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De novo synthesis of plant polyketide plumbagin in yeast: a platform for sustainable production of naphthoquinones

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Abstract

Plumbagin is a natural naphthoquinone with different pharmacological properties and abundantly present in the roots of *Plumbago zeylanica* L. In spite of its therapeutic anti-cancerous potential, its limited availability from plant sources has slowed down its large scale production. In the present study, we report the heterologous reconstruction of the complete plumbagin biosynthetic pathway in *Saccharomyces cerevisiae* using six functionally characterized genes from *P. zeylanica* viz. Polyketide synthase (PKS), Polyketide cyclase, Aldo-keto reductase (AKR1), two cytochrome P450 monooxygenases (CYP81B140 and CYP81B141), and a cytochrome P450 reductase (CPR). Stepwise pathway engineering was performed to evaluate the necessity and sufficiency of individual and combined gene sets. The expression of PKS alone was not able to synthesize measurable products, while the co-expression of PKS, cyclase, and AKR1 (PCA) enabled the biosynthesis of 3-methyl-1,8-naphthalenediol which is a key intermediate and also confirmed by LC–MS/MS. The addition of CYP81B140 and CPR (PCACC) led to the production of 3-methyl-1,8-naphthalenediol and isoshinanolone, and further addition of CYP81B141 (PCACCC) in yeast effectively biosynthesized plumbagin. These results confirm the functional roles of all six genes and demonstrate full pathway reconstruction in yeast from the primary precursors acetyl-CoA and malonyl-CoA. The present study establishes a microbial production platform for plumbagin and provides valuable insights into the biosynthesis of plant-derived naphthoquinones as well as paving the way for the sustainable and scalable production of medicinally important compounds.

Keywords 3-methyl-1,8-naphthalenediol, Isoshinanolone, Plumbagin, Yeast, LC–MS/MS analysis, Heterologous expression

1 Introduction

Plant natural products comprise a structurally diverse class of compounds with fundamental ecological roles and pharmaceutical potential. However, their complex architectures often obstruct efficient chemical synthesis which making plant extraction as a primary source of supply. To meet the increasing demand synthetic biology and metabolic engineering researchers have developed transgenic microorganisms by transferring

metabolic pathways into heterologous hosts. Bioengineering approach has revolutionized natural product biosynthesis by enabling expression and pathway reconstruction in new hosts. For example Szczebara et al. [1] modified yeast to express 13 heterologous genes to establish the full and independent reconstruction of hydrocortisone biosynthesis from basic carbon sources [1]. Another remarkable example involves the microbial production of artemisinin precursors. Ro et al. [2] engineered the mevalonate pathway in *Escherichia coli*, along with amorpha-4,11-diene synthase to achieve high-level production of amorpha-4,11-diene which is a key precursor of artemisinin [2]. Next example is reconstruction of paclitaxel (Taxol) intermediates. Nowrouzi et al. (2020)

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reconstructed 10 enzymatic steps of the paclitaxel pathway in the *S. cerevisiae* to assist the microbial production of taxadiene and its consequent derivatives [3]. In recent years, complete biosynthetic pathways that produce complex alkaloids such as noscapine have been reconstituted in yeast. Li et al. [4] successfully engineered over 30 heterologous genes in the *S. cerevisiae*, achieving an 18,000-fold increase in noscapine production compared with its initial levels [4]. Among various hosts, *S. cerevisiae* remains a favored platform for heterologous biosynthesis because of its eukaryotic nature and well known genetics as well as the compatibility with membrane-bound enzymes (such as cytochrome P450s). Moreover its proven capacity to support multi-step pathway engineering and Generally Recognized as Safe (GRAS) status as well as scalability in industrial fermentation improves its value as a production host for complex plant natural products [5, 6]. Plumbagin is a naturally occurring naphthoquinone predominantly found in the roots of *Plumbago zeylanica* L. (family Plumbaginaceae). With its potent anticancer properties, it is a promising candidate for cancer therapy. Presently commercial supply relies on extraction of plumbagin from *Plumbago* species which is a resource intensive process. Plumbagin has a structurally complex framework. As a result, its stereoselective synthesis remains technically challenging and economically impractical. Plumbagin is a type III polyketide synthesized via the acetate-polymalonate (acetyl-CoA and malonyl-CoA) pathway. Our recent studies showed that six genes participate in plumbagin biosynthesis, and each was functionally validated in *P. zeylanica* through gene silencing and overexpression [7–9].

In the present study we intended to understand the necessity and sufficiency of six previously identified and individually characterized genes involved in plumbagin biosynthesis by co-expressing them in *S. cerevisiae*. These six genes, derived from *P. zeylanica*, comprise a *polyketide synthase* (*PKS*), a *polyketide cyclase*, an *aldo-keto reductase* (*AKR1*), two cytochrome P450 monooxygenases (*CYP81B140* and *CYP81B141*), and a cytochrome P450 reductase (*CPR*). To systematically reconstruct the plumbagin biosynthetic pathway we used a modular approach by introducing different gene combinations into yeast under galactose inducible promoters. Engineered *S. cerevisiae* strains expressing these combinations were *PKS* alone, *PCA* (*PKS*, *cyclase*, *AKR1*), *PCACC* (*PCA*+*CYP81B140*+*CPR*), and *PCACCC* (*PCACC*+*CYP81B141*) cultured under inducible conditions and heterologous transcript expression was analyzed using reverse transcription polymerase chain reaction (RT-PCR) and metabolite analysis was performed with LC–MS/MS. Overexpression of *PKS* alone did not yield any measurable intermediates. However,

co-expression of the *PCA* genes resulted in the production of 3-methyl-1,8-naphthalenediol, an intermediate in naphthoquinone biosynthesis. Adding *CYP81B140* and *CPR* enabled production of isoshinanolone, and introducing *CYP81B141* led to successful plumbagin synthesis. These results confirm that the six genes are necessary and sufficient for the complete biosynthesis of plumbagin in yeast. This study establishes a robust microbial platform for the production of plumbagin. This work provides a foundation for future metabolic engineering. It supports scalable and sustainable production of plant-derived naphthoquinones and related compounds.

2 Material and methods

2.1 Material and culture conditions

Plumbagin was purchased from Sigma-Aldrich, USA. For DNA manipulation and plasmid maintenance, chemically competent *E. coli* (TOP10) was used and grown at 37 °C in Luria–Bertani (LB) Broth with ampicillin as antibiotic to maintain plasmid in the cells. INVSc1 (MAT α his3 Δ 1 leu2 trp1-289 ura3-52/MAT α his3 Δ 1 leu2 trp1-289 ura3-52) yeast strain was used in the study with His[−], Leu[−], Trp[−], Ura[−] phenotype (Invitrogen, USA). The INVSc1 yeast strain was grown in yeast extract, peptone, adenine sulfate and dextrose complex media (YPAD media) (HIMEDIA, India) at 30 °C and 180 rpm on a rotary shaker. For DNA manipulation and plasmid maintenance, INVSc1 yeast strain was grown on Synthetic Dropout (SD) media containing yeast nitrogen base (YNB) without amino acid, with ammonium sulfate (HIMEDIA), 2% dextrose and the appropriate dropout amino acid solutions. SD media with appropriate amino acid dropout was used for the selection of the transformed yeast strain at 30 °C. SD media was prepared as per the manufacturer's instructions. 2% galactose was used for induction of transformed yeast cells.

2.2 Gene amplification, cloning and transformation of candidate genes

All previously identified candidate genes viz. *Polyketide synthase* (*PKS*), *aldo-ketoreductase* (*AKR1*), *cyclase*, *cytochrome P450* (*CYP81B140* and *CYP81B141*) and *cytochrome reductase* (*CPR*) were amplified using iProof high fidelity DNA polymerase (BIO-RAD, USA) with appropriate annealing temperatures, and PCR amplification was performed as per the manufacturer's protocol using a Veriti 96-Well Fast Thermal Cycler (Thermo Fisher Scientific, USA). The primers used for the full-length amplification of all candidate genes are provided in Online Resource 1a. The PCR product was purified using the GeneJET Gel Extraction kit as per the manufacturer's protocol (Thermo Fisher Scientific, USA). The conventional digestion and ligation technique was used to clone

all candidate genes in respective vectors using Fast Digest restriction enzymes (Thermo Fisher Scientific, USA) and T4 DNA ligase (Thermo Fisher Scientific, USA). All the candidate genes were cloned into pESC-Leu⁻, pESC-Ura⁻, pESC-Trp⁻ and pESC-His⁻ vectors with appropriate restriction sites. Details about plasmid construction are provided in Online Resource 1b. All the constructs were confirmed using gene specific PCR, restriction digestion and Sanger sequencing (Bioserve Biotechnologies India Pvt Ltd). Gel images demonstrating full-length amplification of six genes and colony PCR confirmation following cloning are presented in Online Resource 2a and 2b. Additionally, a gel image showing plasmid PCR of a confirmed positive clone is provided in the Online Resource 2c. Yeast competent cells were prepared and sequence confirmed plasmids were transformed into the INVSc1 strain as per the method Gietz et al. [10] and plated on SD media with appropriate amino acid dropout solution, incubated at 30 °C for 3–4 day. Further single transformed colony was inoculated into liquid SD with appropriate amino acid dropout media and grown overnight at 30 °C at 180 rpm on a rotary shaker. Overnight grown yeast culture was used for plasmid isolation as per the manufacturer's instructions (FAVORGEN, plasmid isolation kit). To confirm the presence of candidate genes, plasmid PCR was performed using gene and vector-specific primers. Four combinations of genes in yeast were made as viz. *PKS* alone, *PKS*, *Polyketide cyclase*, *Aldo-keto reductase* (PCA), *PKS*, *Polyketide cyclase*, *Aldo-keto reductase*, *CYP81B140* and *CPR* (PCACC) and *PKS*, *Polyketide cyclase*, *Aldo-keto reductase*, *CYP81B140*, *CYP81B141* and *CPR* (PCACCC). Here onward in the manuscript for better understanding, we used the abbreviations for different gene combinations introduced into yeast as mentioned above.

2.3 Culture conditions for metabolite production in yeast

For metabolites and RNA extraction, single positive transformed yeast (INVSc1) colony was inoculated in 5 ml SD with appropriate amino acid dropout media containing 2% dextrose at 30 °C, 180 rpm on a rotary shaker. To reconstruct the step-wise plumbagin biosynthetic pathway in yeast, 500 µl overnight grown cultures of different combinations of yeast clones (*PKS* alone, PCA, PCACC, PCACCC) were inoculated into 500 ml of fresh SD appropriate amino acid dropout media with 2% galactose. After galactose induction, the cells were grown up to 72 h and used for metabolite extraction. Metabolite extraction from the yeast cell pellet was done using the freezing and thawing in methanol method [11]. The 500 ml supernatant was lyophilized and the dried supernatant was resuspended in 1 ml methanol. Finally 20 µl

samples were injected into LC–MS/MS for targeted and untargeted metabolites analysis.

2.4 RNA isolation and detection of heterologously expressed genes using RT-PCR analysis

The 2% galactose-induced positive cells were harvested by centrifugation and resuspended into 400 µl AE buffer (50 mM Na acetate pH 5.3, 10 mM EDTA). RNA isolation from resuspended cells were done as per the protocol of Schmitt et al. [12]. RNA purity and concentration were checked using a NanoDrop spectrophotometer (Thermo Fisher, USA). One µg of total yeast RNA was used to synthesize cDNA following the manufacturer's protocol. The reverse transcription PCR (RT-PCR) was carried out to check the presence of over-expressed transcripts from the yeast cDNA as per the standard PCR protocol following manufacturer's instructions.

2.5 Metabolite extraction and LC-QTOF-MS/MS analysis

Metabolites from transgenic yeast pellets were extracted using the freezing–thawing in methanol and 20 µl from each sample was injected into LC–MS/MS for analysis. The LC-QTOF-MS/MS analysis was carried out with the HPLC Prime Infinity II 1260 system (800 bar) equipped with Hypersil GOLD C18 (2.1 × 150 mm, 1.9 µm particle size, Thermo Scientific, USA) column with a mass detector 6530 QTOF (Agilent, USA) as an accurate mass detector. The HPLC method and QTOF parameters were kept as reported in our previous study [13]. The time-of-flight MS data were acquired in the 2 GHz extended dynamic range with a mass scan range of 50–1700 m/z. For tandem MS (MS/MS) analysis, the collision energies were set at 10, 20, and 40 eV. A methanol wash was given after the biological replicates to avoid sample carryover.

2.6 Metabolite data analysis

The targeted metabolite analysis was carried out using the MassHunter Workstation and Mass Hunter Navigator software (B.08.00, Agilent). All metabolites were identified based on their Mass to charge ratio, and the corresponding peak areas were extracted and quantified using MassHunter Qualitative Navigator (B.08.00, Agilent). Confirmation of metabolites was done with daughter fragments matching at the MS/MS level with reported fragments of metabolites using MassHunter Qualitative Navigator (B.08.00, Agilent). The CFM-ID web server was used for the prediction of in silico MS/MS fragments for the compounds where standards are not available [14]. The MS-Excel Spreadsheet was used to calculate the fold change and create the bar graphs for the metabolite data.

3 Results

3.1 Functional characterization of six *P. zeylanica* specific genes by heterologous expression in *S. Cerevisiae*

Our previous reports indicate that plumbagin biosynthesis is mediated by six core genes catalyzing the conversion of acetyl-CoA and malonyl-CoA to plumbagin. These comprise *PKS*, a *polyketide cyclase*, *AKR1*, two P450 monooxygenases (*CYP81B140* and *CYP81B141*), and a P450 reductase (*CPR*). An earlier study from our lab proposed that the *PKS* enzyme catalyzes the initial condensation of one molecule of acetyl-CoA with five molecules of malonyl-CoA to produce the first polyketide intermediate. This intermediate then undergoes intramolecular aldol condensation at the C2–C11 and C4–C9 positions, catalyzed by the polyketide cyclase, leading to the formation of a second intermediate. Subsequently, *AKR1* catalyzes the reduction of the carbonyl groups at the C3, C5, and C7 positions, followed by the elimination of the hydroxyl group at C7, forming 3-methyl-1,8-naphthalenediol. This compound then undergoes oxidation at the C1 position and hydroxylation at the C4 position and reactions catalyzed by *CYP81B140* in association with *CPR* to synthesize isoshinanolone. Finally, *CYP81B141*, in conjunction with *CPR*, catalyzes the oxidation at the C4 position of isoshinanolone to produce the final product, plumbagin, as shown in Fig. 1. To evaluate the necessity and sufficiency of previously identified and characterized genes involved in the plumbagin biosynthetic pathway, we reconstructed the pathway in *S. cerevisiae* using various gene combinations: *PKS* alone, *PCA*, *PCACC*, and *PCACCC*. As the *PKS* catalyzes the initial step in the plumbagin biosynthetic pathway, *PKS* alone was expressed in yeast to evaluate its impartial functionality. To ensure plasmid maintenance, the engineered *S. cerevisiae* strain was cultured on synthetic dropout medium lacking leucine (SD -Leu) amino acid. The expression of *PKS* was induced using 2% galactose, as the expression of cloned gene constructs were driven by the *GAL1* and *GAL10* promoters. Transformed yeast cultures were induced for up to 72 h, after which the culture supernatant was harvested, processed, and subjected to LC–MS/MS analysis following the protocols described in the Materials and Methods section. The expression of

PKS alone did not result in the production of key pathway intermediates, such as 3-methyl-1,8-naphthalenediol and isoshinanolone, as shown in Fig. 2a. Additionally, the first hexaketide intermediate could not be detected, probably due to the absence of exact structural information, together with its molecular formula and exact mass. These results suggest that *PKS* by itself is insufficient to produce detectable pathway intermediates and requires the presence of additional accessory enzymes to assist the downstream biosynthetic steps [7, 8].

Based on prior functional analyses of the polyketide cyclase and aldo–keto reductase enzymes involved in plumbagin biosynthesis; we chose a three gene combination *PCA* module, for pathway reconstitution in *S. cerevisiae*. Although the chemical identity and mass of the second hexaketide intermediate remain unknown but the structure, molecular formula, and mass of the downstream compound i.e. 3-methyl-1,8-naphthalenediol is well known. The engineered *S. cerevisiae* strain containing the *PCA* gene cassette was cultured in a SD medium lacking leucine and uracil amino acid to maintain plasmid selection. The 2% galactose was used to induce the gene expression of the transformed yeast strain up to 72 h, followed by the harvest and processing of the culture supernatants for downstream reverse transcription PCR (RT-PCR) and LC–MS/MS analysis. RT-PCR confirmed the successful transcription of all three target genes in the induced yeast cells (Online Resource 2d). Targeted metabolomics analysis using LC–MS/MS recognized the synthesis of 3-methyl-1,8-naphthalenediol, as an ammonium adduct $[M + NH_4]^+$ with an m/z of 192.100 (Fig. 2) and the corresponding empty vectors (*pESC-Leu⁻*, and *pESC-Ura⁻*) did not show any peak corresponding to 3-methyl-1,8-naphthalenediol (Fig. 2). MS/MS fragmentation of the corresponding peak matched the in silico spectra generated by CFM-ID software (Online Resource 3a) to confirm the compounds' identity. These findings reveal that the co-expression of *PKS*, Cyclase, and *AKR1* is adequate to produce 3-methyl-1,8-naphthalenediol, the first structurally known intermediate from the plumbagin biosynthetic pathway. The results suggest that the *PCA* gene combination is capable of catalyzing key reactions, including C2–C11 and C4–C9 intramolecular aldol

(See figure on next page.)

Fig. 1 Predicted plumbagin biosynthetic pathway: Acetyl-CoA (one molecule) and malonyl-CoA (five molecules) undergo iterative condensation and forms hexaketide intermediate backbone catalyzed by polyketide synthase (*PzPKS*). Followed by *PKS*, polyketides cyclase catalyzed the C2–C11 and C4–C9 intermolecular aldol condensation to form hexaketide intermediate. Our previous study proposed that aldo–keto reductase 1 (*PzAKR1*) catalyzed C3, C5 and C7 carbonyl reduction and C7 hydroxyl elimination to form 3-methyl-1,8-naphthalenediol. Further, *CYP81B140* along with cytochrome reductase (*CPR*) which is an accessory enzyme required for functioning of *CYP81B140* and *CYP81B141*; catalyzed the C1 oxidation and C4 hydroxylation of 3-methyl-1,8-naphthalenediol to form isoshinanolone. Moreover addition of *CYP81B141* catalyzed C4 oxidation of isoshinanolone to form plumbagin and complete the plumbagin biosynthetic pathway

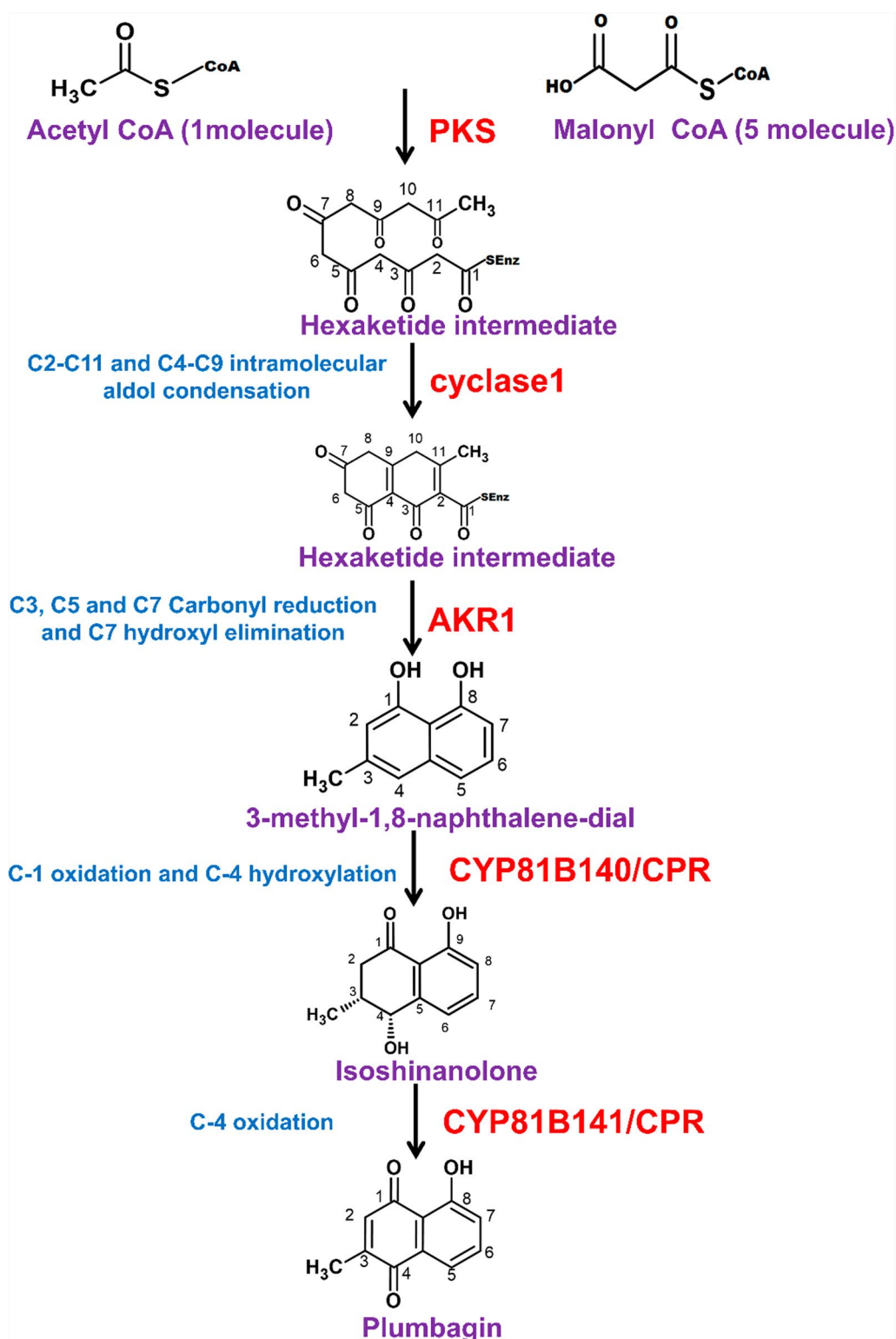


Fig. 1 (See legend on previous page.)

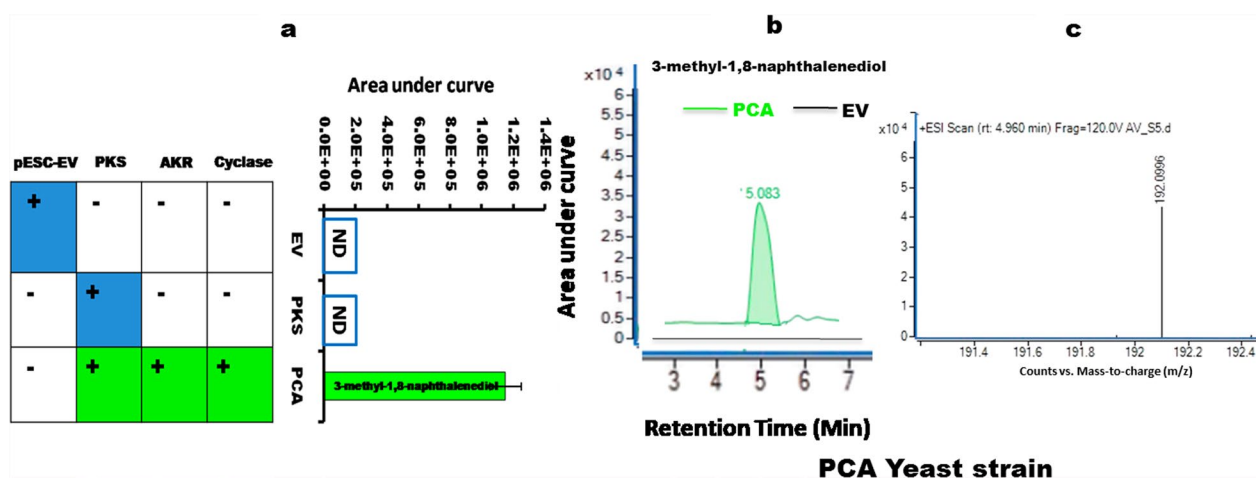


Fig. 2 Detection of 3-methyl-1,8-naphthalenediol in PCA transformed yeast strain: **a** Represents the presence of 3-methyl-1,8-naphthalenediol only in the PCA gene combination. Green color graph denotes the area under the chromatographic peak for 3-methyl-1,8-naphthalenediol, ND denotes metabolite not detected in empty vector (EV) and alone PKS, **b** Chromatographic peak of 3-methyl-1,8-naphthalenediol from PCA yeast strain and black color flat line indicates EV controls ((pESC-Leu⁻ and pESC-Ura⁻) reflecting the absence of the 3-methyl-1,8-naphthalenediol in the negative control. **c** Mass spectra of 3-methyl-1,8-naphthalenediol showing m/z 192.0996 as [M + NH₄]⁺ adduct at RT=5.0 min

condensation and C3, C5 and C7 carbonyl reduction, and C7 hydroxyl group elimination, as depicted in Fig. 1.

3.2 Functional characterization of *CYP81B140* and *CPR* using the PCACC gene combination in yeast

To study the subsequent step in the plumbagin biosynthetic pathway, we introduced two additional genes *CYP81B140* and *cytochrome P450 reductase (CPR)* into the PCA yeast strain, forming a five gene construct namely PCACC. The Engineered PCACC *S. cerevisiae* strain was grown on SD medium lacking leucine, uracil, and tryptophan amino acids. Following induction with 2% galactose for 72 h, the culture supernatant was collected and processed as described in the Materials and Methods section and used for downstream analysis. RT-PCR analysis confirmed the transcriptional activity of all five genes in the engineered strain (Online Resource 2d). LC-MS/MS analysis of the supernatant revealed the presence of both 3-methyl-1,8-naphthalenediol (m/z 192.100) and a isoshinanolone with m/z 210.110 as adduct [M + NH₄]⁺ (Fig. 3) and the EVs (pESC-Leu⁻, pESC-Ura⁻, pESC-Trp⁻), employed as negative controls, exhibited no detectable signals corresponding to 3-methyl-1,8-naphthalenediol or isoshinanolone (Fig. 3) MS/MS spectra of both the compounds matched with in silico fragments generated using CFM-ID and provided in the Online Resource 3b and 3c. These results indicate that the PCACC gene combination enables the conversion of 3-methyl-1,8-naphthalenediol to isoshinanolone. This conversion likely involves C1 oxidation and C4 hydroxylation, catalyzed by *CYP81B140* along with

CPR. As previously described in the literature [13], CPR provides the necessary electrons required for the proper enzymatic function of cytochrome P450. In PCACC yeast strain we were not able to detect plumbagin.

3.3 Stacking of *CYP81B141* enabled conversion of isoshinanolone to plumbagin in the PCACC yeast strain

Previous studies have proposed that the final steps in the plumbagin biosynthetic pathway are mediated by *CYP81B141*, which catalyzes C4 oxidation of isoshinanolone to synthesize plumbagin [13]. To authenticate this function, we introduced *CYP81B141* into the PCACC background. The PCACC strain was cultured in SD medium lacking leucine, uracil, tryptophan, and histidine to maintain all expression plasmids. After induction with 2% galactose for 72 h, the culture supernatants were harvested for analysis. RT-PCR confirmed the successful expression of all genes (Online Resource 2d). LC-MS/MS analysis revealed a low-abundant peak at a retention time (RT) of 9.179 min, corresponding to the [M + NH₄]⁺ adduct of plumbagin (m/z 206.0850). This peak closely matched the RT of 9.823 min (Fig. 4) and the MS/MS fragmentation pattern of a commercially available standard plumbagin as well as in silico MS/MS spectra generated using the CFM-ID software (Online Resource 3d). The negative control EVs (pESC-Leu⁻, pESC-Ura⁻, pESC-Trp⁻, and pESC-His⁻) showed no detectable peak corresponding to plumbagin (Fig. 4). These data confirm that *CYP81B141* catalyzes the oxidation at C4 position of isoshinanolone to form plumbagin. The successful

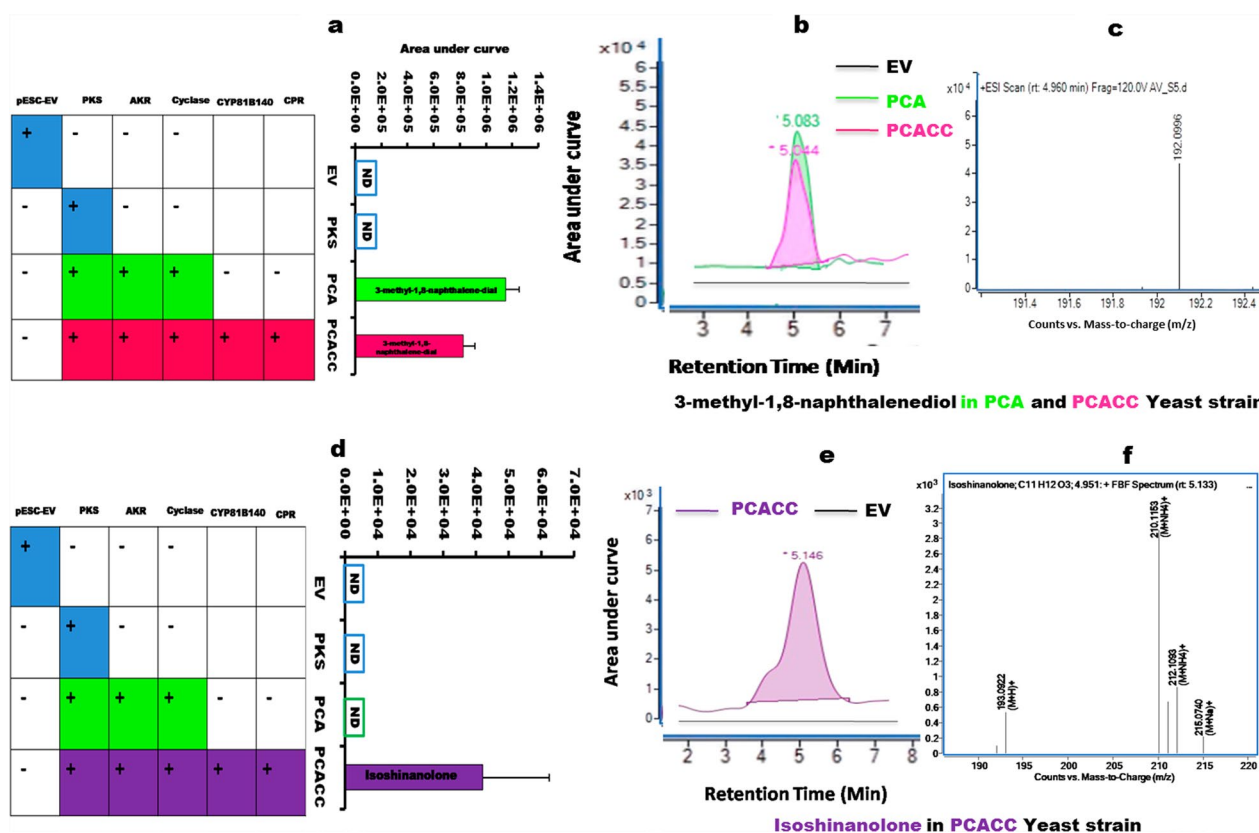


Fig. 3 Biosynthesis of 3-methyl-1,8-naphthalenediol and isoshinanolone in PCACC transformed yeast strain: **a** Green color graph represents chromatographic peak for 3-methyl-1,8-naphthalenediol in PCA yeast strain and pink color graph showed peak for 3-methyl-1,8-naphthalenediol from PCACC yeast strain. In PCACC strain 3-methyl-1,8-naphthalenediol content decreased compared to PCA strain because it is utilized as a substrate to form isoshinanolone in PCACC yeast, **b** Denotes the overlapping extracted peak of 3-methyl-1,8-naphthalenediol from PCA (green graph) and PCACC (pink graph) yeast and flat black lines denote empty vector negative control (pESC-Leu⁻ and pESC-Ura⁻ undetectable peak corresponding to 3-methyl-1,8-naphthalenediol), **c** Mass spectra of 3-methyl-1,8-naphthalenediol showing m/z 192.0996 as [M + NH₄]⁺ adduct at RT-5.0 min, **d** Purple color graph denotes the area under peak for isoshinanolone from PCACC strain and the flat black lines indicates negative control EVs (pESC-Leu⁻, pESC-Ura⁻, and pESC-Trp⁻) with undetectable peak corresponds to isoshinanolone), **e** Extracted ion chromatogram of isoshinanolone and **f** Mass spectra of isoshinanolone showing m/z 210.1163 i.e. [M + NH₄]⁺ adduct at RT 5.1 min

production of plumbagin in yeast confirms that the functional role of all six enzymes in the biosynthetic pathway is necessary.

4 Discussion

The successful reconstruction of the plumbagin biosynthetic pathway in *S. cerevisiae* represents a significant progress in the understanding and engineering of plant specialized metabolites. In this study, we systematically introduced and evaluated combinations of six candidate genes from *P. zeylanica* including *PKS*, *polyketide cyclase*, *AKR1*, *CYP81B140*, *CYP81B141*, and *CPR* to evaluate their individual and combined roles in the production of plumbagin and its pathway intermediates. Expression of the polyketide synthase (*PKS*) gene alone in yeast did not result in the accumulation of any detectable intermediates which includes the expected hexaketide product. This is consistent with observations from similar

biosynthetic pathways; where *PKS* derived polyketide intermediates often require rapid enzymatic tailoring to prevent degradation or polymerization [7, 15, 16]. The inability to detect this chemical may be due to the lack of mass and structural information, which makes targeted LC-MS/MS detection challenging.

To conquer this we focused on the co-expression of *PKS* with downstream accessory enzymes specifically the polyketide cyclase and aldo-keto reductase (*AKR1*) to produce the first stable and structurally known intermediate 3-methyl-1,8-naphthalenediol. Our approach was informed by the previous functional characterization of these enzymes in similar type III polyketide synthase derived pathways [8, 9, 17, 18]. Indeed the co-expression of PCA combination resulted in the production of 3-methyl-1,8-naphthalenediol, which was detected as an [M + NH₄]⁺ adduct with m/z 192.100 and further validated via MS/MS fragmentation and in silico comparison

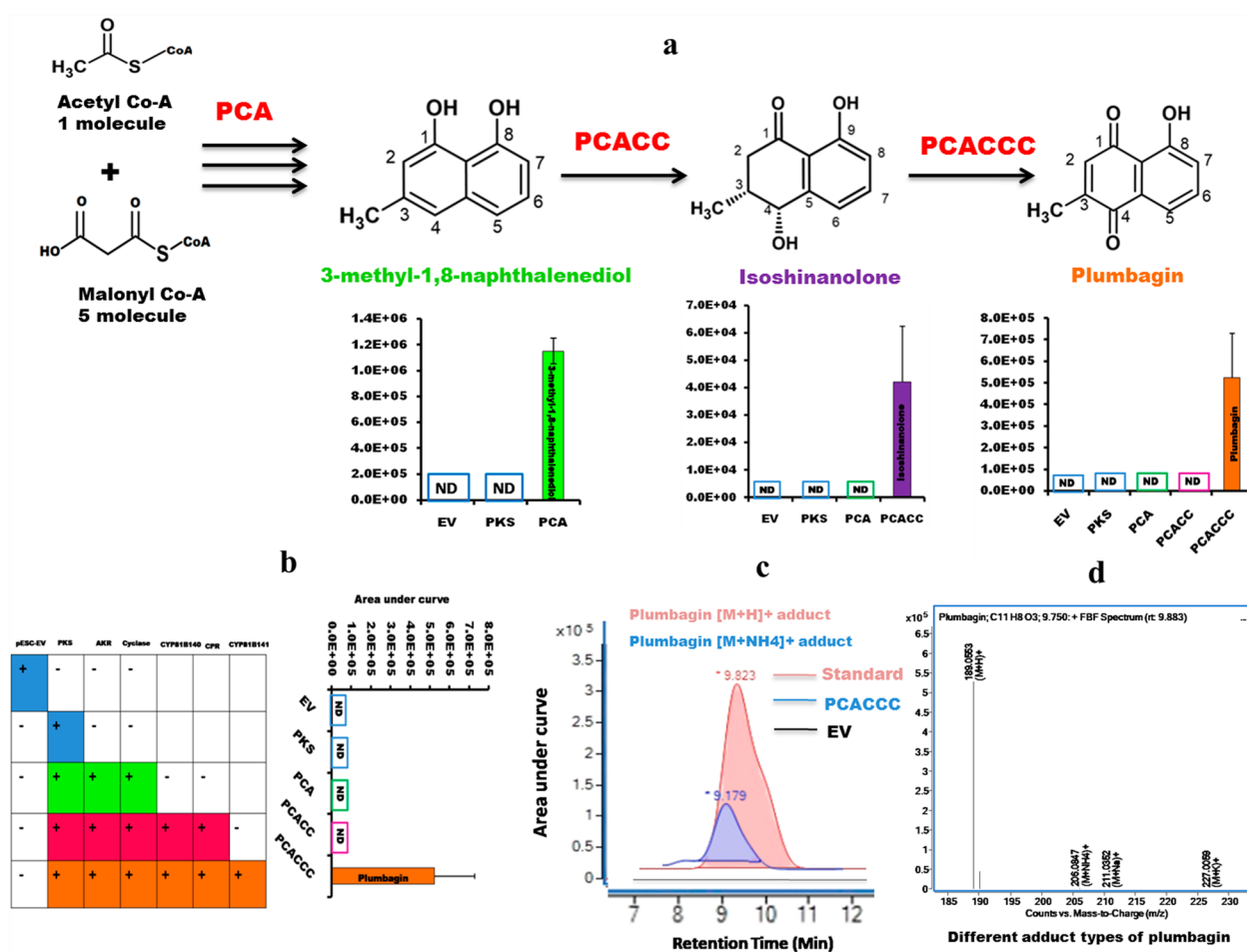


Fig. 4 Detection and confirmation of plumbagin from PCACCC yeast strain: **a** Stepwise reconstruction of the plumbagin biosynthetic pathway representing chromatographic peak area of the intermediate metabolites. **b** Orange color represents PCACCC gene combination in yeast strain. **c** Overlapping extracted ion chromatogram of standard plumbagin (red color peak) at RT 9.8 min and plumbagin detected from PCACCC yeast strain (blue color peak) at RT 9.1 min and black color flat line indicates the empty vector negative controls (pESC-Leu⁻, pESC-Ura⁻, pESC-Trp⁻, and pESC-His⁻) representing absence of detectable peak corresponding to plumbagin. **d** MS spectra of plumbagin indicating both [M+H]⁺ and [M+NH₄]⁺ adducts

with the CFM-ID predicted spectra. These results underline the importance of both the cyclase and AKR1 in converting the unstable polyketide intermediates into a more stable compound. The role of AKR1 in site specific carbonyl reduction and hydroxyl group elimination is very critical to achieve the correct substitution pattern on the naphthalene ring. Remarkably similar strategies have been employed in the reconstruction of the anthraquinone and naphthoquinone pathways in microbial hosts where reductases and cyclases play vital roles in intermediate stabilization and conversion [19, 20].

Our work established 3-methyl-1,8-naphthalenediol as the first confirmed intermediate in the plumbagin biosynthetic pathway. These results also emphasize the importance of rational in the pathway design particularly

when working with partially characterized or structurally uncharacterized intermediates by targeting downstream well characterized metabolites is a useful method. Beyond fundamental insights these results have translational implications. Moreover heterologous production of plant derived bioactive compounds in microbial hosts is a promising approach for sustainable and scalable synthesis. Particularly for medicinally relevant natural products such as plumbagin which exhibits anticancer, antimicrobial, and anti-inflammatory activities [21, 22]. Establishing the fundamental biosynthesis stages in yeast prepares the way for additional pathway optimization which includes insertion of P450 enzymes (CYP81B140 and CYP81B141) and CPR to understand complete plumbagin biosynthesis.

Successful biosynthesis of 3-methyl-1,8-naphthalenediol by co-expression of the PCA gene set, we wanted to further advance the pathway by introducing additional tailoring enzymes namely, the cytochrome P450 monooxygenases (CYP81B140 and CYP81B141) and the cytochrome P450 reductase (CPR) which are predicted to catalyze the final oxidative modifications leading to plumbagin. To investigate their functional roles we engineered yeast strains PCACC (PCA, CYP81B140, CPR) and PCACCC (PCACC, CYP81B141). Expression of the PCACC gene combination aimed to catalyze the oxidation of 3-methyl-1,8-naphthalenediol at the C1 position and hydroxylation at the C4 position to form isoshinanolone. LC–MS/MS analysis of the PCACC expressing yeast biosynthesized isoshinanolone and 3-methyl-1,8-naphthalenediol corresponds to m/z 210.1100 and 193.1100 respectively (Online Resource 3b and 3c). Metabolites confirmation was supported by MS/MS fragmentation patterns that closely matched with the *in silico* predictions (Online Resource 3b and 3c). These findings confirm that CYP81B140 in conjunction with CPR, is functionally active in *S. cerevisiae* and capable of modifying 3-methyl-1,8-naphthalenediol into isoshinanolone.

To accomplish the final conversion to plumbagin we further introduced CYP81B141 into the system (PCACCC strain). This P450 monooxygenase is anticipated to catalyze the final oxidation at the C4 position of isoshinanolone to form plumbagin. LC–MS/MS analysis of the PCACCC strain showed the emergence of a new peak with at m/z 206.0805 corresponding to plumbagin (Fig. 4), and its MS/MS spectrum was consistent with authentic standards and *in silico* fragment data (Online Resource 3d). This demonstrates the successful *in vivo* reconstitution of the complete plumbagin biosynthetic pathway in *S. cerevisiae*, from the central metabolic precursors acetyl-CoA and malonyl-CoA to form final product. It is noteworthy that yeast expressed plant P450 enzymes may operate effectively in a heterologous microbial host, as these enzymes usually need sophisticated electron transport systems and appropriate membrane location. The successful co-expression of CYP81B140, CYP81B141, and CPR in yeast suggests that *S. cerevisiae* provides a suitable cellular environment for P450-mediated oxidation reactions, which is consistent with previous studies on plant P450 function in yeast [23, 24].

Taken together these results showed that all six candidate genes from *P. zeylanica* are necessary and sufficient to produce plumbagin in yeast. This work represents one of the first full biosynthetic reconstructions of a specialized naphthoquinone pathway in a microbial system. This study also highlights the value of stepwise pathway engineering for elucidating enzyme function and identifying

key bottlenecks. Furthermore, the modular design of the expression constructs under the GAL1 and GAL10 promoters provides flexibility for future optimization efforts. This could include promoter fine-tuning or compartmentalization strategies to improve yield and reduce intermediate accumulation. Also the availability of malonyl-CoA to form heterologous metabolites in yeast is bottleneck as it is required for fatty acid metabolism in yeast. In addition, the reconstruction of the pathway in yeast paves the way for exploring the structural analogs of plumbagin through precursor feeding or enzymatic engineering with potential applications in developing novel bioactive compounds [25, 26].

5 Conclusion

The present study showed the stepwise reconstruction of the plumbagin biosynthetic pathway in *S. cerevisiae* using six genes derived from *P. zeylanica*. All genes play a vital role in the chronological conversion of primary metabolites acetyl-CoA and malonyl-CoA into plumbagin. Expression of the PCA genes synthesized the first stable intermediate 3-methyl-1,8-naphthalenediol. Subsequent addition of CYP81B140 and CPR enabled the production of isoshinanolone while inclusion of CYP81B141 completed the pathway and produced plumbagin.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1007/s13659-025-00588-7>.

Supplementary material 1. Online Resource 1a: Primer sequences used for the full length gene amplification and cloning of six genes in yeast. Online Resource 1b: Gene cloning details: vector name and restriction sites used for cloning. Online Resource 2a: Full length amplification and colony PCR of six candidate genes from *P. zeylanica*. Online Resource 2b: Full length amplification and colony PCR of six candidate genes of *P. zeylanica*. Online Resource 2c: Plasmid PCR of positive clones of six gene using vector specific primers. Online Resource 2d: Full length amplification of genes using reverse transcription (RT)-PCR with yeast cDNA as template in the dextrose and galactose media. Online Resource 3a: MS and MS/MS spectra of 3-methyl-1,8-naphthalenediol from induced PCA yeast strain. Online Resource 3b: MS and MS/MS spectra of 3-methyl-1,8-naphthalenediol from PCACC yeast strain. Online Resource 3c: MS and MS/MS spectra of isoshinanolone from the induced PCACC yeast strain. Online Resource 3d: MS and MS/MS spectra of Plumbagin synthesized by PCACCC yeast and standard Plumbagin.

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Author contributions

APV performed all the experimental work and wrote the manuscript. VTB designed the experiment, supervised the study and revised the manuscript. Both the authors read and approved the final manuscript.

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Data availability

All data supporting the findings of this manuscript are available within the paper and its online resources. The plasmid constructs will be made available on reasonable request.

Declarations

Competing interests

Both authors don't have any relevant financial or non-financial interests to disclose. The authors have no competing interests to declare that are relevant to the content of this article.

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References

- Szczębara FM, Chandelier C, Villeret C, Masurel A, Bourot S, Duport C, et al. Total biosynthesis of hydrocortisone from a simple carbon source in yeast. *Nat Biotechnol*. 2003;21(2):143–9. <https://doi.org/10.1038/nbt775>.
- Ro DK, Paradise EM, Ouellet M, Fisher KJ, Newman KL, Ndungu JM, et al. Production of the anti-malarial drug precursor artemisinic acid in engineered yeast. *Nature*. 2006;440(7086):940–3. <https://doi.org/10.1038/nature04640>.
- Nowrouzi B, Li RA, Walls LE, d'Espaux L, Malci K, Liang L, et al. Enhanced production of taxadiene in *Saccharomyces cerevisiae*. *Microb Cell Fact*. 2020;19(1):200. <https://doi.org/10.1186/s12934-020-01458-2>.
- Li Y, Li S, Thodey K, Trenchard I, Cravens A, Smolke CD. Complete biosynthesis of nescapine and halogenated alkaloids in yeast. *Proc Natl Acad Sci U S A*. 2018;115(17):E3922–31. <https://doi.org/10.1073/pnas.1721469115>.
- Shukla B, Saxena S, Usmani S, Kushwaha P. Phytochemistry and pharmacological studies of *Plumbago zeylanica* L.: a medicinal plant review. *Clin Phytoscience*. 2021;7(1):34.
- Abe I, Oguro S, Utsumi Y, Sano Y, Noguchi H. Engineered biosynthesis of plant polyketides: chain length control in an octaketide-producing plant type III polyketide synthase. *J Am Chem Soc*. 2005;127(36):12709–16.
- Jadhav S, Phapale P, Thulasiram HV, Bhargava S. Polyketide synthesis in tobacco plants transformed with a *Plumbago zeylanica* type III hexaketide synthase. *Phytochemistry*. 2014;98:92–100. <https://doi.org/10.1016/j.phytochem.2013.11.017>.
- Vasav AP, Meshram BG, Pable AA, Barvkar VT. Artificial microRNA mediated silencing of cyclase and aldo-keto reductase genes reveal their involvement in the plumbagin biosynthetic pathway. *J Plant Res*. 2023;136(1):47–62. <https://doi.org/10.1007/s10265-022-01415-7>.
- Vasav AP, Godbole RC, Darshetkar AM, Pable AA, Barvkar VT. Functional genomics-enabled characterization of CYP81B140 and CYP81B141 from *Plumbago zeylanica* L. substantiates their involvement in plumbagin biosynthesis. *Planta*. 2022;256(6):102. <https://doi.org/10.1007/s00425-022-04014-x>.
- Gietz RD, Schiestl RH. High-efficiency yeast transformation using the LiAc/SS carrier DNA/PEG method. *Nat Protoc*. 2007;2(1):31–4. <https://doi.org/10.1038/nprot.2007.13>.
- Canelas AB, ten Pierick A, Ras C, Seifar RM, van Dam JC, van Gulik WM, et al. Quantitative evaluation of intracellular metabolite extraction techniques for yeast metabolomics. *Anal Chem*. 2009;81(17):7379–89. <https://doi.org/10.1021/ac900999t>.
- Schmitt ME, Brown TA, Trumpower BL. A rapid and simple method for preparation of RNA from *Saccharomyces cerevisiae*. *Nucleic Acids Res*. 1990;18(10):3091–2. <https://doi.org/10.1093/nar/18.10.3091>.
- Vasav AP, Pable AA, Barvkar VT. Differential transcriptome and metabolome analysis of *Plumbago zeylanica* L. reveal putative genes involved in plumbagin biosynthesis. *Fitoterapia*. 2020;147:104761. <https://doi.org/10.1016/j.fitote.2020.104761>.
- Wang F, Liigand J, Tian S, Arndt D, Greiner R, Wishart DS. CFM-ID 4.0: more accurate ESI-MS/MS spectral prediction and compound identification. *Anal Chem*. 2021;93(34):11692–700. <https://doi.org/10.1021/acs.analchem.1c01465>.
- Abe I, Morita H. Structure and function of the chalcone synthase superfamily of plant type III polyketide synthases. *Nat Prod Rep*. 2010;27(6):809–38. <https://doi.org/10.1039/b909988n>.
- Matsuzawa M, Katsuyama Y, Funa N, Horinouchi S. Alkylresorcylic acid synthesis by type III polyketide synthases from rice *Oryza sativa*. *Phytochemistry*. 2010;71(10):1059–67. <https://doi.org/10.1016/j.phytochem.2010.02.012>.
- Austin MB, Noel JP. The chalcone synthase superfamily of type III polyketide synthases. *Nat Prod Rep*. 2003;20(1):79–110. <https://doi.org/10.1039/b100917f>.
- Funa N, Ohnishi Y, Fujii I, et al. A new pathway for polyketide synthesis in microorganisms. *Nature*. 1999;400:897–9. <https://doi.org/10.1038/23748>.
- Katsuyama Y, Matsuzawa M, Funa N, Horinouchi S. In vitro synthesis of curcuminoids by type III polyketide synthase from *Oryza sativa*. *J Biol Chem*. 2007;282(52):37702–9. <https://doi.org/10.1074/jbc.M707569200>.
- Gagne SJ, Stout JM, Liu E, Boubakir Z, Clark SM, Page JE. Identification of olivetolic acid cyclase from *Cannabis sativa* reveals a unique catalytic route to plant polyketides. *Proc Natl Acad Sci U S A*. 2012;109(31):12811–6. <https://doi.org/10.1073/pnas.1200330109>.
- Wang B, Kong W, Lv L, Wang Z. Plumbagin induces ferroptosis in colon cancer cells by regulating p53-related SLC7A11 expression. *Heliyon*. 2024;10(7):e28364. <https://doi.org/10.1016/j.heliyon.2024.e28364>.
- Powolny AA, Singh SV. Plumbagin-induced apoptosis in human prostate cancer cells is associated with modulation of cellular redox status and generation of reactive oxygen species. *Pharm Res*. 2008;25(9):2171–80. <https://doi.org/10.1007/s11095-008-9533-3>.
- Zhang Y, Nielsen J, Liu Z. Engineering yeast metabolism for production of terpenoids for use as perfume ingredients, pharmaceuticals and biofuels. *FEMS Yeast Res*. 2017. <https://doi.org/10.1093/femsyr/fox080>.
- Lau W, Sattely ES. Six enzymes from mayapple that complete the biosynthetic pathway to the etoposide aglycone. *Science*. 2015;349(6253):1224–8. <https://doi.org/10.1126/science.aac7202>.
- Moses T, Pollier J, Thevelein JM, Goossens A. Bioengineering of plant (tri) terpenoids: from metabolic engineering of plants to synthetic biology in vivo and in vitro. *New Phytol*. 2013;200(1):27–43. <https://doi.org/10.1111/nph.12325>.
- Cravens A, Payne J, Smolke CD. Synthetic biology strategies for microbial biosynthesis of plant natural products. *Nat Commun*. 2019;10(1):2142. <https://doi.org/10.1038/s41467-019-09848-w>.

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