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Identification of growth stage specific transcript profiles in *Fusarium proliferatum* (*Gibberella fujikuroi*, mating population D) by cDNA-AFLP analysis

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Abstract

A cDNA-AFLP approach was developed to identify differentially regulated genes in mycotoxin-producing and non-producing growth stages of *Fusarium proliferatum* (*Gibberella fujikuori* Mating Population-D). Using 160 PCR-primer combinations, a number of fragments which showed strikingly different intensities depending on growth stage were cloned, subjected to Northern analysis and sequenced. Two fragments, amplified from genes up-regulated during spore germination gave significant sequence homology to an amino acid transporter gene from *Neurospora crassa* and a GAL4-like transcriptional activator, respectively. Among the cDNAs derived from late growth stage transcripts, fragments of genes involved in polyol metabolism and cell cycle regulation, respectively were identified. Additional cDNAs (without significant homologies to known sequences) were also isolated and these clearly differentiated between mycotoxin-producing and non-producing growth stages of the fungus.

Introduction

Gibberella fujikuroi is a complex of eight mating populations (MPs) (Leslie, 1991; Klittich et al., 1997; Britz et al., 1999) and a number of asexual lineages, described as distinct morphological species (Nirenberg and O'Donnell, 1998). Most representatives of this species complex have economic importance, but members of mating population-D (anamorph: *Fusarium proliferatum*) are especially worthy of investigation, as these fungi are frequently isolated from a range of diseased plants, including asparagus, date palm, fig, maize, rice, sorghum, tomato and wheat (Shaw et al., 1993; Elmer, 1995; Leslie, 1995; Desjardins et al., 1997; Moretti et al., 1997a, 1998; Abdalla et al., 2000). Strains of *F. proliferatum* secrete mycotoxins, such as fumonisins (Leslie et al., 1992), moniliformin (Marasas et al., 1986), beauvericin and fusaproliferin (Moretti et al., 1997a).

Among the many toxic metabolites produced by *G. fujikuroi*, fumonisins have been studied in most depth. Owing to this intensive research, ample information is available on the molecular genetic background of fumonisin biosynthesis. *FUM5* (renamed later as *FUM1*), which codes for a polyketide synthase, was the first member of the fumonisin gene cluster to be cloned and characterized (Proctor et al., 1999). Subsequently, four additional genes (*FUM6*, *FUM7*, *FUM8*, *FUM9*), adjacent to *FUM1* were identified (Seo et al., 2001) and further components of the cluster (*FUM10–FUM19*) were described by Proctor et al. (2003). Some of these genes code for enzymes, which catalyse substitutions at various positions of the polyketide skeleton, whereas others are only indirectly related to fumonisin production and are predicted to control transportation and self-protection functions.

All wild-type strains of *F. proliferatum* analysed secrete fumonisins, but there are individual

differences in the level of production between strains originating from different geographical or host plant sources (Leslie et al., 1992; Logrieco et al., 1995; Moretti et al., 1997b). The reasons for this variability are poorly understood. Activity of other types of genes, not linked to the fumonisin gene cluster could certainly be among the causes. These genes which are supposed to encode modifying enzymes, transcription factors and transporters may be under developmental control or they could be triggered by specific environmental conditions. One such a gene, *FCC1* which encodes a C-type cyclin, plays a role in fumonisin biosynthesis in *G. fujikuroi* MP-A (*F. verticillioides*) (Shim and Woloshuk, 2001). However, little is known about other developmentally or environmentally regulated genes, which may influence secondary metabolite production or other important phases of the life-cycle, such as germination, hyphal branching, sporulation and reproduction. Similarly, there is little knowledge of the causes of the quantitive differences that can be observed with beauvericin, fusaproliferin, and moniliformin production by *F. proliferatum* or other members of the species complex (Moretti et al., 1997a). Furthermore, great differences can be observed in the production kinetics of all kinds of secondary metabolites of *G. fujikuroi* depending on strains and culture conditions, but information on the regulation of these biosynthetic pathways is limited.

The aim of the present work was to construct a growth curve of *F. proliferatum*, identify characteristic points on this curve and develop an efficient cDNA-AFLP-based approach to tag mRNA fragments which are selectively accumulated during different stages of growth. We expected to identify fragments of developmentally regulated genes and thus improve our knowledge of the life-cycle of this important fungus.

Materials and methods

Fungal strains, media and growth conditions

Fusarium proliferatum, strain ITEM 2287 and *F. verticillioides*, strains ITEM 1776 and ITEM 1148, were obtained from the culture collection of the Institute of Sciences of Food Production, Bari, Italy and stored in 20% (v/v) glycerol at −70 °C. Conidial suspensions were prepared from liquid cultures of the fungi grown on CMC medium (Cappellini and Peterson, 1965). Fumonisin production was assessed in cultures grown in modified Myro medium containing 1% (w/v) corn-hull-extract (Dantzer et al., 1996) for 5 days on a rotary shaker (125 rpm) at 28 °C, in the dark.

The growth curve of *F. proliferatum* ITEM 2287 was constructed by growing the fungus in liquid complete medium (LCM) containing salts of the standard Czapek solution plus sucrose (30.0 g), casein hydrolysate (2.5 g), yeast extract (1.0 g) per litre of distilled water. One hundred ml medium in a 300 ml Erlenmeyer flask was inoculated with microconidia at a final concentration of 10^6 ml^{-1} and grown as shaken culture (180 rpm) at 28 °C, in the dark. Fungal mats were collected by filtration after 6, 9.5, 12, 24, 48, 72, 96, 168, 202 and 220 h and the dry weight of the harvested biomass was measured. The same samples were examined by light microscopy to register the number of conidia, the ratio of germinated to non-germinated conidia, the appearance of hyphal branching and fruiting structures, as well as to estimate the relative proportion of conidial and mycelial cells.

Fumonisin analysis

Fumonisin content of samples taken from 24, 48, 72, 96 and 120-h-old cultures grown on Myro medium was determined by means of a Toxiklon ELISA kit (Barna-Vetró et al., 2000).

cDNA-AFLP analysis

Total RNA was isolated from fungal mats with TRI REAGENT™ (Sigma, Saint Luis, USA) and the integrity of these RNA preparations was checked on a denaturing agarose gel. Oligotex™ was used to isolate mRNA. cDNA was synthesized from 2 μg of mRNA with the Universal Ribo-Clone® cDNA Synthesis System (Promega, Madison, USA). Second strand cDNA was digested by restriction endonucleases, *Eco*RI and *Mse*I and ligated to *Eco*RI and *Mse*I adapters (Vos et al., 1995). Pre-amplification was performed in 19 cycles with primers corresponding to the *Eco*RI and *Mse*I adapters, without extension with a pre-amplification programme (60 s at 94 °C; 19 cycles of 60 s at 56 °C, 60 s at 72 °C, 30 s at

94 °C). The PCR product was then diluted 25× and amplified again with one or two selective base extensions at the 3′ end of the *Mse*I and *Eco*RI primers with a cDNA-AFLP touchdown-selective amplification programme (30 s at 94 °C; 13 cycles of 30 s at 65 °C, 1 min at 72 °C, 30 s at 94 °C; 23 cycles of 30 s at 94 °C, 30 s at 56 °C, 1 min at 72 °C, 30 s at 94 °C; 30 s at 56 °C; 5 min at 72 °C). The *Eco*RI primers were 5′ end labelled with [$^{33}\gamma$P]ATP. Bands which seemed to be differentially expressed on the basis of visual evaluation were cut from the gel and re-amplified (1 min at 94 °C; 22 cycles of 30 s at 94 °C, 30 s at 56 °C, 1 min at 72 °C). The amplified fragments, separated on a 2% agarose gel, were cloned into pBluescriptIIKS (Stratagene, La Jolla, USA) using T4 DNA ligase at 16 °C, overnight. *Escherichia coli* strain DH5α was transformed with the resulting plasmid (Sambrook et al., 1989). Transformed *E. coli* colonies were checked for the presence of the expected insert by PCR with the same primers used in the selective amplification programme. Plasmids were purified with the QIAprep® Miniprep Kit.

Northern analysis

Inserts obtained from these plasmids were labelled with [$^{32}\alpha$P] dCTP with the prime-A-Gene system (Promega) and used as probes in Northern analysis to the RNAs isolated from fungal mats representing different growth stages. Total RNA samples were electrophoresed in denaturing agarose gel, transferred to nylon membranes and hybridized with the $^{32}\alpha$P-labelled probes.

Sequencing protocols

The cloned cDNA-AFLP products were sequenced by the Sequencing Service of the Agricultural Biotechnology Center (Gödöllö, Hungary). DNA sequences were analysed with the Wisconsin Software Package (Devereux et al., 1984) and BLAST searches were obtained against the EMBL database (Altschul et al., 1997).

Results and discussion

Culture conditions were chosen to obtain cells occurring exclusively at a specific growth stage. A nutrient rich medium (LCM), which did not favour conidial production was used and culturing was performed in total darkness. The growth curve, presented in Figure 1, shows the amount of fungal biomass in samples taken at nine time intervals between 6 and 220 h of culture. After 6 h, swollen conidia could only be detected, then an explosive germination started and by 9.5 h almost

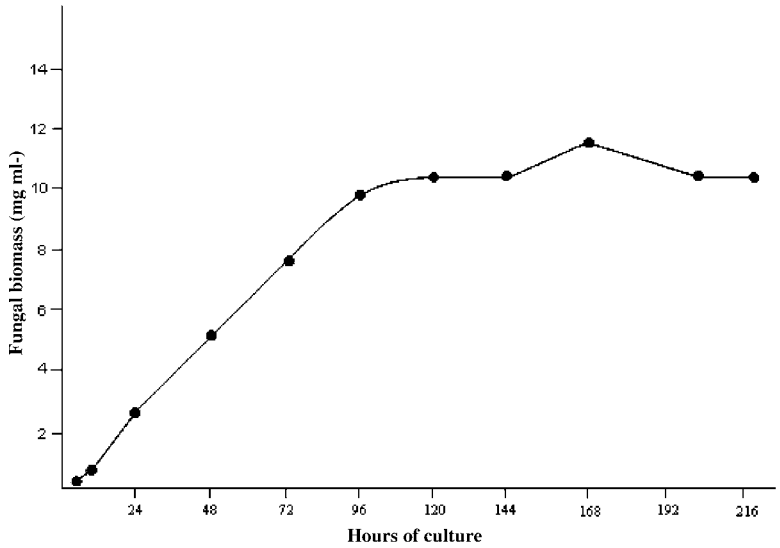

Figure 1. Growth curve of *Fusarium proliferatum*, ITEM-2287 cultured in liquid complete medium. Fungal biomass collected after 6, 9.5, 12, 24, 48, 72, 96, 168, 202 and 220 h of culturing was determined gravimetrically. Growth stages were: acceleration phase, 6–9.5 h; exponential phase, 9.5–96 h; deceleration phase, 96–168 h; stationary phase, 168–216 h; death stage, after 216 h.

all conidia produced germ-tubes, ranging between 10 and 100 μm in length, without the apparent signs of septum formation. Considerable morphological heterogeneity was observed in each additional sampling, except for the 202-h-old sample, which was dominated by mature differentiated mycelium. The clear and characteristic morphological differences detected in the 9.5 and the 202-h-old fungal material were promising in respect of finding significant transcriptional differences. Therefore, these samples were subjected to comparative cDNA-AFLP analysis. An additional advantage of using 9.5-h-old culture as one of the cornerstones, was that at this time no traces of fumonisin production were observed in *F. proliferatum* ITEM 2287, whereas, in subsequent samplings the fumonisin B1 concentration of the culture ranged from 0.5 to 6.0 μg g^{-1} dry weight of the fungal mat.

By using 160 PCR-primer combinations (eight labelled *Eco*RI primers combined with 20 different *Mse*I primers), more than 5000 transcript-derived fragments were generated by cDNA-AFLP. Figure 2 shows an example of the expression pattern differences that can be seen in a 9.5 and a 202-h-old culture of *F. proliferatum* ITEM 2287. Visual evaluation indicated that 310 fragments originated from differentially regulated genes, as their intensity was significantly different in the two fungal samples. These fragments were excised from the gel and subjected to reamplification. Forty-eight fragments which showed the most strikingly different intensities depending on the age of the fungal culture, were selected for further examination. To establish whether the intensity of these fragments correctly reflected the differences in the original mRNA populations of the two growth stages, the fragments were cloned and used as probes in Northern analysis.

Seven of the 48 fragments displayed marked growth stage dependent expression patterns in Northern analysis. Three fragments gave positive signals only in the 9.5-h-old cultures, indicating that they represent sequences up-regulated in germinating conidia. On the other hand, four fragments gave positive signals only in the 202-h-old sample, therefore they were assumed to derive from genes selectively expressed in mature differentiated mycelial cells.

These seven fragments, named according to the selective primer extensions used for their amplification, were sequenced and subjected to database searches. Table 1 summarizes the results of this analysis. Of the three fragments amplified from sequences that had been up-regulated during spore germination of *F. proliferatum*, two are worthy of particular attention. The predicted *Eco*RI + AG/CG1 protein shows significant similarity to an amino acid transport protein described in *Neu-*

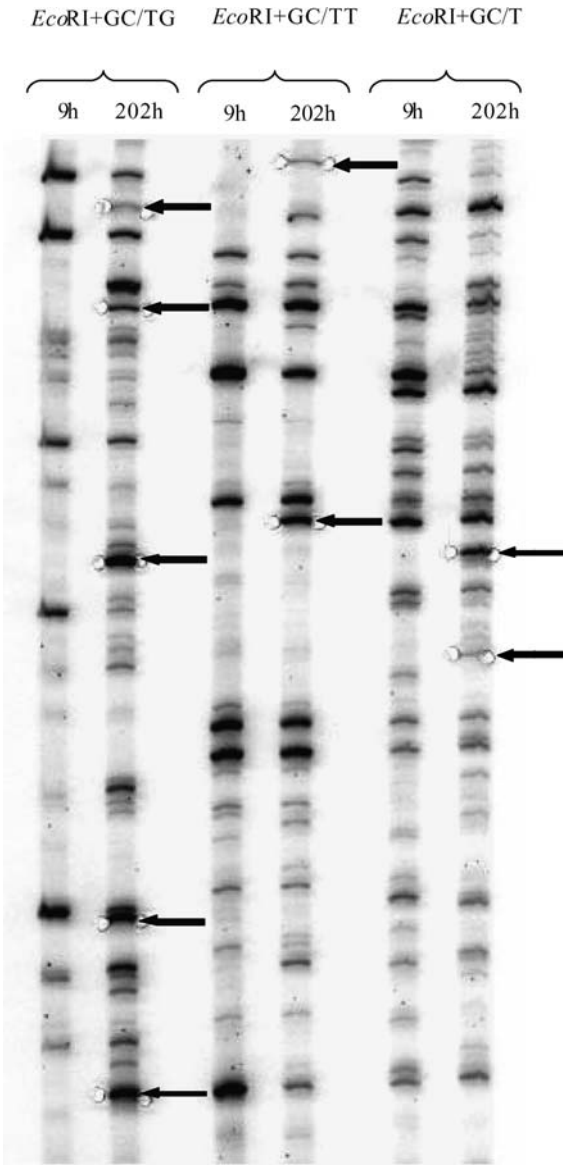

Figure 2. cDNA-AFLP radiogram generated by using labelled *Eco*RI + GC and *Mse*I primer combinations (*Mse*I + TG, *Mse*I + TT and *Mse*I + T). Fragments indicating significantly different expression in *F. proliferatum* ITEM-2287 at 9.5 h and 202 h of culturing are arrowed.

Table 1. Sequence similarities of cDNA-AFLP clones confirmed by Northern hybridisation

Clone	Sample	Length (bp)	Similarity	Identities	Positives
EcoRI-AG/CG1	9 h	352	Amino acid transport system protein (*Neurospora crassa*)	35/105 (33%)	56/105 (53%)
EcoRI-GA/GC2	202 h	562	Related to myo-inositol transport protein (*Neurospora crassa*)	71/170 (41%)	92/170 (53%)
			Mannitol transporter (*Apium graveolens*)	60/179 (33%)	90/179 (50%)
EcoRI-GA/TC2	9 h	555	Putative GAL4-like transcriptional activator (*Colletotrichum lindemuthianum*)	17/42 (40%)	22/42 (51%)
EcoRI-GA/T1	9 h	564	Hypothetical 45.6 kDa protein (*Neurospora crassa*)	26/112 (23%)	50/112 (44%)
EcoRI-GC/TG3	202 h	319	CG5406 protein (*Drosophila melanogaster*)	24/75 (32%)	29/75 (38%)
EcoRI-GC/CC1	202 h	306	Hypothetical protein (*Plasmodium falciparum*)	11/40 (27%)	22/40 (54%)
EcoRI-GC/TT4	202 h	325	SIT4-associating protein SAP155 (*Saccharomyces cerevisiae*)	21/65 (32%)	31/65 (47%)

rospora crassa. Fungal amino acid transporters are known to mediate unidirectional transport from the external milieu into the cytoplasm. Most transporter-encoding genes are constitutively expressed in fungi, except for a few that are activated by nitrogen or sulphur starvation (Gow and Gadd, 1995). A special feature of the putative amino acid transporter identified by cDNA-AFLP is its selective expression in germinating conidia, indicating a developmental control of this specific transport system in *F. proliferatum*. The LCM medium is rich in amino acids, therefore a substrate-derived induction of this transport system cannot be fully excluded. However, it remains difficult to explain why such an external induction was not observed in differentiated mycelial cells collected after 202 h of culturing. The other cDNA fragment, amplified selectively from mRNAs of the 9.5-h-old cultures, shared similarity to the *CLTA1* gene, identified in *Colletotrichum lindemuthianum*. *CLTA1* encodes a putative GAL4-like transcriptional activator and this regulatory gene was found to be involved in the transition between biotrophy and necrotrophy during the life-cycle of this fungus (Dufresne et al., 2000). Another GAL4-like protein encoding gene, named *fl*, has been found to activate conidiation-specific genes in *Neurospora crassa* (Bailey and Ebbole, 1998).

Among the cDNAs derived from late growth stage transcripts, a 562 bp *Eco*RI + GA/GC2 sequence showed convincing similarity to a myo-inositol transport protein from *N. crassa* and a mannitol transporter from *Apium graveolens*. The up-regulation of genes controlling polyol metabolism in the late stationary phase could be expected, as the level of osmotic stress increases after prolonged culturing, although little information is available about osmoregularity mechanisms in filamentous fungi (Hocking and Norton, 1983). Another cDNA clone (*Eco*RI + GC/TT4), amplified from transcripts of the old differentiated mycelium, appeared to share significant homology to the SAP155 protein from *Saccharomyces cerevisiae*. SAP proteins are known to be physically associated with the SIT4 phosphatase in late G_1 stage of the cell cycle. The association results in the formation of separate complexes, which remain stable until the middle of mitosis. SIT4 is involved in a number of late G_1 processes, including cytoskeleton organization, cell wall integrity and ribosomal gene transcription. However, SIT4 exerts these activities only in physical association with the SAPs. The level of free monomeric forms of SIT4 is constant throughout the cell cycle, but association of this phosphatase with SAP phosphoproteins is regulated by cellular growth signals. SAPs are believed to transduce external growth signals (e.g. availability of nutrients) to the cell through their association with SIT4 (Luke et al., 1996), but how this association process is initiated is not yet clear. According to the present study, at least one SAP encoding gene is up-regulated in the late stationary growth phase in *F. proliferatum*, forced probably by density

dependent or nutrient depletion signals. Elevated levels of SAPs may then promote the SAP – SIT4 association, leading to the start of the late G_1 events mentioned above.

The culturing procedures used in this study provided fungal materials suitable for the amplification of growth stage specific transcripts in *F. proliferatum* by cDNA-AFLP. The cDNA fragments described (and others in the course of confirmation) are suitable for tagging genes that are regulated by developmental or environmental signals and are thus selectively involved in the key events of the life-cycle of this fungus. Such an approach should lead to a better understanding of the causes of within-species or growth stage dependent differences of biological traits including secondary metabolite production. This research also resulted in the isolation of some additional cDNAs, which lacked any significant homologies to known sequences, but clearly discriminated between the mycotoxin-producing and the non-producing growth stage and may thus serve as a starting material for developing novel diagnostic DNA probes.

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