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Combining Gal4p-mediated expression enhancement and directed evolution of isoprene synthase to improve isoprene production in *Saccharomyces cerevisiae*

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ABSTRACT

Current studies on microbial isoprene biosynthesis have mostly focused on regulation of the upstream mevalonic acid (MVA) or methyl-erythritol-4-phosphate (MEP) pathway. However, the downstream bottleneck restricting isoprene biosynthesis capacity caused by the weak expression and low activity of plant isoprene synthase (ISPS) under microbial fermentation conditions remains to be alleviated. Here, based on a previously constructed *Saccharomyces cerevisiae* strain with enhanced precursor supply, we strengthened the downstream pathway through increasing both the expression and activity of ISPS to further improve isoprene production. Firstly, a two-level expression enhancement system was developed for the P_{GAL1}-controlled ISPS by overexpression of *GAL 4*. Meanwhile, the native *GAL1*/7/10 promoters were deleted to avoid competition for the transcriptional activator Gal4p, and *GAL80* was disrupted to eliminate the dependency of gene expression on galactose induction. The *IspS* expression was obviously elevated upon enhanced Gal4p supply, and the isoprene production was improved from 6.0 mg/L to 23.6 mg/L in sealed-vial cultures with sucrose as carbon source. Subsequently, a novel high-throughput screening method was developed based on precursor toxicity and used for ISPS directed evolution towards enhanced catalytic activity. Combinatorial mutagenesis of the resulting ISPS mutants generated the best mutant ISPSM4, introduction of which into the *GAL4*-overexpressing strain YXM29 achieved 50.2 mg/L of isoprene in sealed vials, and the isoprene production reached 640 mg/L and 3.7 g/L in aerobic batch and fed-batch fermentations, respectively. These results demonstrated the effectiveness of the proposed combinatorial engineering strategy in isoprene biosynthesis, which might also be feasible and instructive for biotechnological production of other valuable chemicals.

1. Introduction

Isoprene, as the simplest member of isoprenoids, is a key monomer for rubber production. In nature, isoprene emission is ubiquitous among plants, but it is difficult to harvest this volatile product from the extended canopy of leafy plant (Guenther et al., 2006). Therefore, plant-based isoprene production is economically unfeasible for commercial applications. At the moment, isoprene is mainly produced from petroleum-derived feedstocks. However, the unrenewable nature of petroleum resources and pollutions caused by the petrochemical industry have led to serious problems. With development of biotechnology, bio-based isoprene production employing microbial cell factories has become a sustainable and green solution (Ye et al., 2016).

The biosynthesis pathway of isoprene can be decomposed into two modules: the upstream MEP or MVA pathway and the downstream isoprene-forming pathway. In the upstream pathway, dimethylallyl diphosphate (DMAPP) is produced from pyruvate or acetyl-CoA under catalysis of multiple enzymes. In the downstream pathway, isoprene synthase (ISPS) catalyzes the conversion of DMAPP to isoprene (Fig. 1). Isoprene synthases sourced from different plants have been introduced to *Escherichia coli*, *Bacillus subtilis* or *cyanobacteria* for isoprene biosynthesis through the endogenous MEP pathway (Lindberg et al., 2010; Lv et al., 2016a, 2013; Xue and Ahring, 2011; Zhao et al., 2011). More recently, introduction of exogenous MVA pathway into *cyanobacteria* and *E. coli* has been proven as an efficient strategy towards enhanced isoprene production (Bentley et al., 2014;

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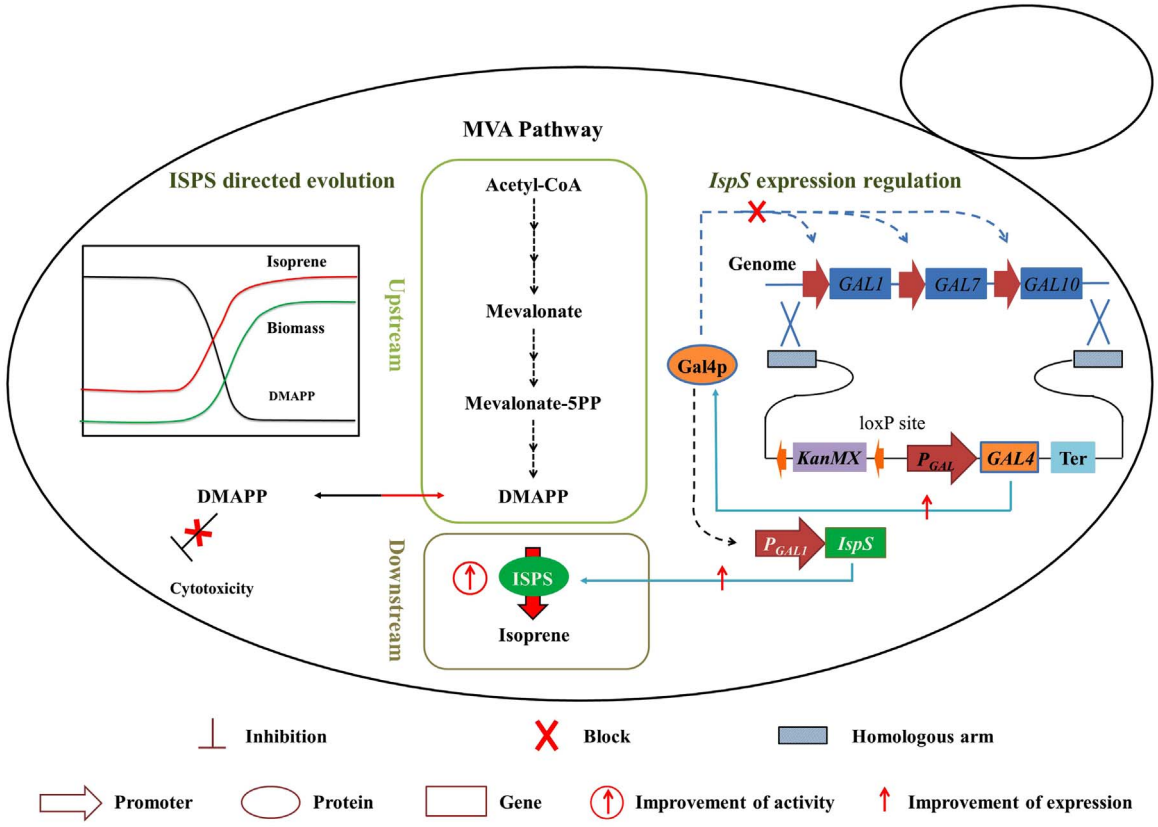

Fig. 1. Strategy of strengthening isoprene-forming pathway for high isoprene production in *S. cerevisiae*. The strategy consists of two parts. Part 1: The isoprene-forming pathway was strengthened by the enhancement of *IspS* expression. *GAL4* was overexpressed with P_{GAL1} and P_{GAL4} respectively to enhance the supply of available Gal4p in the cell. Meanwhile, the endogenous $P_{GAL1/7/10}$ was deleted to provide more Gal4p for activation of the P_{GAL1} controlling *IspS* transcription. Part 2: The isoprene synthesis pathway was further strengthened through directed evolution of ISPS towards enhanced activity. BY4742-C-04 with excessive accumulation of DMAPP was used as the host for ISPS directed evolution. DMAPP could be converted to isoprene by ISPS catalysis, so the growth rate was positively correlated with ISPS activity.

Yang et al., 2016; Zurbriggen et al., 2012). In contrast, there are relatively few studies on biosynthesis of isoprene in eukaryotes harboring the native MVA pathway such as *S. cerevisiae*, which is featured by low risk of contamination during fermentation, great potential for isoprenoids accumulation, and feasibility as possible animal feed (Chang and Keasling, 2006; Hong and Nielsen, 2012; Lv et al., 2014; Lv et al., 2016b).

Regardless of the host species, all studies about isoprene biosynthesis have laid emphasis on the regulation of precursor supply, whereas hardly any attention was paid to the downstream isoprene-forming pathway. After up-regulation of the upstream pathway, the conversion of DMAPP to isoprene becomes the bottleneck in the whole isoprene biosynthesis pathway, the insufficient efficiency of which largely limits isoprene production. Meanwhile, the cytotoxicity of DMAPP upon over-accumulation inhibits cell growth (Lu et al., 2014; Martin et al., 2003; Withers et al., 2007). Therefore, how to improve the strength of isoprene-forming pathway catalyzed by ISPS has become the key to further enhancement of microbial isoprene synthesis.

Copy number adjustment is a frequently adopted approach to enhance the expression of exogenous genes, but it has the risk to cause metabolic burden on microbial growth, exerting negative effects on high density fermentation (Karim et al., 2013). Transcriptional-level regulation is an alternative means to increase gene expression. Especially, inducible expression has become a very prevalent and efficient method for enhancing expression of exogenous genes (Guan et al., 2016; Nakajima et al., 2016). The galactose (*GAL*) regulatory network in *S. cerevisiae* is one of the most well-characterized transcriptional regulation systems whose induction and repression are tightly regulated by galactose and glucose (Johnston et al., 1994; Lohr et al., 1995). *GAL* promoters (P_{GAL}) are activated by binding the transcriptional regulator Gal4p (Giniger and Ptashne, 1988). However, in natural yeast strains, the expression level of *GAL4* gene is quite low and the amount of Gal4p is the rate-limiting factor for transcriptional activity. In a previous attempt to enhance Gal4p level by constitutive overexpression of *GAL4*, the desired regulatable feature of the system was concomitantly lost (Johnston and Hopper, 1982). In a more recent study, the genomic replacement of *GAL1* with *GAL4* (resulting in fusion of *GAL4* with the natural *GAL1* promoter) led to a 4.6-fold increase in the expression of the reporter GFP, demonstrating the potential of appropriate Gal4p overproduction in maximizing heterologous expression of proteins driven by galactose-inducible promoters (Stagoj et al., 2006). These results inspired us to examine whether *GAL4* overexpression could also act as a valid regulatory solution to metabolic bottlenecks in biosynthesis pathways by up-regulation of the P_{GAL}-driven rate-limiting pathway enzyme, here ISPS.

Aside from metabolic regulation towards elevated expression level, protein engineering of the rate-limiting enzyme for enhanced catalytic activity is another approach to eliminate metabolic bottlenecks. Directed evolution and rational design are practical strategies in protein engineering. For rational design, deep understanding on the structure and catalytic reaction mechanism of the enzyme is a prerequisite. The amino acids involved in the ISPS catalysis remain unknown, impeding its rational design, although the X-ray crystal structure of recombinant isoprene synthase from grey poplar leaves has been resolved and the reaction mechanism has been preliminarily revealed (Faraldos et al., 2012; Köksal et al., 2010). In contrast, the advantage of directed evolution lies in its independence on the structure and reaction mechanism. However, to construct an efficient high-throughput screening method is often a challenge. Generally, only distinguished phenotypes that produce color change or distinction in