Overexpression of *erg20* gene encoding farnesyl pyrophosphate synthase has contrasting effects on activity of enzymes of the dolichyl and sterol branches of mevalonate pathway in *Trichoderma* *reesei*.

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**Running title**: Overexpression of FPP synthase in *T.reesei*

**Abstract**

The mevalonate pathway is the most diverse metabolic route resulting in the biosynthesis of at least 30 000 isoprenoid compounds, many of which, such as sterols or dolichols, are indispensable for living cells. In the filamentous fungus *Trichoderma* of major biotechnological interest isoprenoid metabolites are also involved in the biocontrol processes giving the mevalonate pathway an additional significance. On the other hand, little is known about genes coding for enzymes of the mevalonate pathway in *Trichoderma*.

Here, we present cloning and functional analysis of the *erg20* gene from *T. reesei* coding for farnesyl pyrophosphate (FPP) synthase (EC 2.5.1.10), an enzyme located at the branching point of the mevalonate pathway. Expression of the gene in a thermosensitive *erg20-2* mutant of *Saccharomyces cerevisiae* impaired in the FPP synthase activity suppressed the thermosensitive phenotype. The same gene overexpressed in *T. reesei* significantly enhanced FPP synthase activity and also stimulated the activity of *cis*-prenyltransferase, an enzyme of the dolichyl branch of the mevalonate pathway. Unexpectedly, the activity of squalene synthase from the other, sterol branch, was significantly decreased without, however, affecting ergosterol level.

**Keywords:** FPP synthase, *Trichoderma*, *cis*-prenyltransferase, squalene synthase

**Abbreviations:**

DMAPP- dimethylallylpyrophosphate

*erg20*- gene encoding FPP synthase

*erg9*- gene encoding squalene synthase

ERGXX- FPP synthase protein

FPP- farnesyl pyrophosphate

GPP- geranyl pyrophosphate

HMGR - 3-hydroxy-3-methylglutaryl-CoA reductase

IPP- isopentenyl pyrophosphate

*pyr4*-gene encoding orotidine-5'-monophosphate decarboxylase

**Introduction**

The mevalonate pathway (Fig. 1) is the route of synthesis of numerous essential compounds including prenyl groups, coenzyme Q, dolichols and sterols. All these products derive from farnesyl pyrophosphate (FPP) produced by farnesyl pyrophosphate synthase (FPP synthase). This enzyme catalyzes the elongation of dimethylallyl pyrophosphate, itself derived from mevalonate, to geranyl pyrophosphate (GPP) and subsequent GPP elongation to FPP. In yeast cells the end products of FPP synthase are FPP and GPP in a 75:25 ratio (Grabińska and Palamarczyk, 2002). FPP is the last common intermediate of two branches of the isoprenoid biosynthesis pathway leading to, respectively, linear polymers or pentacyclic steroids. Changes in FPP synthase activity alter the flow of FPP towards the two branches of the mevalonate pathway, which implicates that the enzyme could play a crucial regulatory role in this pathway and be a very promising target for a general regulation of the pathway activity (Goldstein and Brown, 1990; Grabowska et al., 1998). FPP synthase is inhibited by bisphosphonates, chemically stable analogues of inorganic pyrophosphate, developed for the treatment of disorders of excessive bone resorption, metastases and osteoporosis (Russell, 2011). The therapeutic effect of bisphosphonates is due to the fact that FPP synthase generates isoprenoids for posttranslational modifications of small GTP-binding proteins essential for osteoclast functioning.

On the other hand, FPP is an important feedback regulator of the mevalonate pathway through its action on 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase (HMGR), an enzyme located at the beginning of the pathway, believed to be its main regulatory point (Hampton et al., 1996; Gardner and Hampton, 1999). It has been shown that FPP is the source of a positive signal for degradation of HMGR isoenzyme Hmg2p in yeast (Gardner and Hampton, 1999). In mammalian cells HMGR degradation required high levels of FPP and sterols (Sever et al., 2003).

In addition to regulating mevalonate synthesis by modulating HMGR level/activity, cells also regulate the FPP distribution between the two branches of the pathway (Goldstein and Brown, 1990). The enzymes of the non-sterol branch have been proposed to have higher affinities for the mevalonate-derived substrates than those of the sterol branch. Those authors hypothesized that *cis*- and *trans*- prenyltransferases, having high affinities for FPP, should be fully saturated even at low concentrations of this substrate and therefore would not be influenced by fluctuations in FPP concentration (Faust et al., 1980; Goldstein and Brown, 1990). On the other hand, Grabowska et al. (1998) showed that polyprenol synthesis was sensitive to FPP level when squalene (sterol precursor) synthesis was inhibited. In a yeast *erg9* (squalene synthase) mutant the substrate was directed exclusively to the synthesis of polyprenols, resulting in their 6-fold higher synthesis (Grabowska et al., 1998). Those results indicate an essential but controversial role of FPP in the regulation of the mevalonate pathway.

Most of the presented information about the mevalonate pathway and its regulation comes from studies on yeast and mammalian cells. Little is known, however, on how the pathway functions in other organisms of practical relevance.

In one such organism, *Trichoderma* sp., the mevalonate pathway was mostly studied as the route of synthesis of terpenoids such as trichodermin, harzianum A, mycotoxin T2, ergokonin A and B, and ergosterol, metabolites playing a major role in the biocontrol activity of some *Trichoderma* spp. (Cardoza et al., 2007, 2006; Nielsen and Thrane, 2001; Sivasithamparam and Ghisalberti, 1998; Tijerino et al., 2011). Due to the contribution of many products of the mevalonate pathway in the biocontrol activity of *Trichoderma*, a thorough functional analysis of genes coding for enzymes of this pathway is of substantial interest as it offers means of enhancing the usefulness of this fungus.

FPP synthase is encoded by *erg20* (*ERG20*) gene which has been cloned and characterized from some fungi such as *Neurospora crassa*, *Gibberella fujikuroi* (Homann et al., 1996), and *Mucor circinelloides* (Velayos et al., 2004), the yeasts *Saccharomyces cerevisiae* (Anderson et al., 1989) and *Candida glabrata* (Nakayama et al., 2011), and plants, e.g., *Arabidopsis thaliana* (Delourme et al., 1994) and *Artemisia annua* (Matsushita et al., 1996). In *S. cerevisiae*, *ERG20* is a single-copy essential gene whose product constitutes up to 0.1% of the cytosolic protein (Anderson et al., 1989). In the rat liver there are five copies of the FPP synthase gene, which suggest a need for its extremely high expression (Clark et al., 1987).

In this study we present cloning and characterization of a putative FPP synthase gene from *Trichoderma*. We confirmed the function of the cloned gene by suppression of a thermosensitive phenotype of an *S. cerevisiae* mutant impaired in FPP synthase activity. We also show that overexpression of this gene in *Trichoderma* elevated the FPP synthase activity in the transformants and stimulated the dolichyl branch of the mevalonate pathway.

**Materials and Methods**

***Strains and growth conditions***

*T. reesei* TU-6, a *∆pyr4* mutant of *T.reesei* QM9414 (ATCC 26921) (Gruber et al., 1990) was used as a recipient strain for transformation. *Escherichia coli* strain JM 109 was used for plasmid propagation (Yanish-Perron et al., 1985). *T.reesei* was cultivated at 30°C on a rotary shaker (250 r.p.m.) in 2-liter shake flasks containing 1 l of minimal medium (MM): 1 g MgSO4 x 7H2O, 6 g (NH4)2SO4, 10 g KH2PO4, 3 g sodium citrate x 2H2O, 1.2 g uridine and trace elements (25 mg FeSO4 x 7H2O, 2.7 mg MnCl2 x 4H2O, 6.2 mg ZnSO4 x 7H2O, 14 mg CaCl2 x 2H2O) per liter and 1% (w/v) lactose as a carbon source. The flasks were inoculated with 42 x 106 conidia/l medium.

*S. cerevisiae* strain CC25 (*Mat* ***a****, erg12-2, erg20-2, ura3-1, trp1-1*), kindly provided by Prof. dr. F. Karst (Institut National de la Recherche Agronomique, Colmar, France) (Chambon et al. 1990) was used for expression of the *erg20* gene (NCBI Acc. No.: JX845568) from *Trichoderma*.

The yeast was grown in SC medium (glucose 2% (w/v), yeast nitrogen base w/o amino acids 0.67% (w/v), mix of amino acids and adenine 0.2% (w/v) without uracil) (Sherman, 1991) or in YPD medium (1% (w/v) yeast extract, 2% (w/v) bactopeptone, and 2% (w/v) glucose). The media were, solidified with 2% agar. When required, ergosterol was added to the cultivation medium (80 µg/ml on agar plates). Ergosterol was supplied by dilution of a stock solution (4 mg/ml) in a mixture of Tergitol NP-40/ethanol (1:1 v/v) (Servouse and Karst, 1986).

***Expression plasmids***

For expression of *T.reesei erg20 gene* in *S.cerevisiae*,a cDNA fragment of 1044 bp containing the *erg20* open reading frame, was amplified by PCR using *T. reesei* cDNA (a gift from prof. dr. M. Saloheimo from VTT, Finland) as a template and Erg20-U and Erg20-L primers (Table 1), subcloned into the pGEM-T Easy Vector (Promega), cut out with NotI restriction enzyme and cloned into the NotI site of pNEV vector carrying *URA3* marker between the *PMA1* (plasma membrane H + ATPase) promoter and terminator (a gift from prof. dr. F. Karst from Laboratoire de Genetique de la Levure, Universite de Poitiers, France).

For yeast transformation, the one-step transformation method of Chen et al. (1992) was used. The yeast *erg20-2* mutant was transformed with *Trichoderma erg20* gene and selected on -Ura minimal medium.

***Integration of additional copies of the* erg20 *gene into the* T.reesei *genome***

The *Trichoderma* *erg20* gene wasintroduced into *T. reesei* TU-6 under the *Aspergillus nidulans* *gpdA* gene promoter and *trpC* terminator using the pAN521N plasmid (NCBI Acc. No.: Z32697). The complete genomic DNA sequence of the *T.reesei erg20* gene (1217 bp DNA) was amplified by PCR with primers UergBam and LergBam (Table 1). The pAN521N plasmid was cleaved between the promoter and the terminator using BamHI, and the PCR product was ligated with the plasmid. The proper orientation and sequence of the *erg20* open reading frame was confirmed by sequencing.

The resultant plasmid together with the pFG1 vector containing the *T. reesei pyr4* gene (Gruber et al., 1990) were used for co-transformation of *T.reesei* TU-6 by the protoplast transformation method as described previously (Kruszewska et al., 1999). The obtained transformants were then cultivated in liquid MM medium for preparation of DNA and RNA.

***Molecular biology methods***

Chromosomal DNA was isolated from *T. reesei* using the Promega Wizard Genomic DNA Purification kit. Total RNA was isolated using the single-step method described by Chomczynski and Sacchi (1987). Other molecular biology procedures were performed according to standard protocols (Sambrook et al., 1989).

Integration of additional copies of *erg20* gene was shown by Southern blotting analysis of DNA from transformants and the control strain cut with PstII restriction enzyme (cutting once inside the *erg20* gene) and hybridized with the 1.1 -kb DNA containing the coding sequenceof *T.reesei erg20* gene.

The radioactive probe was prepared using [32P] dATP and the Fermentas DecaLabel DNA Labeling system according to the standard Fermentas protocol.

###### Biochemical methods

***FPP synthase activity***

The FPP synthase activity was assayed in a cell free extract obtained from transformants and the control *T. reesei* TU-6 strain. After 168 h of cultivation mycelia of *Trichoderma* were harvested by filtration, washed with water and suspended in 50 mM phosphate buffer, pH 7.5 containing 1 mM MgCl2 and 5 mM iodoacetamide. Cells were homogenized in a Beadbeater with glass beads (0.5 mm) and the homogenate was centrifuged at 5,000 x g for 10 min to remove cell debris and unbroken cells. The resulting supernatant was centrifuged again at 100 000 x g for 1 h to remove membrane pellet and the obtained supernatant was used as the source of FPP synthase. The whole procedure was performed at 4oC.

The FPP synthase activity was also analyzed in *S. cerevisiae* CC25 strain transformed with *erg20* cDNA from *Trichoderma*. For this analysis yeast strains CC25 and CC25/*erg20Tr* were cultivated overnight at 28oC in SC medium and then diluted to OD600= 0.8 with SC medium warmed to 36oC and cultivated at this temperature for the next 2 h. Subsequently, yeast cultures were centrifuged at 5,000 x g for 10 min. A membrane-free supernatant was obtained from yeast using the procedure described above for *Trichoderma*.

The FPP synthase activity was measured in a reaction mixture of 100 µl containing 50 mM phosphate buffer, pH 7.5, 1 mM MgCl2, 5 mM iodoacetamide, 60 µM isopentenyl pyrophosphate (IPP), 1x105cpm [14C]IPP (specific activity 52 Ci/mol), 120 µM dimethylallylpyrophosphate (DMAPP), and 150 µg protein (Karst et al., 2004). After 5 min of incubation at 37oC samples were ice-chilled and 0.5 ml water was added, followed by 1 ml hexane and 0.2 ml of 1 M HCl to dephosphorylate the products. The samples were shaken for 30 min at 37oC. The mixture was ice-chilled and vigorously mixed. The upper phase was washed three times with water and subjected to TLC on Silica Gel 60 plates (Merck) in benzene-ethyl acetate 7:1,v/v. Radioactive spots were localized by autoradiography, identified by co-chromatography with unlabeled standards, then scraped off and the radioactivity was measured in a scintillation counter (Karst et al., 2004).

### *Membrane preparation*

Mycelium was harvested by filtration, washed with water and suspended in 50 mM Tris-HCl, pH 7.4, containing 15 mM MgCl2 and 9 mM -mercaptoethanol. Cells were homogenized and clarified homogenate obtained as above homogenate was centrifuged at 100,000 x g for 1 h. The membrane pellet was homogenized in 50 mM Tris-HCl, pH 7.4, containing 3.5 mM MgCl2 and 6 mM β-mercaptoethanol, and used as a source of enzyme. The whole procedure was performed at 4oC (Perlińska-Lenart et al., 2006).

***cis-prenyltransferase activity***

The enzyme activity was assayed in the membrane fraction by incubation (final volume 250 μl) of 500 μg of membrane proteins with 4 μg of FPP, 50 mM sodium phosphate buffer pH 7.4, 0.5 mM MgCl2, 20 mM β-mercaptoethanol, 10 mM KF and 3 x 105 cpm [14C]IPP. After 90 min of incubation at 30oC, the reaction was terminated by addition of 4 ml of chloroform - methanol (3: 2, v/v). The protein pellet was removed by centrifugation and the supernatant was washed three times with 1/5 volume of 10 mM EDTA in 0.9% NaCl. The organic phase was concentrated under a stream of nitrogen and subjected to HPTLC on RP-18 plates developed in 50 mM H3PO4 in acetone. The zone containing the radiolabeled polyprenols was scraped off and the radioactivity was measured in a scintillation counter (Perlińska-Lenart et al., 2006; Szkopińska et al., 1997).

***Squalene synthase activity***

Squalene synthase activity was analyzed in the membrane fractions obtained from *Trichoderma* transformants and the control TU-6 strain by incubation of 200 µg of membrane fraction with 100 mM potassium phosphate buffer pH 7.4, 5 mM MgCl2, 5 mM CHAPS, 10 mM DTT, 2 mM NADPH and 10 µM [3H]FPP in total volume of 100 µl. Incubation was performed in 37oC for 20 min, then the reaction was stopped with 10 µl of 1 M EDTA pH 9.2 and 10 µl of unlabeled 0.5% squalene was added as a carrier. The reaction mixture was applied onto Silica Gel 60 (Merck) thin layer chromatography plate and developed in 5% (v/v) toluene in hexane. Radioactive zone containing squalene (Rf = 0.5) was scraped off and measured in a scintillation counter (Shechter et al., 1992).

***Determination of crude ergosterol in non-saponifiable lipids***

Analysis of the ergosterol content in the mycelia was done according to Szkopińska et al. (2000) and Cardoza et al. (2006). Using this technique ergosterol and ergosta-5-7-enol are measured but in yeast they represent about 70% of the total sterol amount (Szkopińska et al. 2000).

After 168 h of cultivation mycelia of *Trichoderma* were harvested by filtration, dried between filter papers and the wet weight of the mycelia was determined. One gram of the mycelia was suspended in 2 ml of 60% (w/v) KOH and 5 ml of methanol and hydrolyzed at 70oC for 2 h. Then, 10 ml of hexane was added and the mixture was shaken vigorously. After centrifugation the upper hexane phase was saved and the lower extracted again with hexane. Combined hexane phases were evaporated and extracted lipids were dissolved in 1 ml of ethanol. The amount of ergosterol was determined from the absorbance at 281.5 nm (A1%/281.5= 290 for ergosterol).

***Lipid extraction and analysis***

Lipids extraction and analysis were done according to Leszczynska et al. (2009) and Ayme et al. (2014) .

Mycelia (3g) were homogenized and suspended in chloroform: methanol (2:1) to a volume equal to 20 times the volume of mycelia. The mixture was agitated for 8 h at room temperature. The homogenate was then centrifuged to recover the lipid phase. The organic solvent containing lipids was washed three times with 1/5 v of 10 mM EDTA in 0.9% NaCl and evaporated to dryness in a nitrogen stream and suspended in benzene. The lipids were hydrolyzed at 95oC for 2 h in a mixture of ethanol : water (17:3) containing 15% KOH (v/w). Lipophilic products were extracted with diethyl ether, washed with water and evaporated. Isolated lipids were analyzed on Silica gel 60 F254 plates (Merck) developed with toluene/ethyl acetate (95/45 v/v) and visualized with iodine vapor. Squalene and ergosterol were identified based on the migration of standards (Sigma-Aldrich). Plates were scanned using Epson Perfection V700 Photo scanner.

###### Protein concentration assay

Protein concentration was estimated according to Lowry et al. (1951).

###### Quantification of fungal dry weight

Fungal dry weight was quantified by filtering culture samples through G1 sintered glass funnel, washing the biomass with a threefold volume of tap water, and drying to constant weight at 110oC.

**Results**

***Cloning of* T. reesei *ORF with homology to the* S. cerevisiae ERG20 *gene encoding FPP synthase***

The nucleotide sequence of the putative *erg20* gene was identified in the genomic data base of *Trichoderma* (<http://genome.jgi-psf.org/Trire2/Trire2.home.html>) using BLAST (Altschul, et al. 1997) and the sequences of Erg20 protein from *Neurospora crassa* as query (NCBI Acc.No.: XM\_956448). A cDNA fragment encoding the putative *Trichoderma* ERGXX protein was amplified as described in the Methods and sequenced. Comparative analysis of the cDNA and the genomic sequence of the cloned gene revealed one intron (173 bp) in position 121-294 in the 1217 bp sequence of *erg20*. The intron is more than twice longer than the introns found in the *erg20* genes from *N. crassa* (87 bp) and *Gibberella fujikuroi* (71 bp) (Homann et al., 1996). The *Neurospora* and *Glibberella* genes comprise 1131 and 1115 nucleotides, respectively, and both code for 347 amino acids, as does the *Trichoderma* gene. In all three genes the intron is located at the same position, between codons 40 and 41.

Sequence analysis of the 1044-bp open reading frame (ORF) of *erg20* revealed that it encoded a protein of 347 amino acids, a predicted *T. reesei* ERGXX. This protein shows 80% identity with and 93% similarity to the *Neurospora crassa* FPP synthase (Fig. 2) and 56% identity and 75% similarity to the *S. cerevisiae* Erg20p (Altschul et al., 1997).

A phylogenetic tree built for Erg20 proteins (Fig. 3) supported the results from the above protein sequence comparison (Fig. 2) showing the closest relationship of the *Trichoderma* ERGXX with the Erg20 proteins from other filamentous fungi and a more distant relationship with *S. cerevisiae* Erg20p.

A BLAST search (Altschul et al., 1997) identified some conserved domains in the ERGXX sequence recognized as a substrate binding pocket, substrate-Mg2+ binding site or chain length determination region characteristic for isoprenoid biosynthesis enzymes. In the middle and in the C-terminal domain of the ERGXX protein two DDXXD (Fig.2) motifs were found, possibly involved in substrate binding through the formation of magnesium salt bridges between the diphosphate residue of the substrate and the carboxyl groups of the aspartates (Homann et al., 1996). We also found a highly conserved GGKXXR motif in the N-terminal domain (Fig. 2) present in all the FPP synthases analyzed and predicted to be engaged in substrate binding (Homann et al., 1996).

The FPP synthase from *S. cerevisiae* is a soluble, cytosolic enzyme (Eberhardt and Rilling, 1975), which was also predicted with PSORT II (http://psort.nibb.ac.jp/) program for ERGXX from *Trichoderma*.

***Expression of the* Trichoderma erg20 *homolog in* S. cerevisiae erg20-2 *mutant***

To show that the cloned ORF from *Trichoderma* encodes an active FPP synthase we expressed it in the CC25 strain of *S. cerevisiae* which carries an *erg20-2* mutation leading to a lack of FPP synthase activity at 36oC. The CC25 strainof *S. cerevisiae* had previously been used to study the functions of FPP synthases from *Arabidopsis thaliana*, *Euphorbia pekinensis* and basidiomycetous fungus *Ganoderma lucidum* (Cao et al., 2012; Cunillera et al., 1996; Ding et al., 2008). In yeast cells the end products of FPP synthase are FPP and GPP in a 75:25 ratio (Grabińska and Palamarczyk, 2002). The leaky mutation *erg20-2* results in limited condensation of GPP with IPP to yield FPP (Chambon et al. 1990). As a consequence, this strain synthesized GPP as the major product. Subsequently, the excess of GPP is dephosphorylated and excreted as geraniol into the culture medium. To protect the cell against toxicity of geraniol, the CC25 strain carries additional *erg12-2* mutation results in decreased activity of mevalonate kinase limiting accumulation of GPP. Lower affinity of the mutated FPP synthase to GPP causes shortage of FPP and limited production of final products of the mevalonate pathway. As a consequence this strain is auxotrophic for ergosterol at the nonpermissive temperature (Chambon et al. 1990; Cunillera et al., 1996). To demonstrate that the putative *erg20* gene from *Trichoderma* encodes FPP synthase the CC25 strain transformed with the gene was cultivated together with the control untransformed CC25 strain at 36oC on YPD plates with or without ergosterol (Fig. 4). The *erg20* gene from *Trichoderma* clearly complemented the ergosterol auxotrophy of the CC25 strain at 36oC (Fig.4).

We also measured the activity of FPP synthase in the control CC25 strain and the CC25/*erg20Tr* transformant incubated for 2 h at 36oC. The FPP synthase activity was 2.6-fold higher in the transformant (4178±750 cpm/mg protein/5 min) compared to the control untransformed strain (1584±190 cpm/mg protein/5 min).

***Overexpression of the* erg20 *gene in* T.reesei**

We further documented the function of the cloned gene by overexpressing it in *Trichoderma*. The gene in question was amplified on the genomic DNA of *Trichoderma*, cloned into an expression vector as described in Methods and used to transform *T. reesei* strain TU-6. Transformants growing on a selective medium without uridine were collected and isolated by three consecutive transfers from selective to nonselective medium. Subsequently, the transformants were examined by Southern analysis for integration of additional *erg20* gene copies into the genome (Fig. 5). Six transformants SP6/11, SP10/11, SP24/11, SP30/11, SP45/11and SP66/11 exhibiting additional or different bands compared to the non-transformed TU-6 strain were subjected to further analysis by Northern blotting for *erg20* expression. All the examined strains revealed higher levels of the *erg20* mRNA than the control strain (Fig. 6A). The highest expression was found in the SP30/11transformant, three-fold that in the control TU-6 strain. Strains SP24/11, Sp30/11, Sp45/11 and SP66/11, which exhibited the highest expression of the *erg20* gene, were selected for further investigation.

***Enzymatic activity***

To show unequivocally that the putative *Trichoderma* ERGXX protein has an FPP synthase activity we measured such activity in the *Trichoderma* *erg20* transformants. All the strains were cultivated for 168 h and soluble fractions were obtained from their mycelia as described in Methods. Analysis of the FPP synthase activity confirmed that the overexpression of the putative *erg20* gene resulted in an increase of the FPP synthase activity in all the transformed strains. The increase was by 86, 62, 48 and 29% in the SP66/11, SP45/11, SP30/11 and SP24/11 strains, respectively, compared to the control strain (Fig. 6B). The FPP synthase activity was not directly proportional to the level of the *erg20* mRNA, which was the highest in the SP30/11and SP24/11 strains (Fig. 6A). This discrepancy suggests a post-transcriptional regulation of the FPP synthase amount/activity in addition to the regulation at the mRNA level.

This reservation aside, the increased FPP synthase activity in all the strains overexpressing the gene under study provides a strong indication that it encodes the synthase.

Since FPP synthase produces the substrate for the both branches of the mevalonate pathway, its increased activity in the transformants could bring about by a feed-forward mechanism an elevated activity of enzymes utilizing FPP. To check this possibility the activities of *cis*-prenyltransferase and squalene synthase, enzymes of the dolichol and ergosterol branches of the mevalonate pathway, respectively, were measured. The *cis*-prenyltransferase activity was elevated in the membrane fraction from all the transformed strains compared to the wild type strain (Fig. 6C). The most pronounced increase by 61% of the control value, was obtained for the SP24/11 transformant. Contrary to expectations, the activity of squalene synthase, the first enzyme of the sterol branch of the mevalonate pathway was strongly decreased in the transformants, in the SP45/11 strain being as low as 39% of the control activity (Fig. 6D).

Despite the decreased activity od squalene synthase, the content of squalene in the mycelia of transformants and the control strain remained comparable (Fig. 7). Furthermore, the content of ergosterol in the transformants’ mycelia was exactly the same for all the transformants and virtually unaltered comparing to that in the non-transformed strain (93±2 vs. 92±1 mg/g wet weight, respectively) (Fig.7). TLC analysis showed also that patterns of lipids isolated from the mycelia were near the same for transformants and the control strain.

**Discussion**

The aim of this study was to identified and characterize the *T. reesei* gene encoding FPP synthase, a crucial enzyme of the mevalonate pathway. Since we found a sequence in the *Trichoderma* genomic data base coding for a protein up to 80% identical with the Erg20 proteins from other organisms, we assumed that that gene encodes a bona fide FPP synthase, which was proven correct.

Expression of the candidate *erg20* cDNA from *Trichoderma* in the *S. cerevisiae* thermosensitive strain CC25 carrying the *erg20-2* mutation suppressed the defect and thereby confirmed that the isolated ORF from *Trichoderma* codes for FPP synthase.

An additional confirmation that the putative *erg20* gene encodes FPP synthase we provided by its overexpression in *T. reesei.* As expected, transformants had a higher activity of FPP synthase compared to the parental strain.

Since the product of FPP synthase is a common substrate of both branches of the mevalonate pathway (see Fig 1), its increased supply due to an augmented synthase activity seemed likely to affect the both branches.

We therefore determined the activities of two enzymes representing the two branches of the mevalonate pathway in the transformants expressing the *erg20* gene. Rather unexpectedly, we found a markedly different effect of the overexpression on the activities of *cis*-prenyltransferase from the dolichyl branch and squalene synthase from the sterol branch. The former was substantially increased (by up to 60%) compared to the control level, while the latter- decreased (by as much as ca. 60%). That decrease, however, had no effect absolutely on the efficiency of the sterol branch, since the level of its final product ergosterol was unaltered relative to that in the non-transformed control.

It has been reported that in yeast the flux of FPP is directed primarily to the synthesis of ergosterol to keep its level constant (Karst et al., 2004). Accordingly, a drop of the FPP synthase activity to 42% of the wild type level left the level of ergosterol unaffected (Karst et al., 2004). The ergosterol level is similarly tightly protected against an unphysiological increase, since a six-fold increase of the FPP synthase activity in yeast resulted in an 80% increase of the amount of dolichols but only a 32% increase of ergosterol (Szkopińska et al., 2000). The lack of changes in the level of ergosterol observed here in the *erg20*-overexpressing *Trichoderma* strains could be due to this tight regulation of sterol synthesis, possibly mediated by a decrease of the squalene synthase activity. An additional proof of the preferential utilization of FPP for ergosterol synthesis comes from the drop-test shown in our Fig.4. The CC25 strain grows at 36oC almost equally poorly on ergosterol-free and ergosterol-supplemented medium, which convincingly demonstrates that it’s the growth defect is due to a sub-optimal level of mevalonate pathway product(s) other than ergosterol. This result confirmed the previous observation that, ergosterol taken up from medium by auxotrophic mutant strains in aerobiosis cannot enter the site to exert regulatory function and remains associated with membranous structures (Servouse and Karst, 1986).

Conversely, the enhanced activity of FPP synthase in the *erg20* transformants stimulated the activity of *cis*-prenyltransferase in the dolichyl branch of the mevalonate pathway. This stimulation could result from the elevated level of FPP not directed to the sterol branch. In addition, it has been postulated that FPP synthase could be a component of an enzyme complex involved in polyprenol formation (Szkopińska et al., 1997). Overexpression of the *erg20* gene should result in an elevated level of FPP synthase protein which could then form additional complexes with enzymes from the dolichyl branch of the mevalonate pathway, thereby enhancing their activity.

**Summary**

Analysis of the putative *erg20* gene from *Trichoderma* clearly showed that the gene codes for FPP synthase. The *erg20* gene from *Trichoderma* expressed in thermosensitive *S. cerevisiae* strain CC25 defective in FPP synthase activity restored its growth at the non-permissive temperature. Furthermore, overexpression of *erg20* in *T. reesei* additionally confirmed the function of the cloned gene.

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**Figure legends**

Figure 1

Mevalonate pathway

IPP - isopentenyl pyrophosphate; DMAPP - dimethylallyl pyrophosphate; FPP - farnesyl pyrophosphate

Figure 2

Deduced amino acid sequence of *Trichoderma* ERGXX protein and its alignment with sequences of Erg20 proteins of *Aspergillus niger, A. fumigatus, Neurospora crassa, Magnaporthe grisea, Gibberella zeae, Botryotinia fuckeliana and Saccharomyces cerevisiae*. Two DDXXD motifs and GGKXXR motif probably involved in substrate binding are marked.

Figure 3

Phylogenetic tree of putative Erg20 proteins. Phylogenetic analysis was performed using the Geneious 6.1 program and the UPGMA tree building method (Sneath and Sokal, 1973). Genetic distances calculated based on Jukes-Cantor method (Jukes and Cantor, 1969) are marked on the scale below.

Figure 4

Functional complementation of *erg20-2* defect in *S. cerevisiae* strain CC25.

Control strain CC25 [CC25] and transformed CC25 strain carrying e*rg20* gene from *Trichoderma* [CC25/*erg20Tr*] were cultivated on YPG plates supplemented or not with ergosterol (80 µg/ml) and incubated at 28oC or 36oC for 3 days.

Figure 5

Presence of additional copies of *erg20* gene in *T. reesei* TU-6 genome.

A - Localization of PstI restriction sites in the *erg 20* gene locus in *T. reesei*

B - Southern analysis of PstI-digested DNA of the control strain TU-6 and the *erg20* transformants hybridized with coding sequence of *T. reesei erg20* gene. Lines with DNA from transformants SP6/11, SP10/11, SP24/11, SP30/11, SP45/11 and SP66/11 (6, 10, 24, 30, 45, 66) used for further analysis are marked with arrows.

Figure 6

A - Transcription of the *erg20* gene in the transformants and the control strain.

Level of *erg20* mRNA in transformants SP6/11, SP10/11, SP24/11, SP30/11, SP45/11 and SP66/11 (6, 10, 24, 30, 45, 66) and the untransformed host strain TU-6 (TU) were analyzed after 168 h of cultivation in lactose-based medium. Columns show signal intensity as percentage of the control expression after normalization for the actin probe. Below are shown Northern blots hybridized with *erg20* and *act1* probes.

B - Activity of FPP synthase in cell free extracts obtained from transformants SP24/11, SP30/11, SP45/11 and SP66/11 (24, 30, 45, 66) and the control TU-6 strain (TU) cultivated for 168 h in lactose-based medium. Data are presented as mean ± standard deviation from four independent experiments, each determined in triplicate.

C - Activity of *cis*-prenyltransferase measured in the membrane fraction obtained from the transformants SP24/11, SP30/11, SP45/11 and SP66/11 (24, 30, 45, 66) and the control TU-6 strain (TU) cultivated for 168 h in lactose-based medium. Data are presented as mean ± standard deviation from six independent experiments each determined in triplicate.

D - Activity of squalene synthase in membrane fraction obtained from transformants SP24/11, SP30/11, SP45/11 and SP66/11 (24, 30, 45, 66) and the control TU-6 strain (TU) cultivated for 168 h in lactose-based medium. Data are presented as mean ± standard deviation from four independent experiments each determined in triplicate.

All differences between the control TU-6 strain and transformants are statistically significant (P < 0.05; *t* test)

Figure 7

TLC analysis of lipids

Lipids were extracted from mycelia of transformants SP24/11, SP30/11, SP45/11 and SP66/11 (24, 30, 45, 66) and the control TU-6 strain (TU) cultivated for 168 h in lactose-based medium. They were analyzed on Silica gel 60 F254 plates as described in the Materials and Methods. Bands represented squalene and ergosterol in the extracts were identified compared to the migration of standards. Sterol esters, triacylglycerols and free fatty acids were identified based on literature (Ayme et al. 2014).

Abbreviations: vertical arrow indicates the direction of migration; O - origin of migration; SQ - squalene; SEs – sterol esters; TAGs – triacylglycerols; FFAs – free fatty acids; E – ergosterol

The experiment was repeated three times.