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Fine-Tuned Transcription Factor Engineering and Precursor Rewiring Drive Enhanced Production of Berberine in *Nicotiana benthamiana*

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ABSTRACT

Berberine is a pharmacologically active benzylisoquinoline alkaloid (BIA) with broad therapeutic potential. Although its heterologous biosynthesis has been achieved in microbial hosts, large-scale production remains hindered by the pathway complexity, metabolic burden, and inefficient expression of plant-derived cytochrome P450 enzymes. Here, we report the complete *de novo* biosynthesis of berberine in *Nicotiana benthamiana* through systematic pathway reconstruction and metabolic engineering. By increasing the availability of precursors, such as dopamine and 3,4-dihydroxyphenylacetaldehyde (3,4-DHPAA), and enhancing *L*-tyrosine biosynthesis via plastid-targeted strategies, we achieved a (S)-reticuline yield of 17.76 $\mu\text{g}\cdot\text{g}^{-1}$ dry weight (DW). Introduction of downstream biosynthetic genes enabled the production of berberine at suboptimal titers. The accumulation of *L*-tyrosine-derived metabolites in competing pathways demonstrates substantial potential for flux optimization within the target pathway. However, additional transcription factors (TFs) lead to excessive transcriptional activation, resulting in leaf necrosis. This issue was resolved by fine-tuning TF expression using promoters of varying strengths. Subsequent enzyme engineering of the berberine bridge enzyme (BBE), specifically the *AmBBE1*^{A400I-I431F} variant, further increased the titer. Coexpression of this optimized BBE with the finely tuned TF CjWRKY1 culminated in a final berberine yield of 13.91 $\mu\text{g}\cdot\text{g}^{-1}$ DW, which further increased to 15.03 $\mu\text{g}\cdot\text{g}^{-1}$ DW after postharvest heat treatment. To our knowledge, this represents the first complete reconstruction of the berberine biosynthetic pathway in a nonnatural plant chassis, establishing a robust and scalable platform for alkaloid production. Although current yields remain lower than those of microbial systems, our study provides a generalizable framework for the plant-based biosynthesis of complex natural products.

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1. Introduction

Benzylisoquinoline alkaloids (BIAs) represent a structurally diverse class of plant-derived natural products with significant pharmacological importance [1,2]. Well-known BIAs such as morphine, noscapine, berberine, cepharanthine, and papaverine have long served as clinically relevant medicines [3]. Among these compounds, berberine is notable for its more than 3000-year history of

use in traditional medicine and its broad-spectrum pharmacological activities, as supported by modern studies [4]. As a quaternary ammonium-type BIA, berberine is naturally found in the roots, stems, and bark of plants in the *Berberidaceae*, *Ranunculaceae*, and *Papaveraceae* families [5]. Many pharmacological and clinical studies have demonstrated the multifaceted bioactivities of berberine, such as anti-inflammatory [6], anticancer [7], antiobesity [8,9], antidepressant, neuroprotective [10,11], hepatoprotective [12], and cardioprotective effects [13].

Currently, berberine production relies mainly on extraction from medicinal plants such as *Coptis chinensis*. However, the long growth cycle (6–8 years), low metabolite abundance, and stringent cultivation requirements limit sustainable and scalable production

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[14]. Although chemical synthesis provides an alternative route, it often involves high-pressure hydrogenation and expensive palladium-based catalysts, resulting in harsh reaction conditions, high costs, and environmental concerns [15].

Synthetic biology offers a promising alternative for the sustainable and environmentally friendly production of BIAs [16–21]. The complete biosynthetic pathway of berberine in plants has been elucidated, and efforts to reconstruct this pathway in microbial chassis such as *Escherichia coli* (*E. coli*) and *Saccharomyces cerevisiae* (*S. cerevisiae*) have achieved initial success. The berberine biosynthetic pathway originates from *L*-tyrosine (Fig. 1). In plants, dopamine and 4-hydroxyphenyl acetaldehyde (4-HPAA), two tyrosine derivatives, are condensed into (*S*)-norcoclaurine by norcoclaurine synthase (NCS) [22]. Alternatively, a microbial monoamine oxidase (MAO) has been employed in the BIA pathway for the synthesis of norlaudanosoline via NCS to skip the step of plant cytochrome P450 hydroxylase (CYP80B2) in the microbial chassis [23]. For instance, engineered *E. coli* strains produce up to 16.81 mg·L⁻¹ (*S*)-canadine [24] and 1.65 mg·L⁻¹ berberine in shake flask cultures (unpublished data from our previous work), and *S. cerevisiae* yields up to 16.9 mg·L⁻¹ berberine [25]. Nevertheless, these titers remain far from commercially viable. The low efficiency is due primarily to challenges such as the pathway complexity, poor functional expression of plant cytochrome P450 enzymes, and the toxicity of pathway intermediates and products to microbial hosts [26]. A one-pot whole-cell biocatalysis system in *E. coli* yielded 3.19 g·L⁻¹ (*S*)-scoulerine, a key berberine precursor. However, this strategy depends on costly exogenous substrates and cofactors (e.g., dopamine and adenosine triphosphate (ATP)), thereby limiting its economic feasibility [27].

Plant chassis provide unique advantages for BIA production, such as the innate biosynthesis of aromatic amino acids (AAAs), subcellular compartmentalization, and compatibility with plant-specific enzymes. Among them, *Nicotiana benthamiana* (*N. benthamiana*) has emerged as a highly versatile host for transient expression, allowing for rapid and modular reconstitution of complex biosynthetic pathways. Notably, heterologous production of momilactone B in *N. benthamiana* reached 167 μg·g⁻¹ dry weight (DW) within days, exceeding its natural abundance in rice hulls

[28]. Furthermore, coinfiltration of multiple *Agrobacterium* strains enables efficient pathway assembly without the need for complex DNA constructs [29]. While *N. benthamiana* has been successfully used to produce various classes of natural plant products, the heterologous biosynthesis of BIAs, particularly berberine, has not yet been achieved.

In this study, we established a transient expression-based platform for the efficient biosynthesis of berberine in *N. benthamiana*. Initial attempts to reconstruct the (*S*)-reticuline pathway yielded no detectable product, highlighting a critical bottleneck in precursor supply. To overcome this, we implemented targeted precursor enhancement strategies, which successfully enabled the accumulation of (*S*)-reticuline. Subsequent integration of the downstream enzymatic modules led to the first demonstration of *de novo* berberine biosynthesis in *N. benthamiana*. To further improve the titers, we optimized the expression of rate-limiting enzymes and implemented promoter-guided fine-tuning of transcription factor (TF) activity. This combinatorial strategy significantly enhanced metabolic flux through the pathway, ultimately resulting in increased berberine accumulation.

2. Methods and materials

2.1. Plant materials

N. benthamiana plants were cultivated in soil under greenhouse conditions with a 16 h light/8 h dark photoperiod at 25 °C.

2.2. Plasmid construction

Genes involved in berberine biosynthesis were either synthesized *de novo* (AnHui, China) or cloned from cDNA templates. DNA fragments were amplified using PrimeSTAR Max DNA Polymerase (R045A; TaKaRa, Japan) with the gene-specific primers listed in Table S1 in Appendix A. Polymerase chain reaction (PCR) products were inserted into the pEAQ-HT vector via one-step seamless cloning (10911ES50; Yeasen, China) following restriction digestion.

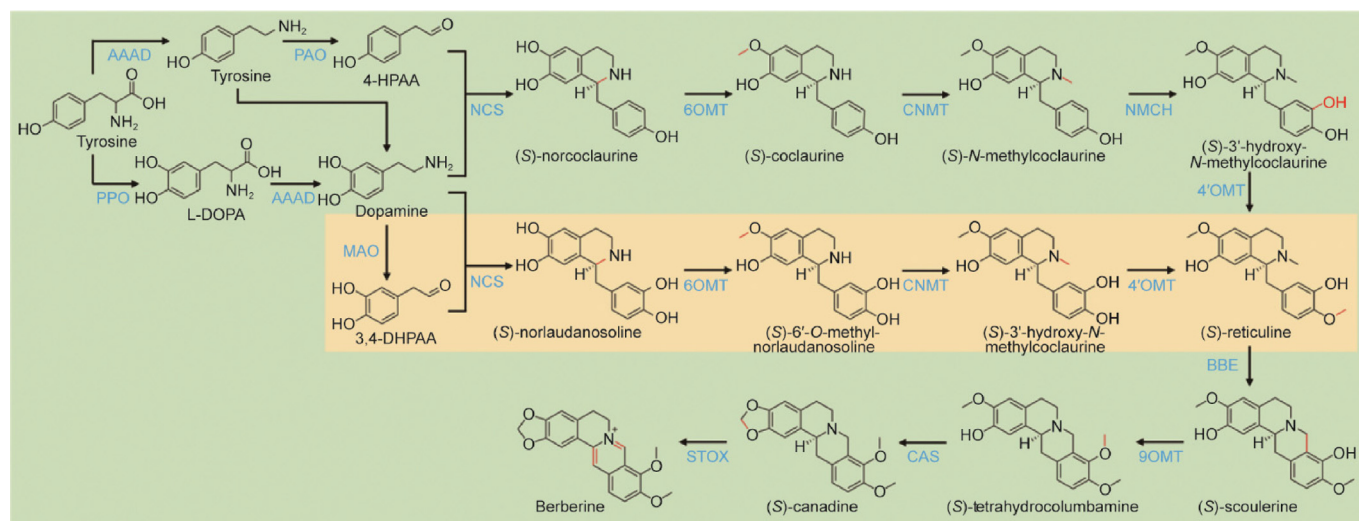


Fig. 1. Berberine biosynthetic pathways in plants and engineered microbes. In the native plant pathway (green), *L*-tyrosine is converted into (*S*)-reticuline via the sequential action of polyphenol oxidase (PPO), aromatic *L*-amino-acid decarboxylase (AAAD), primary amine oxidase (PAO), norcoclaurine synthase (NCS), norcoclaurine 6-*O*-methyltransferase (6OMT), coclaurine *N*-methyltransferase (CNMT), (*S*)-*N*-methylcoclaurine-3'-hydroxylase (NMCH), and 3'-hydroxy-*N*-methylcoclaurine-4'-*O*-methyltransferase (4'OMT), yielding (*S*)-reticuline. Downstream tailoring in plants occurs via the berberine bridge enzyme (BBE), (*S*)-scoulerine-9-*O*-methyltransferase (9OMT), canadine synthase (CAS) and, optionally, tetrahydroprotoberberine oxidase (STOX) to produce berberine. The engineered microbial route (yellow) focuses on optimizing the precursor supply and expression of key enzymes to increase (*S*)-reticuline and downstream intermediate accumulation. MAO: monoamine oxidase.

For subcellular localization studies, target genes were fused to green fluorescent protein (GFP) using a GGGGG linker to generate chimeric constructs. fine-tuning TF sequences were codon-optimized for *N. benthamiana* and synthesized by Sangon Biotech (Shanghai, China), flanked by *Bsa*I-HFv2 recognition sites (AATG and GCCT; #R3733, New England Biolabs, USA). TF expression cassettes were driven by distinct promoters, terminated with the *Arabidopsis thaliana* Hsp terminator, and subsequently cloned and inserted into the pCF001 vector as previously described [30]. A full list of plasmids used in this study is provided in Table S2 in Appendix A.

2.3. Transient expression in *N. benthamiana*

All binary plasmids were subsequently transformed into *Agrobacterium tumefaciens* strain GV3101. Single colonies were cultured overnight at 28 °C and 220 revolutions per minute (rpm) in Luria broth (LB) medium supplemented with 50 mg·L⁻¹ kanamycin, 25 mg·L⁻¹ rifampicin, and 25 mg·L⁻¹ gentamycin. Cells were harvested by centrifugation at 8000 rpm for 5 min and resuspended in infiltration buffer containing 10 mmol·L⁻¹ (mM) 2-(*N*-morpholino)ethanesulfonic acid (MES; Sangon Biotech, China), 10 mM MgCl₂, and 200 μM acetosyringone to an optical OD₆₀₀ of 1.2. Equal volumes of different strains harboring individual constructs were mixed and incubated at room temperature for 3 h before infiltration into the abaxial side of 5-week-old *N. benthamiana* leaves.

2.4. Subcellular localization

For subcellular localization, *A. tumefaciens* suspensions carrying GFP-tagged target genes and an endoplasmic reticulum (ER)-localized mCherry marker were coinfiltrated into tobacco leaves. At 48 h post-infiltration, the leaf samples were mounted on microscope slides and examined under a confocal laser scanning microscope (Zeiss LSM880, Germany). Excitation wavelengths of 405, 488, and 556 nm were used, and images were captured using ZEN BLACK software.

2.5. Metabolite extraction

Leaf tissues (0.05 g) were collected from infiltrated areas six days post-infiltration, placed in 1.5 mL microcentrifuge tubes with grinding beads, and flash-frozen in liquid nitrogen. The samples were ground using a tissue grinder (Wonbio-48RS, WONBIO, China) and homogenized in 250 μL of methanol.

For quantitative metabolite analysis, 0.2–0.5 mg of infiltrated leaf tissue was collected, snap-frozen, and lyophilized overnight at –80 °C using a FreeZone Plus 6 L freeze dryer (Labconco, USA). The dried tissue was pulverized and extracted with methanol (0.1 g of tissue per 1 mL of solvent). Extractions were performed by shaking at 55 Hz for 1 min, followed by centrifugation at 12 000 rpm for 5 min. The supernatant was collected, and the extraction was repeated three times. The combined extracts were concentrated under reduced pressure and redissolved in 50 μL of methanol. The final samples were filtered through 0.2 μm membranes prior to ultra-performance liquid chromatography–mass spectrometry (UPLC MS) analysis.

2.6. In vivo imaging of betanin accumulation

To visualize betanin accumulation, the infiltrated leaves were imaged using an IVIS Spectrum *in vivo* imaging system (PerkinElmer, USA) with an excitation wavelength of 535 nm and an emission wavelength of 600 nm. Fluorescence quantification was performed using Living Image software (v4.4; Caliper Life Sciences,

PerkinElmer). Circular regions of interest (ROI) were defined manually on each leaf image, and fluorescence intensity values were extracted. All the imaging parameters were kept constant across the samples for comparative analysis.

2.7. Targeted metabolite analysis

Qualitative analysis was performed using an Agilent 6545 Q-TOF mass spectrometer coupled to an Agilent 1260 Infinity high performance liquid chromatography (HPLC) system. Separation was achieved using an Agilent 300 Extend-C18 column (4.6 × 150 mm, 3.5 μm) at 30 °C with a flow rate of 0.35 mL·min⁻¹ and a 1 μL injection volume. The mobile phases were A (ddH₂O + 0.1% formic acid) and B (acetonitrile + 0.1% formic acid), with the following gradient: 0–1 min, 5% B; 1–20 min, 5%–98% B; 20–25 min, 98% B; and 25–26 min, 98%–5% B. Mass spectrometry was conducted in positive ion mode with a scan range of *m/z* 100–1700 and collision energies of 0, 10, 20, and 40 V.

Quantitative analysis was conducted using a SCIEX Triple Quad 6500 + mass spectrometer coupled to a Shimadzu Ultra HPLC system (DGU-405, LC-40D × 3, SCL-40, CTO-40C). Chromatographic separation was achieved on an Agilent RRHD SB-C18 column (2.1 × 100 mm, 1.8 μm) at 30 °C, with a 0.3 mL·min⁻¹ flow rate and 2 μL injection volume. The mobile phase and gradient were 0–3 min, 5% B; 3–5 min, 5%–30% B; 5–22 min, 30%–70% B; 22–24 min, 70%–100% B; 24–29 min, 100% B; 29–29.1 min, 100%–5% B; and 29.1–36 min, 5% B. Mass spectrometry (MS) acquisition was performed in multiple reaction monitoring (MRM) mode with the optimized parameters listed in Table S3 in Appendix A.

The quantification of key metabolites, such as (*S*)-reticuline, (*S*)-scoulerine, (*S*)-tetrahydrocolumbamine, (*S*)-canadine, and berberine, was performed using calibration curves constructed from standard solutions (10–1000 ng·mL⁻¹). Triplicate measurements were performed at each concentration to generate linear regression models, and metabolite concentrations in samples were calculated on the basis of the peak area using the appropriate standard curve (Fig. S1 in Appendix A).

2.8. Untargeted metabolomics analysis

Leaf samples (100 mg) were homogenized with 400 μL of 80% aqueous methanol using a chilled tissue grinder, followed by low-temperature ultrasonication. After centrifugation (15 min), the supernatant was collected for liquid chromatography tandem mass spectrometry (LC MS/MS) analysis using a Thermo UHPLC-Q Exactive system. The raw data were processed with Progenesis QI software to generate a three-dimensional feature matrix. Metabolites were annotated using the MJDBPM plant-specific metabolite database.

3. Results and discussion

3.1. Reconstitution of the (*S*)-reticuline pathway in *N. benthamiana*

Efficient biosynthesis of (*S*)-reticuline, a central intermediate in BIA production, is essential for generating valuable downstream BIAs such as berberine. In analogy with Kyoto Encyclopedia of Genes and Genomes (KEGG) annotations of *Nicotiana tabacum*, *N. benthamiana* harbors native *L*-tyrosine metabolic pathways that potentially supply critical BIA precursors, including dopamine, 4-HPAA, and 3,4-dihydroxyphenylacetaldehyde (3,4-DHPAA) (Fig. S2 in Appendix A). To reconstruct this pathway in *N. benthamiana*, five key downstream genes from *Coptis japonica* were transiently expressed via *Agrobacterium*-mediated infiltration. These precursors condense via NCS and are subsequently converted to

(S)-reticuline through the sequential actions of norcoclaurine 6-O-methyltransferase (6OMT), coclaurine N-methyltransferase (CNMT), (S)-N-methylcoclaurine-3'-hydroxylase (NMCH, optional), and 3'-hydroxy-N-methylcoclaurine-4'-O-methyltransferase (4'OMT) (Fig. 1).

Despite confirmed enzyme expression at 6 d post-infiltration (dpi) (Fig. S3 in Appendix A) and proper subcellular localization to the ER (Fig. 2(a)), LC MS/MS analysis revealed no detectable (S)-reticuline in the infiltrated leaves. This absence suggested a metabolic bottleneck, likely caused by insufficient precursor supply. The expression of CjNCS alone failed to yield either (S)-norcoclaurine or (S)-norlaudanosoline, reinforcing the hypothesis that the endogenous dopamine and aldehyde pools were inadequate to initiate the pathway.

Previous studies in microbial chassis have demonstrated that the use of dopamine and 3,4-DHPAA as substrates leads to efficient BIA biosynthesis [23]. To ensure a more specific and efficient supply of these precursors, we implemented L-tyrosine-derived precursor enhancement strategies by introducing a set of exogenous genes encoding *Beta vulgaris* tyrosine hydroxylase (BvCYP76AD5) [31], *Pseudomonas putida* L-3,4-dihydroxyphenylalanine (L-DOPA) decarboxylase (PpDDC) [32], and *Micrococcus luteus* MAO (MMAO) [33] (Fig. 2(b)). BvCYP76AD5 was selected for its high specificity toward L-tyrosine hydroxylation and its minimal production of cyclo-DOPA, a substitution previously shown to increase BIA yields in yeast [31,34,35]. In parallel, PpDDC and MMAO were chosen because of their proven high efficiency in (S)-reticuline biosynthesis in *E. coli*, thereby providing a validated and reliable route for the generation of dopamine and 3,4-DHPAA [24]. This gene set was designed to supplement endogenous polyphenol oxidase (PPO), AAA decarboxylase (AAD), and primary amine oxidase (PAO), which primarily mediate the formation of 4-HPAA and, to a lesser extent, 3,4-DHPAA. Coexpression of CjNCS with these exogenous genes led to the detectable accumulation of (S)-norlaudanosoline at m/z 288.1230 $[M + H]^+$, whereas (S)-norcoclaurine remained undetectable under such conditions (Fig. 2(c)). These results suggest a preferential flux through the 3,4-DHPAA branch, potentially driven by the higher catalytic efficiency of BvCYP76AD5 toward L-tyrosine and reduced metabolic competition relative to the 4-HPAA route.

Building on this, we optimized the 3,4-DHPAA-dependent pathway by replacing CjNCS1 with a truncated PR10A variant from *C. japonica* (trCjPR10A) [34], which was previously reported to improve (S)-norlaudanosoline formation. Coexpression of trCjPR10A with Cj6OMT, CjCNMT, CjCYP80B2, and Cj4'OMT successfully produced (S)-reticuline *de novo*, as confirmed by LC MS/MS detection of the characteristic peak at m/z 330.1700 $[M + H]^+$, with a yield of 2.44 $\mu\text{g}\cdot\text{g}^{-1}$ DW (Figs. 2(d)–(f)). Notably, omission of CjNMCH had a negligible effect on (S)-reticuline accumulation (Fig. 2(f)), further supporting the dominance of the dopamine and 3,4-DHPAA condensation route in this system. These insights informed subsequent efforts to increase the L-tyrosine precursor supply and direct metabolic flux toward (S)-reticuline synthesis.

3.2. Plastid engineering enhances the supply of endogenous L-tyrosine

In plant cells, L-tyrosine is synthesized primarily in plastids via the shikimate pathway. This pathway is subject to complex regulation, including enzyme isoform diversity and feedback inhibition, posing challenges for metabolic engineering aimed at increasing the supply of endogenous L-tyrosine [36,37]. To bypass these regulatory constraints, we targeted key enzymes downstream of chorismate. Plastid-localized chorismate mutase 1 (CM1), prephenate aminotransferase (PPT), and arogenate dehydrogenase (ADH) catalyze sequential conversion to L-tyrosine through prephenate and arogenate [37]. Alternatively, plastid-derived isochorismate

may be exported to the cytosol and processed via a microbial-like pathway involving cytosolic chorismate mutase 2 (CM2), prephenate dehydrogenase (PDH), and tyrosine aminotransferase (TAT), reminiscent of bacterial systems such as *E. coli*, which utilize bifunctional TyrA^{fb} (CM2/PDH) and TyrB (TAT homolog) enzymes [38,39]. Leveraging this knowledge, we constructed plastid- and cytosol-targeted metabolic modules to increase L-tyrosine availability for (S)-reticuline biosynthesis (Fig. 3(a)). A visible reporter system based on betanin biosynthesis (BvCYP76AD1, BvDODA, and Mj5GT) was used to monitor L-tyrosine levels in planta (Fig. S4 in Appendix A).

Key enzymes were chosen on the basis of substrate affinity and feedback insensitivity: cytosolic *A. thaliana* AtCM2 was favored over plastidial AtCM1 because of its higher affinity and lower feedback sensitivity, whereas plastidial AtADH1 was preferred over AtADH2 because of its superior catalytic efficiency (P1 in Fig. 3(a)). To further circumvent native regulation, the feedback-insensitive microbial enzymes EcTyrA^{fb} [40] and EcTyrB were incorporated (P2 in Fig. 3(a)). These enzymes, with the exception of AtADH1, were fused to the transit peptide of *A. thaliana* Rubisco small subunit 1A (Rs1A) to ensure plastid targeting, which was confirmed by subcellular localization assays (Fig. S5 in Appendix A). Among the constructs tested, the coexpression of plastid-targeted Rs1A-EcTyrA^{fb} and Rs1A-EcTyrB significantly increased betanin pigmentation in leaves, indicating elevated L-tyrosine pools (Fig. 3(b) and Fig. S6 in Appendix A). In contrast, the coexpression of plastid-targeted Rs1A-AtCM2 and AtADH1 modestly decreased. This superior performance of microbial enzymes likely stems from their orthogonality and reduced sensitivity to host feedback inhibition.

Considering that BIA biosynthesis, like betalain production, occurs mainly outside the plasmid, we assessed cytosolic L-tyrosine enhancement strategies (C1 and C2 in Fig. 3(a)), including direct cytosolic expression of EcTyrA^{fb} and EcTyrB and plastid-targeted enzymes combined with a plastid-redirection variant of the *A. thaliana* AtCAT6 transporter (Rs1A-AtCAT6) to facilitate L-tyrosine transport to the cytosol. The cytosolic approaches did not significantly increase L-tyrosine levels relative to those in the controls (Fig. 3(c)), likely because of limited cytosolic isochorismate and/or chorismate availability, suboptimal transporter activity, or efficient endogenous export.

Together, these results establish plastid engineering as a superior strategy for enhancing the endogenous L-tyrosine supply. The incorporation of plastid-targeted L-tyrosine biosynthetic modules substantially increased (S)-reticuline production, from 2.84 to 17.76 $\mu\text{g}\cdot\text{g}^{-1}$ DW (Fig. 3(d)). This spatial organization, with upstream L-tyrosine biosynthesis in plastids and downstream BIA biosynthesis in the cytosol, recapitulates native cellular compartmentalization and aligns with previous successful heterologous alkaloid pathway reconstructions in *N. benthamiana* [41].

3.3. Reconstitution of the berberine pathway in *N. benthamiana*

Building upon the enhanced (S)-reticuline biosynthesis achieved via plastid-targeted L-tyrosine pathway engineering, we reconstituted the complete berberine biosynthetic pathway by introducing four downstream tailoring enzymes that convert (S)-reticuline into berberine: berberine bridge enzyme (BBE), scoulerine 9-O-methyltransferase (9OMT), canadine synthase (CYP719As, also known as CAS), and tetrahydroberberine oxidase (STOX) (Fig. 4(a)). Using the high-yield (S)-reticuline construct as a platform, we sequentially introduced *Papaver somniferum* PsBBE1 [42], *C. japonica* Cj9OMT [43], CjCYP719A1 [44], and *Berberis wilsonae* BwSTOX [45], which have been efficiently characterized and applied in other platforms for protoberberine alkaloid biosynthesis [24,46]. LC MS analysis confirmed the production of pathway

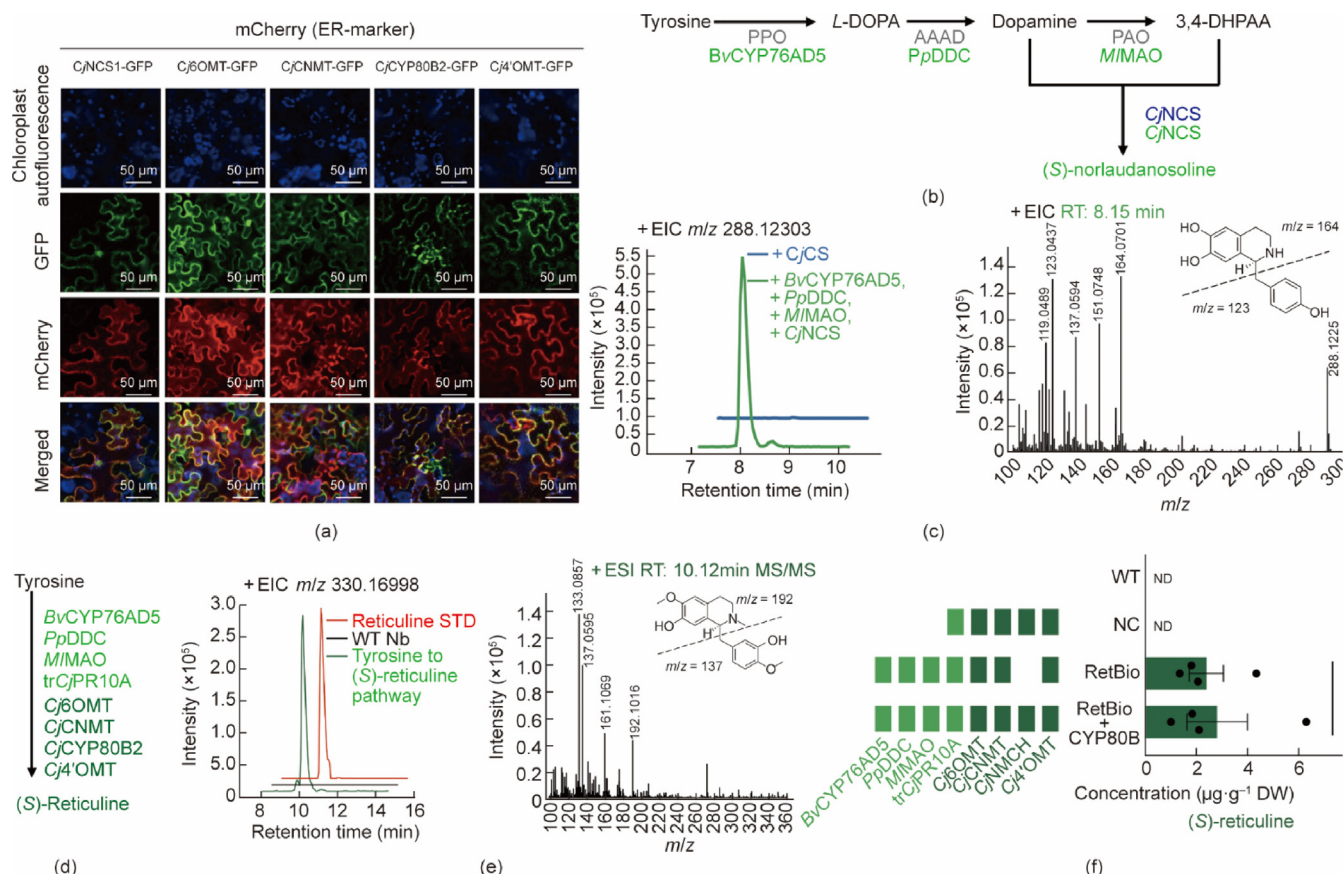


Fig. 2. Reconstruction of (S)-reticuline biosynthesis in *N. benthamiana*. (a) Subcellular localization of biosynthetic enzymes involved in BIA production. Enhanced GFP (EGFP)-tagged target proteins and ER-localized mCherry markers were transiently coexpressed in *N. benthamiana* leaves and visualized by confocal microscopy. Scale bars, 50 μ m. (b) Strategies to increase endogenous precursor supply from tyrosine. Gray enzymes indicate putative endogenous enzymes based on the *N. tabacum* tyrosine metabolic network (Fig. S1); blue and green indicate the use of exogenous enzyme combinations, respectively. (c) Detection of the biosynthetic intermediate (S)-norlaudanoline. The extracted ion chromatogram (EIC) at m/z 288.12303 shows a peak at 8.15 min; the corresponding MS/MS fragmentation spectrum is shown on the right. (d) Heterologous reconstruction of the L-tyrosine-to-(S)-reticuline pathway via stepwise expression of eight biosynthetic genes in *N. benthamiana*. (e) The EIC at m/z 330.1700 identifies (S)-reticuline with a retention time of 10.12 min; the MS/MS fragmentation pattern is shown on the right. (f) Quantification of (S)-reticuline by UPLC MS/MS. Data represent the means $\pm$ standard errors of the means (s.e.m.) of four biological replicates. Statistical analysis was performed using two-tailed Student's *t* tests. ns: not significant; NC: negative control; ND: not detected.

intermediates and ultimately berberine in the infiltrated leaves (Figs. 4(b)–(e) and Figs. S7–S10 in Appendix A).

The expression of *PsbBE1* nearly depleted (S)-reticuline, with the accumulation of (S)-scoulerine at $0.49 \mu\text{g}\cdot\text{g}^{-1}$ DW, corresponding to an approximately 2.6% conversion efficiency (Fig. 4(f)). This low efficiency likely results from the substantial diversion of (S)-scoulerine into competing endogenous pathways in *N. benthamiana*. In support of this, glycosylated derivatives of (S)-scoulerine (e.g., m/z 490.2070 $[\text{M} + \text{H}]^+$) were detected by LC MS/MS (Fig. S11 in Appendix A), indicating host-mediated metabolic modifications. Encouragingly, *Cj9OMT* expression efficiently redirected flux toward (S)-tetrahydrocolumbamine ($6.53 \mu\text{g}\cdot\text{g}^{-1}$ DW), confirming effective 9-O-methylation and partial mitigation of host interference. The subsequent introduction of *CjCYP719A1* yielded $7.84 \mu\text{g}\cdot\text{g}^{-1}$ DW (S)-canadine and $2.60 \mu\text{g}\cdot\text{g}^{-1}$ DW berberine, with a conversion rate of approximately 25% (Fig. S12 in Appendix A). In *N. benthamiana*, the conversion of (S)-canadine to berberine may not rely solely on the spontaneous oxidation observed in microbial systems [24]. BLASTp analysis using *BwSTOX* revealed multiple candidate oxidases with high sequence similarity, several of which were expressed in leaf tissues, suggesting potential enzymatic contributions to this reaction (Table S4 in Appendix A). Unexpectedly, the coexpression of *BwSTOX* reduced (S)-canadine ($2.03 \mu\text{g}\cdot\text{g}^{-1}$ DW) and berberine ($0.61 \mu\text{g}\cdot\text{g}^{-1}$ DW) levels. This

interference may result from competition for common cofactors or the formation of unstable oxidation intermediates that subtly perturb pathway flux [45,47]. Additionally, as a flavin adenine dinucleotide (FAD)-dependent oxidase, STOX generates hydrogen peroxide as a byproduct, which can transiently affect cellular redox homeostasis and the activity of BBE, similar to what has been reported in yeast [25]. Despite these effects on metabolite levels, the infiltrated leaves did not show visible phenotypic changes. Consequently, STOX was excluded from further construction to increase the yield of berberine. At each enzymatic step, upstream substrates were nearly completely consumed, indicating efficient metabolic flux in planta. This contrasts with microbial systems, where membrane-associated enzymes often limit downstream conversion despite high (S)-reticuline titers [20,47].

To identify potential metabolic bottlenecks, untargeted metabolomics was performed on *N. benthamiana* leaves expressing the full pathway. A total of 632 differentially accumulated metabolites were detected compared with those in the controls, with 380 significantly upregulated (Table S5 in Appendix A). Elevated levels of dopamine, shikimate, and arogenate aligned with increased flux toward BIA biosynthesis (Fig. 4(g)). However, KEGG enrichment analysis revealed significant accumulation of phenylpropanoid pathway metabolites, representing major competing branches downstream of the shikimate pathway (Fig. S13 in Appendix A).

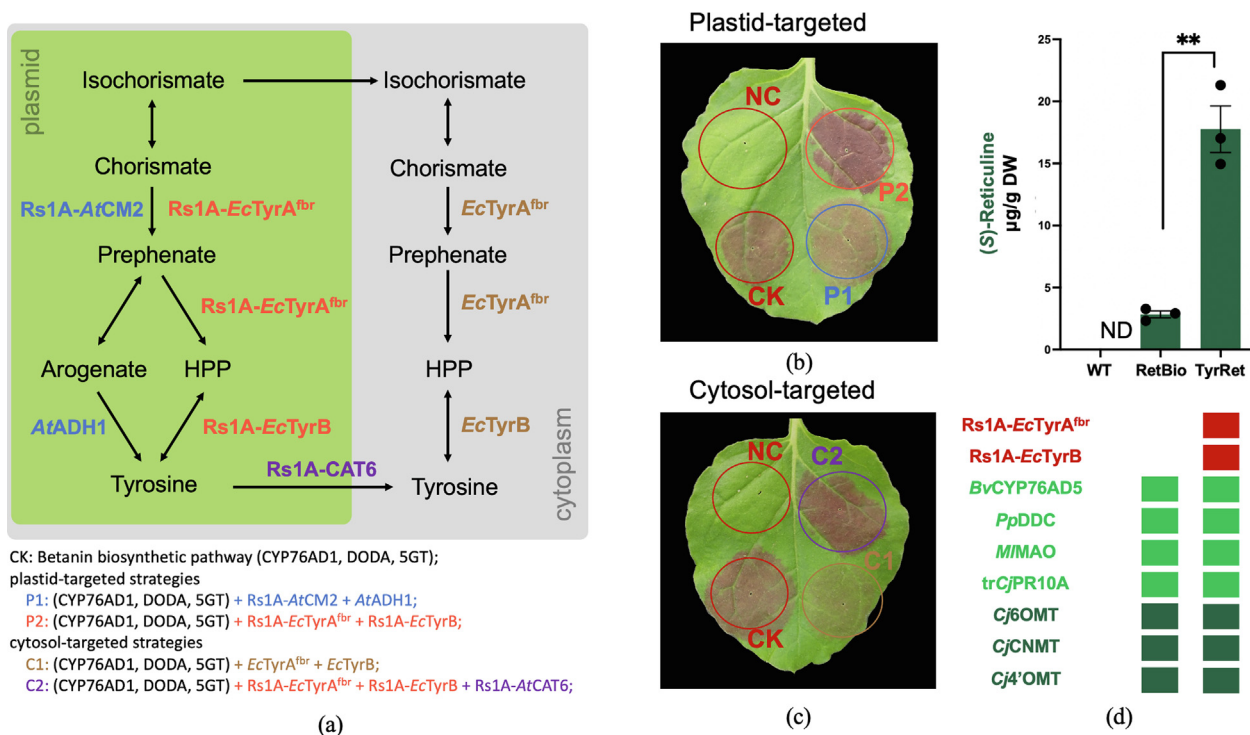


Fig. 3. Plastid engineering enhances the endogenous *L*-tyrosine supply in *N. benthamiana*. (a) Schematic representation of plastid- and cytosol-targeted metabolic engineering strategies designed to increase *L*-tyrosine levels. (b) Betanin production in *N. benthamiana* leaves following plastid-targeted enhancement of *L*-tyrosine biosynthesis. The fluorescence-based radiant efficiency was measured using *in vivo* imaging (Fig. S6). (c) Betanin production under cytosol-targeted tyrosine enhancement strategies. (d) Quantification of (S)-reticuline levels by UPLC MS/MS under different tyrosine enhancement strategies. Data represent the means $\pm$ s.e.m. from four biological replicates. Statistical significance was determined using a two-tailed Student's *t* test. $P < 0.05$ (*), $P < 0.01$ (**).

These findings indicate substantial flux diversion into competing endogenous pathways. Despite successful pathway reconstruction, only a fraction of the enhanced *L*-tyrosine precursor pool was channeled into berberine biosynthesis, highlighting considerable potential for further flux optimization.

3.4. TF engineering promotes the synthesis of berberine

TFs are key regulators of secondary metabolism in plants and provide effective tools to increase metabolite accumulation. Typically, pathway-specific TFs control biosynthetic flux; for instance, WRKY TFs participate in BIA biosynthesis [48], whereas MYB family members regulate phenylpropanoid pathways [49]. To increase berberine production in *N. benthamiana*, we employed a TF-based metabolic engineering approach that both strengthened the BIA pathway and reduced competing phenylpropanoid flux. We selected six TFs with known involvement in tyrosine-derived pathways: *CjWRKY1* from *C. japonica* [50], *PsWRKY* from *Papaver somniferum* [51], *NnWRKY70a* and *NnWRKY70b* from *Nelumbo nucifera* [52], which activate BIA biosynthesis; *AmMYB308* from *Antirrhinum majus* [53], a repressor of phenylpropanoid metabolism; and *AtMYB12* from *A. thaliana* [54,55], which is reported to increase *L*-DOPA and flavonoid levels (Fig. 5(a)). Each TF was expressed under the strong constitutive promoter *P_{StUbi}* [30] and coinfiltrated individually with the (S)-canadine biosynthetic pathway (CP).

Coexpression of *CjWRKY1*, *NnWRKY70a*, *NnWRKY70b*, *AmMYB308*, or *AtMYB12* resulted in severe leaf necrosis, whereas the introduction of *PsWRKY* had no obvious effect on leaf morphology or growth (Fig. 5(b)). Notably, the CP + *PsWRKY* leaves accumulated berberine at $5.49 \mu\text{g}\cdot\text{g}^{-1}$ DW and (S)-canadine at $2.68 \mu\text{g}\cdot\text{g}^{-1}$ DW (Fig. 5(c)). Although *CjWRKY1* induced necrosis, its coexpression still yielded $3.80 \mu\text{g}\cdot\text{g}^{-1}$ DW berberine and

$4.76 \mu\text{g}\cdot\text{g}^{-1}$ DW (S)-canadine, a trend similarly observed for *NnWRKY70a* and *NnWRKY70b*, albeit with relatively lower productivity (Fig. 5(c)). The CP + *AtMYB12* combination resulted in lower berberine levels ($0.8 \mu\text{g}\cdot\text{g}^{-1}$ DW) but increased (S)-canadine levels ($6.91 \mu\text{g}\cdot\text{g}^{-1}$ DW) (Fig. 5(c)). *AmMYB308* coexpression led to negligible accumulation of both metabolites (Fig. 5(c)). Across all combinations, pathway intermediates such as (S)-reticuline, (S)-scoulerine, and (S)-tetrahydrocolumbamine remained at trace levels ($<0.8 \mu\text{g}\cdot\text{g}^{-1}$ DW), indicating efficient flux under permissive cellular conditions (Fig. 5(c)).

Compared with that in the CP group, the addition of *PsWRKY* increased the berberine content by 167 %, while the total content of (S)-canadine and berberine decreased (Fig. S14 in Appendix A). Although *CjWRKY1* caused tissue necrosis, the yield of berberine was comparable to that of CP, and the total yields of (S)-canadine and berberine were comparable to those of *PsWRKY*, suggesting that biosynthesis occurred rapidly before cell death and indicating a strong positive regulatory role of *CjWRKY1*. Although these TFs do not directly activate the promoters of heterologous biosynthetic genes, their effects likely arise from indirect reprogramming of endogenous metabolic networks that modulate precursor supply and flux partitioning toward BIA biosynthesis. To investigate this possibility, we performed transcriptome sequencing of *N. benthamiana* leaves infiltrated with the CP + *CjWRKY1* combination, using wild-type leaves as a control. A total of 7059 genes were significantly upregulated, and 7940 genes were downregulated (Table S6 in Appendix A). The upregulated genes were predominantly enriched in mitogen-activated protein kinase (MAPK) signaling, hormone signal transduction, and secondary metabolism pathways, including phenylpropanoid, glutathione, flavonoid, and tryptophan metabolism (Fig. S15 in Appendix A). In contrast, genes associated with photosynthesis, starch and sucrose metabolism, and glyoxylate/dicarboxylate metabolism were downregulated.

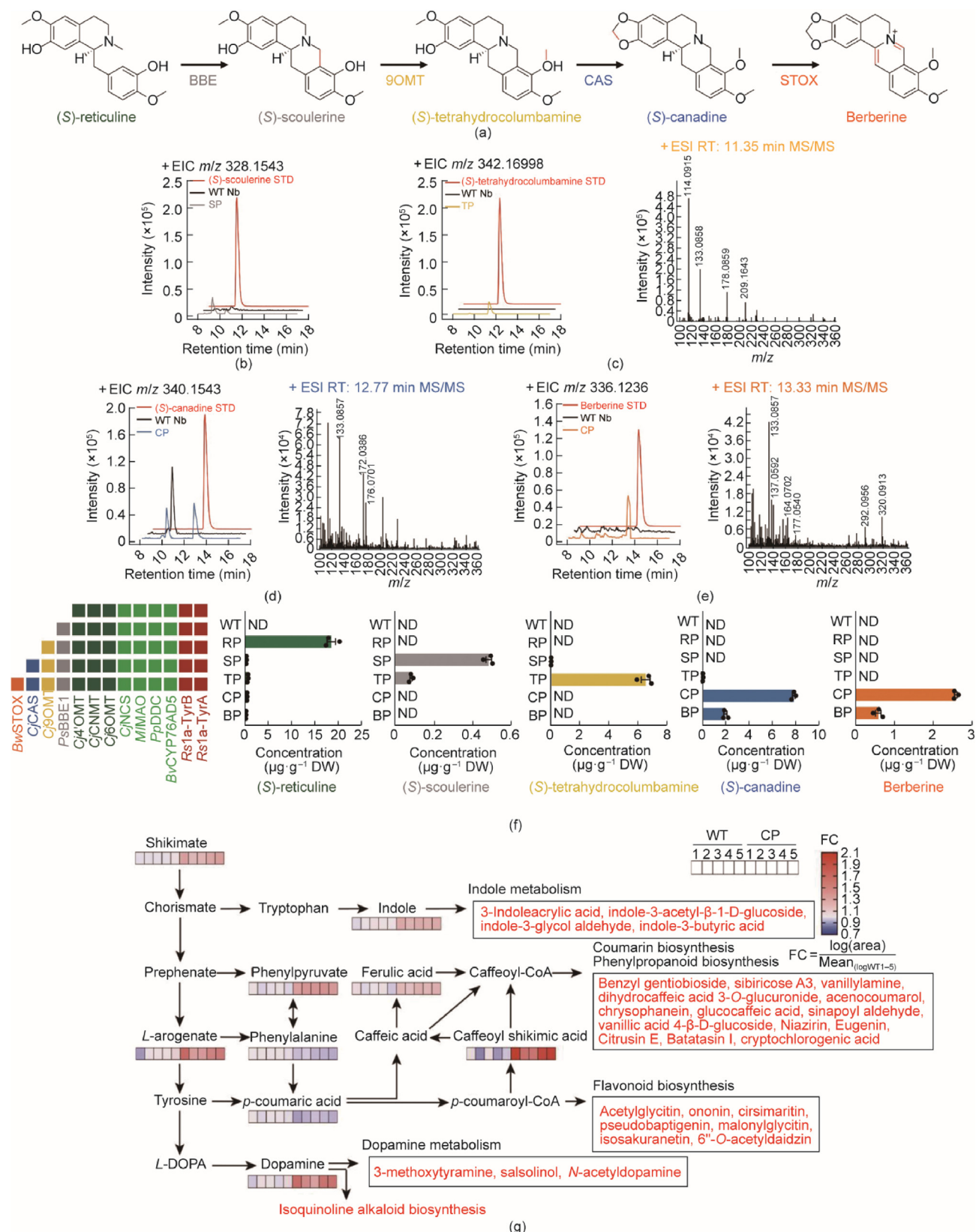


Fig. 4. Reconstitution of the berberine biosynthetic pathway in *N. benthamiana*. (a) Enzymatic steps catalyzing the conversion of (S)-reticuline to berberine. (b) Transient expression of the (S)-scoulerine biosynthetic pathway (SP) combination produced a peak at m/z 328.1543 with a retention time of 10.54 min, corresponding to (S)-scoulerine. (c) Transient expression of the (S)-tetrahydrocolumbamine biosynthetic pathway (TP) combination produced a peak at m/z 342.1700 with a retention time of 11.34 min, corresponding to (S)-tetrahydrocolumbamine. (d) Transient expression of the CP combination yielded a peak at m/z 340.1543 at 12.77 min, corresponding to (S)-canadine. (e) Transient expression of the CP gene combination further resulted in a peak at m/z 336.1236 at 13.33 min, corresponding to berberine. (f) Quantification of pathway intermediates and berberine accumulation by UPLC MS/MS. The data represent the means $\pm$ s.e.m. of three independent replicates. RP: (S)-reticuline biosynthetic pathway. BP: berberine biosynthetic pathway. (g) Comparative metabolomic analysis revealed coordinated upregulation of shikimate pathway-derived metabolites following infiltration with the CP combination. Metabolites with increased accumulation are labeled in red.

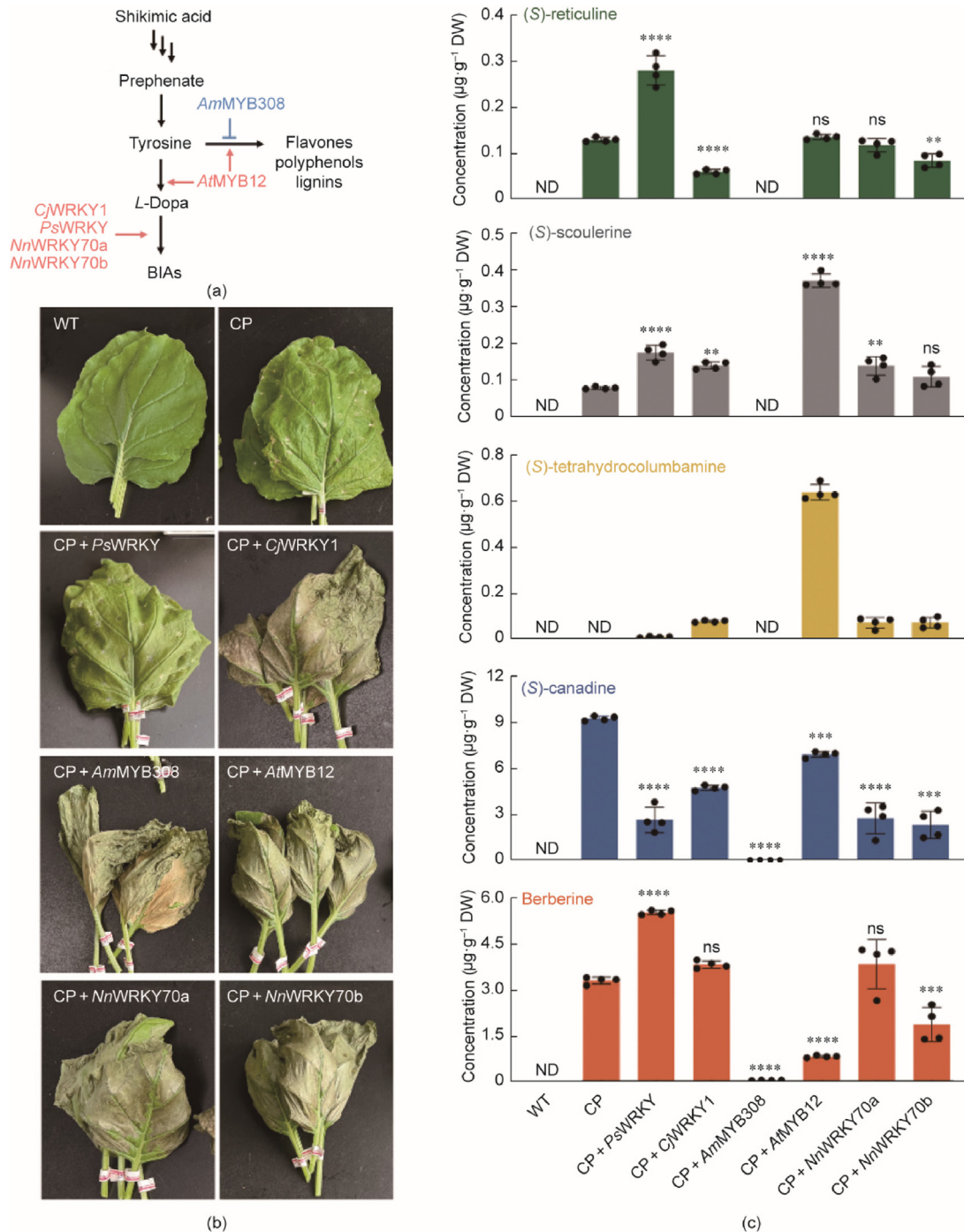


Fig. 5. TF engineering promotes the synthesis of berberine. (a) Schematic diagram of TF-targeted metabolic engineering strategies to increase berberine biosynthesis. (b) Phenotypes of *N. benthamiana* leaves at 6 d post-infiltration with CP alone and coexpressed with individual TFs (*PsWRKY*, *CjWRKY1*, *AmMYB308*, *AtMYB12*, *NnWRKY70a*, and *NnWRKY70b*), all driven by the StUbi promoter. (c) Accumulation of berberine, (S)-canadine, and relevant pathway intermediates in leaves coinfiltrated with CP and the indicated TFs. The data represent the means $\pm$ s.e.m. of four independent replicates. The *P* values were determined by one-way analysis of variance (ANOVA) using CP as the control. ****, $p < 0.0001$.

Furthermore, *N. benthamiana* may harbor endogenous enzymes that are functionally similar to berberine biosynthetic genes. BLASTp analysis of the upregulated gene set against key berberine pathway enzymes (*BvCYP76AD5*, *CjNCS*, *Cj6OMT*, *Cj4'OMT*, *PsBBE1*, *Cj9OMT*, *CjCAS*, and *BwSTOX*) revealed homologs with relatively high similarity to all the tested enzymes except *CjCNMT* (Table S7 in Appendix A). These results suggest that TF-induced

activation of endogenous homologs could partially complement or substitute the introduced heterologous pathway, contributing to the observed increase in berberine accumulation.

Notably, while individual TFs exerted variable effects on the accumulation of (S)-canadine and berberine, their combined levels consistently decreased relative to those of the CP control under all the TF expression conditions (Fig. S14). These results underscore

the necessity of precisely tuning TF expression to achieve balanced pathway activation while minimizing host metabolic disruption, as supported by previous studies [56].

3.5. Boosting berberine biosynthesis through fine-tuned TF engineering

To address this challenge of TF-induced cytotoxicity, we employed a promoter titration strategy using a set of quantitative promoters to fine-tune TF expression levels (Fig. 6(a)). Specifically, three promoters with graded strengths were selected to modulate TF dosage: P_CsVMV (medium, ~50% of P_StUbi), P_AtWRKY6 (weak, ~9% of P_StUbi), and P_DAISY (very weak, ~2% of P_StUbi), as accurately quantified in our previous study [30]. Reducing TF expression effectively mitigated necrosis symptoms, although the optimal expression level varied among the TFs (Fig. 6(b)). Under P_CsVMV control, CP + CjWRKY1 and CP + AtMYB12 caused only localized necrosis, whereas AmMYB308 still induced complete leaf death. At weak promoter strength (P_AtWRKY6), the differences became more pronounced: CP + CjWRKY1 leaves exhibited normal growth, CP + AtMYB12 caused mild lesions, and CP + AmMYB308 continued to induce full necrosis. At the weakest promoter level (P_DAISY), leaves infiltrated with all the TF combinations displayed a normal morphology.

At the product level, all four TFs increased the accumulation of berberine at specific expression levels. CP + PsWRKY achieved 5.97 $\mu\text{g}\cdot\text{g}^{-1}$ DW berberine and 6.78 $\mu\text{g}\cdot\text{g}^{-1}$ DW (S)-candarine under P_CsVMV, whereas CP + CjWRKY1 yielded up to 7.33 $\mu\text{g}\cdot\text{g}^{-1}$ DW berberine and 6.52 $\mu\text{g}\cdot\text{g}^{-1}$ DW (S)-candarine under the same pro-

motor. In contrast, CP + AmMYB308 resulted in a maximum of 4.99 $\mu\text{g}\cdot\text{g}^{-1}$ DW berberine and 6.39 $\mu\text{g}\cdot\text{g}^{-1}$ DW (S)-candarine under P_DAISY, and CP + AtMYB12 resulted in 6.86 $\mu\text{g}\cdot\text{g}^{-1}$ DW berberine and 6.18 $\mu\text{g}\cdot\text{g}^{-1}$ DW (S)-candarine when expressed with P_AtWRKY6 (Fig. 6(c) and Fig. S16 in Appendix A). The intermediates accumulated at trace levels, consistent with the previous results (Fig. S17 in Appendix A). These results demonstrate that each TF requires a distinct optimal expression level to maximize berberine biosynthesis. With respect to PsWRKY, expression driven by P_StUbi and P_CsVMV resulted in comparable berberine levels, whereas a further reduction in the expression level decreased the output. CjWRKY1 increased the accumulation of berberine as its expression decreased from P_StUbi to P_CsVMV, with the yield of berberine with the latter reaching 222% of that in the CP group. However, a further decrease in expression led to decreased yield. In contrast, AmMYB308 expression under P_StUbi or P_CsVMV was too strong, causing leaf necrosis and no accumulation of the target product. As the expression strength decreased, the yield of berberine gradually increased, and the highest yield occurred under P_DAISY. For AtMYB12, the optimal promoter was P_AtWRKY6, under which berberine levels reached 204% of those in the CP group.

To explore potential additive effects, we coexpressed the highest-performing construct (CP + P_CsVMV::CjWRKY1) with P_StUbi::PsWRKY and P_AtWRKY6::AtMYB12. Unexpectedly, compared with single-TF combinations, this triple-TF combination triggered systemic leaf necrosis (Fig. S18 in Appendix A) and reduced berberine production (Fig. S19 in Appendix A). This likely resulted

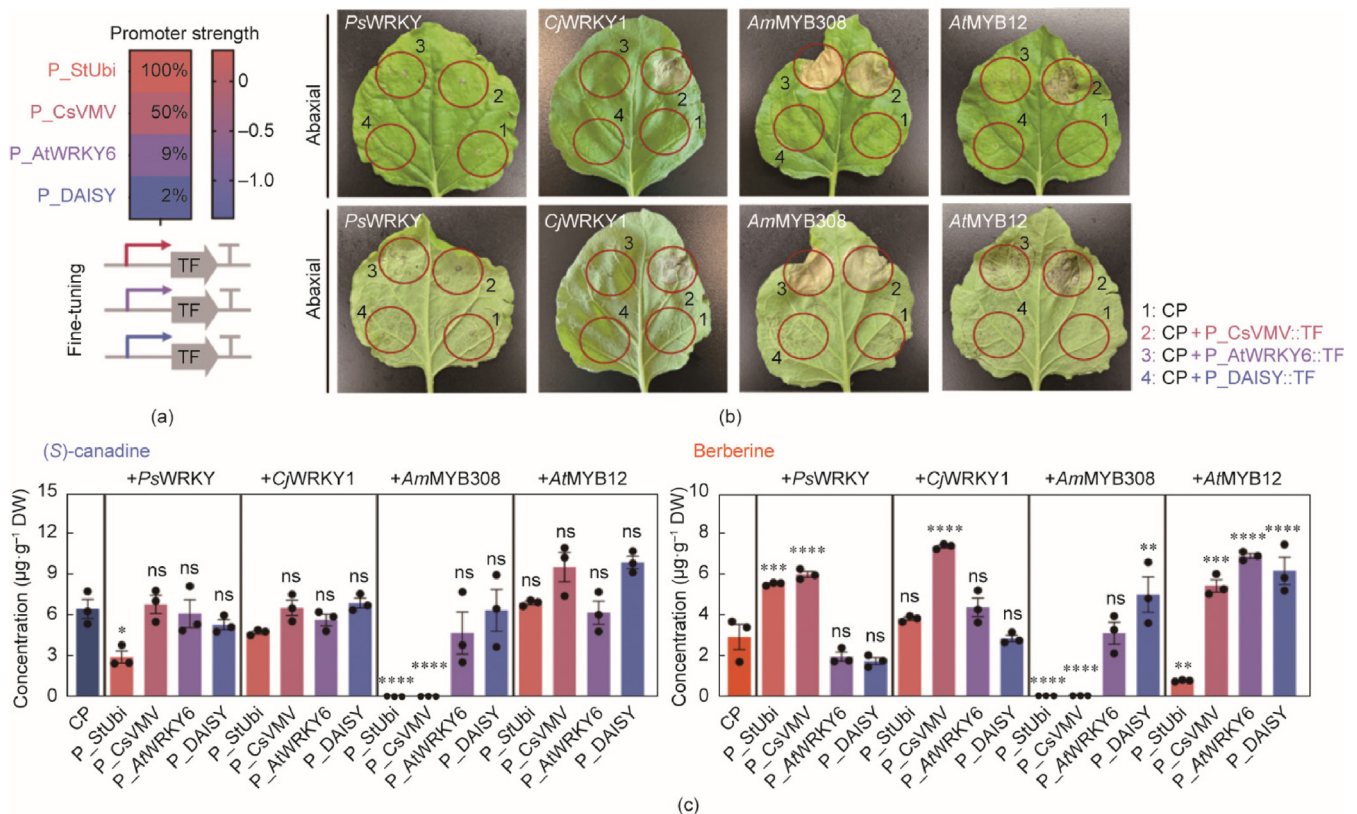


Fig. 6. Fine-tuning engineering of TFs. (a) Schematic overview of fine-tuned TF expression strategies using four promoters of varying strengths. The heatmap shows the relative promoter activities of StUbi (100%), CsVMV (~50%), AtWRKY6 (~9%), and DAISY (~2%). Each color indicates a specific promoter driving the corresponding TF. (b) Phenotypic changes in *N. benthamiana* leaves after modulation of TF expression levels using three quantitative promoters. (c) Berberine and (S)-candarine yields in leaves coinfiltreated with the CP pathway and individual TFs driven by promoters of differing strengths. The data represent the means $\pm$ s.e.m. of three independent replicates. The bar colors correspond to the promoters depicted in (a). The *P* values were calculated by one-way ANOVA with CP as a control. *, *p* < 0.05; **, *p* < 0.01; ***, *p* < 0.001; ****, *p* < 0.0001.

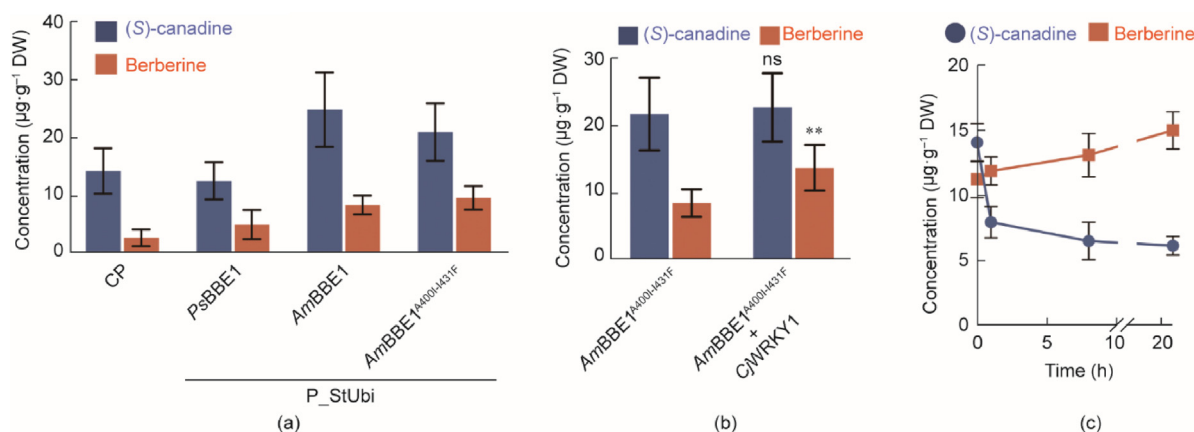


Fig. 7. Implementation of a catalytically superior BBE variant increases berberine production. (a) Comparison of the (S)-canadine and berberine yields in *N. benthamiana* leaves expressing CP combined with PsBBE1, AmbBBE1, or the engineered mutant AmbBBE1^{A400I-I431F}, all driven by the P_StUbi promoter. Replacement of PsBBE1 with AmbBBE1 variant significantly increased the accumulation of berberine. (b) (S)-canadine and berberine yields in leaves coinfiltrated with the optimized CP combination (containing AmbBBE1^{A400I-I431F} under P_StUbi control) and the TF CjWRKY1. (c) Changes in (S)-canadine and berberine yields under heating conditions at 98 °C. The data represent the means $\pm$ s.e.m. of three independent replicates. *P* values of (a) were calculated by one-way ANOVA with CP as the control. *P* values of (b) were calculated by one-way ANOVA with AmbBBE1^{A400I-I431F} as the control. *, *p* < 0.05; **, *p* < 0.01.

from disruption of the delicate growth–metabolism equilibrium achieved through promoter fine-tuning, reflecting the cytotoxicity observed in TF-overexpressing cells.

3.6. Implementation of a catalytically superior BBE variant increases berberine production

BBE catalyzes the first committed step in the conversion of (S)-reticuline into the tetrahydropprotoberberine scaffold, thus funneling metabolic flux into berberine biosynthesis. In our initial CP construct, *P. somniferum* PsBBE1 was expressed under the P_CaMV35S-UTR promoter in pEAQ-HT, yet the accumulation of berberine remained low. Replacing the P_CaMV35S-UTR with the stronger P_StUbi promoter for PsBBE1 yielded only a marginal, nonsignificant increase (Fig. 7(a)), indicating that simply increasing transcript levels was insufficient to overcome this flux bottleneck.

To resolve this issue, we adopted an enzyme-centric approach. The catalytic efficiency of *Argemone mexicana* AmbBBE1 is greater than that of PsBBE1, and its engineered variant AmbBBE1^{A400I-I431F} has achieved higher conversion *in vitro* [24]. Since all the enzymes involved in the upstream (S)-reticuline biosynthetic pathway are localized to the ER (Fig. 2(a)), we performed subcellular localization predictions for PsBBE1 and AmbBBE1, which similarly suggested ER localization (Fig. S20 in Appendix A). Substituting PsBBE1 with AmbBBE1 increased berberine titers 2.8-fold, whereas the AmbBBE1^{A400I-I431F} mutant resulted in a 3.85-fold improvement, reaching 10.14 $\mu\text{g g}^{-1}$ DW (Fig. 7(a)). Coexpressing AmbBBE1^{A400I-I431F} with the optimally titrated TF CjWRKY1 further increased the titer of berberine to 13.91 $\mu\text{g g}^{-1}$ DW (Fig. 7(b)). These results confirm that BBE is a key flux control point. Strategic replacement with a catalytically superior enzyme, when combined with transcriptional fine-tuning, enables synergistic enhancement of pathway throughput.

Downstream of BBE, the terminal conversion of (S)-canadine to berberine occurs primarily via spontaneous oxidation under ambient conditions, although endogenous STOX-like oxidases may also contribute. The spontaneous chemical conversion of (S)-canadine can be optimized through high-temperature treatment [57]. To explore whether post-harvest process optimization could increase yields, we heated the leaves at 98 °C for 24 h and collected samples at 1, 8, and 24 h for LC MS/MS analysis. During the treatment, the berberine concentration gradually increased, while (S)-canadine

was continuously consumed (Fig. 7(c)). After 24 h of heating, the berberine yield increased from 11.25 to 15.03 $\mu\text{g g}^{-1}$ DW, accompanied by a residual concentration of 6.15 $\mu\text{g g}^{-1}$ DW of (S)-canadine. Compared with the heating-mediated conversion observed in microbial chassis, the postharvest heating treatment in *N. benthamiana* resulted in only a modest improvement in berberine yield [25,57]. The limited improvement is likely attributable to the restricted availability of (S)-canadine and rapid tissue dehydration compared with those in aqueous microbial systems. A more sustainable solution lies in pathway engineering: identification and optimization of STOX homologs with higher catalytic efficiency, coupled with tailored subcellular compartmentalization, may alleviate reactive oxygen species (ROS)- and cofactor-related stress and enable more efficient enzymatic conversion in planta.

Together, these combined metabolic and process engineering strategies will be essential to fully exploit the *N. benthamiana* chassis for scalable berberine biosynthesis and offer a generalizable framework for the heterologous production of other complex plant alkaloids.

4. Conclusions

This study exploited the capacity of *N. benthamiana* as a robust plant chassis for the heterologous biosynthesis of BIAs. By addressing critical bottlenecks in precursor supply and pathway flux through systematic metabolic engineering, we achieved the first reconstitution of the complete berberine biosynthetic pathway in a nonnative plant host. We began by reconstituting (S)-reticuline synthesis and overcoming insufficient precursor supply through a heterologous module that redirected *L*-tyrosine flux into dopamine and 3,4-DHPAA. Plastid-targeted engineering of *L*-tyrosine biosynthesis further increased (S)-reticuline titers from 2.84 to 17.76 $\mu\text{g g}^{-1}$ DW, representing a 6.26-fold increase (Fig. 3(d)), while the final berberine yield reached 2.60 $\mu\text{g g}^{-1}$ DW (Fig. 4(f)). Introduction of BBE, 90MT, and CAS yielded (S)-canadine, which underwent spontaneous or endogenous enzyme-mediated oxidation to berberine in planta, rendering STOX dispensable under these conditions. TF-mediated regulatory strategies and precise expression control exhibited groundbreaking potential in overcoming metabolic bottlenecks for heterologous berberine biosynthesis. Crucially, the implementation of a promoter titration strategy—utilizing a gradient of expression strengths (P_StUbi, P_CsVMV, P_AtWRKY6, P_DAI5Y)—enabled

fine-tuned TF regulation, revealing that subtle adjustments in transcriptional activity are essential to balance pathway activation with host viability. For example, reducing CjWRKY1 expression from high (P_{StUbi}) to moderate (P_{CsVMV}) levels eliminated cytotoxicity while increasing berberine production to 7.33 $\mu\text{g}\cdot\text{g}^{-1}$ DW, an increase of 222% (Fig. 6(c)). Although combinatorial TF coexpression failed to synergistically increase yields, the success of individual TF optimization underscores the importance of dose-responsive control in plant metabolic engineering. Finally, replacing PsBBE1 with the high-activity AmbBE1^{A400L-I431F} variant and combining it with optimized TF expression resulted in a maximal berberine titer of 13.91 $\mu\text{g}\cdot\text{g}^{-1}$ DW. Postharvest thermal treatment of leaves at 98 °C for 24 h further facilitated the spontaneous conversion of (S)-canadine to berberine, increasing the yield to 15.03 $\mu\text{g}\cdot\text{g}^{-1}$ DW. These results establish a robust, sustainable *N. benthamiana* chassis for complex BIA production and illustrate a generalizable framework for reconstructing intricate natural-product pathways in plant.

CRedit authorship contribution statement

Chenfei Tian: Writing – review & editing, Writing – original draft, Visualization, Validation, Project administration, Methodology, Formal analysis, Data curation. **Qian Zhang:** Writing – original draft, Validation, Project administration, Methodology, Investigation. **Yulian Zhang:** Validation, Methodology. **Zhaotao Yan:** Validation, Methodology. **Yuhan Wu:** Validation, Methodology. **Haili Liu:** Writing – review & editing, Supervision, Conceptualization. **Yong Wang:** Writing – review & editing, Supervision, Resources, Project administration, Conceptualization.

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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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.eng.2025.10.021>.

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