PH.D. THESIS

FUNCTIONAL ANALYSIS OF HMG-COA REDUCTASES IN *MUCOR CIRCINELLOIDES*

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INTRODUCTION

Isoprenoids or terpenes are one of the largest and most diverse classes of naturally occurring organic chemicals. They have role in formation of membrane structure (e.g. cholesterol and ergosterol) and different biochemical processes in the cell. They may be pigments (e.g. carotenoids) playing an important role in photosynthesis and photooxidative protection. Because of their important roles in living cell, their biosynthesis is intensively researched.

Phycomyces blakesleeanus, *Blakesle trispora* and *Mucor circinelloides* belonging to zygomycetes fungi (Mucoromycotina) are model organisms for studying microbial carotenoid biosynthesis. Regulation of carotenoid biosynthesis is studied mostly in *Phycomyces*. Carotenoids and other isoprenoids share the early steps of biosynthesis. This common pathway is called mevalonate biosynthetic pathway (MEV), in which isopentenyl pyrophosphate (IPP) is synthesized from acetyl-coenzyme A. The central step of MEV is the conversion of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) to mevalonic acid catalysed by HMG-CoA reductase (HmgR). Thus, this enzyme regulates the production of isoprenoids. Statins are selective inhibitors of the enzyme and used in human medication as anti-cholesterolemic drugs, but their antifungal effects were also examined.

The number of HMG-CoA reductase genes is variable in the different organisms. Genome of mammals contains generally one reductase gene, but the number of genes varies in plants. Zygomycetes genomes encode one or two genes, however *M. circinelloides* has three *hmgR* genes, *hmgR1*, *hmgR2* and *hmgR3*. In this study, we would like to clarify the specific role of each reductase in the isoprene biosynthesis in *M. circinelloides*. After isolation of the genes we elevated their copy number and silenced them to analyse their effect on the fungus. With gene expression studies we planned to examine the regulation of *hmgR* genes and to find differences in their expressions.

HMG-CoA reductase may have role in many different biological processes in the cell, such as morphogenesis, apoptosis or stress-response pathways. Although they may be important for potential biotechnological applications or in the study of fungal pathogenesis, little information is available from these processes in *Mucor*. Investigation of the regulatory processes of the terpene biosynthesis may contribute to create new carotenoid producing strains for industry. Clarification of the role of the enzymes in the ergosterol biosynthesis could be useful in developing new antifungal agents, in study of adaptation to the environment and the formation of membrane structure.

AIMS

1. Isolation and characterization *of M. circinelloides* HMG-CoA reductase genes

The whole genome sequence of *M. circinelloides* was completed in 2010 (http://genome.jgi-psf.org/Mucci2/Mucci2.home.html), which facilitated the identification and characterization of genes involved in the isoprene biosynthesis. Our aims were to search gene sequences in the *M. circinelloides* genome, which are homologous with known fungal *hmgR* genes, and analyse the sequences *in silico*. We also planned the isolation and cloning of the found sequences.

2. Analysis of the transcription of the *hmgR* genes under different growth conditions.

We planned to examine the transcription of the identified genes under different environmental and growing conditions by the quantitative PCR method. During the analysis, we focused on the differences in the induction and expression of the three reductase genes under specific conditions

3. Overexpression and silencing of the *hmgR* genes

After isolation of genes, two different types of vectors were designed, one series of vectors was built to overexpress the *hmgR* genes by elevating the copy number and another series was constructed to silence them for testing the function of genes. After the successful transformation procedure we could examine the role of *hmgR* genes in cell growing, sensitivity to statins and production of carotenoid and ergosterol.

4. Subcellular localization of the HmgR proteins.

We planned to examine the intracellular localization of HmgR proteins. This requires the construction of vectors, which are able to express the transmembrane region of the proteins fused with green fluorescent protein gene (*gfp*). Following dying of endoplasmic reticulum and mitochondria, co-localization of cell organelles and HmgR-GFP proteins may be investigated.

METHODS

DNA and RNA based techniques:

- DNS extraction
- RNA extraction, cDNA synthesis
- DNA/RNA electrophoresis
- Polymerase chain reactions (PCR), real-time PCR (qPCR)
- Cloning of DNA fragments, DNS sequencing
- Plasmid construction
- Transformation of bacteria
- Plasmid DNA extraction
- Northern hybridization

Analysis of nucleotide and amino acids sequence data:

- *In silico* analysis and comparison of the nucleotide sequences
- Deduction of the amino acid sequences from nucleotide sequences
- Alignment of the nucleotide and the amino acid sequences

Genetic transformation of fungi:

- Generation of fungal protoplasts
- PEG-mediated protoplast transformation using autonomously replicating plasmids
- Analysis of sensitivity to statins

Analytical methods:

- Fungal carotenoid and ergosterol extraction
- Analysis of carotenoid content using spectrophotometry
- Analysis of ergosterol content using high performance liquid chromatography (HPLC)

Microscopy:

- Light-, fluorescent-, confocal microscopy
- Detection of apoptotic processes
- Expression of HmgR-GFP fusion proteins
- Examination of localization of HmgR-GFP proteins in the cells
- Isolation and examination of the membrane fraction

RESULTS

1. Isolation and characterization *of M. circinelloides* HMG-CoA reductase genes.

Using the *hmgR* genes of *P. blakesleeanus* and *R. miehei*, searches for homologous sequences were performed in the genome database of *M. circinelloides* f. *lusitanicus*. Three *hmgR* genes (*hmgR1*, *hmg2* and *hmgR3*) were identified. The genes were isolated and their cDNA sequences were determined. In *hmgR1* and *hmgR2*, five introns were identified, while *hmgR3* had only two introns. We analysed the amino acid sequences of the putative proteins as well. All three proteins belong to type I reductases with two main domains, the variable N-terminal region and the conserved C-terminal region. Between them there is a short linker region. NAD(P)H and HMG-CoA binding motifs and the sterol sensing domain (SSD) could be identified in all three proteins. SSD is responsible for the post-translational regulation of proteins. In *hgmR1* six, in *hmgR2* nine, while in *hmgR3* five transmembrane helices were identified.

2. Analysis of the transcription of the *hmgR* genes under different growth conditions.

Relative transcription levels of the three *hmgR* genes were determined under various culture conditions by quantitative real-time PCR. It was found that *hmgR1* is transcribed only under aerobic conditions. At the same time, the transcription level of *hmgR3* was relatively high under anaerobiosis, while *hmgR2* showed high transcription under both conditions. Expression of *hmgR1* was detected only in the hyphae and reached the maximum level at 48 hours after inoculation showing a temperature-dependent transcription.

In the germinating spores *hmgR2* and *hmgR3* were active and did not show temperature dependence. Expression of both genes was inhibited by maltose and trehalose and *hmgR2* responded to salt stress. Our results show that transcription of *hmgR2* was highly affected by the environmental factors.

3. Overexpression and silencing of the *hmgR* genes.

Two different types of vectors were constructed, one series of vectors was built to overexpress the *hmgR* genes by elevating the copy number and changing their promoter to that of the *gpd1* gene (pNG series) and another series to silence them by antisense RNA-based gene silencing (pAS series). Vectors were transformed into the recipient MS12 strain using the PEG-mediated protoplast transformation method and transformed colonies were isolated. After analysis of the transformants to prove the presence of the transformed DNA, growing rate,

macro- and micromorphology, carotenoid and ergosterol content and sensitivity to statins were tested.

Over-expression and silencing of *hmgR1* had no significant effect to the majority of the examined features. However, cytoplasm effusions could be detected during the microscopic examination of the silenced transformants and their ergosterol content was slightly decreased. In both types of transformants, necrotic cells and hyphae were detected.

Over-expression of *hmgR2* resulted in increased carotenoid and ergosterol contents and decreased sensitivity to statins in the transformants. Silencing of the gene also led to cytoplasm effusions and we could detect necrotic cells and hyphae as well.

Similarly to the changes detected in case of *hmgR2*, over-expression of *hmR3* increased the carotenoid and ergosterol content and decreased the sensitivity to statins of the transformants. Silencing of this gene caused altered morphology in the transformants with irregular, swollen hyphae and cytoplasm effusions and increased hyphal branching. Carotenoid and ergosterol content decreased significantly in these strains and we could detect the early stage of apoptosis. It seems that *hmgR3* has role in spore germination and morphogenesis.

4. Subcellular localization of the HmgR proteins.

To analyse the subcellular localization of the HmgR proteins, the transmembrane region of each reductase was fused with the green fluorescent protein (GFP). With these fusion proteins we could follow the localization of the reductases in the cell. After PEG mediated protoplast transformation the transformed colonies expressing the fusion proteins were isolated. We have started to analyse the transformed strains with fluorescent microscopy and it was found that all three proteins are localized in large regions in the cell, but the HmgR2 also appeared in a number of not well-defined small compartments. To get the exact localization of the proteins we stained the endoplasmic reticulum and the mitochondria, and samples were analysed by confocal microscopy. The HmgR2 and HmgR3 showed a clear co-localization to the endoplasmic reticulum, but not to the mitochondria.

SUMMARY

In this study, the following main similarities and differences were identified between the *M. circinelloides hmgR* genes:

1. Under aerobic conditions the *hmgR1* gene is active in hyphae and during sporulation. We managed to identify temperature dependent gene expression only with *hmgR1* and the relative transcription level of this gene was lower compared to the *hmgR2* and *hmgR3*. Under anaerobic conditions the activity of this gene was significantly decreased. Ergosterol and carotenoid production of fungus was effected by the altered function of the gene (increase of copy number, silencing) and may have role in cytoplasmic integrity. HmgR1 typically appeared in spores and germ tubes and possibly localizes to the endoplasmic reticulum.

2. The *hmgR2* gene showed strong activity under aerobic and anaerobic conditions and *hmgR2* had the highest transcription level under aerobic cultivation condition during the whole life cycle of the fungus. Relative transcription level of this gene was high under spore germination time. Expression of *hmgR2* increased proportionally with increasing salt concentration, but the trehalose and maltose inhibited the expression. As a result of gene overexpression the carotenoid and ergosterol content of the transformant strains increased significantly and their sensitivity to the statins decreased. As HMG-CoA reductase has important role in ergostreol biosynthesis, functional testing of *hmgR2* may be useful in finding and developing new antifungal agents. Ergosterol biosynthesis is one of the most important target for antifungal therapies and the HMG-CoA reductase inhibitors are intensively researched (Galgóczy et al. 2011). With the silencing of the gene, carotenoid and ergosterol content of the transformant strains decreased and cytoplasmic integrity was also effected. HmgR2 is associated in the endoplasmic reticulum but may be localized in other small compartments in the cell.

3. Oxygen content of the environment affected expression of *hmgR3*, because the relative transcription level of the gene significantly increased under anaerobic conditions, and the gene seems to be important during spore germination and sporulation. Temperature dependent gene expression or role in adaptation to osmotically changed environment were not found, but the maltose and trehalose also inhibited the gene epxression. *hmgR3* plays important role in the caroteinoid biosynthesis but did not have influence in the

ergosterol biosynthesis. *hmgR3* and *hmgR2* are possible targets to create new carotenoid producing strains. After silencing the gene the carotenoid content of transformants decreased significantly, macro morphology of strains changed and we could detect apoptotic processes too. The spores lost germinating ability and germination time was delayed. In Zygomycetes apoptotic processes are not investigated so *hmgR3* is an excellent target for apoptotic researches. It seems HmgR3 localizes in the endoplasmic reticulum.

Results summarized in the Ph. D. thesis were published in the following articles:

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CUMULATIVE IMPACT FACTOR: 11,877