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Comparative proteome analysis of *Penicillium verrucosum* grown under light of short wavelength shows an induction of stress-related proteins associated with modified mycotoxin biosynthesis

Dominic A. Stoll, Sebastian Link, Sabine Kulling, Rolf Geisen, Markus Schmidt-Heydt *

Max Rubner-Institut, Federal Research Institute of Nutrition and Food, Department of Safety and Quality of Fruits and Vegetables, Haid-und-Neu-str. 09, D-76131 Karlsruhe, Germany

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ABSTRACT

In this study the differentially expressed protein population of *Penicillium verrucosum* grown either in the dark or under light with a wavelength of 450 nm has been analyzed. Light of short wavelength led to oxidative stress in the fungal cell; under this condition the mycotoxin biosynthesis revealed a mutual shift from ochratoxin A to citrinin. Using a proteomic approach combining an optimized protein extraction method with 2-dimensional SDS-PAGE followed by HPLC-ESI-TOF-MS/MS mass spectrometric analysis, initially 56 significantly differential proteins (light vs. dark) were detected comprising proteins of a broad range of isoelectric points and molecular masses. In total, 46 proteins could be identified further by database query, most of these proteins are assumed to be involved in response to stress (e.g. antioxidative proteins, heat shock proteins) and general metabolic processes (e.g. glycolysis, ATP supply). Proteome analyses are necessary to unravel the regulation of secondary metabolite biosynthesis at a translational level. This may enable identification of proteins which are involved in mycotoxin biosynthesis, adaption processes or even stress compensation mechanisms. This study depicts the first proteome analysis of *P. verrucosum*.

1. Introduction

Ochratoxin A (OTA) and citrinin are mycotoxins produced by different *Aspergillus* and *Penicillium* species. From the Aspergilli, the most important producing species are *Aspergillus carbonarius*, *Aspergillus westerdijkiae*, *Aspergillus steynii* and *Aspergillus ochraceus* (Frisvad et al., 2004; Gil-Serna et al., 2009), whereas from the genus *Penicillium*, only *Penicillium nordicum* and *Penicillium verrucosum*, are described as producing OTA (Castella et al., 2002; Larsen et al., 2001). *P. verrucosum* produces citrinin as well as OTA, whereas *Penicillium citrinum* and *Penicillium expansum* are able to produce citrinin. *P. verrucosum* occurs on food such as cereal grains, and salt rich food such as brined olives and fermented meat and is the ultimate species responsible for the occurrence of OTA and citrinin in cereals (Lund and Frisvad, 2003). OTA is a dihydro-isocoumarin polyketide molecule, chlorinated at position 5 and coupled via peptide linkage to the amino acid phenylalanine (Höhler, 1998). Like ochratoxin, citrinin belongs to the benzopyrone carbonic acids, has a polyketide structure and shows some similarity to the ochratoxin molecule, but does not contain chlorine or phenylalanine (Hajjaj et al., 1999). OTA is rated by the WHO/FAO as a type II carcinogen (Petzinger and Ziegler, 2000). The prolonged ingestion even of small amounts of these nephrotoxic and hepatotoxic mycotoxins could lead to several health defects in humans and animals (Petzinger and Ziegler, 2000; Pfohl-Leszkowicz and Manderville, 2007).

It has been shown previously that the biosynthesis by fungi of the mycotoxins OTA and citrinin is strongly regulated by environmental factors such as temperature (Belli et al., 2004; Mitchell et al., 2004), water activity (Valero et al., 2006), pH (Arroyo et al., 2005), substrate (Skrinjar and Dimic, 1992), light of visible wavelength (Schmidt-Heydt et al., 2011) and preservatives (Schmidt-Heydt et al., 2007), but could also be induced by environmental factors imposing stress on the fungus (Jayashree and Subramanyam, 2000; Ellner, 2005; Schmidt-Heydt et al., 2008). The mycotoxins OTA and citrinin are often mutually regulated in *P. verrucosum*. High osmotic conditions, especially when attributed to high NaCl concentrations, shift the secondary metabolite profile of *P. verrucosum* towards OTA, whereas conditions imposing oxidative stress for the fungus shift secondary metabolite biosynthesis towards citrinin.

Light, especially blue light with a wavelength of 450 nm is able to induce oxidative cell stress by the generation of Reactive Oxygen Species (ROS) which further leads to a reduction in OTA biosynthesis in *P. nordicum*, *P. verrucosum* (Schmidt-Heydt et al., 2011) and in *Aspergillus niger* (Fanelli et al., 2012), but strongly increases citrinin biosynthesis in *P. citrinum*, *P. expansum* and *P. verrucosum* (Schmidt-Heydt et al., 2011) and fumonisin biosynthesis in *A. niger* (Fanelli et al., 2012). There exist no data that describe the changes which occur at the proteome level under oxidative stress conditions induced by light of short wave length which led further to the mutual induction of citrinin biosynthesis.

* Corresponding author. Tel.: +49 721 6625 459; fax: +49 721 6625 453.
E-mail address: Markus.Schmidt-Heydt@mri.bund.de (M. Schmidt-Heydt).

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The transcriptional regulation of mycotoxin biosynthesis has been under examination for several years and could be monitored by techniques such as Real-Time-PCR and microarray. However, only a few genes of the biosynthesis pathways of OTA and citrinin have been reported in *Penicillium*. It has been repeatedly observed that the transcriptional activity of mycotoxin biosynthesis genes does not correlate routinely with phenotypical mycotoxin biosynthesis. One reason for this circumstance is based on the fact that an important part of the regulation mechanisms which affect fungal physiology and secondary metabolism takes place on translational and post-translational level (Rizwan et al., 2010). While genome sequencing and transcriptional analysis of fungi started years ago, there is still a deficiency in analyses at the proteome level especially of mycotoxigenic Penicillia. To understand in general the regulation of mycotoxin biosynthesis under a specific growth condition to get further insights into the role of adaptive changes on fungal physiology affecting secondary metabolite biosynthesis, it is necessary to use a three-pillar-model, which means the comprehensive analysis of the fungus on a transcriptional level, proteome level and secondary metabolite level. In this study we used a proteomic approach using 2D-SDS-PAGE with subsequent mass spectrometry analyses to identify new proteins in *P. verrucosum* which are associated with modified growth rate and mycotoxin biosynthesis.

2. Materials and methods

2.1. Fungal strains and growth conditions

P. verrucosum BFE808, a strong OTA and citrinin producing fungal strain from the culture collection of the Max Rubner-Institut was used for the experiments. The strain was routinely sub-cultured on malt extract agar (Merck, Darmstadt, Germany) at 25 °C. For the experiments, a 7-day old culture of *P. verrucosum* BFE808 was single point inoculated in biological triplicates on YES agar plates (20 g/L yeast extract, 150 g/L sucrose, 15 g/L agar) with a spore suspension containing 10^7 spores/mL. Inoculated agar plates were placed immediately into the light irradiated chamber (450 nm) of the incubation box and into a non-irradiated, dark chamber and incubated for 7 days at 25 °C at a relative humidity of 85%. Samples were taken after 1 h, 2 h, and 3 h and at day 7.

2.2. Light incubation equipment

The light incubation device used was the same as that described in Schmidt-Heydt et al. (2011).

2.3. Growth assessment

For analyzing growth and morphology, the colony diameters were measured two times, at right angles to each other. These measurements were performed at the beginning of the growth experiments (diameter of inoculum) and at the end of each incubation of 7 days, the obtained differences corresponding to the growth within the incubation experiments. Furthermore, the standard deviation between the biological triplicates was calculated.

2.4. Cell lysis and protein extraction

Three different protocols for cell lysis were carried out and the respectively obtained amount of protein was compared on SDS-PAGE in order to determine the most efficient extraction method. The three different methods were as follows. The initial step was the same in all methods: After incubation the mycelium was detached from the media without remaining agar residues. 700 mg of mycelium was weighed in a prepared tube with 1 mL protein extraction buffer (1 M TRIS; 1 M $MgCl_2$; 50 mM EDTA; pH 7.5) containing protease- and phosphatase-inhibitor (cOmplete, Mini, EDTA-free Protease Inhibitor Cocktail resp. PhosSTOP, Roche, Mannheim, Germany). All further steps were carried out in a climatized room with controlled temperature.

2.4.1. Method 1

The mycelium was sonicated twice for 5 min in ice water in a Bandelin Sonorex Super RK 255H sonicator at a frequency of 35 kHz (BANDELIN electronic GmbH & Co. KG, Berlin). After spinning for 10 min at 4000 rpm, 200 μL of 50% trichloracetic acid was added to the supernatant. After incubation on ice for 30 min the samples were spun for 7 min at 13,000 rpm. The pellet was resuspended in 150 μL Laemmli-buffer (0.5 M TRIS; 192 mM SDS; 20% glycerol; 10% ß-mercaptoethanol; bromophenol blue) and evaporated with ammonia gas to yield deprotonated proteins. The protein extracts were vortexed for 30 min, denatured for 3 min at 95 °C and stored at −80 °C until further analysis.

2.4.2. Method 2

The mycelium was disintegrated as in method 1, except that stainless steel beads in a Tissue-Lyzer (Qiagen, Hilden, Germany) at room temperature were used instead of ultrasonication.

2.4.3. Method 3

The mycelium was disintegrated as in method 1, except that glass beads in an Ultra Turrax Tube drive (IKA, Staufen, Germany) were used instead of ultrasonication.

2.5. One-dimensional gel electrophoresis and staining

Proteins were separated by gel electrophoresis using a 12.5% acrylamide gel, SDS buffer (25 mM TRIS; 192 mM glycine; 0.1% SDS) in a Mini-Protean Tetra System (Bio-Rad Laboratories GmbH, Munich). The gels were loaded with the protein extracts and an electric field of 80 V was applied for concentration and 120 V for separation of the proteins. The gel was stained overnight by using a Coomassie staining solution (73% H_2O; 20% ethanol; 7% acetic acid; 1 g Coomassie Brilliant Blue R250). After staining the dye was rinsed out using a decoloration solution (60% H_2O; 30% ethanol; 10% acetic acid). The gels were scanned using the Molecular Imager ChemiDoc XRS + System (Bio-Rad Laboratories GmbH, Munich).

2.6. Preparation of protein extracts for isoelectric focusing and 2-dimensional gel electrophoresis

Mycelium (700 mg) was weighed into a prepared tube with 1 mL of protein extraction buffer (1 M TRIS; 1 M $MgCl_2$; 50 mM EDTA; pH 7.5) containing protease- and phosphatase-inhibitor (cOmplete, Mini, EDTA-free Protease Inhibitor Cocktail resp. PhosSTOP, Roche, Mannheim, Germany). The mycelium was sonicated for 2 × 5 min on ice. After spinning for 10 min at 4000 rpm, 200 μL of 50% trichloracetic acid was added to the supernatant. After incubation on ice for 30 min the samples were spun for 7 min at 13,000 rpm. The pellet was washed three times with ice-cold 80% acetone and air-dried after the last washing step. The protein extracts were dissolved in 150 μL lysis buffer (13.5 g urea; 0.5 mL Triton X-100; 500 mg DTT; 0.5 mL ampholytes; 35 mg phenylmethane sulfonylfluoride; dissolved in 25 mL H_2O bidest) and stored at −80 °C until further analysis.

2.7. Protein quantification

Protein extracts for isoelectric focusing and 2-dimensional gel electrophoresis were initially quantified by using a NanoDrop-1000 spectrophotometer (Peqlab, Erlangen, Germany). Values were verified by using a modified Bradford-Assay according to (Ramagli and Rodriguez, 1985).

2.8. Isoelectric focusing

Isoelectric focusing and 2-dimensional gel electrophoresis were carried out in biological triplicates for each growth condition. Isoelectric focusing was done in a PROTEAN IEF Cell (Bio-Rad Laboratories GmbH, Munich) according to the specifications of the manufacturer: An amount of 100 μg of proteins was loaded on one 7 cm ReadyStrip with an immobilized pH-Gradient, linear from pH 3–10 (Bio-Rad Laboratories GmbH, Munich). The dry strips were actively rehydrated overnight at 20 °C in an electric field of 50 V to allow a uniform distribution of the proteins in the whole strip. Subsequently, isoelectric focusing was conducted overnight at 20 °C. Maximum voltage of 4000 V was reached within 2 h and held for 20,000 Vh. After focusing the strips were cooled and stored at 500 V to prevent deviation of the focused proteins until they were stored at −80 °C or used directly for electrophoresis.

2.9. 2-dimensional gel electrophoresis

Prior to electrophoresis, the strips were equilibrated stepwise in 2 buffers based on equilibration buffer (1.5 M Tris–HCl 3.3 mL; urea 36 g; glycerol 20 mL; 20% SDS 10 mL; solved in 100 mL H_2O bidistilled) for 10 min under moderate agitation. Buffer 1 was composed of 200 mg dithiothreitol (DTT) dissolved in 10 mL equilibration buffer, whereas buffer 2 consisted of 250 mg iodoacetamide in 10 mL equilibration buffer. After equilibration the strips were washed in the same SDS buffer (25 mM TRIS; 192 mM glycine; 0.1% SDS) that was used for horizontal electrophoresis. Proteins were separated by electrophoresis by using a self-casted 12.5% acrylamide gel of 1 mm thickness in a Mini-Protean Tetra System (Bio-Rad Laboratories GmbH, Munich). The equilibrated strips and 10 μL of denaturated protein ladder were positioned at the top of the gels with 1% agarose (Serva, Heidelberg, Germany) in SDS-buffer. For separation an electric field of 80 V was applied.

2.10. Staining and data analysis

The gels were stained by using a modified Coomassie-protocol as described by Candiano et al. (2004), Dyballa and Metzger (2009) and Kang et al. (2002), attaining a level of sensitivity almost comparable with classical silver staining. The gels were washed twice in bidistilled H_2O and then incubated overnight in a modified Coomassie staining solution (25 g ammonium sulfate; 50 mL EtOH; 100 mg Coomassie Brilliant Blue R250; 12 mL orthophosphoric acid; solved in 500 mL bidistilled H_2O). After staining the gels were washed twice as mentioned above, followed by an incubation of 60 min in discoloration solution (50 mL EtOH; 438 mL bidistilled H_2O; 12 mL orthophosphoric acid) and washed again. The gels were scanned with the Molecular Imager ChemiDoc-XRS + System (Bio-Rad Laboratories GmbH, Munich) and statistically analyzed using the PDQuest 2-D Analysis Software Version 8.0.1 (Bio-Rad Laboratories GmbH, Munich). Protein spot scan intensities were analyzed by using a *t*-test with implemented normalization, proteins were classified as significantly regulated when p-value <0.05.

2.11. In gel digestion of selected protein spots

Protein spots of interest were manually excised from the gels with a spot-picker (Biostep, Jahnsdorf, Germany) and washed twice for 15 min in bidistilled H_2O. Afterwards the spots were incubated twice for 15 min in 100 μL sterile 50 mM ammonium-bicarbonate/50% acetonitrile to remove staining residues, dehydrated by incubation for 10 min in 100 μL of 100% acetonitrile, and lyophilized. The supernatants after each step were discarded. For In-gel digestion, 20 μL of 0.1 μg/μL sequencing grade modified trypsin (Promega Corporation, USA) was mixed with 180 μL of 25 mM ammonium-bicarbonate and each spot was rehydrated in 10–15 μL of this solution. After 20 min the spots were overlayed with 25 mM ammonium-bicarbonate. The digest was incubated over night at 37 °C. After digestion the supernatant was collected. For extraction the spots were incubated for 15 min in 60% acetonitrile/0.1% TFA. The last extraction was carried out for 15 min by using 100% acetonitrile which was added to the combined supernatants of all extraction steps. Both extraction steps were carried out under sonication. The extracts were lyophilized and stored at −20 °C until MS analysis.

2.12. HPLC-ESI-TOF-MS/MS analysis of protein spots

Lyophilized peptides were reconstituted in 30 μL of 5% aqueous acetonitrile with 1% formic acid, and 20 μL injected onto the column. Peptides were separated on an Aeris Peptide 3.6 μm XB-C18 250 × 4.6 mm column (Phenomenex Ltd., Aschaffenburg, Germany) in an Agilent 1290 UPLC System (Agilent, Waldbronn, Germany) hyphenated to an AB-Sciex Triple-TOF 5600 (AB-Sciex, Framingham, Massachusetts, USA) equipped with Dual-Source. With minor modifications from validated in-house LC–MS methods, the gradient was set to 5% to 35% B in 12 min (A = 1% aqueous acetonitrile with 0.1% FA, B = acetonitrile with 10% tri-fluoroethanol and 0.1% FA (Reagents from VWR GmbH, Darmstadt, Germany), followed by a 3 min column regeneration and a 4 min equilibration phase at a flow rate of 600 μL/min. Mass spectrometric detection was performed in ESI positive mode with a TOP3 IDA method in 1.25 Hz cycles. Peptides were fragmented from 300 to 1250 m/z with charge >1 with rolling collision energy and dynamic exclusion setting according to system properties.

2.13. Protein identification

Online MASCOT (Matrix Science) sequence query using peptide mass values and corresponding fragment peak lists was used to search for matching protein sequences against the NCBInr (National Center for Biotechnology Information) database. The search parameters were: enzyme digestion with trypsin, no taxonomic restriction. As fixed modification we set Carbamidomethyl (C), as variable modifications we selected Deamidated (NQ), Oxidation (M) and Propionamide (C). Peptide charge state was set to 2+, 3+ and 4+, the peptide mass tolerance to 15 ppm and the fragment mass tolerance to 0.1 Da. Proteins were positively identified either with at least 2 identified peptides with a score above 40 each or if one identified unique peptide was found with score >70 and manual inspection of the respective spectra for low noise and properly assigned peaks as mentioned in Carr et al. (2004). Proteins where only one unique peptide have been found with a score <70 were manually positively inspected using the spectra as mentioned above and included “in brackets” in the results part. In general the pI and MW of the candidate proteins were compared to the gel analysis values for an additional dimension of reliability.

2.14. Determination of oxidative stress by measuring the amount of reactive oxygen species (ROS)

Oxidative stress was determined by using the OxiSelect-In-Vitro ROS/RNS Assay Kit (Cell Biolabs Inc., San Diego, USA). The samples, the DCF Standard Curve and the assays were prepared according to the manufacturer's manual. The samples were obtained by sonication of 50 mg mycelium in 1 mL PBS for 2 × 5 min on ice. For measurement the samples were diluted 1:100 in PBS. The fluorescence was analyzed by using a Tecan-Spectra-Fluor-Plus fluorescence and absorbance reader (Tecan Group Ltd., Männedorf, Swiss). In this assay, increasing ROS-concentrations led to an induction of the formation of fluorescent DCF, therefore the ROS-concentration could be determined on the basis of the fluorescence of the DCF Standard Curve. The standard deviation was also calculated.

2.15. HPLC analysis of OTA and citrinin

For determination of OTA and citrinin biosynthesis, BFE808 was grown after pre-culture at 25 °C for 7 days at the respective

experimental conditions. Two agar plugs (diameter 1 cm, 0.25 g) were taken with the aid of a sterile corer after 1 h, 2 h, and 3 h respectively depending on the experiment and after 7 days from the region between center and edge of the colony. These agar plugs with the adhering mycelium were transferred into 2 mL micro-reaction tubes and 1 mL of chloroform was added. The fungal mycelia were extracted for 30 min at room temperature on a rotary shaker; the mycelia were discarded and the chloroform extract was evaporated to dryness in a vacuum concentrator (Speed Vac, Savant Instruments, Farmingdale, USA). Quantitative determination of OTA and citrinin was performed as described by Sato et al. (2010) using a Hitachi D-7000 HPLC system (Merck, Tokio, Japan) equipped with an auto-injector, column oven and fluorescence detector. The column oven was set to 40 °C; the fluorescence detector was set to an excitation of 331 nm and an emission of 500 nm. The flow rate was 0.7 mL/min and the injection volume 10 μL. Solvent A consisted of 250 mM ortho-phosphoric acid in H_2O and solvent B of methanol. Separation was carried out on a LiChrospher 100, C18 (250 mm, Ø 4 mm i.d., particle size 5 μm) reversed phase column (VWR International GmbH, Darmstadt, Germany) using the following gradient: 0 min – A 60%, B 40%; 7 min – A 40%, B 60%; 12 min – A 35%, B 65%; 16 min – A 5%, B 95%; 27 min – A 60%, B 40%. The limit of quantification was 25 pg on column. Data collection and handling was done with EZ-Chrome Elite 3.2. All standards were obtained from Sigma (Taufkirchen, Munich, Germany) with a purity of ≥98%. The standard deviation was calculated between the biological replicates.

2.16. Statistical analyses

Statistical calculation of the obtained data from growth, mycotoxin biosynthesis via HPLC and ROS-level was performed by using Sigma Plot 2.3 (Systat Software GmbH, Erkrath, Germany). For the comparison between growth in the dark and under irradiation with light (450 nm) the one-tailed *t*-test has been used. In case of the oxidative stress level measurement after 7 days of growth in constant darkness with further inoculation under irradiation with light (450 nm) for 1 h, 2 h and 3 h, the one way ANOVA has been used. The given p-values for the prolonged treatment are related to the initial value before exposure. p-values are indicated in the respective figures.

3. Results and discussion

The present work had three objectives. First, to develop a protein extraction method optimized for Penicillia, especially *P. verrucosum*; second, to understand if the light driven physiological changes namely the mutual mycotoxin biosynthesis and the growth retardation of *P. verrucosum* are associated with different protein expression; and third, to identify in general new *P. verrucosum* related proteins and in detail those that are up-, or down-regulated under the respective growth condition.

3.1. Development of an optimized protein extraction method for P. verrucosum

Various protocols for fungal protein extractions have been developed, e.g. for black microcolonial fungi such as *Exophiala jeanselmei* (Isola et al., 2011), *Fusarium graminearum* (Milles et al., 2007) and *A. ochraceus* (Rizwan et al., 2010). The extraction of fungal proteins is a challenge as fungi have an exceptionally resistant cell wall and the fungal kingdom comprises a high variety of diverse characteristics negatively influencing protein yield and leading to artifacts during proteomic studies. Therefore, protein extraction protocols have to be developed and optimized for each fungal species and their specific properties. Standard extraction protocols include lysis of the cell wall, protein extraction and electrophoresis, removal of interfering substances followed by solubilisation and desalting of the proteins. For the purpose of cell disruption several research groups used either mechanical grinding with glass or stainless steel beads to liberate cytoplasmic proteins (Grinyer et al., 2004; Melin et al., 2002; Nandakumar et al., 2003; Strom et al., 2005), grinding in liquid nitrogen using mortar and pestle (Grinyer et al., 2005; Hernandez-Macedo et al., 2002; Kniemeyer et al., 2006; Shimizu and Wariishi, 2005; Yajima and Kav, 2006) or a combination of chemical or enzymatic extraction methods (Nandakumar and Marten, 2002). To ascertain an effective protein extraction method for Penicillia, *P. verrucosum* BFE808 was cultivated on YES medium. The time to collect samples for protein analysis was selected as day 7 at 25 °C after starting the incubation period under irradiation with light (450 nm) and in the dark. Using an amount of 700 mg mycelium for protein extraction proved optimal. Three different sample preparation protocols were used and compared regarding their efficiency for preparation of total protein extracts from *P. verrucosum*. The first method was cell disruption by ultrasonic, the second disintegration by stainless steel beads using a Tissue-Lyzer (Qiagen) and the third glass beads in an Ultra Turrax Speed Drive (IKA). Disintegration of the mycelium using ultrasonic was found to be most suitable to obtain strong, well defined protein bands in subsequent SDS-PAGE analysis (Fig. 1a). This method was defined by an appropriate reproducibility. The standard deviation between the signal saturation of distinctive bands within triplicates was from 0.2% to 3.1%, ascertained using the Image Lab-

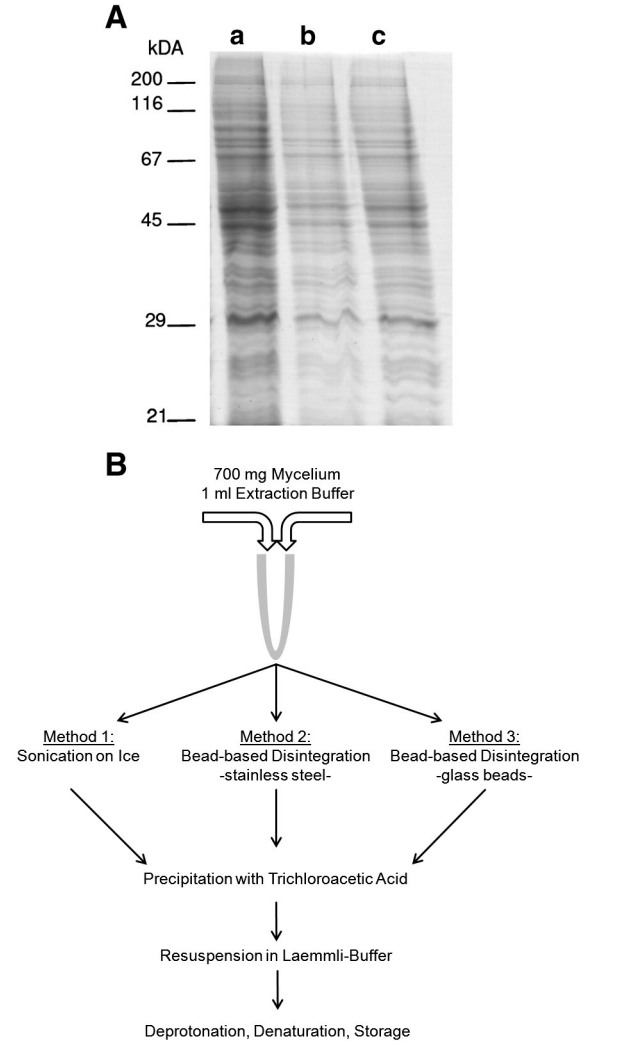

Fig. 1. (A) SDS-PAGE of protein extracts from *P. verrucosum*. Lane "a" depicts the protein extract achieved with extraction method number 1, lane "b", with method number 2 and lane "c" with method number 3. It could be observed that the three methods differ substantially regarding their efficiency to isolate a particular amount of total proteins. (B) Schematic visualization of the different protein extraction methods.

Software (Bio-Rad Laboratories GmbH, Munich). Using glass beads together with an Ultra Turrax Tube Drive (IKA) resulted in only weak protein bands (Fig. 1b), disintegration with stainless steel beads in a Tissue Lyzer (Qiagen) showed only slightly better results than method 3 (Fig. 1c).

3.2. Influence of light irradiation on growth and the biosynthesis of OTA and citrinin

In order to analyze differences in the growth rate and the citrinin and OTA biosynthesis in *P. verrucosum* grown under irradiation with light (450 nm) and in the dark, this strain was incubated 7 days at 25 °C under the respective conditions. As shown in Fig. 2a, the growth rate of *P. verrucosum* was markedly reduced under irradiation with light (450 nm) of sublethal intensity to a growth diameter of 2.5 cm/7 days compared to the dark grown control (3.35 cm/7 days). The metabolite profile shifted from the biosynthesis of OTA to citrinin (Fig. 2b). In detail, the citrinin production increased from 3116 ng/g in the dark (low oxidative stress) to 5507 ng/g under irradiation with light (high oxidative stress), whereas the OTA biosynthesis decreased from 5613 ng/g (dark) to 206 ng/g (light). According to these light-driven effects (degradative and physiological) which have been described earlier (Schmidt-Heydt et al., 2011; Schmidt-Heydt et al., 2012) *P. verrucosum* adapts to different growth conditions inter alia by changing the secondary metabolite profile. While the biosynthesis of OTA is down-regulated and the molecule itself is degraded by short wavelength light to toxic by-products such as chlorinated coumarin derivatives which could act as fungicides (Brooker et al., 2008; Schmidt-Heydt et al., 2012), the mycotoxin citrinin instead acts as a light protectant (Størmer et al., 1998) and could in this way support the competitiveness of *P. verrucosum* on its natural, light-exposed habitat on the field.

3.3. Influence of light irradiation on intracellular ROS generation in P. verrucosum

To find out if a duration of 1 h, 2 h and 3 h irradiation with light of 450 nm wavelength leads to an induction of oxidative stress in cells of *P. verrucosum*, a mycelial fraction was taken at the respective time points from a 7 day-old culture grown either at 25 °C under light irradiation or in the dark and used for the determination of intracellular ROS. 7 days after incubation the cellular ROS-level was significantly reduced compared to the control sample (Fig. 2c). The kinetics over 3 h (Fig. 2d) showed a more detailed picture. Transferring a colony of *P. verrucosum* in the light chamber after 7 days of growth in the dark, led in the first 2 h of light irradiation to a significant induction of ROS generation (100% to 126%; $p = 0.036$) in the fungal cell followed by a decrease back to slightly above 100% after 3 h of incubation (Fig. 2d).

3.4. Comparative analysis of the proteome profile of P. verrucosum obtained under light irradiation or in the dark — possible functional roles of the identified proteins

To understand if the observed physiological reaction becomes apparent on protein level also, a 2-dimensional separation of the total protein extract of *P. verrucosum* was carried out under the respective comparative growth conditions (Fig. 3). Using Coomassie staining, 141 proteins could be clearly discriminated on the gels (Fig. 3a light; Fig. 3b dark). Three replicate gels were used to generate the master gel. Discrete spots could be distinguished from low to high *pI* as first dimension as well as from low to high molecular weight as second dimension. The spots had a range of sequence coverage from 2% to 36% and MASCOT-scores from 34 to 911. In comparison to growth of the fungus either in the dark or under irradiation with light (450 nm), 90 proteins out of the 141 were highly prominent or differentially expressed. Based

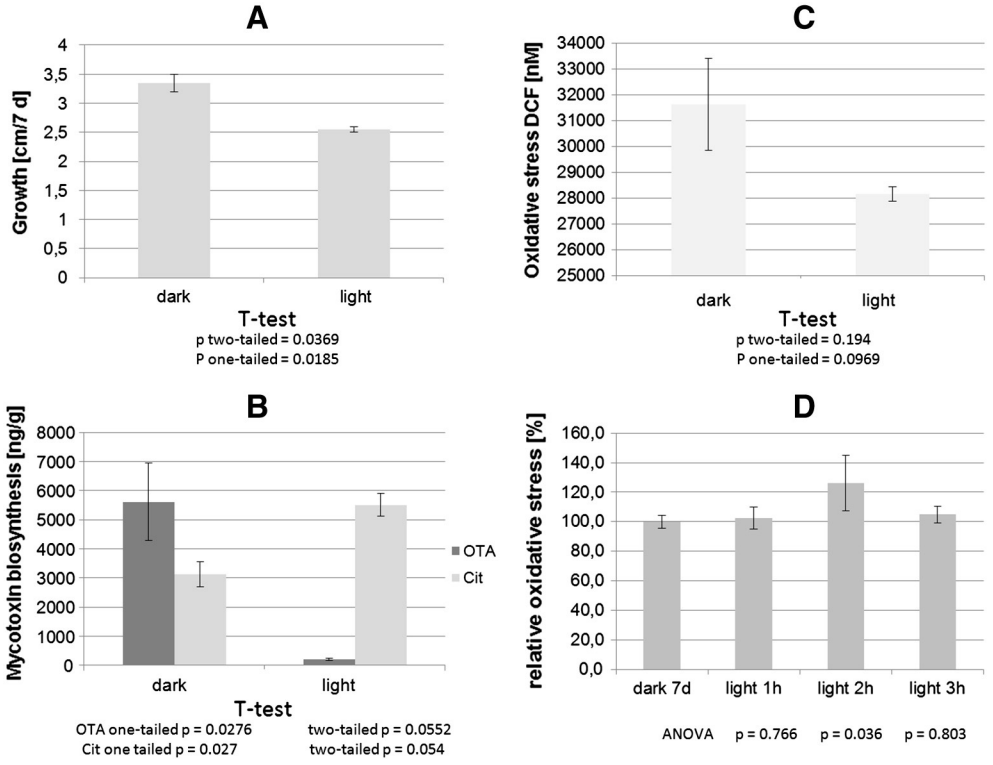

Fig. 2. (A) Growth of *P. verrucosum* BFE808 for 7 days at 25 °C on YES medium either in the dark or under irradiation with light (450 nm). (B) HPLC analysis of ochratoxin A and citrinin biosynthesis of *P. verrucosum* BFE808 after 7 days of incubation on YES medium at 25 °C in the dark or under irradiation with light (450 nm). (C) Oxidative stress; DCF in nano-molar scale, rated by measuring of total ROS generation in mycelial cells of *P. verrucosum* BFE808 after 7 days of incubation on YES medium at 25 °C either in the dark or under irradiation with light (450 nm). (DCF=) Dichloro-fluorescein is the fluorescence marker of oxidative stress. (D) Oxidative stress in percental scale after 7 days of incubation on YES medium at 25 °C in the dark followed by incubation under irradiation with light (450 nm) for 1 h, 2 h and 3 h.

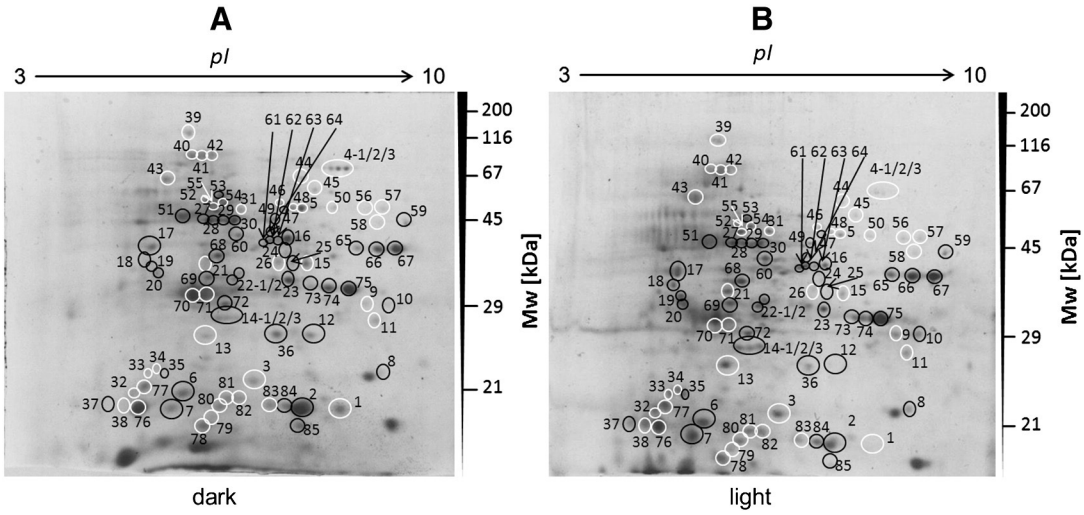

Fig. 3. (A) 2-dimensional gel electrophoretic separation of the total protein extract of *P. verrucosum* BFE808 grown in the dark. 100 μg of the protein extract were subjected to IEF followed by SDS-PAGE and Coomassie Blue staining. Numbers refer to protein identifications (see Table 1). White circles designate proteins which have been identified; black circle remain unidentified and will subject to de novo sequencing in further studies. (B) 2-dimensional gel electrophoretic separation of the total protein extract of *P. verrucosum* BFE808 grown under irradiation with light (450 nm). 100 μg of the protein extract were subjected to IEF followed by SDS-PAGE and Coomassie Blue staining. Numbers refer to protein identifications (see Table 1). White circles designate proteins which have been identified; black circle remain unidentified and will subject to de novo sequencing in further studies.

on statistical analysis using the student-*T*-test, 56 spots were regarded as significantly differential regulated ($p < 0.05$). In detail of the 141 spots, 44 spots were down-regulated (15 with significance), 78 spots were up-regulated (41 with significance). The remaining proteins were abundant, but neither up- nor down-regulated. A total of 46 protein spots out of the chosen 90 spots were identified using MS/MS-Ion search against a protein database and are listed in Table 1. Out of these 46 identified proteins, 16 were significantly regulated and separately depicted in Fig. 4. The remaining 44 Spots out of the 90 are subjected to de novo sequencing for further studies. The genome of *P. verrucosum* has not been sequenced so far; only genome sequences of the Penicillia *P. chrysogenum* and *P. marneffei* are available. Despite this fact, the function of the identified proteins in this study was assigned by sequence homology with related fungal species. This circumstance does not matter since most proteins are highly conserved throughout the fungal kingdom. Some proteins appear more than once on the gels. This was true e.g. for the Cu–Zn superoxide–dismutase (Spots 2 & 84), aspartic protease pep1 (Spots 7 & 18), S-adenosylmethionine synthase 1 (Spots 10 & 60), uridylate kinase (Spots 14-1 & 14-2), formate dehydrogenase (Spots 16 & 62), heat-shock 70 kD protein (Spots 19 & 20), fructose-bisphosphate aldolase 1 (Spots 22-1 & 22-2), 3-isopropylmalate dehydrogenase B (Spots 24 & 64), heat-shock protein SSB1 (Spots 27 & 28), enolase (Spots 29 & 30), YcgL domain-containing protein MS1047 (Spots 61 & 63), endo-polygalacturonase A (Spots 65, 66 & 67) and subtilisin-like protease (Spots 73, 74 & 75). One obvious reason for these repetitions might be the fact that more than one homologue of the respective genes in *P. verrucosum* exists and some proteins have posttranslational modifications such as phosphorylation, glycosylation, etc. Also a strong binding of ampholytes to specific proteins may introduce changes of the isoelectric point. This is especially the case for spots that are aligned in one level of molecular weight and show minor variations in *pI*. Another observation that has to be mentioned was that in some cases the mass as the *pI* of the protein-spots on the SDS-PAGE differs to the theoretical data of the original protein. Examples are e.g. the HSP70 proteins which were found at a smaller molecular mass or a lower *pI* as expected, or the aspartic protease. The reason in the case of the HSP70 protein could be that the respective spot is an observed breakdown product of this protein, which is derived in general out of a 44 kD amino-terminal ATPase domain and a 28 kD carboxy-terminal domain that is further subdivided in one 18 kD peptide binding domain and one variable 10 kD carboxy-terminal domain. Again posttranslational modifications would lead to the observed disparities, too.

Proteins whose expression was induced under irradiation with light (450 nm), were e.g. enzymes which are involved in oxidative stress response like superoxide-dismutase (Cu–Zn) which is necessary for degradation of reactive oxygen species (ROS) and peroxi-redoxin (pmp20). Peroxi-redoxins comprise a family of highly conserved antioxidant proteins and are needed for the reduction of hydrogen-peroxide to control intracellular peroxide levels. Furthermore these enzymes are essential for circadian rhythmicity in human blood cells and their posttranslational modification could be induced by light (O'Neill and Reddy, 2011). Moreover, 5 members of the heat-shock protein family could be identified; in detail two isoforms of HSP-SSB1, two HSP70 and one HSP60. The latter one was not differentially regulated, both HSP-SSB1 were slightly up-regulated and the HSP70 proteins were strongly induced. Members of the HSP70 family are generally activated by heat stress, toxic chemicals, heavy metals such as arsenic, cadmium, copper, mercury, and under conditions which impose oxidative stress. We have previously shown that the biosynthesis of OTA and citrinin by *P. verrucosum* is dependent on light (Schmidt-Heydt et al., 2011). It has been described previously that light of short wavelength could lead to oxidative stress (Miyazaki et al., 2001). The biosynthesis of ochratoxin is thereby strongly reduced under light of short wavelength, especially royal-blue light (450 nm), whereas the biosynthesis of citrinin is increased under these conditions. One explanation for the light-driven induction of citrinin biosynthesis could be that the citrinin molecule itself acts as a light protectant and as radical scavenger as it has been described for the citrinin producing fungal strain *P. citrinum* (Heider et al., 2006; Størmer et al., 1998). That means that the biosynthesis of citrinin under light conditions may be of advantage for the fungus and consequently the biosynthesis of citrinin has to be increased under short wavelength light or other conditions which impose oxidative stress for the fungal cell. In contrast under blue light irradiation OTA-biosynthesis was decreased. The need for this mutual regulation lies in the fact, that OTA is degraded under light of short wavelength to genotoxic decomposition products (Gillman et al., 1998, Schmidt-Heydt et al., 2012). Moreover OTA and citrinin are structurally highly related therefore share the same progenitor molecules. In summary we could demonstrate that irradiation with light (450 nm) leads to an

Table 1
Chart of the proteins which could be identified in *Penicillium verrucosum* BFE808, ordered by spot number. From left to right: spot number; assignment by MASCOT; organism; accession number; MASCOT score; number of matched peptides; number of unique peptides; total sequence coverage (%); the observed and calculated physical parameters molecular weight (kD) and PI (isoelectric point); ratio between incubation in the dark versus irradiation with light (450 nm) and if student's *t*-test was passed (marked by X). Proteins identified with only one unique peptide and MASCOT score <70 are given in brackets […], see Section 2 for further details. The ratio is marked with an asterisk if the respective spot appears new under irradiation with light (450 nm).

Spot no.	Protein name MASCOT	Organism	Acc. number NCBI	MASCOT score	Peptides matched	Unique peptides	Coverage [%]	Exp. Mr. [kD]	Exp. *pI*	2D ~ Mr. [kD]	2D ~ *pI*	Ratio—dark vs. light	*t*-test passed
2	Superoxide dismutase [Cu–Zn]	*Aspergillus flavus*	GI:51702125	70	1	1	6	15,886	6,03	16	7,7	1,33	
6	Putative peroxi-redoxin pmp20	*Emericella nidulans*	GI:74680531	104	2	1	23	18,515	5,58	20	5,2	1,83	X
7	Aspartic protease pep1	*Penicillium roqueforti*	GI:74676291	91	2	1	4	41,086	4,52	16	5	2,73	X
8	Peptidyl-prolyl cis-trans isomerase B	*Emericella nidulans*	GI:74657233	115	2	2	11	23,392	6,89	22	8,9	1,22	
10	S-adenosyl-methionine synthase 1	*Saccharomyces cerevisiae*	GI:1346525	102	1	1	3	42,077	5,04	29	9,2	8,71	X
12	Superoxide dismutase [Mn], mitochondrial	*Penicillium chrysogenum*	GI:7388256	128	2	2	9	23,114	6,79	24	7,8	0,38	X
14-1	[Uridylate kinase]	*Saccharomyces cerevisiae*	GI:137024	[54]	1	1	3	22,919	6,19	26	6	1,31	
14-2	[Uridylate kinase]	*Saccharomyces cerevisiae*	GI:137024	[42]	1	1	3	22,919	6,19	26	6,2	2,48	X
14-3	[ATP synthase gamma chain]	*Nocardioides sp.*	GI:166224075	[41]	1	1	2	33,575	5,63	26	6,3	2,96	X
16	Formate dehydrogenase	*Neurospora crassa*	GI:729469	84	2	2	4	41,216	5,93	41	7,4	0,72	
17	Vacuolar protease A	*Neosartorya fumigatus*	GI:74675969	87	1	1	4	43,556	4,81	40	4,8	1,17	
18	Aspartic protease pep1	*Penicillium chrysogenum*	GI:327488354	109	2	2	5	41,403	4,88	35	4,7	59,92*	X
19	Heat shock 70 kD protein (Fragment)	*Penicillium citrinum*	GI:14423733	369	7	3	11	55,206	5	32	4,9	141,95*	X
20	Heat shock 70 kD protein (Fragment)	*Penicillium citrinum*	GI:14423733	290	6	3	10	55,206	5	31	4,95	191,97*	X
22-1	Fructose-bisphosphate aldolase 1	*Paracoccidioides brasiliensis*	GI:257051062	82	2	2	4	39,721	6,09	32	6,3	2,3	X
22-2	[Fructose-bisphosphate aldolase]	*Aspergillus oryzae*	GI:94730356	[40]	1	1	2	39,81	5,8	33	6,4	1,29	
23	Malate dehydrogenase, mitochondrial	*Schizosaccharomyces pombe*	GI:74626323	101	2	2	5	35,879	8,9	32	7,5	1,18	
24	[3-isopropylmalate dehydrogenase B]	*Aspergillus niger*	GI:6647557	[38]	1	1	2	40,272	5,27	37	7,4	0,85	
25	[Guanine nucleotide-binding protein subunit beta-like protein]	*Neurospora crassa*	GI:3023852	[58]	1	1	2	35,508	6,79	35	7,4	0,74	
27	Heat shock protein SSB1	*Candida albicans*	GI:353526248	265	4	3	7	66,61	5,25	45	5,8	1,28	
28	Heat shock protein SSB1	*Candida albicans*	GI:353526248	307	5	4	11	66,61	5,25	45	6	1,45	
29	Enolase	*Penicillium chrysogenum*	GI:74662366	712	11	2	29	47,264	5,14	45	6,2	0,9	
30	Enolase	*Penicillium chrysogenum*	GI:74662366	911	11	3	36	47,264	5,14	45	6,3	1,51	
35	ATP synthase subunit beta, mitochondrial (Fragments)	*Penicillium glabrum*	GI:182676425	76	1	1	7	12,025	9,89	22	5	2,52	
36	[Ornithine carbamoyl-transferase, catabolic]	*Haemophilus influenzae*	GI:1171930	[45]	1	1	2	37,908	6,23	24	7,2	0,49	X
37	[ATP synthase subunit d, mitochondrial]	*Aspergillus terreus*	GI:124007118	[40]	1	1	4	19,444	7,88	16	3,7	240,17*	X
47	NADP-specific glutamate dehydrogenase	*Penicillium chrysogenum*	GI:41017058	306	4	3	11	50,029	6	50	7,4	0,9	
51	ATP synthase subunit beta, mitochondrial	*Saccharomyces cerevisiae*	GI:84028178	388	5	2	12	54,817	5,52	48	5,2	2,18	
53	Heat shock protein 60	*Emericella nidulans*	GI:74680843	651	11	3	20	61,98	5,53	57	6,1	1,01	
59	Citrate synthase, mitochondrial	*Aspergillus niger*	GI:1705871	356	5	1	11	52,406	8,35	45	9,5	1,18	
60	S-adenosyl-methionine synthase 1	*Saccharomyces cerevisiae*	GI:1346525	201	2	1	7	42,077	5,04	41	6,4	1,98	
61	[YcgL domain-containing protein MS1047]	*Mannheimia succiniciproducens*	GI:238056545	[41]	1	1	11	12,09	9,52	40	6,9	108,67*	
62	[Formate dehydrogenase]	*Neurospora crassa*	GI:729469	[48]	1	1	2	41,216	5,93	41	7	0,93	
63	[YcgL domain-containing protein MS1047]	*Mannheimia succiniciproducens*	GI:238056545	[60]	1	1	11	12,09	9,52	41	7,2	0,93	
64	[3-isopropylmalate dehydrogenase B]	*Aspergillus niger*	GI:6647557	[34]	1	1	2	40,272	5,27	42	7	0,69	X
65	[Probable endo-poly-galacturonase A]	*Neosartorya fumigata*	GI:294956677	[45]	1	1	9	38,542	4,22	40	8,7	1,22	
66	Probable endo-poly-galacturonase A	*Neosartorya fumigata*	GI:294956677	70	1	1	9	38,542	4,22	40	9,1	1,29	X
67	Poly-galacturonase 1	*Penicillium olsonii*	GI:7388013	100	1	1	12	38,496	6,53	40	9,4	0,98	
68	Enolase	*Penicillium chrysogenum*	GI:74662366	852	11	4	29	47,264	5,14	37	6	1,35	
69	Inorganic pyrophosphatase	*Emericella nidulans*	GI:357528811	77	1	1	3	32,513	5,21	32	5,8	2,39	X
72	Probable NADP-dependent mannitol dehydrogenase	*Davidiella tassiana*	GI:85701146	152	3	3	7	28,731	5,9	28	6,2	1,21	
73	Subtilisin-like protease	*Coccidioides posadasii*	GI:327488488	105	1	1	2	53,014	6,47	31	7,8	263,01*	X
74	Subtilisin-like protease	*Coccidioides posadasii*	GI:327488488	296	4	4	8	53,014	6,47	31	8,2	0,83	

(*continued on next page*)

Table 1 (*continued*)

Spot no.	Protein name MASCOT	Organism	Acc. number NCBI	MASCOT score	Peptides matched	Unique peptides	Coverage [%]	Exp. Mr. [kD]	Exp. *pI*	2D ~ Mr. [kD]	2D ~ *pI*	Ratio— dark vs. light	*t*-test passed
75	Subtilisin-like protease	*Coccidioides posadasii*	GI:327488488	359	5	5	8	53,014	6,47	31	8,5	1,5	
84	Superoxide dismutase [Cu–Zn]	*Aspergillus flavus*	GI:51702125	80	1	1	6	16	6,03	16	7,4	1,84	
85	Ubiquitin-conjugating enzyme E2-16 kD	*Colletotrichum gloeosporioides*	GI:27805755	170	4	3	20	16,688	6,4	14	7,7	2,45	

increase in ROS generation in *P. verrucosum* which was followed, maybe as kind of compensation reaction, by an upregulation of citrinin biosynthesis and an induction of stress associated proteins such as the superoxide dismutase (SOD) and heat-shock proteins (HSP). It could be hypothesized that these mechanisms maintain further in subsequent reduction of total amount of ROS in the fungal cell. The action of the citrinin molecule as antioxidant has consequences on the physiology of the producing fungus which result in adaptation processes such as the shift of the metabolite profile from ochratoxin to citrinin.

Further proteins could be identified, for example proteins which are involved in the degradation of enzymes, such as the highly induced aspartic proteases “pep1” and subtilisin-like protease, but also enzymes that are necessary for degradation of polysaccharides like the endo-poly-galacturonases. Some proteins which play a role in glycolysis were also induced, namely the fructose-bisphosphate-aldolase-1 and some forms of enolase. Interestingly the latter has been described as a highly allergenic protein which acts as fungal antigen and has been identified earlier in *Aspergillus* species where it is expressed during invasive aspergillosis (Denikus et al., 2005). Moreover we found an increase in mitochondrial citrate synthase activity. Taking into account that several subunits of ATPases that are necessary for ATP-generation and energy supply for the fungal cell are also activated under light irradiation, it is obvious that the mycelium shows higher requirement for energy resources under this stress condition. For regeneration of NADPH we identified the moderately expressed putative NADP-dependent mannitol-dehydrogenase that may be involved in mannitol cycle. Mannitol itself was also described as potent stress protectant with antioxidative functions (Stoop et al., 1996) and may also be relevant in *P. verrucosum* to compete oxidative stress. Numerous regulatory proteins have been characterized, in detail the peptidyl-prolyl cis-trans

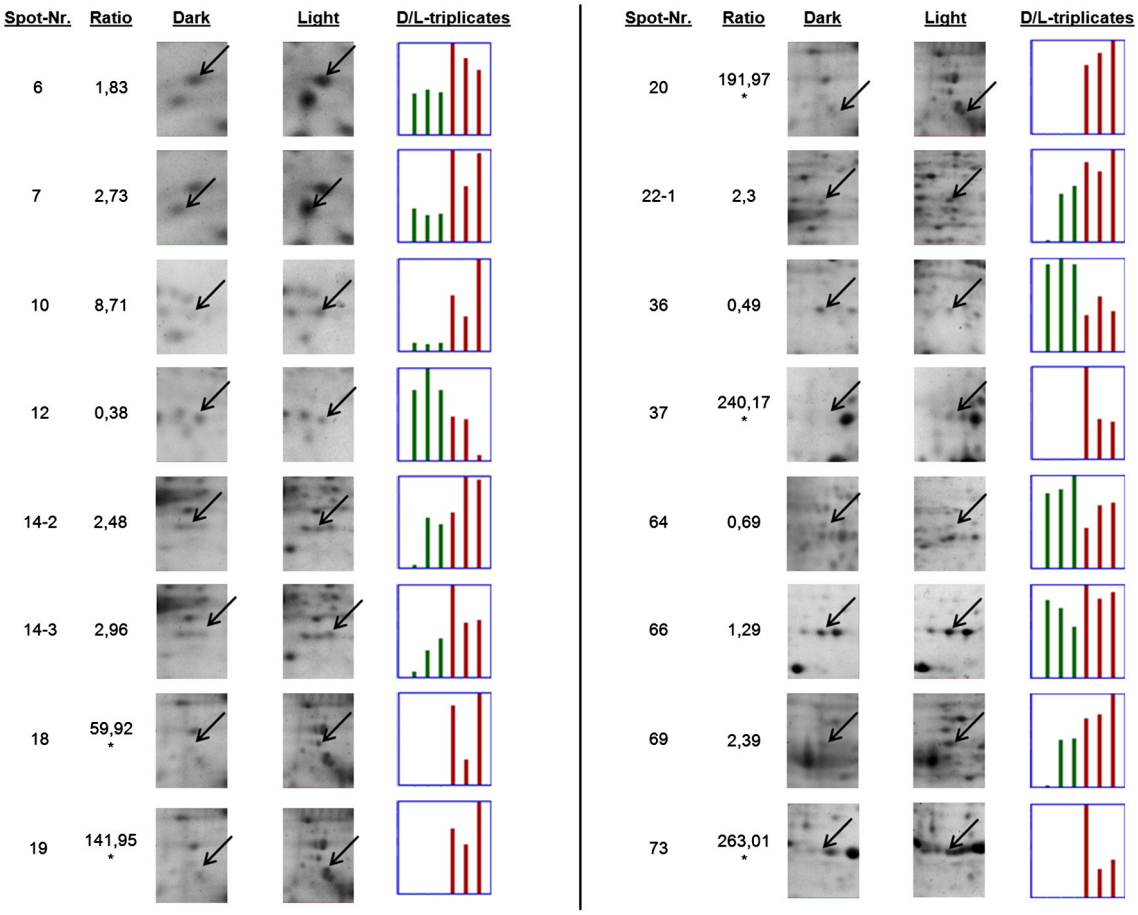

Fig. 4. Significant differentially expressed proteins which could be identified by peptide mass analyses. The given ratios reflect the regulatory level of the respective spots under light in comparison to the darkness. For visualization one representative spot out of the triplicates was chosen. First three bars correspond to biological triplicates grown in the dark; second three bars correspond to biological triplicates grown under irradiation with light (450 nm).

isomerase B, two S-adenosyl-methionine synthases, vacuolar protease A and ubiquitin-conjugating enzyme E2-16 kD. However one guanine nucleotide-binding protein subunit beta-like protein was decreased. Proteins that are involved in amino-acid metabolism were down-regulated under light stress, such as 3-isopropyl-malate dehydrogenase B and ornithine carbamoyl-transferase. Additionally NADP-specific glutamate dehydrogenase, which is necessary in nitrogen-metabolism, was down-regulated, too. Further proteins that were identified and induced under irradiation with light (450 nm) were malate dehydrogenase, uridylate kinase, inorganic pyrophosphatase and the not further characterized YcgL-domain containing proteins. A poor down-regulation could be observed in the case of formate dehydrogenase. Conclusionary (Fig. 5), proteins whichever are involved in "metabolic processes" such as "amino acid metabolism" and "protein anabolism/catabolism" were most abundant (56%). Other classes of identified proteins include "response to stress" (21%), "general regulator proteins/cellular homeostasis" (19%), and other not further specified proteins (4%). Highly induced proteins under light irradiation were SOD and HSP proteins as a compensation reaction on oxidative stress. Possibly the increased citrinin biosynthesis under oxidative stress is driven by the induction of stress regulated proteins, too. To uncover the molecular mechanism behind this observation will be the intention for further investigations, e.g. comparison of intraspecific variation between different strains of *P. verrucosum* or the influence of other stress factors on protein expression in correlation to phenotypical mycotoxin biosynthesis.

4. Conclusion

This study is the first proteome analysis of *P. verrucosum*, a mycotoxigenic and facultative pathogenic fungal strain. In a comparative analysis several proteins could be identified which are associated with diverging mycotoxin biosynthesis under different growth conditions. Besides proteins which are generally involved in physiological processes or stress response, the highly allergenic protein enolase which has been described in other fungal genera as a virulence or colonization factor has been identified now also in *P. verrucosum*. Comparative proteome analysis of mycotoxigenic fungi will open another view on proteins which are involved in growth and toxin biosynthesis. This will enable possibilities to develop case-sensitive prevention strategies to inhibit fungal growth and mycotoxin biosynthesis. Moreover in further studies we plan to identify all spots in order to create a reference map to provide a comprehensive insight into the regulation of growth and mycotoxin biosynthesis of *P. verrucosum* on a protein level.

Acknowledgments

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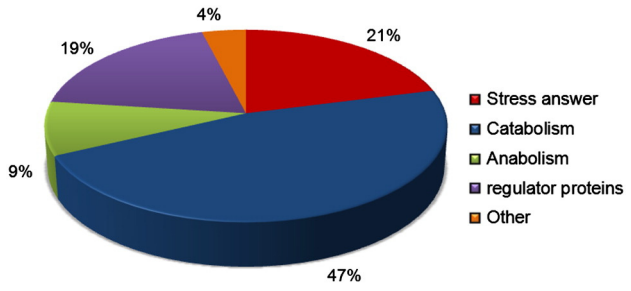

Fig. 5. Percental assignment of the respective proteins to a physiological function.

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