

**Functional characterization of the mevalonate-
isoprenoid biosynthesis pathway genes in *Mucor
circinelloides***

Ph.D. thesis

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INTRODUCTION

Members of the subphylum Mucoromycotina, order Mucorales (such as *Lichtheimia*, *Mucor*, *Rhizomucor* and *Rhizopus* species) are saprotrophic fungi, which also have medical, industrial, biotechnological and agricultural importance. Some species may cause post-harvest damage in agriculture; or are used as producers of extracellular enzymes, organic acids and carotenoids. Several species belonging to this fungal group are also considered opportunistic human pathogens, which can cause fatal systemic infections (zygomycosis or mucormycosis) in immunocompromised patients with neutropenia, diabetic ketoacidosis or serious skin injuries. *Mucor* and *Lichtheimia* are, after *Rhizopus*, the clinically most relevant genus of Mucoromycotina. Today ergosterol and its biosynthetic pathway are the major targets of the antifungal agents used in clinics to treat infections caused by Mucoromycotina fungi. The therapy of mucormycosis is still limited because of the intrinsic resistance of these fungi to the majority of the currently clinically applied antimycotics.

Metabolites synthesized via the mevalonate-isoprenoid pathway (such as sterols, functional groups of proteins and carotenoids) play an important role in signal transduction, morphogenesis, adaptation to environmental change and protection against free radicals. In the pathway three molecules of acetyl-CoA are condensed by thiolase and 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthase to form HMG-CoA, which is then reduced to mevalonate by HMG-CoA reductase. Next, conversion of mevalonate to isopentenyl pyrophosphate (IPP, which is the binding block of all isoprenoids) is catalyzed by three enzymes: mevalonate is phosphorylated by mevalonate kinase and mevalonate-5-phosphate kinase, and then decarboxylated to IPP by diphosphomevalonate decarboxylase (or mevalonate-5-diphosphate decarboxylase). In the isoprenoid pathway, formation of dimethylallyl pyrophosphate (DMAPP) is catalyzed by IPP isomerase, followed by repetitive condensations with further IPP units, result in elongation of the prenyl chain. These steps are catalyzed by prenyltransferases, such as farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP) synthases, and form the intermediate geranyl pyrophosphate (GPP), FPP and GGPP. FPP and GGPP are the precursors of ergosterol, carotenoids and functional groups of farnesylated and geranylgeranylated proteins.

AIMS OF THE STUDY

Till the date limited information is available about the function and regulation of the genes playing role in mevalonate-isoprenoid pathway in Mucoromycotina fungi. Characterization of these genes can serve valuable knowledge for the improvement of secondary metabolite production by Mucoromycotina fungi and may lead us to better understanding of biological processes, including morphogenesis, protection against free radicals, response to environmental change (e.g. changes in the soil composition, oxygen concentration or salinity of the environment) or pathogenesis.

Mucor circinelloides (Mucoromycotina, Mucorales) is a carotenoid producing fungus, which is a model organism of several carotenogenic studies. In this study six genes involved in the mevalonate-isoprenoid biosynthesis in *M. circinelloides*, encoding the HMG-CoA synthase (*hmgS*), mevalonate kinase (*mvk*), diphosphomevalonate decarboxylase (*dmd*), IPP isomerase (*ipi*), FPP and GGPP synthases (*isoA* and *carG* genes, respectively) were selected for characterization. One of our goals was to improve the carotenoid production of the fungus with overexpression and silencing of the genes. Our next aim was to examine the effect of the overexpression and silencing of the genes on the ergosterol content of *M. circinelloides* and its effect on the susceptibility to different antifungals, which may allow the identification of potential novel drug targets. Little is known about the efficiency of gene silencing can be achieved with different plasmid constructs in *M. circinelloides*, thus among our aims was to compare the characteristics of the mutants harbouring different plasmids for gene knockdown as well.

The following specific objectives have been addressed to present study:

1. Investigation of the transcription of selected six genes under different cultivation conditions involved in the mevalonate-isoprenoid biosynthesis in *M. circinelloides*.

Effect of cultivation conditions, such as temperature, oxygen tension, light sources, medium composition and incubation time on the transcription of the *hmgS*, *mvk*, *dmd*, *ipi*, *isoA* and *carG* genes.

2. Overexpression and silencing of selected six genes involved in the mevalonate-isoprenoid biosynthesis in *M. circinelloides* and characterization of the transformants.

Development of different plasmid constructs for overexpression and silencing of the *hmgS*, *mvk*, *dmd*, *ipi*, *isoA* and *carG* genes in *M. circinelloides*. Transformation experiments with the *M. circinelloides* MS12 strain. Characterization of the transformants in detail, such as investigation of micromorphology, carotenoid and ergosterol content, antifungal susceptibility and interaction with macrophages. Comparison of the overexpression and gene silencing efficiency achieved with the different plasmid constructs.

METHODS

Molecular techniques:

- DNA extraction
- RNA extraction, cDNA synthesis
- Polymerase chain reaction (PCR), quantitative real-time PCR (qPCR)
- Agarose gel electrophoresis
- Gene cloning
- Construction of plasmids
- Transformation of *E. coli*
- Plasmid DNA extraction

Fungal transformation and characterization of the *M. circinelloides* mutants:

- Protoplast formation of *M. circinelloides*
- PEG/CaCl₂-mediated transformation of *M. circinelloides*
- Light and fluorescence microscopy
- Spectrophotometry and high performance liquid chromatography (HPLC) to determine the carotenoid and ergosterol content
- Antifungal susceptibility test
- Phagocytosis assay

RESULTS

1. Investigation of the transcription of selected six genes under different cultivation conditions involved in the mevalonate-isoprenoid biosynthesis in *M. circinelloides*.

Before the present thesis, our research group designed primers to study the transcription of *hmgS*, *mvk*, *dmd*, *ipi*, *isoA* and *carG* genes. The *M. circinelloides* MS12 strain was cultivated under different cultivation conditions, such as on different media composition, different temperature, light conditions, oxygen tension, which was followed by RNA extraction and cDNA synthesis; the relative transcription level was analyzed with qPCR. The *Mucor actA*, encoding an actin was used as reference gene. The transcription of six mevalonate-isoprenoid pathway genes was compared to each other. The fungus was cultivated in liquid or on solid YNB medium for four days. In both cases, the isoprenoid pathway genes (*ipi*, *isoA* and *carG*) showed higher transcription level than mevalonate pathway genes (*hmgS*, *mvk* and *dmd*), moreover we found that *mvk* shows the lowest, while *ipi* the highest transcription level. Light played key role, and reduced the transcription level of almost all genes when the fungus was cultivated in continuous dark, in comparison with cultures cultivated in continuous light. The short light exposure increased significantly the transcription of *carG*. The *isoA* showed higher transcription level, when *M. circinelloides* MS12 was cultivated under warm white (contains more components in the yellow-red wavelength range), than under daylight (contains more components in the blue wavelength range) source; while the *carG* showed higher transcription level under daylight condition. Previous study verified that blue light induce the transcription of *carG*, moreover our findings presume that red color has similar effect to the transcription of *isoA*.

M. circinelloides is a dimorphic fungus, which can grow in yeast-like morphology under anaerobic conditions. All six genes showed an increased transcription level upon cultivation under anaerobic conditions in comparison with cultivation under aerobic conditions. Interestingly, anaerobic followed by one hour aerobic growth increased the transcription of *dmd* significantly and decreased the transcription of *carG* compared to cultures under continuous anaerobic condition. *M. circinelloides* grows intensively on 25 °C. Growth temperature at 35 °C increased the transcription of all six genes, moreover similar effect was observed at suboptimal

temperature at most of the genes in comparison with the control conditions (25 °C). Genes showed the highest transcription level at four hours and eight hours postinoculation, except *carG*, which showed the highest transcription in 96 hours old cultures.

The increased glucose concentration generally resulted decrease in most of the gene transcription, while DHA (in previous studies modified and increased the carotenoid production of mutant *M. circinelloides* strains) significantly reduced the transcription of *hmgS* and *mvk*, and increased that of *ipi* in comparison with cultivation on glucose. Addition of NaCl to the media generally did not changed the transcription of the genes significantly, at the same time fluvastatin (statins are cholesterol-lowering drugs and are competitive inhibitors of the HMG-CoA reductase) significantly increased the transcription of all six genes in comparison with the control.

2. Overexpression and silencing of selected six genes involved in the mevalonate-isoprenoid biosynthesis in *M. circinelloides* and characterization of the transformants.

For overexpression and silencing of the *hmgS*, *mvk*, *dmd*, *ipi*, *isoA* and *carG* genes five plasmid were constructed for all six genes. For overexpression the genes were amplified with their own promoter and terminator regions or were placed under the control of the *M. circinelloides* glyceraldehyde-3-phosphate dehydrogenase 1 (*gpd1*) promoter and terminator (*own* and *gpd* plasmid constructs, respectively). For gene silencing three different plasmids were constructed for all six genes: (1) the genes were inserted between the *Mucor gpd1* and *zrt1* (encoding a ZIP zinc transporter protein) promoter regions (*pMAT* constructs); (2) a fragment of a gene was inserted between the *Mucor gpd1* promoter and terminator in inverted orientation (*as* constructs); (3) a fragment of a gene and its reverse complement together with an intron were placed under the regulation of the *gpd1* promoter and terminator (*hpRNA* constructs). The *Mucor pyrG* and *leuA* genes were used as selection markers, which complement the uracil and the leucine auxotrophy, respectively. Successful transformation of *M. circinelloides* MS12 strain was carried out with the plasmids; the mutants were selected based on the auxotrophy complementation.

The presence of the plasmids in the mutants was verified with PCR. The plasmid copy number was investigated in the mutants and found as to be 0.3–10 copy/genom; at the same time fluctuation in the copy number was observed. The relative transcription levels of the overexpressed and silenced genes were also analyzed. We observed an increase in the relative transcription level of the overexpressed genes, which was more prominent in those transformants, harbouring the genes in extra copies under the control of the *gpd1* promoter and terminator, while gene silencing was most effective when the *hpRNA* and *as* constructs were used. Significant decrease was found in the colony forming unit in all transformants, including those harbouring the plasmids with the *pyrG* and *leuA* genes, in comparison with *M. circinelloides* MS12, which can be due to mitotic instability of the plasmids. This was more prominent in transformants harbouring the *hpRNA* plasmids. In case of several transformants (mainly in four days or older colonies) increased number of hyphae branching and cytoplasmic effusion was observed.

Overexpression of all six genes (primary the *mvk*, *ipi* and *carG*) increased the carotenoid content, while silencing of mevalonate pathway genes decreased that in comparison with the wild type. Similarly, significant decrease was found in the ergosterol content with silencing of the genes with *hpRNA* plasmids. Antifungal susceptibility of the mutants was also tested. Primarily modification of the *dmd* and *ipi*, responsible for the formation and isomerization of IPP, respectively, resulted significant differences in the susceptibility to azoles and statins in comparison with *M. circinelloides* MS12. Overexpression of the *hmgS*, *ipi* and *carG* and silencing of the *mvk* and *isoA* resulted decrease in phagocytic indexes, while overexpression of the *mvk* and *dmd* increased phagocytic indexes.

CONCLUSIONS

Six genes (*hmgS*, *mvk*, *dmd*, *ipi*, *isoA* and *carG*) involved in the *M. circinelloides* mevalonate-isoprenoid pathway were selected for characterization and the transcription of the genes was determined under different cultivation conditions, which can contribute in further studies to modify the metabolite production (such as carotenoids and ergosterol) of the fungus with optimization of the culturing conditions. Characteristics of the mutant *M. circinelloides* strains were also compared to each other and found that overexpression and silencing of the genes are the most

effective with plasmids harbouring the genes under the control of the *gpd1* promoter and *hpRNA* constructs, respectively.

Carotenoid overproducing mutants were isolated with overexpression of the *mvk*, *ipi* and *carG* genes, which can be used in further studies to improve the β -carotenoid production of *Mucoromycotina* fungi. Moreover, 40 – 54% decrease in the ergosterol content was detected with silencing of all six genes and increased susceptibility to azoles and statins of mutants harbouring the *hpRNA* plasmids for silencing of *dmd* gene was determined, which may serve as potential novel drug target in future.

A. PUBLICATIONS SERVING AS THE BASIS FOR THE DOCTORAL PROCESS

Kumar D, Rizvi SI (2015) Black tea extract improves anti-oxidant profile in experimental diabetic rats. *Archives of Physiol Biochem* 121:109-115.

Kumar D, Rizvi SI (2014) Markers of oxidative stress in senescent erythrocytes obtained from young and old age rats. *Rejuvenation Res* 17:446-452.

Kumar D, Singh SK, Singh AK, Rizvi SI (2012) Pomegranate (*Punica granatum*) peel extract provides protection against mercuric chloride-induced oxidative stress in Wistar strain rats. *Pharmaceutical Biol* 1-6.

B. COMPLETE LIST OF PUBLICATIONS IN REFERRED JOURNALS

Kumar D, Rizvi SI (2017) Black tea supplementation augments redox balance in rats: relevance to aging. *Archives of Physiol Biochem* 123:212-218.

Kumar D, Sharma B, Rizvi SI (2016) Protective role of black tea and vitamin C during subacute toxicity of carbofuran in rats. *Progress in Health Sci* 6.

Mishra N, Kumar D, Rizvi SI (2016) Protective Effect of *Abelmoschus esculentus* Against Alloxan induced Diabetes in Wistar Strain rats. *J Diet Suppl* 13:634-646.

Kumar D, Rizvi SI (2015) Black tea extract improves anti-oxidant profile in experimental diabetic rats. *Archives of Physiol Biochem* 121:109-115.

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Kumar D, Singh SK, Singh AK, Rizvi SI (2012) Pomegranate (*Punica granatum*) peel extract provides protection against mercuric chloride-induced oxidative stress in Wistar strain rats. *Pharmaceutical Biol* 1-6.

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Rizvi SI, Kumar D, Chakravarti S, Singh P (2010) Erythrocyte plasma membrane redox system may determine maximum life span. *Medical Hypothesis* 76:547-549.

C. CONFERENCE ABSTRACTS RELATED WITH THE RESULTS OF THE PH. D. THESIS

Kumar D, Csernetics Á, Jager O, Nagy G, Voigt K, Vágvölgyi Cs, Papp T (2017) Modification of the mevalonate isoprenoids biosynthesis pathway and its outcome on the ergosterol and carotenoid biosynthesis and antifungal susceptibility of *Mucor Circinelloides*. 6th Hungarian Mycological conference, Hungary. Abstracts p. 113.

Nagy G, Hassan M, Kumar D, Vaz AG, Bartha E, Csernetics Á, Vágvölgyi Cs, Voigt K, Papp T (2016) Characterisation of virulence genes in opportunistic human pathogenic Mucoralean fungi. 13th European conference on fungal genetics. Paris, France, Abstracts p. 161.

Csernetics Á, Kumar D, Nagy G, Jager O, Voigt K, Vágvölgyi Cs, Papp T (2016) overexpression and silencing of the *Mucor circinelloides* mevalonate isoprenoid pathway genes and their effects on the biosynthesis of ergosterol and antifungal susceptibility. Interdisciplinary forum on virulence mechanisms of phyto and human pathogenic fungi. Erlangen, Germany. Abstracts p. 6.

Nagy G, Hassan M, Kumar D, Vaz AG, Bartha E, Vágvölgyi Cs, Voigt K, Papp T, Csernetics Á (2016) Molecular background of virulence in human pathogenic Mucoralean fungi. Biospectrum, Jena, Germany. Abstracts p. 84.

DECLARATION

I declare that, the contribution of Dileep Kumar was significant in the listed publications and the doctoral process is based on the publications listed in part (A).

The results reported in the PhD dissertation and the publications have not been used to acquire any PhD degree previously and will not be used in the future either.

Szeged, 18.06.2018

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Prof. Dr. Csaba Vágvolgyi