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Efficient diterpene production in yeast by engineering Erg20p into a geranylgeranyl diphosphate synthase

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ABSTRACT

Terpenes have numerous applications, ranging from pharmaceuticals to fragrances and biofuels. With increasing interest in producing terpenes sustainably and economically, there has been significant progress in recent years in developing methods for their production in microorganisms. In *Saccharomyces cerevisiae*, production of the 20-carbon diterpenes has so far proven to be significantly less efficient than production of their 15-carbon sesquiterpene counterparts. In this report, we identify the modular structure of geranylgeranyl diphosphate synthesis in yeast to be a major limitation in diterpene yields, and we engineer the yeast farnesyl diphosphate synthase Erg20p to produce geranylgeranyl diphosphate. Using a combination of protein and genetic engineering, we achieve significant improvements in the production of sclareol and several other isoprenoids, including *cis*-abienol, abietadiene and *β*-carotene. We also report the development of yeast strains carrying the engineered Erg20p, which support efficient isoprenoid production and can be used as a dedicated chassis for diterpene production or biosynthetic pathway elucidation. The design developed here can be applied to the production of any GGPP-derived isoprenoid and is compatible with other yeast terpene production platforms.

1. Introduction

Terpenes are a large class of natural compounds whose numerous members have attracted industrial interest as flavors and fragrances, pharmaceuticals, or biofuels. According to the number of carbon atoms in their skeleton, basic terpene structures are classified into different groups, such as monoterpenes (C_{10}), sesquiterpenes (C_{15}), or diterpenes (C_{20}). These are formed by the action of terpene synthases on prenyl diphosphate substrates of the same size, i.e., geranyl diphosphate (GPP) for monoterpenes, farnesyl diphosphates (FPP) for sesquiterpenes, or geranylgeranyl diphosphate (GGPP) for diterpenes (Fig. 1). The prenyl diphosphate substrates themselves are formed by the sequential addition of the 5-carbon building block isopentenyl diphosphate (IPP) initially on dimethylallyl diphosphate (DMAPP), and, subsequently, on the resulting GPP and FPP, by specific prenyl diphosphate synthases of varying specificity (e.g. FPP synthase in mammals, GGPP and FPP synthases in plants, or Erg20p and Bts1p in *Saccharomyces cerevisiae*). Further modification of the various terpene scaffolds produced by this modular biosynthetic process gives rise to a vast diversity of natural structures with more than 50,000 members (Dewick, 2009; McGarvey and Croteau, 1995).

Several members of the 20-carbon diterpene group have found industrial application. For example, paclitaxel (or taxol; from the pacific yew *Taxus brevifolia*) prevents microtubule de-polymerization and is widely used as a chemotherapeutic agent (Goldspiel, 1997; Schiff et al., 1979). Total paclitaxel synthesis is feasible, but not

Abbreviations: geranyl diphosphate, GPP; dimethylallyl diphosphate, DMAPP; isopentenyl diphosphate, IPP; farnesyl diphosphate, FPP; geranylgeranyl diphosphate, GGPP; nerolidol, NOH; farnesol, FOH; geraniol, GOH; linalool, LOH; geranylgeraniol, GGOH; geranyllinalool, GLOH; 8-hydroxycopalyl diphosphate, 8OH-CPP; *Salvia sclarea* labdenediol diphosphate synthase, *Ss*LPP; *Salvia sclarea* sclareol synthase, *Ss*SCLS; *Cistus creticus* geranylgeranyl diphosphate synthase, *Cc*GGPPS; *Cistus creticus* 8-hydroxycopalyl diphosphate synthase, *Cc*CLS; *Salvia pomifera* copalyl diphosphate synthase, *Sp*CDS; *Salvia fruticosa* copalyl diphosphate synthase, *Sf*CDS; *Nicotiana tabacum* abienol synthase, *Nt*ABS; abietadiene synthase, PaLAS; *X. dendrorhous* phytoene desaturase, crtI; *X. dendrorhous* phytoene synthase/lycopene cyclase, crtYB; *X. dendrorhous* GGPP synthase, crtE; dry cell weight, DCW

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commercially viable, and medicinal paclitaxel can be produced either by semi-synthesis from 10-deacetyl baccatin or by *Taxus* cell cultures (Bringi et al., 1993; Gibson, 1999). Another diterpene-derived high-value compound is ambroxan (Fig. 2), an important base molecule in perfumery which serves as a substitute for ambergris, a substance excreted from the injured intestines of whales (Ohloff, 1982). Currently, ambroxan is synthesized from sclareol, a diterpene diol obtained from *Salvia sclarea* plants (Barton et al., 1994). Abienol (Fig. 2), a related labdane-type diterpene alcohol, is also used as a precursor for ambroxan synthesis (Barrero et al., 1993).

The above examples also highlight an important limitation in the industrial application of many natural products; the low yield and high cost associated with their chemical synthesis or extraction from natural sources, which has prompted the application of biotechnological methods for their production (Ajikumar et al., 2008; Lai et al., 2009; Mora-Pale et al., 2014; Xu et al., 2013). Metabolic engineering efforts for the production of terpenoids have been particularly successful in the case of the antimalarial sesquiterpene lactone artemisinin (Paddon et al., 2013; Ro et al., 2006; Westfall et al., 2012), achieving industrial production of semi-synthetic artemisinin using engineered *S. cerevisiae* (Peplow, 2013). *S. cerevisiae* is an advantageous host for the production of isoprenoids, as it is robust and compatible with existing infrastructure, it can be readily modified by well-established genetic tools, and it provides a favorable environment for the functional expression of downstream biosynthetic activities, such as cytochrome P450s (Kampranis and Makris, 2012; Kirby and Keasling, 2009; Krivoruchko et al., 2011; Renault et al., 2014). The latter is of particular importance for the production of more structurally complex molecules, such as paclitaxel, tanshinones or carnosic acid, which require extensive modifications.

Although yeast has successfully been used for the production of several sesquiterpenes, including amorphadiene (40 g/L in fed-batch fermentation (Westfall et al., 2012)), artemisinic acid

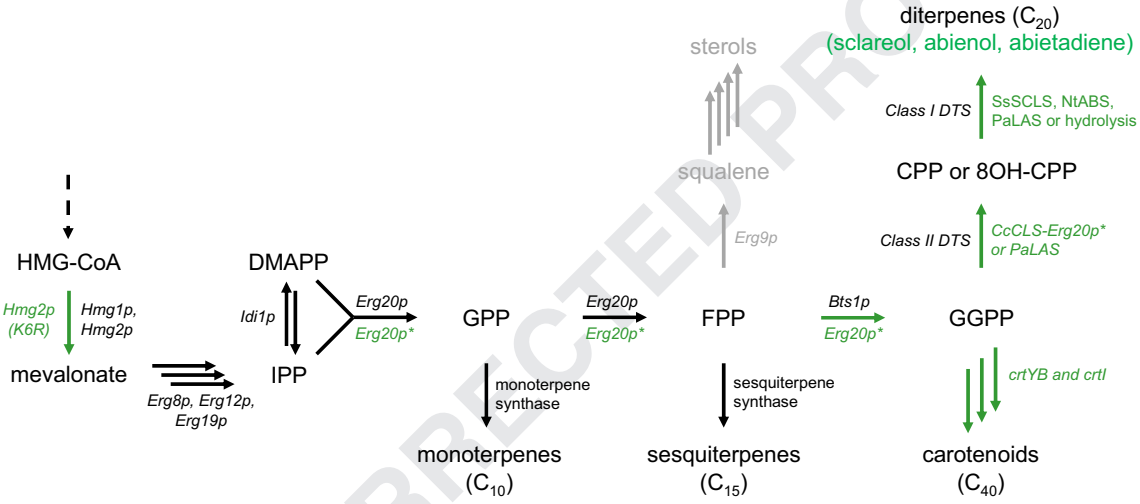

Fig. 1. Description of terpene biosynthesis in yeast indicating major intermediates and products. Steps upregulated in this study are indicated in green, downregulated in gray. Steps catalyzed by the different Erg20p variants, denoted collectively as Erg20p*, are also illustrated. (CPP, copalyl diphosphate; 8OH-CPP, 8-hydroxycopalyl diphosphate; DTS, diterpene synthase.) (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

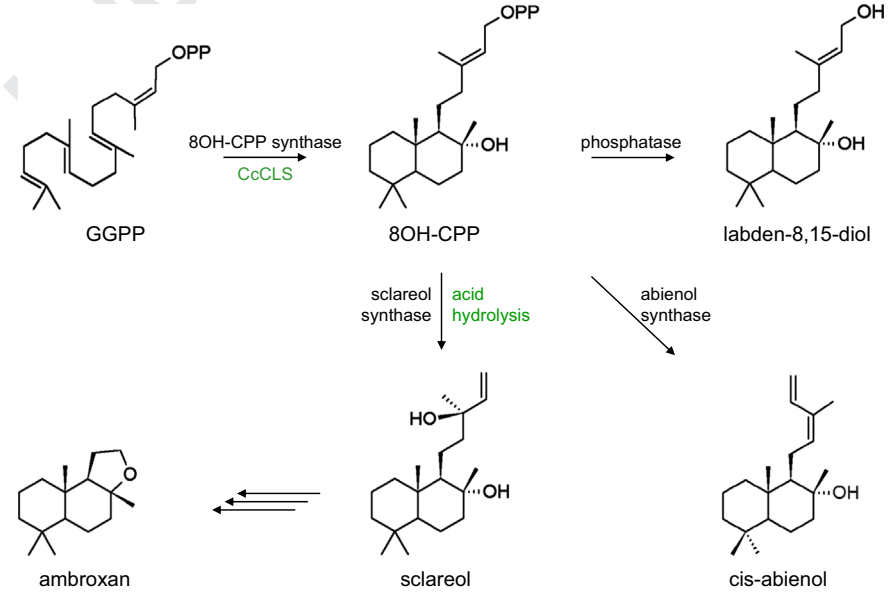

Fig. 2. Pathway describing the synthesis of sclareol, abienol, and ambroxan. Dedicated synthases, such *Ss*LPPS1 from *S. sclarea* or *Cc*CLS from *C. creticus* (*Cc*CLS), catalyze cyclization of GGPP to 8-hydroxycopalyl diphosphate (8OH-CPP), which is further converted to sclareol (either enzymatically or by acid hydrolysis), to *cis*-abienol (enzymatically), or to labd-13E-en-8α,15-diol (by the action of yeast phosphatases). Sclareol or *cis*-abienol serves as the starting compound for the synthesis of ambroxan, a central molecule in perfumery.