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Screening for improved isoprenoid biosynthesis in microorganisms

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ABSTRACT

The production of isoprenoids in recombinant microbes for flavor & fragrance, pharmaceutical, agricultural or fuel applications is a booming research field. Isoprenoid extraction from natural resources and chemical synthesis is frequently neither ecological nor commercially profitable. However, recombinant microbes also show severe limitations in specific isoprenoid synthesis. Therefore, diverse directed evolution strategies have been developed for recombinant microbes. The focus has been laid either on the overall engineering of recombinant hosts or on the improvement of isoprenoid synthases. Currently, the most prominent and advanced approaches are based on carotenoid-producing strains, which can be screened by simple colorimetric readout. Other screening strategies are based on spectrophotometric analyses of colored by-products, fluorescence applications, growth selection and, to a minor extent, the use of biosensors indicating the pool of isoprenoid precursors. Although the number of approaches is still small, we observe a trend towards rigorous and highly creative assays that, however, often rely on the indirect detection of the evolved enzyme activities or host strains. We conclude that the use of whole-cellular systems is clearly favored over cell extracts and predict that next-generation screening assays need to be developed towards broader applicability and more direct assessment of isoprenoid production levels.

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1. Introduction

Progress in molecular biotechnology heavily depends on directed enzyme or strain evolution strategies and a variety of applications is taking profit from optimized enzyme or strain performance (Dalby, 2011; Davids et al., 2013; Goldsmith and Tawfik, 2012). Most importantly, the heart and prerequisite of each directed evolution approach is the availability of a stable and reliable high-, or at least medium-throughput assay. But what if the screening assay needs to be established on challenging substrates and products that are, among others, water-insoluble,

Abbreviations: *Aa*ADS, *Artemisia annua* amorpha-4,11-diene synthase; *Aa*FS, *Artemisia annua* β-farnesene synthase; CAT, chloramphenicol acetyltransferase; DMAPP, dimethylallyl diphosphate; DXP, deoxyxylulose-5-phosphate; FCM, fluorescence-activated cell sorting by flow cytometry and magnetic cell sorting; FPP, farnesyl diphosphate; GPP, geranyl diphosphate; GGPP, geranylgeranyl diphosphate; GGPPS, GGPP synthase; IPP, isopentenyl diphosphate; HTS, high-throughput screening; MEV, mevalonate; PPi, inorganic diphosphate; TEAS, *Nicotiana tabacum* 5-*epi*-aristolochene synthase.

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volatile and toxic to the production host? A commercially interesting substance class that combines all of these characteristics is isoprenoids. These compounds are also referred to as terpenoids for their first isolation from conifer turpentine secretions, but may be summarized as isoprenoids since they are metabolically derived from five-carbon isoprene units (Croteau et al., 2000). Volatile isoprenoids are primarily produced by plants, and, to a lower extent, by microorganisms (Quin et al., 2014; Yamada et al., 2015) and insects (Dewick, 2002; Dubey et al., 2003; Sobotník et al., 2010). Isoprenoid isolation from natural sources may be rather unsatisfactory on a commercial scale due to low abundances, contaminations with (isoprenoid) impurities and, thus, unpredictable seasonal variations in product quality (Ishida and Chapman, 2009; Lapkin et al., 2006). Therefore, isoprenoid overproduction with recombinant microbes has been established as attractive alternative (Keasling, 2010). Isoprenoid biosynthesis is initiated by the condensation of the two 5-carbon precursors isopentenyl diphosphate (IPP) and its isomer dimethyl allyl diphosphate (DMAPP) by prenyl diphosphate synthases, e.g. geranyl diphosphate (GPP), farnesyl diphosphate (FPP) and geranylgeranyl diphosphate (GGPP) synthases. Eukaryotes and prokaryotes have developed distinct pathways for the generation of IPP and DMAPP, namely the mevalonate-dependent (MEV) pathway (Buhaescu and Izzedine, 2007) and the mevalonate-independent or deoxyxylulose-5-phosphate (DXP) pathway (Wanke et al., 2001). Plants use both, the MEV pathway and the DXP pathway (Eisenreich et al., 2004; Rohmer, 1999). The condensation products of IPP and DMAPP termed polyprenyl diphosphates, e.g., GPP, FPP and GGPP, are converted to more complex isoprenoid structures by isoprene synthases (Dewick, 2002; Tholl, 2006) and enzymatic hydroxylation and oxidation reactions (Fig. 1). Hence, isoprene synthases are important regulators at metabolic branch points and often compete with other metabolic enzymes for the prenyl diphosphate pool. For example, FPP is the precursor to many cellular molecules including squalene (sterols), dolichols, ubiquinones and the heme cofactor. Isoprene synthases form a multitude of isoprene carbon skeletons and their reaction mechanisms and product specificities have been heavily studied (Cane et al., 1997; Gao et al., 2012; Mathis et al., 1997; Miller and Allemann, 2012; Segura et al., 2003). Isoprene synthases are slow enzymes with turnover rates of less than 0.5 per second (Cane, 1990; Liang, 2009) and, therefore, often constitute the bottlenecks of isoprenoid metabolic pathways. Given that prenyl diphosphates are essential for diverse cellular processes, this is hardly surprising. Cells will most likely not be viable if isoprene synthases extensively deplete the prenyl diphosphate pool. Additionally, isoprenoid synthesis can be limited by substrate

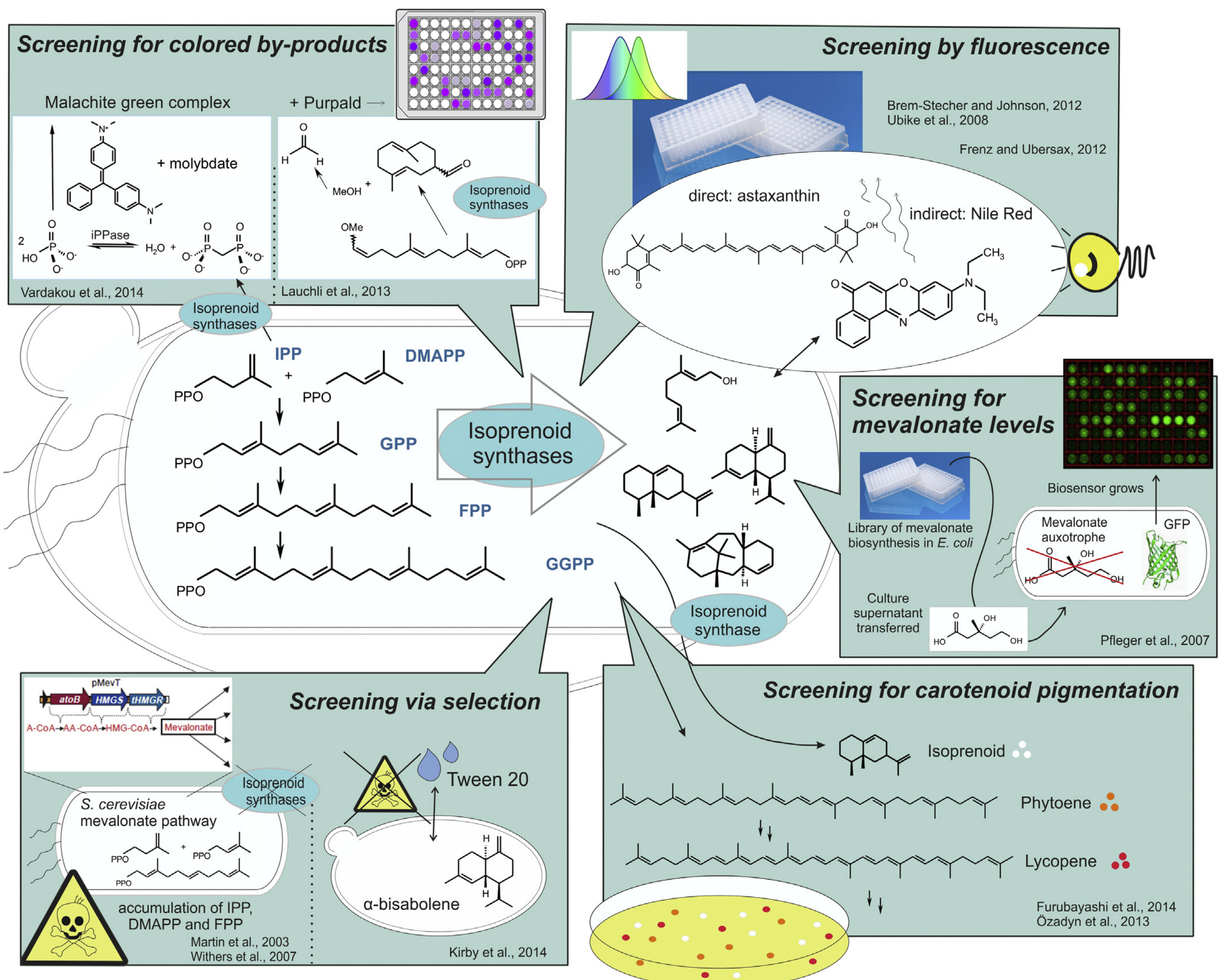

Fig. 1. Overview of screening strategies applied to screen for higher microbial and enzymatic isoprenoid production levels.

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