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•Commentary•

Identification of missing CYP450 enzymes involved in paclitaxel biosynthesis and heterologous reconstitution of baccatin III

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Paclitaxel, a tetracyclic diterpenoid, has garnered attention for its potent anti-cancer properties and intricate molecular structure, making it a significant target for chemical synthesis and biosynthesis [1]. However, its natural sources are extremely limited, as it is derived exclusively from the bark of endangered genus *Taxus* plants, which contain paclitaxel in very low concentrations (0.01%−0.05%) [2-3]. Recent advances in synthetic biology present promising opportunities to enhance paclitaxel levels in *Taxus* cell cultures or to enable the reconstitution of its production in heterologous hosts, such as yeast or tobacco (*Nicotiana benthamiana*).

The complete elucidation of the paclitaxel biosynthetic pathway is a prerequisite for the practical application of synthetic biology or metabolic engineering strategies to facilitate paclitaxel production. In 2021, YAN's group established the first high-quality reference genome map of *Taxus chinensis* at the chromosome level to address the aforementioned challenges [4]. Recently, YAN and LEI's group published an article titled "Characterization and heterologous reconstitution of *Taxus* biosynthetic enzymes leading to baccatin III" in *Science* [5]. This pivotal research identified the previously elusive CYP450 enzymes, taxane oxetanase 1 (TOT1) and taxane 9*α*-hydroxylase 1 (T9*α*H1), and achieved the *de novo* biosynthesis of baccatin III using a minimal gene set (Fig. 1).

In their investigation, JIANG *et al.* identified the enzyme TOT1 from 58 candidate CYP450s using an efficient multi-gene screening method in *N. benthamiana*. Their findings demonstrated that TOT1, which belongs to the CYP725A subfamily specific to *Taxus*, was capable of catalyzing the formation and rearrangement of the oxetane ring. Moreover, the knockdown of *TOT1* in *Taxus* cells resulted in a significant decrease in the levels of baccatin III and paclitaxel. The oxetane moiety is crucial for the anti-tumor activity of paclitaxel, acting as a conformational lock that facilitates its interaction with microtubules [6]. A previous report has indicated that the formation of the oxetane ring involved the transformation of the 5*α*-acetyl 4,20-ene moiety into the 4(20) *β*-epoxy-5*α*-acetoxy intermediate, which then rearranged to form the oxetane ring [7]. However, JIANG *et al.* proposed a novel mechanism by performing molecular dynamics (MD) simulations and density functional theory (DFT) calculations on the TOT1 enzyme along with its substrate. Their results revealed that TOT1 does not merely convert epoxide intermediates into oxetane rings but simultaneously catalyzes the transformation of the double bond into both ternary and tetrad rings, challenging the previous assumption that epoxides are necessary intermediates in oxetane ring formation. This breakthrough provides a clear understanding of the oxetane ring's biosynthesis, resolving a long-standing debate in the scientific community.

Over the past three decades, identifying enzymes responsible for C9 mono-oxidation in the biosynthesis of baccatin III has presented a substantial challenge. This difficulty arises from the simultaneous occurrence of oxidation reactions at various positions and the inability to isolate intermediates undergoing mono-oxidation at C9 [8]. Subsequently, JIANG *et al.* identified the enzyme responsible for C9 oxidation, T9*α*H1, from 17 candidate CYP725As by engineering the taxusin biosynthetic plant chassis and analyzing tissue-

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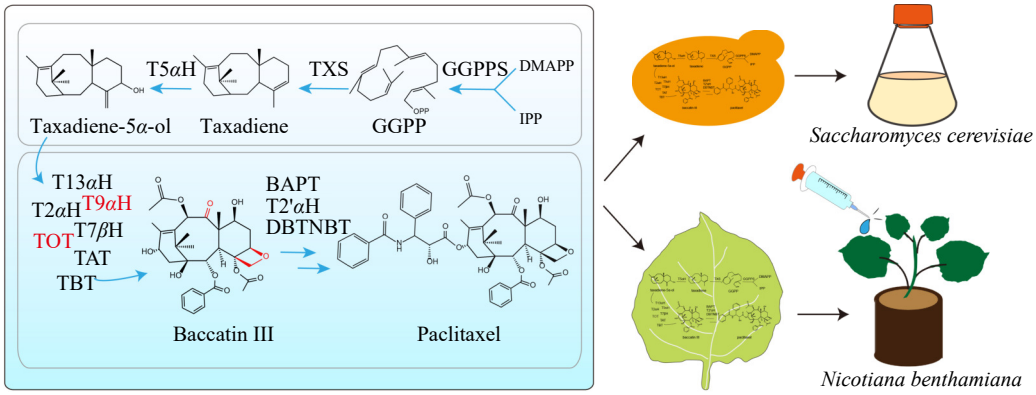

Fig. 1 The synthetic biology strategy for paclitaxel production in yeast or tobacco. JIANG *et al.* reported the characterization of missing enzymes T9*α*H1 and TOT1 in the biosynthetic pathway of paclitaxel. Their work led to the reconstitution of baccatin III in tobacco by co-expressing nine core genes. Future research efforts will focus on improving the catalytic efficiency of these enzymes and integrating the entire paclitaxel biosynthetic pathway into tobacco or yeast, thereby facilitating the production of paclitaxel. IPP: isopentenyl pyrophosphate; DMAPP: dimethylallyl pyrophosphate; GGPP: geranylgeranyl pyrophosphate; GGPPS: geranylgeranyl diphosphate synthase; TXS: taxadiene synthase; T5*α*H: taxane 5*α*-hydroxylase; T13*α*H: taxane 13*α*-hydroxylase; T2*α*H: taxane 2*α*-hydroxylase; T9*α*H: taxane 9*α*-hydroxylase; TOT1: taxane oxetanase 1; T7*β*H: taxane 7*β*-hydroxylase; TAT: taxadien 5*α*-ol-*O*-acetyl transferase; TBT: taxane 2*α*-*O*-benzoyltransferase; BAPT: baccatin III 3-amino-3-phenylpropanoyl transferase; T2'*α*H: 3'-*N*-debenzoyl-2'-deoxytaxol 2'*α* hydroxylase; DBTNBT: 3'-*N*-debenzoyl-2'-deoxytaxol *N*-benzoyl transferase.

specific expression patterns. This identification marks a pivotal advancement in understanding the biosynthesis of baccatin III.

Subsequently, the researchers employed a heterologous reconstitution strategy to co-express the newly identified *TOT1* and *T9αH1* genes with seven other well-established biosynthesis-related genes (*TXS*, *T5αH*, *T13αH*, *T2αH*, *T7βH*, *TAT* and *TBT*) in *N. benthamiana* to produce baccatin III (Fig. 1). The analysis of expression patterns and subcellular localization of these genes underscored the necessity of precise functional cooperation among these nine core genes in both temporal and spatial dimensions for the biosynthesis of baccatin III. These findings indicated that the biosynthesis of baccatin III could be achieved in geranylgeranyl pyrophosphate (GGPP) by co-expressing nine core genes in *N. benthamiana*, which was significantly fewer than the previously estimated requirement of at least 13 enzymes [9].

In summary, the research [5] identified the crucial enzymes, TOT1 and T9αH1, which were previously unknown components of the paclitaxel biosynthetic pathway. Through comprehensive omics analyses and extensive functional validations, this study not only filled a crucial gap in our understanding of paclitaxel biosynthesis but also revealed a novel mechanism underlying the formation of oxetane structures in plants. Furthermore, it achieved the biosynthesis of baccatin III within the plant chassis utilizing only nine enzymes in *N. benthamiana*. This research resolved a long-standing mystery in paclitaxel biosynthesis and laid a solid foundation for further understanding of the biosynthetic mechanism, optimizing the production process, and conserving *Taxus* resources.

Meanwhile, despite the successful *de novo* biosynthesis of baccatin III using a minimal gene set in this study, the yield remains relatively low (50 $ng \cdot g^{-1}$). Therefore, further enhancements are necessary in subsequent investigations to achieve industrial-scale production. Furthermore, future research will focus on optimizing key regulatory elements and coordinating enzyme catalysis to enhance the efficiency and yield of paclitaxel production in yeast or tobacco.

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