

Full Length Research Paper

Identification of thirteen up-expressed sequence tags from *Monascus pilosus* mutant MK-1

Mingyong Zhang^{1,2}, Xing Yang^{1,3}, Tsuyoshi Miyake², Yaqing Wang^{4*} and Kuaifei Xia¹

¹Key Laboratory of Plant Resources Conservation and Sustainable Utilization, South China Botanical Garden, Chinese Academy of Sciences, Guangzhou 510650, China. ²Industrial Technology Center of Okayama Prefecture, Haga 5301, Okayama, Japan. ³Graduate University of Chinese Academy of Sciences, Beijing 100049, China. ⁴College of Life Science, South China Normal University, Guangzhou 510631, China.

Accepted 19 March, 2019

Monascus pilosus MK-1 is a mutant of *M. pilosus* IFO4520, which is used to brew the red yeast rice for food industry as functional food additives or food colorants. The obvious phenotypes of the mutant MK-1 showed a high productivity of lovastatin and red pigments, slow growth of the fungal mycelia comparing with the wild type IFO4520. Through the suppression subtractive hybridization, we identified 30 up-expressed sequence tags (up-EST) (AB193486 to AB193498) from the mutant MK-1. Among them, 10 of the up-ESTs were homologues of the known-functional genes. Two up-ESTs (AB193498 and AB193494) are the transcript factor-related gene homologues, and they were homologous with the phosphatidylinositol-4-phosphate 5-kinase and the transcription factor respectively. Three up-ESTs (AB193487, AB193496 and AB193497) were homologous with pyruvate carboxylase, glyceraldehydes-3-phosphate dehydrogenase and ATP-dependent protease respectively, and the three genes are involved in the carbon metabolism. Four up-ESTs (AB193486, AB193488, AB193491 and AB193495) were homologous with the ADP-ribosylation factors. An up-EST (AB193490) was a homologue of penicillin-binding protein. These up-ESTs might indicate to explain the phenotypic differences between the wild type IFO4520 and the mutant MK-1.

Key words: *Monascus pilosus*, lovastatin, EST, red pigment.

INTRODUCTION

Monascus genus is used to brew a lot of oriental fermented foods (such as: Red Yeast Rice), and to treat hypercholesterolemia for a long time in Asia (Samiee et al., 2003), since some *Monascus* spp. can synthesize lovastatin and the red pigments. The red pigments are used as food additives and colorants for food industry around the world. Lovastatin can block cholesterol synthesis by inhibiting the HMG-CoA reductase and highly effective in lowering levels of plasma total and LDL cholesterol (Endo, 1992; Kennedy et al., 1999), therefore, the Red Yeast Rice brewed with *Monascus* is also used as the functional food additives in food industry. The biosynthesis pathway and the genes for lovastatin

biosynthesis pathway and the genes for lovastatin biosynthesis have been identified and characterized in *Aspergillus terreus* (Kennedy et al., 1999; Yoshizawa et al., 1994) and *Monascus* was suspected of possessing a similar lovastatin biosynthetic pathway and these genes have been cloned recently from *Monascus pilosus* (Chen et al., 2008). Fungal growth (Omamor et al., 2008) and regulation of lovastatin biosynthesis depends on culture conditions, including the composition of the medium, the incubation parameters, and light, amongst other things in *Aspergillus* (Hajjaj et al., 2001; Casa-Lopez et al., 2003) and *Monascus* (Wang et al., 2003; Miyake et al., 2005; Zhang and Miyake, 2009). We bred a *M. pilosus* mutant MK-1 with high lovastatin and red pigments productivity comparing with the wild type *M. pilosus* IFO4520 by chemical treatment and cerulenin selection (Miyake et al., 2005). In order to characterize some genes which might be related with the phenotypes of the mutant MK-1, we

*Corresponding author. E-mail: yangchw@scnu.edu.cn. Tel. +86-20-8521 0855.

identified 13 up-expressed sequence tags (up-ESTs) from the mutant MK-1 by using the suppress subtractive hybridization.

MATERIALS AND METHODS

M. pilosus strains, culture conditions and secondary metabolite analysis

M. pilosus MK-1 is a mutant of *M. pilosus* IFO4520 bred by Miyake et al. (2005). The fungal strains (IFO4520 and MK-1) were routinely maintained on the potato dextrose agar (PDA), Difco medium. The maltose-glycerol-peptone (MGP) medium was used to grow fungi for the different analysis, since the MGP medium leads *M. pilosus* to produce higher lovastatin and the red pigments than the PDA medium did (Zhang et al., 2009). The MGP medium consists of 1% maltose, 7% glycerol, 3.8% peptone, 0.1% MgSO₄·7H₂O and 0.2% NaNO₃. Fungal biomass was determined based on the fresh-weight of the filtrated cells from cultures. Red pigment production was estimated and expressed as absorbance units (U) at 490 nm. The contents of lovastatin were determined by chromatograms detected at 237 nm and 260 nm respectively, under established conditions, as described previously by Zhang and Miyake (2009), and the peak of lovastatin was identified by mass spectra. All data presented were the averages of results obtained from three independent experiments.

Identification the up-ESTs by the suppress subtractive hybridization

Total RNA was isolated from *M. pilosus* mycelia with RNeasy Plant Mini Kit (Qiagen) and treated with DNase I (RNase free) (TaKaRa, Japan). mRNA was purified from the total RNA with Oligotex™-dT30 (Super) mRNA Purification Kit (TaKaRa, Japan) for the suppress subtractive hybridization (SSH). SSH was performed with Clontech PCR-Select™ cDNA Subtraction Kit (BD Biosciences, USA), and mRNA of the IFO4520 and the MK-1 used in SSH experiment was isolated from the fungi incubated in the MGP broth medium for 14 days.

Expression confirmation by Northern dot blot

Northern dot blot was used to confirm the up-expression of the up-ESTs identified by the SSH. RNA of the fungi was isolated from different growth times, and they were transferred on to the charged nylon membrane (Roche) after electrophoresis in a 1.5% agarose-formaldehyde gel. DIG-labeled full-length anti-sense RNA probes of cDNAs were generated *in vitro* transcription from the cloned ESTs in pGEM-T easy vector (Promega) by SP6 RNA polymerase, by following the protocols of DIG Northern Starter Kit

(Roche). Pre-hybridization, hybridization, washing and chemiluminescent detection were also carried out as describing in this manual. Stringent washes were given as 2 × SSC / 0.1% SDS at RT for 10 min twice, and 0.1 × SSC / 0.1% SDS at 68°C for 20 min twice. Other molecular methods were following the protocols of Sambrook and Russell (2001).

RESULTS AND DISCUSSION

Main phenotypes of *M. pilosus* mutant MK-1

The filament fungus, *M. pilosus* can produce lovastatin

red pigments and citrinin as secondary metabolites (Chen et al., 2008; Zhang and Miyake, 2009). To breed an *M. pilosus* with high lovastatin productivity, a mutant MK-1 was bred from *M. pilosus* IFO4520. The spores of *M. pilosus* IFO4520 were treated with a chemical mutagen N-methyl-N'-nitro-N-nitrosoguanidine, and further selected with cerulenin (Miyake et al., 2006). Three main phenotypes of the mutant MK-1 were found comparing with its wild type IFO4520 (Figure 1). First, the mutant MK-1 had the ability of 10 fold high lovastatin production comparing with the IFO4520 (Figure 1B). The fermentation conditions for high lovastatin production were established, and the optimal cultivation for high lovastatin was the growth of the fungi in the maltose-glycerol-peptone (MGP) medium containing 1% maltose, 7% glycerol, 3.8% peptone, 0.1% MgSO₄·7H₂O and 0.2% NaNO₃ at 28°C for 14 days. Secondly, the mutant MK-1 could produce higher red pigments comparing with the IFO4520 (Figure 1D). Thirdly, the mutant MK-1 grew slowly in the PD medium and in the MGG medium comparing with the IFO4520 (Figure 1B).

Identification of 13 up-ESTs from *M. pilosus* mutant MK-1

To isolate the up-expressed genes in the mutant MK-1, a subtractive cDNA library was constructed from the IFO4520 and the mutant MK-1 grown in the MGP media at 28°C for 14 days by the suppression subtractive hybridization (SSH). After the subtractive selection, a total of 96 EST clones showed differential expression between *M. pilosus* mutant MK-1 and the IFO4520 by the reverse Northern dot blot (Figure 2). Among them, 13 clones were found to show most-up-expression in the mutant MK-1 and their up-expression was further confirmed by Northern dot blot analysis (Figure 3). Then these up-ESTs were sequenced and were homologous searching by Blast. The accession No. of the 13 up-EST in GeneBank was listed in Table 1.

Homologous analysis of the up-ESTs of the mutant MK-1

Homologous search of the differentially expressed ESTs was done by Blast, and a summary of the database homologous search was given in Table 1. Ten up-ESTs could be found the homologues which were known- or predicated-functions; other three ESTs could not be found any homologue in the database. Two ESTs (AB193494 and AB193498) were homologous with the genes involved in the expression regulation for the genes. They may play regulation roles for gene transcription in the mutant MK-1. The EST (AB193498) was a homologue of phosphatidylinositol-4-phosphate 5-kinase, which was secreted by *Fusarium oxysporum* during colonization of tomato xylem vessels (Houterman et al., 2009). The EST

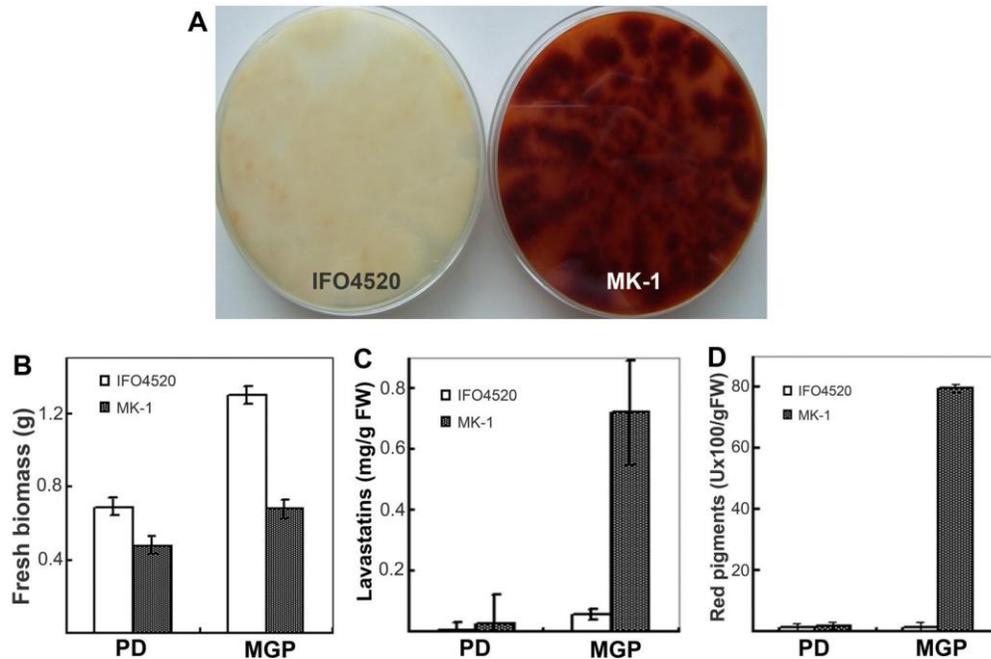


Figure 1. Phenotypes of *M. pilosus* IFO4520 and its mutant MK-1. A. Colony of IFO4520 and MK-1. The fungi were grown on the MGP (maltose-glycerol-peptone) solid medium (1% maltose, 7% glycerol, 3.8% peptone, 0.1% MgSO₄·7H₂O, 0.2% NaNO₃ and 2% agar) at 28°C for 7days. B-D. Biomass, production of lovastatin and red pigments of the IFO4520 and the mutant MK-1. The fungi were grown in 50 ml of the PD (potato dextrose, DIFCO) broth medium or in 50 ml of the MGP broth medium at 28°C for 14 days.

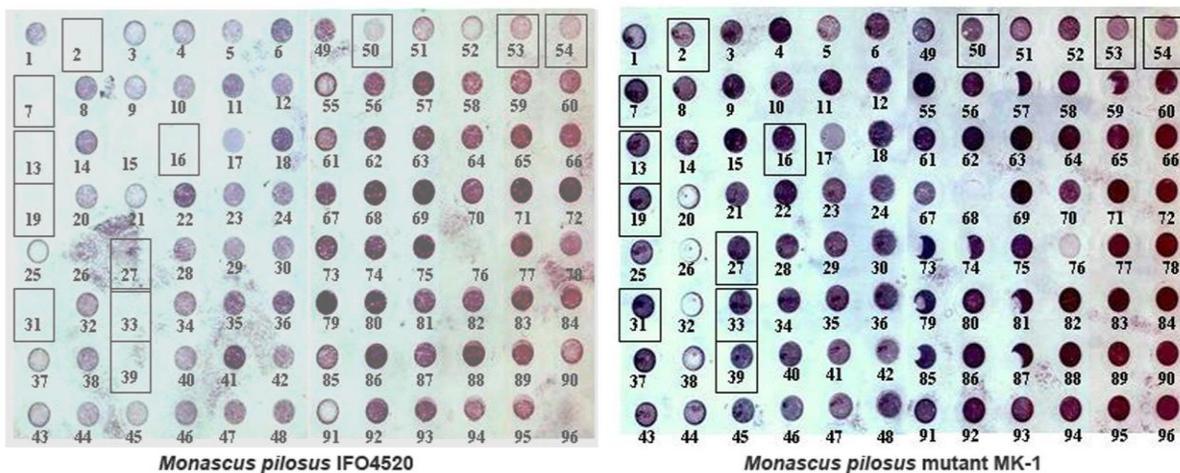


Figure 2. Up-expressed sequence tags (up-ESTs) in *M. pilosus* mutant MK-1 comparing with its wild type *M. pilosus* IFO4520 by reverse Northern dot blot. Equal plasmids of the subtracted ESTs cloned in pGEM-T easy vector from the mutant MK-1 and the IFO4520 were blotted on the Nylon membrane, and probed with the DIG-labeling total cDNAs from the IFO4520 and the MK-1. The clones in the boxes are the most-differential expression between the IFO4520 and the MK-1.

tomato xylem vessels (Houterman et al., 2009). The EST (AB193494) was a homologue of a rice transcription factor *OsRS2* (Tattersall et al., 2005). Another three ESTs (AB193487, AB193496 and AB193496) were homologues with the genes involved in carbon and protein metabolism,

and they may be related with the slow-growth phenotype of the mutant MK-1. The EST (AB193487) was a homologue of pyruvate carboxylase (Jitrapakdee et al., 2008), and the EST (AB193496) was homologous with glyceraldehyde-3-phosphate dehydrogenase, these

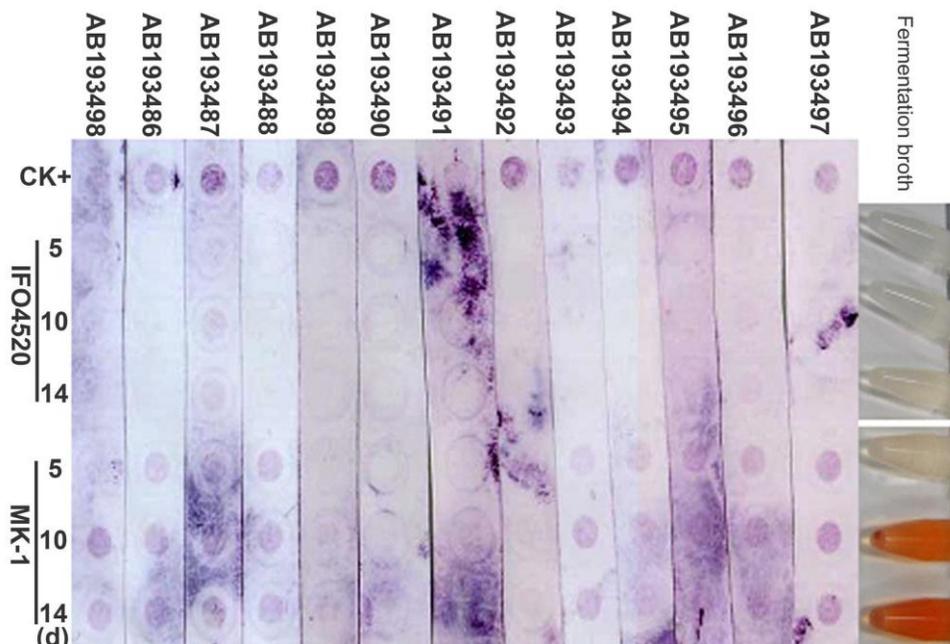


Figure 3. Confirmation of the differential expression of the 13 up-ESTs between *M. pilosus* IFO4520 and its mutant MK-1 by Northern dot blot. Fifteen μ g of total RNA from the IFO4520 and the MK-1 grown in the MGP broth medium at different growth times were blotted on the nylon membrane, and probed with the corresponding cloned up-EST respectively.

Table 1. Predicated functions of the up-expressed sequence tags in *M. Pilosus* mutant MK-1.

Up-ESTs	Homologues	Predicated function
AB193498	NM106158	Phosphatidylinositol-4-phosphate 5-kinase
AB193486	NM191065	ADP-ribosylation factor
AB193487	AF097728	Pyruvate caboxylase
AB193488	NM191065	ADP-ribosylation factor
AB193489	-	-
AB193490	AB119947	Penicillin-binding protein
AB193491	NM191065	ADP-ribosylation factor
AB193492	-	-
AB193493	-	-
AB193494	AB071600	Transcription factor OsRS2
AB193495	NM191065	ADP-ribosylation factor
AB193496	Z32524	Glyceraldehyde-3-phosphate dehydrogenase
AB193497	M76451	ATP-dependent protease

- no homologue could be found by Blast searching in GeneBank.

up-ESTs might be involved in the carbohydrate metabolism. The EST (AB193497) was a homologue of an ATP-dependent protease (Baker and Sauer, 2006), which may be involved in the protein degradation. The EST (AB193490) was a homologue of a penicillin-binding protein (Sauvage et al., 2008), which may play roles in the secondary metabolism. Four ESTs (AB193486, AB193488, AB193491 and AB193495) were homologous

with ADP-ribosylation factor, whose function in mammalian cells is as regulator of vesicular traffic and actin remodeling (Lee et al., 2008).

In summary, 13-up-expressed sequence tags (up-ESTs) have been isolated from the mutant MK-1 of *M. pilosus* IFO4520. These up-ESTs might be related with the three main phenotypes of the mutant MK-1, that is: high productivity of lovastatin and red pigments, slow growth of

the fungal mycelia.

ACKNOWLEDGMENTS

This work was supported by Foundation of Guangdong Province, China (2010B011000009, 2007A0201000001), and Japan Society for the Promotion of Science. It is also partly supported by the fund of Key Laboratory of Plant Resources Conservation and Sustainable Utilization, South China Botanical Garden, Chinese Academy of Sciences

REFERENCES

- Baker TA, Sauer RT (2006). ATP-dependent proteases of bacteria: recognition logic and operating principles. *Tred. Biochem. Sci.*, 31: 647-653.
- Casa-Lopez JL, Sanchez-Perez JA, Fernandez-Sevilla JM, Acien-Fernandez FG, Molina-Grima M, Chisti Y (2003). Production of lovastatin by *Aspergillus terreus*: effects of C:N ratio and the principal nutrients on growth and metabolite production. *Enzyme Microbiol. Technol.*, 33: 270-277.
- Chen YP, Tseng CP, Liaw LL, Wang CL, Chen IC, Wu WJ, Wu MD, Yuan GF (2008). Cloning and characterization of lovastatin biosynthetic gene cluster from *Monascus pilosus*. *J. Agric. Food Chem.*, 56: 5639-5646.
- Endo A (1992). The discovery and development of HMG-CoA reductase inhibitors. *J. Lipid Res.*, 33: 1569-1582.
- Hajjaj H, Niederberger P, Duboc P (2001). Lovastatin biosynthesis by *Aspergillus terreus* in a chemically defined medium. *Appl. Environ. Microbiol.*, 67: 2596-2602.
- Houterman PM, Ma L, Ooijen GV, De Vroomen MJ, Cornelissen BGC, Takken FLW, Rep M (2009). The effector protein Avr2 of the xylem-colonizing fungus *Fusarium oxysporum* activates the tomato resistance protein I-2 intracellularly. *Plant J.*, 58: 970-978.
- Jitrapakdee S, Maurice MS, Rayment I, Cleland WW, Wallace JC, Attwood PV (2008). Structure, mechanism and regulation of pyruvate carboxylase. *Biochem. J.*, 413: 369-387.
- Kennedy J, Auclair K, Kendrew SG, Park C, Vederas JC, Hutchinson CR (1999). Modulation of polyketide synthase activity by accessory proteins during lovastatin biosynthesis. *Science*, 284: 1368-1372.
- Lee I, Doray B, Govero J, Kornfeld S (2008). Binding of cargo sorting signals to AP-1 enhances its association with ADP ribosylation factor 1-GTP. *J. Bio. Chem.*, 180: 467-472.
- Miyake T, Mori A, Kii T, Okuno T, Usui Y, Sato F, Sammoto H, Watanabe A, Kariyama M (2005). Light effects on cell development and secondary metabolism in *Monascus*. *J. Ind. Microbiol. Biotechnol.*, 32: 103-108.
- Miyake T, Uchitomi K, Zhang M, Kono I, Nozaki N, Sammoto H, Inagaki K (2006). Effects of principal nutrients on lovastatin production by *Monascus pilosus*. *Biosci. Biotech. Biochem.*, 70: 1154-1159.
- Omamor IB, Eziashi EI, Adekunle AA (2008). Carbon nutrition in relation to growth of three *Monascus* species isolated from decaying date fruits. *Afr. J. Microbiol. Res.*, 2: 153-155.
- Sambrook J, Russell DW (2001). *Molecular Cloning: A Laboratory Manual*. 3rd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Samiee SM, Moazami N, Haghghi S, Mohseni FA, Mirdamadi S, Bakhtiari MR (2003). Screening of lovastatin production by filamentous fungi. *Iran Biomed. J.*, 7: 29-33.
- Sauvage E, Kerff F, Terrak M, Ayala JA, Charlier P (2008). The penicillin-binding proteins: structure and role in peptidoglycan biosynthesis. *FEMS Microbiol. Rev.*, 32: 234-258.
- Tattersall AD, Turner L, Knox MR, Ambrose MJ, Ellis THN, Hofer JMI (2005). The mutant *crispa* reveals multiple roles for PHANTASTICA in pea compound leaf development. *Plant Cell*, 17: 1046-1060.
- Wang JJ, Lee CL, Pan TM (2003). Improvement of lovastatin, gamma-aminobutyric acid and citrinin production ratio as a function of environmental conditions of *Monascus purpureus* NTU 601. *J. Ind. Microbiol. Biotechnol.*, 30: 669-676.
- Yoshizawa Y, Witter DJ, Liu Y, Vederas JC (1994). Revision of the biosynthetic origin of oxygens in mevinolin (lovastatin), a hypocholesterolemic drug from *Aspergillus terreus* MF4645. *J. Am. Chem. Soc.*, 116: 2693-2694.
- Zhang MY, Miyake T (2009). Development and media regulate alternative splicing of a methyltransferase pre-mRNA in *Monascus pilosus*. *J. Agric. Food Chem.*, 57: 4162-4167.