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A microarray for monitoring the production of mycotoxins in food

Markus Schmidt-Heydt, Rolf Geisen *

Federal Research Centre for Nutrition and Food, Location Karlsruhe, Haid-und-Neu-Str. 9 76131 Karlsruhe, Germany

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Abstract

A microarray, which covers most of the known relevant mycotoxin biosynthesis genes, has been developed. The microarray carries oligonucleotides of the fumonisin, the aflatoxin, the ochratoxin, the trichothecene (type A and B) and the patulin biosynthesis pathways. For trichothecene producing Fusaria the biosynthesis cluster of trichothecene producing *Fusarium sporotrichioides* (type A) and of *Gibberrella zeae* (type B, teleomorph of *F. graminearum*) have been spotted. The aflatoxin cluster carries oligonucleotides specific for *Aspergillus flavus*. The ochratoxin pattern is specific for ochratoxin A producing *Penicillia*, the fumonisin cluster is specific for *G. moniliformis* (teleomorph of *F. verticillioides*) and the patulin genes have been obtained from *Penicillium expansum*. The microarray is designed in a way that newly identified pathway genes can be added easily at any time. The microarray was used to detect the activation of all gene clusters under conditions conducive for mycotoxin biosynthesis. According to the results the obtained signals were specific under the hybridization conditions used and only insignificant cross-hybridizations occurred. The microarray was used to demonstrate differences in mycotoxin pathway gene expressions after growth on various media for trichothecene and ochratoxin A biosynthesis. It was used further to study and compare the expression kinetics of the trichothecene biosynthesis genes of *Fusarium* on different trichothecene supporting media. An expression pattern indicative for trichothecene biosynthesis could be identified.

Keywords: Microarray; Gene expression; Trichothecene; Aflatoxin; Ochratoxin; Fumonisin

1. Introduction

Mycotoxins are secondary metabolites produced by various fungi. About 300 different secondary metabolites are known (Bhatnagar et al., 2002), however only a few of them play a role as contaminants in food. These are especially aflatoxins, trichothecenes, fumonisins, ochratoxin A and patulin (Bennett et al., 2003). For all of them statutory limits have been set or are under discussion within the EU. The most important fungal genera, which produce these mycotoxins, belong to the genera *Aspergillus*, *Penicillium* or *Fusarium.* Aflatoxin is produced mainly by *Aspergillus flavus* or *Aspergillus parasiticus*. The trichothecenes are produced among other *Fusarium* species by *F. graminearum* (type B) and *F. sporotrichioides* (type A). *F. verticillioides* is the most important species for the production of fumonisins and ochratoxin A is produced by different species of the genus *Aspergillus* (*A. ochraceus*, *A. carbonarius* and *A. niger*) and the genus *Penicillium* (*P. verrucosum* and *P. nordicum*). Patulin is mainly produced by *P. expansum*. For nearly all of the important mycotoxin biosynthesis pathways most or part of the genes are known (Beck et al., 1990; Karolewiez and Geisen, 2005; Kimura et al., 2003; O'Callaghan et al., 2003; Proctor et al., 2003; Penalva and Arst Jr. et al., 2002; Yu et al., 2004) and can be used for diagnostic PCR (Edwards et al., 2002) or differentiation purposes (Niessen et al., 2004). However it is a well known fact that the presence of a mycotoxigenic fungus in a food sample does not ultimately indicate the production of the respective mycotoxin. The biosynthesis of secondary metabolites, like the mycotoxins, is tightly regulated depending on environmental conditions like substrate, pH, water activity or temperature (Hope et al., 2005). These facts cause the situation, that for a complete assessment of the mycotoxicological status of a food, not merely the detection of a putative mycotoxin producing fungus is

* Corresponding author. Tel.: +49 721 6625 450; fax: +49 721 6625 453.
E-mail address: rolf.geisen@bfel.de (R. Geisen).

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important, but the knowledge about the ability of the fungus to activate mycotoxin biosynthesis genes under the environmental conditions suitable for the food chain.

It has been shown for several times that the mycotoxin biosynthesis genes are induced and not expressed constitutively (Peplow et al., 2003; Price et al., 2005). Their induction can be measured some time before the mycotoxin can be detected by analytical methods (Mayer et al., 2003; Xu et al., 2000). For this reason, the activation of these genes can be used as an early indication for mycotoxin biosynthesis. However only some genes of a given mycotoxin biosynthesis pathway can be regarded as key genes, whose activation is directly coupled to the mycotoxin biosynthesis. This has been demonstrated clearly by RT-PCR for a range of single genes of the aflatoxin biosynthesis pathway of *A. flavus* (Scherm et al., 2005). In order to predict whether mycotoxin biosynthesis may be possible under certain environmental conditions in a food sample, monitoring of the whole pathway genes would therefore be favourable. A certain expression pattern can be expected to indicate the onset of mycotoxin biosynthesis within the fungus.

Due to this situation, it should be possible to use such a system to systematically analyse the environmental conditions in a food, which allows the activation of mycotoxin biosynthesis genes. The obtained data will lead to the ability to predict, if mycotoxin production under a given situation can be expected, because the genes are going to be activated in a food environment. This in turn can lead to measures to reduce mycotoxin production, simply by avoiding, counteracting or minimizing conducive conditions. The environmental conditions which allow the induction of mycotoxin biosynthesis genes can be regarded as "molecular critical control points" (MCCPs) in terms of an HACCP concept. In contrast to the official analytical methods for the detection of mycotoxins, which can be used to control or assess the final product in relation to statutory mycotoxin limits and which may lead to the rejection of foods in the worst case, the microarray technology can be regarded as a preventive approach to avoid mycotoxin production and to ensure food safety.

In the current manuscript we describe the development of a microarray suitable for the analysis of the activation of most important food relevant mycotoxin biosynthesis genes in a food related context. The functionality of the microarray is demonstrated and an expression pattern is established which is indicative for trichothecene biosynthesis.

2. Materials and methods

2.1. Strains and culture conditions

The following strains from the culture collection of the Federal Research Centre for Nutrition and Food have been used throughout the experiments: *A. flavus* BFE96 as an aflatoxin producer, *F. culmorum* BFE928 as a trichothecene producer, *F. verticillioides* BFE313 as a fumonisin producer and *P. nordicum* BFE487 as an ochratoxin A producer. The following growth conditions have been routinely used to support mycotoxin biosynthesis:

A. flavus: incubation for 5 days at 30 °C on YES agar (20 g/l yeast extract [Merck, Darmstadt, Germany], 150 g/l sucrose, 15 g/l agar).
F. culmorum BFE928: incubation for 5 to 30 days at 25 °C on maize agar (50 g/l maize [Alnatura, Karlsruhe, Germany], 10 g/l agar), wheat agar (50 g/l wheat [Alnatura, Karlsruhe, Germany], 10 g/l agar), YES agar or MGA agar (15 g/l peptone, 1 g/l KH_2PO_4, 0.5 g/l $MgSO_4 \times 7H_2O$, 20 g/l agar and 0.0025 g/l malachite green oxalate).
F. verticillioides BFE313: incubation for 5 days at 25 °C on maize agar.
P. nordicum was grown as described previously (Geisen, 2004).

Other growth conditions, if used, are described underneath the results.

2.2. Isolation of RNA

To perform microarray experiments, RNA has been isolated by using the RNAeasy plant mini kit (Qiagen, Hilden, Germany). An amount of 0.5–1.0 g of the mycelium was used for isolation of total RNA. The mycelium was resuspended in 750 µl lysis buffer, mixed with 7.5 µl β-mercaptoethanol and about 100 glass beads with a diameter of 1 mm (B. Braun Biotech International GmbH, Melsungen, Germany) in a RNase free micro-reaction tube. The probe was mixed thoroughly and incubated for 15 min at 60 °C and 42 kHz in an S10H ultrasonic bath (Elma, Singen, Germany). All further procedures were essentially the same as suggested by the manufacturer of the kit.

In case of natural wheat kernels, an amount of 2.5–5 g wheat kernels was crushed in liquid nitrogen in a mortar. The further procedure was essentially the same as described above.

2.3. cDNA synthesis and labeling

For cDNA synthesis and labeling an amount of 7 µg of the DNase I treated total RNA was used according to the producer's specifications of the Micromax cDNA direct labeling kit (Perkin Elmer Life And Analytical Sciences, Inc. Boston, USA). After cDNA synthesis and labeling, the cDNA was purified with a QiaQuick MinElute-Kit (Qiagen, Hilden, Germany).

2.4. Hybridization and scanning of the microarray

The labeled and purified cDNA was evaporated to dryness in a Speed Vac concentrator (Savant Instruments, Farmingdale, USA), resuspended in 60 µl hybridization buffer (Scienion, Berlin, Germany), heated for 2 min at 95 °C and hybridized for 18 h at 42 °C to the microarray by using an automatic hybridization station (Perkin Elmer, Boston, USA). After hybridization the array was scanned with a confocal laser system (Scanarray lite, Perkin Elmer) at a resolution of 5 µm. The analysis of the results was performed using the Scanarray software (Perkin Elmer, Boston, USA). The results were normalized using the Lowes algorithm (subtraction of

background) and in addition against the constitutive expressed beta tubulin genes.

2.5. Thin layer chromatography (TLC) of deoxynivalenol (DON)

In order to confirm that the used growth conditions do indeed support the biosynthesis of the analysed mycotoxins, a part of the biomass which was used for microarray analysis has been withdrawn and subjected to TLC. For that purpose 10 g of an agar plate, overgrown with mycelium were harvested from the plate by a sterile scalpel and transferred into a reaction tube. An amount of 100 ml of acetonitril:water (84:16, [v:v]) was added. The fungal mycelia were extracted for 2 h at room temperature on a rotary shaker. The mycelia were discarded and the extract was evaporated to dryness in a vacuum concentrator. The residues were dissolved in 1 ml of methanol and 10 µl were spotted onto a TLC plate (Silicagel 60, Merck, Darmstadt, Germany). As the mobile phase, toluol:acetone:methanol (40:20:10, [v:v:v]) was used. Pure DON (1 mg toxin in 1 ml methanol, Sigma, St. Louis, USA) was used as a standard. After development the TLC plate was sprayed with $AlCl_3$ (20% dissolved in methanol) and heated for 7 min at 120 °C to derivatize the toxin. After this procedure it could be visualized under UV light (366 nm).

In case of toxin extraction from natural grown wheat kernels, an amount of 10 g wheat kernels were pulverized with a coffee mill. Then 100 ml of acetonitril:water (84:16, [v:v]) were added, the mixture was extracted overnight on a rotary shaker. To reduce copurified contaminations, the extract was filtered through activated carbon for several times. Each further treatment was the same as described above.

2.6. Quantitative determination of ochratoxin A by HPLC

Detection and quantitative determination of ochratoxin A from fungal colonies was performed according to the method described in the ISO 15141 standard (1998, www.iso.ch). For this purpose 100 mg of the fungal colony were extracted under shaking conditions in 500 µl chloroform at room temperature for 30 min. The mycelial residue was discarded and the chloroform was evaporated to dryness in a Speed Vac concentrator (Savant Instruments, Farmingdale, USA). The residue was redissolved in 500 µl methanol and subjected to HPLC analysis (20 µl) in a Pharmacia HPLC apparatus LKB 2150 (Pharmacia, Uppsala, Sweden). A nucleosil 100-5 C18 CCV 250/4 column has been used for separation (Machery and Nagel, Düren, Germany) at a flow rate of 1 ml/min with acetonitril:water: acetic acid (40:60:1, [v:v:v]). The peak was determined with a fluorescence detector (Shimazu RF551, Düsseldorf, Germany).

3. Results

3.1. Layout of the microarray

The microarray carries oligonucleotides with a length of about 60 nucleotides, which have been selected and generated by an bioinformatics service (Scienion, Berlin, Germany; Eurogentec, Liège, Belgium). The microarray carries most known pathway genes which are available in public databases and important with respect to food safety issues. The ochratoxin A cluster is not yet complete but contains oligonucleotides from genes involved in ochratoxin A biosynthesis (Geisen et al., 2006). The pathways are spotted in subarrays. The microarray carries oligonucleotides for type A trichothecenes (*F. sporotrichioides*), for type B trichothecenes (*Gibberrella zeae*) for fumonisins (*G. moniliformis*), for ochratoxin A (*P. nordicum*) for aflatoxin (*A. flavus*) and for patulin (*P. expansum*). The microarray contains standard negative controls and oligonucleotides of α and β-actin genes from the genera mentioned above as positive controls. The nucleotide sequences and the gene origin of the oligonucleotides are shown in Table 1.

3.2. Functionality and specificity of the microarray

To test the functionality and specificity of the microarray, fungal species which produce the respective mycotoxins, have been grown for 5 days under conditions conducive for mycotoxin production. In particular *F. verticillioides* which produces fumonisin has been grown on maize medium at 25 °C. The same was true for *F. culmorum* which produces the type B trichothecene deoxynivalenol. *A. flavus*, an aflatoxin producing strain has been grown on YES medium at 30 °C and *P. nordicum*, a strong ochratoxin A producing species, has also been grown on YES medium, however at 25 °C. Until now only two genes of the patulin biosynthesis pathway are known, which are already placed on the array, however no microarray experiments with a patulin producer (e.g. *P. expansum*) have been performed yet. After 5 days of incubation the RNA was isolated and subjected to microarray analysis. The results are shown in Fig. 1. According to these results the mycotoxin pathway genes are actively expressed under conditions conducive for mycotoxin production. These results indicate that the sensitivity and the specificity of the microarray is high enough to detect the expression of the targeted mycotoxin biosynthesis pathways. Because of their homologous sequences, cross-hybridization occurs between the type A and type B trichothecene biosynthesis genes.

3.3. Analysis of differential expression of trichothecene biosynthesis genes from F. culmorum and ochratoxin A biosynthesis genes of P. nordicum

To demonstrate that the microarray is suitable to analyse differential expression of mycotoxin biosynthesis genes under different growth conditions, *F. culmorum* and *P. nordicum* have been grown on different media for 5 days. In case of *Fusarium*, maize medium has been used to simulate natural conditions and YES medium has been used to simulate laboratory growth conditions. Both media are conducive for trichothecene biosynthesis. MGA medium has been used as a negative control. MGA medium is a selective medium for *Fusarium* species (Castella et al., 1997) and *F. culmorum* BFE928 does not produce measurable amounts of trichothecenes on this medium. *P. nordicum* was grown on YES medium as an ochratoxin A conducive medium and on minimal medium as a non-conducive medium. After the time of incubation the mRNA was isolated from

Table 1
Oligonucleotides applied to the microarray

Gene sequences and GenBank accession numbers		
Gene	Oligonucleotide sequence[a]	Accession#
Aflatoxin/*A. flavus* [orange][b]		
*nor*B	GTGATGCAGAAAGCGCCATATGTGTTTCCCATTGTTGGAGTCCGCAAGGTCGACCATCTCACGGGCGT	AY510452
*cyp*A	CCACCCGCGATGGGAACAACTTGCATCGGTATCTGATTGTCTTTAGCAAAGGCAGTCGGCATTGCCTC	AY510452
*afl*T	TCAGGTGGCCCTAATTTGCAGCTGTCTTAGCATTCTCGGCGCCGTCGGGATCGAATGGAGAAGCGTTA	AY510453
*pks*A	CAGACGGTGCTAATCATTTTACGTTGATGCAAAAGGAACATGTCTCTATAATTAGCGATCTGATCGACCGGG	AY510453
nor-1	ACTACATGGTGCGCAAGTTCCACTTTGAGAACAAATGGCTCACGGCCTTCATCATCGATCCAGGACAT	AY510453
*fas*2	CACGGTGGGCGTGGACCTGGTTCCTCTCGCCTCCTTCGATGCATACAAGAATGCCATCTTTGTTGAGC	AY510451
*fas*1	CAACATCAACTCGCAGCAATACGTCTGCGCAGGCCATTTCCGAGCCCTTTGGATGCTGGGTAAGATAT	AY510451
*afl*R	TGGAGACGATGCAGCAGGGATACCGGCGCACCATCCAGCGTCCCCTTTCTCACTACTCGGGTTTAGTG	AY510451
*afl*J	GCGGGAAGTTCGCTCTCCCACTAACGCCGTTATTGCATTTGAGATTCAGTACCGTGTGGATAACGATG	AY510453
*adh*A	GGGAGTACGCTGTAACCTTCTTGCTCCTTGGTTCATCGACACCCCACTGATTGCGCCTATGAAGAAGG	AY510453
*est*A	ATGAAGGGGGCGTTAGACACTGCTAAGGTGCCGGTCATGTACGATGCCTATCCGGGATATC	AY510453
*nor*A	CCGTACTAACATGACGAGCAAGGATATATGGCAGCTGTCCTGCAACACTCGGCTAGAAACAGTGCCCA	AY510451
ver-1	GGCTGTGTCGCGGGAGTATATCCCCAATGGTGAGACTTTCACCGATGAGCAGGTAGACGAGTGCGCCG	AY510453
*ver*A	ACGATGAGTGGGATAGCATTCACCCGGCCACTACTTCCAAAGAGGTTAATGGGCATCGTGCCTACCAG	AY510453
*avn*A	CGTGGAAATGCGGTTGATCCTAGTCAAGCTTCTCTGGCATTTCGACCTACGATTAGATACTACGCAGA	AY510453
*ver*B	GAGCAACAGTTTGTGGCCGATTCGTGCCCGAAGAGACTAGGGTCGGTGTTAGCCATCTTGGCGCCTAC	AY510453
*avf*A	CGGCTCGTTTGAGGGAGAAAATAGCGAAGAAGACAGTAGTTGTCGGGATTGTGTGTTCTTTCCTGGTA	AY510453
*omt*B	GATATTCCTCAAACTCTAGCTAACGCGCAGCCAGCGGCAGGGGTCGAGTTCATGGAGCACAATTTCTT	AY510453
*omt*A	GTTGAGGACACTGATAAAGTCACCATGATGGAGCATGACATTCGGCGCCCCAACCCGGTGCGTG	AY510453
*ord*A	CGTGATCAGTCAACCGGGCGCCTTTGACCTCAATGTGGTTCCTCGCAGCCCTGCTCACGAAGAATTGA	AY510453
vbs	TCCACAATACGACGGCAAGAATTATTTTAGTATGTCTGCCGCTCTGATGACCCCCTTCAGTCGTGGTA	AY510453
*cyp*X	TGTCGTTACGCAGAACTGGAGTCTGCACGGCAATCCGAAGGTGTGGAAAGACCCTCATACATTTGATC	AY510453
*mox*Y	TACGTGGTTCAGTGGATTAAGAAGGCCCAGAATGAGAATCTCCGTAGCTTCGTGCCGCGGC	AY510453
*ord*B	GTACAGTGACCTAGCGAAGGCCGAGAAGATCTTACGTGCTCAGAAGCATTGGGTGTCCACAACTTTCG	AY510452
*hyp*A	AGAAGGCAGACTTTCTAACACTGGCACACATGACTATGGATTCGGTCGAGCGCATGTTGGATCCAAAT	AY510453
*nad*A	GTCAGGAGCCATTCCTTTGCCACGACATATTGAGTGGCAAGATACCTGGCGCCATAATTCCGGTTATG	AY510453
*hxt*A	AGAAATGATCAGACCCTCCATGTTCGAGCTTATATTCTCGCCGACATTCTTCGCAACTATAGGCGGTT	AY510455
*glc*A	ACGGCTGCATATCCCAACGACACAGAGCTTCACAAGGTGACATTAGAGCAATTCCGCGTAAAGTCTCG	AY510451
*sug*R	CCATTCAGTCAGGAACGGCATCTGTTCGTCACCGGCGACTTCAAAGGCTGGTATCGCCCTGGATGAGT	AY510451
Fumonisin/*G. moniliformis* [green]		
*fum*19	CGCAATCCATGTCAGAACCAGCGCCCCAAGGTGGGCTTCTCCTACTCGATGAGCCTAGTTCTGCT	AF155773-19
ORF20	CGGATTGTTGTGGAGTAGATACACACGATTCCGCCTTTATCTACGTGCAGCTCACTTCGAAACAT	AF155773-20
*fum*14	TTTACAGAAGCAGGGCCAGTTGCGACTTCGAATGTACTATCAGTCGCAGCTCCTGAGTCACGATA	AF155773-14
*fum*11	CCACGGGCTGAGTGGTTTCTTTCAAGGATTCTGGCCTACTACCGCTCGACAGTCCGCAGGCAGCG	AF155773-11
*fum*13	GCCCAAGGGACTAGCAGCAACACCATCCGATGTGACGAATGGCAGTAACATCTTCGGTATTGGTC	AF155773-13
*fum*1	AATTCGCCTCCTAGTCGCCTCATGTGAGGAAGACCCGGGTATCTTGGAGGATCCGGCAACGGTTG	AF155773-5
*zpd*1	GCGCGGAACTATTTCGGTGCTGAGAAGATCATTTCAACAGTCTCGACGCCAAAGATGGGCTTGGT	AF155773-26
*zrt*1	CGCTGCCGCCTGTGGTCAGAACATCCACGAGGAGTGTTTCAGAATGTGGGCGCAGACAAAGG	AF155773-3
*png*1	TTCTCCATTGATGGAGCTACTGATGTCACTCGACGATACGTGCGCAAGAATCAGCATGCT	AF155773-25
*wdr*1	TGGCGGTGAACGCAAGAGCTGGTGGATGACTTTCAAGTACAGACCCATCTTCGGTGTCGTCCCTC	AF155773-24
*npt*1	TCGTCAAGATTCCGGCGACCCTGCAGAGTACACCAAGTGGATGAAAGAGTTCTACGATAAGCAGG	AF155773-23M
*fum*8	AGGGTATATCATTAGCAGTGCCTCATTCATTGAAGTACACACTGTAAGTTCCAAGAGCTTTGTCT	AF155773-8
*mpu*1	CGCTGTCTTTAACTACCTGGCCGGTTCTTTGTCTCGAATCTTCACAACCCTCCAGGAAGTTGACG	AF155773-27
*fum*18	AGTGTATACTCAGATGTTCCTCGAATAATCGAGCCGTCTTGTTTCATGGGCTCTGCCAATGACCT	AF155773-18
*fum*17	ACCGTTGGTCCTTTCGAGCTTGACTGGGAGGCAGAACAGTACAAATGCCAATTATCTCAGTTCAT	AF155773-17
*fum*16	TGTCAAAATGCAAGGTGGCGAGTATATCGCCCTGGAAAAGCTTGAATCCATCTACAGGACATCTC	AF155773-16
*fum*12	GCTACCAGGCACTGGGTATGAAAGTGACCGCAAGGGCATTCTGCAGCCTTTTTTGATCGGTCCCC	AF155773-12
*fum*10	GAGATTTGTCTTCGGGGTAAGAATATCATGGCGGGTTATACTAATAACCCTGCCGCGAACAGGGA	AF155773-10
*fum*3	TTAGTATGGGCACCGCCAAGAAGCTTCCGGTCCGCTCCCAGCGTTGCCTGGGATTTCGATCTCA	AF155773-9
*fum*7	GCCCAGTTTCATCAGATAGCAGAAAACGCTATGGAGGATGTCTGCACACAAGTTAATCCGGTTGA	AF155773-7
*fum*6	ACCTGTGATTATGGTGGGAGCCGGAACTGGACTGGCTCCGTTCAGAGCCTTTCTACAAGAGCGCA	AF155773-6
ORF21	GACGCTGTCTGCAGTGCGCTGGTCCAAACGATCGACCTTCACGTGCAACATGGAATAAAGTCACT	AF155773-21
*fum*15	GTGTCTCGAAGTTATGCGGTACTTCGCCCCTATACCACTCACAATGCGAGAGGCAACGTGTGACA	AF155773-15
Patulin/*P. expansum* [not shown]		
IDH	TGTCCCACTTCTTAGGCCCGAAAGGCATTCGCGTCAATGCTATCTCACCGGGACCTATTTTGTGC	DQ084388
MSA	GTTGTATTGCGCAGTTTGGCATTTAATGAGGGTGAGGCTCTTCCCATACCGATCTTGAACGACAT	DQ084387
Trichothecene/*F. sporotrichioides* [dark blue]		
fpp	ATCCCTCTCGGAGAGTACTTCCAGATCCAGGACGACTACCTTGACAACTTT	FPP-0

Table 1 (*continued*)

Gene sequences and GenBank accession numbers		
Gene	Oligonucleotide sequence[a]	Accession#
*tri*5	ATTGGCGAATGTTCGATCCAAGGATGTGAAGGAGGTTCAGAAGCCTTTTCTGAGCTCAATTG	AF359360
*tri*4	CTCTAGACAGTGCATCGGTTACACAATGGCCTTTGCTGAGATGTATCTTG	U22462
*tri*101	CTACTGAATTCTGCCGCGCTGTCGACATGCGGGGCCCAATGGGCGTATCAAGCACATACCCAGGCCTT	AF127176
*tri*11	GCAAGAACAGTTTGGGAGTTTGATATTCGTCTTTCTGAAGGTAGCCGGAAT	AF011355
*tri*3	GTTGTAAACCAGTCTTTGCCATATCTCTGGAGGGACGCTTCTACCCTCAAC	U22463
*tri*7	GGCATTCGTACTGTCGCTGGACTCTCACCACGATCAGTAGTTTTATATGC	U22463
*tri*8	CACTACGAGGAAACCAACAATAGTTATAAGGCGGCTGTTCAGTGGCTTGAG	AY032744
*tri*1	GATGTTGCGGTTAGCGTTGGAGGATATTACTCTCGAAGACGGCACCTTTATTCCTAAAGGTCATC	AY040587-0
*tri*9	TCGCCGTTGTAAACTTCAACTACAGAAACCTAGCAAAACCATCTATATCACCCA	AF359360
*tri*12	TTGGACTGCTACTTAACTGACCACGTTCCGCATCAGCTTTATGACAGAAAG	AF011355
*tri*13	TCTCTAGCTACCGTACCATCGAAGGTTTGACTAGCATGCCGGAACATTCCTACATCAGTCTGGCG	AF330109-0
*tri*14	GCCGAGAACATGACACCGGATTATCCCTACGGTGGCGTGTCGGTTTATCAGGATAAGACTAAGCA	AF326571-0
*tri*15	TCCCGACACTCAAGTGTTGACCAGACGCGAGAAGCGACAAAGGGCTACTGCCGCGCATCAGC	AF327521-0
*tri*16	TCAACAAGTGTTGCAAATGCACCATTCTACGAATGTGACTTTGGGCCATCGCTTAGCGAAGATTC	AY187275-0
*tri*6	CAATGTCTTAGAGTCTTCAGCCGCGTGGATAACATGAGGGATCACTATAGG	U22150
*tri*10	GGTCTTATGAAGCGACATTCAGCTCGTTAAAGAAGTACGACGGAGTTCTTGAGGTGTTGGAAGATGCT	AF359360
p450	GAAGAACCCTGACAAGTTCGTTCCGGAGCGTTGGCTTGACATTTACTCGCAAAATCAGACGTCTT	AY226098-0
Trichothecene/*G. zeae* [turquoise]		
orfF	ACCTGCACTATTCACGCGAGTATGGAACTACTCCCAGGATCAAGTCATGGTTTTAATGGAAGGCGTGA	AF359361
orfE	AAACAATGCCATTCTCATCGAGAACTGTTACAACCTAGCTATCCGAGATGGTGTCGTTAACGGAGGCG	AF359361
orfD	GATTCCTTACATAAAATACCCGTTCTTCACACTCAGCGAAACTGACAAAACGGAATTGGTGCTTT	AY102584
orfC	TGCGACATTGCCATCACAAACGGTTTCCCCTACTGGCAGGGTGTTCCCATCAAGGATGCTCTCCGCCT	AF359361
orfB	ATCTGGACCTCAACACCGCAAGCACCAAGTATGTTAATGCTATTGGCCAAGAGAACGCGGATAAATAC	AF359361
*tri*5	CGTGGCACTTGTGCGACGCTAGATACCGCCTCCATGAGATTTATGAAAAGGTCAAGGATCAGGATACA	AY102584
*tri*4	AACTTGAAGTACAAGGAGCATGTCATCCCTAAAGGAACTCCCATTTCTCAGTCCACCTATTTCATGCA	AF359361
*tri*11	GAGATGCGCGTTTCAGTAGTGACCGACTGGATGCGGTCAAGCCATTCTCAATCGGACCACGGAATTGT	AF359361
*tri*3	AGCTCTAGAGAAGGCTTGCAGGGATATCAAGAAATGTTACGATCAATGGCTTGGAAATCCGTTCCTGG	AF359361
*tri*7	AGCTCTATGGCGCCGCATCACCGGTGCCACAAAGCAAGACGGAGACGAAGGAGTCCCTCTATGGCACA	AF336365
*tri*8	AGACTGGCTTTCTCGCTGCTGAGGGTTTTACCATCAACGAAGCTGGCGTCGCCTTTTATGGAATTGAT	AF359361
*tri*9	CTCATATGAGATGGACCCTGATGTCTCGTGGCTCGAGGTTTTCGCATACTCGGGAGTTAGCGCTGCTT	AF359361
*tri*12	TATGATAGAAATAAAGCGGATAAGGACGTACTTGAGGGAGATTCCGATTCGCAGTCATCTCCAACTA	AF359361
*tri*13p	TCGCACCATAGAAGGTCTGACTAGCATGCCAGAGTATTTATATATCAGGCTGGCAAAGAGCGATTTGA	AB060689
*tri*14	TTGCCGAGAATATGACACCGGATCATCCCTTCGGTGGCGTGTCAGTTTACCAGGATAAGACTCAGCAG	AF359361
*tri*6	ACGGGACTTTAGGCGGCATTACCGGCAACACTTCAAGCGCTTTTTCTGTCGCTACTCAGAATGCCCTC	AF359361
*tri*102	AACAACCAGTACCCAAAAGAAATCGGAAGCCATCTGACTTCAGCCCTGCGTGGTACGGATATTCCGCA	AF336365
*tri*10	GATAGTGTGGCCGGGACGCTTCAATTGTTGGAGAGGCTCCCAAATCTTGAAGCTGTCACGAGCGTTAC	AF359361
orfG	CTATTCGTCTTTTGGCGGTGCCGAACCCTCTCTAGACTTGTACAACGCTGGCCTAAACCATGGAGGCT	AF359361
orfH	TTCATAGGTAAACCTACGATCGAAAGCTGGGAAGATTTACCGAACTTGCGAGTTAACGTCTCGGGCAA	AF359361
orfI	TTGAGGAGATAAAATTCCACCCCTATGTAGCTCGCAAAGAGTCGACTGGCTTCATCTACAACCGTCTT	AF359361
orfJ	CGGTACCCAAAGAACTTCAAAGTTTATTACATGTTAGATGAACCGCCTAGCGATTGGGAGCATGGCTC	AF359361
*tri*13	TGACCACGACATTGGAGCTTTCAAGCCCGAAAGATGGCTCATACAGAAGGAGTCATCGACTGGAACCG	AF336365
orfK	ATATGATCCTACCAGGAGGATATCGTCTCCCAAAGGGTGCAGTAGTTATTTCAGCGCTTCATCACATG	AF359361
orfL	GTCTTTAACGGAAACGGATACAAGAAATTTAATGGTACCATCAGCTGTGATGCACTCGAATGGAGAGA	AF359361
Trichothecene genes/*F. poae* [not shown]		
*tri*5_FP	AACTGGCAAACGTTCGATCCAAGGATGTGAAGAATGTGAAGCAGATTGAAAAGCCTCTGCTGAGTTCA	AY130294
Positive control genes (β-actin genes) from the important fungal species [not shown]		
*btb*PV	CGGACAGTAAGTTTTAATGGTGATGTTGGTTTCTGGTGGATTGCACGTCTGATATCTTGCTAGGT	AF001205-0
*btb*PN	GCGTTGGGTATCAATTGACAAGTTACTAACTGGATTACAGGCAAACCATCTCTGGCGAGCACGG	AY674319
*btb*AO	ATGGACAGTAAGTTTTAACTGTGATGGGGGTTCCGGTAGATCATACATCTGATATCTTCCTAGGT	AY160979
*btb*F	CATGAGCGTCTACTTCAACGAGGCCTCTGGCAACAAGTACGTCCCTCGCGCCGTCCTCGTCGATCTTG	AY725266
*atb*GZ	CCCCACTGGTTTCAAGCTTGGTATCTGCTACCAGGCTCCCGAGAACGTGCCCAACGGCGACCTCGCCA	AY860418
*btb*FP	ATGCGTGAGATTGTTCACCTTCAGACCGGTCAGTGCGGTAACCAAATCGGTGCTGCTTTCTGGCAGAC	U85572
*btb*FS	TCTGGCGAGCACGGTCTCGACAGCAATGGTGTTTATCACGGTACCTCCGAGCTCCAGCTCGAGCGCAT	AF404188
Ochratoxin genes *P. nordicum* [red]		
*otapks*PN1	CACCTATTTATTTCAAGTGGGACTCGTCGCTGTTCTTGATTCATTGGGTCTACAGCCCAATGCGATAA	AY557343
*otapks*PN2	TCTCACCTATTTATTTCAAGTGGGACTCGTCGCTGTTCTTGATTCATTGGGTCTACAGCCCAATGCGA	AY557343
*nps*PN1	TCTTTATAGGACCTGCTTTCTAAGATTGTTGTGGAAATCGTTCACACCCGCCAAAAGACACAGCGAGA	AY557343
*nps*PN2	TTTATAGGACCTGCTTTCTAAGATTGTTGTGGAAATCGTTCACACCCGCCAAAAGACACAGCGAGAAA	AY557343
otach/PN1	TGCTCGACAAAGTCGGGAAGAGAGCCATGGTGGCATACTTGGGAAGTGTTCTGATAGTGTCACTTGGT	DQ100374
otach/PN2	ACAATCCCTCCATAGGTTGTGACTTGACTTGTCACTGCCGATACCAGCCCCCAAGCGCAGACGAAGAA	DQ100374

(continued on next page)

Table 1 (*continued*)

Gene sequences and GenBank accession numbers		
Gene	Oligonucleotide sequence[a]	Accession#
otach/PNNc	GGGCACACGACGAATTCAAGAATGTATGTGCTGAAACATTATCAAGAAGGAT	BX294091
*otatra*PN1	GGAACTGGCGCTGCGCTCTTGAGCACAGTTGCGTTGGCTAGTGTACCTCAATGGTTTGATCGAAAGGC	otaTraPn1
*otatra*PN2	GGCTAGTGTACCTCAATGGTTTGATCGAAAGGCAGCCTTTGCAATTGGGATTTCGATGTCGGGCGCCG	otaTraPn2
*ntra*PN	GGTTGCTCACTTGATCAGATGGAAACTAGTGAAGAACAGGGACAATTTCCC	ntraPN
*nreg*PN1	CCGAGAAACCTCATTACTCCACGGCTATGGGTGTTTGCATTGCGCTGCAGCTAGTTGGTGGTCTTTCG	AY557343
*nreg*PN2	CGATCTGGACCCCCTACACGTATTTCGAAACCGAGAAACCTCATTACTCCACGGCTATGGGTGTTTGC	AY557343
*pks*PUrtic	GGAGTCATCCGACTGTCAGCCATCTGGCGGTGTGTTTGCTGAGAAGCTTGCCAAATGATTGACCTTATAAGAACG	pksp_urticae
*asp*1PN	TGTTATTCGTGGAAGCGTACTGATGGTATTACTACAGTCGACCTCTATGCTCCTGGGGAGGCCATCACGGCCGCA	AY557343
*asp*2PN	GTCCCTTGCGACTCTGGCAGTCGTCAATGCCGGTAAGCTCCTTACCGCCAGTGATGCCCACGCTGTCATT	AY557343
*t*01_a	GCGAAGGTGTGGGCAAAAACGCGAGTCCCGGGATATCACGATCACGTGCTTCTATTACATAGGACAATACTGTTT	T-01_A
*t*02_b	TGGACCCAAGTGCAGATCTAGAGTATTAAATGTTTCCCTGCATGCGAAATACCCATCTTGAAATCATGAA	T-02_B
*t*03_b	TTACGTTCACGAAACGAGTGTGAAGTGCATTTGTCAAGAAATCATTGATCATCAAAGCTGCATAAACCCCCACCG	T-03_B
*t*04_b	TGCCCCCCAAAGCCCTGGGCAGAACAGGTCCCCAATATCGCCGGCGCTATGACCGATTTAGTGAG	T-04_B
*t*06_b	CTCATGACGAGATTTCGGGCAACGTTGATTTACATGTAACCTAACAACTACATTTGGAGCCAAAAGTCTGTCCGT	T-06_B
*t*07_a	ATTAACCCTCACTAAATCGGTCATAGACCTTGAACCACAGTCCCTCTGCCCACCAAAGTACCCCCAGAACCACAA	T-07_A
*t*08_ab	ATATAACAGAGTAATAGATTAGCTCTAGTCCTTTCTATGGCTGTCGCAGGCGTCATTTCGAAGA	T-08_aB
*t*08_bb	CATCTATATACCTACGTACATTACTCAGTCTTTTTGTGCACAGCAGGCGACTTCAGAAAGCCGAAAACCGCCAGT	T-08_bB
*pks*PV	AGCGATGTTTTTGCTTTCGATGCTCGTGATGGGTCACTTGTTGAAGTTGCTCTTGGTATTAGTTA	PKS_PV
*pks*AO	ATTAACTTCTGTTTCGAATTTGCCGGACCCAGCTATACCAATGACACGGCCTGTTCATCCAGTCT	AY320070

[a]The oligonucleotides are given in the same order as spotted from left to right in the mycotoxin subarrays.
[b]The colour given here corresponds to the colour scheme of the layout in Fig. 1. "Not shown" means that for clarity these spots are not shown in Fig. 1, but are present on the microarray.

all cultures and subjected to microarray analysis. The production of DON or ochratoxin A was determined by TLC or HPLC in parallel. The results are shown in Fig. 2. The medium clearly has a strong effect on gene expression. In case of *F. culmorum* no activation could be found after growth on MGA medium. Despite the fact that YES medium is a conducive medium for trichothecene biosynthesis, only several genes are activated at that growth phase (see next paragraph). Just the opposite was observed for maize medium, which resembles natural conditions. Nearly the whole pathway cluster is activated after growth on this medium. The data correlate well with the DON biosynthesis. No DON could be detected after growth on MGA medium, however detectable amounts of DON could be measured after prologened growth on YES or maize medium (Fig. 2A).

In case of *P. nordicum* the situation is similar, but not that absolute. After growth of *P. nordicum* on non-conducive minimal medium, some of the ochratoxin A pathway genes are activated, however after growth on conducive YES medium nearly all genes are induced. This result is in correlation with the production of ochratoxin A. *P. nordicum* is able to synthesize about 20 μg ochratoxin A per gram of mycelium on YES medium, but only about 0.120 μg/g on minimal medium (Fig. 2B).

The results clearly show that there is a strong influence of external conditions on the activation of mycotoxin biosynthesis genes, which in turn have an influence on mycotoxin production and that these influences can be measured and visualized by the microarray.

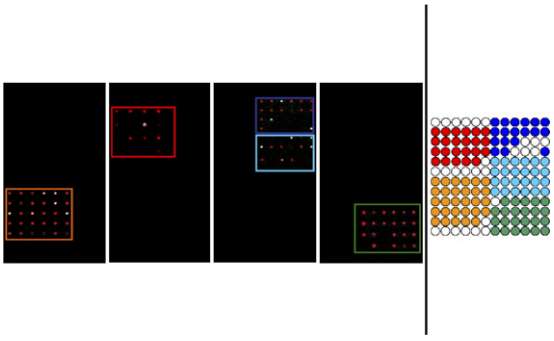

Fig. 1. Demonstration of the functionality of the microarray. Mycotoxin producing fungi (*A. flavus*/aflatoxin; *P. nordicum*/ochratoxin A, *F. culmorum*/trichothecene, *F. verticillioides*/fumonisin) have been grown under conditions which support mycotoxin biosynthesis. RNA has been isolated and subjected to microarray analysis (left side of the line). Part of the microarray layout with the mycotoxin gene subarrays is shown on the right side of the line (A complete list of the oligonucleotides spotted on the array is given in Table 1). In this figure the different subarrays are indicated by different colours (red = ochratoxin A, light brown = aflatoxin, dark blue = trichothecenes (type A), dark green = fumonisins), which correspond to the colours of the frames surrounding the hybridized spots after microarray analysis.

3.4. Expression kinetics of the trichothecene biosynthesis genes determined by microarray analysis

The microarray has been used to study the expression kinetics of the trichothecene biosynthesis genes after growth of *F. culmorum* on YES and maize medium for 30 days. Both media support trichothecene biosynthesis, albeit on YES medium biosynthesis starts earlier and leads to higher amounts. The results are shown in Fig. 3. After 5 days of incubation the whole gene cluster is completely activated on maize medium. Obviously under these conditions the cluster as a whole is induced after activation of the secondary metabolism. Five days later (day 10) only a subset of genes are still detectable, in

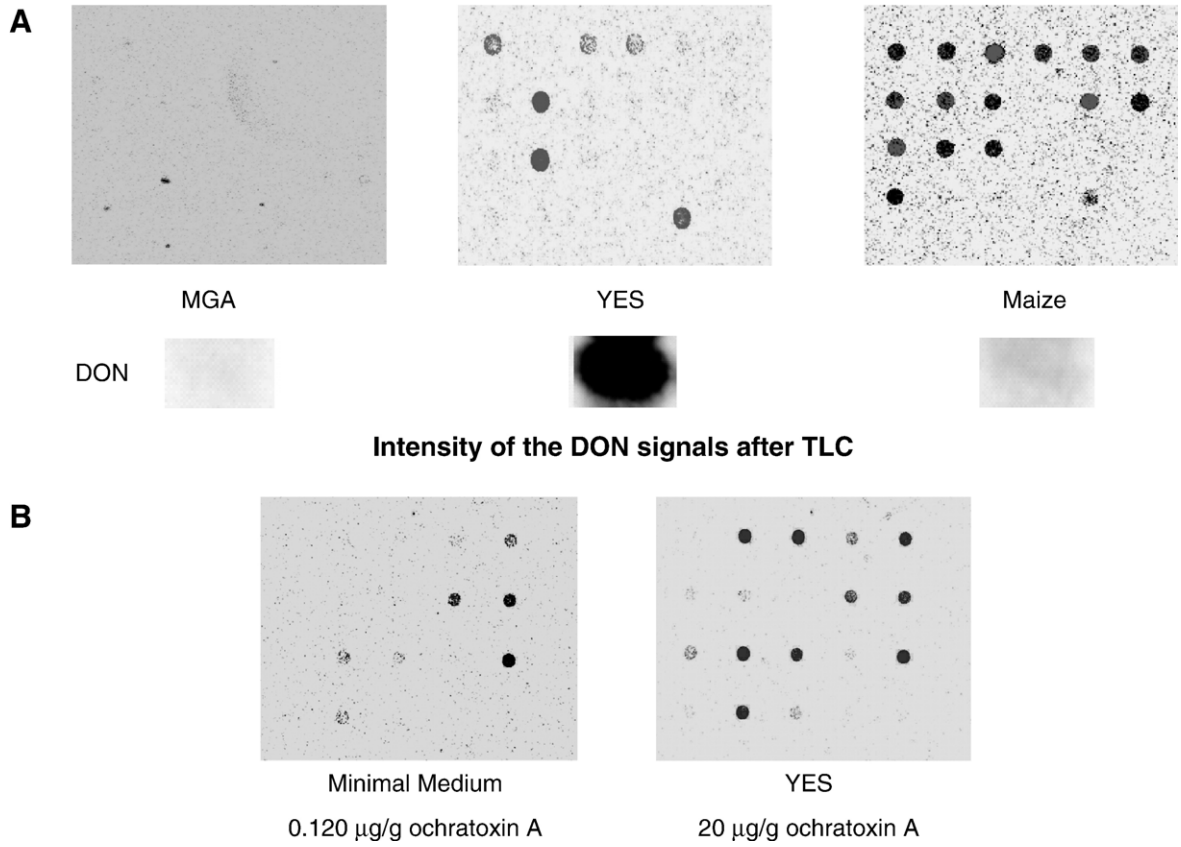

Fig. 2. Differential expression of trichothecene biosynthesis genes of *F. culmorum* (A) and ochratoxin A biosynthesis genes of *P. nordicum* (B) after growth for 5 days on different media. The production of DON by *F. culmorum* is indicated by the intensity of the signal after TLC (A) and the amount of ochratoxin A by *P: nordicum* determined by HPLC is indicated (B).

particular *tri*4, *tri*5, *tri*6, *tri*8, *tri*12, *tri*15, *tri*101, p450 and FPP (farnesylpyrophosphate synthetase). Another 10 days later the same genes are still active, except *tri*6 and p450. The genes *tri*5, *tri*12, *tri*8, *tri*15 and *tri*101 are still highly induced at day 20. At day 30 no actively transcribed gene could be detected. A very similar kinetics of gene expression could be observed after growth on wheat medium (data not shown).

In contrast to maize medium, *F. culmorum* expresses only a small subset of 4 (in particular *tri*5, *tri*8, *tri*15 and *tri*101) genes after 5 days of growth in YES medium, indicating a different type of expression activation on this medium. Interestingly some additional genes are upregulated during the next 5 days and the same expression pattern arose at day 10 as could be identified on maize medium (and also on wheat medium, data not shown). From that time point on nearly the same expression behaviour could be observed for both media. After further incubation, different genes became downregulated until only 4 genes were still active late in the growth phase (20 days). In contrast to maize medium, this subset of genes is still expressed at day 30 in YES medium. Also the amount of trichothecenes increased until day 30 to a higher rate in YES medium compared to maize medium.

These results imply a clear timely coordinated expression of trichothecene biosynthesis genes in *F. culmorum*, which is dependent on the growth medium. The expression pattern that occurs after 10 days of growth is identical under both growth conditions and seems to be an indication for trichothecene biosynthesis.

The phenotypic trichothecene production is different in both types of media. The first traces of trichothecene could be determined analytically after 10 days of incubation on YES medium, but only after about 15 to 20 days of incubation on maize medium. In both situations the fungus still synthesizes and accumulates trichothecene until day 30. At that time high amounts (Fig. 3B) of DON were produced.

These results show that the activation of the cluster is different in both media and can be determined before active mycotoxin biosynthesis. During later phases, a unique expression pattern is generated under both growth conditions (day 10) and can be regarded as an indicative pattern for trichothecene biosynthesis.

3.5. Microarray analysis of natural contaminated wheat

In order to demonstrate that expression analysis of the trichothecene genes with the developed microarray is also possible under natural conditions, the RNA was isolated from two different natural samples of wheat [Ihringerhof, University Hohenheim, Germany]. One sample contained high amounts of DON (947 µg/kg), whereas the DON content of the other sample was below the detection limit. The RNA was isolated from both samples and subjected to microarray analysis. Semi-quantitative results of this analysis are shown in Fig. 4. The sample with the high DON content clearly showed a much

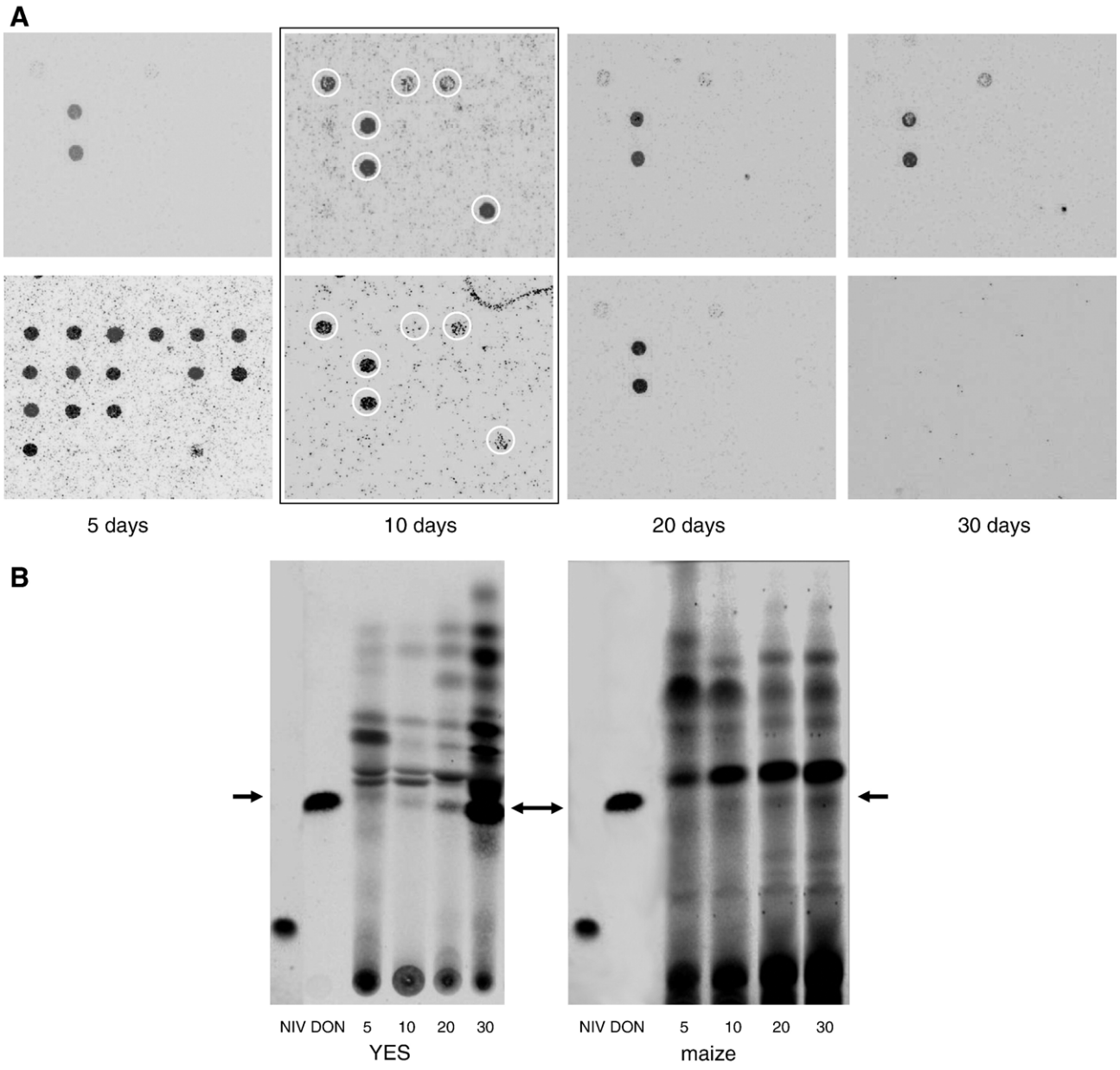

Fig. 3. Expression kinetics of the trichothecene biosynthesis genes of *F. culmorum* after growth on YES medium (upper row) and on maize medium (lower row) (A). Both media are supportive for trichothecene biosynthesis. The indicative pattern for trichothecene biosynthesis is framed and the pattern is indicated by circles. The production kinetics of DON on the two media determined by TLC is shown (B). The incubation times are indicated in days. The position of DON is indicated by an arrow.

higher RNA concentration of all trichothecene cluster genes as the sample with non-detectable amounts of trichothecenes. These results which have been confirmed by Real Time PCR analysis (data not shown), clearly demonstrate that the developed microarray is sensitive enough also to measure and semi-quantitatively compare the RNA content of natural food samples. In addition there seems to be a correlation between the amount of detected trichothecene cluster mRNA and the trichothecene content of the sample.

4. Discussion

A microarray for the analysis of the regulation of biosynthesis genes of the most important mycotoxins has been developed. Initial application of this microarray demonstrates that it is specific, that it can be used to study the influence of growth parameters on the regulation of the genes (demonstrated after growth on different media), to analyse the temporal regulation of the genes during growth and that it can be applied to detect mycotoxin biosynthesis gene mRNA in natural food environments.

This approach offers new possibilities to study the influence of environmental parameters like substrate, pH, temperature and water activity on the activation of the mycotoxin biosynthesis genes and thereby on mycotoxin biosynthesis. It is a well known fact, that growth parameters have a profound effect on the biosynthesis of mycotoxins. Häggblom (1982) demonstrated the effect of growth phase, temperature and inoculum size on ochratoxin A production by *P. verrucosum*. Arroyo et al. (2005)

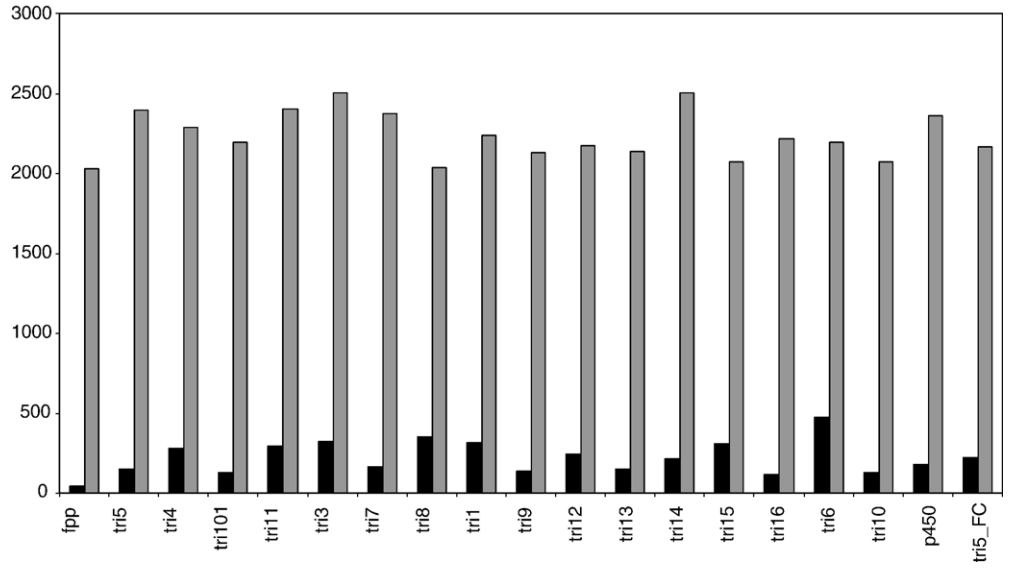

Fig. 4. Semi-quantitative microarray analysis of RNA isolated from two wheat samples. One sample did not contain detectable amounts of DON and NIV (black bars) and the other sample contained 947 μg/kg DON (grey bars). The relative expression values are given in arbitrary units measured as grey levels of the spots by the Scan Array software. The graph shows the hybridization results against the oligonucleotides of the *F. sporotrichioides* trichothecene gene cluster. These oligonucleotides obviously also react with type B trichothecene producers (as shown already with *F. culmorum*, see text). The *tri*5 gene of *F. culmorum* which is located on the array too, gave results very similar to the *tri*5 gene of *F. sporotrichioides*. The gene names given in the graph represent the oligonucleotides of the trichothecene genes of *F. sporotrichioides* unless indicated as FC = *F. culmorum*.

described the effects of water acitivity and the presence of preservatives on the production of ochratoxin A by *P. verrucosum*. Hope et al. (2005) and Llorens et al. (2004) analysed the influence of water activity and temperature on the production of trichothecenes by *F. culmorum* and *F. graminearum*.

This demonstrated high variability of mycotoxin biosynthesis, ranging from high to non-detectable levels. This is of course due to regulation at the molecular level.

The presented microarray is suitable for analysis of mycotoxin biosynthesis gene expression. It could be demonstrated, that the oligonucleotides spotted on the array are specific. Only some minor cross-hybridizations of single genes (e. g. polyketid synthases) between the clusters occurred. It could be demonstrated that the microarray can be used to analyse the influence of environmental parameters on mycotoxin biosynthesis gene expression. After growth of *F. culmorum* on three different media, different expression patterns became obvious. The same was true for *P. nordicum*. In both cases a correlation between expression behaviour and mycotoxin production could be demonstrated. These results clearly show the strong influence of external parameters on gene expression. During this analysis the influence of the substrate was demonstrated. In future experiments systematical analysis to measure the influence of the substrate, the pH, the temperature and the water activity will be performed. The microarray also seems to be suitable to measure the influence of other technological measures, like fungicides or biocontrol agents on the activity of mycotoxin biosynthesis genes.

Especially the generation of an expression kinetics is a convincing feature of the microarray. Two expression kinetics of the trichothecene biosynthesis genes of *F. culmorum* after growth on different media have been generated with the microarray. One laboratory medium (YES) and one maize medium mimicking natural conditions. Both media support trichothecene production. Interestingly the activation of the pathway differs strongly, however the later kinetics are the same in both media. According to this situation a general pattern which is an indication for trichothecene biosynthesis could be identified at day 10. Despite this similarity in the gene expression patterns, the rate of phenotypical production of trichothecene is higher on YES medium than on maize medium. However the absolute amount of trichothcene gene specific mRNA is also higher on YES medium compared to maize medium (data not shown). From a food safety point of view, the emergence of the indicative pattern should be avoided in a production chain by controlling the relevant technological parameters.

From the perspective of gene regulation it is interesting to note, that the *tri*5 gene, one of the key genes of the pathway, is activated instantly in both media and is active nearly thoughout the whole observation period. This gene codes for the trichodiene synthase, one of the key enzymes of that pathway (Hohn and Desjardins, 1992) and it is expectable that this key gene is highly expressed during the period of biosynthesis. Interestingly during the late growth phase only 5 genes are still detectable by the microarray. Most of them encode for self-protection activities like *tri*12, which is an efflux pump to excrete the trichothecene produced (Alexander et al., 1999), *tri*101 which encodes for an 3-*O*-acetyltransferase and protects the cell by acetylation of the trichothecene molecule (McCormick et al., 1999) and *tri*15 which has some activity as a negative regulator protein for trichothecene biosynthesis (Alexander et al., 2004). This regulation behaviour makes biological sense. In later growth phases the concentration of the trichothecenes increases which urges the cell to initialize or maintain self-defense mechanisms.

According to the results, the trichothecene gene oligonucleotides, adapted from *F. sporotrichioides*, also work with *F. culmorum* (and other type B trichothecene producing species as shown with the wheat samples), indicating high homology of the nucleotide sequences. This high homology also leads to cross-hybridization between type A and type B trichothecene genes, which was expected, but has no influence on the data output.

The microarray approach is functional also with natural food commodities as demonstrated by the wheat samples. The amount of trichothecene cluster mRNA detected correlates with the trichothecene content of the sample. This demonstrates the functionality of the approach even under natural conditions.

It has been shown that the microarray is suitable for the analysis of the activation of biosynthesis genes of most relevant mycotoxins under various conditions. It will be used in further work to systematically analyse the influence of relevant parameters on pathway gene expression.

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