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Manipulation of GES and ERG20 for geraniol overproduction in Saccharomyces cerevisiae

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ABSTRACT

Manipulation of monoterpene synthases to maximize flux towards targeted products from GPP (geranyl diphosphate) is the main challenge for heterologous monoterpene overproduction, in addition to cell toxicity from compounds themselves. In our study, by manipulation of the key enzymes geraniol synthase (GES) and farnesyl diphosphate synthase (Erg20), geraniol (a valuable acyclic monoterpene alcohol) overproduction was achieved in *Saccharomyces cerevisiae* with truncated 3-hydroxy-3-methylglutaryl-coenzyme reductase (tHMGR) and isopentenyl diphosphate isomerase (IDI1) overexpressed. The expressions of all above engineered genes were under the control of Gal promoter for alleviating product toxicity. Geraniol production varied from trace amount to 43.19 mg/L (CrGES, GES from *Catharanthus roseus*) by screening of nine GESs from diverse species. Further through protein structure analysis and site-directed mutation in CrGES, it was firstly demonstrated that among the high-conserved amino acid residues located in active pocket, Y436 and D501 with strong affinity to diphosphate function group, were critical for the dephosphorylation (the core step for geraniol formation). Moreover, the truncation position of the transit peptide from the N-terminus of CrGES was found to influence protein expression and activity significantly, obtaining a titer of 191.61 mg/L geraniol in strain with CrGES truncated at S43 (t3CrGES). Furthermore, directed by surface electrostatics distribution of t3CrGES and Erg20^{WW} (Erg20$^{F96W\text{-}N127W}$), co-expression of the reverse fusion of Erg20^{ww}/t3CrGES and another copy of Erg20^{WW} promoted the geraniol titer to 523.96 mg/L at shakes flask level, due to enhancing GPP accessibility led by protein interaction of t3CrGES-Erg20^{WW} and the free Erg20^{WW}. Eventually, a highest reported titer of 1.68 g/L geraniol in eukaryote cells was achieved in 2.0 L fed-batch fermentation under carbon restriction strategy. Our research opens large opportunities for other microbial production of monoterpenes. It also sets a good reference for desired compounds overproduction in microorganisms in terms of manipulation of key enzymes by protein engineering and metabolic engineering.

1. Introduction

Geraniol (3,7-dimethylocta-trans-2,6-dien-1-ol), an acyclic monoterpene alcohol, is widely used in flavor and fragrance industries (Chen and Viljoen, 2010). Besides, geraniol has exhibited good performance as a kind of plant-based insect repellent (Barnard and Rui-DeXue, 2004; Papachristos et al., 2004), antimicrobial agent (Togashi et al., 2008; Unlu et al., 2010), antitumor drug (Burke et al., 1997; Kim et al., 2011) and a gasoline alternative as well (Peralta-Yahya and Keasling, 2010). However, the limited and unstable yield of geraniol produced through plant extraction could not satisfy the market command at all. Therefore, microbial cell factories by metabolic engineering and synthetic biology provide promising alterative to ease the situation. Even though we have achieved much progress in other terpenes production in microbial factories (Paddon and Keasling, 2014; Yang et al., 2016), most of monoterpenes production in microbials is still relatively low (Zebec et al., 2016). Manipulation of monoterpene synthases to maximize flux towards targeted products from GPP is still the main challenge for heterologous monoterpene overproduction, in addition to cell toxicity from compounds themselves (Zhao et al., 2016). In last decades, several efficient approaches have been proposed and explored to engineer key enzymes involved in terpenes synthesis,

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mainly focusing on protein engineering and metabolic engineering: (1) selection of enzyme counterparts with same function (Chen et al., 2016; Ma et al., 2016). Ding and her colleagues improved the taxadiene yield to 7.2-fold through selection of six geranylgeranyl diphosphate synthases from different species (Ding et al., 2014); (2) transit peptide truncation from the N-terminus of heterologous enzymes. It was reported that Ajikumar et al. achieved more than 98% taxadiene conversion to taxadien-5α-ol and the byproduct 5(12)-Oxa-3(11)-cyclotaxane (OCT) by N-terminus truncation at a proper length in taxadien-5α-ol hydroxylase (Ajikumar et al., 2010); (3) co-location of the endogenous and heterologous enzymes by fusion, scaffold or compartments to control over local concentrations of important intermediates (Lee et al., 2016; Pham et al., 2015). Baadhe and his colleagues improved the amorphadiene production by 4-fold in yeast through coupling farnesyl diphosphate synthase and amorphadiene synthase (Baadhe et al., 2013). Definitely, systematic integration of above strategies for key monoterpenes synthases engineering could make higher monoterpenes outputs enabled.

Most monoterpene synthases contain N-terminal transit peptide that targets the initial translation product towards the plastids (Turner et al., 1999). The transit peptide would be proteolytically removed when the translated preprotein is imported into the plastids to promote the formation of mature enzyme (Bohlmann et al., 1998). *S. cerevisiae* lacks the mechanism to get rid of the plastidial transit peptide which might result in decreased enzymatic activity. Recently, Zhao showed that strain containing truncation of VoGES could get higher geraniol titer, about 3-fold increase compared with that containing full-length of VoGES in engineered yeast (Zhao et al., 2016). However, it is difficult to find out the correct transit peptide-mature protein cleavage position because no common sequence elements have been identified (Bohlmann et al., 1998; von Heijne et al., 1989) and the plastidial proteolytic processing to the native form in plants is quite imprecise (Williams et al., 1998). What' more, the N-terminal domain might act as a scaffold facilitating proper folding of the catalytically active C-terminal domain as well (Köllner et al., 2004). However, more thorough work on the mechanism of heterogenous expressed terpene synthases was rarely reported and a highest reported geraniol titer of 293 mg/L in *S. cerevisiae* is still far away from industrialization (Zhao et al., 2016). So, acquiring the right GES from more diverse species, figuring out the proper truncated N-terminus of GES accordingly, and then coupling GES and Erg20 as fusion protein could lead to a big leap and breakthrough in geraniol production.

GPP obtained from condensation of isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) is known to be the last common precursor for monoterpenes (Withers and Keasling, 2007). IPP and DMAPP are synthesized through the mevalonate (MVA) pathway (Agranoff et al., 1960) in which both tHMGR and IDI1 were verified as rate-limiting enzymes (Liu et al., 2013). Therefore, we overexpressed the tHMGR and IDI1 in *S. cerevisiae* strain CEN.PK2-1C, obtaining JGZ1 as the host for further research (Fig. 1A). In the following work, we enhanced the geraniol production significantly in *S. cerevisiae* through manipulation of GES and Erg20. To this end, three aspects of works were accomplished. Firstly, nine different species of codon-optimized geraniol synthases were selected and a dynamic geraniol production pool ranging from trace amount to 43.19 mg/L (CrGES) was obtained accordingly. Two essential amino acid residues Y436 and D501 located in active pocket of CrGES were firstly identified to be critical for the dephosphorylation (the core step for geraniol formation) due to their strong affinities to diphosphate function group by protein structure analysis and site-directed mutation. Secondly, the effect of the truncation position of the transit peptide from the N-terminus of CrGES was also investigated. The strain harboring t3CrGES achieved a titer of 191.61 mg/L geraniol, attributed much for better protein expression and activity. Finally, guided by surface electrostatics distribution of t3CrGES and Erg20^{WW} (Erg20$^{F96W-N127W}$), co-expression of the reverse fusion of Erg20^{WW}/t3CrGES and another copy of Erg20^{WW} presented the best performance in geraniol accumulation as 523.96 mg/L. Ultimately, 1.68 g/L of geraniol was obtained through fed-batch fermentation, which was the highest reported titer in eucaryote cells up to now. Our study provides a good example for monoterpenes overproduction in microbials through key enzymes manipulation, which would have profound impact on other terpenes biosynthesis. Importantly, the present study also highlights the importance of engineering proteins along with pathways as a key strategy in achieving microbial biosynthesis and overproduction of pharmaceutical and chemical products.

2. Materials and methods

2.1. Strains and medium

E. coli DH5α, which was used for plasmids construction and amplification, was purchased from BEIJING Biomed Co., Ltd and cultured at 37 °C in Luria-Bertani (LB) medium (1% tryptone, 0.5% yeast extract, and 1% NaCl). When needed, 100 μg/ml of ampicillin or 50 μg/ml of kanamycin was supplemented into the media.

S. cerevisiae CEN.PK2-1C (*MAT a, ura3-52, trp1-289, leu2-3,112, his3Δ1, MAL2-8C, SUC2*) (Entian and Kötter, 2007), used for construction of geraniol-producing strains (listed in Table 1), was obtained from EUROSCARF (Frankfurt, Germany). *S. cerevisiae* strains without plasmid were cultured in yeast extract-peptone-dextrose (YPD) medium and strains with plasmids were cultured in synthetic complete (SC) drop-out media. All the medium formulations were the same to the previous work (Su et al., 2015).

2.2. Strains and plasmids construction

The plasmids and primers used in this study were listed in Table S1 and Table S2 respectively. Plasmid pRS415K from our laboratory, which is derived from pRS415 (Addgene, USA) by substitution of kanamycin resistant gene for ampicillin resistant gene, was used to construct all the cassettes utilized in this study. The yeast inducible promoters (*GAL1*, *GAL7*) and terminators (*GPM1*, *GPD*, *FBA1*) were amplified from the genomic DNA of *S. cerevisiae* BY4741. The PCR products were assembled according to the desired order by overlap extension PCR (OE-PCR), obtaining two cassettes named T_{GPM1}-P_{GAL7}-T_{GPD} and T_{GPD}-P_{GAL1}-T_{FBA1}, respectively. Then these cassettes were digested with *Not*I-HF (New England Biolabs, USA) and inserted into the same site of pRS415K by T4 DNA ligase (New England Biolabs, USA), respectively, producing plasmids pJGZ1 and pJGZ2 with two back-to-back *Bsa*I sites between the promoter and the second terminator.

The starting *S. cerevisiae* strain yJGZ1 was constructed as follows: $P_{GAL1,10}$ promoter, T_{ADH1} and T_{TDH2} terminator and *HIS3* marker as well as the up and down 400 bp homologous arm of Gal80p were amplified from BY4741 genomic DNA. The PCR products were assembled according to the desired order by OE-PCR, obtaining the cassette named Gal80up-T_{ADH1}-$P_{GAL1,10}$-T_{TDH2}-*His*-Gal80down. Then the cassette was digested with *Not*I-HF and inserted into the same site of pRS415K, producing plasmids pJGZ3 with two back-to-back *BsmB*I sites between T_{ADH1} and $P_{GAL1,10}$ and two back-to-back *Bsa*I sites between $P_{GAL1,10}$ and T_{TDH2}. *tHMGR* amplified from BY4741 genomic DNA was digested with *BsmB*I (New England Biolabs, USA) and ligated into the same digested pJGZ3 to produce pJGZ4. The fragment Gal80up-T_{ADH1}-*IDI1*-$P_{GAL1,10}$-*tHMGR*-T_{TDH2}-*His*-Gal80down was cleaved from pJGZ4 by *Not*I-HF and inserted into a reconstructed pEASY-Blunt vector without kanamycin resistant gene and *Bsa*I sites to produce pJGZ5. Subsequently, *IDI1* amplified from BY4741 genomic DNA was inserted into pJGZ5 through *Bsa*I restriction sites to obtain pJGZ6. Finally, the integrated fragment Gal80up-T_{ADH1}-*IDI1*-$P_{GAL1,10}$-*tHMGR* T_{TDH2}-*His*-Gal80down cleaved by *Not*I-HF from pJGZ6 was transformed into CEN.PK2-1C