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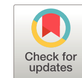
Comparative proteomic analyses of *Hyphozyma roseonigra* ATCC 20624 in response to sclareol



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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 up-regulated 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 up-regulated proteins under sclareol stress are involved in carbon metabolism; and nitrogen metabolism; and replication, transcription, and translation processes. Eighteen up-regulated 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.

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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

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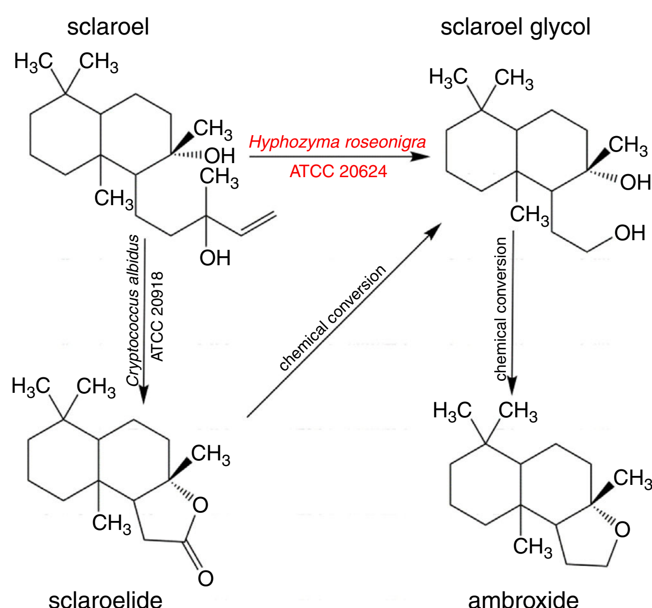

Fig. 1 – Diagram of conversion from sclareol to ambroxide.

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 source. 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 (MD, 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 KH_2PO_4, 14.04 g $Na_2HPO_4 \cdot 12H_2O$, 2 g NH_4Cl, 0.2 g $MgCl_2 \cdot 6H_2O$, 1 mg $CaCl_2 \cdot 2H_2O$, 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 $FeCl_2 \cdot 4H_2O$, 0.5 g $MnCl_2$, 0.1 g $NaMoO_4 \cdot 2H_2O$, 0.05 g $CuCl_2$, 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 up-regulation and down-regulation 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 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,

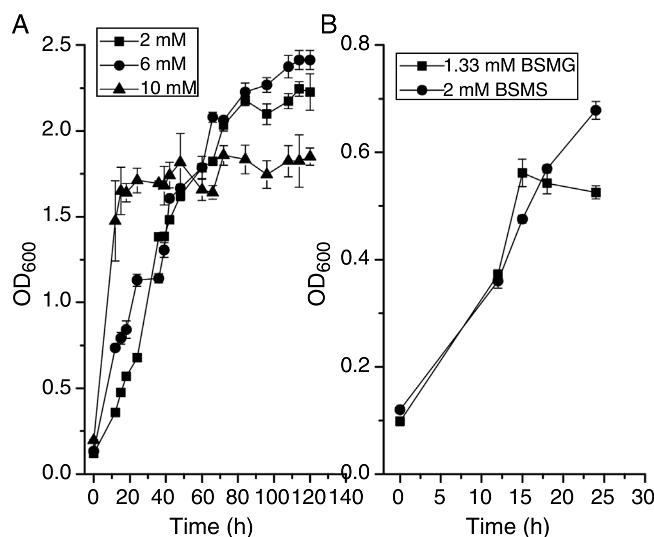

Fig. 2 – Effects of sclareol stress on the cell growth of *Hyphozyma roseonigra* ATCC 20624. (A) Effects of sclareol on cell growth. (B) Growth curves of *H. roseonigra* ATCC 20624 in BSMS and BSMG media. Experiments were performed in triplicate. Error bars represent the standard deviations of the means of three independent experiments.

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 cultivation. *H. roseonigra* ATCC 20624 also grew much faster on the medium with glucose than in that with sclareol from 12 h 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 (Supplementary data, Fig. I).

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 up-regulated 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, antibioticbiosynthesis, 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 up-regulated 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 folds. The observed up-regulation 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 up-regulated ALDHs may protect *H. roseonigra* ATCC 20624 from damage induced by active aldehydes. Eighteen up-regulated spots were identified as ALDHs, which indicated that ALDHs may play an important role in sclareol metabolism.

Besides the above-mentioned protein narrative, two other proteins, that is, heatshock proteins SSB2 and SSA2, were found up-regulated (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 up-regulated. 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 up-regulated. This protein homolog

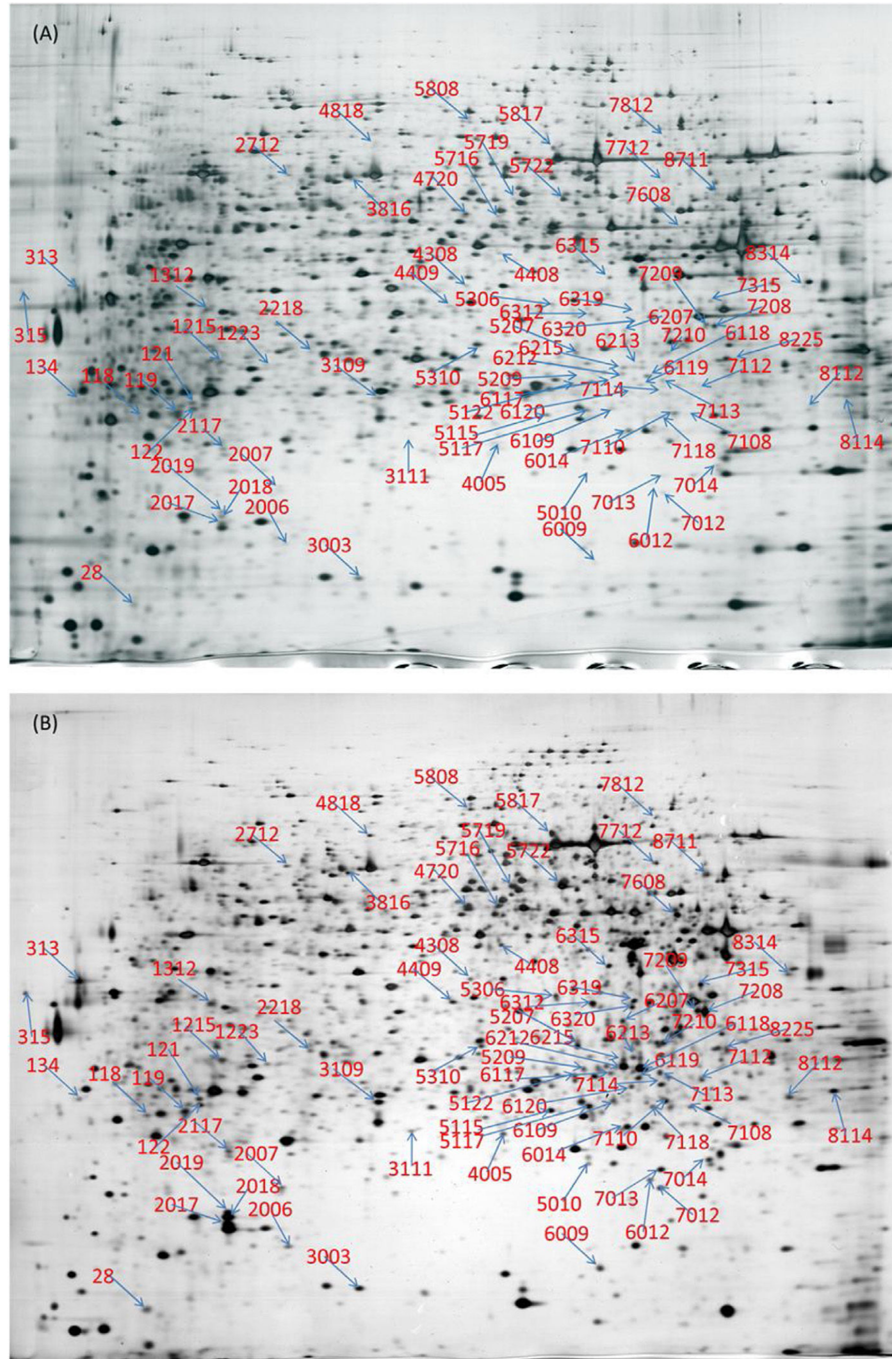

Fig. 3 – Comparative proteomic profiling of *H. roseonigra* ATCC 20624 exposed to (A) 1.33 mM glucose and (B) 2 mM sclareol. The differentially expressed proteins were labeled with spot numbers. Experiments were performed in triplicate.

Table 1 – Proteins identified by mass spectrometry.

Spot number[a]	Protein identification	Fold change[b]
2007, 5722, 6117, 7208, 7209, 7210, 7315, 4720, 5122, 7113, 2017, 2019, 7712, 5719, 7608, 3111, 5817, 6109	Aldehyde dehydrogenase (ALD5)	11.62, 3.37, 33.63, 2.40, 2.91, 2.97, 5.00, 2.85, 3.23, 3.67, 12.66, 9.35, 4.30, 8.81, 2.70, 6.52, 4.82, 3.86
5806, 6119, 7108	Nicotinic acid transporter (TNA1)	2.91, 8.25, 3.50
8114	Meiotic recombination protein (REC114)	4.43
3109	Polyamine oxidase (FMS1)	2.33
6014	Histone-lysine N-methyltransferase (SET1)	3.76
6212	Peroxisomal 2,4-dienoyl-CoA reductase (SPS19)	4.89
6213	Enolase 1 (ENO1)	3.54
6215, 6306	Transcriptional regulator (URE2)	4.10, 4.16
6313	Citrate synthase, mitochondrial (CIT1)	3.35
6319, 7114, 7118, 6320	DNA replication licensing factor (MCM3)	11.15, 4.81, 8.72, 10.35
7014	Mitochondrial peroxiredoxin Prx1 (PRX1)	6.12
119, 6118	Actin (ACT1)	2.89, 2.89
5115	Heat shock protein (SSB2)	2.58
3003, 122, 313, 121	Fructose-bisphosphate aldolase (FBA1)	3.59, 3.74, 3.16, 3.91
134	Saccharopepsin (PEP4)	3.16
1215	78 kDa Glucose-regulated protein homolog (KAR2)	6.97
2218	Structural maintenance of chromosomes protein (SMC4)	2.60
3816	GTPase (MTG2)	6.52
4005	Heat shock protein (SSA2)	7.96
1312, 4409, 4818, 5010, 7110, 6312	Malate dehydrogenase (MDH1)	3.10, 4.58, 6.37, 110.69, 7.80, 6.54
5117	Flocculation protein (FLO11)	3.40
7812	Peroxisomal catalase A (CTA1)	3.89
8112	Acetyl-coenzyme A synthetase 2 (ACS2)	5.39
2006, 6118, 8314, 1223	NADP-dependent 3-hydroxy acid dehydrogenase (YMR226C)	2.40, 2.40, 16.42, 4.60
1312	Succinate dehydrogenase (SDH1)	6.19
2117	Eukaryotic translation initiation factor (HYP2)	3.48
5716	Glutamine synthetase (GLN1)	6.13
6009	Cerevisin (PRB1)	17.27
28, 2712, 6012, 7012, 7013	Phosphoenolpyruvate carboxykinase (PEPCK1)	13.54, 5.45, 15.45, 13.45, 9.74
7108, 7112	ATP synthase subunit (ATP1)	5.59, 16.42

[a] Consistent with the protein number as shown in Fig. 3.
[b] Fold changes in protein expression were calculated using the cells from the BSMG medium as reference.

is active as a homodimer and exhibits ATPase activity. Meanwhile, GTPase is required for mitochondrial protein synthesis.[16] The up-regulation 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 up-regulated 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.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bjm.2018.04.001.

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