Comparative proteomic analyses of *Hyphozyma roseonigra* ATCC 20624 in response to sclareol

Xiwen Wang¹ · Xiaohua Zhang¹ · Qingshou Yao¹ · Dongliang Hua² · Jiayang Qin¹

Received: 21 August 2017 / Accepted: 3 April 2018 / Published online: 15 January 2019
© Sociedade Brasileira de Microbiologia 2019

Abstract
Sclareol is an important intermediate for ambroxide synthesis industries. *Hyphozyma roseonigra* ATCC 20624 was the only reported strain capable of degrading sclareol to the main product of sclareol glycol, which is the precursor of ambroxide. To date, knowledge is lacking about the effects of sclareol on cells and the proteins involved in sclareol metabolism. Comparative proteomic analyses were conducted on the strain *H. roseonigra* ATCC 20624 by using sclareol or glucose as the sole carbon source. A total of 79 upregulated protein spots with a > 2.0-fold difference in abundance on 2-D gels under sclareol stress conditions were collected for further identification. Seventy spots were successfully identified and finally integrated into 30 proteins. The upregulated proteins under sclareol stress are involved in carbon metabolism and nitrogen metabolism, and replication, transcription, and translation processes. Eighteen upregulated spots were identified as aldehyde dehydrogenases, which indicating that aldehyde dehydrogenases might play an important role in sclareol metabolism. Overall, this study may lay the fundamentals for further cell engineering to improve sclareol glycol production.

Keywords Comparative proteomic · *Hyphozyma roseonigra* · Sclareol · Sclareol glycol · Ambroxide

Introduction
Sclareol, a diterpene alcohol isolated from *Salvia sclarea* (clary sage), can be applied to medicine, cosmetics, health products, flavors and fragrances, and pesticides [1–3]. The main application of sclareol is for synthesizing high-end substitutes of ambergris in the perfume industry [4]. Given the supply shortage and price inflation of ambergris, a number of substitutes have been developed. Ambroxide is the most appreciated substitute of ambergris and is obtained from the semi-synthesis of sclareol [5–7]. Sclareol can be transformed to ambroxide through redox and cyclization, during which sclareol glycol and sclareolide are important intermediates (Fig. 1). Thus, the biological production of sclareol glycol or sclareolide to complete the biosynthesis pathway has gained much interest due to the increasing demand of natural fragrances.

Numerous strains reportedly transform sclareol, whereas most strains merely introduce hydroxyl at the main ring rather than modify the branched chain. Only few strains can transform sclareol to sclareol glycol or sclareolide.
Cryptococcus albidus ATCC 20918 converts sclareol to sclareolide at a high yield of more than 100 g/L and has been exploited for industrialization [8]. To date, Hyphozyma roseonigra ATCC 20624, which can exist in both yeast-like and filamentous forms, is the only reported strain capable of degrading sclareol to the main product of sclareol glycol [9]. However, the explicit conversion mechanism from sclareol to sclareol glycol and other products has not been reported. Knowledge is also lacking about the effects of sclareol on cell growth, considering that sclareol is a labdane diterpene with a high antimicrobial activity [10]. These problems hinder the further improvement of conversion rate and the expanded application of the biotechnology. H. roseonigra ATCC 20624 can survive in basic inorganic salt medium with sclareol as the sole carbon source and is therefore a good candidate for exploring the mechanism to some extent.

In the present study, comparative proteomic analyses were conducted on the strain H. roseonigra ATCC 20624, with sclareol or glucose as the sole carbon sources. The difference in protein expression between the two conditions is expected to enrich our knowledge on sclareol stress in microorganisms.

**Material and methods**

**Microorganism and cultivation conditions**

H. roseonigra ATCC 20624 was purchased from ATCC (Maryland, USA) and grown on yeast-malt medium (per liter: 3 g yeast extract, 3 g malt extract, 10 g glucose, and 5 g tryptone at pH 6.2) at 24 °C and 180 rpm. The seed culture was incubated in two kinds of basic inorganic salt medium (BSM) containing sclareol (BSMS) or glucose (BSMG) to monitor the cell growth rates.

The modified BSM medium contained (per liter) 2.44 g KH₂PO₄, 14.04 g Na₂HPO₄·12H₂O, 2 g NH₄Cl, 0.2 g MgCl₂·6H₂O, 1 mg CaCl₂·2H₂O, 0.01 g yeast extract, 5 mL metal ion mixture, and 0.2 mL vitamin mixture. Meanwhile, the metal ion mixture included (per liter) 0.5 g FeCl₂·4H₂O, 0.5 g MnCl₂, 0.1 g NaMoO₄·2H₂O, 0.05 g CuCl₂, and 120 mM HCl. The vitamin mixture contained (per liter) 2 g calcium pantothenate, 1 g creatine, 2 g nicotinic acid, 2 g pyridoxamine, 2.5 mg cobalamin, and 1 g para-aminobenzoic acid.

Tween 80 (0.8%, v/v) and sclareol (2 mM) were added to BSM medium to prepare the BSMS medium, whereas glucose (1.33 mM) was added to BSM medium to produce the BSMG medium. Cells grown in the BSMG medium were run in parallel with the samples in the BSMS medium.

**Preparation of protein extracts and comparative proteomic analysis**

Cells in the exponential growth phase were harvested from 50 mL cultures by centrifugation at 4000×g for 10 min at 4 °C. The cell pellets were frozen in liquid nitrogen and stored at −80 °C until proteome analysis.

Comparative proteomic analysis by 2-D gel was carried out as described in literature [11]. Cells were suspended in lysis buffer of 2DE (Sangon Co., Shanghai, China) and ultrasonically disrupted at 80–100 W for 3 min. The debris was removed by centrifugation, and the supernatant was incubated overnight with ice-cold acetone. After centrifugation at 12,000×g and 4 °C for 30 min, the precipitated proteins were solubilized in sample lysis buffer. Total protein concentrations were determined using the Non-interference Protein Assay Kit (Sangon Co., Shanghai, China). Protein spots on the gels were visualized by silver staining in accordance with the protocol [12]. Each set of samples was independently analyzed in triplicate.

**Analyses of 2-D gels**

2-D gels were scanned at a resolution of 300 dots per inch (dpi) by an Image Scanner III (GE healthcare). Statistically significant differences were discerned by performing Student’s t test. Protein spots showing significant changes in upregulation and downregulation were extracted, digested, rehydrated, and identified in a 5800 Plus MALDI TOF/TOFTM analyzer (Applied Biosystems). Data were acquired in a positive mass spectrometry (MS) reflect or by using a CalMix5 standard to calibrate the instrument (ABI 5800 Calibration Mixture).
Results

Growth characteristics of H. roseonigra ATCC 20624

The growth rates of H. roseonigra ATCC 20624 on media containing different sclareol concentrations (2, 6, and 10 mM) were investigated. The growth curves of H. roseonigra ATCC 20624 on media containing 2 and 6 mM sclareol were similar, and the optical density (OD$_{600}$) of 2.23 ± 0.11 for 2 mM sclareol and 2.41 ± 0.05 for 6 mM sclareol were obtained at 120 h. During the first 50 h of incubation, H. roseonigra ATCC 20624 grew well on the medium containing 10 mM sclareol, and prolonged cultivation times resulted in retarded growth rates (Fig. 2a). Glucose is a carbon source preferred by most microorganisms. Hence, the cell growth on the medium containing 1.33 mM glucose was also investigated; this concentration was set to provide the same number of moles of carbon as that lost if 2 mM sclareol was completely transformed to sclareol glycol. The cell mass of sclareol cultures reached the same value observed as those in the glucose cultures during the first 12 h of cultivation. H. roseonigra ATCC 20624 also grew much faster on the medium with glucose than in that with sclareol from 12 to 15 h (Fig. 2b). However, further incubation in glucose decreased cell growth, and the OD$_{600}$ value of H. roseonigra ATCC 20624 on the glucose-containing medium reached 0.53 ± 0.04 at 24 h. Interestingly, the growth of H. roseonigra ATCC 20624 continued to increase after 15 h of cultivation in BSMS medium. This result indicates the predominant sclareol-tolerant capacity of H. roseonigra ATCC 20624. Cells cultured in BSMG medium and BSMS medium were collected at 12 h and used for subsequent proteomic analyses. At this time point, sclareol had not been completely converted to sclareol glycol (Fig. S1).

Protein expression alterations in response to sclareol stress

The global proteomic response of H. roseonigra ATCC 20624 to sclareol was profiled by 2-D gel electrophoresis. Glucose cultures were then used as the control group, whereas sclareol cultures were adopted as the experimental group. In three independent experiments, protein abundances were analyzed using the PDQuest software. Protein abundances were significantly altered ($p < 0.05$) in 117 spots (Table S1 and Fig. 3) from the BSMS and BSMG media. This change suggests that sclareol affected the fungus’s cellular physiology. Among these spots, 79 upregulated spots with a >2.0-fold difference in abundance on 2-D gels were collected for further mass spectrometric identification. Seventy spots were successfully recognized, whereas several spots were identified as the same protein after Omics Bean analysis. Finally, 70 protein spots were integrated into 30 proteins (Table 1).

Discussions

Many of the differentially expressed proteins in Table 1 are common to several categories. Approximately 27% of the differentially expressed proteins belonged to carbon metabolism, followed by 10% belonging to the metabolic pathway, and 10% belonging to protein processing in the endoplasmic reticulum. Meanwhile, 3% of the proteins each belonged to the cell cycle, phagosome formation, DNA replication, glutathione metabolism, peroxisome formation, lysine degradation, β-alanine metabolism, antibiotic biosynthesis, and glyoxylate metabolism.

The proteins involved in carbon metabolism (including glycolysis and the citric acid cycle), such as fructose bisphosphate aldolase (FBA), enolase (ENO), citrate synthase (CIT), malate dehydrogenase (MDH), succinate dehydrogenase (SDH), and phosphoenolpyruvate carboxykinase, were all upregulated in response to sclareol stress. Notably, the fold change of MDH, which catalyzes the conversion of malate and oxaloacetate [13], was the largest, at approximately 111-fold. The observed upregulation of these proteins indicated that the H. roseonigra ATCC 20624 cells expressed additional enzymes for carbon metabolism to metabolize sclareol more efficiently than usual. Additionally, sclareol stress induced significant changes in the expression of aldehyde dehydrogenases (ALDHs). ALDHs, generally considered as a superfamily of NADP-dependent enzymes and participating in aldehyde metabolism, catalyze the oxidation of exogenous aldehydes into corresponding carboxylic acids [14]. The upregulated ALDHs may protect H. roseonigra ATCC 20624 from damage induced by active aldehydes. Eighteen upregulated spots were identified as
ALDHs, which indicated that ALDHs may play an important role in sclareol metabolism.

Besides the abovementioned protein narrative, two other proteins, that is, heatshock proteins SSB2 and SSA2, were found upregulated (2.58- and 7.96-fold increases, respectively). SSB2 and SSA2 are molecular chaperones belonging to the 70 kDa heat-shock proteins (Hsp70s). The two enzymes exhibited low intrinsic ATPase activities in the native protein, whereas the isolated ATPase domains manifested much greater ATPase activities [15]. Both proteins participate in the transport of polypeptides but function distinctly. Furthermore, proteins acting on cellular processes, such as the nicotinic acid transporter (TNA), meiotic recombination protein (REC), DNA replication licensing factor (MCM), and eukaryotic translation initiation factor (HYP) were upregulated. This finding indicates that sclareol stress also forced *H. roseonigra* ATCC 20624 to strengthen its life activities.

*H. roseonigra* ATCC 20624 also expressed additional structural proteins, such as actins, structural maintenance of chromosomes protein 4, and flocculation protein FLO11, to survive under sclareol stress. Three proteins, that is, 78 kDa glucose-regulated protein homolog, GTPase, and ATP synthase subunit α, were also upregulated. This protein homolog is active as a homodimer and exhibits ATPase activity. Meanwhile, GTPase is required for mitochondrial protein synthesis [16]. The upregulation of these proteins implies that
H. roseonigra ATCC 20624 can generate additional ATP or GTP by expressing additional enzymes related to energy metabolism when exposed to sclareol stress.

**Conclusions**

In summary, the results indicated that proteins involved in carbon metabolism, nitrogen metabolism, and replication, transcription, and translation processes are upregulated when sclareol is the sole carbon source relative to that when glucose is the sole carbon source. Some of the identified proteins are known to serve heat-inductive functions and play general roles in cross-protecting cells against diverse stress conditions. These cross-protecting proteins may play important roles in the sclareol tolerance of H. roseonigra ATCC 20624. Overall, the present study may lay the fundamentals for further cell engineering to improve sclareol glycol production.

**Funding information**  This work was supported by the National Natural Science Foundation of China (31200078), the Shandong Province Science and Technology Project (No. ZR2017MC023 and 2015GSF117034), and the Jinan Youth Science and Technology Star Project (No. 201406021).

**Compliance with ethical standards**

**Conflict of interest**  The authors declare that they have no conflicts of interest.
Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

References

1. Bhatia SP, Mcginty D, Letizia CS, Api AM (2008) Fragrance material review on sclareol. Food Chem Toxicol 46:S270–S274
2. Li F, Tao F (2015) Recent advances in biotechnological production of sclareol. Guangzhou Chem Ind 43:5–7
3. Wang L, He HS, Yu HL, Zeng Y, Han H, He N, Liu ZG, Wang ZY, Xu SJ, Xiong M (2015) Sclareol, a plant diterpene, exhibits potent antiproliferative effects via the induction of apoptosis and mitochondrial membrane potential loss in osteosarcoma cancer cells. Mol Med Rep 11:4273–4278
4. Pan XW, Han L, Zhang YH, Chen DF, Simonsen HT (2015) Sclareol production in the moss physcomitrella patens, and observations on growth and terpenoid biosynthesis. Plant Biotechnol Rep 9:149–159
5. Aranda G, Lallemand JY, Mammoumi A, Azerad R (1991) Microbial hydroxylation of sclareol by Mucor plumbeus. Tetrahedron Lett 32:1783–1786
6. Barrero AF, Alvarez-Manzaneda EJ, Altarejos J, Salido S, Ramos JM (1993) Synthesis of ambrox® from (−)-sclareol and (+)-cis-abienol. Tetrahedron 49:10405–10412
7. Schalk M, Pastore L, Mirata MA, Khim S, Schouwey M, Deguerry F, Pineda V, Rocci L, Daviet L (2012) Toward a biosynthetic route to sclareol and amber odorants. J Am Chem Soc 134:18900–18903
8. Farbood MI, Morris JA, Downey AE (1990) Process for producing diol and lactone and microorganisms capable of same. US Patent 4,970,163
9. Farbood MI, Willis BJ (1986) Process for producing diol and furan and microorganism capable of same. US Patent 4,798,799
10. Trikka FA, Nikolaidis A, Athanasakoglou A, Andreadelli A, Ignea C, Kottas K, Argiriou A, Kampranis SC, Makris AM (2015) Iterative carotenogenic screens identify combinations of yeast gene deletions that enhance sclareol production. Microb Cell Factories 14:60–79
11. Wang H, Wang L, Yang H, Cai Y, Sun L, Xue Y, Yu B, Ma Y (2015) Comparative proteomic insights into the lactate responses of halophilic Salinicoccus roseus W12. Sci Rep 5:13776
12. Shevchenko A, Wilm M, Vorm O, Mann M (1996) Mass spectrometric sequencing of proteins from silver-stained polyacrylamide gels. Anal Chem 68:850–858
13. Steffan JS, McAlister-Henn L (1991) Structural and functional effects of mutations altering the subunit interface of mitochondrial malate dehydrogenase. Arch Biochem Biophys 287:276–282
14. Marchitti SA, Brocker C, Stagos D, Vasiliou V (2008) Non-P450 aldehyde oxidizing enzymes: the aldehyde dehydrogenase superfamily. Expert Opin Drug Metab Toxicol 4:697–720
15. Lopez-Buesa P, Pfund C, Craig EA (1998) The biochemical properties of the ATPase activity of a 70-kDa heat shock protein (Hsp70) are governed by the C-terminal domains. Proc Natl Acad Sci U S A 95:15253–15258
16. Dutta K, Fuentes JL, Maddock JR (2005) The yeast GTPase Mrg2p is required for mitochondrial translation and partially suppresses an rRNA methyltransferase mutant, mrm2. Mol Biol Cell 16:954–963