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**New functions of a HOG-type MAP kinase, *Fphog1* in  
*Fusarium proliferatum***

Abstract of PhD dissertation

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## Introduction

In eukaryotic organisms, like in all organisms it is indispensable to respond adequately to changes of their environment. This requires a continuous communication with the extra or intracellular environment by applying multiple signaling pathways in response to different intra- or extracellular stimuli.

In molecular level most processes of signal transduction involve ordered sequences of biochemical reactions inside the cell resulting in a signal transduction pathway. One of the most prominent signal transduction pathways is the mitogen activated protein kinase (MAP kinase) pathway. The MAP kinases are serine/threonine-specific protein kinases that play an universal role in regulation of various cellular activities, such as the regulation of cell wall integrity in yeast (Herskowitz, 1995), signaling auxin responses in plant cells (Mizoguchi et al., 1994), programmed cell death during hypersensitive reaction (Ádám et al., 1997), and regulating vulva differentiation in *Caenorhabditis elegans* (Eisenmann and Kim, 1994).

MAP kinases with 35-50 kDa molecular weight, are regulated transcriptionally or post-translationally by phosphorylation-dephosphorylation events in all organism. They are activated by a conserved three-step MAPK phosphorylation cascade. The stimulus sensed by a receptor, firstly activates the MAP kinase kinase kinase (MAPKKK). The MAPKKK phosphorylates the MAP kinase kinase (MAPKK) on the [ST] X<sub>3-5</sub> [ST] motif, which finally activates the MAPK. The prerequisite of the specific phosphorylation by kinases and the specific deactivation by phosphatases is the presence of the kinase interaction motif localized on the N-terminal region of the MAP kinase (Jin et al., 2003; Zuniga et al., 1999). Out of the 11 conserved subdomains, the VIII. subdomain containing the (TXY) dual phosphorylation motif, plays a crucial role in the post-translational activation of MAPKs. The phosphorylated treonine (T) and tirozine (Y) residues are required also for the enzymatic activity and interaction with the docking motifs of the transcription factors (Lee et al., 2004). MAP kinases can regulate their substrates by phosphorylation, protein-DNA and protein-protein interactions either on a level of transcription translation or post-translation.

Based on sequence alignment and phylogenetic analysis of MAPK genes from yeast species fungal MAP kinases have been divided into three subgroups, namely YERK1 (yeast and fungal *extracellular regulated kinase*) YERK2 and YSAPK (yeast and fungal *stress activated protein kinase*) (Kültz, 1998). In a wider context, YERK1 and YERK2 belong to the plant/fungal/animal ERK (*extracellular regulated kinase*) group playing an important role in

sexual recombination and pathogenesis-related mechanisms. The YSAPK MAPKs have been assigned to the fungal/animal SAPK (stress activated protein kinase) group (Kültz, 1998), and they are mostly transducing abiotic stimuli. The first characterized YSAPK MAPK was the *Saccharomyces cerevisiae* HOG1 (high osmolarity glycerol) MAPK (Brewstert et al., 1993), playing a role in adaptation to hyperosmotic stress, therefore we use HOG-type MAPK instead of YSAPK MAPK, because it is more prevalent in the literature.

In the present work we investigated the functions of the HOG-type MAPK of *Fusarium proliferatum* (teleomorph: *Gibberella intermedia*). This fungus is an economically important fungus, occurs globally and is associated with a diverse range of host plants including maize, wheat, asparagus, rice, sorghum, date and garlic as a weak parasite or secondary invader (Abdalla et al., 2000; Desjardins et al., 1997; Dugan et al., 2003; Leslie, 1995; Moretti et al., 1997). Moreover Herbrecht and co-workers (2004) reported about a pneumonia caused by *F. proliferatum* in a 62 year-old lung transplant recipient. The wide geographic distribution and the great variety of substrates utilized by this fungus suggest that *F. proliferatum* is especially successful in adapting itself to different conditions, including environmental stress conditions and, therefore seems to be a suitable organism for studying stress responses.

*Fusarium proliferatum* secretes a wide range of secondary metabolites including dangerous mycotoxins which also enhances the economical importance of this fungus. Fumonisin including fumonisin B1, are among the most dangerous fungal toxins, as they have reportedly been associated with various animal diseases and human esophageal cancer (Yoshizawa et al., 1994; Marasas, 2001; Myburg et al., 2002).

In our work we investigated the molecular mechanisms of the HOG type MAPK mediated adaptation to environmental stresses and the signal transduction of factors affecting the secondary metabolism in *Fusarium proliferatum*.

The aims of our experiments were:

1. Using multiple sequences alignment we could set up a subgroup specific cloning approach for cloning HOG-type MAPKs
2. Isolation of *Fphog1* a HOG-type MAPK encoding gene from *F. proliferatum*
3. Generation of  $\Delta Fphog1$  knock out mutants applying PEG mediated protoplast transformation
4. Phenotypic characterization of *Fphog1* MAPK gene using mutant strains, in consideration of:

- sexual recombination and the pathogenicity
  - abiotic stress tolerance during heat-, UV-, osmotic-, oxidative, and cell wall stresses
  - programmed cell death (PCD) under hyperosmotic stress.
5. Study of the role of *Fphog1* in regulation of the fumonisin metabolism under nitrogen depletion and nitrogen starvation conditions.

## Methods

### Strains and culture conditions

ITEM 2287 and FGSC 7615, wild type strains of *F. proliferatum*, obtained from Institute of Sciences of Food Production, CNR, Bari, Italy and Fungal Genetic Stock Center, University of Missouri-Kansas City, Kansas City, MO, USA, respectively were maintained on potato dextrose agar at 4 °C. Conidial suspensions were prepared from liquid cultures grown on CMC medium (Cappellini and Peterson, 1965).

### cDNA synthesis

Total RNA was extracted from fungal samples using TRI reagent (Sigma St. Louis, MO, USA). The RNA was then treated with DNaseI (MBI Fermentas, Vilnius, Lithuania). cDNA was synthesized with the RevertAid cDNA Synthesis Kit, (MBI Fermentas, Vilnius, Lithuania) from 1 or 2,5 µg DNA free total RNA following the manufacturer's instructions.

### PCR reactions

PCR amplification reactions were carried out in T3 thermocycler (Biometra, FRG) in a volume of 25 or 50 µl. The PCR reaction was performed in a reaction buffer containing 1x PCR buffer (MBI Fermentas, Vilnius, Lithuania), 1,5 mM MgCl<sub>2</sub>, 0,2 mM concentration of each deoxynucleoside triphosphate, 25 pmol of each primer, 1U Taq polimerase (MBI Fermentas, Vilnius, Lithuania) and about 20 ng of fungal DNA. Initial denaturation was done at 94 °C for 5 min, followed by 35-40 cycles consisting of (i) 3 min at 95 °C; (ii) 15s at 94°C, 30s at 60 °C, 30s-5 min at 72 °C, depending on the length of the reaction product; (iii) and the final extension step was at 72 °C for 5 minutes.

### **Quantitative real time (qrt-) and RT-PCR reactions**

One  $\mu\text{l}$  of the 10 times diluted first-strand cDNA reaction mixture (equivalent to 10 ng total RNA) was used as a template for qrt-PCR in a standard PCR mixtures.

Qrt-PCR was carried out using the ABI PRISM SDS 7000 system (Applied Biosystem, Foster City, CA, USA) with SYBR Green (Bio-Rad, Hercules, CA, USA) detection. Amplification conditions were: (i) 95 °C for 10 min and (ii) 40 cycles for 15 s at 94 °C and 60 s at 57 °C. In RT-PCR amplifications initial denaturation at 95 °C lasted for 3 min, and the number of cycles was reduced to 30. A 377 bp fragment of the *histone H3* gene (Glass and Donaldson; 1995) was used as a reference. Reference gene expression was validated for each experiment to demonstrate that *histone H3* expression was unaffected by experimental conditions. To determine the relative change in the expression of the examined gene, the comparative  $C_T$  ( $\Delta\Delta C_T$ ) method was used (Livak and Schmittgen, 2001). To check the validity of the  $\Delta\Delta C_T$  calculations, the target genes and the *histone H3* gene amplification efficiencies ( $E$ ) were compared. For this purpose, serial dilutions of cDNA (cDNA equivalent to 0.1–10 ng total RNAs) were amplified by qrt-PCR, using the above-mentioned gene specific primers. The  $\Delta C_T = (C_{T, Fphog1} - C_{T, histone H3})$  value was calculated for each cDNA dilution. A plot of cDNA dilution versus  $\Delta C_T$  was made. The absolute value of the slope ( $s$ ) of the regression line was close to zero in different runs, indicating that the efficiencies of the examined genes and *histone H3* gene amplifications were similar and the  $\Delta\Delta C_T$  calculation is suitable for measuring the target gene expression (Livak and Schmittgen, 2001). However, individual PCR efficiencies ( $E_i$ ) were neglected by the above approach. Therefore  $E_i$  values for each sample were calculated directly from the PCR kinetic curves by the linear regression method (Ramakers et al., 2003). Finally, gene expression data were also assessed according to gene expression's  $C_T$  difference (GED) formula based on correction of expression data with individual PCR efficiencies (Scheffe et al., 2006).

### **Sequence analysis**

DNA fragments were sequenced by the Sequencing Service of the Agricultural Biotechnology Center, in BIOMI Ltd. (Gödöllő, Hungary). Primers were designed and the sequence data were analyzed with the Lasergene (DNASar Inc., USA) software package and the FGENES program (<http://www.softberry.com>). Blast searches (Altschul et al., 1997) were done with the NCBI database using the BLAST program (<http://www.ncbi.nih.gov>).

### **Southern blot**

Genomic DNA samples were isolated and digested with restriction endonucleases or kept undigested to examine the DNA fragmentation during PCD. The DNA samples were separated in agarose gel electrophoresis and then blotted to Hybond-N membrane. After 6-8 hour of blotting the DNA were fixed to the membrane by UV irradiation. Southern hybridization was carried out in CHURCH buffer at 65 °C according to Sambrook and Russel (2001).

### **Cloning and disruption of the *Fphog1* gene**

A full length (2089 bp) copy of the gene was then amplified by PCR using primers, YSfor and YSrev and cloned into pGEM-t Easy (pYS). A 369 bp *XbaI-KpnI* fragment of pYS was replaced with a 3805 bp hygromycin expression cassette (*hph*) containing the hygromycin *B* phosphotransferase (*hygB*) gene (Punt et al., 1987); this yielded the plasmid pGemFpKYS. A 5739 bp PCR generated fragment of this plasmid was used to transform protoplasts of *F. proliferatum* ITEM 2287 isolated from exponentially growing cultures. PEG-mediated transformation was carried out as described by Proctor et al. (1997). Hygromycin (Duchefa, Haarlem, The Netherlands) resistant transformants were passed and selected in the presence Hygromycin. Site-specific integration of the *hph* cassette was confirmed by PCR, using the primer pairs CHPH1-CHPH2 and CHOGfor-CHOGrev and Southern hybridization with *Fphog1* and *hph* probes, respectively.

### **Complementation of the $\Delta Fphog1-24$ mutant**

The neomycin phosphotransferase gene (*nptII*) fused to a 2089 bp PCR amplified fragment of the *Fphog1* genomic clone of *F. proliferatum* (Gen-Bank accession number: **EF467357**) containing 304 bp of its own promoter and 116 bp of its own terminator region was used to complement the hygromycin resistant  $\Delta Fphog1-24$  mutant strain. Protoplast transformation was performed according to Proctor and co-workers (1997) with modifications. Two geneticin resistant strains, rescue-1 (R1) and rescue-2 (R2) were selected. Complemented strains maintained hygromycin resistance supporting the idea that complementation events happened at other locations. RT-PCR was used to prove the expression of the *Fphog1* gene in complemented genotypes.

### **Sexual crossing**

Strains were crossed according to Klittich and Leslie (1988) using the mating type tester strain, FGSC 7615 as crossing partner. Strains tested for female fertility were grown on carrot agar for

one week then sprayed with conidial suspension ( $10^6$  cells/ml) of the opposite mating type partner, used as male. Plates were incubated for 5-6 weeks at 25°C with a photoperiod of 12 h dark/light. Cultures were scored regularly for the presence of perithecia. Mature perithecia were crushed under a stereoscope, and the liberated ascospores transferred aseptically to water agar and spore germination evaluated microscopically.

### **Pathogenicity assay**

Invasive growth was measured by inoculation of 10 µl conidial suspensions ( $5 \times 10^7$  conidia ml<sup>-1</sup>) into tomato fruits sterilized by using 70% ethanol according to Di Pietro et al. (2001). The diameter of the lesions were measured daily.

### **Stress treatments**

Fungi, subjected to stress treatments were grown in LCM as shaken cultures (200 rpm) at 25°C or on CM agar. The amount of fungal biomass and optical density (OD<sub>600nm</sub>) of the mycelial suspension showed linear relationship between OD<sub>600nm</sub> 0.0 and 0.4. Samples with higher OD values were diluted and measured by JENEWAY (UK), GENOVA spectrophotometer. Salt, osmotic, cell wall and oxidative stresses were induced by adding NaCl (2–8 %), sorbitol (0.8–1.8 M), Congo red (40 mg/l), SDS (0,014%) hydrogen peroxide (25–100 mM), diamide (0.5–0.75 mM) menadion (0,1mM) and methylglyoxal (12-50 mM) to fungal cultures grown in LCM for overnight (OD<sub>600 nm</sub>=0.3-0.4) or CM agar. Heat and UV stresses were induced by exposing conidia in LCM ( $10^6$  ml<sup>-1</sup>) to 42 or 45 °C for 0–2 h and on CM agar to UV irradiations at U V-C (210-280 nm), monochromatic UV-C (254 nm) or UV-B (312 nm) wavelengths for 0–10 min with VL-6MC UV lamp (Vilber Lourmat, Marne La Vallée, France), respectively. All assays were carried out in triplicate and repeated at least three times.

For experiments examining fumonisin biosynthesis under N-depletion or N-starvation, 500 ml,  $10^6$  db/ml conidial suspension were inoculated in WM+AF (pH 3,3) media. After one day incubation the cultures were transferred to the same media or the same media but without any nitrogen source.

### **Microscopy and fluorescent techniques**

Cells were stained with 0.1% Evans blue dye as described earlier (Ádám et al., 1989). The percentage of blue-stained dead cells was determined by light microscopic examination. Nuclei, stained with 4',6'-diamidino-2-phenylindole (DAPI) according to Harris et al. (1994)

were examined under fluorescent microscope (Olympus BH2 RFCA, Japan) equipped with digital camera.

Intracellular ROS production was monitored by capturing 2',7'-dichlorodihydrofluorescein (DCHF) fluorescence at 520 nm. Samples, taken at 0.5, 3 and 24 h from non-stressed and osmotically stressed cell suspensions grown in 5 ml LCM ( $OD_{600}=0.4$ ) were incubated in 50  $\mu$ M 2',7'-dichlorodihydrofluorescein diacetate (DCHFDA) for 20 min, in the dark. Samples were then washed two times in phosphate buffered saline (0.1 M PBS, pH = 7.4); the pellet, harvested by centrifugation was re-suspended in PBS and cell density was adjusted to  $OD_{600}=0.4$  in all samples. Fluorescence intensity of 200  $\mu$ l samples was measured by luminescence spectrometer LS 50B (PerkinElmer, Norwalk, CT, USA).

Staining of apoptotic cells under osmotic stress conditions was assayed by Mito PT Kit (Immunochemistry Technologies, Bloomington, MN, USA) according to the manufacturer's instruction using JC-1 fluorescent cationic dye (5,5',6,6-tetrachloro-1,1',3,3-tetraethylbenzimidazolocarboyanin iodide). Samples were exposed to excitation at 490 nm in fluorescence microscope or in luminescence spectrometer. The ratio of relative fluorescence intensity (RFU) was calculated by comparing the ratios of green and red fluorescence maxima:  $RFU_{527}/RFU_{590nm} \times 100$ .

### **Determination of ammonium ion concentration**

Concentrations of ammonium ion in culture filtrates were determined by the indophenol blue method (Solorzano, 1969). Ammonium ions react with hypochlorite to form monochloramine. In the presence of phenol and excess of hypochlorite, monochloramine forms blue indophenol, if nitroprusside is used as catalyst. The concentration of ammonium was determined spectrophotometrically at 680 nm. Calibration curves were prepared using ammonium chloride stock solution.

### **HPLC analysis of fumonisin B1**

FB1 content of culture filtrates was determined by HPLC according to the method of Shephard et al. (1990) with small modifications. 200  $\mu$ l o-phthaldialdehyde and mercaptoethanol containing reagent was added to 50  $\mu$ l centrifuged (4000 rpm for 10 min) and sterile filtered. The fluorescent derivatives of fumonisins were determined as previously described (Fazekas et al., 1998). The detection limit of FB1 was 0,1  $\mu$ g/ml.

## Results and discussion

Based on multiple sequence analysis of fungal MAPK genes, a subgroup specific cloning approach was developed and used for cloning of HOG-type MAP kinase genes from different filamentous species. With this approach we successfully isolated and annotated to the Gen-Bank ([www.ncbi.nih.gov](http://www.ncbi.nih.gov)) HOG-type MAPK representing gene tags from *Fusarium proliferatum* (teleomorf: *Gibberella intermedia* Gen-Bank no.: DQ071423), *F. culmorum* (teleomorf: *Hypocrea lixii* Gen-Bank no.: DQ065608) and *Trichoderma harzianum* (Gen-Bank no.: DQ071424). This technique can be advised for isolation of HOG-type MAPK genes from other filamentous fungi.

Using YSAPK MAPK subgroup-specific degenerate primers in nested PCR, an 880 bp fragment was amplified from *F. proliferatum* ITEM 2287. This putative MAPK encoding fragment was 82.1% identical to an *Fgos-2* nucleotide sequence from *Fusarium graminearum* (Gen-Bank no.: XM389788; Ochiai et al., 2007). A 2089 bp amplicon, obtained by single oligonucleotide nested (SON) PCR (Antal et al., 2004), contained the entire Hog-type MAPK encoding gene, *Fphog1* of *F. proliferatum*. *Fphog1* (Gen-Bank no.: EF467357) has a 1071 bp ORF interrupted with eight introns. The deduced FpHOG1 protein is 357 aa in length and has 98.9, 95.3 and 77.9 % identity to FgOS-2, OSM1 and HOG1 proteins from *F. graminearum*, *M. grisea* and *Saccharomyces cerevisiae*, respectively.

Semi-quantitative RT- and qrtPCR data demonstrated that *Fphog1* is constitutively expressed both in dormant and germinating conidia, as well as in differentiated mycelial cells. The constitutive expression of the *Fphog1* gene allowed to study stress responses in different developmental stages (i.e. ungerminated conidia and mycelia) of this fungus. Comparison of the  $\Delta\Delta C_T$  method and the GED formula, two methods used to analyze qrt-PCR data demonstrated that the  $\Delta\Delta C_T$  method results in a moderate overestimation of the expression differences of *Fphog1*, although the trend of the data was not affected. Similar conclusion was obtained when these methods were used in human medical systems (Scheffe et al., 2006).

For functional analysis of *Fphog1* MAPK gene we generated  $\Delta Fphog1$  knock out mutants using PEG mediated protoplast transformation (Proctor et al., 1997). Complementation of the mutants with the wild type *Fphog1* MAPK gene controlled by its' own promoter resulted in two rescued (R1 and R2) strains. An ectopic integration (Ec) mutant was also generated by using only the antibiotic resistance encoding *hph* cassette. All mutants grew normally on CM agar plates and no signs of reduction or delay of sporulation were observed in these mutants.

Germination capacity of the mutant spores was also normal. The mating capability of  $\Delta Fphog1$  mutants showed no detectable decline either, indicating that this HOG-type MAPK gene is dispensable for growth and reproduction under optimum culture conditions. Invasive growth of the wild type, three gene replacement mutants, the complemented R1, R2 and the ectopic integration mutant (Ec) were assessed on tomato fruits. All strains behaved similarly, i.e. they produced visible symptoms on the second day after conidial inoculation and the diameter of the lesions were nearly the same after six day of incubation.

Salt, osmotic and oxidative stressors were tested on agar plates. External oxidative ( $H_2O_2$ ), methylglyoxal, menadion, sorbitol and NaCl stressors strongly blocked the germination of conidia and/or the hyphal growth of the  $\Delta Fphog1$  mutants in a concentration dependent manner, whereas the R1, R2 and Ec mutant showed a phenotype, identical to that of the wild type. Conidial germination was blocked similarly by diamide (0.5-0.75 mM) treatment in both the wild type and mutant strains. Among *Fusarium* species we demonstrated first the role of a HOG-type MAPK in cell wall stress signaling. All the mutant strains were sensitive to Congo red and SDS cell wall stressors. Thermotolerance and UV light sensitivity of the mutants were tested on conidia. The  $\Delta Fphog1$  mutants showed decreased conidial viability after heat, UV-C (210-280 nm), monochromatic UV-C (254 nm) and UV-B (312 nm) stresses demonstrating that *Fphog1* is indeed a HOG-type MAPK gene with multistress signaling functions. Complementation of the  $\Delta Fphog1-24$  mutant with the wild type *Fphog1* gene, rescued the multistress sensitivity of the mutant strains. Another novelty of the our study was that the effects of a wide range of abiotic stressors including heat, salt, cell wall stress, hyperosmolarity, external and internal oxidative stressors and UV-irradiations were tested on the same fungus.

Expression of *Fphog1* during adaptation to salt (4% w/v) and sorbitol (1,2 M) stresses was studied by qrtPCR in wild type *F. proliferatum* mycelial cells. No up-regulation of *Fphog1* expression was observed under salt and osmotic stress conditions as assessed by two different qrtPCR data analysis methods, the comparative  $\Delta\Delta C_T$  method and the GED formula.

Sorbitol and NaCl treatment caused significant increase in the cell death indicated by Evans blue dye in the mutant strains. To demonstrate apoptotic-like cell death, triggered by hyperosmotic shock in  $\Delta Fphog1-24$  HOG-type MAPK mutant strain, we investigated different markers of PCD including levels of intracellular ROS accumulation, mitochondrial membrane permeability transition, nuclear disintegration and DNA fragmentation. All these markers showed significant increases under hyperosmotic stress conditions in a null mutant of *Fphog1* in comparison to the wild type, indicating that this HOG-type MAPK gene plays a prominent

role in regulating apoptotic-like responses. In filamentous fungi a wide range of deleterious environmental effects were shown to trigger PCD, a cascade of events when the cells actively participate in their own death. However, the role of the HOG-type MAPK gene in PCD has not been experimentally demonstrated. We demonstrated for the first time the role of a HOG-type MAPK gene in attenuating apoptotic-like phenotypes under salt and hyperosmotic stress conditions.

A little is known about the molecular background of the regulation of secondary metabolism in *Fusarium* species. In *Fusarium graminearum* a HOG1 homologue MAP kinase, Fgos2 positively regulates the trichotecene biosynthesis (Ochiai et al., 2007). In *F. verticillioides* *FUM1* (a polyketide synthase) and *FUM8* (an aminotransferase) the two prominent members of the fumonisin biosynthesis gene cluster are transcriptionally regulated (Brown et al 2007). It has been reported that N-starvation induces fumonisin B1 biosynthesis in *Fusarium verticillioides* (Shim and Woloshuk, 1999). Therefore we investigated the role of *Fphog1* in fumonisin induction under N-depletion and N-starvation stresses.

The wild type strain of *F. proliferatum* ITEM 2287 was grown in WM medium (modified after Shim and Woloshuk, 1999). Both N-depletion of the culture medium and expression levels of the two *FUM* genes were continuously measured throughout the experiment. *FUM1* and *FUM8* were repressed until four days of culturing, but by day five expression levels showed a sudden and significant increase. This increase, approaching nearly an order of magnitude occurred when ammonium concentration of the culture medium decreased below 10 mM. This experiment provided molecular evidence of the effect of limited N-supply (N-depletion) on *FUM* gene expression.

We compared the growth of the wild type strain and its *Fphog1* deleted  $\Delta Fphog1-24$  mutant of *F. proliferatum* ITEM 2287 in WM media containing either 30 or 0 mM ammonium phosphate. The two strains grew equally well under abundant nitrogen supply. N-starvation reduced the growth of both strains indicating that fungi suffered severe stress, when they were transferred from an optimum N-supply state to N-starvation conditions. These results suggest that *Fphog1* plays a role in adaptation to N-starvation induced stress similarly to its function in reducing deleterious cellular consequences of other stress influences.

To determine whether *Fphog1*, a HOG-type MAP kinase gene is involved in the regulation of *FUM* genes, we compared transcript levels of *FUM1* and *FUM8* in the wild type strain of *F. proliferatum* ITEM 2287, its  $\Delta Fphog1-24$  mutant and the R1 strain, complemented with the wild-type *Fphog1* gene. *FUM1* and *FUM8* expression was measured by real-time PCR at four intervals during a nine days' culturing. *FUM1* expression started to increase from day

four, as intracellular N-reserves depleted and N-starvation progressed. This increase was much stronger in the  $\Delta Fphog1-24$  mutant, than in the wild type or the R1 strain indicating that N-starvation causes much stronger stimulus on *FUM1* in the absence of an intact MAPK pathway. The same tendency was observed when *FUM8* expression was measured: much stronger *FUM8* expression occurred in the *Fphog1* deleted mutant strain than its wild type or the complemented R1 strain. However, no differences in basic expression levels of the two *FUM* genes were observed among these three strains at the start of the experiment (day 0) suggesting that although stimulation of biosynthesis genes by N-starvation stress is enhanced in the absence of an intact HOG-type MAPK pathway, this pathway has no influence on *FUM* gene expression under normal N-supply.

By day five, significant amounts of FB1 were measured by HPLC in culture filtrates of the  $\Delta Fphog1-24$  mutant and the mycotoxin concentration continuously increased in this culture. On the other hand only traces of FB1 could be detected in filtrates either of the wild type or the R1 strain throughout the experiment.

With these experiments we demonstrated that adaptation to N-starvation stress, accompanied with *FUM* gene expression and fumonisin production is regulated on a HOG-type MAPK dependent manner.

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