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Research Highlights

Bioproduction of monoterpene indole alkaloids in a single cell factory

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ABSTRACT

The *de novo* biosynthesis of vindoline and catharanthine, the direct precursors used for industrial production of the anti-cancer drug vinblastine, has been achieved in the yeast cell factory. To date, this is the longest natural product biosynthesis pathway that has been successfully transferred from plants to microorganisms, indicating the possibility of producing more than 3,000 other monoterpene indole alkaloids in yeast by synthetic genome engineering.

The discovery of medicinal plants and the isolation of active plant natural products are the principal driving forces for developing pharmaceuticals for human use [1]. However, many active compounds are present in plants as secondary metabolites and can be synthesized in minimal amounts in specific tissues of plants at the appropriate developmental stage. Therefore, the shortage of drugs from plant-derived natural products has always been a major issue. In the past decades, chemical synthesis based on compound structure and plant metabolic engineering based on biosynthetic pathways have contributed to numerous exciting achievements in this field. For example, the total synthesis of complex natural products, Taxol (paclitaxel) [2–4], artemisinin [5], and vinblastine [6], has been achieved. Furthermore, artemisinin production was significantly enhanced in transgenic *Artemisia annua* [7]. Nevertheless, considering the high cost, low yield, and difficulty of total synthesis, or the substantial land and time investment of medicinal plant cultivation, developing an efficient production platform is essential to ensure global access to quality medicines.

Synthetic biology is a rigorous engineering discipline that uses molecular biology tools and techniques to forward-engineer cellular behavior [8], bringing constructive approaches to drug production [9–11]. Based on understanding the biosynthetic mechanisms of species-specific plant natural products, synthetic biologists have enabled heterologous bioproduction of the desired phytochemicals by transplanting the specific biosynthesis pathway genes from native organisms into an industrial model host, such as *Escherichia coli* or *Saccharomyces cerevisiae*. In one exemplary illustration, commercial production of the antimalarial drug artemisinin was achieved by the fermentation of engineered *S. cerevisiae* expressing biosynthetic genes identified in *A. annua* to produce artemisinic acid, followed by extraction and chemical conversion to artemisinin [12]. In the relatively short trajectory of synthetic biology applications to natural product synthesis, the heterologous production of compounds with complex structures has been achieved by increasing the pathway length. In a landmark publication by the Keasling laboratory in 2003, the amorphadiene synthase gene identified in *A. annua* and the mevalonate isoprenoid pathway from *S. cerevisiae* were used to engineer *E. coli* for high-level production of amorphadiene, the sesquiterpene olefin precursor to artemisinin [13]. In 2006, the same group succeeded in producing artemisinic acid in engineered yeast by expressing three enzymes from *A. annua* [14]. The number of enzymes introduced into heterologous host increased to 25 for the *de novo* noscapine biosynthesis in 2018 [15] and 26 for scopolamine bioproduction in 2020 [16]. It seems that the only limiting factor in determining whether a complex natural product of interest can be synthesized using synthetic biology is whether its biosynthetic genes have been identified from plants. However, as the pathway length increases, is it still guaranteed that the final product will be obtained in appreciable yield in a heterologous host?

The anti-cancer drug vinblastine is a monoterpene indole alkaloid (MIA) produced by *Catharanthus roseus* (Madagascar periwinkle) [17]. About 0.32 and 1.03 μg/g of vinblastine can be obtained from *C. roseus* dried leaf and root, respectively [18]. The increasing demand for vinblastine in cancer treatment and the low yield of this alkaloid in plant have led to severe drug shortages [19]. Recently, Keasling and colleagues successfully engineered a yeast strain for *de novo* production of vindoline and catharanthine, the direct precursors used for industrial production of vinblastine, through 56 genetic edits, including the expression of 34 biosynthetic enzymes from plants [20] (Fig. 1). To date, this is the longest plant biosynthetic pathway effectively reconstructed in a heterologous host, marking a new milestone in synthetic biology.

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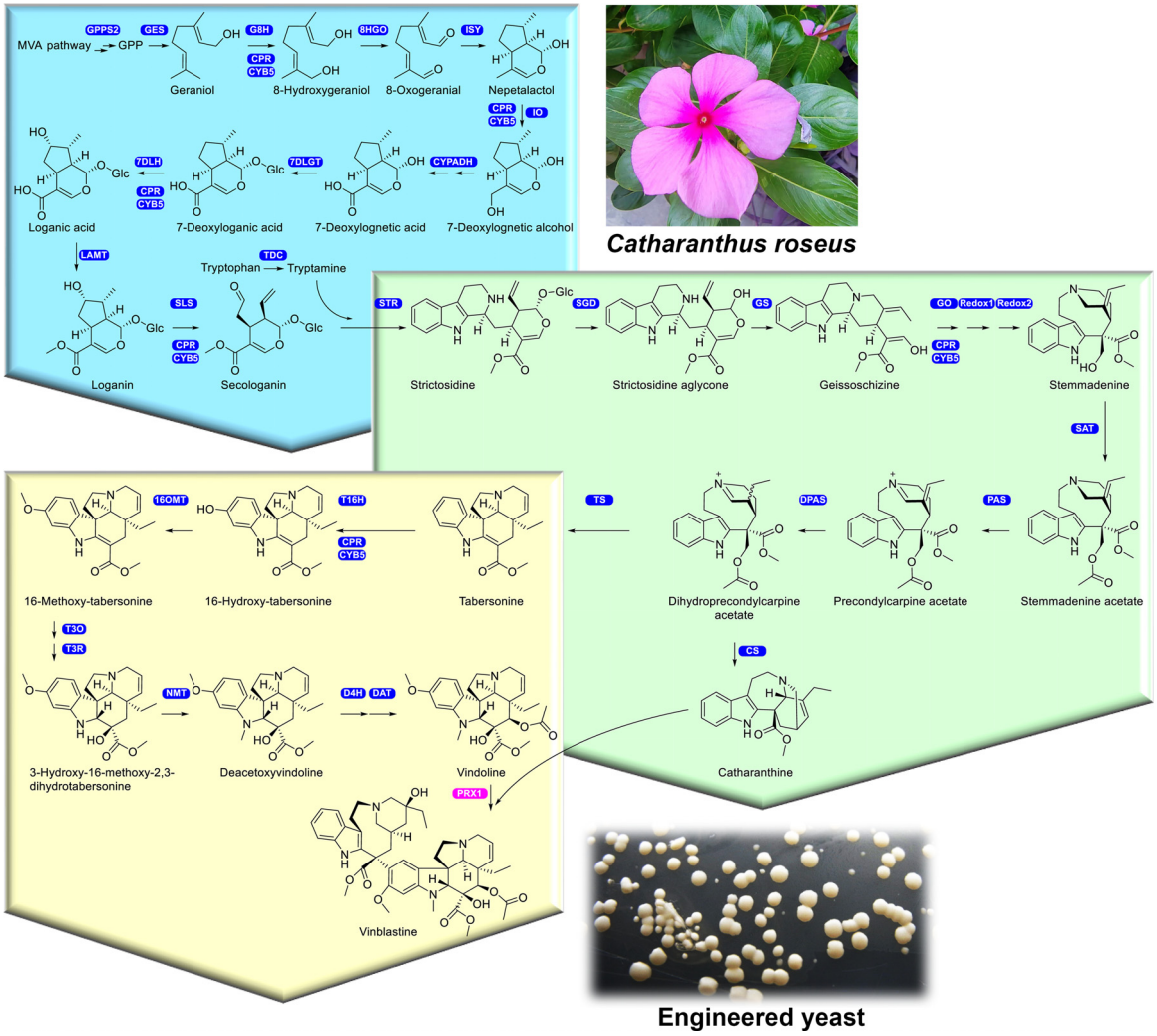

Fig. 1. The complete biosynthetic production pathway of vinblastine in yeast. Abbreviations for biosynthetic intermediates and enzymes: the MVA pathway, mevalonate pathway; GPP, geranyl pyrophosphate; GPPS, GPP synthase; CPR, NADPH-cytochrome P450 reductase; CYB5, cytochrome b5; GES, geraniol synthase; G8H, geraniol 8-hydroxylase; 8HGO, 8-hydroxygeraniol oxidoreductase; ISY, iridoid synthase; IO, iridoid oxidase; CYPADH, alcohol dehydrogenase 2; 7DLGT, 7-deoxyloganetic acid glucosyl transferase; 7DLH, 7-deoxyloganic acid hydroxylase; LAMT, loganic acid *O*-methyltransferase; SLS, secologanin synthase; TDC, tryptophan decarboxylase; STR, strictosidine synthase; SGD, strictosidine-*O*-β-D-glucosidase; GS, geissoschizine synthase; GO, geissoschizine oxidase; Redox1, protein redox 1; Redox2, protein redox 2; SAT, stemmadenine-*O*-acetyltransferase; PAS, precondylocarpine acetate synthase; DPAS, dihydroprecondylocarpine acetate synthase; CS, catharanthine synthase; TS, tabersonine synthase; T16H, tabersonine 16-hydroxylase; 16OMT, tabersonine 16-*O*-methyltransferase; T3O, tabersonine 3-oxygenase; T3R, 16-methoxy-2,3-dihydro-3-hydroxytabersonine synthase; NMT, 3-hydroxy-16-methoxy-2,3-dihydrotabersonine-*N*-methyltransferase; D4H, deacetoxyvindoline 4-hydroxylase; DAT, deacetylvindoline-*O*-acetyltransferase; PRX1, class III peroxidase. The colored boxes indicate strictosidine (blue), tabersonine/catharanthine (green), and vindoline (yellow) modules.

More importantly, given that MIAs share common biosynthetic intermediates, this accomplishment offered a yeast platform for achieving a high-level synthesis of more than 3,000 natural MIAs.

At the beginning of the reconstruction of the vinblastine biosynthetic machinery in yeast, the researchers divided the original pathway for vinblastine biosynthesis in *C. roseus* into three modules (Fig. 1): a module focusing on the overproduction of strictosidine, a well-established last common biosynthetic intermediate for all MIAs, from yeast native metabolites (geranyl pyrophosphate and tryptophan); a module responsible for the conversion of strictosidine to tabersonine/catharanthine through ten enzymatic steps and a vinblastine module dedicated to converting tabersonine to vindoline and the formation of vinblastine by coupling vindoline and catharanthine. In principle, the modules were designed for beginning and ending with commercially available compounds. With this design, the researchers can promptly and independently test whether each module could effectively perform its intended catalytic function by exogenous substrate feeding and product quantification. Furthermore, and very importantly, to enable iterative engineering and refactoring of these long biosynthetic pathways, the authors included a set of unique 20-nucleotide guide-RNA sequences in front of the promoter of each expression cassette for easy swapping of target promoters or entire expression cassettes iteratively by CRISPR/Cas9. The pathway optimizations included deletions, knockdowns, and overexpression of yeast genes for overproduction of pathway precursors, replacement of pathway enzymes with their more efficient homologs from other plants, and introduction of additional copies of rate-limiting enzymes. Unfortunately, with catharanthine and vindoline as substrates, non-enzymatic leurosine formation was detected in yeast, rather than peroxidase (PRX1)-catalyzed vinblastine production [21]. Therefore, the anti-cancer drug vinblastine production was achieved by *de novo* microbial biosynthesis of vindoline (13.2 μg/L, 29.0 nM) and catharanthine (91.4 μg/L, 271 nM) using this highly engineered yeast, followed by extraction and chemical coupling to vinblastine.

According to this landmark achievement in yeast metabolic engineering, the success of reconstructing long biosynthetic pathways to produce complex natural products in heterologous hosts depends on three main factors. (i) The complete biosynthetic pathway of the compound of interest is established. While equipping enzymes with unnatural functions has been achieved in the laboratory, building artificial biosynthetic pathways for complex natural products is challenging. Enzymes identified from the native plant host remain essential tools for pathway reconstruction. (ii) Improve the pathway precursor supplies by rational channeling the metabolism fluxes in heterologous hosts. The inherent nature of enzymatic conversion rate and the catalytic promiscuity of native or genetically introduced enzymes determine that as the biosynthetic route is extended, the yield of the final product becomes very low. To ensure high-level production of the target products, the supply of precursors must be sufficient, which requires efficient reprogramming of the yeast metabolic network. (iii) Comprehensive profiling of plant genomes and transcriptomes based on protein sequence similarities could provide candidate enzymes exhibiting higher catalytic activity for pathway optimization. The enzymes introduced into the heterologous host may not function properly due to low expression levels, altered subcellular localization, or incorrect folding/posttranslational modifications. Although the optimization of enzymatic steps could be achieved by integrating additional gene copies for critical enzymes, changing the subcellular location of target enzymes by truncation or signal peptide replacement, substituting the target catalytic enzymes with their homologous proteins is also a concise and effective way. In the future, directed evolution of enzymes at bottlenecks and artificial designs to reconstruct streamlined biosynthetic pathways for complex natural products will be the basis for the high-level production of drugs based on synthetic biology. The realization of these goals depends on inspiration from the biosynthetic machinery of natural products and the functional mechanisms of natural enzymes.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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