Molecular characterization of *Fusarium poae*

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Fusarium poae is a wide-spread cereal pathogen with the ability to produce trichothecenes. Molecular characterization of 15 geographically different strains of this fungus was performed. Electrophoretic karyotype analysis revealed the presence of five co-migrating chromosome-sized DNA molecules between 4.6 and 7.5 Mb in size; strains were uniform in this respect. On the other hand, almost all isolates contained one or two mini-chromosomes, smaller than 2.0 Mb, and marked individual differences were observed in the mini-chromosome patterns. A linear mitochondrial plasmid, 1.9 kb in size, was found in one of the strains and this plasmid showed no homology with other plasmids known in the genus *Fusarium*. The majority of strains harbored double-stranded RNA (dsRNA) elements with great individual differences in numbers and sizes. *F. poae* has a trichodiene-synthase gene with high sequence similarity (> 88 %) to the *Tox5* gene of *Fusarium sporotrichioides*.

Keywords: double-stranded RNA, electrophoretic karyotype, *Fusarium poae*.

Fusarium poae (Peck) Wollenw. is a weak pathogen of cereals and also may infest stored grains. Some strains of this fungus have been reported to produce toxic trichothecenes, primarily diacetoxy-scirpenol (DAS) (Logrieco & al., 1990), others proved to be nontoxigenic (Tóth & al., 1993), still others were found to synthesize mainly nivalenol, fusarenon-X and DAS (Pettersson & Olvang, 1995). The species is strictly asexual, like its close relative, *Fusarium sporotrichioides* Sherb. and has never been the subject of classical or molecular genetic studies.

Recent surveys indicate that *F. poae* is common in grain samples from North America (Wilcoxson & al., 1988) and Europe (Tóth & al., 1993). Considering its growing importance on the one hand, and the limited knowledge on its genetic properties on the other, geographically different strains of the fungus were subjected to electrophoretic karyotype analysis and were screened for the presence of extranuclear genetic elements: a potential trichodiene-synthase gene was cloned from one isolate of *F. poae* and its sequence was compared with that of the *Tox5* gene from *F. sporotrichioides*.

Materials and methods

Strains

Ten isolates (A-11, A-13, A-14, A-15, A-18, TA-3, TAPO-2, TAPO-4, TAPO-5, 22-189) of *F. poae* were collected from wheat kernels, surface-sterilized with sodium hypochlorite. Five additional isolates of different geographic origins were also included, namely: Nos. 10 and 11 (from Japan), 72-187 (from Finland), BC-1 (from Canada), NZ-194 (from New Zealand) kindly sent by Drs. Y. Ueno, A. Ylimäki, R. M. Clear, and M. Di Menna, respectively.

Separation of chromosome-sized DNA

Protoplasts were isolated, embedded into low melting point agarose (Sigma, St. Louis, USA), treated with Proteinase K (Serva, Heidelberg, Germany); chromosome-sized DNAs were separated by pulsed-field gel electrophoresis as described by Fekete & al. (1993a). Electrophoresis was performed using the contour-clamped homogeneous electric field dynamically regulated II (CHEF-DR II) system (Bio-Rad) at 9 C in circulated 1 x TAE buffer under the following conditions: (1) in 0.6% agarose at 40 V for the whole run with 3 sec pulse time for 48 h, followed by 53 min/96 h, 45 min/72 h, and 37 min/72 h to separate the large chromosomes; (2) in 0.7 % agarose 40 V/1600 sec/24 h, 40 V/1200 sec/24 h, 180 V/120 sec/12 h, and 180 V/180 sec/12 h to separate the small chromosomes. Gels were stained with 0.5 mg ml^{-1} ethidium bromide, visualized and photographed under UV illumination at 312 nm. *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* chromosome size standards (Bio-Rad) served as markers.

Purification of nucleic acids

Fungi were grown on a complete medium (CM, salts of Czapek solution, glucose, 20 g, yeast extract, peptone, and casein hydrolysate, 3 g per litre of each) in 100 ml volume inoculated with 10^6 ml^{-1} conidia and incubated on a rotary shaker for 45–48 h at 120 rev. min^{-1}, at 25 C. Mycelium was harvested by filtration, washed several times with sterile cold water, blotted dry and freeze-dried. The lyophilized material (0.2 g) was powdered in liquid nitrogen, extracted in 2 ml lysis buffer [50 mM TrisHCl, 50 mM EDTA, 2% (w/v) N-lauryl-sarcosine (Sigma) and 1% (v/v) 2-mercaptoethanol] for 1 h at 65 C, centrifuged to remove cell debris; the supernatant was extracted sequentially with equal volumes of phenol, phenol-chloroform-

isoamyl alcohol (25:24:1), chloroform–isoamyl alcohol (24:1), and diethylether. DNA was precipitated by adding 0.1 volume of 3 M sodium acetate and 2 vol ethanol (–70 C, 15 min), centrifuged and washed in 70% (v/v) ethanol, dried and dissolved in 200 ml distilled water. Samples of this material were treated with various nucleases to identify their nature. These treatments were: DNase I (Sigma) and RNase I (Sigma). Electrophoretic fractionation of the samples was performed in 1% (w/v) agarose in TBE (89 mM Tris-borate, 2.5 mM EDTA), at 0.5 V cm^{-1}. Size standards were phage λ DNA digested with *Hind*III or *Pst*I.

Isolation of mitochondria

Mitochondria were isolated by the method of Mirfakhrai & al. (1990) on a Percoll (Sigma) gradient, then treated with DNase I (Sigma, 20 mg ml^{-1}) and lysed in TE-SDS [10 mM Tris-HCl, 1 mM EDTA, 1% (w/v) sodium lauryl sulfate (Sigma), pH 8.0] at 65 C for 1 h to extract DNA.

Hybridization conditions

Total nucleic acid samples were digested with *Bam*HI (Amersham International, Amersham, United Kingdom) and electrophoresed in 1% (w/v) agarose. Gels were transferred onto Hybond-N nylon membrane (Amersham) according to Southern (1975). A 1.1 kb *Xho*I–*Sac*I fragment of the *Tox5* gene from *F. sporotrichioides* (kindly provided by Dr. T. M. Hohn, USDA, ARS, Peoria, Illinois, USA) was labelled with [a^{32}P]dCTP by the multiprime DNA labelling system (Amersham). Plasmids, pCK2 (kindly supplied by Prof. Kistler, H. C., University of Florida, Gainesville, USA) and pFSEK34 were similarly labelled.

Cloning and sequencing the *Tox5* gene from *F. poae*

Two oligonucleotide primers were constructed on the basis of known *Tox5* sequences from *F. sporotrichioides* (Hohn & Beremand, 1989) and synthesized at the Institute for Biochemistry, Agricultural Biotechnology Center, Gödöllo, Hungary. The left primer was 5´-CCAATATGGAGAACTTTCCCACC-3´ and the right primer was 5´-CGGTCACTCCACTAGCTCAATTG-3´ (positions 360–383 and 1530–1553, respectively on the original *Tox5* map). Using these primers a 1.2 kb fragment was amplified from *F. poae*, strain TAPO-5 by means of the polymerase chain reaction. DNA amplification was

carried out according to Saiki & al. (1988) with some modifications: the 50 µl mixture contained 100 ng DNA, 200 nM of each primer, 100 µM of the four dNTPs, and 2.5 unit of *Taq* DNA polymerase (Amersham). The mixture was covered with a drop of mineral oil and subjected to 30 PCR cycles (1 min, 94 C; 1 min, 55 C; 1 min, 72 C) and one final incubation (7 min, 72 C). PCR-products that gave positive signals when probed with *Tox5* from *F. sporotrichioides* were directly cloned into the Bluescript vector (Stratagene, La Jolla, California) as described by Marchuk & al. (1991). DNA sequencing was performed by the dideoxy chain termination method (Sanger & al., 1977) using Sequenase, Version 2.0 DNA sequencing kit (United States Biochemical, Cleveland, Ohio). The nucleotide sequence identified in this study appears in EMBL, GenBank and DDBJ nucleotide sequence databases under the accession number U15658. The nucleotide sequence as well as the deduced polypeptide sequence were compared with the EMBL sequence library (release 38.0) using the GCG software package (Devereux & al., 1984).

Restriction endonucleases *Bam*HI, *Hind*III, *Sac*I and *Xho*I were obtained from Amersham and used according to the manufacturer's recommendations.

Results and discussion

In an earlier study (Fekete & al., 1993a) in which electrophoretic karyotypes of eight species from *Fusarium* sections Arthrosporiella and Sporotrichiella were compared, the single *F. poae* isolate had an unusual distribution of chromosome-sized DNA molecules. Two large, intensively stained bands were found in the upper regions between 4.6 and 7.5 Mb and these bands were assumed to be composed by five co-migrating chromosomes. We confirmed these results for nine additional strains of *F. poae*. All strains contained the two characteristic, thick bands comprising four or five chromosomes. Medium-sized chromosomes were not resolved under electrophoretic conditions suitable for separating chromosome-sized DNA molecules within the size-ranges of *S. pombe* chromosomes, but almost all strains had one or two bands smaller than 2.0 Mb (Fig. 1) under short-run electrophoresis. These bands represent mini-chromosomes with sizes between 1.0 to 1.9 Mb with the size and number varying according to strain. Intraspecific karyotype differences were thus limited to the mini-chromosome range like in *F. sporotrichioides* (Nagy & al., 1995).

When total DNA samples of the 15 strains of *F. poae* were subjected to traditional gel electrophoresis in a 1.0% agarose gel, one or more low-molecular-weight bands appeared in addition to the

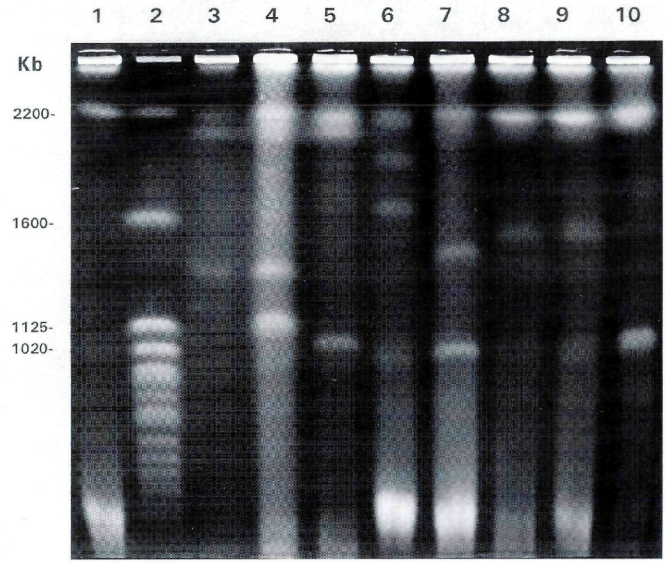

Fig. 1. – Mini-chromosome patterns of *F. poae* strains. Separation conditions are given in 'Materials and Methods'. – Lanes (from left): 1, *F. poae*, 72 187, 2, *Saccharomyces cerevisiae* (size marker), 3–10, *F. poae* strains TAPO-4, TAPO-5, A-13, A-11, TAPO-2, A-14, A-15 and A-18, respectively.

upper band composed of high-molecular-weight nuclear and mitochondrial DNA (Fig. 2). If the preparations were treated with RNase I, then these smaller bands disappeared in all but one isolate, indicating that they were RNA molecules. Subsequently, these bands were identified as virus-like particles with double-stranded RNA genome (Fekete & al., 1995). As with the mini-chromosome patterns there were great individual differences in the dsRNA composition of the various strains, but the number and sizes of the dsRNA bands remained constant for a given isolate even after repeated sub-culturing.

The strain 72–187 that contained an RNase-resistant extra-chromosomal element was isolated in Finland 23 years ago and supplied by A. Ylimäki. The extrachromosomal element in this strain was 1.9 kb in size and was located in the mitochondria. When intact mitochondria were isolated on Percoll gradient, treated with DNase to remove outer DNA contamination, and then lysed in TE-SDS, the 1.9 kb

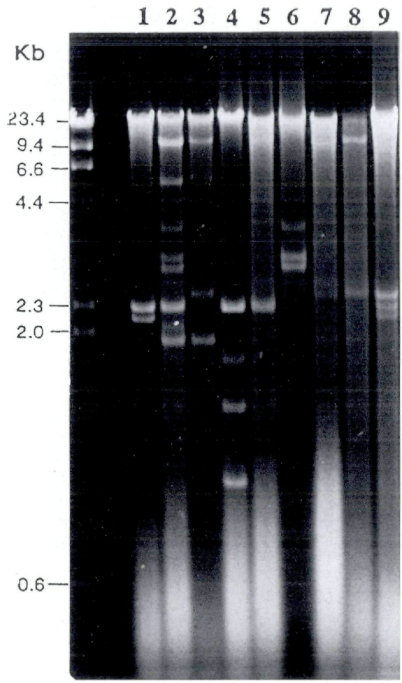

Fig. 2. – Double-stranded RNA patterns of *F. poae* strains. – Lanes (from left): λ phage, digested with *Hind*III, size marker; 1–9: TAPO-2, TAPO-4, TAPO-5, A-11, A-13, A-14, A-15, A-18, and 72 187.

element – a mitochondrial plasmid – remained unchanged. This plasmid, in its intact form, was resistant to exonuclease digestion. After a treatment with Proteinase K, however, it could readily be digested with *Bal*-31 indicating that its DNA was linear. Total DNA samples from strain 72–187 were blotted and probed with other linear *Fusarium* plasmids, pCK2 from *Fusarium oxysporum* Schlecht. (Kistler & Leong, 1986) and pFSEK34 from *Fusarium pallidoroseum* (Cooke) Sacc. (Fekete & el., 1993b), respectively. No detectable hybridization occurred with pCK2 and only a very weak signal was observed with pFSEK34, suggesting that our element is not closely related to either of these two plasmids.

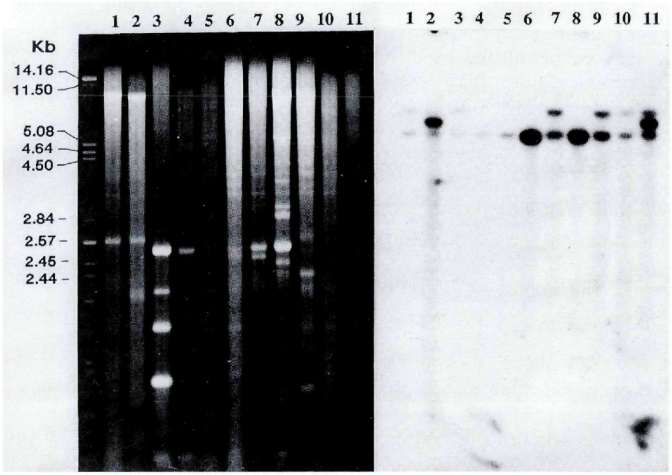

Fig. 3. – *Bam*HI digested total nucleic acid samples of selected strains of *F. poae*. – Lanes (from left): λ phage DNA digested with *Pst*I; 1-11: A-18, A-15, A-11, A-13, A-14, 72 187, TAPO-2, TAPO-4, TAPO-5, TA-3, 22-189. Left part: ethidium-bromide stained gel; right part: Southern blot of the gel probed with the *Tox5* gene of *F. sporotrichioides*.

No morphological alterations were observed in dsRNA- or plasmid-containing isolates; sporulation was normal and there were no signs of senescence. None of the strains containing dsRNA and/or plasmid elements were trichothecene producers, but, since no dsRNA- (or plasmid-) free, toxin-producing isolates were found in our collection a causal relationship between the presence of extra-chromosomal nucleic acid elements and the inability to synthesize trichothecenes cannot be confirmed.

DNA from the 15 dsRNA- (or plasmid-) containing strains of *F. poae* was probed with a 1.1 kb *Xho*I–*Sac*I fragment of the trichodiene synthase gene (*Tox5*) of *F. sporotrichioides* to determine whether or not this gene was present in these nontoxigenic isolates. In hybridizations of this probe to *Bam*HI digested total DNA (Fig. 3), one, two or three bands appeared depending on the strain. The original *Tox5* sequence does not contain a recognition site for *Bam*HI and is present in a single copy in *F. sporotrichioides* (Hohn & Beremand, 1989), implying that multiple alleles or multiple loci with this sequence occur in *F. poae*.

To determine the sequence similarity between the *Tox5* region of *F. poae* and *F. sporotrichioides* the appropriate segment of the *F. poae* genome was amplified by PCR using synthetic primers based on the *F. sporotrichioides Tox5* sequences. The *Tox5* region of *F. poae* was 88.77 % identical to the original gene from *F. sporotrichioides* and the similarity of the predicted amino acid sequences exceeded 95%. When aligned sequences of *F. poae* and *F. sporotrichioides* were examined (Fig. 4), *F. poae* was found to contain three additional amino acid residues near the C-terminus of the predicted gene product.

A

F. poae 1126 TCC**AAGGATGTG**AAGAATGTGAAACAGATT 1155

F. sporotrichioides 1491 TCC**AAGGATGTG**AAGGAG.....GTT 1511

B

F. poae 351 LANVRS**KDV**KNVKQIEKPLLSSIELVE 377

F. sporotrichioides 351 LANVRS**KDV**...KEVQKPLLSSIELVE 374

Fig. 4. – Details of the 3´ regions of the *Tox5* sequences from *F. poae* and *F. +sporotrichioides* (**A**) and the C-termini of the predicted gene products (**B**).

In summary, *F. poae* contains five to six chromosomes, most of which co-migrate in the 5–7 Mb range. Within-species karyotype differences are limited to mini-chromosomes, and extrachromosomal elements, especially dsRNA molecules occur frequently. In spite of the absence of trichothecene producing isolates in our collection (Tóth & al., 1993) the species itself harbor an unharmed trichodiene synthase gene similar to the same locus in *F. sporotrichioides*, a fungus in which almost all strains are strong toxin producers (Logrieco & al., 1990; Nagy & al., 1995; Thrane, 1986). Differences in toxin producing ability and patterns between these closely related species are caused by factors other than the integrity of the *Tox5* region.

Acknowledgments

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