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Cloning and Characterization of a Panel of Mitochondrial Targeting Sequences for Compartmentalization Engineering in *Saccharomyces cerevisiae*

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ABSTRACT

Mitochondrion is generally considered as the most promising subcellular organelle for compartmentalization engineering. Much progress has been made in reconstituting whole metabolic pathways in the mitochondria of yeast to harness the precursor pools (i.e. pyruvate and acetyl-CoA), bypass competing pathways, and minimize transportation limitations. However, only a few mitochondrial targeting sequences (MTSs) have been characterized (i.e. MTS of *COX4*), limiting the application of compartmentalization engineering for multi-gene biosynthetic pathways in the mitochondria of yeast. In the present study, based on the mitochondrial proteome, a total of 18 MTSs were cloned and the efficiency of these MTSs in targeting heterologous proteins, including the *Escherichia coli* FabI and enhanced green fluorescence protein (EGFP) into the mitochondria was evaluated by growth complementation and confocal microscopy. After systematic characterization, seven of the well-performed MTSs were chosen for the colocalization of complete biosynthetic pathways into the mitochondria. As proof of concept, the full α-santalene biosynthetic pathway consisting of 10 genes capable of converting acetyl-coA to α-santalene was compartmentalized into the mitochondria, leading to a 3.7-fold improvement in the production of α-santalene. The newly characterized MTSs should contribute to the expanded metabolic engineering and synthetic biology toolbox for yeast mitochondrial compartmentalization engineering.

KEYWORDS: Mitochondrial targeting sequences, *Saccharomyces cerevisiae*, Compartmentalization engineering, Metabolic engineering, α-Santalene

1. INTRODUCTION

Saccharomyces cerevisiae is the most well-characterized and widely used model eukaryotic organism and has been developed as a platform cell factory via various metabolic engineering strategies (Borodina and Nielsen 2014; Botstein and Fink 2011; Lian et al. 2018). Its well-characterized genetic background, abundant genetic tools, fast growth rate, and robust fermentation performance are highly conducive to recombinant strain construction and subsequent mass production processes. Numerous high value-added compounds such as artemisinic acid (Ro et al. 2006), thebaine (Galanie et al. 2015), and farnesene (Meadows et al. 2016) have been successfully produced by reconstructing their metabolic pathways in *S. cerevisiae*.

While most engineered metabolic pathways are located in the yeast cytoplasm, compartmentalization engineering that redirects the whole metabolic pathways to a subcellular organelle also represents an attractive metabolic engineering strategy. Yeast compartmentalization engineering has demonstrated advantages for the production and accumulation of the desirable compounds in several cases, i.e., reconstructing the synthetic pathway in the endoplasmic reticulum (ER) for morphine or plant terpenoid production (Arendt et al. 2017; Thodey et al. 2014), insulating a toxic enzyme into peroxisome to alleviate cellular toxicity for alkaloid overproduction (Grewal et al. 2020), and utilizing the abundant precursors such as pyruvate and acetyl-CoA to synthesize terpenoids in the mitochondria (Farhi et al. 2011; Lv et al. 2016; Yuan and Ching 2016). The various yeast organelles can provide isolated spaces and specific microenvironments that are beneficial for some specific enzymes or pathways in metabolic engineering applications.

Mitochondrion, working as the cellular powerhouse (Gray et al. 1999), has been equipped with some unique traits, i.e. carrying their own genetic machinery, containing abundant precursors (such as acetyl-CoA, ATP, NADH, and fatty acyl-AC), and showing a more reducing redox microenvironment with higher pH and lower oxygen concentration (Avalos et al. 2013; Galdieri et al. 2014; Hu et al. 2008; Orij et al. 2009; Schnell et al. 1992). Together with the advantages of higher local enzyme concentration and minimized competing pathways, mitochondrial compartmentalization had been demonstrated to improve the production of various value-added compounds such as valencene (Farhi et al. 2011), isobutanol (Avalos et al. 2013), isoprene (Lv et al. 2016), and amorpha-4,11-diene (Yuan and Ching 2016). To enable mitochondrial compartmentalization, N-terminal mitochondrial targeting sequences (MTSs) are required to relocalize the target enzymes (generally synthesized in the cytoplasm as cytosolic pre-proteins) into the mitochondria. However, only a few MTSs have been characterized, limiting the application of mitochondrial compartmentalization for whole multi-gene biosynthetic pathways. Currently, the MTSs of *COX4* (subunit IV of the cytochrome c oxidase) and *COQ3* (O-methyltransferase in Coenzyme Q biosynthesis) were the best characterized, with the MTS of *COX4* used most frequently for mitochondrial compartmentalization. Although the same MTS could be used repeatedly to localize the whole pathway (i.e. 8 genes of the mevalonate pathway) into the mitochondria (Lv et al. 2016; Yuan and Ching 2016), there are considerable concerns for genome instability with regard to the high recombinant efficiency of *S. cerevisiae* and potential competition during the mitochondria importing process. Therefore, systematic characterization of a panel of mitochondrial targeting sequences is highly desirable for expanding the yeast metabolic engineering toolbox for mitochondrial compartmentalization.

In the present study, a panel of yeast MTSs were cloned, characterized, and adopted for mitochondrial compartmentalization. Firstly, based on the mitochondrial proteome study, 18 MTSs of the most abundant mitochondrial proteins were chosen and cloned. MTSs were evaluated for their capability to relocalize the *Escherichia coli* FabI protein into the mitochondria of a respiration deficiency strain (*etr1* deletion) for growth complementation. Then, 10 highly active MTSs that restored the cell growth in non-fermentable medium (YPE) were tagged to enhanced green fluorescence protein (EGFP) for fluorescence confocal microscopy analysis. Finally, 6 MTSs with excellent performance were chosen for mitochondrial compartmentalization of multi-gene pathways. As a proof of concept, a total of 10 expression cassettes containing the complete biosynthetic pathway for the conversion of acetyl-CoA to α-santalene were colocalized into the mitochondria, resulting in a 3.7-fold improvement in the production of α-santalene. The newly characterized MTSs should enable the development of sophisticated compartmentalization engineering strategies to harness the yeast mitochondria for the production of high value-added compounds.

2. MATERIALS AND METHODS

2.1 Strains, cultivation conditions, and reagents

E. coli DH5α was used for plasmid cloning and propagation and recombinant *E. coli* strains were cultured with Luria-broth medium supplemented with 100 μg/mL ampicillin at 37°C. CEN.PK2-1C was the host for growth complementation, fluorescence confocal microscopy analysis, α-santalene pathway assembly, and α-santalene fermentation. Wild type yeast strain was cultured in YPD medium (1% yeast extract, 2% peptone, and 2% glucose). Recombinant yeast strains were maintained in synthetic medium (SCD-LEU and SCD-HIS-URA), containing 1.7 g/L YNB (Difco,

Boom, The Netherlands), 5 g/L ammonium sulfate, 20 g/L glucose, and CSM-LEU or CSM-HIS-URA (MP Biomedicals, Solon, Ohio). Growth complementation using non-fermentable carbon source was tested in YPE medium (1% yeast extract, 2% peptone, and 2% ethanol). Q5 polymerase, restriction enzymes, and T4 DNA ligase were purchased from NEB (Ipswich, MA) and all chemicals were purchased from Sigma (Sigma Aldrich, St. Louis, MO) unless stated otherwise.

2.2 Plasmid construction

18 MTSs of the most abundant mitochondrial proteins (Sickmann et al. 2003) were PCR amplified from the genome of CEN.PK2-1C and cloned into the *FabI* expression plasmid pRS425-*ENO2p-NheI-BamHI-FabI-PGK1t*, resulting in the construction of a series of pRS425-*ENO2p*-MTSs-*FabI-PGK1t* plasmids. To visualize the mitochondrial localization via confocal microscope, the selected MTSs were cloned into *EGFP* expression plasmid pH3 (pRS425-*ENO2p-NheI-BamHI-EGFP-PGK1t*). To facilitate the cloning and assembly of mitochondrial targeting pathways, a series of mitochondria helper plasmids (pRS425-promoter-*NheI*-MTSs-*BamHI-EGFP*-terminator) were constructed based on the previously developed helper plasmids (Lian and Zhao 2015). For mitochondrial pathway assembly, the mevalonate pathway genes and α-santalene synthase encoding gene were PCR amplified from the yeast genome and pRS425-*SanSyn* (Dong et al. 2020), respectively, and subcloned to the mitochondrial helper plasmids. Individual gene expression cassettes, *GMP1p*-MTS1-*ERG10-ADH1t*, *GDPp*-MTS17-*MVD1-CYC1t*, *ENO2p*-MTS4-*IDI1-PGK1t*, *TPI1p*-MTS8-*tHMGR1-TPI1t*, *TEF1p*-MTS12-*SanSyn-TEF1t*, *GMP1p*-MTS1-*tHMGR1-ADH1t*, *GDPp*-MTS17-*ERG8-CYC1t*, *ENO2p*-MTS4-*ERG13-PGK1t*, *TPI1p*-MTS12-*ERG20-TPI1t*, and *TEF1p*-MTS16-*ERG12-TEF1t*, were further amplified and assembled with the linearized pRS413 or pRS416 using DNA Assembler (Shao et al. 2009), leading to the

construction of two mitochondrial pathway plasmids pRS413-Mito1 and pRS416-Mito2. Yeast plasmids were extracted using the Zymoprep Yeast Plasmid Miniprep II Kit (Zymo Research, Irvine, CA) and amplified in *E. coli*. All plasmids used in this study were listed in Table 1 and oligonucleotides (TSINGKE Biological Technology, Hangzhou, China) used for PCR amplification, plasmid assembly, and diagnostic PCR verification were listed in Supplementary Table S1.

2.3 Strain construction

The *etr1* deficient yeast strain was constructed using the *loxP-KanMX-lopX* method and selected on YPD agar plates supplemented with 200 μg/mL G418. The deletion of the target gene was verified by diagnostic PCR. Yeast strains were transformed using the LiAc/ssDNA/PEG method (Gietz and Schiestl 2007), and transformants were selected on the appropriate selection plates.

2.4 Fluorescence confocal microscopy analysis

Fluorescence visualization and organelle staining were carried out by following the previously established protocols (Baggett et al. 2003) or manufacturer's instructions. For mitochondrial staining, overnight culture was inoculated into 5 mL SCD-LEU medium with an initial OD600 of 0.05, and cultivated at 30°C and 250 rpm for ~18 hours when OD600 reached ~0.6-1.0. Mitotracker Red CMXRos (Molecular Probes, 1 mM solution in DMSO) was added at a final concentration of 200 nM. The yeast cells were incubated at 30°C (covered with foil, 250 rpm) for 10 min, pelleted, and resuspended in 100 μL phosphate buffer (135 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, and 1.4 mM KH_2PO_4, pH=7.4) with 2% glucose. After staining, cells were mounted onto poly-L-lysine coated slides and visualized using a 63X oil immersion

objective lens on a Nikon A1 Confocal Microscope (Nikon, Tokyo, Japan) with an Argon 488 nm laser and a HeNe 543 nm laser.

2.5 α-Santalene fermentation and quantification

α-Santalene producing strains with different combinations of plasmids were pre-cultured in SCD-HIS-URA and inoculated to 5 mL fresh medium with 0.5 ml *n*-dodecane for a two-phase fermentation. After cultivation for 3 days in a 30°C and 250 rpm shaker, the supernatant *n*-dodecane layer was collected for α-santalene quantification. 1 μL diluted sample (with an equal volume of *n*-dodecane, containing caryophyllene as the internal standard) was injected into the SHIMADZU QP2010SE GC-MS system (SHIMADZU, Tokyo, Japan) for analysis, following a previously established protocol (Dong et al. 2020). α-Santalene levels were determined via the internal standard method and normalized to the cell density.

3. RESULTS

3.1 Selection and cloning of 18 MTSs

The yeast mitochondria harbor a minimized proteome of ~700 proteins (Sickmann et al. 2003). While only a few mitochondria proteins are encoded by the mitochondrial genome, most of the mitochondrial proteins (~95%) are synthesized in the cytosol and redirected to the mitochondria generally with an N-terminal mitochondrial targeting sequence (MTS). Assuming that abundant proteins should have efficient MTSs for mitochondrial targeting, 16 mitochondrial proteins with the highest abundance were selected for subsequent studies in the present study. In addition, the two commonly used MTSs of *COX4* and *COQ3* were included as positive controls. MTSs of the selected proteins were obtained from NCBI annotation as well as predicted by the online tool MITOPROT (https://ihg.gsf.de/ihg/mitoprot.html). The longer MTSs were

cloned for further characterization, with an additional amino acid (aa) included for MTSs removal after redirecting the target proteins (Maarse et al. 1984) (Table 2 and Supplementary Table S2).

3.2 Characterization of MTSs via growth complementation

The mitochondrial targeting efficiency of the selected MTSs was evaluated by growth complementation of the *etr1* deficient yeast strain on non-fermentable carbon sources (i.e. ethanol and glycerol) via importing its *E. coli* orthologue (FabI) into the mitochondria (Fig. 1A). *ETR1* encodes a mitochondrial 2-enoyl thioester reductase involved in mitochondrial fatty acid biosynthesis, whose deletion resulted in growth failure on non-fermentable carbon sources (Martinez et al. 2004; Torkko et al. 2001). FabI (NADPH-dependent 2-enoyl-acyl carrier protein reductase), the *E. coli* orthologue of Etr1p, should be tagged with MTSs to complement the growth of the *etr1* deletion strain (Torkko et al. 2001). Therefore, the 18 selected MTSs were cloned into the *FabI* expression plasmid pRS425-*ENO2p-BamHI-FabI-PGK1t* and the resultant plasmids were transformed into the *etr1* null strain, followed by the evaluation of the growth on non-fermentable medium YPE. As shown in Figure 1B, all strains showed cell growth recovery although to different degrees when compared with the negative control strain (expressing FabI in the cytosol of yeast), indicating all MTSs were able to redirect FabI into the mitochondria with various efficiencies. MTS1, MTS3, MTS4, MTS8, MTS9, MTS10, MTS12, MTS16, MTS17, and MTS18 enabled the best growth complementation and were selected for further characterization.

3.3 Characterization of selected MTSs via confocal microscopy analysis

After the growth complementation assays, the selected MTSs were further characterized via fluorescence confocal microscopy. Thus, the selected MTSs were

cloned into *EGF*P expression plasmid pH3 (pRS425-*ENO2p-BamHI-EGFP-PGK1t*). A yeast strain expressing cytosolic EGFP was included as a negative control. Fluorescence distribution of EGFP was visualized using a fluorescence confocal microscope and a MitoTracker Red dye was used to indicate the location of mitochondria. As shown in Fig. 2, the fluorescence of EGFP was highly condensed and matched the location of the mitochondria for MTS1, MTS4, MTS8, MTS9, MTS12, MTS16, and MTS17, while the fluorescence was distributed in both cytoplasm and mitochondria for MTS3, MTS10, and MTS18. These results indicated that MTS1, MTS4, MTS8, MTS9, MTS12, MTS16, and MTS17 were able to import most of EGFP to the mitochondria, while MTS3, MTS10, and MTS18 only enabled partial mitochondrial targeting. Considering the high efficiency in redirecting both FabI and EGFP to the mitochondria, MTS1, MTS4, MTS8, MTS9, MTS12, MTS16, and MTS17 could be used for mitochondrial compartmentalization of the whole multi-gene biosynthetic pathways.

3.4 Mitochondrial compartmentalization of the whole α-santalene biosynthetic pathway using multiple MTSs

Finally, compartmentalization of a complete metabolic pathway into the mitochondria was attempted using the multiple MTSs characterized in the present study. To facilitate the assembly of multi-MTS-gene pathways, six mitochondrial helper plasmids (pMH101, pMH102, pMH103, pMH104, pMH105, and pMH106, harboring MTS1, MTS17, MTS4, MTS8, MTS12, and MTS16, respectively) were constructed, based on the previously developed helper plasmids (Lian and Zhao 2015). Using the additional promoters or terminators as the homologous recombination arms, multi-MTS-gene pathways can be constructed in a single step using via the DNA Assembler method (Shao et al. 2009).

To demonstrate the utility of these characterized MTSs and helper plasmids for mitochondria compartmentalization engineering, the mevalonate (MVA) pathway and the α-santalene biosynthesis pathway were reconstituted to enable the conversion of acetyl-CoA to α-santalene in the yeast mitochondria. The yeast MVA pathway is located in the cytosol (Kuzuyama and Seto 2012) and responsible for producing the important precursor, farnesyl pyrophosphate (FPP), for the synthesis of numerous terpenoid compounds. α-Santalene is a plant sesquiterpene synthesized from FPP by a santalene synthase (*SanSyn*) and serves as the intermediate for the biosynthesis of santalol (an important traditional medicine and perfumery ingredient) (Scalcinati et al. 2012). FPP could be translocated from the cytosol into the mitochondria, which had been proved to be a limiting step for subsequent conversions (Farhi et al. 2011; Yuan and Ching 2016). By relocalizing the whole MVA pathway into the mitochondria with abundant acetyl-CoA supply, α-santalene production should be significantly improved. The complete MVA pathway genes (*ERG10*, *ERG13*, *tHMGR*, *ERG12*, *ERG8*, *ERG19*, *IDI1*, and *ERG20*) and the α-santalene synthase gene (*SanSyn*) were cloned into the mitochondrial helper plasmids (pMH101–pMH105) (Table 2). As tHMGR has been determined to be the rate-limiting enzyme, two copies of MTS-*tHMGR* were included in two separate plasmids (pRS413-Mito1 and pRS416-Mito2, Table1).

The α-santalene production level was evaluated in recombinant strains harboring different combinations of plasmids using two-phase fermentation. As showed in Fig.3, the targeting of *SanSyn* into mitochondria resulted in a 50% decrease in the production level of α-santalene, indicating that the supply of FPP might be limiting in the mitochondria. The compartmentalization of the full MVA pathway into mitochondria significantly enhanced FPP supply and accordingly the production level of α-santalene was increased by more than 3.7-fold. The compartmentalization of a partial MVA

pathway (p416-Mito2) had no effect on the production level of α-santalene, indicating that the full MVA pathway was required for the synthesis of FPP from acetyl-CoA in the yeast mitochondria. Considering that most of the enzymes and intermediates of the MVA pathway were absent in the yeast mitochondria, the enhancement of α-santalene production by the complete pathway but not the incomplete pathway indicated that all the enzymes of the full pathway were successfully compartmentalized into the mitochondria.

5. DISCUSSION

In this study, a total of 18 MTSs were cloned and evaluated via growth complementation and fluorescence confocal microscopy analysis. Seven MTSs demonstrated excellent performance in targeting both the *E. coli* FabI and EGFP to the mitochondria, indicating their potential application in the compartmentalization of other heterologous proteins or pathways into the mitochondria. In addition, a series of mitochondrial helper plasmids (pMH101-pMH106, Table 1) were constructed to facilitate the assembly of multi-MTS-gene pathways for metabolic engineering and synthetic biology applications. As a proof of concept, the complete MVA pathway and the α-santalene synthesis pathway were compartmentalized into the yeast mitochondria, leading to ~4-fold improvement in the production of α-santalene.

The *E. coli* FabI tagged with the 18 MTSs could recover the growth of the *etr1* deficient strain to various degrees, indicating their difference in the mitochondrial targeting efficiency for heterologous proteins. Notably, MTS18 (MTS of *COQ3*) was commonly used in previous studies to either simply relocate proteins into the mitochondria (Gurvitz 2009; Hsu et al. 1996) or enable mitochondrial compartmentalization of multi-gene pathways (Yuan and Ching 2016). However, MTS18 was found to relocate FabI and EGFP to the mitochondria at a much lower

efficiency than those characterized in the present study (i.e. MTS1, MTS4, MTS8, MTS9, MTS12, MTS16, and MTS17) and should be excluded in future compartmentalization engineering studies.

In the proof-of-concept study, the targeting of SanSyn to the mitochondria turned out to lower the α-santalene production level, when compared with the untagged SanSyn in the cytosol (Fig. 3B). In previous studies, the amorpha-4,11-diene production level was significantly improved when relocalizing the amorpha-4,11-diene synthase (ADS) into the mitochondria (Farhi et al. 2011), while the isoprene productivity was lower with mitochondrially targeted isoprene synthase (ISPS) (Lv et al. 2016). The discrepancy in the effects of mitochondrial targeting on the production level might result from the unique microenvironment of the mitochondria matrix, which was favorable for some proteins (Blumhoff et al. 2013; Farhi et al. 2011). In other words, the effects of compartmentalization on heterologous enzyme activities or terpenoid production levels should be evaluated case by case.

In conclusion, a panel of MTSs was systematically characterized for the first time, many of which demonstrated excellent mitochondria targeting performance. Together with the constructed mitochondrial helper plasmids, a metabolic engineering toolbox was established to facilitate fast assembly and efficient colocalization of a full pathway into the mitochondria. This study should contribute to the fast-developing compartmentalization engineering to harness the unique microenvironment of the mitochondrial matrix and the abundant mitochondria acetyl-CoA pool.

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AUTHOR CONTRIBUTIONS

J.L. and H.Z. conceived the study. C.D., Z.S., and J.L. performed the experiments. C.D. drafted the manuscript. L.H., J.L., Z.X., and H.Z. revised the manuscript. All authors approved the manuscript.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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417

418 **Table 1.** List of plasmids used in this study

Name	Description	Reference
pRS413	*HIS3* marker with CEN/ARS4 origin	This lab
pRS416	*URA3* marker with CEN/ARS4 origin	This lab
pMTS1-FABI	pRS425-*ENO2p*-MTS1-*FabI-PGK1t*	This study
pMTS2-FABI	pRS425-*ENO2p*-MTS2-*FabI-PGK1t*	This study
pMTS3-FABI	pRS425-*ENO2p*-MTS3-*FabI-PGK1t*	This study
pMTS4-FABI	pRS425-*ENO2p*-MTS4-*FabI-PGK1t*	This study
pMTS5-FABI	pRS425-*ENO2p*-MTS5-*FabI-PGK1t*	This study
pMTS6-FABI	pRS425-*ENO2p*-MTS6-*FabI-PGK1t*	This study
pMTS7-FABI	pRS425-*ENO2p*-MTS7-*FabI-PGK1t*	This study
pMTS8-FABI	pRS425-*ENO2p*-MTS8-*FabI-PGK1t*	This study
pMTS9-FABI	pRS425-*ENO2p*-MTS9-*FabI-PGK1t*	This study
pMTS10-FABI	pRS425-*ENO2p*-MTS10-*FabI-PGK1t*	This study
pMTS11-FABI	pRS425-*ENO2p*-MTS11-*FabI-PGK1t*	This study
pMTS12-FABI	pRS425-*ENO2p*-MTS12-*FabI-PGK1t*	This study
pMTS13-FABI	pRS425-*ENO2p*-MTS13-*FabI-PGK1t*	This study
pMTS14-FABI	pRS425-*ENO2p*-MTS14-*FabI-PGK1t*	This study
pMTS15-FABI	pRS425-*ENO2p*-MTS15-*FabI-PGK1t*	This study
pMTS16-FABI	pRS425-*ENO2p*-MTS16-*FabI-PGK1t*	This study
pMTS17-FABI	pRS425-*ENO2p*-MTS17-*FabI-PGK1t*	This study
pMTS18-FABI	pRS425-*ENO2p*-MTS18-*FabI-PGK1t*	This study
pMTS1-EGFP	pRS425-*ENO2p*-MTS1-*EGFP-PGK1t*	This study
pMTS3-EGFP	pRS425-*ENO2p*-MTS3-*EGFP-PGK1t*	This study
pMTS4-EGFP	pRS425-*ENO2p*-MTS4-*EGFP-PGK1t*	This study
pMTS8-EGFP	pRS425-*ENO2p*-MTS8-*EGFP-PGK1t*	This study
pMTS9-EGFP	pRS425-*ENO2p*-MTS9-*EGFP-PGK1t*	This study
pMTS10-EGFP	pRS425-*ENO2p*-MTS10-*EGFP-PGK1t*	This study
pMTS12-EGFP	pRS425-*ENO2p*-MTS12-*EGFP-PGK1t*	This study
pMTS16-EGFP	pRS425-*ENO2p*-MTS16-*EGFP-PGK1t*	This study
pMTS17-EGFP	pRS425-*ENO2p*-MTS17-*EGFP-PGK1t*	This study
pMTS18-EGFP	pRS425-*ENO2p*-MTS18-*EGFP-PGK1t*	This study
pMH101	pRS425-*GMP1p*-*NheI*-MTS1-*BamHI*-*EGFP*-*XhoI*-*PYK1t*	This study
pMH102	pRS425-*AvrII*-*ADH1t*-GPDp-*NheI*-MTS17-*BamHI*-EGFP-*XhoI*-*CYC1t*-SbfI-*ENO2p*	This study
pMH103	pRS425-*SbfI*-*ENO2p*-*NheI*-MTS4-*BamHI*-EGFP-*XhoI*-*PGK1t*-*NotI*-*TPI1p*	This study
pMH104	pRS425-*NotI*-*TPI1p*-*NheI*-MTS8-*BamHI*-EGFP-*XhoI*-*TPI1t*-*SacII*-*TEF1p*	This study
pMH105	pRS425-*SacII*-*TEF1p*-*NheI*-MTS12-*BamHI*-EGFP-*XhoI*-*TEF1t*-*XmaI*	This study
pMH106	pRS425-*TEF1t*-*PGK1p*-*NheI*-MTS16-*BamHI*-EGFP-*HindIII*-*HXT7t*	This study
pERG10mt	pMH101-*ERG10*	This study
pMVD1mt	pMH102-*MVD1*	This study
pIDI1mt	pMH103-*IDI1*	This study

ptHMGR1mt	pMH104-*tHMGR*	This study
pSanSynmt	pMH105-*SanSyn*	This study
ptHMGR2mt	pMH101-*tHMGR*	This study
pERG8mt	pMH102-*ERG8*	This study
pERG13mt	pMH103-*ERG13*	This study
pERG20mt	pMH104-*ERG20*	This study
pERG12mt	pMH105-*ERG12*	This study
pRS413-Mito1	pRS413-*GMP1p*-MTS1-*ERG10-PYK1t-GPD*p-MTS17-*MVD1-CYC1t-ENO2p*-MTS4-*IDI1-PGK1t-TPI1p*-MTS8-*tHMGR-TPI1t-TEF1p*- MTS12-*SanSyn-TEF1t*	This study
pRS413-Mito2	pRS416-*GMP1p*-MTS1-*tHMGR-PYK1t-GPD*p-MTS17-*ERG8-CYC1t-ENO2p*-MTS4-*ERG13-PGK1t-TPI1p*-MTS8-*ERG20-TPI1t-TEF1p*-MTS12-*ERG12-TEF1t*	This study
pRS413-SanSyn	pRS413-*TEF1p-SanSyn-TEF1t*	This study
pRS413-SanSynmt	pRS413-*TEF1p*-MTS12-*SanSyn-TEF1t*	This study

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420 **Table 2.** Mitochondrial targeting sequences (MTSs) cloned for characterization

MTS	Target Genes	Predicted Targeting Sequences		Cloned for Characterization
		NCBI	MITOPROT	
MTS1	*MMF1*	1-17 aa	1-25 aa	1-26 aa
MTS2	*HSP10*	NA	1-26 aa	1-27 aa
MTS3	*SOD2*	1-26 aa	1-26 aa	1-27 aa
MTS4	*HSP60*	1-25 aa	1-22 aa	1-26 aa
MTS5	*SSC1*	1-23 aa	1-23 aa	1-24 aa
MTS6	*KGD2*	NA	1-71 aa	1-72 aa
MTS7	*LAT1*	1-28 aa	1-28 aa	1-29 aa
MTS8	*LSC2*	NA	1-38 aa	1-39 aa
MTS9	*ALD4*	1-24 aa	1-31 aa	1-32 aa
MTS10	*ACO1*	NA	1-23 aa	1-24 aa
MTS11	*CIT1*	1-37 aa	1-37 aa	1-38 aa
MTS12	*LPD1*	1-21 aa	1-28 aa	1-29 aa
MTS13	*ILV5*	1-47 aa	1-34 aa	1-48 aa
MTS14	*ADH3*	1-27 aa	1-24 aa	1-28 aa
MTS15	*IDH2*	1-15 aa	1-26 aa	1-27 aa
MTS16	*MDH1*	1-17 aa	1-17 aa	1-18 aa
MTS17	*COX4*	1-25 aa	1-25 aa	1-26 aa
MTS18	*COQ3*	1-32 aa	1-31 aa	1-33 aa

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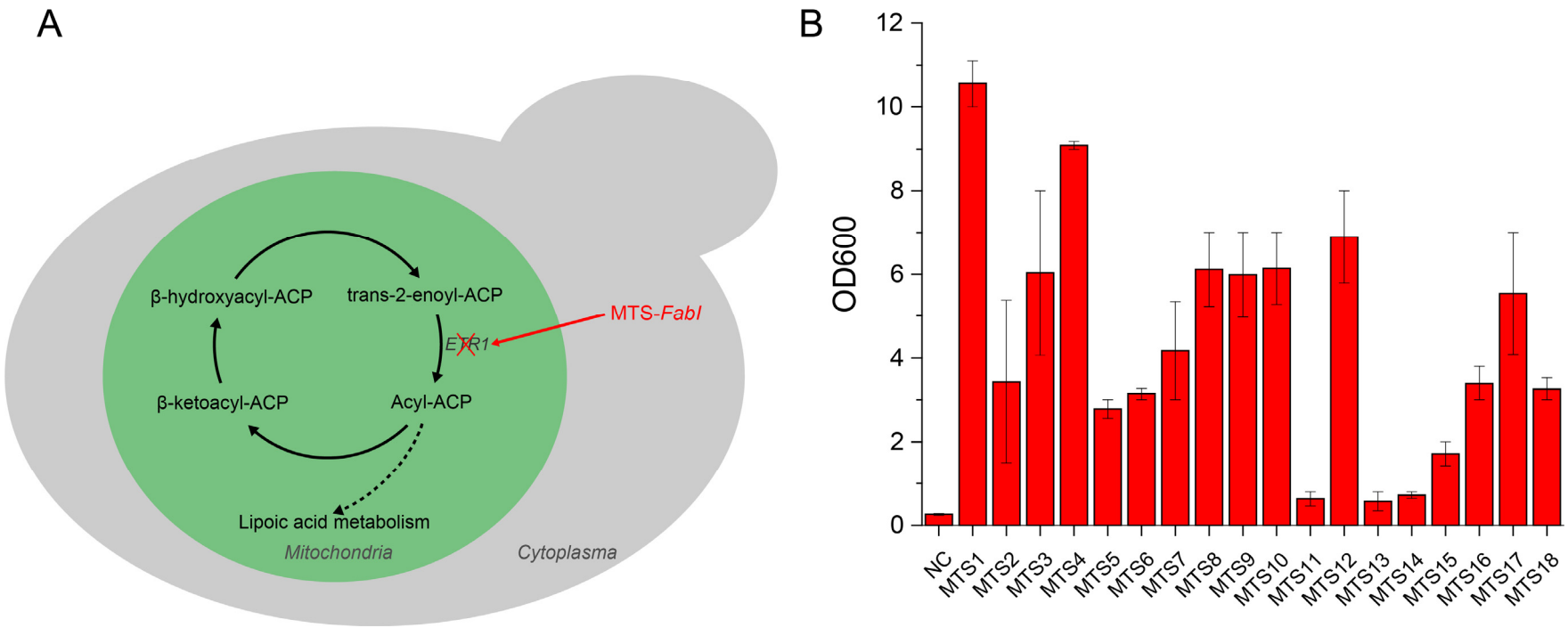
Figure legends

Figure 1. Characterization of MTSs via growth complementation. (A) A schematic view of the growth complementation by various MTS tagged FabI proteins in the *etr1* null strain. (B) Cell growth of recombinant strains harboring various MTS-*FabI* plasmids. The cell density (OD600) was determined after cultivation in the non-fermentable medium YPE for 72 h. Error bars represented the mean ± s.d. of biological triplicates.

Figure 2. Fluorescence microscopy of yeast strains expressing MTS tagged EGFP (MTS1, MTS3, MTS4, MTS8, MTS9, MTS10, MTS12, MTS16, MTS17, and MTS18). The untagged EGFP expressed in the cytosol was included as control for comparison.

Figure 3. Compartmentalization of the full pathway into the yeast mitochondria for α-santalene biosynthesis. (A) Reconstructed mitochondrial α-santalene biosynthesis pathway. HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; GPP, geranyl diphosphate; FPP, farnesyl diphosphate. The 10 expression cassettes, with *ERG10*, *ERG13*, 2x*tHMGR*, *ERG12*, *ERG8*, *MVD1*, *IDI1*, *ERG20*, and *SanSyn* tagged with their corresponding MTSs, were assembled into two plasmids, pRS413-Mito1 (harboring genes marked in red) and pRS416-Mito2 (harboring genes marked in blue). (B) Production of α-santalene in recombinant CEN.PK2-1C strains harboring different combinations of plasmids. Cytosol SanSyn: pRS413-*SanSyn* and pRS416; Mitochondrial SanSyn: pRS413-MTS12-*SanSyn* and pRS416; Partial mitochondrial pathway: pRS413-MTS12-*SanSyn* and pRS416-Mito2; Full mitochondrial pathway: pRS413-Mito1 and pRS416-Mito2; Error bars represented the mean ± s.d. of biological triplicates.

446 **Figure 1**



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449 **Figure 2**

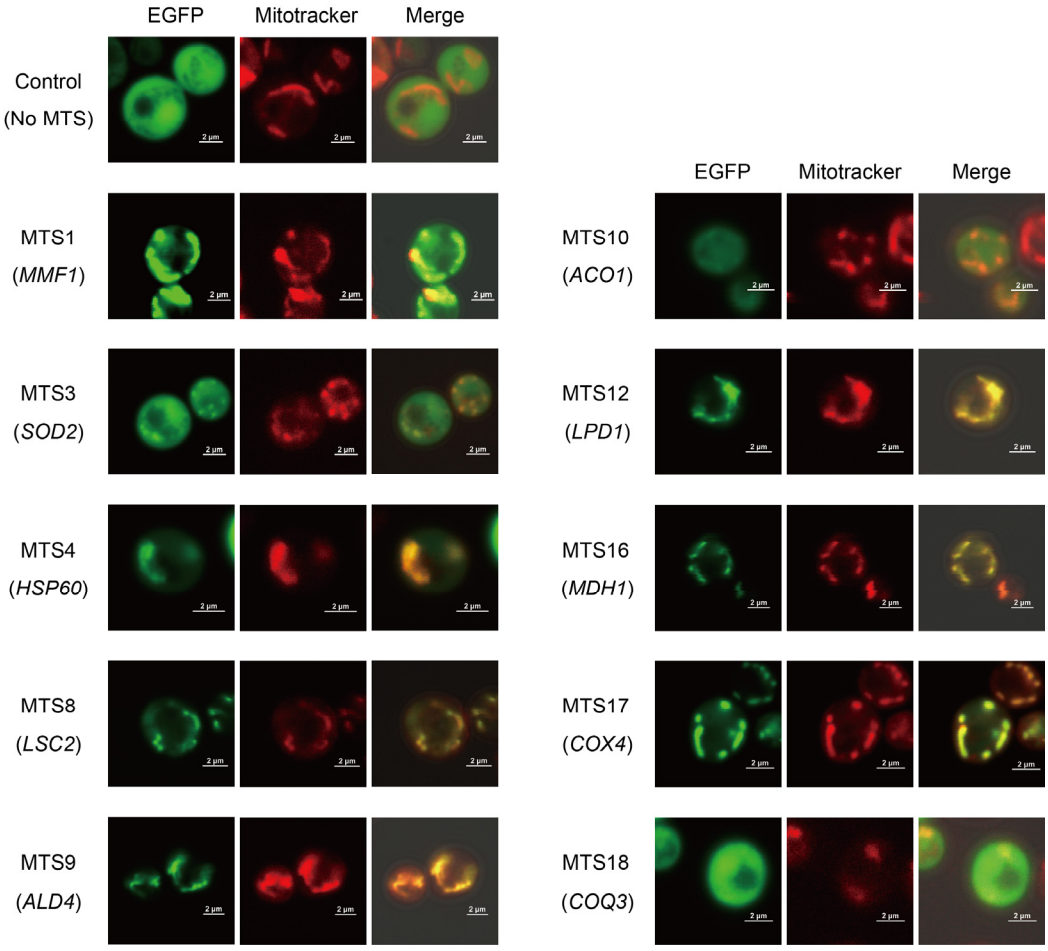

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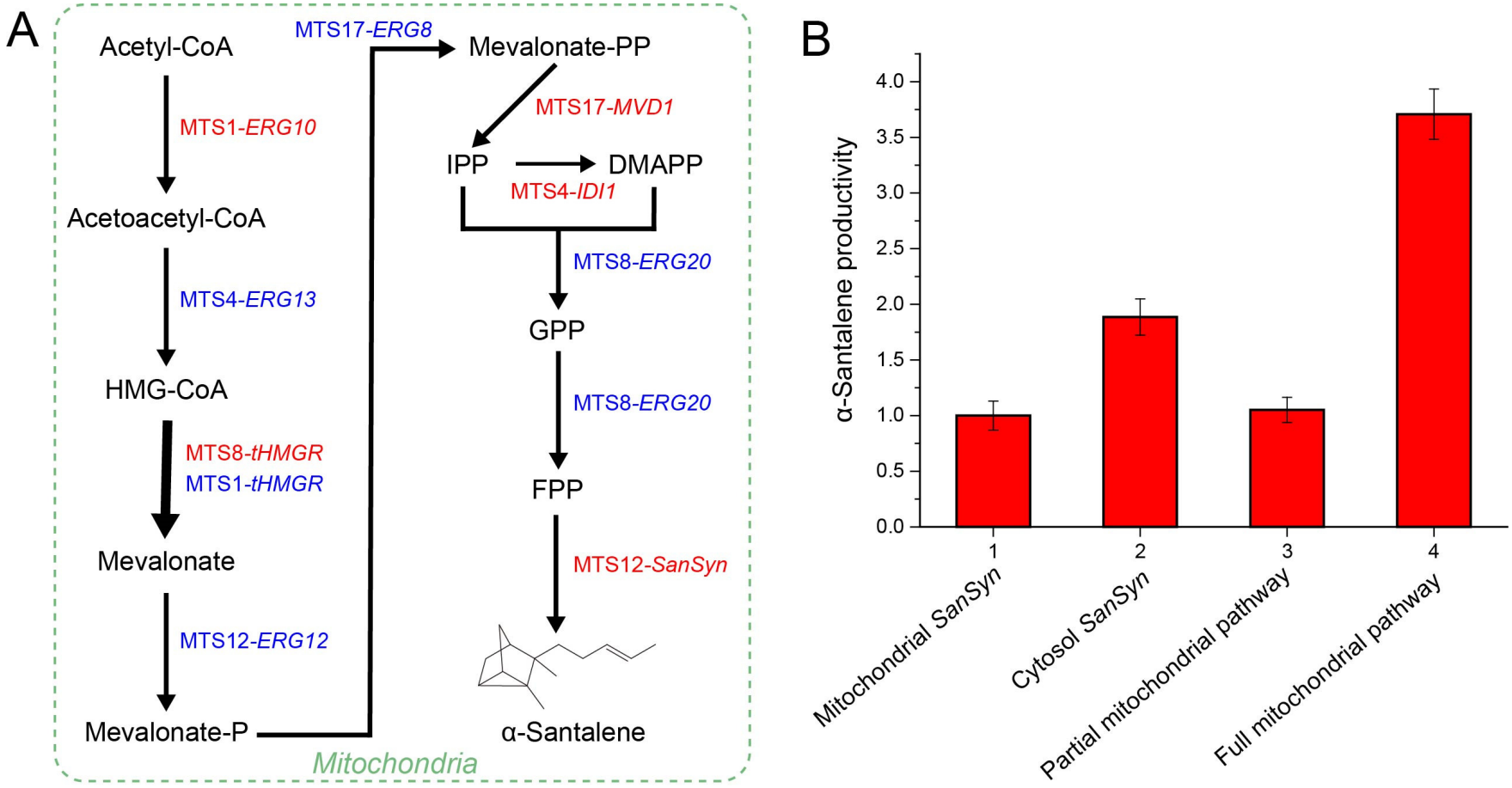
Figure 3


A
Acetyl-CoA
MTS1-ERG10
Acetoacetyl-CoA
MTS4-ERG13
HMG-CoA
MTS8-tHMGR
MTS1-tHMGR
Mevalonate
MTS12-ERG12
Mevalonate-P
MTS17-ERG8
Mevalonate-PP
MTS17-MVD1
IPP
DMAPP
MTS4-IDI1
MTS8-ERG20
GPP
MTS8-ERG20
FPP
MTS12-SanSyn
α-Santalene
Mitochondria
B
α-Santalene productivity
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
1
2
3
4
Mitochondrial SanSyn
Cytosol SanSyn
Partial mitochondrial pathway
Full mitochondrial pathway