

Effect of Disruption of the Enniatin Synthetase Gene on the Virulence of *Fusarium avenaceum*

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Production of the phytotoxic compound enniatin has been proposed to play a role during the infection process of plants by enniatin-synthesizing *Fusarium* species. Enniatins are cyclohexadepsipeptides synthesized by the multifunctional enzyme enniatin synthetase. To test the hypothesis that enniatin contributes to pathogenicity, enniatin-nonproducing mutants were constructed by gene disruption of the enniatin synthetase gene of a virulent *Fusarium avenaceum* strain. Four independent enniatin-nonproducing mutants were characterized that did not express enniatin synthetase, as proved by RNA and protein blot analysis. The virulence on potato tuber tissue of the enniatin-nonproducing strains was significantly reduced compared with the virulence of the parent strain and three enniatin-producing transformants. Therefore, we conclude that enniatin production contributes to the virulence of *Fusarium avenaceum*.

Additional keywords: homologous recombination, phytotoxin.

The molecular mechanisms determining plant pathogenicity of fungi are poorly understood. The ability of fungi to penetrate the external barrier of plants has drawn attention to a diversity of fungal enzymes capable of decomposing components of plant cell walls (Chasan 1992). Relatively little is known about the possible involvement of compounds produced by fungi that are toxic to plants.

Many species of *Fusarium* are serious pathogens and produce phytotoxic substances in culture (Booth 1971). The known toxins produced by *Fusarium* are non-host-specific and may affect a wide range of plants (Drysdale 1982). Enniatins are a group of cyclohexadepsipeptides consisting of alternating residues of D-2-hydroxyisovaleric acid and a branched chain amino acid linked by peptide and ester bonds. They are produced by several species of *Fusarium* and are postulated to play a role during the infection of plants (Walton 1990).

Enniatins affect the water uptake of tomato shoots, causing wilt followed by necrosis of leaves (Gäumann et al. 1960), and reduce the growth of germinating wheat seeds, resulting in decreased root and leaf development (Burmeister and Plattner 1987). An enniatin-producing isolate of *Fusarium avenaceum* isolated from *Centaurea maculosa* (spotted knapweed)

also produces the phytotoxin acetamido-butenolide, and the two toxins act synergistically to cause necrotic lesions on leaves of different plant species (Hershenhorn et al. 1992). Enniatins also induce necrosis of potato tuber tissue (Herrmann et al. 1996) and have antibiotic and insecticidal activity (Gäumann et al. 1960; Grove and Pople 1980). The toxicity of enniatins presumably derives from their ability to increase the permeability of membranes to ions and from their action as uncouplers of oxidative phosphorylation (Shemyakin et al. 1969).

The multienzyme enniatin synthetase, a polypeptide of 347 kDa, synthesizes enniatins by means of a nonribosomal thio-ester template mechanism. The enzyme harbors all functions necessary for synthesizing enniatin from the branched chain L-amino acid, D-2-hydroxyisovaleric acid, S-adenosylmethionine, and ATP (Zocher et al. 1982; Zocher et al. 1983; Billich and Zocher 1987; Pieper et al. 1995). Enniatin synthetases with distinct substrate amino acid specificities have been isolated from *F. scirpi* ETH 1536, *F. sambucinum* BBA 63933, and *F. lateritium* BBA 65090 (Pieper et al. 1992).

The enniatin synthetase gene (*esyn1*) from *F. scirpi* ETH 1536 encoding an open reading frame of 9,393 bp has been isolated, sequenced, and further characterized by heterologous expression (Haese et al. 1993; Haese et al. 1994). The enniatin producer *F. avenaceum* BBA 64338 was isolated from seeds of winter barley in Württemberg, Germany, and induces necrosis of potato tuber tissue. The enniatin synthetase gene of *F. avenaceum* BBA 64338 resembles the isolated *esyn1* gene of *F. scirpi* ETH 1536 (Herrmann et al. 1996).

To determine directly the role of enniatin synthesis in the infection process of *F. avenaceum* BBA 64338, we constructed enniatin synthetase-deficient mutants and characterized their virulence. The results indicate an influence of enniatin production on virulence of *F. avenaceum* BBA 64338 on potato tuber tissue.

RESULTS

Characterization of the enniatin synthetase gene in *F. avenaceum* BBA 64338.

The strain *F. avenaceum* BBA 64338 used for gene disruption experiments produced predominantly enniatin B (100 to 250 µg/ml) on solid *Fusarium* complete media (FCM), as shown by high-performance liquid chromatography analysis (A. Stindl, personal communication). The strain was virulent

on potato tuber tissue and enniatin could be isolated from the infected tissue.

The use of an internal *esyn1* fragment of *F. scirpi* to drive disruption of the enniatin synthetase gene of *F. avenaceum* BBA 64338 by homologous recombination requires a high degree of similarity of their DNA sequences. Similarity of the enniatin synthetase genes of the two strains was studied by means of Southern blot analysis of *F. avenaceum* and *F. scirpi* chromosomal DNA digested with 10 restriction enzymes. Three *F. scirpi* DNA sequences were used as probes: an internal 7,102-bp *SalI* fragment, a 2,834-bp *SalI* fragment from the 5' end, and a 1,225-bp *PstI* fragment from the 3'-flanking region of the *esyn1* gene. The DNA of the two organisms showed an identical hybridization pattern for all restriction enzymes and probes and almost identical patterns of repetitive DNA sequences (data not shown).

An internal 1,004-bp fragment of the *esyn1* gene of *F. avenaceum*, corresponding to bp 4,882 to 5,886 of the *esyn1* gene of *F. scirpi*, was amplified by polymerase chain reaction (PCR). Sequencing of the subcloned PCR product showed only one nucleotide difference in the 1,004-bp product, indicating that the sequences of the *esyn1* genes of *F. avenaceum* and *F. scirpi* are 99.9% identical.

Disruption of the *esyn1* gene.

The plasmid used for gene disruption, pGPC1-2.3HIII, contains an internal 2.3-kb *HindIII* fragment of the *F. scirpi* *esyn1* gene corresponding to bp 3,076 to 5,405, and was derived from plasmid pTK10 (Weltring 1995). The vector pTK10 carries the hygromycin B phosphotransferase (*hph*) gene from *Escherichia coli* as selection marker and a 399-bp regulatory element of *Gibberella pulicaris*, conferring resistance to hygromycin B. Polyethylene glycol (PEG)-mediated transformation of *F. avenaceum* protoplasts with pGPC1-2.3HIII DNA yielded eight transformants per μg of plasmid DNA. Approx. 5.5% of 350 hygromycin B-resistant transformants failed to produce enniatin on solid FCM. Ten of them were passaged five times on hygromycin B-free medium and found to be mitotically stable under nonselective conditions.

Single-spores were isolated from 19 enniatin-nonproducing and three enniatin-producing transformants. These transformants were further characterized by Southern blotting of *SalI*-restricted chromosomal DNA and by means of the 2,329-bp *HindIII* *esyn1* fragment inserted into pTK10 as a hybridization probe. Results for six transformants representing three types of vector integration events are shown in Figure 1. The DNA hybridization pattern of transformants T23, T75, T82, and T114 indicates integration of at least two plasmid copies into the enniatin synthetase gene as shown in Figure 2A and B. Instead of the 7.1-kb *SalI* *esyn1* fragment of the wild-type DNA that would hybridize to the 2,329-bp *HindIII* fragment, three fragments of 6.3, 7.7, and 8.5 kb, respectively, were detected. A hybridization pattern expected for the integration of at least two copies of pGPC1-2.3HIII was also observed when *XhoI*-restricted DNA was subjected to Southern blot analysis by means of the same hybridization probe (data not shown). Besides a 2.9-kb fragment that was also present in the wild-type DNA, an additional fragment of 7.7 kb was found (Fig. 2C). In the case of the enniatin-nonproducing transformant T100, the 7.1-kb *SalI* fragment was also not detectable, indi-

cating disruption of the enniatin synthetase gene. However, the integration event could not be explained by homologous recombination. Enniatin synthesis was still detectable in transformant T224, in which pGPC1-2.3HIII had apparently integrated ectopically.

Integration of a single copy of the transformation vector into the enniatin synthetase gene was observed for none of the 19 enniatin-nonproducing transformants.

RNA and protein blot analysis of the transformants.

The effect of disruption on transcription of the enniatin synthetase gene was determined by RNA blot analysis using an internal 7.1-kb *SalI* radiolabeled *esyn1*-fragment of *F. scirpi* as probe. A transcript of approximately 9.5 kb was detectable in the wild-type and the transformant T224 RNA (Fig. 3A). The size of the *esyn1* transcript of *F. avenaceum* was indistinguishable from that of *F. scirpi* (Haese et al. 1993). RNA of both null mutants T23 and T82 showed no hybridization.

Protein blot analysis of crude protein extracts was performed to test for the enniatin synthetase protein in the transformants T23 and T82. The blots were incubated with a polyclonal antiserum directed against enniatin synthetase purified from *F. scirpi*. As shown in Figure 4, a protein of the same molecular mass as enniatin synthetase of *F. scirpi* was detected in the protein extracts of the wild-type and ectopic transformant T224, but not in those of T23 and T82.

Virulence assay.

Enniatins at 5 to 100 μg per slice cause necrotic damage of potato tubers in vitro and *F. avenaceum* BBA 64338 induces necrosis of potato tuber tissue (Herrmann et al. 1996). Seven

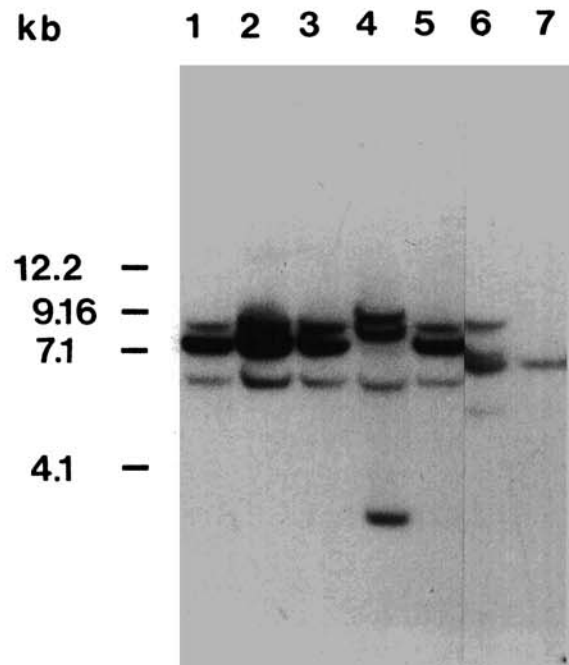


Fig. 1. Southern blot analysis of *SalI*-digested chromosomal DNA of *Fusarium avenaceum* wild-type strain and transformants. The lanes are T23 (1), T75 (2), T82 (3), T100 (4), T114 (5), T224 (6), and *F. avenaceum* wild type (7). The Southern blot was probed with the 2.3-kb *HindIII* *esyn1* fragment inserted into pTK10. DNA size markers are indicated on the left.

null mutants, three enniatin-producing hygromycin B-resistant transformants, and the *F. avenaceum* wild type were tested for their ability to attack potato tubers. Of the seven enniatin-nonproducing mutants, T23 and T82 are characterized with respect to expression of the *esyn1* gene and the integration event. T75 and T114 were also obtained by homologous recombination in the *esyn1* gene, while the integration event in T3, T100, and T107 is unknown. All the strains caused some dry rot of the potato tuber tissue (Fig. 5A, B). Enniatins could be recovered from the tissue decomposed by the ectopic transformants T224, T234, and T242 and the wild type (Fig. 5B). No enniatin was detected in tissues infected with the null mutants. Virulence of the group of the seven enniatin-nonproducing transformants was significantly reduced ($P = 0.05$), as shown by the *t* test, compared with the virulence of the group of enniatin-producing transformants and the wild type.

DISCUSSION

By inserting a vector containing an internal fragment of the *esyn1* gene of *F. scirpi* ETH 1536, we disrupted the gene encoding enniatin synthetase in *F. avenaceum*. The virulence of the enniatin-nonproducing transformants on potato tuber tissue was significantly reduced. These results indicate a contribution of enniatin production to virulence of *F. avenaceum*.

In a previous study, 36 *Fusarium* strains from a wide range of host plants were characterized with respect to enniatin pro-

duction and virulence on potato tuber tissue (Herrmann et al. 1996). The results did not indicate a contribution of enniatin production to virulence of *Fusarium*. Several strains that produced large amounts of enniatins were nonpathogenic on potato tuber tissue whereas some enniatin nonproducers exhibited strong virulence. However, the results obtained here from the enniatin-nonproducing mutants of *F. avenaceum* indicate an involvement of enniatin production in the virulence of this strain.

In *Fusarium* species only a few possible pathogenicity factors have been studied. Among the nonspecific toxins produced by *Fusarium* the trichothecenes, which are sesquiterpene epoxides, were found in different plant tissues infected by *Fusarium* species (Marasas et al. 1984). The first unique step in biosynthesis of these secondary metabolites is catalyzed by trichodiene synthase. After disruption of the corresponding gene, *tox5*, in *Gibberella pulicaris* (anamorph: *F. sambucinum*; Hohn and Desjardins 1992), transformants were obtained that showed a significantly reduced virulence on parsnip root slices but not on potato tuber tissue (Desjardins et al. 1992). Similar results were obtained for *tox5* mutants of *G. zeae*, which showed a reduced virulence on seedlings of one wheat cultivar and winter rye but only a small inconsistent reduction in virulence on other cultivars (Proctor et al. 1995). These results indicate that the influence of trichothecene production on pathogenesis depends on the host species.

The influence of naphthazarin production on the virulence of *N. haematococca* (anamorph: *F. solani* var. *martii*) on pea

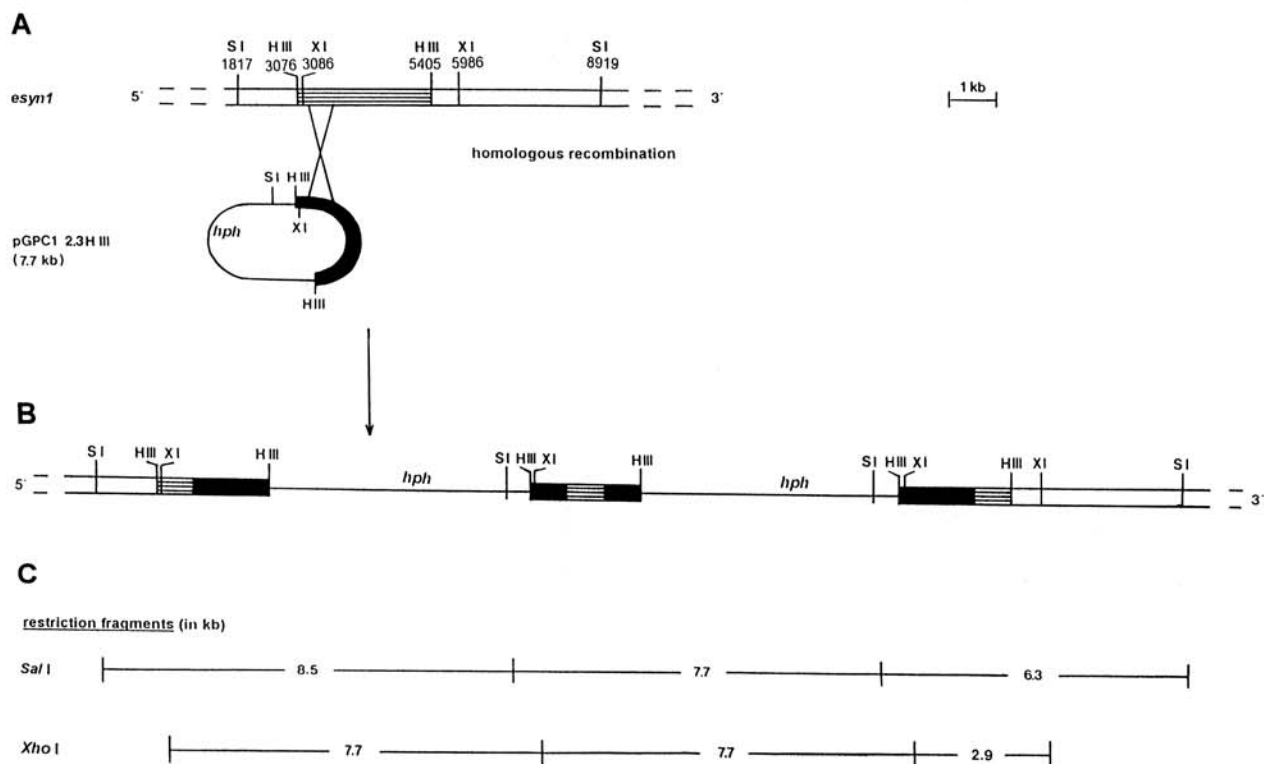


Fig. 2. Transformation-mediated disruption of the *esyn1* gene by tandem integration. **A**, Restriction maps of the enniatin synthetase wild-type gene and of the transformation vector pGPC1-2.3HIII. The boxes indicate the 2.3-kb *Hind*III regions of the genomic (striped) and the plasmid DNA (black) involved in the homologous recombination event. **B**, Restriction map of the mutated *esyn1* locus of the transformants T23, T75, T82, and T114. **C**, Sizes of restriction fragments that would hybridize to the 2.3-kb *Hind*III-fragment after digesting chromosomal DNA of transformants generated by vector tandem integration. S I (*Sal*I), H III (*Hind*III), X I (*Xho*I), *hph* (hygromycin phosphotransferase gene).

stems was determined by crossing a naphthazarin-producing, highly pathogenic strain with a non-naphthazarin-producing slightly pathogenic mutant strain (Holenstein and Défago 1983). The virulent strain produced several toxic naphthazarin compounds of the polyketide type. Because highly virulent segregants were obtained that did not produce naphthazarins, they are apparently not a major virulence factor.

In contrast to the toxins produced by *Fusarium* species, HC-toxin, a cyclic tetrapeptide produced by the maize-

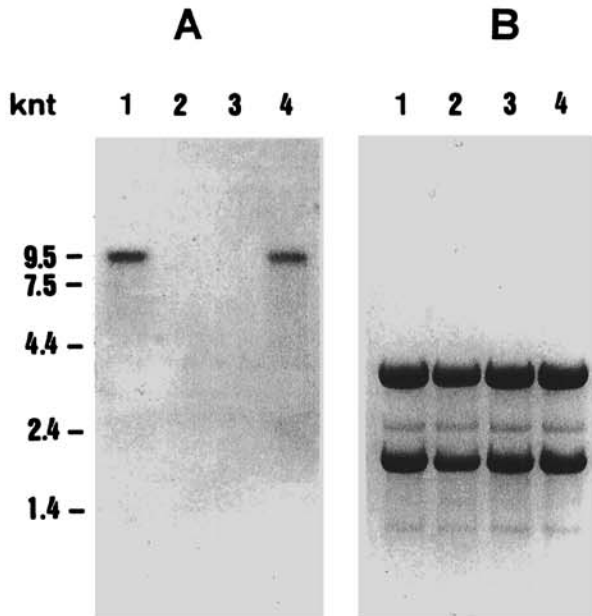


Fig. 3. Transcriptional analysis of *Fusarium avenaceum* wild type strain and transformants. **A**, Northern hybridization of total RNA with an internal *F. scirpi* *esyn1*-fragment (7.1 kb *SalI*). The lanes are as follows: *F. avenaceum* wild type (1), T23 (2), T82 (3), and T224 (4). Sizes of RNA marker are indicated at left. **B**, Methylene Blue-stained agarose gel of total RNA.

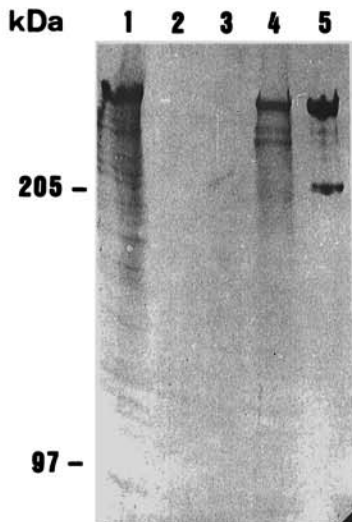


Fig. 4. Analysis of proteins from *Fusarium avenaceum* wild-type strain and transformants. Immunoblot with antiserum directed against enniatin synthetase purified from *F. scirpi*. Crude protein extracts (100 µg of protein) of *F. avenaceum* wild-type strain (lane 1), transformants T23 (2), T82 (3), and T224 (4), and 10 µg of enniatin synthetase from *F. scirpi* (lane 5) were separated on a 5% sodium dodecyl sulfate-polyacrylamide gel. Protein size markers are indicated on the left.

pathogen fungus *Cochliobolus carbonum*, represents a host-selective toxin that is required for infection of particular genotypes of maize. Like enniatins, it is synthesized by a large, multifunctional enzyme (Scott-Craig et al. 1992). Mutants in which both copies of the HC-toxin synthetase gene were disrupted via homologous recombination could not infect maize plants susceptible to the wild type (Panaccione et al. 1992).

Because the enniatin null mutants of *F. avenaceum* still affect potato tubers, although to a lesser extent compared with the enniatin-producing transformants and the wild type, they probably express additional pathogenicity factors. Besides the production of toxic low-molecular-weight metabolites, the pathogenicity of *Fusarium* species might be influenced by the resistance to phytoalexins. The gene encoding pisatin demethylase (*pda*) that detoxifies the phytoalexin pisatin produced by pea in response to fungal infection has been isolated from *Nectria haematococca* (Weltring et al. 1988). Transfor-

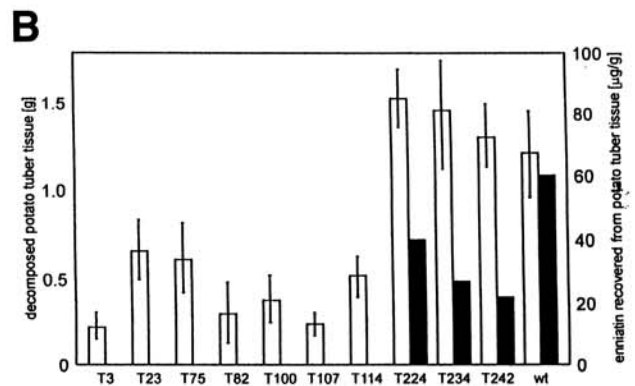
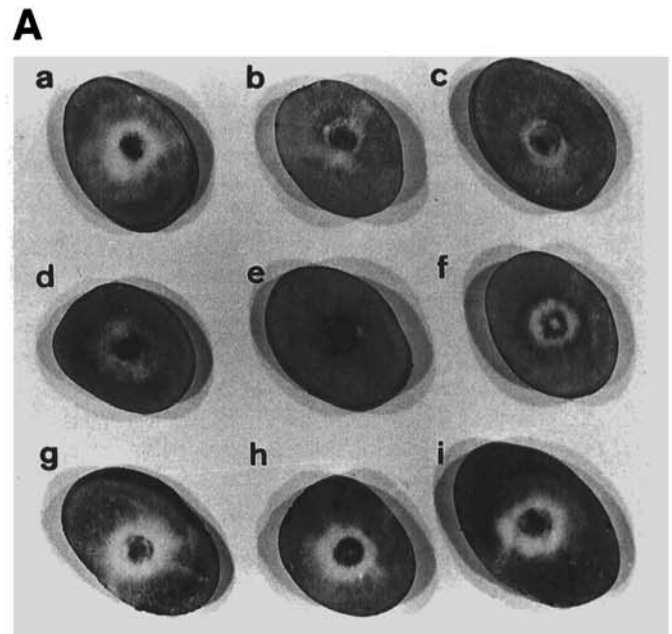


Fig. 5. Virulence assay of *Fusarium avenaceum* on potato tuber tissue. **A**, Slices of potato tubers six days after infection. *F. avenaceum* wild type (a), and transformants T23 (b), T75 (c), T82 (d), T100 (e), T114 (f), T224 (g), T234 (h), and T242 (i). **B**, Mean amounts of decomposed potato tuber tissue per slice (white bars with standard deviations), from 24 single values and means of enniatin recovered from the decomposed tissue (black bars). Values were obtained from four independent tests with six slices per test for all mutants and the wild-type strain.

mation of the pisatin demethylase gene into a pisatin-sensitive, avirulent strain of *N. haematococca* (anamorph: *F. solani*) resulted in pisatin-tolerant mutants that could infect pea plants (Ciuffetti et al. 1988). *Nectria haematococca* mutants disrupted in the *pda* gene did not show the expected reduction in virulence (VanEtten et al. 1994). Two other phytoalexins, lubimin and rishitin, occur in potato tuber tissue infected with *F. sambucinum*. The ability of different *F. sambucinum* isolates to metabolize these compounds is correlated with virulence on potato tuber tissue in general. Some isolates that metabolize lubimin and rishitin to a high extent in vitro do not infect potato tubers (Desjardins et al. 1989; Desjardins and Gardner 1989).

In conclusion, the results obtained with enniatin-nonproducing mutants suggest that enniatin production contributes to the virulence of *F. avenaceum* on potato tuber tissue.

MATERIALS AND METHODS

Fungal cultures.

Fusarium avenaceum BBA 64338 was obtained from the Biologische Bundesanstalt, Berlin. For DNA isolation, *F. avenaceum* was grown in liquid FDM (Madry et al. 1983): 100 ml of medium in a 500-ml Erlenmeyer flask was inoculated with approximately 5×10^7 spores and incubated for 68 h at 26°C and 120 rpm (Environmental incubator shaker, New Brunswick, Edison, NJ). For RNA and protein isolation, *F. avenaceum* was cultivated on solid FCM (3% molasses, 1% cornsteep liquor, and 2% agar). Agar plates (145 mm in diameter) were covered with a filter paper circle (cellulose 589/2, 125 mm in diameter, Schleicher and Schüll, Dassel, Germany) soaked with 2 ml of distilled water containing approximately 2×10^7 spores. After 3 days of incubation at 26°C, the mycelium was scraped off and frozen in liquid nitrogen.

DNA isolation and Southern blotting.

Nuclear DNA was purified from 200 mg of lyophilized mycelium according to Möller et al. (1992). Gel electrophoresis, Southern blotting, and hybridization were performed following standard protocols (Sambrook et al. 1989). *SalI* and *XhoI* (GIBCO BRL, Eggenstein, Germany) restricted chromosomal DNA was separated on 0.5% agarose gels in 1× TAE (40 mM Tris-acetate and 1 mM EDTA pH 7.8) buffer at 20 V overnight and after capillary transfer in 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) crosslinked to nylon membrane (Hybond-N, Amersham, Braunschweig, Germany) using UV light. The 2.3-kb *HindIII* insert of pGPC1-2.3HIII was labeled with alpha-[³²P]-dCTP to a specific activity of approximately 1×10^8 cpm per 25 ng of DNA (Random Primers DNA Labeling System, GIBCO BRL, Eggenstein). Hybridization was performed in 5× SSPE (1× SSPE is 0.15 M NaCl, 10 mM NaH₂PO₄, 1 mM EDTA pH 7.4), 5× Denhardt's solution, and 0.1% (wt/vol) sodium dodecyl sulfate (SDS) at 65°C overnight. Blots were washed at high stringency (0.1× SSPE, 0.1% SDS, 65°C).

PCR and DNA sequencing.

Sequences for degenerate primers were deduced from the nucleotide sequence of *esyn1* of *F. scirpi* ETH 1536 J5 (*esyn1* accession number Z18755 of the EMBL nucleotide sequence

database) as follows. 5' primer (starting at nucleotide 4,882 of *esyn1*): 5'-GGAATT⁴⁸⁸²CT C/T AA A/G GCI A/G A/G CCT IGCITATCTTCC C/T CT C/T GA-3'; 3' primer (ending at nucleotide 5,886 of *esyn1*): 5'-TGGATCC⁵⁸⁸⁶TTICCICGI AT T/C TT A/G AA-3'. Primers had 5' *EcoRI* and *BamHI* restriction sites, respectively, in order to facilitate the cloning of the amplified DNA fragments. PCR was performed with 2 µg of chromosomal DNA in a total volume of 100 µl with 300 pmol of both primers, 200 µM each dNTP, 2.5 U *Taq* DNA polymerase (Pharmacia, Freiburg, Germany), and the buffer supplied by the manufacturer. Amplification with an automated thermal cycler (Gene ATAQ Controller, Pharmacia, Freiburg) included 28 cycles of the following: denaturation for 30 s at 95°C, annealing for 2 min at 37°C during the first three cycles, further annealing at 45°C, and extension for 2 min at 72°C. The PCR product was cloned and further subcloned in pBluescript SK (Stratagene, Heidelberg, Germany) with *E. coli* XL1-Blue (Bullock et al. 1987) used as host. Both strands were sequenced (Sequenase-7-deaza-dGTP Sequencing Kit, Amersham, Braunschweig).

Protoplast isolation and fungal transformation.

Transformation of *F. avenaceum* was done by the protocol of Weltring (1995). Conidiospores (2×10^8) were washed twice in 10 ml of water and incubated in 40 ml of YEPD (0.3% yeast extract, 1% Bacto-peptone, and 2% glucose) broth for 6.5 h at 30°C and 80 rpm (Environmental incubator shaker, New Brunswick). Germinated spores were collected by centrifugation at $1,000 \times g$ for 10 min and washed twice with 0.8 M KCl. The cell walls were digested with 1.3% Novozym 234 (Sigma, Deisenhofen, Germany), 2.2% driselase (Sigma), and 0.1% chitinase (Sigma) in 10 ml of 0.8 M KCl overnight at 30°C and 80 rpm. The protoplasts were collected by centrifugation at $1,000 \times g$ for 5 min at room temperature and washed once in 0.8 M KCl. After resuspending the pellet in 8 ml of 0.8 M KCl, 4 ml of STC (1.2 M sorbitol, 10 mM Tris-HCl, pH 7.5, and 50 mM CaCl₂) was added, centrifuged as above, and washed twice in 10 ml of STC. Protoplasts were resuspended in 0.5 ml of STC and diluted to a final concentration of 3×10^8 protoplasts/ml. Plasmid DNA (100 µg) in 1 ml of STC were mixed with 3×10^8 protoplasts followed by the addition of 500 µl of 25% PEG 6000 (25% PEG 6000, 50 mM CaCl₂, and 10 mM Tris, pH 7.5). After incubation at room temperature for 20 min, 20 ml of 25% PEG 6000 were added, mixed, and incubated for another 5 min. The transformation mixture was diluted with 40 ml of STC and completely mixed. Protoplasts were plated by mixing samples of 600 µl with 4.5 ml of molten regeneration medium (0.1% yeast extract, 0.1% casein-enzyme-hydrolysate, 1 M sucrose, and 1.1% agar; 44°C), and poured into petri dishes (94 mm in diameter) containing 20 ml of regeneration medium. After incubation overnight at 26°C, plates were overlaid with 5 ml of top agar (1% agar in distilled water) containing 2.5 mg of hygromycin B to yield a final concentration of 100 µg of agar per ml. Transformants became visible after 2 days of incubation at 26°C.

Selection of transformants and mitotic stability test.

Five days after plating the protoplasts, hygB-resistant colonies were transferred to FCM-agar containing 100 µg of hygromycin B per ml and incubated for another 10 days at

26°C. An agar slice of each culture (10 mm in diameter) was extracted with 2 ml of chloroform and enniatin was determined photospectrometrically as described by Audhya and Russel (1973). In order to determine the mitotic stability of the transformants, 100 conidiospores were spread on FCM agar plates and incubated for 3 days at 26°C. Conidiospores were harvested again and passaged under nonselective conditions five times. Then 100 spores were spread in parallel on FCM-agar plates with and without hygromycin B (75 µg/ml).

RNA isolation and RNA blotting.

Total RNA was purified with the RNeasy Total RNA Plant Kit (Qiagen, Hilden, Germany). Fresh mycelium (200 mg) was ground in liquid nitrogen, suspended in the supplied RLC (lysis) buffer and sonicated for 3 × 10 s. After RNA isolation, glyoxylated total RNA, 40 µg each lane for blotting and 10 µg for staining, was separated on an 1% agarose gel for Northern blotting. For staining, gel was shaken for 1 h in a solution of 0.5 M sodium acetate containing 0.04% methylene blue and destained overnight in water. Transfer to nylon membrane (Hybond-N, Amersham) via capillary blotting was performed following standard protocols (Sambrook et al. 1989). An internal 7.1-SalI fragment of *esyn1* of *F. scirpi* (bp 1,817 to 8,919), radiolabeled as described for Southern blotting, was used as probe. The blot was hybridized in 6× SSC, 2× Denhardt's solution, and 0.1% SDS (wt/vol) at 65°C overnight and washed as described above for Southern blotting.

Enzyme purification and protein blotting.

Crude protein extracts were prepared from 100 mg of lyophilized mycelium with buffer A (0.3 M KCl, 50 mM Tris-HCl, pH 7.2, 4 mM DTT, 1 mM benzamidine, and 15% glycerol) including PEI (polyethylenimine) precipitation and ammonium sulfate precipitation (60% saturation) as described (Zocher and Kleinkauf 1978). Crude extract pellets were suspended in 50 mM Tris-HCl, pH 7.2, 1 mM EDTA, and 4 mM DTT, desalted on Ultrogel ACA 54 (Serva, Heidelberg, Germany) columns (4 × 75 mm), and eluted with the same buffer. Crude protein extracts were separated by SDS-polyacrylamide gel electrophoresis (PAGE) in 5% Laemmli gels (Laemmli 1970). Electrophoretic transfer to nitrocellulose and immunoblotting were performed according to Billich et al. (1987) with polyclonal anti-enniain synthetase antiserum raised in rabbits as first antibody. As second antibody anti-rabbit IgG-AP conjugate (Promega, Heidelberg) was used with Nitro Blue Tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) as substrates (Sambrook et al. 1989).

Virulence assay on potato tuber tissue.

Virulence of the fungal strains on potato tuber tissue was determined by a slightly modified protocol described for *Fusarium* species by Desjardins et al. (1989). New potatoes (cv. Sieglinde) were surface sterilized for 5 min in 1% sodium hypochlorite and washed three times in sterile water. After drying, the potatoes were cut into slices approximately 6 mm thick and placed in petri dishes on Whatman 3MM paper soaked with water. Each slice was infected with a piece (10 mm in diameter, mycelial side down) of a 6-day-old culture of *F. avenaceum* wild type and transformants grown on FCM agar without HygB and incubated for 6 days at 25°C in the

dark. After 6 days of incubation at 26°C, petri dishes with potato tuber tissue were weighed before and after removal of the decomposed tissue.

For calculation of means and standard deviations the highest and the lowest value from the total of 24 values for each transformant were not considered. The statistical significance of the results was determined by the *t* test comparing the group of enniatin-nonproducing transformants with the group of enniatin-producing transformants and the wild type.

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