Article

Engineering Plant Metabolism for Synthesizing Amino Acid Derivatives of Animal Origin Using a Synthetic Modular Approach

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ABSTRACT: The biosynthesis of amino acid derivatives of animal origin in plants represents a promising frontier in synthetic biology, offering a sustainable and eco-friendly approach to enhancing the nutritional value of plant-based diets. This study leverages the versatile capabilities of *Nicotiana benthamiana* as a transient expression system to test a synthetic modular framework for the production of creatine, carnosine, and taurine-compounds typically absent in plants but essential for human health. By designing and stacking specialized synthetic modules, we successfully redirected the plant metabolic flux toward the synthesis of these amino acid derivatives of animal origin. Our results revealed the expression of a standalone creatine module resulted in the production of 2.3 μg/g fresh weight of creatine in *N. benthamiana* leaves. Integrating two modules significantly carnosine yield increased by 3.8-fold and minimized the impact on plant amino acid metabolism compared to individual module application. Unexpectedly, introducing the taurine module caused a feedback-like inhibition of plant cysteine biosynthesis, revealing complex metabolic adjustments that can occur when introducing foreign pathways. Our findings underline the potential for employing plants as biofactories for the sustainable production of essential nutrients of animal origin.

KEYWORDS: amino acid derivatives, creatine, carnosine, taurine, biosynthetic modules, feedback inhibition

INTRODUCTION

Primary metabolites, such as amino acids, fatty acids, and nucleotides, serve as the fundamental building blocks that give rise to a diverse range of small molecules, each conferring unique biological functions to the organism. Among the primary metabolites, proteinogenic amino acids are the main constituents of proteins and also serve as biosynthetic precursors for numerous bioactive small molecules, including nonproteinogenic amino acids and a variety of nitrogen-containing chemical groups.[1−3] These derived compounds often exhibit distinct biological activities and are pivotal in various physiological processes.[4,5]

A significant divergence exists between plants and animals in the biosynthesis of certain amino acid derivatives. Some, synthesized exclusively by animals, are not naturally present in plants. This difference presents a nutritional challenge for vegetarians who may not receive adequate amounts of essential substances like creatine, carnosine, and taurine from plant-based diets alone. Creatine is known for enhancing muscle strength, buffering acid−base balance in the blood, and promoting recovery.[6] In animal systems, creatine is produced from glycine and arginine via the sequential action of two enzymes: glycine amidinotransferase (AGAT) and guanidinoacetate methyltransferase (GAMT) (illustrated in Figure 2a).[7] Carnosine offers antiaging benefits and protects against oxidative stress, typically found in animal tissues, and is a dipeptide made from nonproteinogenic amino acid β-alanine and histidine catalyzed by carnosine synthase (CARNS1) (Figure 3a).[8,9] Taurine, a compound crucial for nerve function, endocrine health, and immune, is produced from cysteine.[10]

Two animal-specific enzymes, cysteine dioxygenase (CDO) and cysteine sulfinic acid decarboxylase (CSAD) convert cysteine into hypotaurine and ultimately to taurine through spontaneous oxidation (Figure 4a).[11] Insufficient intake of these compounds can lead to health issues, including muscle weakness, cognitive fatigue, and hormonal imbalances, potentially accelerating aging and contributing to various health problems.[12−14] Consequently, vegetarians often need additional nutritional supplements, which are mostly synthesized drugs and may impose a burden on the body with long-term use.[12,14]

Plant synthetic biology has been a key strategy for producing "healthier foods", complementing traditional plant breeding methods to develop and cultivate nutrient-enriched crops and vegetables.[15−17] Significant efforts have been made in engineering plant amino acid pathways, leading to the creation of soybeans with higher levels of sulfur-containing amino acids (methionine/cysteine),[18,19] lysine-rich in maize,[20] and soybeans with enhanced tryptophan production.[21] By genetically engineering plants to express specific biosynthetic pathways, we can transform them into biofactories capable of producing amino acid derivatives of animal origin.[22−24]

However, the biosynthesis of target compounds in the synthetic biology chassis is often constrained by the availability of primary metabolites, which serve as their biosynthetic precursors. These essential substances are present in limited concentrations and are subject to stringent regulation to maintain cellular homeostasis.[25] Diverting these metabolites toward the production of non-native compounds could potentially disrupt this balance, adversely affecting plant growth and development.

Our study introduces a synthetic modular approach to address this challenge. We present a proof-of-concept study in *Nicotiana benthamiana* leaves for producing amino acid derivatives of animal origin. Employing modular synthetic biology, we first establish modules for substrate amino acid synthesis, followed by the integration of additional modules for converting these substrates into the desired derivatives. This tailored assembly of synthetic modules aims to maximize the output of target compounds while minimizing disruption to the native amino acid metabolism of the plant hosts. Through the strategic stacking of these modules, we explore the biosynthesis of creatine, carnosine, and taurine in plants, providing insights into the challenges and feasibility of our design.

■ MATERIALS AND METHODS

Plant Materials and Growth Conditions. The leaves *N. benthamiana*, a tobacco species native to Australia, were served as the biological chassis to evaluate the efficacy of the synthetic modular approach. Seedlings of *N. benthamiana* were from seeds preserved in our laboratory, which can be cultivated and harvested in the plant growth chamber year-round. The plant growth chamber holds a controlled environment setting to temperature 21−25 °C, relative humidity 60−70%, light intensity 90 μmol m^{-2} s^{-1} photosynthetic photon flux density, and 16 h light/8 h dark cycle. Seedlings were carefully transferred to individual pots following the emergence of cotyledons, approximately 7−10 days postsowing. Regular watering was conducted to maintain adequate nutrient supply. Selection for experimental use was based on healthy morphological criteria, such as vibrantly green and robust leaves, at the developmental stage of 5−6 weeks.

Construction of Plant Transient Transformation Vector and Protein Expression Vector. The genes of nonplant origin utilized in our experiments were synthesized chemically. This set included human glycine amidinotransferase (*HsAGAT*: NM_001482.3) and guanidinoacetate methyltransferase (*HsGAMT*: NM_000156.6), the L-aspartate-α-decarboxylase (ADC) from *Bacillus subtilis* (*BspanD*: NC_000964.3), the carnosine synthase (CARNS) from *Bos taurus* (*BtCRANS1*: XM_024987631.1), the cysteine dioxygenase (*CcCDO*: BAE73112) and the cysteine sulfinic acid decarboxylase (*CcCSAD*: BAE73113) from *Cyprinus carpio*. At the same time, the serine acetyltransferase (*SlSAT*: Solyc02g082850) and the O-acetylserine-(thiol)lyase (*SlOAS*: Solyc09g082060) from *Solanum lycopersicum* were amplified via PCR using primers detailed in Supporting Information Table S1. The pEAQ and pET28b vectors were linearized using PCR with respective primers, also itemized in Supporting Information Table S1. The resulting gene fragments and vector backbones were fused using the One Step Fusion Cloning Mix kit (TOROIVD, FCM-050), according to the manufacturer's instructions for homologous recombination.

Transient Expression of *N. benthamiana*. The *N. benthamiana* transient expression system was employed to evaluate the effectiveness of our modular strategy for synthesizing amino acid derivatives of animal origin. The pEAQ vectors carrying the correct inserts were introduced into *Agrobacterium tumefaciens* strain GV3101. Transformants were selected on LB agar plates supplemented with 50 mg/L kanamycin and 25 mg/mL rifampicin. Positive colonies were confirmed by PCR using primers listed in Supporting Information

Table S1. Positive *Agrobacterium* colonies were then cultured in LB liquid medium with the same antibiotics at 28 °C until the optical density at 600 nm (OD$_{600}$) reached 1.0 to 1.2. For constructs that required coexpression of multiple genes, *Agrobacterium* cultures corresponding to each gene were mixed in a single centrifuge tube. The volume of each strain was calculated based on their actual OD$_{600}$ values to ensure that the final OD$_{600}$ for each was adjusted to 0.5. The combined cultures were centrifuged at 5000g for 10 min at room temperature. The pellet was washed with washing buffer (10 mM MES, pH 5.6, 10 mM MgCl$_2$) and repelleted by centrifugation. The resulting pellet was then resuspended in infiltration buffer (10 mM MES, pH 5.6, 10 mM MgCl$_2$, and 200 mM acetosyringone) to prepare the inoculum, which was then incubated at room temperature for 2 h.

Following incubation, the inoculum was loaded into a syringe and gently infiltrated into the abaxial side of *N. benthamiana* leaves. Plants aged 5 to 6 weeks were selected for transformation, focusing on the 3 to 5 leaves immediately below the apex. Postinfiltration, the leaves displayed a transiently wet appearance. The infiltrated leaves were harvested 4−5 days postinfiltration, immediately frozen in liquid nitrogen, and stored at −80 °C for subsequent analyses.

Extraction and Detection of Amino Acids and Derivatives. Approximately 100 mg of the ground *N. benthamiana* samples were extracted using 400 μL of isotopically labeled amino acid extraction buffer (detailed composition provided in Supporting Information Table S2). The samples were vortexed for 10 s and incubated at 90 °C for 10 min. The extraction solution was then cooled on ice for 5 min and centrifuged at 13,000g for 10 min at 4 °C. The supernatant was carefully transferred to a new 1.5 mL microcentrifuge tube and filtered using a 0.45 μm hydrophilic polytetrafluoroethylene centrifugal filter to prepare the samples for liquid chromatography, with interim storage at 4 °C if necessary.

Amino acids and their derivatives were separated and quantified using an Agilent 6495 Triple Quadrupole LC/MS System equipped with a Waters ACQUITY UPLC BEH Amide column (1.7 μm particle size, 2.1 × 100 mm). Chromatographic separation commenced with a mobile phase consisting of 10% solvent A (10 mM ammonium acetate and 0.2% formic acid in water) and 90% solvent B (acetonitrile). The column temperature was maintained at 40 °C, and the flow rate was set at 0.4 mL/min. The elution gradient was as follows: initial conditions were held at 90% solvent B for the first minute, followed by a reduction to 50% solvent B from 1 to 10 min, a hold at 50% solvent B up to 14 min, an increase to 90% solvent B at 14.5 min, and then a hold at 90% solvent B until 17 min.

Quantification of amino acids and their derivatives in the *N. benthamiana* leaf samples was carried out in multireaction monitoring (MRM) mode, employing the isotope dilution method for accurate quantification. The isotopically labeled standards in the extraction buffer facilitated the quantification of peaks by MRM. The residence times, collision energies, fragmentor voltages, and parent-to-daughter ion transitions were optimized and are listed in Supporting Information Table S3. The concentrations of 20 standard amino acids and 5 derivatives were calculated by the system, based on the peak areas and the internal standards, each tobacco plant counts as one biological replicate, with three replicates per treatment.

Protein Expression and Purification Using the *Escherichia coli* System. To investigate the effects of taurine and hypotaurine on the enzymatic activity of plant-derived serine acetyltransferase (SAT) and O-acetylserine(thiol)lyase (OAS), we carried out protein expression and subsequent *in vitro* enzyme assays. The *S. lycopersicum* SlSAT and SlOAS were cloned into the pET28b expression vector and transformed into *E. coli* Rosetta (DE3) cells. Transformants were selected on LB agar plates supplemented with 50 mg/L kanamycin and 34 mg/L chloramphenicol, using primers detailed in Supporting Information Table S1 for verification. The positive *E. coli* strains were cultivated in liquid LB medium containing the antibiotics. Cultures were grown at 37 °C until an OD$_{600}$ of 0.5−0.6 was reached. Protein expression was induced by the addition of 0.05 mM IPTG, and the cultures were then incubated at 16 °C with shaking at 120 rpm for about 14 h. Cells were harvested by centrifugation at 5000g for 15
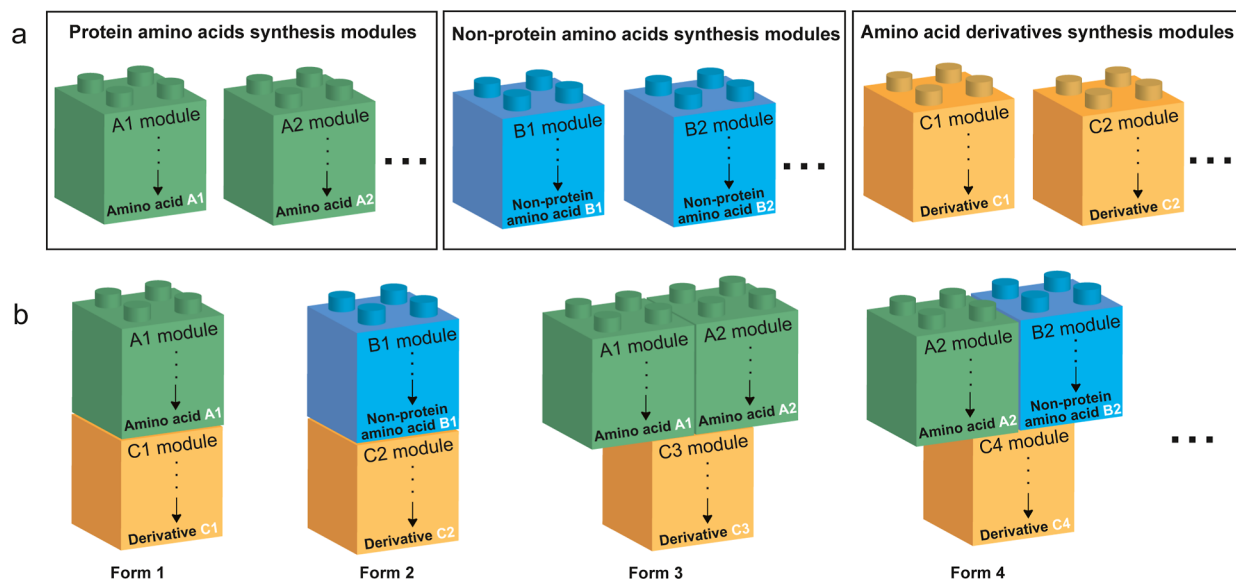
Figure 1. Design and configuration of synthetic modules for the production of valuable amino acid-derived compounds. (a) Biosynthetic module categories. Modules depicted in green represent those that synthesize proteinogenic amino acids in plants (e.g., A1, A2, etc.), blue modules are for nonproteinogenic amino acids (e.g., B1, B2, etc.), and yellow modules pertain to amino acid derivatives (e.g., C1, C2, etc.). Within each module, one or several key enzymes are involved in catalyzing the conversion of precursor molecules into desired target compounds. (b) Modular stacking strategies for the synthesis of target derivatives: Form 1 combines a module generating a substrate amino acid (A1) with a module dedicated to the production of a derivative (C1). Form 2 pairs a module for a nonproteinogenic amino acid (B1) with a module for a derivative (C2). Forms 3 and 4 depict a more intricate arrangement where the synthesis of the target compound relies on a combination of proteinogenic and nonproteinogenic amino acids, necessitating two modules for the biosynthesis of the substrate amino acids and one for the derivative.

min at 4 °C, and the pellet was resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10% glycerol, pH 8.0). The resuspended cells were lysed by sonication, and the cell debris was removed by centrifugation at 20,000g for 30 min at 4 °C. The clarified supernatant was incubated with Ni-NTA resin pre-equilibrated with the lysis buffer at 4 °C for 2 h to facilitate protein binding. The resin was then collected by gentle centrifugation at 1000g for 5 min at 4 °C, transferred to a gravity column, and washed with lysis buffer to remove unbound proteins. The bound proteins were eluted with a buffer containing increasing concentrations of imidazole. Protein purity was assessed using SDS-PAGE and Western blot analysis. For short-term storage, the purified proteins were mixed with glycerol to a final concentration of 40% and stored at −20 °C until further use.

***In Vitro* Enzyme Assays for SlSAT and SlOAS.** The *in vitro* enzyme assays for SlSAT and SlOAS were performed to elucidate the functionality of these enzymes in cysteine biosynthesis. The SlSAT assay was adapted from previously described methods.[26] A reaction mixture was prepared containing 50 mM Tris−HCl (pH 7.6), 1 mM EDTA, 20 mM L-serine, and 0.1 mM acetyl-CoA. The introduction of purified recombinant SlSAT proteins initiated the catalysis, which proceeded at 20 °C for 25 min. The production of coenzyme A (CoA) was measured by adding 50 μL of 1 mM 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), allowing the reaction to continue for an additional 5 min. The formation of the yellow 5-thio-2-nitrobenzoic acid was monitored spectrophotometrically at 412 nm. A standard curve was established using known concentrations of CoA (0, 12.5, 25, 50, 100, and 200 μM) in the reaction matrix. To assess the inhibitory impacts of hypotaurine and taurine, 100 μM hypotaurine or 50 μM taurine were introduced into the SlSAT assay alongside the control experiments.

The SlOAS assay, which measures the second step in plant cysteine biosynthesis, was based on established protocols with modifications.[26] The reaction mixture consisted of 50 mM Tris−HCl (pH 7.5), 5 mM DTT, 5 μM pyridoxal phosphate (PLP), 5 mM *O*-acetylserine, and 10 mM Na_2S. The reaction was started by the addition of purified SlOAS proteins and was maintained at 25 °C for 10 min. Termination of the reaction was achieved by adding 100 μL of acetic anhydride. The mixture was then treated with 200 μL of ninhydrin reagent (25 mg/

mL in acetate: HCl, 60:40, v/v) and heated to 95 °C for 10 min, followed by rapid cooling. The final step to halt the reaction involved the addition of 200 μL of anhydrous ethanol. Cysteine production was quantified by measuring the absorbance at 560 nm. To explore the inhibitory effects of hypotaurine and taurine on SlOAS activity, either 100 μM hypotaurine or 50 μM taurine was added to the reaction. Each treatment was repeated 3 times, and each experiment was independently repeated 3 times.

RNA-Sequencing Analysis of *N. benthamiana* Leaves Infiltrated with Different Synthetic Modules. To illustrate the metabolic adjustments associated with amino acid biosynthesis in plant expressing diverse synthetic modules, we conducted RNA-sequencing on *N. benthamiana* leaves infiltrated with these modules in comparison to control leaves infiltrated with the pEAQ empty vector.

Each tobacco plant was considered a biological replicate, with three replicates per treatment. Total RNA was extracted utilizing the TRIzol reagent (Invitrogen, CA, USA) in accordance with the manufacturer's protocol. The purity and concentration of the RNA were determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA), and RNA integrity was verified using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Subsequent library preparation was completed using the VAHTS Universal V6 RNA-seq Library Prep Kit, following the manufacturer's guidelines.

The sequencing was performed on an Illumina NovaSeq 6000 platform, producing 150 bp paired-end reads. The raw data has been deposited to the National Center for Biotechnology Information (NCBI) under the accession number PRJNA1117068. The initial processing of raw FASTQ files was accomplished using FASTQ tools, with low-quality reads being filtered out to procure high-quality clean reads. These clean reads were then aligned to the reference genome via HISAT, and gene expression levels were quantified as FPKM values, with read counts obtained using HTSeq-count. Differential expression analysis was carried out employing DESeq2, with a *Q* value ≤0.05 and an absolute fold change ≥2 or ≤0.5 serving as the criteria for significant differential expression. Enrichment analyses of differentially expressed genes (DEGs) for Gene Ontology terms, KEGG pathways, Reactome, and WikiPathways were performed based on the
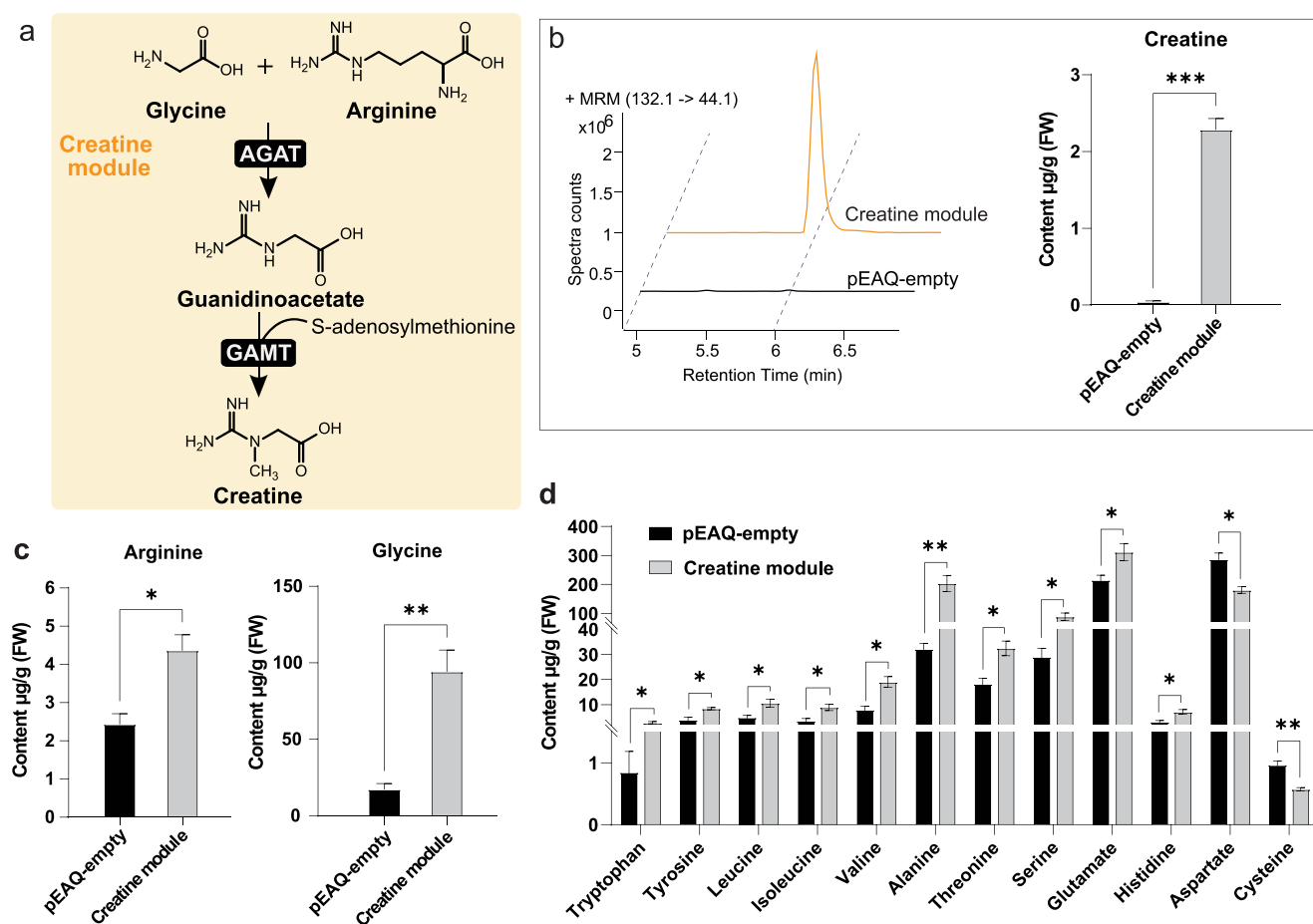
Figure 2. Applying a creatine synthesis module in *N. benthamiana* to channel amino acid precursors into creatine production. (a) Schematic representation of the creatine synthesis module designed to convert glycine and arginine into creatine, catalyzed by the enzymes AGAT and GAMT. (b) Representative LC/MS chromatograms and the quantification of creatine levels in *N. benthamiana* leaves after expressing the creatine module. (c) Elevated levels of arginine and glycine in *N. benthamiana* leaves following the introduction of the creatine module, suggesting successful redirection of amino acid flux. (d) Alterations in the abundance of 12 other amino acids in *N. benthamiana* leaves, with 10 amino acids showing a significant increase and two amino acids (aspartate and cysteine) exhibiting a notable decrease. The presented data are mean ± SEM from three biological replicates; each expressed plant is considered a separate biological entity. Statistical significance between control and treated groups was determined by an unpaired t-test, with asterisks denoting the level of significance (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

hypergeometric distribution using R software (version 3.2.0). These analyses aided in the identification of significantly enriched terms. Key genes involved in amino acid metabolism were subsequently pinpointed, and alterations in their expression patterns were meticulously examined.

Reverse Transcription and Quantitative PCR Analysis. To validate the RNA sequencing data, reverse transcription reactions were performed on the RNA samples using a reverse transcription kit (TOROIVD, RTQ-101) according to the manufacturer's instructions. Real-time quantitative PCR experiments were conducted to quantify the relative expression levels of 25 target genes (primers listed in Supporting Information Table S1) using a qPCR kit (TOROIVD, QST-100) following the manufacturer's protocol. The stability of the reference genes *EF1a*, *PP2A*, and *Actin* was assessed using the RefFinder web tool (http://blooge.cn/RefFinder/), alongside Norm-Finder, BestKeeper, and geNorm software. *EF1a* and *Actin*, which demonstrated better stability as evidenced in Supporting Information Table S4, were chosen as the housekeeping genes. The geometric mean of their expression levels was employed for the normalization of the target gene expression data. The reverse transcription and quantitative PCR (RT-qPCR) results were then compared with the fold change of FPKM values obtained from the RNA transcriptome data.

Data Analysis. All the statistical analyses were performed using GraphPad Prism 9 (GraphPad Software Inc.; San Diego, CA, USA).

The data of the mean ± SEM is derived from three biological replicates. Asterisks indicate significant differences between groups using unpaired t-test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

■ RESULTS

Conceptualizing Modular Biosynthetic Pathways for Amino Acid-Derived Compound Production. The efficient production of amino acid-derived valuable compounds in synthetic biology is often constrained by the availability of the biosynthetic precursors in the host organisms. To overcome this limitation and boost the production of target compounds, it is essential to enhance the precursor amino acid pool and divert the metabolic route toward the desired chemical products. Here, we have devised a biosynthetic modular framework specially tailored for the production of amino acid-derived chemicals using plants as the synthetic biology chassis. Our design principle allows for each module to specialize in the boost production of a particular amino acid or its derivative.

As illustrated in Figure 1, three categories of biosynthetic modules were designed, which are named by the products they generate: modules for proteinogenic amino acids in plants (labeled A1, A2, etc.), modules for nonproteinogenic amino
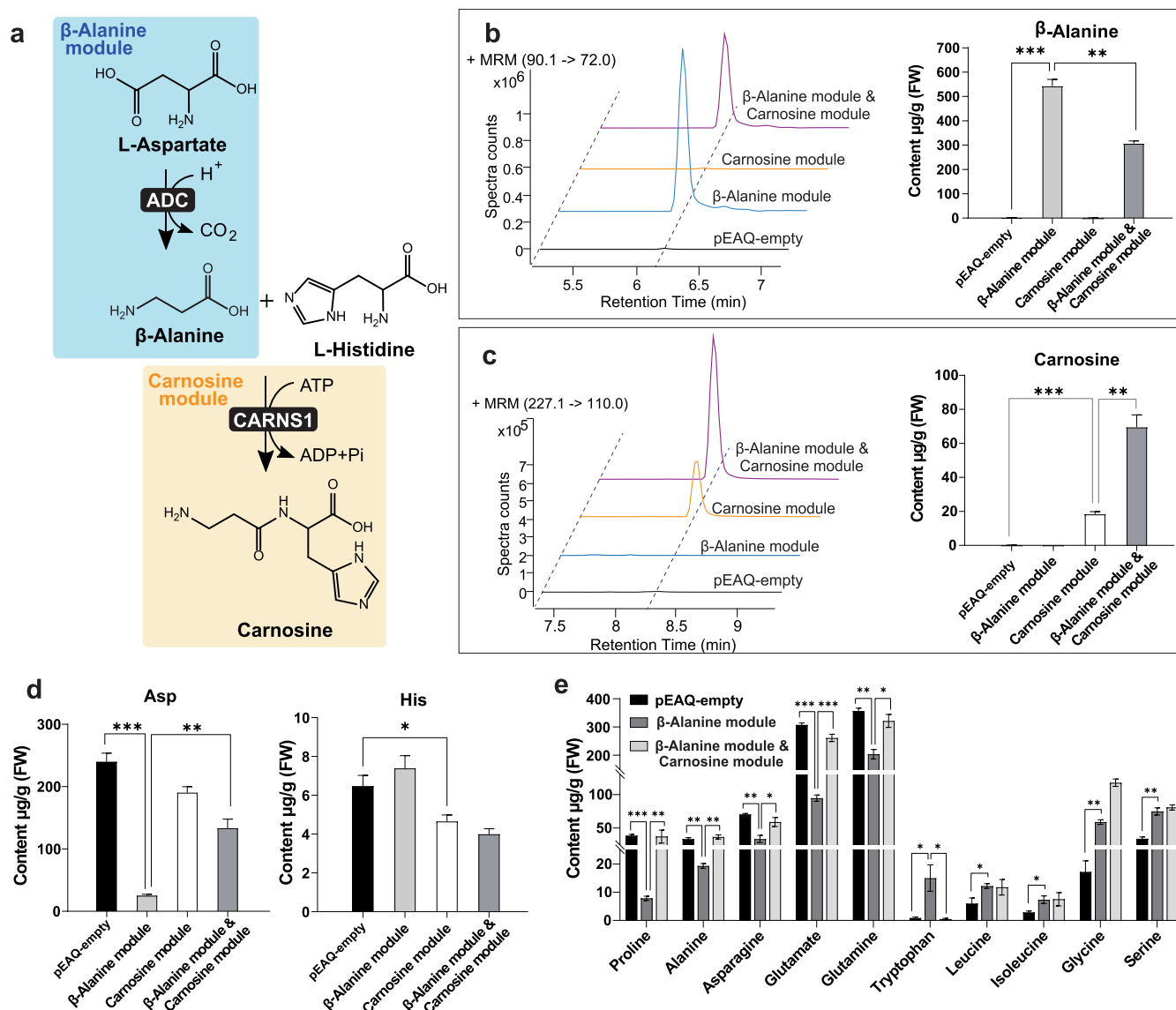
Figure 3. Enhanced carnosine synthesis in *N. benthamiana* leaves through combined biosynthetic modules. (a) Diagram illustrating the synergistic stacking of *β*-alanine and carnosine biosynthetic modules, with the enzymes ADC and CARNS1 facilitating carnosine production in *N. benthamiana* leaves. (b) LC/MS chromatograms and quantification of *β*-alanine in leaves after individual and combined module infiltration. *β*-Alanine levels rose significantly with the *β*-alanine module alone and then diminished upon the addition of the carnosine module. (c) LC/MS chromatograms and quantification of carnosine in *N. benthamiana* leaves, showing carnosine synthesis following the introduction of the carnosine module and a subsequent increase with the combined infiltration of both *β*-alanine and carnosine modules. (d) The levels of L-aspartate (Asp) and histidine (His), precursors for *β*-alanine and carnosine respectively, displayed a decreasing trend upon the application of the carnosine module. (e) Changes in the levels of 10 additional amino acids in *N. benthamiana* leaves, with significant reductions in 5 amino acids (proline, alanine, asparagine, glutamate, glutamine) upon expression of only the *β*-alanine module, which then rose again after coexpression with the carnosine module. The displayed data are mean ± SEM from three biological replicates, with each infiltrated plant considered a separate biological entity. Statistical significance between groups was assessed using an unpaired *t*-test, with asterisks marking significance levels (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

acids (labeled B1, B2, etc.), and modules for amino acid-derived compounds with nonplant origins (labeled C1, C2, etc.). Each of these modules incorporates one or several pivotal enzymes capable of catalyzing the transformation of a precursor molecule into a designated target compound.

A critical aspect of the design is the selection of key enzymes based on an in-depth understanding of plant amino acid biosynthetic pathways. This plant-centric approach ensures that modules such as A1 and A2 are equipped with enzymes that naturally participate in plant metabolism, thereby enhancing the synthesis of plant-derived proteinogenic amino acids. In contrast, modules designed for the production of

nonproteinogenic amino acids and their derivatives (B1, B2 and C1, C2) incorporate enzymes sourced from microorganisms or animals, adapted to plant systems to extend the biosynthetic capabilities beyond traditional plant metabolism. Furthermore, the modules are designed to be stackable, allowing for flexible assembly into various configurations, as demonstrated in Figure 1b. This stack ability is key to customizing the biosynthetic pathways for the efficient and scalable production of both simple and complex amino acid derivatives.

In the subsequent experimental section, we focus on evaluating the performance of individual synthetic modules

and the combined efficacy of stacked synthetic module pairs using the *N. benthamiana* transient expression system. These proof-of-concept experiments are designed to validate our modular strategy. The goal is to validate the capability of the platform to facilitate the biosynthesis of a broad array of amino acid-derived compounds of non-plant origin within a plant-based context.

Testing a Creatine Synthesis Module in *N. benthamiana* to Redirect Amino Acid Flux. To explore the potential of plant systems in producing compounds typically not synthesized by their native metabolic pathways, a creatine synthesis module was tested in *N. benthamiana*. We engineered a synthetic module for creatine production in *N. benthamiana* by leveraging the human enzymes HsAGAT and HsGAMT (Figure 2a). Each gene coding for these enzymes was cloned into the plant transient expression vector pEAQ. Coexpression of these constructs into *N. benthamiana* leaves led to the successful production of creatine, with concentrations reaching 2.3 μg/g fresh weight (FW) in the assayed tissue (Figure 2b). This result confirmed the functional integration of the creatine synthesis module within a plant system.

Considering that glycine and arginine are the precursors for creatine synthesis, we hypothesized that the diversion of these amino acids toward creatine production might influence the metabolic equilibrium of the host plant. To evaluate this, a comprehensive LC−MS/MS analysis of the 20 essential amino acids was performed for the transformed *N. benthamiana* leaves. Contrary to expectations that substrate levels would decrease with increased product formation, a significant elevation in the precursor amino acids glycine and arginine was observed. Glycine concentrations increased nearly 5-fold, from 17.2 to 97.2 μg/g, compared to the control, and arginine levels also rose from 2.4 to 4.4 μg/g (Figure 2c). This unexpected increase might reflect a compensatory response to the overexpressed biosynthetic enzymes or a broader metabolic realignment by the plant to stabilize its internal amino acid pool.

The introduction of the creatine synthesis pathway not only affected the direct substrates but also had broad implications for the plant amino acid metabolism. Other ten amino acids showed a significant increase in concentration, whereas aspartate and cysteine levels were markedly reduced (Figure 2d and Supporting Information Table S5). The overall accumulation of endogenous amino acids suggested that metabolic adjusted events might be occurring within the plant system to adjust to the altered amino acid flux caused by the foreign biosynthetic pathway.

Dual Biosynthetic Modules Application Could Further the Production of Carnosine than Using Single Module in *N. benthamiana* Leaves. After the successful implementation of a single synthetic module for metabolic pathway testing, we hypothesized that combining multiple modules could lead to even greater yields of target compounds. Such stackable modules offer the flexibility to construct increasingly complex compounds from simple amino acid precursors. To test this, we aimed to synthesize carnosine and the CARNS1 from *B. taurus* was selected to design the first module for carnosine biosynthesis (Figure 3a). Additionally, to ensure the availability of the precursor β-alanine, which is not a proteinogenic amino acid and is present in low amounts in plants, we introduced a second module consisting of ADC from *B. subtilis*. This enzyme transforms aspartate to β-alanine,

thus supplying the necessary precursor for carnosine synthesis (Figure 3a).

When each module was introduced individually into *N. benthamiana* leaves, carnosine production was measurable, reaching 18.3 μg/g FW with the carnosine module alone (Figure 3c). However, when both the β-alanine and carnosine modules were simultaneously expressed, carnosine production increased significantly, by 3.8-fold, to 69.5 μg/g FW (Figure 3c). This marked improvement in yield was attributed to the heightened availability of β-alanine provided by the coexpressed ADC enzyme. Although β-alanine is naturally produced in plants, it is barely detectable in *N. benthamiana* leaves under normal conditions. The β-alanine module alone elevated β-alanine levels to 542.3 μg/g FW. Interestingly, these levels dropped to 306.2 μg/g FW when both the β-alanine and carnosine modules were present (Figure 3b). This decrease suggests that the plant effectively utilized the additional β-alanine for carnosine biosynthesis, resulting in a higher overall production of carnosine compared to when the carnosine module was expressed in isolation (Figure 3c). These findings support the hypothesis that integrating multiple biosynthetic modules can substantially enhance the production of complex compounds such as carnosine in plant systems.

To elucidate the effects of dual-module expression on the overall metabolism of *N. benthamiana* plants, we conducted a comprehensive analysis of the endogenous free amino acid concentrations after introduction of the two synthetic modules. The expression of the single carnosine module led to a reduction in the content of histidine (from 6.5 to 4.6 μg/g), the other precursor for carnosine synthesis, and this decrease persisted to 4.0 μg/g when both the carnosine and β-alanine modules were coexpressed (Figure 3d). Aspartate, the immediate precursor for β-alanine synthesis, showed a significant decline when the β-alanine module was expressed alone (from 239.8 to 25.0 μg/g). Intriguingly, this decrease was reversed upon to 133.0 μg/g when the concurrent expression of both the β-alanine and carnosine modules (Figure 3d). A similar pattern was observed for several other amino acids, including proline, alanine, asparagine, glutamate, glutamine, and tryptophan. The levels of these amino acids decreased or increased with the single-module expression of β-alanine but returned to baseline when both modules were introduced (Figure 3e). These results suggest that the overproduction of β-alanine initially disrupted the metabolic balance of certain amino acids. However, when the two modules were combined, the excess β-alanine was efficiently channeled toward carnosine synthesis, thereby mitigating the disruption and restoring the balance of other affected amino acids. This illustrates a key advantage of using dual modules: the potential metabolic burden introduced by accumulating intermediates from the first module can be alleviated by the subsequent conversion into the desired end product by the second module.

Conversely, the expression of either the β-alanine module alone or in conjunction with the carnosine module consistently led to increased levels of four amino acids—leucine, isoleucine, glycine, and serine (Figure 3e and Supporting Information Table S6). These changes, which were not reversed by the dual-module approach, likely reflect a more complex metabolic response to the engineered disturbance of amino acid homeostasis within the plants. Besides, the results obtained by expressing only the carnosine module were similar to those obtained by expressing only the creatine module in the
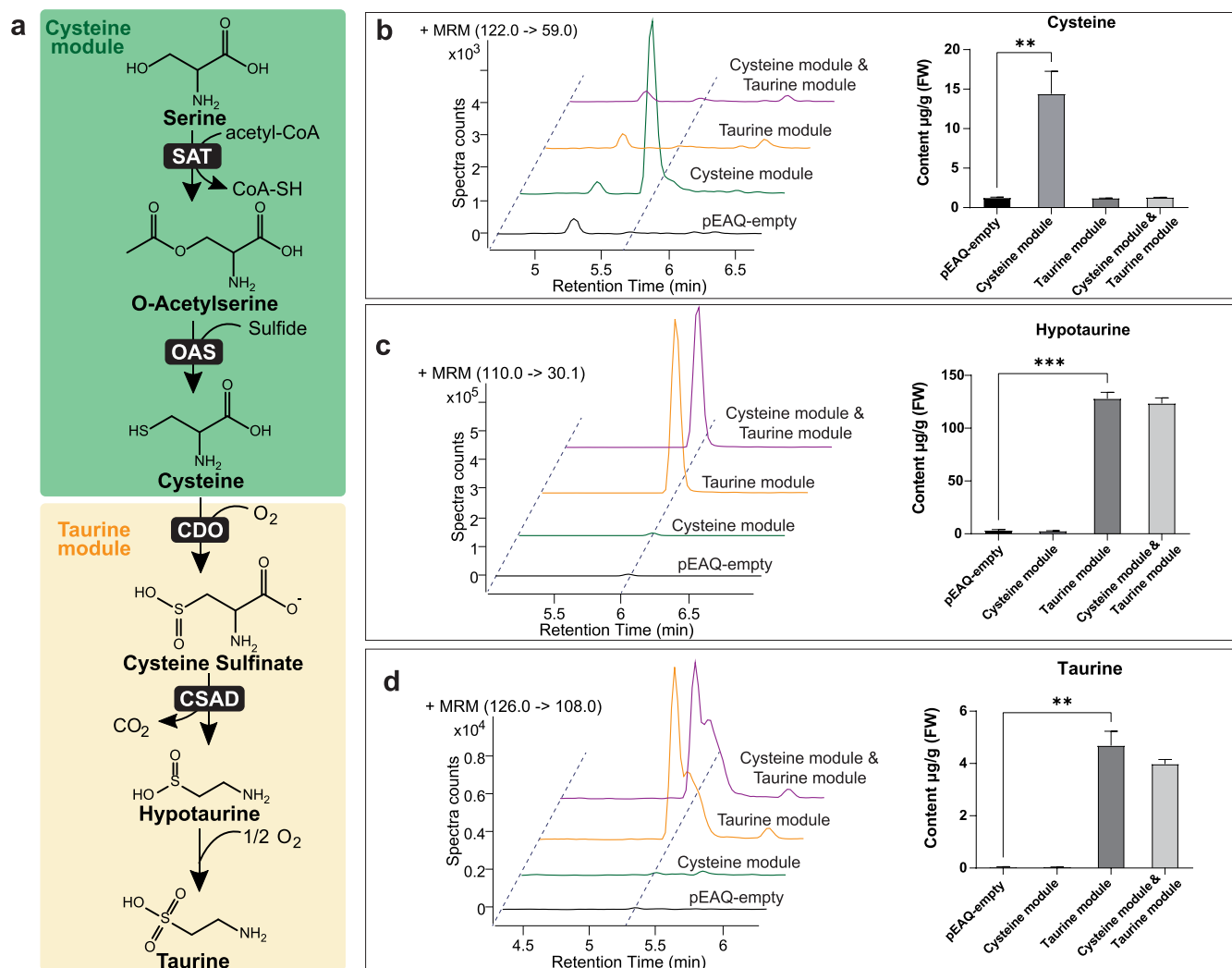
Figure 4. Dual module-based taurine synthesis in *N. benthamiana* leaves. (a) Diagram showing the integration of two modules for taurine synthesis in *N. benthamiana* leaves. The cysteine module involves the enzymes SAT and OAS, while the taurine module incorporates the enzymes CDO and CSAD converted cysteine to hypotaurine, subsequently undergoing spontaneous oxidation to form taurine. (b) LC/MS chromatograms and quantification of cysteine in leaves following separate module expression show a significant increase in cysteine with the cysteine module alone, with no noticeable change upon the addition of the taurine module. (c) LC/MS chromatograms alongside quantification data demonstrate the successful detection of hypotaurine and (d) taurine following expression with the taurine module. Notably, the levels of both compounds did not show additional increases upon subsequent integration with the cysteine module. The data presented are mean ± SEM from three biological replicates, with each infiltrated *N. benthamiana* plant considered a separate biological replicate. Statistical significance between different groups was determined using an unpaired *t*-test, with asterisks indicating the levels of significance (**$p < 0.01$, ***$p < 0.001$).

application of synthesizing creatine, indicating significant interference with the metabolism of plant endogenous amino acids. Among them, the content of 5 amino acids increased significantly and the content of two amino acids significantly decreased (aspartic acid and phenylalanine) (Supporting Information Figure S1).

Taurine and Hypotaurine Produced by the Taurine Module Inhibit the Cysteine Biosynthetic Enzymes in *N. benthamiana*. Leveraging the dual-module synthetic approach that previously enhanced carnosine production, we turned our focus to synthesizing taurine, an amino sulfonic acid typically absents in plant metabolism. Utilizing *N. benthamiana* as a host, we aimed to channel the naturally higher levels of serine into the production of cysteine, the direct precursor of taurine. The cysteine synthesis module, comprising SAT and OAS from *S. lycopersicum*, was introduced to facilitate this conversion. Subsequently, in the taurine

module, CDO and CSAD from *C. carpio* were used to convert cysteine into hypotaurine and ultimately to taurine through spontaneous oxidation (Figure 4a).

The expression of the individual cysteine module in *N. benthamiana* leaves yielded an 11-fold increase in cysteine levels, from 1.2 to 14.4 μg/g (Figure 4b), while the taurine module introduction resulted in detectable levels of hypotaurine (127.4 μg/g) and a modest amount of taurine (4.7 μg/g) (Figure 4c,d). However, simultaneous expression of both modules did not enhance taurine production as hypothesized. Instead, the levels of hypotaurine (123.4 μg/g) and taurine (4.0 μg/g) were slightly lower than those achieved with the taurine module alone, and cysteine level (1.3 μg/g) remained comparable to the control (Figure 4b).

To elucidate the unexpected attenuation of cysteine accumulation when both the cysteine and taurine modules were coexpressed in *N. benthamiana*, we postulated an
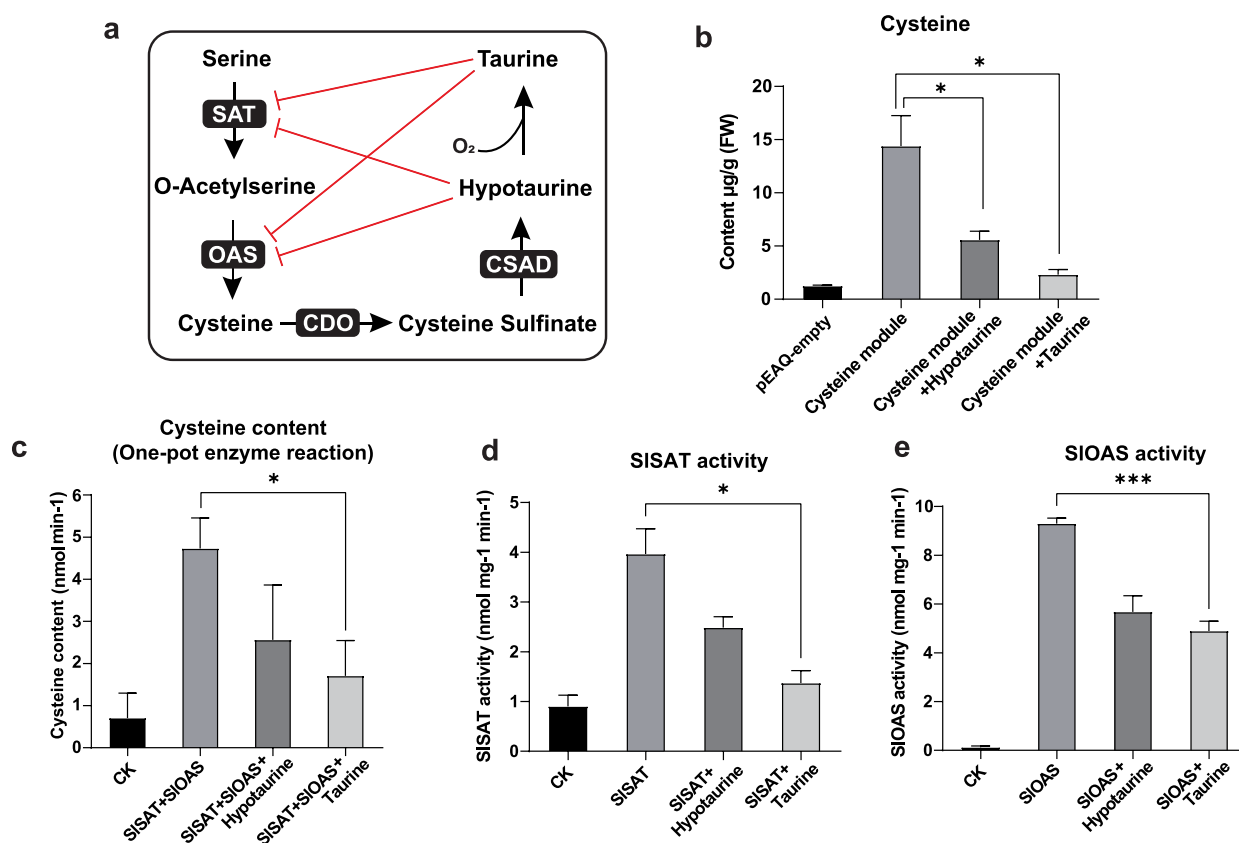
Figure 5. Taurine and hypotaurine regulate plant cysteine biosynthesis through inhibiting SAT and OAS enzymes. (a) Illustrative diagram showing the inhibitory effects of taurine and hypotaurine on the cysteine biosynthetic enzymes SAT and OAS in plants. (b) Application of 100 μM hypotaurine or 50 μM taurine resulted in a significant reduction of cysteine levels compared to the leaves infiltrated with only the cysteine module. (c) In a one-pot enzymatic assay combining two enzymes SlSAT and SlOAS, the production of cysteine was notably decreased upon the addition of either taurine or hypotaurine. (d) Separate enzyme assays demonstrated a reduction in SlSAT activity, as assessed by the decreased formation of its product, CoA. (e) SlOAS activity was diminished in the presence of taurine or hypotaurine, as determined by the reduced synthesis of cysteine. Data are presented as mean $\pm$ SEM from three biological replicates, with each treated *N. benthamiana* plant serving as an individual replicate. Asterisks denote significant differences between treatment groups as determined by an unpaired *t*-test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

inhibitory influence of the metabolites taurine and hypotaurine on the biosynthesis of cysteine. To investigate this, we coexpressed *N. benthamiana* leaves with the cysteine module along with either hypotaurine or taurine and subsequently measured the amino acid content. The results suggested that 100 μM hypotaurine or 50 μM taurine efficiently inhibited the function of cysteine module in *N. benthamiana* leaves. Compared to individually expressing cysteine module, the content of cysteine decreased significantly when hypotaurine was coinjected, from 14.4 to 5.6 μg/g (Figure 5b). Taurine seems to further inhibit cysteine production when it was coinjected with the cysteine module, with the cysteine content maintained at 2.3 μg/g, close to that of the pEAQ empty vector control group (Figure 5b). This data supports the hypothesis that the metabolites produced by the taurine module can interfere with the cysteine synthesis pathway.

To elucidate the inhibitory mechanism of hypotaurine and taurine on the cysteine biosynthesis pathway, we proposed that these metabolites could directly inhibit the enzymatic activities of SAT and OAS, key enzymes in the cysteine module (Figure 5a). To investigate this hypothesis, we conducted *in vitro* enzyme assays using purified SlSAT and SlOAS proteins (Supporting Information Figure S2), assessing the potential inhibitory effects of taurine and hypotaurine. We initiated the assays with a one-pot reaction combining SlSAT and SlOAS,

an approach that simulates the *in vivo* condition of applying a single cysteine module in plants. The introduction of 100 μM hypotaurine or 50 μM taurine into this reaction mixture resulted in a significant decrease in cysteine production, as compared to the control reactions without these metabolites (Figure 5c). Further assays conducted separately with SlSAT or SlOAS enzymes corroborated the initial findings, demonstrating that both hypotaurine and taurine indeed possess the capacity to inhibit the catalytic functions of these key enzymes involved in cysteine synthesis (Figure 5d,e). These results provide a mechanistic explanation for the observed reduction in cysteine levels in planta and underscore the complexity of engineering synthetic pathways that intersect with native metabolic processes.

To mitigate this inhibition, we experimented with staggered module introduction, administering the cysteine module 2 days prior to the taurine module. This sequential expression alleviated the inhibition on cysteine synthesis, with an increase in cysteine content (9.5 μg/g) observed (Supporting Information Figure S3). Nonetheless, the levels of taurine (0.9 μg/g) and hypotaurine (33.7 μg/g) were substantially reduced compared to when the taurine module was expressed alone, suggesting compromised efficacy likely due to diminished leaf vitality postagrobacterial expression (Supporting Information Figure S3).
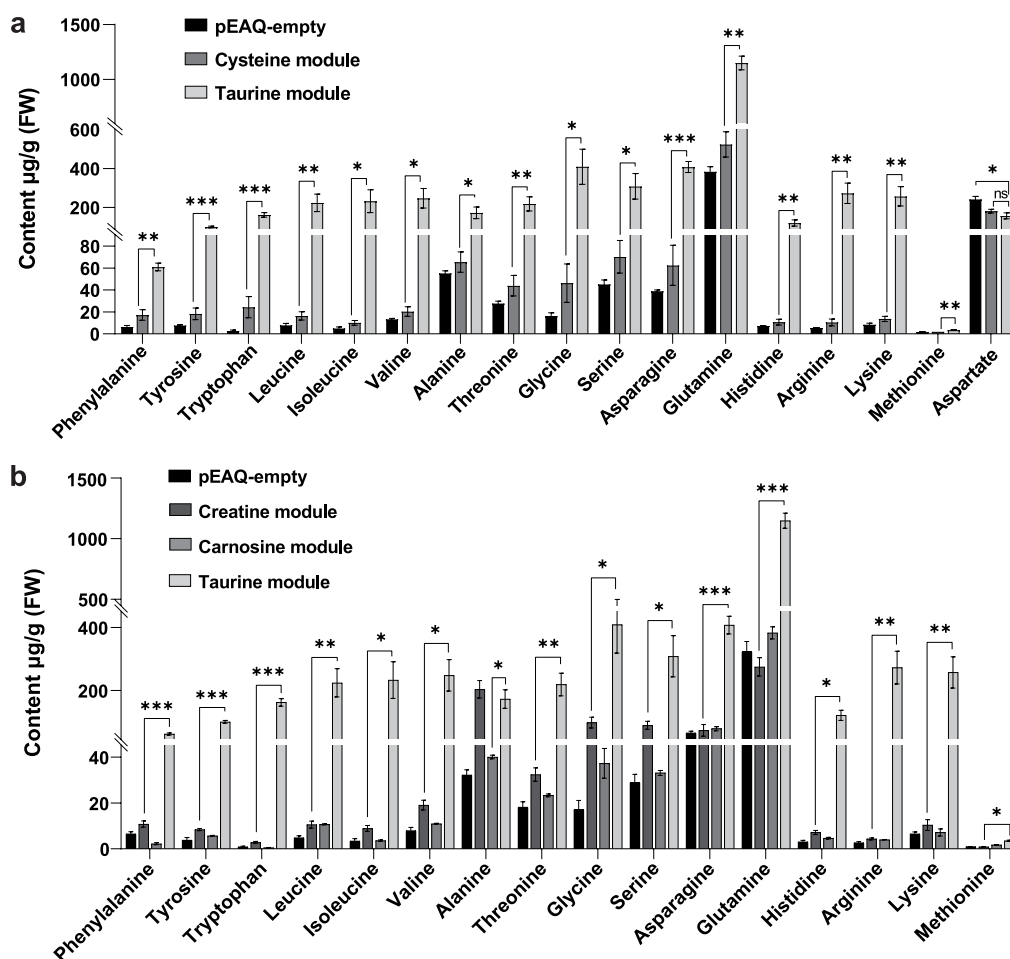
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Figure 6. Taurine module triggers the most significant alterations in amino acid profiles of *N. benthamiana* leaves compared to creatine and carnosine modules. (a) *N. benthamiana* leaves infiltrated with the taurine module exhibited a pronounced increase in the levels of 16 free amino acids, showcasing significantly higher concentrations when compared to leaves infiltrated with the cysteine module. (b) Of the three modules—creatine, carnosine, and taurine—designed to produce different amino acid derivatives of animal origin, the taurine module triggered the most extensive array of significant amino acid fluctuations. The data depicted in the figure are presented as mean ± SEM derived from three biological replicates, with each *N. benthamiana* plant counted as a single biological replicate. The asterisks denote the presence of significant differences between the comparison groups as determined by an unpaired *t*-test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

The effects of the taurine module on the endogenous free amino acid metabolism in *N. benthamiana* leaves were investigated. Consistent with our hypothesis, the amino acid profiles resulting from the coexpression of both the cysteine and taurine modules closely resembled those observed with individually expressing the taurine module (Supporting Information Figure S4). Strikingly, expression with the taurine module alone led to a significant upregulation in the levels of 15 amino acids compared with the leaves expressing only the cysteine module (Figure 6a and Supporting Information Table S7). This drastic difference suggests that hypotaurine or taurine may exert a direct effect on amino acid metabolism, potentially through the inhibition of SAT and OAS enzyme activities. When comparing the impacts of expressing creatine, carnosine, and taurine modules on the amino acid metabolism of *N. benthamiana* leaves, the taurine module caused the highest number of amino acids to show a significant increase in content (Figure 6b). These results indicate that complex metabolic adjustments occur in plant cells, rebalancing amino acid metabolism upon expression of the synthetic module.

Metabolic Adjustments of Branched Chain and Aromatic Amino Acids Biosynthesis in *N. benthamiana*

after Exogenous Synthetic Pathway Integration. To investigate the systemic metabolic alterations induced by the introduction of exogenous biosynthetic pathways into *N. benthamiana*, we performed a comparative transcriptomic analysis of leaves expressed with various synthetic modules. Additionally, the expression levels of several key genes were validated by RT-qPCR (Supporting Information Figure S5), confirming the accuracy of the RNA sequencing data. We chose to analyze leaves expressed with the creatine and cysteine/taurine modules as they triggered more pronounced alterations in endogenous amino acid levels compared to the carnosine module (Figure 6b). This analysis, coupled with the quantification of free amino acid content, allowed us to dissect the biochemical adjustments occurring within the plant host metabolism following the application of synthetic biology techniques aimed at producing compounds of animal origin.

Our analysis identified two prominent metabolic adjusted events consistent across plants expressed with creatine, cysteine, and taurine modules. First, we observed an upsurge in threonine levels, which paralleled with an upregulation of threonine synthase (THS) gene expression (Figure 7 and Supporting Information Tables S8 and S9). Increased
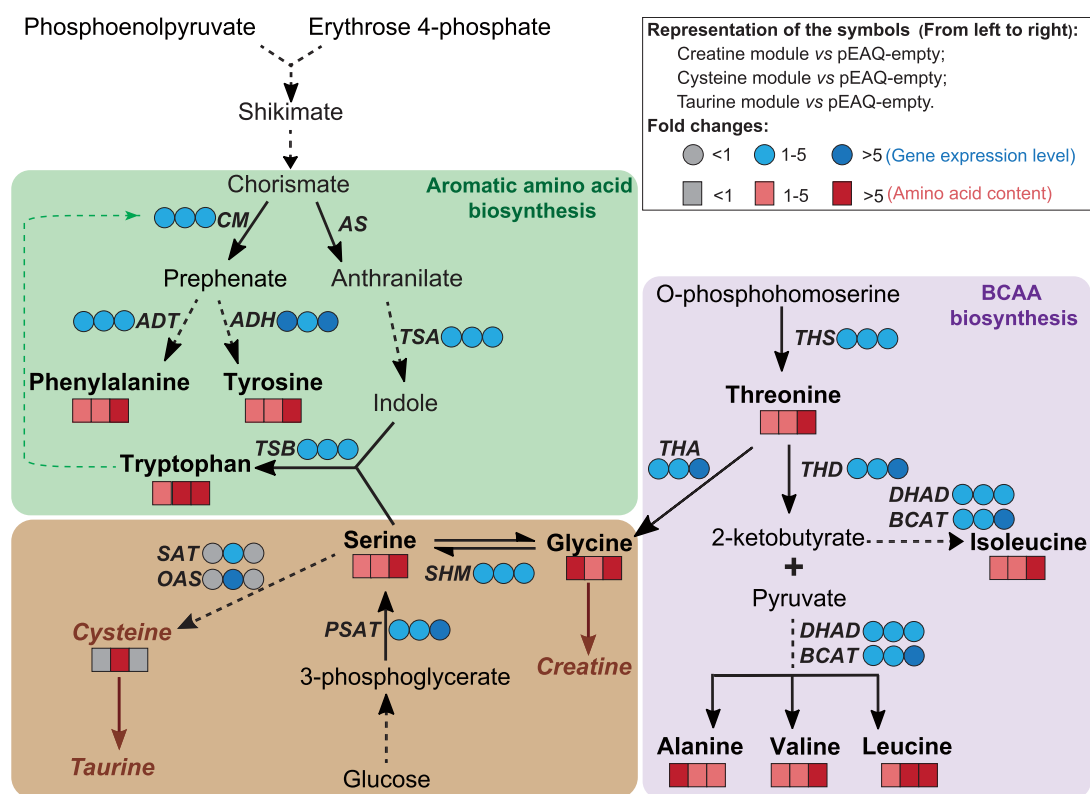
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Figure 7. Metabolic adjustments of branched-chain and aromatic amino acid biosynthesis in *N. benthamiana* after introducing the creatine, cysteine, and taurine modules. The red blocks beneath each amino acid denote the fold changes in amino acid content, while the blue circles adjacent to the gene names indicate the fold changes in gene expression levels, as described in the legend box in the upper right corner. In the green section that highlighted the biosynthetic pathway of aromatic amino acids, there was a significant upregulation in the expression of genes such as CM, ADH, ADT, and TSB (tryptophan synthase beta chain). This upregulation correlated with a marked increase in the levels of phenylalanine, tyrosine, and tryptophan. The dotted green line denoted the allosteric regulation of CM by tryptophan. In the brown highlighted area, SAT and OAS/cysteine synthase were significantly upregulated only in the cysteine module, leading to a high content of cysteine. The content of serine and glycine showed an increased pattern in all situations, which corresponding to the upregulation of PSAT, serine hydroxymethyltransferase (SHM), and THA. The purple area denoted the upregulated biosynthesis of BCAAs. There was a correlation with increased expression of genes such as THS, threonine dehydratase (THD), DHAD, and BCAT, highlighting an overall stimulation of the BCAA metabolic pathway.

threonine appears to enhance downstream biosynthesis of branched-chain amino acids (BCAAs) such as isoleucine, leucine, valine, and alanine, supported by the upregulation of genes encoding enzymes like threonine aldolase (THA), dihydroxy-acid dehydratase (DHAD), and branched-chain-amino-acid aminotransferase (BCAT) in Figure 7 (highlighted in the purple area). The second adjusted event involved an upsurge in serine concentration, which correlated with the upregulated expression of phosphoserine aminotransferase (PSAT), the enzyme responsible for converting 3-phosphogly-cerate to serine (highlighted in the brown area). In conjunction, the content of tryptophan, a secondary metabolite deriving from serine and indole, also showed an increase pattern. This was in line with the elevated expression of the tryptophan synthase *β* subunit (TSB), the enzyme catalyzing this conversion. Additionally, phenylalanine and tyrosine, which share a common biosynthetic pathway with tryptophan, exhibited increased levels, corresponding with the upregulation of enzymes such as chorismate mutase (CM), arogenate dehydrogenase (ADH), and arogenate dehydratase (ADT), as highlighted in the green area in Figure 7.

The observed metabolic adjustments in *N. benthamiana* leaves upon the introduction of synthetic modules can be elucidated by examining the DEGs from a biochemical network perspective. The introduction of the creatine module

led to a temporary reduction in glycine levels as it was utilized as a substrate for creatine. To compensate for this decrease, the expression of genes encoding SHM and THA, both key in converting serine and threonine into glycine, was upregulated. This shift likely resulted in a transient depletion of the threonine pool due to its rerouting toward glycine production by THA. In response, the plant upregulated THS to replenish threonine levels. Enhanced activity of THA also indirectly stimulated THS activity, leading to increased synthesis of threonine and, consequently, higher levels of downstream BCAAs such as valine, leucine, isoleucine, and alanine. Simultaneously, the augmented activity of SHM, which catalyzes the reversible conversion of serine to glycine, could have temporarily diminished serine levels. This, in turn, led to increased expression of PSAT, which diverts 3-phosphogly-cerate from the glycolytic pathway toward serine biosynthesis. An accumulation of serine may have triggered a rise in expression of TSB, channeling excess serine into tryptophan production, an activator of CM. CM is known to undergo allosteric feedback regulation, modulating the synthesis balance between anthranilate-derived tryptophan and prephenate-derived phenylalanine and tyrosine. Consequently, an over-abundance of tryptophan led to enhanced production of phenylalanine and tyrosine, as indicated in the green area of Figure 7.

For plants expressed with the cysteine or taurine modules, indicated in the second and third mini-blocks of Figure 7, a comparable series of events can be deduced. The biosynthesis or consumption of cysteine can lead to a temporary reduction in serine availability, prompting increased expression of SHM to convert glycine back into serine. Hence, expressing these modules is expected to induce similar metabolic shifts as observed with the creatine module, promoting the biosynthesis of both BCAAs and aromatic amino acids, demonstrating a consistent response pattern to synthetic module integration within the plant metabolism.

DISCUSSION

Exploiting Synthetic Modular Approach to Product Compounds of Animal Origin in the Plant Host. The utility of plants as platforms for synthetic biology offers distinct advantages over microbial systems. Plant inherent capabilities to utilize atmospheric carbon and nitrogen not only bolster the safety profile of the production process but also enable large-scale, standardized cultivation. These attributes made them eco-friendly and sustainable long-term development.[15,16] Traditionally, the focus of plant synthetic biology has been the production of plant-native compounds, such as flavonoids, terpenoids, sclareol and so on, primarily through heterologous pathway transfers.[27−29] However, applications involving the synthesis of amino acid derived compounds that are non-plant origin remain relatively scarce, primarily confined to large molecules such as peptide hormones, antibodies and vaccines. Noteworthy efforts include expression of human proinsulin in tomatoes,[30,31] and vaccines for diseases like cholera and malaria produced in *N. benthamiana*,[32] along with recent COVID-19 vaccine development.[33] These studies underscore the feasibility of utilizing plants as chassis for synthesizing amino acid-derived products.

This study further validates the concept of bioengineering plants to synthesize amino acid derivatives of animal origin—namely, creatine, carnosine, and taurine—via transient transformation in *N. benthamiana*. Our research introduces a strategic modular approach to amino acid synthesis, with the carnosine synthesis serving as a prime example. We have shown that the initial enhancement of precursor amino acid concentrations via one synthetic module, followed by the introduction of a subsequent module to catalyze the conversion to the desired product, significantly boosts the production of the target compound (Figure 3b). Importantly, this sequential module deployment serves to mitigate disturbances in the plant endogenous amino acid metabolism, thereby reducing the risk of adverse metabolic consequences (Figure 3f). The flexibility and effectiveness of this modular system suggest its broad applicability in the expanding field of plant synthetic biology and its potential to significantly enhance the nutritional profile of plant-derived foods.

This study relies on transient expression in *N. benthamiana* leaves, a method commonly used for testing metabolism, biochemical manipulations, and discovering new activities.[34] While this approach is valuable for proof-of-concept studies, it is important to acknowledge its limitations. Transient expression does not result in stable transformation events, which are necessary for the long-term production of introduced synthetic modules in plants. Establishing stably transformed plants through techniques such as *Agrobacterium*-mediated transformation is essential for sustainable and large-scale production of these valuable compounds.[35,36] However,

creating stable plant lines is time-consuming and requires extensive characterization and optimization, which is beyond the scope of the current study. Future research should focus on translating the findings from this transient expression system into stable plant lines, paving the way for the long-term production of the amino acid derivatives of animal origin in plants.

Feedback-like Inhibition Induced by Introducing Compounds of Animal Origin to Plants. Feedback inhibition is a well-established mechanism whereby the accumulation of end products inhibits the activity of enzymes early in their biosynthetic pathways, thus regulating metabolic flux to maintain equilibrium within biological systems.[37] This regulatory mechanism helps to prevent the overaccumulation or underutilization of metabolites, thereby maintaining homeostasis.[38] For example, general metabolites like isocitrate and citrate modulates the activity of isocitrate dehydrogenase within the citric acid cycle, thereby regulating the metabolic throughput in accordance with cellular demands.[39,40] Similarly, in the plant tryptophan biosynthesis pathway, the accumulation of tryptophan can exert feedback inhibition on multiple upstream enzymes to curtail its own overproduction.[41,42]

Our investigation reveals an unexpected instance of feedback-like inhibition in plant-based synthetic biology. We observed that taurine and its precursor hypotaurine, when synthesized in plants, can inhibit the SAT and OAS, which are critical to the biosynthesis of the amino acid cysteine (Figure 5). This finding aligns with classic feedback inhibition but occurs in a context where such regulation was previously uncharacterized. Taurine, a sulfur-containing amino acid, is vital for several physiological functions in humans, although it typically does not integrate into the primary metabolic pathways.[10,43] The feedback inhibition mechanisms involving taurine are not found in animals, likely due to its distinct biosynthetic route. In animals, cysteine is synthesized from homocysteine, which is derived from methionine through a series of reactions involving cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE).[11,44,45]

Our data suggest that the introduction of taurine and hypotaurine synthesis into plants can disrupt native amino acid metabolism. The inhibition of plant SAT and OAS by these compounds (Figure 5) constrains cysteine synthesis, forcing the plant to enhance the activation of alternative enzymes involved in amino acid biosynthesis. This compensatory response can lead to the accumulation of metabolites such as serine, ultimately resulting in a pronounced imbalance in the plant amino acid profile (Figure 6). This disruption presents a significant challenge for metabolic engineering efforts, which must be carefully addressed to ensure the successful integration of exogenous pathways without adverse effects on the metabolism of host organism.

Adjustments of Amino Acid Metabolism in Plants after Introducing Foreign Metabolic Steps. The incorporation of exogenous metabolic pathways into plant systems often induces adjustments of native metabolic networks to accommodate the biosynthesis of foreign compounds.[46,47] Our study provides a clear illustration of the complexity involved in integrating biosynthetic steps of animal origin into plant metabolism, particularly concerning amino acid synthesis. The adjustments were not merely additive but resulted in a systemic reorganization of metabolic fluxes, which can have profound implications for plant physiology and metabolic homeostasis.

Upon the introduction of synthetic modules for the production of animal-originated amino acid derivatives in *N. benthamiana*, we observed a pronounced shift in the metabolic landscape of the host plant. The engineered pathways for creatine, carnosine, and taurine synthesis did not operate in isolation; rather, they engaged in a dynamic interplay with the inherent metabolic processes (Figure 6). For instance, the diversion of precursor amino acids toward the synthesis of these non-native compounds triggered compensatory mechanisms within the host, leading to altered levels of various amino acids. The metabolic adjustments reflect the plasticity of plant metabolic networks. However, it also underscores the importance of a systemic understanding of metabolic interactions when introducing foreign pathways. The elevated levels of amino acids such as serine, following the expression of synthetic modules, highlight a cascade of effect through the metabolic network, impacting pathways that are not directly tied to the engineered route (Figures 6 and 7). Such changes could indicate a stress response or an adaptive rebalancing act by the host to maintain metabolic equilibrium.

Our findings emphasize the need for an integrative approach in synthetic biology that goes beyond the targeted biosynthetic steps. The manipulation of one metabolic pathway can have unforeseen consequences on related pathways due to the interconnected nature of metabolism.[25,46,48] Thus, the introduction of foreign metabolic steps should be accompanied by a comprehensive analysis of the metabolic state of selected host plant, ensuring that the synthetic pathway aligns with the metabolic capabilities of the host. Furthermore, the inhibition of key enzymes by the synthesized compounds suggests that the temporal and spatial expression of synthetic modules should be finely tuned.[49,50] A staggered introduction of the modules or the use of inducible promoters could provide a means to mitigate the inhibitory effects and enhance the efficiency of the engineered pathways.

In conclusion, the adjustments of plant amino acid metabolism by the introduction of foreign metabolic steps presents both a challenge and an opportunity. It challenges our capacity to predict and control metabolic outcomes in synthetic biology, but it also offers an opportunity to deepen our understanding of plant metabolic flexibility. By decoding these complex interactions, we can refine our engineering approaches to harness the full potential of plant synthetic biology for sustainable production of valuable compounds.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.4c05719.

Primer sequences used in the current study; detailed composition of isotopic labeling buffers for amino acid extraction; optimized MRM parameters for electrospray ionization (positive mode); analysis of the expression stability of three housekeeping genes in *N. benthamiana* leaves after injection of different modules; amino acid profiles post creatine module expression in *N. benthamiana* leaves; amino acid concentrations following β-alanine and carnosine modules expression in *N. benthamiana* leaves; amino acid levels after introduction of cysteine and taurine modules in *N. benthamiana* leaves; fold changes in amino acid content after expressing different modules compared with the pEAQ

empty vector control; FPKM values and fold changes for amino acid-related biosynthetic genes showed in Figure 7; amino acid content alterations in *N. benthamiana* leaves by carnosine and creatine module expression; protein expression and purification of SlSAT and SlOAS in *E. coli* Rosetta (DE3); influence of staggered infiltration of cysteine and taurine modules on cysteine, taurine and hypotaurine in *N. benthamiana* leaves; comparative analysis of amino acid levels with individual and coexpression of taurine and cysteine modules in *N. benthamiana* leaves; validation of RNA-seq gene expression using RT-qPCR analysis (PDF)

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS AND NOMENCLATURE

AGAT, glycine amidinotransferase; GAMT, guanidinoacetate methyltransferase; ADC, L-aspartate-α-decarboxylase; CARNS, carnosine synthase; SAT, serine acetyltransferase; OAS, O-acetylserine(thiol)lyase; CDO, cysteine dioxygenase; CSAD, cysteine sulfinic acid decarboxylase; THS, threonine synthase; THA, threonine aldolase; THD, threonine dehydratase; DHAD, dihydroxy-acid dehydratase; BCAT, branched-chain-amino-acid aminotransferase; PSAT, phosphoserine aminotransferase; TSB, tryptophan synthase β subunit; CM, chorismate mutase; ADH, arogenate dehydrogenase; ADT, arogenate dehydratase; CBS, cystathionine β-synthase; CSE, cystathionine γ-lyase; BCAAs, branched-chain amino acids; MRM, multireaction monitoring; CoA, coenzyme A; IPTG, isopropyl β-D-1-thiogalactopyranoside; EDTA, ethylenediaminetetraacetic acid; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; PLP, pyridoxal phosphate

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