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# Expression of a *gfp* gene in *Penicillium nordicum* under control of the promoter of the ochratoxin A polyketide synthase gene

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ABSTRACT

A *gfp* reporter gene strain of *Penicillium nordicum* was constructed in order to be able to study the influence of environmental parameters on ochratoxin A biosynthesis. To introduce the *gfp* gene an *Agrobacterium tumefaciens* mediated transformation system (ATMT) for *P. nordicum* was established resulting in a transformation efficiency of about 60 transformants per µg of DNA. The selection principle was based on the hygromycin B resistance gene located on the TI-DNA fragment of the binary vector system. PCR and Southern blot hybridization revealed that the TI-DNA was integrated into the chromosome of *P. nordicum*. To show that the GFP protein can be used as a reporter gene in *P. nordicum*, this species was subsequently transformed with a vector, carrying a *gfp* gene under the control of the strong constitutive *gpd* promoter of *A. nidulans*. Moreover in this vector construction the *gfp* gene contained a *stu*A nuclear localization domain. Successful transformed strains showed a strong GFP activity located in the nuclei after light stimulation in contrast to the wild type which showed only very weak unspecific auto fluorescence under these conditions. Based on this proof of principle a vector was constructed in which the promoter of the *otapks*PN gene, the gene of the ochratoxin A polyketide synthase of *P. nordicum* was placed in front of the *gfp* gene. This construct was transformed into *P. nordicum* by ATMT and the resulting transformants were analysed by fluorescence microscopy. In these transformants the whole mycelial cells showed GFP activity after light stimulation, whereas the wild type strain did not. When the transformed strains were grown on medium which suppressed ochratoxin A biosynthesis, a very low level of fluorescence could be detected, whereas a high level of fluorescence was seen after growth on medium supportive for ochratoxin A biosynthesis.


## 1. Introduction

*Penicillium nordicum* is an important and reliably strong ochratoxin A producing fungus. It occurs regularly on salt rich fermented meats and cheeses (Lund and Frisvad, 2003; Bogs et al., 2006; Battilani et al., 2007). Ochratoxin A is a mycotoxin which has nephrotoxic, immunotoxic and hepatotoxic activities. Moreover it is categorised as a group 2 carcinogen by the WHO. In many countries regulatory limits regarding the presence of ochratoxin A in certain food commodities have been set. For this reason the control of the production of ochratoxin A under food relevant conditions is of utmost importance. It is well known that the biosynthesis of mycotoxins in general and therefore also ochratoxin A in particular are strongly dependent on the environmental conditions. Substrate composition, water activity and temperature are the most important parameters which have an influence on ochratoxin A biosynthesis. Skrinjar and Dimic (1992) showed that the medium has a profound influence on the biosynthesis of ochratoxin A by *Penicillium*. They found highest biosynthesis after growth of *P. verrucosum* on YES medium. Arroyo et al. (2005) described a strong influence of pH and water activity on the biosynthesis of ochratoxin A by *P. verrucosum*. Cairns-Fuller et al. (2005) analysed the influence of water activity, temperature and gas composition on ochratoxin A biosynthesis by *P. verrucosum* on wheat grain. They could show that 50% $CO_2$ reduce ochratoxin A biosynthesis by 75%. Beside these phenotypical approaches, molecular approaches have also been described to analyse the influence of external parameters on ochratoxin A biosynthesis by *Penicillium*. It was shown that the expression of the ochratoxin A polyketide synthase gene (*otapks*PN) is tightly correlated to ochratoxin A biosynthesis (Geisen et al., 2004) and that the level of expression of that gene is dependent on external growth parameters (Geisen, 2004). If the transcription level of this gene is high enough, the influences of external parameters on the activity of ochratoxin A biosynthesis genes should be visualizable by using reporter gene assays like the *gfp* approach. Under a food safety point of view, these reporter gene strains are for example useful to determine conditions during food processing which allow ochratoxin A biosynthesis and which should therefore be avoided. Reporter gene strains have been developed for the aflatoxin producer *Aspergillus flavus* (Flaherty et al., 1995; Brown

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et al., 1997), for *Aspergillus parasiticus* (Xu et al., 2000) and for the trichothecene producer *Fusarium graminearum* (Ochiai et al., 2007) in order to study mycotoxin gene activation direct in the food product.

In this work we describe the development of an ATMT system for the ochratoxin A producing species *P. nordicum* as well as the use of this system to introduce a *gfp* reporter gene driven by the promoter of the ochratoxin A polyketide synthase. It was shown that indeed this strain fluoresced under conditions suitable for ochratoxin A biosynthesis but not under restrictive conditions.

## 2. Materials and methods

### 2.1. Strains and culture conditions

The strain *P. nordicum* BFE487 was used throughout the study. This is a strong and reliable ochratoxin A producing strain. For strain maintenance this strain was routinely grown on malt extract medium (Merck, Darmstadt, Germany). For extraction of DNA *P. nordicum* BFE487 was grown in liquid YES medium (yeast extract 20 g/L, sucrose 150 g/L, pH 6.5). For optimal *gfp* expression the strain was grown on YES agar, however for differential *gfp* expression *P. nordicum* was grown on ochratoxin A supportive minimal medium ($KH_2PO_4$ 3.8 g, $MgSO_4 \times 7\ H_2O$ 0.5 g, NaCl 0.1 g, $CaCl_2$ 0.1 g, KOH 0.75 g, agar 15.0 g, $H_2O$ dest. ad 1000 ml, pH 6.5, glycerol 5.0 ml, and $NH_4^+$ 2.5 g) or on restrictive minimal medium ($KH_2PO_4$ 3.8 g, $MgSO_4 \times 7\ H_2O$ 0.5 g, NaCl 0.1 g, $CaCl_2$ 0.1 g, KOH 0.75 g, agar 15.0 g, $H_2O$ dest. ad 1000 ml, pH 6.5, glucose 5.0 g, $KNO_3^-$ 1.2 g). Incubation was carried out at 25 °C.

### 2.2. Construction of the reporter gene vector pOTAPKS::GFP

The vector pPK2, which is widely used as a general vector for ATMT (Covert et al., 2001) carrying a hygromycin B resistance cassette between the right and left border of the TI-DNA was obtained from the Fungal Genetics Stock Center, Kansas City, USA. This vector was used as a basis for the construction of the pOTAPKS::GFP-vector. The vector pNRPK2::GFP carries a *gfp* gene under the control of the strong constitutive *A. nidulans gpd* promoter. In addition the *gfp* gene in this construct carries an *A. nidulans stu*A sequence at its 3′end (Helber and Requena, 2007). This sequence is a nuclear localization sequence and should direct the heterologous protein into the nucleus of *P. nordicum*. This vector was kindly provided by N. Requena (University of Karlsruhe, Institute for Applied Biosciences). This vector was used to establish the transformation protocol and as a proof of principle to demonstrate the functionality of the *gfp* approach in *P. nordicum*. The third vector used in this study pOTAPKS::GFP was constructed during this work to analyse activation of the ochratoxin A polyketide synthase promoter in relation to environmental factors. For the construction of this vector a 968 bp promoter fragment (position 1–968, 5′ from the AUG of the *otapks*PN gene) was amplified from the genomic DNA of *P. nordicum* by PCR as well as the 792 bp *zsgfp* gene fragment, which was amplified out of the commercial available plasmid pZsGreen (Clontech, Mountain View, Canada) and ligated together via primer mediated insertion of a PacI restriction side. The resulting promoter/*gfp* construct was ligated into the EcoRV cleaved pPK2 vector. For the ligations T4 ligase were used (Promega, Melsungen, Germany). A map of the important elements of the vector pOTAPKS::GFP is shown in Fig. 1.

### 2.3. Agrobacterium tumefaciens mediated transformation (ATMT)

*A. tumefaciens* AGL-1 cells which carry the binary vector (pPK2) were grown at 28 °C for 48 h in minimal medium which was supplemented with kanamycin (50 μg $mL^{-1}$) and streptomycin (50 μg $mL^{-1}$). The cells were then diluted reaching an OD660 of 0.15 in induction medium (IM) (10 mmol $L^{-1}$ $K_2HPO_4$, 10 mmol $L^{-1}$ $KH_2PO_4$, 2.5 mmol $L^{-1}$ NaCl, 2 mmol $L^{-1}$ $MgSO_4$, 0.7 mmol $L^{-1}$ $CaCl_2$, 9 mmol $L^{-1}$ $FeSO_4$, 4 mmol $L^{-1}$ $NH_4SO_4$, 10 mmol $L^{-1}$ glucose, 40 mmol $L^{-1}$ 2-[N-morpholino] ethan sulfonic acid, pH 5.3, and 0.5% glycerol) in the presence of 200 mmol $L^{-1}$ acetosyringone (AS). The cells were then grown for 9 h before mixing with an equal volume of a spore suspension from *P. nordicum* BFE487. This solution was plated onto cellophane sheets which were placed on the agar plates containing the co-cultivation medium (IM + AS supplemented with 5 mmol $L^{-1}$ instead of 10 mmol $L^{-1}$ of glucose). After the co-cultivation procedure at 28 °C for 36 h, the cellophane sheets were transferred to M-100 plates (55 mmol $L^{-1}$ glucose, and 30 mmol $L^{-1}$ $KNO_3$) plus mineral solution (117 mmol $L^{-1}$ $KH_2PO_4$, 28 mmol $L^{-1}$ $Na_2SO_4$, 1.7 mmol $L^{-1}$ KCl, 8 mmol $L^{-1}$ $MgSO_4$–7 $H_2O$, 9 mmol $L^{-1}$ $CaCl_2$, 7.8 mmol $L^{-1}$ $H_3BO_3$, 5.6 mmol $L^{-1}$ $MnCl_2$–4 $H_2O$, 2.3 mmol $L^{-1}$ $ZnCl_2$, 1.3 mmol $L^{-1}$ $Na_2MnO_4$–$2H_2O$, 2.9 mmol $L^{-1}$ $FeCl_3$–6 $H_2O$, and 12.8 mmol $L^{-1}$ $CuSO_4$–5 $H_2O$) and 1.5% agar supplemented with hygromycin B (100 μg $mL^{-1}$) as selection medium for fungal transformants, and cefoxitin (150 μg $mL^{-1}$) to inhibit the growth of *A. tumefaciens* cells. After incubation for 4 days at 28 °C, the number of hygromycin B-resistant colonies was counted and analysed.

### 2.4. PCR for detection of the genes for hph B resistance and gfp in P. nordicum

For identification of successfully transformed *P. nordicum* strains carrying the pOTAPKS::GFP-vector construct with the hygromycin B resistance gene, isolated DNA of fungal strains were subjected to PCR with specific primers targeted against these genes. For amplifying the *hph* B fragment in putative transformants the primer pair hph_for (5′-TTCGATGTAGGAGGGCGTGGAT-3′) and hph_rev (5′-CGC-GTCTGCTGCTCCATACAAG-3′) was used in a polymerase chain reaction described as follows: template DNA 5.0 μl (0.1 μg/ml); 10 × PCR buffer 5.0 μl; dNTP mix 8.0 μl (2.5 mM each nucleotide), each primer 1.25 μl (5 pM); Taq polymerase 0.5 μl (5 u/μl, Amersham-Pharmacia, Uppsala, Sweden), $H_2O$ bidest 29.0 μl; PCR cycle scheme: initial denaturation (95 °C, 2 min), 35 × (95 °C, 45 s; 60 °C, 1 min; 72 °C, 1.5 min); and terminal elongation 72 °C, 5 min. For amplifying the *gfp* gene in transformed *P. nordicum* BFE487 strains the primer pair zsgfp_for (5′-CGA-TCGCTCGCCACCATGGCTCAGTCAAAG-3′) and zsgfp_rev (5′-GTA-CCCCTATTATTTTTGACACCAGAC-3′) was used. The PCR conditions were the same as with the hph_for/hph_rev primer pair except for the annealing temperature which was 55 °C in case of the *zsgfp* primer pair. These PCRs resulted in fragments of 660 bp for the *hph*B gene and 792 bp for the *zsgfp* gene (*gfp* gene of *Zooanthus* sp.).

### 2.5. Isolation of DNA from pure liquid cultures

The isolation of DNA from pure fungal strains was performed according to a modified method originally described by Yelton et al. (1984). For this purpose the strains were grown for 7 days under shaking conditions (180 rpm) in YES broth. The mycelia were harvested by filtration, transferred to a mortar, frozen in liquid nitrogen and ground to


**Fig. 1.** Map of the TI-DNA fragment of the vector pOTAPKS::GFP. LB, left border; RB, right border; Pgpd, promoter of the *A. nidulans gpd* gene, TtrpC, terminator of the A. nidulans *trp*C gene; *zsgfp*, *gfp* gene of a *Zooanthus* species. The direction of transcription driven by the *otapks*PN promoter is indicated by an arrow.

a powder. The powder was resuspended in 7 ml 50 mM lysis buffer (pH 8.5) vortexed and incubated for 15 min at 65 °C in a water bath. After centrifugation at 13,000 rpm and 4 °C for 20 min, 9 ml of the supernatant was transferred into a new centrifuge tube and 1 ml of sodium acetate (4 M) was added. This solution was placed on ice for 1 h and centrifuged for 20 min at 13,000 rpm and 4 °C. After centrifugation the supernatant was transferred into a fresh tube. The solution was precipitated by the addition of 2.5 volumes of absolute ethanol. After precipitation the DNA was purified using the DNeasy Plant Mini kit as suggested by the manufacturer (Qiagen, Hilden, Germany). The DNA concentration was measured with a spectrophotometer (Pharmacia Gene Quant, RNA/DNA Calculator).

### 2.6. Southern blotting

For Southern blotting experiments the *Eco*RI restriction enzyme digested DNA was separated on a 0.8% agarose gel and transferred onto a nylon membrane filter according to the method of Southern (1975). Hybridization was accomplished under stringent conditions as described by Sambrook and Russel (2001). The *hph* B fragment was labelled in a PCR reaction using the primers hph_for and hph_rev. The nucleotide mixture contained 11-DIG-UTP. The same procedure was carried out for labelling the *gfp* fragment using the primers zsgfp_for and zsgfp_rev. Hybridization and staining of the filter were performed according to the recommendations of the manufacturer of the DIG-UTP Labelling and Detection kit (Boehringer, Mannheim, Germany).

### 2.7. Analysis of GFP fluorescence of the transformed P. nordicum strains

For fluorescence analysis of the putative transformed *P. nordicum* strains, 7 day old mycelia were taken from agar plates with an inoculation loop and placed under a fluorescence microscope (Leica, Germany) and photographed either under phase contrast or under fluorescent excitation. For differential activation of ochratoxin A gene expression minimal medium with ammonia and glycerol was used (MM-S). For ochratoxin A gene suppression minimal medium with nitrate and glucose was used (MM-R). YES medium without supplement was used as control as it is strongly supportive for ochratoxin A biosynthesis. A fluorescence filter set with an excitation at 450 nm were used along with filters of 480 nm (dichromatic) and 510 nm (emission).

### 2.8. Quantitative determination of ochratoxin A by HPLC

Detection and quantitative determination of ochratoxin A from fungal colonies were performed according to the method described in the ISO 15141 standard (1998, www.iso.ch). For this purpose 100 mg of the fungal colony was extracted under shaking conditions in 800 µl chloroform at room temperature for 20 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 Merck Hitachi HPLC apparatus L7000 (Merck, Darmstadt, Germany). 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 and 25 °C with acetonitril: water:acetic acid (40:60:1, [v:v:v]). The peak was determined with a fluorescence detector Merck Hitachi L7485.

## 3. Results

### 3.1. Agrobacterium tumefaciens mediated transformation of P. nordicum

During initial experiments to determine the sensitivity of *P. nordicum* against hygromycin B it could be shown that at a concentration of 100 µg/ml no growth of *P. nordicum* was possible. Therefore this concentration was used in all subsequent transformation experiments. For both cell types (*A. tumefacium* cells and *P. nordicum* spores) a concentration of about $10^6$ cells/ml gave best results. From obtained primary transformants of *P. nordicum* single spores were transferred to new selective medium to get homokaryons of the transformants. The number of the obtained primary transformants was dependent on the number of *P. nordicum* spores used for transformation. At a spore concentration of $10^5$ spores/ml about 1 –5 transformants could be obtained in a transformation experiment. If however the spore concentration was increased to $10^6$ spores/ml the transformation frequency also increased to 40–60 transformants per experiment. All of the strains which were able to grow on hygromycin B containing plates gave a band of 660 bp after PCR with the *hph* B specific primer pair and a band of 792 bp with the *gfp* specific primer pair (data not shown). A subsequent Southern blot with the genomic DNA of a selected transformed *P. nordicum* strain revealed a hybridization signal different from the pure vector DNA, indicating that the TI-DNA had integrated into the genome of *P. nordicum*. Moreover the fact that only one hybridizing band is visible suggests that only one copy of the TI-DNA integrated in this transformant (Fig. 2). These results clearly show, that *P. nordicum* is transformable by ATMT with a reasonable transformation frequency.

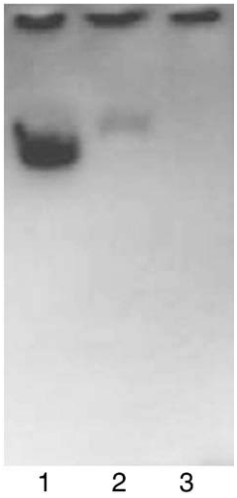


**Fig. 2.** Southern blot of a pOTAPKS::GFP transformed strain. As hybridization probe, a DIG labelled *gfp* gene fragment was used. Lane 1, pOTAPKS::GFP vector; lane 2 genomic DNA of *P. nordicum* transformant T1 digested with EcoRI, lane 3 genomic DNA of the *P. nordicum* wt digested with EcoRI.

### 3.2. Expression of the gfp gene carrying a nuclear localization domain in P. nordicum

In order to show that the GFP protein can be functionally expressed in the ochratoxigenic species *P. nordicum*, an ATMT was carried out with the *stu*A plasmid pNRPK2::GFP. Initial primary transformants were grown to homokaryons by single spore transfer and were grown on YES medium for 7 days. After this time the difference in fluorescence between the wild type and one of the transformants were detected by fluorescence light microscopy. Fig. 3 shows the result of that experiment. It is obvious that the transformed strain showed a strong fluorescence of the nuclei (Fig. 3A), whereas the wild type does not show any fluorescence at all under these conditions (Fig. 3B). These results indicate that the transformant is capable of expressing the GFP protein and that the *stu*A domain is functional in *P. nordicum*. Moreover it can be seen that each nucleus of the hyphae is tagged with *gfp* indicating the homokaryotic nature of the transformant. In this vector the GFP protein is under the control of the strong constitutive *A. nidulans gpd* promoter. The obtained results clearly indicate that under these conditions, e. g.

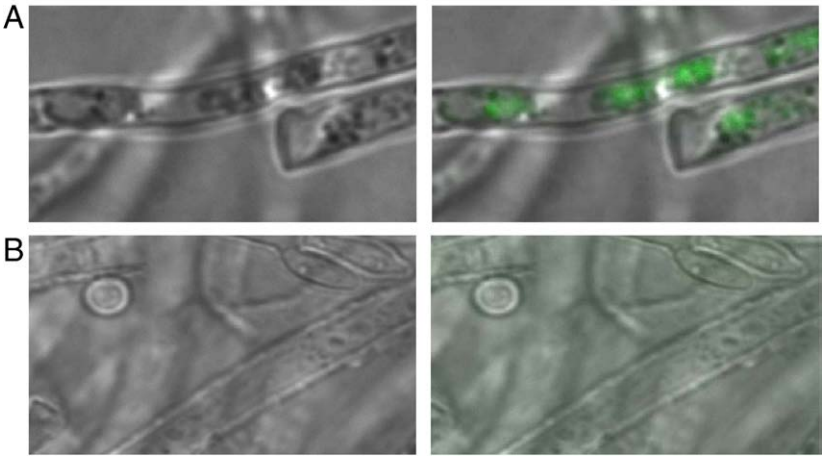


**Fig. 3.** Nuclear located fluorescence of a strain of *P. nordicum* transformed with pNRPK2::GFP (A) compared to the non-fluorescent wild type (B). Fluorescence appeared after excitation (right) and is not visible under phase contrast (left).

expression under the control of a strong constitutive promoter, the fluorescence is intense and clearly differentiate between a transformed strain and the wild type.

### 3.3. Expression of the gfp gene in P. nordicum under the control of the ochratoxin A polyketide synthase promoter

The 5′ region of the *otapks*PN gene with the putative promoter was cloned in front of the *gfp* gene. The construction was performed in a way that the 968 bp promoter fragment of the *otapks*PN gene was placed directly in front of the AUG sequence of the *gfp* gene. The resulting vector pOTAPKS::GFP was transformed by ATMT into *P. nordicum*. The transformation frequency with this vector was similar as shown above. Two of the transformants were analysed for the presence of the transforming TI-DNA by two different PCR reactions, one specific for the *hph*B resistance gene and one for the *gfp* gene (data not shown). Both transformants gave a positive PCR signal in both reactions indicating the presence of the TI-DNA. The fluorescence of the strains was analysed as described above after microscopic examination of 7 day old hyphae grown on YES medium. YES is a very supportive medium for ochratoxin A biosynthesis. For this reason the *otapks*PN gene should be fully expressed and if the approach is functional a bright fluorescence should be visible. Fig. 4 shows the results of this analysis. It is obvious that the transformed *P. nordicum* strain shows a bright green fluorescence after light activation (Fig. 4A). Fig. 4B shows the same detail in phase contrast. Compared to the transformant carrying the *gfp::stu*A construct, the fluorescence is distributed within the whole cytoplasm of the cell. This result indicates that the *otapks*PN promoter is strong enough to exert a fluorescent signal after expression of the *gfp* gene if it is fully induced due to growth on YES medium.

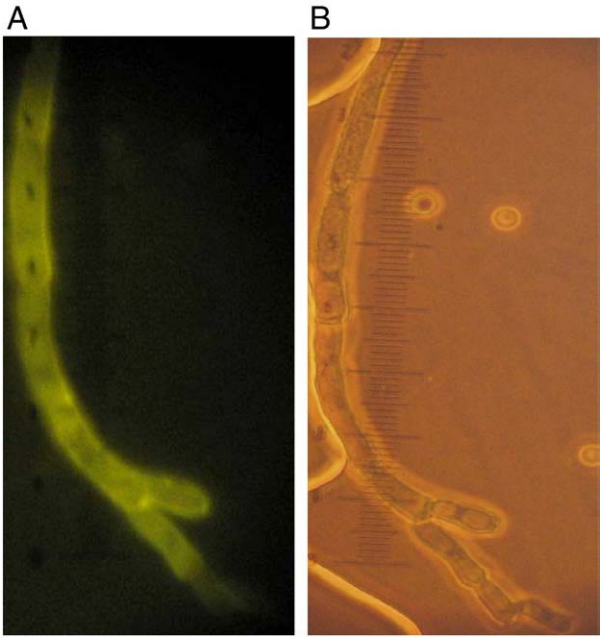


**Fig. 4.** Cytoplasmatically located fluorescence of a strain of *P. nordicum* transformed with pOTAPKS::GFP. A: fluorescence excitation; and B: phase contrast.

### 3.4. Differential expression of the otapksPN controlled gfp gene after growth on different media

In earlier experiments two different minimal media were developed which either induce or nearly complete repress ochratoxin A biosynthesis. The supportive medium (MM-S) contains glycerol as the carbon source and ammonium as the nitrogen source. These substances are replaced by glucose and nitrate in the repressive medium (MM-R). All other substances were the same in both media. It was already previously shown that the expression of the *otapks*PN gene in the restrictive medium is much lower compared to expression of this gene in the supportive medium. This results in a strongly reduced biosynthesis of ochratoxin A on repressive medium, but an increased production on

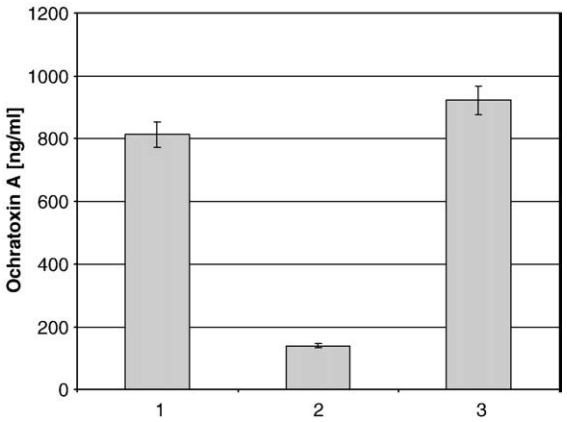


**Fig. 5.** Ochratoxin A biosynthesis by *P. nordicum* after growth on YES medium (1), restrictive minimal medium (MM-R; lane 2) and supportive minimal medium (MM-S, lane 3). Ochratoxin A production was determined by HPLC. The figure shows the average values of 3 independent experiments. Error bars are indicated.

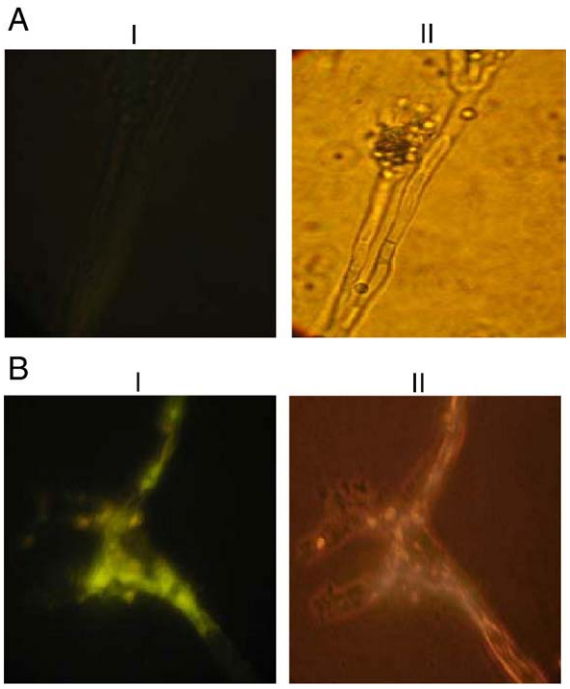


**Fig. 6.** A pOTAPKS::GFP transformant of *P. nordicum* grown on ochratoxin A restrictive medium (A) or supportive medium (B) under fluorescence excitation conditions (I) and phase contrast conditions (II).

supportive minimal medium by a factor of about 10 (Fig. 5). This should be visualizable by *otapks*PN controlled *gfp* gene expression if the approach is functional. Fig. 6 shows the results of the experiment under fluorescence light (left picture) and phase contrast (right picture). After growth of *P. nordicum* on non-supportive medium no fluorescence occurred after excitation (Fig. 6A), however a strong fluorescence signal could be observed after growth on supportive medium (Fig. 6B). These results suggest, that the developed *gfp* approach can detect the induction of the ochratoxin A polyketide synthase gene under ochratoxin A supportive conditions and can differentiate between the two physiological conditions of ochratoxin A biosynthesis and non-biosynthesis.

Unexpectedly the *gfp* activity decreased in strains subcultured from the original homokaryotic transformant indicating some instability in *Penicillium*.

## 4. Discussion

An *A. tumefaciens* mediated transformation system has been developed for the ochratoxin A producing species *P. nordicum* for the first time. Several fungi have been transformed using the ATMT technique, but only scarce information is available about the usage of this technique for the transformation of *Penicillium* species (Michielse et al., 2005). Very recently two ATMT systems have been described for one medical and another agricultural important *Penicillium* species, e. g. the human pathogenic fungus *P. marneffei* (Zhang et al., 2008) and the fruit rot fungus *P. digitatum* (Wang and Li, 2008). In case of *P. digitatum* a similar transformation frequency as with *P. nordicum* was observed. The transformation of *P. nordicum* with a vector carrying the *gfp* protein with a nuclear localization domain leads to transformants with fluorescent nuclei, but no fluorescence was observed in the cytoplasm, indicating that the nuclear localization signal of *A. nidulans* is also functional in *P. nordicum*. This result definitively demonstrate that this approach is functional for *P. nordicum* if the heterologous promoter is strong enough. This was also the case for the *otapks*PN promoter. This is a promoter for a biosynthesis gene of a secondary metabolite. For this reason it should be influenced by the growth phase or growth conditions. Compared to the expression level of a constitutively expressed house keeping gene (*P. nordicum* ß-actin gene), the expression level of the *otapks*PN gene is only at a moderate level (Geisen et al., 2006). Even among the known ochratoxin A biosynthesis genes of *P. nordicum*, the *otapks*PN gene is the gene with the lowest expression level. Despite this moderate expression level, the *otapks*PN promoter produced a clear fluorescent signal in the *otapks*PN::*gfp* transformant.

When the transformant was grown on two different minimal media, one supportive for ochratoxin A biosynthesis the other restrictive a clear differential expression of the *otapks*PN gene could be visualized. In the former case a clear fluorescence could be observed, whereas in the latter case almost no signal arose, indicating the non-induced status of the gene. It has been shown previously by Real Time PCR that the *otapks*PN gene is by a factor of about 10 more induced on the supportive medium than on the restrictive medium (Geisen et al., 2006). This is also reflected at the gene expression level as shown in this work ( Fig. 6). These results indicate that activation of the *otapks*PN gene can be detected and at least a 10 fold difference in gene expression in *P. nordicum* can be visualized by using this approach. Several reporter gene approaches in relation to mycotoxin gene expression have been described, however only a few using GFP as a reporter. Annis et al. (2000) described a system in which the ß-glucuronidase gene was used as a reporter with an aflatoxin biosynthesis gene promoter to study substances from pepper on the transcription of aflatoxin genes. In another approach Brown et al. (1997) ligated the promoter of the ß-tubulin gene of *A. flavus* in front of the ß-glucuronidase gene to study growth of that species in maize kernels. A similar approach in which the promoter of the *nor*-1 gene was cloned in front of the ß-glucuronidase gene was followed by Xu et al. (2000) to study infection and colonization of *A. parasiticus* in peanut pods. Recently Ochai et al. (2007) constructed a *Fusarium graminearum* strain carrying a *gfp* reporter gene controlled by the *tri*5 gene promoter. This strain was used to study the effect of abiotic factors like fungicides or NaCl on *tri*5 expression. In a study done by Hong and Linz (2008) the *ver*-1 promoter was cloned in front of a *gfp* gene. This construct was used to analyse the location of the aflatoxin biosynthesis machinery. It could be shown by this approach that the late steps of aflatoxin biosynthesis are located in the vacuoles. These examples show the applicability of reporter gene strains to study the influence of environmental factors on the induction of mycotoxin biosynthesis genes directly in the food. With this tool critical control points with regard to mycotoxin biosynthesis can be analysed. Moreover growth and mycotoxin biosynthesis can be directly monitored under *in situ* conditions and the impact of food components on mycotoxin biosynthesis can be monitored.

The fact that *gfp* transformed strains of *P. nordicum* are unstable cannot be completely explained yet. However according to our experience instabilities of the phenotype after transformation (irrespective of the introduced vector) are common in ochratoxin A producing Penicillia and might be due to an effective defense mechanism of these species against foreign DNA.

In the current work it could be shown that the *gfp* approach is functional for ochratoxin A producing *P. nordicum* strains. Differences between non-induced and fully induced ochratoxin A biosynthetic genes can be visualized. In future experiments this approach will be used to study the influence of several external factors on ochratoxin A gene activation.

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