Increased activity of the sterol branch of the mevalonate pathway elevates glycosylation of secretory proteins and improves antifungal properties of *Trichoderma atroviride*

Sebastian Graczyka, Urszula Perlińska-Lenarta, Wioletta Górka-Niec, Renata Lichota, Sebastian Piłsyk, Patrycja Zembeka, Jacek Lenartb, Przemysław Bernat, Elżbieta Gryza, Justyna Augustyniak, Grażyna Palamarczyka, Joanna S. Kruszewska

*Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Pawiińskiego 5a, 02-106 Warsaw, Poland*

*Mossakowski Medical Research Centre, Polish Academy of Sciences, Pawiińskiego 5, 02-106 Warsaw, Poland*

*Faculty of Biology and Environmental Protection, University of Łódź, Banacha Street 12/16, 90-237, Łódź, Poland*

**ARTICLE INFO**

**Keywords:**
- Trichoderma
- Antifungal activity
- Yeast ERG20 gene
- FPP synthase
- Squalene synthase

**ABSTRACT**

Some *Trichoderma* spp. have an ability to inhibit proliferation of fungal plant pathogens in the soil. Numerous compounds with a proven antifungal activity are synthesized via the terpene pathway.

Here, we stimulated the activity of the mevalonate pathway in *T. atroviride* P1 by expressing the *Saccharomyces cerevisiae* ERG20 gene coding for farnesyl pyrophosphate (FPP) synthase, a key enzyme of this pathway. ERG20-expressing *Trichoderma* strains showed higher activities of FPP synthase and squalene synthase, the principal recipient of FPP in the mevalonate pathway.

We also observed activation of dolichyl phosphate mannose (DPM) synthase, an enzyme in protein glycosylation, and significantly increased O- and N-glycosylation of secreted proteins. The hyper-glycosylation of secretory hydrolases could explain their increased activity observed in the ERG20 transformants.

Analysis of the antifungal properties of the new strains revealed that the hydrolases secreted by the transformants inhibited growth of a plant pathogen, *Pythium ultimum* more efficiently compared to the control strain. Consequently, the biocontrol activity of the transgenic strains, determined as their ability to protect bean seeds and seedlings against harmful action of *P. ultimum*, was also improved substantially.

1. **Introduction**

*Trichoderma* spp. are used as biocontrol agents against fungal plant pathogens (Hjeljord and Tronsmo, 1998). Their antimicrobial activity is connected with the secretion of cell-wall-degrading enzymes but also with the production of antifungal substances including antibiotics, volatile metabolites, terpenes, peptaboils and piperazine derivatives (Sivasithamparam and Ghisalberti, 1998; Keszler et al., 2000; Benitez et al., 2004; Cardoza et al., 2007; Reino et al., 2008; Druzhinina et al., 2011; Crutcher et al., 2013). The mevalonate pathway (Fig. 1) product farnesyl diphosphate (FPP) is a substrate for the synthesis of the terpene compounds involved directly in the biocontrol (ergokonins and viridins), ergosterol essential for proper functioning of the cell membranes, and dolichol mainly acting as a carrier of carbohydrate residues for protein glycosylation. In this study we engineered the mevalonate pathway in *Trichoderma atroviride* towards an elevated activity of farnesyl diphosphate synthase.

Most of the information concerning the activity and regulation of the mevalonate pathway enzymes comes from studies on the yeast *Saccharomyces cerevisiae*. The pathway is mainly regulated at the level of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR), the key regulated enzyme. HMGR is regulated at the transcriptional, translational and protein stability levels (Goldstein and Brown, 1990; Hampton et al., 1996; Donald and Hampton, 1997). The amount of the HMGR protein is directly dependent on the level of farnesyl diphosphate (FPP), a major product of the mevalonate pathway and a source of a positive signal for degradation of HMGR (Gardner and Hampton, 1999). Although HMGR is commonly accepted to play the key role in
the mevalonate pathway, some authors tend to see FPP synthase as a pivotal enzyme (Dhar et al., 2012). FPP synthase catalyzes the formation of geranyl pyrophosphate (GPP) from two C5 monoterpenoid units, dimethylallyl pyrophosphate (DMAPP) and isopentenyl pyrophosphate (IPP), and its subsequent elongation to C15 FPP. In the yeast cell the end products of FPP synthase are FPP and GPP in a 75:25 ratio (IPP), and its subsequent elongation to C15 FPP. In the yeast cell the end products of FPP synthase are FPP and GPP in a 75:25 ratio (Grabińska and Palamarczyk, 2002).

Since FPP serves as a substrate for the production of almost all isoprenoids larger than monoterpenes its unlimited availability is crucial for the overall productivity of the mevalonate pathway. Moreover, FPP is the last common substrate of two branches of the mevalonate pathway leading to linear polysoprenoids (dolichyl branch) or pentacyclic sterols (ergosterol branch). Consequently, the distribution of FPP between these two branches needs to be regulated by the cell cyclic sterols (ergosterol branch). Consequently, the distribution of FPP is the last common substrate of two branches of the mevalonate pathway. Moreover, FPP is the last common substrate of two branches of the mevalonate pathway leading to linear polysoprenoids (dolichyl branch) or pentacyclic sterols (ergosterol branch). Consequently, the distribution of FPP between these two branches needs to be regulated by the cell (Szkopińska et al., 1997; Grabińska and Palamarczyk, 2002; Ro et al., 2006; Paradise et al., 2008). In yeast the flux of FPP is directed primarily to the synthesis of ergosterol to keep its level constant (Karst et al., 2004). Also in Trichoderma limiting the activity of early steps of the mevalonate pathway induces overexpression of the erg1 and erg7 genes encoding squalene epoxidase and lanosterol:oxidosqualene cyclase, respectively (Cardoza et al., 2006; Cardoza et al., 2007), thereby channeling the mevalonate produced to the synthesis of triterpenoids which are essential components of the cell membrane and hence are required for cell survival. Thus, changes in the activity of the early steps of the mevalonate pathway affect differently the productivity of the dolichol and ergosterol branches of the pathway.

In this study, we stimulated the activity of the mevalonate pathway in T. atroviride to improve the supply of the substrate for the synthesis of all branches of the pathway. The S. cerevisiae ERG20 gene coding for FPP synthase was expressed in the biocontrol strain of T. atroviride P1. Integration of the ERG20 gene into the Trichoderma genome had a significant effect on FPP synthase activity. The ERG20- transformants also had a substantially enhanced DPM synthase activity and significantly higher O- and N-glycosylation of secretory proteins. The ERG20 expression also improved the antifungal and biocontrol activities of T. atroviride.

2. Materials and methods

2.1. Strains and cultivation media

*T. atroviride* strain P1 (“Trichoderma harzianum” ATCC 74058) was used as the recipient strain for transformation. *Escherichia coli* strain JM 109 was used for plasmid propagation (Yanish-Perron et al., 1985). *T. atroviride* was cultivated at 28 °C on a rotary shaker (250 r.p.m.) in 2-l shake flasks containing 1 l of minimal medium (MM): 1 g MgSO₄·7H₂O, 6 g (NH₄)₂SO₄, 10 g KH₂PO₄, 3 g sodium citrate-2H₂O, and trace elements (25 mg FeSO₄·7H₂O, 2.7 mg MnCl₂·4H₂O, 6.2 mg ZnSO₄·7H₂O, 14 mg CaCl₂·2H₂O) per liter and 1% (w/v) lactose or glucose as a carbon source. The flasks were inoculated with 42 × 10⁹ conidia/l medium.

*Pythium ultimum* MUCL 16164, from the MUCL Culture Collection, Leuven, Belgium, was grown on potato-dextrose agar (PDA) (BioShop, Burlington, Canada).

2.2. Analysis of fungal growth

Fungal dry weight was determined by filtering culture samples through G1 sintered glass funnels, washing the biomass with water, and drying to a constant weight at 110 °C.

2.3. Expression of the S. cerevisiae ERG20 gene in *T. atroviride*

*T. atroviride* P1 was cotransformed with the yeast ERG20 gene fused under the A. nidulans gpdA gene promoter (glyceraldehyde-3-phosphate dehydrogenase) and trpC (indole-3-glycerol phosphate synthase) terminator, using pAN52-1NotI plasmid (NCBI Acc. No. Z32697). The complete coding sequence of the S. cerevisiae ERG20 gene was amplified by PCR using primers ErgBam-U and ErgBam-L (Table 1). The pAN521N plasmid was cut between the promoter and the terminator with BamHI, the PCR product was ligated with the plasmid and then the plasmid was cut with Ncol, the sticky ends were blunted with Mung Bean nuclease (Promega, Mannheim, Germany) and selfligated to remove the additional ATG from the Ncol restriction site (Fig. 1S). The pRMLex_30 plasmid with *E. coli* hygromycin B phosphotransferase gene (*hph*) fused between the promoter and terminator elements of the *Trichoderma pkiI* (coding for pyruvate kinase) and cbh2 (encoding cello-biohydrolase II) genes, respectively, was used as a partner in cotransformation (Mach et al., 1994). Transformants were selected for hygromycin B resistance on plates containing 1.2 M sorbitol and 200 μg/ml hygromycin B (Sigma-Aldrich, St. Louis, MO, USA). The transformants were then cultivated in liquid MM medium for preparation of DNA.

2.4. Molecular biology methods

Genomic DNA was isolated from *T. atroviride* using the Wizard Genomic DNA Purification kit (Promega, Mannheim, Germany). 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).

2.4.1. ERG20 copies integrated into *T. atroviride* genome

Quantitative Real-Time PCR (qRT-PCR) analysis was performed to estimate the copy number of the transgenes in the *Trichoderma* genome.
using the relative standard curve method (Joshi et al., 2008; Ma and Chung, 2015; Salas-Marina et al., 2015). Serial 1:10 dilutions of standard samples containing from 300,000 to 300 copies of the ERG20 gene were prepared by mixing the wild-type Trichoderma genomic DNA with pGEM-ERG20 plasmid. Each reaction contained adequate amount of DNA calculated using the online tool (DNA Copy Number and Dilution Calculator, Thermo Fisher Scientific), 0.5 µM of each primer (Table 1), and 7.25 µl 3color 2xHS-qPCR Master Mix Sybr (A&A Biotechnology, Gdańsk, Poland), in a total volume of 15 µl. All reactions were run on a LightCycler® 96 (Roche Diagnostics GmbH, Mannheim, Germany) with an amplification efficiency of 95 °C for 10 s, cooling to 65 °C for 60 s, and then heating at 0.1 °C/s up to 97 °C. The amplification and melting curve data were collected and analyzed using the LightCycler96® software 1.0.

2.5. Biochemical methods

2.5.1. FPP synthase (EC 2.5.1.10) activity

The FPP synthase activity was assayed in a cell free extract obtained from transformants and the control T. atroviride P1 strain. After 120 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 × g for 10 min to remove cell debris and unbroken cells. The resulting supernatant was centrifuged again at 100,000 × 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 4 °C.

The FPP synthase activity was measured in 100 µl of reaction mixture containing 50 mM phosphate buffer, pH 7.5, 1 mM MgCl₂, 5 mM iodoacetamide, 60 µM isopenoteryl diphosphate (IPP), 1x10⁵cpm [14C] IPP (specific activity 52 Ci/mol) (ARC, 101 ARC Drive, St. Louis, MO, USA), 120 µM dimethylallyldiphosphate (DMAPP), and 150 µg protein (Karlo et al., 2004). After 5-min incubation at 37 °C 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 37 °C. 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 in benzene-ethyl acetate 7:1. Radioactive spots were localized by autoradiography, identified by co-chromatography with unlabeled standards, then scraped off and the

<table>
<thead>
<tr>
<th>Primer name</th>
<th>5’ sequence – 3’</th>
<th>Amplification efficiency</th>
<th>Amplicon size bp</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>ErgBam-U</td>
<td>CGGATTCCTACGGTTCAGAA</td>
<td>1075</td>
<td></td>
<td>Screening of T. atroviride ERG20 transformants</td>
</tr>
<tr>
<td>ErgBam-L</td>
<td>CGGATTCCTATTTGGTCCTTCC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Erg20F,a</td>
<td>TAATCGACGACTTGAAGAGCAA</td>
<td>2.04</td>
<td>109</td>
<td>Expression of ERG20 gene from S. cerevisiae</td>
</tr>
<tr>
<td>Erg20R,a</td>
<td>GCCAGACGAGAGTAG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Erg20F,b</td>
<td>TTGACGACGACGACGAATT</td>
<td>2.02</td>
<td>98</td>
<td></td>
</tr>
<tr>
<td>Erg20R,b</td>
<td>GCTGCACATTGACGAGTAG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Erg20F,c</td>
<td>ACCAGACGAAAGCCTTCA</td>
<td>2.03</td>
<td>81</td>
<td></td>
</tr>
<tr>
<td>Erg20R,c</td>
<td>GCCACGACAGCCTTCA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LergTa</td>
<td>TGCGGAGATCTTCTGAGTCC</td>
<td>2.05</td>
<td>203</td>
<td>Expression of ERG20 gene from T. atroviride</td>
</tr>
<tr>
<td>RergTa</td>
<td>TCTTGAGACTCCGGTCCTTCC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hmrTaF1</td>
<td>CAAGGAGTAAGCCGAGAGG</td>
<td>2.03</td>
<td>125</td>
<td>Expression of 3-hydroxy-3-methylglutaryl-coenzyme A reductase encoding gene from T. atroviride</td>
</tr>
<tr>
<td>hmrTaR1</td>
<td>GATGGCAGCTCGAGG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>dmpsTaF1</td>
<td>TTTCCGGACCCCACTAAGGT</td>
<td>1.98</td>
<td>135</td>
<td>Expression of dolichyl diphosphate mannose synthase encoding gene from T. atroviride</td>
</tr>
<tr>
<td>dmpsTaR1</td>
<td>GCTGACAACTTGGCTTCA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ergTaF1</td>
<td>ACCCCCGACTTCTTGGCC</td>
<td>1.98</td>
<td>116</td>
<td>Expression of squalene synthase (erg7) encoding gene from T. atroviride</td>
</tr>
<tr>
<td>ergTaR1</td>
<td>GTTTGGGGAGGAGGAGGGAAG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ergTaF1</td>
<td>ACCCAAGTTGCGAGG</td>
<td>2.19</td>
<td>77</td>
<td>Expression of lanosterol synthase (erg7) encoding gene from T. atroviride</td>
</tr>
<tr>
<td>ergTaR1</td>
<td>CGTAGCGCCACTATGGGGA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ergTaF1</td>
<td>GATCCGTTGACGGAGG</td>
<td>2.12</td>
<td>113</td>
<td>Expression of squalene monoxygenase (erg1) encoding gene from T. atroviride</td>
</tr>
<tr>
<td>ergTaR1</td>
<td>ATCCACCGCTGAGGGGA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ActTa</td>
<td>AAGCCTGGAAGGATGACG</td>
<td>1.93</td>
<td>98</td>
<td>Expression of act1 gene from T. atroviride</td>
</tr>
<tr>
<td>ActTa</td>
<td>CCGGCGGACGGAATG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>gapBdTaF</td>
<td>GGCTCTCCTCAAGCGCTTCT</td>
<td>1.95</td>
<td>108</td>
<td>Expression of glyceraldehyde 3-phosphate dehydrogenase encoding gene from T. atroviride</td>
</tr>
<tr>
<td>gapBdTaR</td>
<td>TAGGAAGTGAGCGTTGGCTAT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>eflTaF1</td>
<td>ACAGAAAACCTTTGGGAGG</td>
<td>1.99</td>
<td>147</td>
<td>Expression of elongation factor 1 alpha encoding gene from T. atroviride</td>
</tr>
<tr>
<td>eflTaR1</td>
<td>GATTTGGGACCTTTGGG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>uceTaF1</td>
<td>AGCGGAGAAGCTTGAGGAA</td>
<td>1.96</td>
<td>130</td>
<td>Expression of ubiquitin conjugating enzyme encoding gene from T. atroviride</td>
</tr>
<tr>
<td>uceTaR1</td>
<td>GTAGCTCTCTGGGGCTCTGAT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c3ulTaF1</td>
<td>TTGGACACTAACTACCCCGG</td>
<td>2.10</td>
<td>83</td>
<td>Expression of E3 ubiquitin ligase encoding gene from T. atroviride</td>
</tr>
<tr>
<td>c3ulTaR1</td>
<td>TGTAACTAGTGGGGCTGCG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>oxdcTaF1</td>
<td>GCCCTTGGACGGAGAAGTA</td>
<td>2.25</td>
<td>100</td>
<td>Expression of oxalate decarboxylase encoding gene from T. atroviride</td>
</tr>
<tr>
<td>oxdcTaR1</td>
<td>GGGTATGAGCGATCCAGTGG</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
radioactivity was measured in a scintillation counter (Karst et al., 2004).

2.5.2. Membrane preparation

After 120 h of cultivation mycelia were harvested by filtration, washed with water and suspended in 50 mM Tris-HCl, pH 7.4, containing 15 mM MgCl₂ and 9 mM β-mercaptoethanol. Cells were homogenized and processed as above. Following centrifugation at 100,000 × g for 1 h the membrane pellet was collected, homogenized in 50 mM Tris-HCl, pH 7.4, containing 3.5 mM MgCl₂ and 6 mM β-mercaptoethanol, and used as the source of enzyme. The whole procedure was performed at 4 °C (Perlińska-Lenart et al., 2006).

2.5.3. Cis-prenyltransferase (EC 2.5.1.20) 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 FPP, 50 mM sodium phosphate buffer pH 7.4, 0.5 mM MgCl₂, 20 mM β-mercaptoethanol, 10 mM KF and 3 × 10⁵ cpm [14C]IPP (specific activity 52 Ci/mol) (ARC, 101 ARC Drive, St. Louis, MO, USA). After 90-min incubation at 30 °C, 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 vol of 10 mM EDTA in 0.9% NaCl. The organic phase was concentrated under a stream of nitrogen and subjected to thin-layer chromatography on HPTLC RP-18 plates (Merck Group, Darmstadt, Germany) 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 (Szkopińska et al., 1997; Perlińska-Lenart et al., 2006).

2.5.4. Squalene synthase (EC 2.5.1.21) activity

Squalene synthase activity was analyzed 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 [1-14C]IPP (sp. act. 15–50 Ci/mmol) (ARC, 101 ARC Drive, St. Louis, MO, USA) in a total volume of 100 µl. Incubation was performed at 37 °C for 20 min, then reaction was stopped with 10 µl of 1 M EDTA pH 9.2 and the radioactivity was measured in a scintillation counter (Szkopińska et al., 1997; Perlińska-Lenart et al., 2006).

2.5.5. Dolichyl phosphate mannose (DPM) synthase (EC 2.4.1.83) activity

DPM synthase activity was measured in the pelletted membrane fraction by incubating it with GDP[14C] Mannose (sp. act. 50–60 Ci/mol) (ARC, 101 ARC Drive, St. Louis, MO, USA) and 5 ng of dolichyl phosphate (Dol-P) (Danilov and Chojnacki, 1981) according to Kruszewska et al. (1989).

2.5.6. Ergosterol and squalene determination

Sterols were extracted according to the method proposed by Bernat et al. (2014) with some modifications. Lyophilized biomass (30 mg) was transferred into Eppendorf tube containing glass beads and 1 ml of methanol:chloroform (2:1, v/v). The homogenization process using a ball mill (FastPrep-24, MP-Biomedicals) was carried out for 2 min. Next, the sample was centrifuged (2 min, 6000 × g). The mixture was transferred to another Eppendorf tube. In order to facilitate the separation of two layers, 0.2 ml of H₂O was added. The lower layer was collected, evaporated and dissolved in 1 ml of methanol:chloroform (4:1, v/v). Sterols were measured using an Agilent 1200 HPLC system (Santa Clara, CA, USA) and a 3200 Q-TRAP mass spectrometer (Sciex, Framingham, MA, USA) equipped with an atmospheric-pressure chemical ionization (APCI) source operating in the positive ionization mode. Lipid extract (10 µl) was injected onto a Kinetex C18 column (50 mm × 2.1 mm, particle size: 5 µm; Phenomenex, Torrance, CA, USA) heated at 40 °C with the flow rate of 0.8 ml min⁻¹. Water (A) and methanol (B) were applied as a mobile phase, both containing 5 mM ammonium formate. The solvent gradient was initiated at 40% B, increased to 100% B during 1 min, and maintained at 100% B for 3 min before returning to the initial solvent composition over 2 min. The following instrumental settings were applied: curtain gas 25.0, ion spray voltage 5500, nebulizer gas 50, auxiliary gas 50, and ion source temperature of 550 °C, entrance potential 10.0. The monitored multiple reaction monitoring (MRM) pairs were m/z 379.3–691.7, 379.3–81.3 for ergosterol and 411.4–231.4, 411.4–163.3 for squalene. The data analysis was performed with the Analyst™ v1.5.2 software (Sciex, Framingham, MA, USA).

2.5.7. Protein concentration assay

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

2.5.8. Identification and quantification of carbohydrates bound to secretory proteins

Secreted proteins were precipitated from post-culture medium with two volumes of 96% ethanol, washed twice with 70% ethanol (Ma et al., 1996). O-linked carbohydrates were removed from the protein pellet using mild alkaline hydrolysis according to Duk et al. (1997) and analyzed in the supernatant and then the N-linked carbohydrates were determined in the pellet. O- and N-linked carbohydrates obtained as above were hydrolyzed with 2 M trifluoroacetic acid (TFA) at 100 °C for 16 h. Monosaccharides were determined by high performance anion-exchange chromatography using Dionex ICS-3000 Ion Chromatography System (Thermo Fisher Scientific, Waltham, MA, USA) with a Carbo Pac PA10 analytical column. Neutral sugars were eluted with 18 mM NaOH at 0.25 ml/min (Zdebska and Kościcielak, 1999).

2.5.9. Cellulase activity

The activity of cellulases was measured in the cultivation medium by incubation of 0.5 ml of carboxymethylcellulose (10 g/l) in 50 mM sodium citrate buffer, pH 5.0 at 50 °C for 10 min with 0.2 ml of culture filtrate. The reaction was stopped by boiling for 5 min. The amount of reducing sugars formed was determined by the method of Bernfield (1955) and estimated using a standard curve prepared with glucose.

2.6. Growth inhibition of Pythium ultimum by hydrolyases and metabolites secreted by Trichoderma strains to cultivation medium

To determine antagonistic activity, Trichoderma was grown on minimal medium with lactose covered with cellophane or dialyzing membrane (cut-off 10 kDa). Following the removal of the membrane with the mycelia, the effect of post-culture medium containing secreted hydrolytic enzymes and metabolites (cellophane) or only metabolites (dialyzing membranes) on the growth of P. ultimum was analyzed.

A 5-mm mycelial disk of P. ultimum was placed in the center of the Trichoderma-pretreated plate. Plates were incubated at 28 °C for eight days and the area of growth was measured. Three replicates were assayed for each Trichoderma strain. As a control, P. ultimum was cultivated on plates containing fresh minimal medium and Trichoderma strains were cultivated on Trichoderma-pretreated plates to exclude lack of nutrients in the medium (Fig. 3S).

2.7. Plate confrontation assay

Mycelial disks (5 mm) of T. atroviride ERG20 transformants and P. ultimum were placed at opposite sides (7 cm apart) of agar plates with MM supplemented with 1% glucose. The plates were incubated at 28 °C and photographs were taken after 3 days of incubation. Three replicates were prepared for each experiment and for each transformant.
2.8. Biocontrol assay

Bean seeds (Phaseolus vulgaris L. (var. nanus L.)) were coated with $5 \times 10^8$ spores of T. atroviride per 10 g of seeds. Pathogen-infected soil was prepared by adding 2.2 g of fungal biomass of P. ultimum to 1 l of sterile soil. Seeds were allowed to germinate in the moist soil for ten days and the number and height of the seedlings was determined.

3. Results

3.1. Expression of S. cerevisiae ERG20 gene in T. atroviride

The biocontrol strain T. atroviride P1 was transformed with the ERG20 gene from S. cerevisiae under the control of the gpdA gene promoter (encoding glyceraldehyde-3-phosphate dehydrogenase) from A. nidulans. Stable transformants were analyzed for the presence of the yeast gene by PCR using ErgBam-U and ErgBam-L primers and genomic DNA of the transformants as template. As positive and negative controls the PCR reactions were performed on the templates of DNA from S. cerevisiae and T. atroviride P1, respectively. Three transformants carrying the ERG20 gene, SGUL59/11, SGUL125/11 and SGUL133/11 were selected for further analysis. qRT PCR analysis revealed that eight copies of ERG20 gene were integrated into the genome of SGUL125/11, and three and one copy were found in the SGUL59/11 and SGUL133/11 transformants, respectively. Expression of the heterologous ERG20 gene measured by RTqPCR showed very high expression of the gene in SGUL125/11 and much lower in the other two transformants (Fig. 2). These results are in agreement with the number of copies of ERG20 integrated into the genome of the transformants. In addition, we found that expression of the heterologous gene from S. cerevisiae did not alter the expression of the native erg20 gene.

3.2. Characterisation of the T. atroviride ERG20 strains

The activity of FPP synthase encoded by the ERG20 gene from S. cerevisiae was measured in cell-free extracts obtained from the transformants and compared with the endogenous FPP activity in the control non-transformed strain. All the transformed strains exhibited higher activity of FPP synthase compared to the control P1 strain (Fig. 3A). The increase in activity ranged from 55 to 104% of the control activity. The highest activity was detected for SGUL125/11 having the highest expression of the ERG20 gene.

Since the higher activity of FPP synthase could affect the production of sterols and/or dolichols, we analyzed the activity of two enzymes representing the sterol and the dolichyl branch of the mevalonate pathway, squalene synthase (Erg9p) and cis-prenyltransferase (Rer2p), respectively, in membrane fractions obtained from the transformants and the control P1 strain. The activity of cis-prenyltransferase was unchanged in the SGUL59/11, SGUL125/11 and SGUL133/11 transformants compared to the control (Fig. 3B). A markedly different effect was found for squalene synthase. Its activity was increased substantially in all the transformed strains, by between 78 and 110% of the control activity for SGUL125/11 and SGUL133/11, respectively (Fig. 3C).

3.3. Ergosterol and squalene production

Since the higher activity of squalene synthase should alter sterol synthesis we analyzed ergosterol and squalene levels in the cells of transformants compared to the control. Although significant differences of the squalene level were found, the concentration of ergosterol in the mycelia remained unchanged in all the transformants. The level of squalene was 100% higher in the mycelium of SGUL133/11 compared to the control strain (Fig. 4). In SGUL59/11 and SGUL125/11 the squalene level increased by 51% and 46%, respectively, compared to the control. Although the activity of squalene synthase (Erg9p) (Fig. 3C) and the level of squalene (Fig. 4) were significantly elevated in the transformants, expression of the erg9 gene was only slightly increased (Fig. 5). Since we did not observe an elevated level of ergosterol (Fig. 4), utilization of squalene had to be partially inhibited in the transformants. The decreased expression of erg7 gene and only moderately increased expression of erg1 (statistically significant only for the SGUL133/11 transformant) (Fig. 5) confirmed this hypothesis.

3.4. Activity of DPM synthase and glycosylation of secreted proteins

The higher activity of the sterol branch of the mevalonate pathway led to a higher production of squalene. Spanova et al. (2021) demonstrated that squalene in combination with ergosterol was an important parameter modulating the fluidity of the ER and plasma membrane. The fluidity of the cell membrane could in turn affect the activity of membrane-bound enzymes. One such membrane-bound enzyme is DPM synthase involved in the processing of hydroxases secreted by Trichoderma. DPM synthase is a key enzyme of the process of protein O-glycosylation and is also engaged in the assembly of the lipid-linked oligosaccharide substrate for protein N-glycosylation. The DPM synthase activity was measured in the membrane fraction and found to be higher by between 26 and 70% in SGUL133/11 and SGUL125/11, respectively, compared to the control strain (Fig. 6A).

We reasoned that the increased activity of DPM synthase found in the ERG20 transformants could affect protein glycosylation and/or secretion (Kruszewska et al., 1999; Perlińska-Lenart et al., 2006; Zembek et al., 2011). To verify this assumption, protein secretion to the culture medium was examined during cultivation. Medium samples were collected every 24 h for 168 h and the concentration of secreted proteins was determined. The amount of proteins was elevated by 19% compared to the control (Fig. 2SA) in the culture medium of the SGUL133 (120 h of cultivation) and SGUL59/11 (168 h of cultivation) strains.

Proteins secreted by Trichoderma, mainly hydrolytic enzymes, are highly glycosylated (Kruszewska et al., 2008-rev). The significantly increased activity of DPM synthase in the transformants should result in higher production of dolichyl phosphate mannose conductive to hyper-glycosylation of the proteins. To analyzed protein glycosylation Trichoderma strains were cultivated in minimal medium with lactose as a carbon source, proteins were precipitated from the medium after 72 and 96 h of cultivation, and the amount of protein-bound carbohydrates was determined. We found the highest amount of carbohydrates on proteins secreted after 96 h of cultivation (Fig. 6B,C). The increase, relative to the untransformed strain, in the amount of N-linked carbohydrates was more pronounced compared to the O-linked sugars. The highest level of protein glycosylation was found for strain SGUL125/11,
and it was 5.3-fold higher for N-glycosylation and 2.2-fold higher for O-glycosylation compared to the parental strain. The other two transformed strains also produced secretory proteins significantly more extensively O- and N-glycosylated than in the control P1 strain.

A qualitative analysis of the carbohydrates by high performance anion-exchange chromatography revealed that in both O- and N-linked carbohydrates mannose was the dominant sugar. The amount of mannose bound to proteins secreted by SGUL125/11 was 4.3- and 3.3-fold higher than for the P1 strain in N- and O-linked carbohydrates, respectively. This result correlates with the highest activity of DPM synthase in SGUL125/11. The both types of carbohydrates of the secretory proteins also contained glucose and galactose.

3.5. Hydrolytic activity of the ERG20-expressing strains

Since a major fraction of the proteins secreted to the cultivation medium are hydrolytic enzymes enabling the uptake of nutrients from complex carbon sources, and the secretory proteins were highly over-
glycosylated, we decided to check the hydrolytic capacity of the transformants.

Analysis of the cellulolytic activity secreted into the cultivation medium showed that all the transformants exhibited maximal cellulolytic activity after 72 and 96 h of cultivation (Fig. 7). The highest activity was noticed for SGUL133/11 after 72 h of cultivation and it was higher by 45% than that of the control strain after 96 h. The lowest activity among the transformants was found for SGUL125/11 and it was only 9% higher than in the control.

3.6. Antifungal activity of ERG20 transformants

To determine the antifungal potential of the ERG20 transformants we studied the effect of compounds liberated by the transformants on the growth of the plant pathogen Pythium ultimum. Trichoderma strains were cultivated on plates covered with cellophane or dialysis membranes (cut-off 10 kDa), then the membrane or cellophane was removed together with the Trichoderma and P. ultimum was inoculated on the pretreated plates containing both metabolites and hydrolases secreted by Trichoderma (cellophane) or only the metabolites (dialysis membrane). The growth of the pathogen on the Trichoderma-pretreated plates was compared with the growth on clean non-pretreated plates. The hydrolases and metabolites secreted through cellophane by all strains inhibited the growth of Pythium significantly, albeit the control strain inhibited the growth of Pythium slightly less effectively - by 70% while the transformants - by 88% (Fig. 8).

In contrast no differences in the inhibition of Pythium growth were found on plates containing only the secreted metabolites (covered with dialysis membrane) (data not shown). In fact the low-molecular-weight metabolites secreted through the dialysis membrane had little inhibitory effect (8%) on Pythium growth regardless of the Trichoderma strain used.

In addition, we made a confrontation assay between the ERG20-transformed Trichoderma strains and P. ultimum (Fig. 9). When Trichoderma and Pythium were cultivated on a single plate from separate inocula, the Pythium colony was overgrown more rapidly by all the transformants than by the control P1 strain, and the SGUL133/11 transformant was the most efficient. Since the cell wall of Pythium...
contains β-(1,4) glucan (cellulose) (Latijnhouters et al., 2003) and SGUL133/11 revealed the highest cellulolytic activity this result was expected. Inspection of the Pythium-overgrown area under a microscope showed that some hyphae of Pythium were devoid of organelles and contained mycelia of Trichoderma inside (Fig. 10).

3.7. Biocontrol properties of the ERG20-transformed strains

Since our transformants revealed a stronger antifungal activity and higher activity of the secretory hydrolases compared to the control strain it could be expected that also their biocontrol activity was more pronounced. To check this we tested seed germination and seedling growth of bean (Phaseolus vulgaris L. (var. nanus L.)) in the presence of P. ultimum. In the absence of Trichoderma, the pathogenic fungus decreased the bean germination rate to 28% of that observed in sterile soil (Fig. 11A). Pre-treatment of the seeds with the control Trichoderma strain P1 increased the germination rate in the presence of P. ultimum to 44% while with the SGUL125/11 transformant the rate was 84%. Poorer protection was observed for SGUL133/11 and SGUL59/11, but still the rate was 72 and 63%, respectively. The presence of P. ultimum in the soil inhibited the plant growth by 64% (Fig. 11B). Trichoderma largely prevented that inhibition: with the untransformed P1 strain the growth was poorer by only 9% compared to fungi-free control, while the SGUL125/11 transformant actually increased the growth rate in the presence of P. ultimum by 13% above the control (P1) value.

4. Discussion

The aim of this study was to engineer T. atroviride toward improving its antifungal properties. The importance of the mevalonate pathway products in the antifungal activity had been shown before using T. harzianum with a partially silenced expression of the gene encoding 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) (Cardoza et al., 2007). The HMGR-silenced transformants showed a reduced antifungal activity against the plant-pathogen fungi Rhizoctonia solani and Fusarium oxysporum. Since we intended to obtain an opposite effect we up-regulated the mevalonate pathway in the biocontrol T. atroviride strain P1 by expression of the yeast ERG20 gene encoding a key enzyme of this pathway, FPP synthase.

We chose a heterologous system since the yeast enzyme exhibits a much higher specific activity than the homologous enzyme from Trichoderma (Szkopińska et al., 2000; Piłsyk et al., 2014).

The increased activity of FPP synthase caused further changes in the mevalonate pathway mainly by increasing the rate of sterol synthesis, while the activity of the dolichol branch represented by cis-prenyltransferase remained unchanged. Szkopińska et al. (2000) showed that overexpression of the ERG20 gene in yeast elevated almost three-fold the rate of dolichol synthesis, while sterol synthesis was elevated only by 30%. The contrasting consequences of the ERG20 overexpression in S. cerevisiae and T. atroviride could reflect its homologous and heterologous origins. Enzymes from the dolichyl branch of the mevalonate pathway have been postulated to form an enzymatic complex with FPP synthase (Szkopińska et al., 1997). The cis-prenyltransferase activity is found in the membrane fraction, while FPP synthase was initially claimed to be cytosolic, which would make formation of such a complex unlikely; however, later studies (Runquist et al., 1992) found the FPP synthase activity also in the microsomal fraction and thus removed that caveat. Since the same yeast FPP synthase expressed in S. cerevisiae and T. atroviride had different effects on the respective endogenous cis-prenyltransferase activities one could infer that the Erg20 protein could only form an active complex for dolichol synthesis with the homologous (S. cerevisiae) but not the heterologous (Trichoderma) cis-prenyltransferase. Furthermore, the
unchanged cis-prenyltransferase activity in the Trichoderma transformants could create a surplus of FPP for the sterol branch of the mevalonate pathway. This could explain the significant increase in the activity of squalene synthase in the transformants.

On the other hand, overexpression of the homologous erg20 gene in T. reesei did not activate the two branches of the mevalonate pathway (Piłsyk et al., 2014). Although cis-prenyltransferase was activated in these transformants the activity of squalene synthase actually decreased 2-fold. Such an effect could be disadvantageous when attempting to improve the antifungal properties of Trichoderma. The level of sterols in the fungal cells could be crucial for activity of membrane-bound enzymes and secretion of proteins.

Fig. 10. Microscopic analysis of Pythium-overgrown area in the P. ultimum and T. atroviride confrontation assay. A- Pythium hyphae, B- Pythium and Trichoderma in the contact area, C- Pythium and Trichoderma in the Pythium-overgrown area, D- hyphae of Pythium attacked by Trichoderma Arrow marks Trichoderma hyphae inside of Pythium mycelium.

Fig. 11. Protective effect of Trichoderma on bean germination (A) and bean seedling growth (B) in soil infected with P. ultimum. A- Efficiency of germination presented as percentage of seeds sown was analyzed after ten days. Seeds were sown in sterile (black bars) or P. ultimum-infected (dark gray bars) soil in the absence or presence of Trichoderma strains as indicated. Each bar represents % of germinated seeds out of 32 seeds used in experiments and 100 seeds for the control with Pythium but no Trichoderma. B- Protective effect of Trichoderma on bean seedling growth. Seedlings height was measured after ten days. Seeds were sown in sterile (black bars) or P. ultimum- infected (dark grey bars) soil in the absence or presence of Trichoderma strains as indicated. Each bar represents mean ± standard deviation from different number of independent experiments, with 28 seedlings for control with P. ultimum but no Trichoderma and 30 seedlings for control without P. ultimum. Difference statistically significant (P < 0.05; t test).
It is known that sterols can affect the whole metabolism of the fungal cell since sterols, major lipids of the plasma membranes, interact with other lipids of the membrane bilayer influencing its rigidity, fluidity and permeability (Abe and Hiraki, 2009; Baumann et al., 2011; Dupont et al., 2011). It has been demonstrated that squalene in combination with ergosterol is an important modulator of the fluidity of ER and plasma membranes (Spanova et al., 1821). Our results revealed that the squalene content in membranes was nearly doubled in the ERG20-expressing T. atrovire. Squalene has a stabilizing effect making membranes more rigid, but the crucial parameter is the ratio of ergosterol to squalene (Spanova et al., 1821).

The changes in membrane properties could in turn modify the activity of membrane-bound enzymes such as DPM synthase, found to be optimal to raise the FPP synthase activity, which resulted in a moderate one copy of the yeast ERG20 gene expressed in T. atrovire. The most pronounced differences between the ERG20 transformants and the non-transformed Trichoderma concerned their biocontrol activity against the plant pathogen P. ultimum. The upper antifungal activity of the transformants compared to the control strain doubled the germination rate of bean seeds and slightly improved the growth of P. ultimum-infected soil.

5. Conclusions

Present results indicate a crucial role of the mevalonate pathway for the biocidal properties of Trichoderma. By a simple manipulation of that pathway we have constructed Trichoderma strains with greatly enhanced antifungal and biocontrol properties. Our results showed that one copy of the yeast ERG20 gene expressed in Trichoderma strain was optimal to raise the FPP synthase activity, which resulted in a moderately higher activity of DPM synthase and protein glycosylation. This ERG20 transformant showed the highest secreted hydrolytic activity, was the most efficient in confrontation with P. ultimum and had the best antifungal and biocontrol properties.

CRediT authorship contribution statement


Acknowledgements

This article is in memory of its co-author dr. Jacek Lenart who recently passed away. We thank Prof. Tadeusz Chojnacki and Ewa Kula-Świeżewska for their gift of dolichyl phosphate. This work was supported by Operational Programme - Innovative Economy, Poland, grant No. USA-POIG.01.03.01-14-038/09 to J.S.K.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fgb.2020.103334.

References


Goldstein, J.L., Brown, M.S., 1986. Regulation of the mevalonate pathway for the biocidal properties of Trichoderma. By a simple manipulation of that pathway we have constructed Trichoderma strains with greatly enhanced antifungal and biocontrol properties. Our results showed that one copy of the yeast ERG20 gene expressed in Trichoderma strain was optimal to raise the FPP synthase activity, which resulted in a moderately higher activity of DPM synthase and protein glycosylation. This ERG20 transformant showed the highest secreted hydrolytic activity, was the most efficient in confrontation with P. ultimum and had the best antifungal and biocontrol properties.

CRediT authorship contribution statement


