient (21). The UV-fluorescent band was taken, and the dye was extracted with *n*-butanol. DNA that was to be cloned was then twice centrifuged in CsCl-bisbenzimide buoyant density gradients (9) to separate the majority of nuclear DNA from satellite A- and T-rich DNAs. The bisbenzimide dye was extracted with water-saturated isopropanol. DNA was dialyzed against TE buffer and concentrated by precipitation with ammonium acetate and ethanol (21).

A cosmid clone library of *F. solani* f. sp. *phaseoli* DNA was prepared in the *E. coli-Emericella nidulans* shuttle vector pKBY2 (41) as described by Maniatis et al. (21), with modifications previously described (30). After ligation of fragments to the vector, the DNA was packaged in lambda coat proteins, using Gigapack Gold (Stratagene), the resulting particles were used to infect *E. coli* VCS257 cells (Stratagene), and the transductants were selected for ampicillin resistance. Approximately 12,000 primary cosmid clones were obtained.

DNA sequence determination. All DNA sequencing was done by using the Sequenase kit from United States Biochemicals Corp. (Cleveland, Ohio) according to recommended protocols (36). Single-stranded DNA was obtained from pIBI76 and pBluescript KS(−) subclones, using the helper bacteriophage M13K07 (39). Sequencing primers were synthesized by using a 380B DNA synthesizer (Applied Biosystems, Foster City, Calif.). Nested unidirectional deletions were generated by the method of Henikoff (11), using

exonuclease III and mung bean nuclease. All sequences were determined on both strands of the DNA clones, and all subcloning sites were crossed except for the *Bam*HI site at position 91 of the *F. solani* f. sp. *phaseoli* coding sequence (see Fig. 4).

Miscellaneous procedures. RNA blots were by the method of Thomas (35). Low-stringency hybridization was done at 42°C overnight in hybridization buffer (25% formamide, 5× SSC [1× SSC is 0.15 M NaCl plus 0.15 M sodium citrate], 5× Denhardt solution [5× Denhardt solution is 0.1% Ficoll, 0.1% polyvinylpyrrolidone, and 0.1% bovine serum albumin], 0.1% SDS, sonicated, denatured salmon sperm DNA [100 μg/ml]). Membranes were washed at room temperature twice with 2× SSC–0.1% SDS and then at 65°C with 0.5× SSC–0.1% SDS. High-stringency hybridization was done in essentially the same way except that the hybridization buffer contained 50% formamide, and the final wash was 0.1× SSC–0.1% SDS at 65°C. To quantitate the levels of hybridizing RNA, a low-exposure autoradiograph was analyzed by laser densitometry, using an Ultroscan XL (LKB, Bromma, Sweden).

DNA blots and DNA-DNA hybridization were performed by standard methods (21). DNA probes were labeled by the method of Feinberg and Vogelstein (7). RNA slot blots were performed by using GeneScreen Plus positive nylon filters (Dupont, NEN Research Products, Boston, Mass.) as instructed by the manufacturer. Restriction endonuclease digestions were performed as previously described (29).

Hybrid selection was performed by the method of Jagus (12). In vitro transcription of the cloned cDNA was done by the method of Melton et al. (22). The transcripts generated with T7 or SP6 RNA polymerase were used for in vitro translation without prior addition of cap analogs. In vitro translation was done with use of a rabbit reticulocyte lysate system (Bethesda Research Laboratories) as instructed by the manufacturer but without addition of KCl. An equal amount of trichloroacetic acid-precipitable, [^{35}S]methionine-labeled product was applied to each lane of an SDS-polyacrylamide gel and subjected to electrophoresis (18) and fluorography (31).

RESULTS

Alterations in mRNA expression. Figures 1A and 1B illustrate in vitro translation analysis of mRNA from mycelia of *F. oxysporum* f.sp. *cucumerinum* and *F. solani* f. sp. *phaseoli*, respectively, and alterations in profiles of the translation products associated with treatment of the fungal cultures with ethanol and with phaseollinisoflavan plus ethanol. One species, encoding a polypeptide of estimated M_r 37,000, was observed at a high level among the products of mRNA from ethanol-treated *F. oxysporum* f. sp. *cucumerinum*. Since a corresponding minor band could be observed in the control lane, it was possible that this represented a species that was expressed at a significant basal level and was induced to very high levels by ethanol. A product of similar size was also observed among the translation products of ethanol-induced *F. solani* f. sp. *phaseoli* mRNA (Fig. 1B), but the level of expression after ethanol induction was much lower than in *F. oxysporum* f. sp. *cucumerinum*.

To examine the effects of another stress factor, both organisms were treated with phaseollinisoflavan. This compound is one of the major antifungal products from *P. vulgaris*, the plant host of *F. solani* f. sp. *phaseoli*. In these experiments, the isoflavonoid was added as an ethanol solution. Treatment of *F. solani* f. sp. *phaseoli* with the

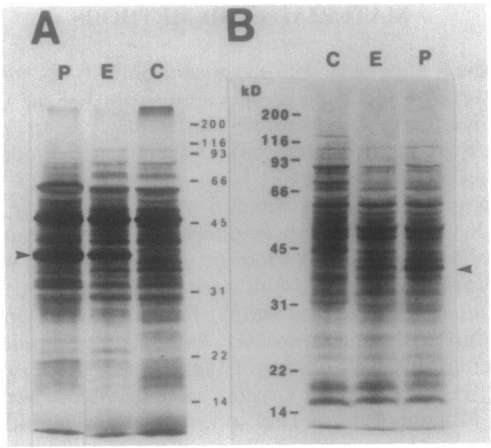

FIG. 1. In vitro translation of poly(A)$^+$ RNAs from *F. oxysporum* f. sp. *cucumerinum* (A) and *F. solani* f. sp. *phaseoli* (B). Mycelia were treated for 12 h with 70 μM phaseollinisoflavan in ethanol (1.5%, vol/vol) (lanes P) or with ethanol alone (1.5%, vol/vol) (lanes E). Control lanes (C) represent mRNAs from nontreated mycelia. The location of the product from the major induced mRNA is indicated by arrows.

isoflavonoid resulted in a very high level of the M_r-37,000 translation product over that in the ethanol control (Fig. 1B). In *F. oxysporum* f. sp. *cucumerinum*, which already showed a high level of this species in response to ethanol, little or no additional increase resulted from treatment with phaseollinisoflavan (Fig. 1A). Although there were several lesser changes also observed among the translation products from both *Fusarium* species, the majority of translatable mRNAs apparently remained unaffected by the chemical treatments.

cDNA clone of an ethanol-induced mRNA. Approximately 600 cDNA clones of mRNA from ethanol-treated *F. oxysporum* f. sp. *cucumerinum* were screened by differential hybridization. Three clones gave stronger signals when hybridized with [^{32}P]cDNA made from ethanol-induced poly(A)$^+$ RNAs. The cDNA inserts in the three clones shared sequence homology (data not shown). One of those identified, designated pKAES010, had a cDNA insert of 1.2 kb.

The cDNA clone pKAES010 was used for hybrid selection of complementary mRNA from ethanol-treated *F. oxysporum* f. sp. *cucumerinum* mycelia. pKAES010-selected mRNA gave a translation product of estimated M_r 37,000 (Fig. 2A). This product comigrated with the major band previously identified and associated with ethanol induction in total mRNA translations. To determine whether the cDNA insert in pKAES010 encoded the entire open reading frame, transcripts of the cDNA were generated in vitro, using T7 and SP6 RNA polymerases, and in vitro translations of the transcripts were performed (Fig. 2B). Since their respective promoters flank the cloning sites, the T7 and SP6 polymerases generate transcripts of complementary strands of the cDNA. Among the translation products of the T7 RNA polymerase-generated transcripts, the strongest band comigrated with the major product associated with ethanol induction. In contrast, there was no detectable translation product from the SP6 RNA polymerase-generated transcripts. The results served to determine the orientation of the cDNA insert and indicated that the cDNA insert represented a near-full-length clone of the message. Since the cDNA sequence (see below) indicated an open reading frame encoding a polypeptide of 34,556 Da, the gene for this

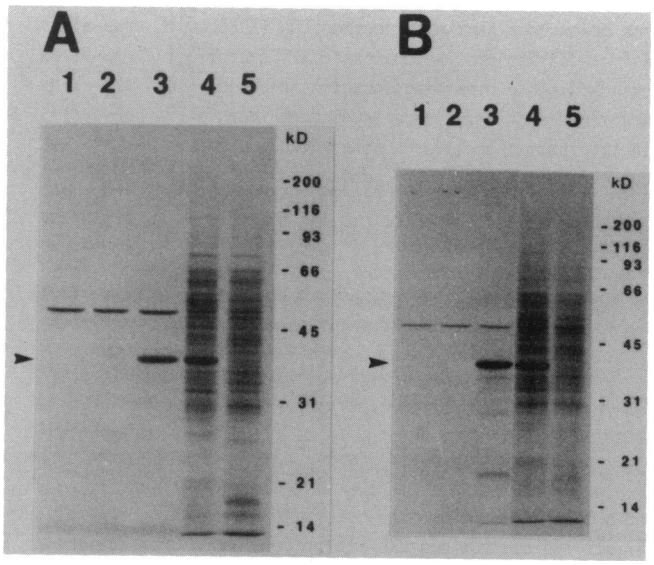

FIG. 2. In vitro translation of pKAES010-selected mRNA from *F. oxysporum* f. sp. *cucumerinum* (A) and RNA synthesized in vitro by transcription of pKAES010 (B). (A) No RNA added to the in vitro translation reaction (lane 1), mock selection using the cloning vector pIBI76 (lane 2), mRNA selected with pKAES010 (lane 3), total mRNAs from ethanol-treated *F. oxysporum* (lane 4), and total mRNAs from untreated *F. oxysporum* (lane 5). (B) No RNA added to the in vitro translation reaction (lane 1), transcripts from an SP6 RNA polymerase reaction using pKAES010 (lane 2), transcripts from a T7 RNA polymerase reaction (lane 3), and total mRNAs from ethanol-treated (lane 4) and untreated (lane 5) *F. oxysporum* mycelia.

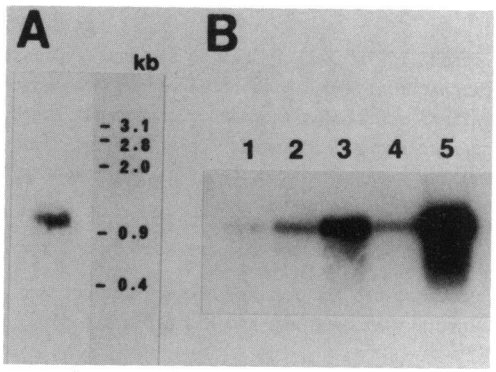

FIG. 3. Northern blot analysis of mRNAs from *F. oxysporum* f. sp. *cucumerinum* and *F. solani* f. sp. *phaseoli*. (A) Size determination of *sti*35 mRNA of *F. oxysporum*. Markers were the RNAs of peanut stunt virus and its satellite virus (from S. Ghabrial, University of Kentucky). (B) Each of lanes 1, 2, and 3 contains 5 μg of poly(A)$^+$ RNA from *F. solani* mycelia which were untreated (lane 1), ethanol treated (lane 2), or phaseollinisoflavan (in ethanol) treated (lane 3). Each of lanes 4 and 5 contains 1 μg of *F. oxysporum* mRNAs from mycelia untreated (lane 4) or ethanol treated (lane 5). The membranes were probed with the 972-bp *Pst*I-to-*Eco*RI fragment of the pKAES010 cDNA insert under high (A)- or low (B)-stringency conditions.

mRNA was designated *sti*35 (stress-inducible mRNA, 35-kDa protein) on the basis of its response to ethanol and other stresses.

The enhancement of the M_r-37,000 in vitro translation products indicated that ethanol induction and, in the case of *F. solani* f. sp. *phaseoli*, phaseollinisoflavan induction were causing increased accumulation of the corresponding mRNAs. This was confirmed by Northern (RNA) blot analysis of poly(A)$^+$ RNAs from the two *Fusarium* species (Fig. 3). In each, a 1.2-kb mRNA was detected by hybridization with the pKAES010 insert (Fig. 3). In *F. solani* f. sp. *phaseoli*, this mRNA was induced by ethanol and further induced by phaseollinisoflavan. Additional tests (data not shown) indicated that the levels of *sti*35 RNA were increased by treatment with copper(II) chloride or dimethyl sulfoxide and that the *F. oxysporum* gene but not the *F. solani* gene was heat induced (37°C, 12 h).

***sti*35 sequences.** The sequence of the entire *F. oxysporum* f. sp. *cucumerinum* *sti*35 cDNA is presented in Fig. 4. This sequence includes a 960-nucleotide major open reading frame, and the predicted amino acid sequence (assuming the universal genetic code) is also shown. The molecular size of the predicted polypeptide is 34,556 Da.

Cosmid clone pKAES018, containing the homologous *F. solani* f. sp. *phaseoli* gene, was identified by hybridization with the *sti*35 cDNA isolated from pKAES010. The restriction endonuclease-cleavage fragments containing the gene were identified by DNA hybridization, and a 5.5-kb *Kpn*I fragment was subcloned into pIBI76 to give pKAES052. Further subclones were generated and sequenced. The *sti*35 coding sequence was identified by comparison with that of the *F. oxysporum* cDNA (Fig. 4). A likely intron was identified which divided codon 294 and contained sequences similar to the introns of *Neurospora crassa* (24). These sequences were canonical 5′(GTGAGT) and 3′(CAG) splice sites and a possible branch site (GCTAACT). The deduced amino acid sequences were highly homologous from residues 1 to 17 and from residues 37 to 322 of the 324-codon sequence of the *F. solani* gene. Predicted amino acid residues 18 to 36 were divergent in both sequence and length (19 residues in *F. solani*; 15 in *F. oxysporum*).

Computer searches of nucleic acid and protein data bases (GenBank and EMBL) failed to find any sequence significantly homologous to the cloned *sti*35 genes or to the deduced polypeptide products. Restriction endonuclease mapping and genomic DNA blot hybridization to fragments of the pKAES010 and pKAES052 inserts (data not shown) indicated that *sti*35 was a single-copy gene in both *Fusarium* species.

***sti*35 homologies in other species.** DNA hybridization analysis (Fig. 5) was undertaken to determine whether *sti*35 sequences were common to other eucaryotes. Total DNAs from a number of fungal species were cleaved with *Eco*RI, electrophoresed, and blotted onto a positive nylon filter. The filter was hybridized at low stringency with a radiolabeled probe consisting of the cloned 859-bp *Bam*HI-to-*Eco*RI fragment of the *F. solani* f. sp. *phaseoli* *sti*35 gene. DNA from each organism exhibited at least one *Eco*RI fragment which hybridized to the probe, demonstrating that the *sti*35 gene probe contained sequences commonly present in these eucaryotic genomes. These sequences do not appear to be highly repetitive and may be single copy in some or all of the genomes analyzed.

DISCUSSION

This report describes the identification and sequence analysis of homologous stress genes from two phytopathogenic *Fusarium* species. The *sti*35 genes contain open reading frames for polypeptide products of approximately 35,000 Da. Levels of *sti*35 mRNAs were increased by treatment of cultures with ethanol and, in the case of *F.solani* f. sp. *phaseoli*, phaseollinisoflavan. Furthermore, DNA hybridiza-

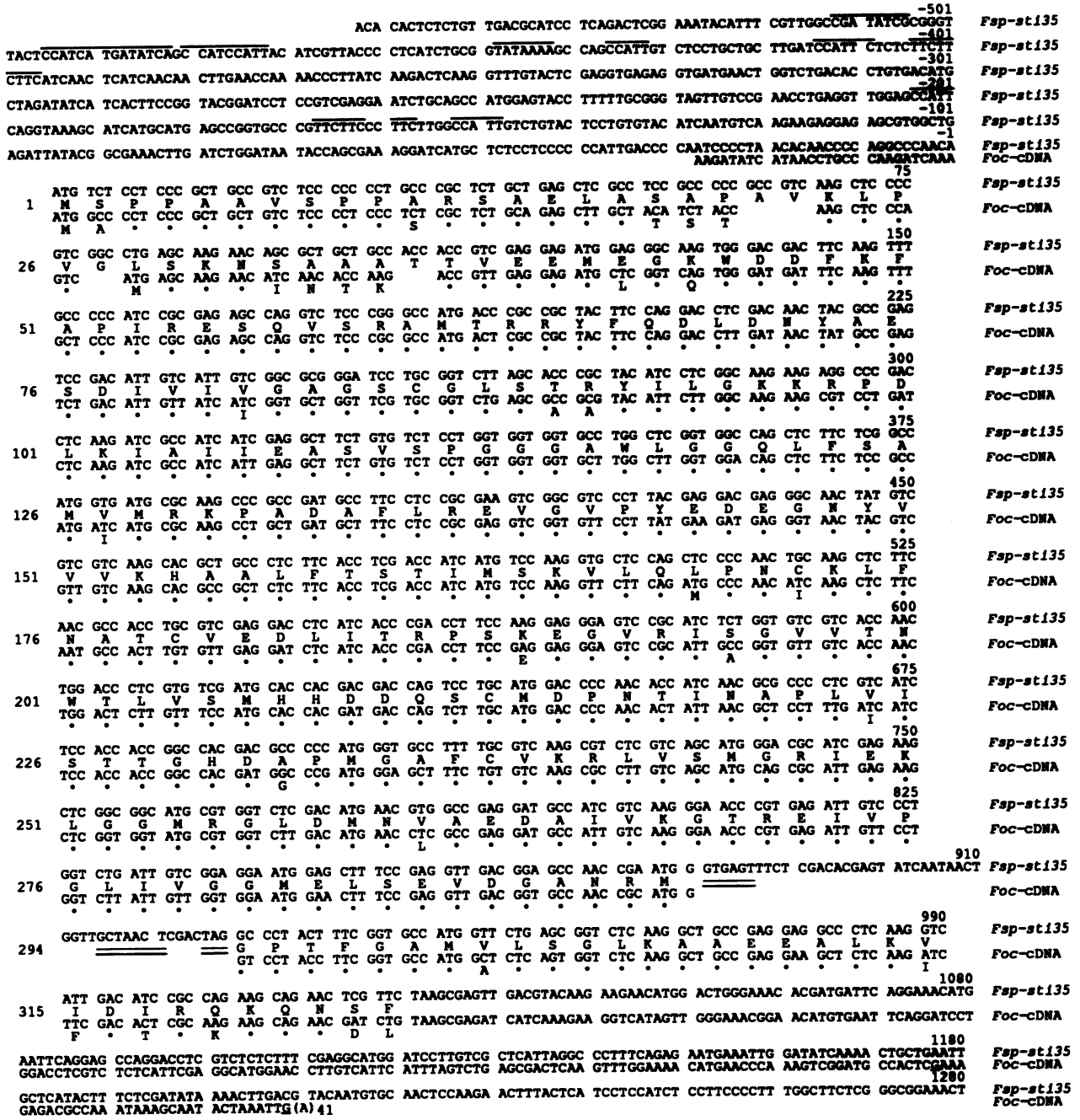

FIG. 4. Sequences of the *F. solani* f. sp. *phaseoli* genomic DNA clone of *sti*35 (*Fsp-sti*35) (GenBank accession number M33642) and the cloned *F. oxysporum* f. sp. *cucumerinum sti*35 cDNA sense strand (*Foc*-cDNA) (GenBank accession number M33643). The predicted polypeptide sequences are indicated by their single-letter amino acid codes beneath the nucleotide sequences. Numbers above the *F. solani* gene sequence indicate nucleotide positions relative to the presumed translation start codon. Numbers to the left indicate codons of the *F. solani* gene. Dots indicate identical predicted amino acids. Underlined bases in the 3′ untranslated *F. oxysporum* cDNA sequence indicate the two polyadenylation sites identified from three independent clones. Overlined sequences indicate dyad symmetries, frequently occurring motifs (TTC and CCATT), and a TATA-box-like sequence at −447. Double-underlined sequences match consensus sequences of nuclear introns.

tion analysis suggested that homologous genes are present in other fungi and in plants. Therefore, it is possible that *sti*35, like other known eucaryotic stress-responsive genes, may serve an essential cellular function.

Although *sti*35 was characterized as a stess-responsive gene, it did not show similar responses to every stress. For example, ethanol was a potent inducer of the mRNA in *F. oxysporum* f. sp. *cucumerinum*, but phaseollinisoflavan had little effect. In contrast, the *F. solani* f. sp. *phaseoli* homolog was induced somewhat by ethanol and further induced by phaseollinisoflavan. This difference in response to phaseollinisoflavan is of interest because the compound is an antifungal phytoalexin produced by the host plant of *F. solani* f. sp. *phaseoli*, *P. vulgaris* (34). It is possible that the

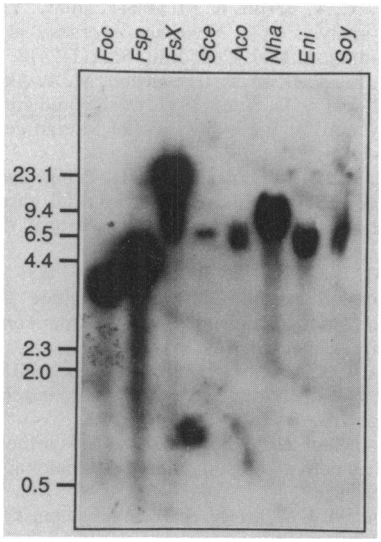

FIG. 5. Hybridization of *Eco*RI-cleaved eucaryotic genomic DNAs to the *F. solani* f. sp. *phaseoli sti*35 gene sequence. DNAs were from *F. solani* f. sp. *phaseoli* (*Fsp*), *F. oxysporum* f. sp. *cucumerinum* (*Foc*), *F. solani* isolate X (*FsX*), *S. cerevisiae* (*Sce*), *A. coenophialum* (*Aco*), *N. haematococca* T9 (*Nha*), *E. nidulans* (*Eni*), and *G. max* (soybean). Samples of DNA (5 μg each) were cleaved with *Eco*RI, electrophoresed, blotted, and probed with the 859-bp insert from pKAES066 (see Materials and Methods). Molecular sizes of *Hin*dIII fragments of phage lambda are indicated in kilobase pairs.

difference in response to the phytoalexin is one manifestation of the adaptation of *F. solani* f. sp. *phaseoli* but not *F. oxysporum* f. sp. *cucumerinum* to this particular host. The results did not indicate whether the different responses were due to differences in the induction mechanisms.

Analysis of the *F. oxysporum sti*35 cDNA sequence (see Fig. 4) revealed features common to other fungal genes. The context of the first ATG (ATCAAAATGG) is characteristic of efficiently translated eucaryotic mRNAs (16), which most often possess purines at positions −3 and +4 (A in ATG is +1). This context also matches the *N. crassa* consensus translation initiation sites (A/G)TCA(A/C)AATGG (28). The corresponding positions of the *F. solani* gene also contained an A at −3, but the +4 nucleotide was T, not G. Overall codon usage of *sti*35 is much like that of highly expressed fungal genes, such as the *am* (15) or *tub*-2 (24) genes of *N. crassa*. For example, inspection of the *F. oxysporum* cDNA sequence reveals a strong preference (91%) for pyrimidines at the third position in four or six codon families. In a majority of families, C or G was found more often in the third position than was T or A. Glycine codon usage, however, showed a very strong preference for T or A in the third position. Codon usage in the *F. solani* gene was similar, with a slightly greater tendency for G or C to occur in the third position.

Gene bank searches for nucleic acid and protein homologies were negative. Detailed comparisons were directed to known stress- and ethanol-regulated genes from other organisms. For example, in *E. nidulans*, ethanol induces mRNAs for alcohol dehydrogenase I and aldehyde dehydrogenase (20, 25). However, no homology was found to strictly conserved amino acid residues in characterized alcohol-polyol dehydrogenases (13). There was also no significant homology to the 35-kDa enzyme glyceraldehyde-3-phosphate dehydrogenase, which is heat inducible in yeast cells (19). In hamsters, ethanol induces expression of mRNA encoding cytochrome P-450-IIe1 (17). Furthermore, cytochromes P-450 are responsible for metabolism of a wide variety of endogenous and exogenous substances (17), and at least one is involved in phytoalexin detoxification (40). Although cytochromes P-450 are not highly conserved, residues of the heme-binding domain, including an essential cysteine, are invariant (1, 14). No such residues were identified from analysis of the *F. oxysporum* and *F. solani sti*35 reading frames.

Sequences homologous to *sti*35 were identified in a variety of eucaryotes, including *S. cerevisiae* and soybean. Freeman et al. (8) recently observed an M_r-35,000 protein that was induced by heat treatment of *F. oxysporum* f. sp. *niveum*. Similar-sized proteins are also induced in *N. crassa* (26) but have not been commonly observed as heat shock proteins in plants (19). However, it is certainly possible that *sti*35-related genes are present in plants and other eucaryotes but, like many heat shock-related genes, are not heat inducible. In fact, the *sti*35 mRNA was not heat inducible in *F. solani* f. sp. *phaseoli* (data not shown). The commonality of *sti*35-homologous sequences may indicate that the gene serves a basic biological function that remains to be explored. Furthermore, since no homologous sequences were identified in the data bases, it appears that *sti*35 represents the first sequenced member of this stress gene family.

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