agronomy

MDPI

Article

De Novo Synthesis of Anticholinergic Hyoscyamine and Scopolamine in *Nicotiana benthamiana* Based on Elucidating Tropane Alkaloid Biosynthetic Pathway of *Anisodus luridus*

Mengling Wen [1,2], Junlan Zeng [1], Fei Qiu [1], Fangyuan Zhang [1] and Zhihua Liao [1,*]

[1] Integrative Science Center of Germplasm Creation in Western China (CHONGQING) Science City, SWU-TAAHC Medicinal Plant Joint R&D Centre, School of Life Sciences, Southwest University, Chongqing 400715, China
[2] Faculty of Agriculture, Forestry and Food Engineering, Yibin University, Yibin 644000, China
* Correspondence: zhliao@swu.edu.cn

Abstract: *Anisodus luridus*, a perennial herb belonging to the genus *Anisodus* of the Solanaceae family, is an important Tibetan medicinal plant that produces pharmaceutical tropane alkaloids (TAs) including hyoscyamine and scopolamine. Its high yield of hyoscyamine makes *A. luridus* a valuable plant source for commercially producing TAs. In this study, we conduct homologous gene research across transcriptome data of different tissues together with functionally tested sequences in *Atropa belladonna* as a reference and identify 13 candidate genes for TAs biosynthesis in *A. luridus*. The results show that these 13 TAs biosynthesis genes identified in *A. luridus* were highly conserved in terms of sequence similarity and gene expression patterns compared to *A. belladonna*, suggesting that the two species may share the same biosynthetic pathway for TAs biosynthesis. Furthermore, scopolamine was detected in *Nicotiana benthamiana* leaves when these 13 enzymes were co-expressed in *N. benthamiana*, which confirmed that these 13 TAs biosynthesis genes are involved in the biosynthesis of TAs. The results of our study not only systematically elucidate the tropane alkaloid biosynthetic pathway of *A. luridus*, but also realize the de novo synthesis of TAs in *N. benthamiana* for the first time. It is now possible to make *N. benthamiana* a potential source for TAs production through synthetic biology techniques.

Keywords: *Anisodus luridus*; tropane alkaloids; biosynthetic pathway; de novo synthesis; transient expression

Citation: Wen, M.; Zeng, J.; Qiu, F.; Zhang, F.; Liao, Z. De Novo Synthesis of Anticholinergic Hyoscyamine and Scopolamine in *Nicotiana benthamiana* Based on Elucidating Tropane Alkaloid Biosynthetic Pathway of *Anisodus luridus*. *Agronomy* **2024**, *14*, 2460. https://doi.org/10.3390/agronomy14112460

Academic Editor: Mirosław Tyrka

Received: 20 September 2024
Revised: 11 October 2024
Accepted: 15 October 2024
Published: 22 October 2024

1. Introduction

Anisodus luridus (which used to be called *Scopolia lurida*), a perennial herb belonging to the genus *Anisodus* of the Solanaceae family, originating from Tibet, Yunnan, Nepal, is an important Tibetan medicinal plant, which produces pharmaceutical tropane alkaloids (TAs) including hyoscyamine and scopolamine [1,2]. Compared to other plant species that produce TAs, such as *Atropa belladonna*, *A. luridus* is one of the most effective producers of TAs due to its high biomass and hyoscyamine content [2]. Therefore, *A. luridus* has become a valuable plant source for commercially producing TAs. However, the enzymes involved in TAs biosynthesis have not all been identified, and the TAs biosynthetic pathway has not been systematically studied.

Due to their anticholinergic activity, TAs are widely used in the treatment of postoperative nausea and vomiting, motion sickness, organophosphate poisoning, and Parkinson's symptoms [3,4]. After more than a century of tireless efforts by generations of scientists, the scopolamine biosynthetic pathway was fully identified in 2021 [5]. The complete biosynthetic pathway of TAs involves a total of 13 enzymes and can be divided into three parts [6,7]. One part is the synthesis of tropine, beginning with ornithine, and ornithine decarboxylase (ODC [8]), putrescine *N*-methyltransferase (PMT [9]), *N*-methylputrescine oxidase (MPO [6]), type III polyketide synthase (PYKS [10,11]), tropinone synthase (CYP82M3 [11]),

and tropinone reductase I (TRI [6,12]) are involved. The other part is the synthesis of phenyllactylglucose, starting from phenylalanine, and aromatic amino acid aminotransferase 4 (AT4 [13]), phenylpyruvic acid reductase (PPAR [14]), and phenyllactate UDP-glycosyltransferase (UGT1 [15]) are involved. The last part is the condensation of tropine and phenyllactylglucose, with a total of four enzymes involved, which are littorine synthase (LS [15]), littorine mutase (CYP80F1 [16]), hyoscyamine dehydrogenase (HDH [17]), and hyoscyamine 6β-hydroxylase (H6H [18]).

In the past two years, the whole genomes of six TA-producing Solanaceae species, such as *A. belladonna*, *Datura stramonium*, *Anisodus tanguticus*, *Brugmansia arborea*, *Mandragora caulescens*, and *Anisodus acutangulus*, have been sequenced, assembled, and annotated [6,7,19,20]. At the same time, the biosynthetic pathway of TAs has been thoroughly and comprehensively analyzed in phylogenetic evolution. Research shows that the TAs biosynthetic pathway likely emerged in the ancestral lineage shared by all Solanaceae plants. However, the absence or pseudogenization of these genes in non-TAs-producing species have led to a constrained distribution of TAs among Solanaceae members [7]. Furthermore, the five species producing TAs (*A. belladonna*, *D. stramonium*, *A. tanguticus*, *B. arborea*, and *M. caulescens*) share a common biosynthetic pathway for scopolamine biosynthesis even though they are distantly related lineages within the Solanaceae family [6,7].

The species most intensively studied in relation to the biosynthetic pathway of TAs is *A. belladonna* in which all genes involved in the biosynthesis of TAs have been identified. Yang et al. employed TAs biosynthesis genes functionally tested in *A. belladonna* as a template to search for homologous genes in the genomes of 15 plant species [7]. The results show that homologous genes could be detected in TAs-producing plants, such as *A. tanguticus*, *B. arborea*, and *M. caulescens*, and with high sequence identity (>80%). However, homologous genes underwent pseudogenization or multiple losses in non-TAs-producing plants, indicating that complete and functional TAs genes are a necessary condition for TAs production. Thus, whether *A. luridus* possesses all homologous genes for TAs biosynthesis and whether TAs are synthesized by the same biosynthetic pathway as *A. belladonna* remain unclear. However, only *PMT*, *TRI*, *CYP80F1*, and *H6H* have been reported in *A. luridus*, among which *TRI* and *H6H* have been studied in more depth [1,2]. In 2017, The *TRI* cDNA sequence was cloned and performed a phylogenetic analysis, tissue profiling, an enzyme assay, and metabolic engineering [2]. In 2018, Lan et al. cloned the full-length *H6H* cDNA and performed metabolic engineering in hairy root cultures of *A. luridus* [1]. To date, the other nine genes related to scopolamine biosynthesis in *A. luridus* have not been reported.

Nicotiana benthamiana has been used in fundamental research for decades as a model plant. The infiltration of *Agrobacterium* strains carrying pathway genes into leaf tissues enables the conversion of endogenous metabolites, derived from the products of photosynthesis, to target compounds. The utilization of *N. benthamiana* has now broadened to encompass the elucidation and reconstitution of diverse biosynthetic pathways [21]. To date, several classes of secondary metabolites have been successfully produced in *N. benthamiana*, such as terpenes [22–24], phenolics [25–27], and alkaloids [28]. *N. benthamiana* has been employed as a plant chassis to clarify the biosynthetic pathways of a large number of alkaloids, including strychnine [29], the benzoxazinoid 2-(2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one)-b-d-glucopyranose [30], the colchicine precursor *N*-formyldemecolcine [31], cocaine [28], diosgenin [32], and piperine [33]. To date, the de novo synthesis of TAs in *N. benthamiana* has not been reported.

In this study, based on transcriptome data from different tissues of *A. luridus*, we identified 13 candidate TAs biosynthesis genes, which are highly consistent with the amino acid sequences of functionally tested TAs genes in *A. belladonna*. Finally, 13 candidate TAs biosynthesis genes were confirmed to be involved in the biosynthesis of TAs in *A. luridus*, and the de novo synthesis of TAs was performed for the first time in *N. benthamiana*.

2. Materials and Methods

2.1. Plant Materials

A. luridus plants were cultivated in the medicinal plant garden of the Xizang Agricultural and Animal Husbandry College (Nyingchi, Tibet, China). *N. benthamiana* was grown in a greenhouse under a photoperiod of 16 h/8 h day/night at 25 °C and used for functional verification in vivo.

2.2. RNA Sequencing and Expression Profiling Analyses

Total RNA was extracted from different tissues of the secondary root (SR), primary root (PR), stem, leaf, flower, and fruit derived from 3 independent *A. luridus* plants using the RNAsimple Total RNA Kit (TIANGEN) according to the manufacturer's instructions. And total RNA was subjected to RNA-seq analysis using an Illumina Genome Analyzer at Biomarker Technologies (Beijing, China). Based on FPKM values, the expression patterns of the candidate TAs biosynthesis genes in different tissues were analyzed, and a heat map was generated using TBtools-II [34].

2.3. Real-Time PCR Analysis

Real-time PCR (RT-PCR) was performed using a CFX Connect™ Real Time PCR System (BIO-RAD) with SYBR-Green (TIANGEN, Beijing, China). The primer sequences used are shown in Supplementary Table S1. Each reaction was carried out in a 10 µL volume consisting of 5 µL of SYBR, 4 µL of diluted template (1 µL of the generated first strand cDNA diluted by 19 µL of RNase-Free ddH_2O), and 0.5 µL of each of the two gene-specific primers. Two steps were used for RT-PCR: 95 °C for 3 min, followed by 40 cycles at 95 °C for 10 s and 60 °C for 30 s. With *PGK* as internal reference, relative gene expression values were calculated using the $2^{-\Delta\Delta Ct}$ method.

2.4. Homologous Gene Retrieval

To search for homologs in the *A. luridus* transcriptome database, we utilized the functionally tested TAs biosynthesis genes of *A. belladonna* as a reference [6,17,35], employing BLAST search and the Hidden Markov Model (HMM). The obtained gene sequences from both methods were integrated, and we manually corrected the errors in the automatic annotations.

2.5. Bioinformatics Analysis

Candidate TAs biosynthesis genes of *A. luridus* were analyzed using Expasy-ProtParam for their physical and chemical properties and WoLF PSORT [36] for prediction of subcellular localization.

2.6. Functional Verification of Candidate Genes in N. benthamiana

The pEAQ-HT plasmid was used for the transient expression of heterologous proteins in *N. benthamiana* [37]. Full-length open reading frames of candidate TAs biosynthesis genes of *A. luridus* were recombined into the expression vector pEAQ-HT, and the detailed methods for vector construction were the same as previously described [28]. Gene-specific primers are listed in Supplementary Table S1. The verified recombinant plasmids were successfully transformed into *A. tumefaciens* GV3101 using the freeze–thaw method. The positive colonies were cultured overnight in the YEP medium containing antibiotics, and the collected bacteria were re-suspended in MMA buffer (10 mM MES pH = 5.6, 10 mM $MgCl_2$, 150 µM acetosyringone), with each strain adjusted to an optical density (OD600) of 0.3. After incubation at room temperature for 2 h, the relevant *Agrobacterium* suspensions were mixed in an equal volume and then infiltrated into the leaves of *N. benthamiana* using a 1 mL syringe. The leaves were harvested 4 days later and lyophilized to determine the dry weight of the leaves.

2.7. Quantification of TAs by LC-MS

An amount of 25 mg dry powder of freeze-dried *N. benthamiana* leaves was extracted with 1 mL of extract solution (20% methanol + 0.1% formic acid). After centrifuges, the supernatant was filtered through a 0.22 μm needle filter. The filtrate was diluted 50 times with extract solution and detected by LC-MS. The TAs content in leaf extract was measured using an Orbitrap Exploris 120 LC-MS (Thermo Scientific, Pittsburgh, PA, USA) according to the methods described previously [6].

3. Results

3.1. Transcriptome Sequencing and Quality Assessment

To identify the genes involved in the biosynthesis of TAs, transcriptome sequencing was carried out in six tissues of *A. luridus*, namely the secondary root (SR), primary root (PR), stem, leaf, flower, and fruit, and the quality of the sequencing is shown in Table 1. After quality control, a total of 115.77 Gb of Clean Data was obtained, and the Clean Data of each sample was greater than 5.7 Gb, the GC content was about 42%, and the Q30 was above 94%. Overall, the database was of high quality and could be used for the subsequent analysis.

Table 1. Transcriptome sequencing quality results of different tissues from *A. luridus*.

Samples	Clean Reads	Clean Bases	GC Content	% ≥ Q30	Total Reads
SR-1	21,416,963	6,412,990,514	42.12%	96.15%	42,833,926
SR-2	21,508,499	6,439,821,856	43.19%	95.27%	43,016,998
SR-3	21,400,303	6,406,596,650	42.29%	94.68%	42,800,606
Flower-1	22,920,435	6,861,817,636	42.60%	94.50%	45,840,870
Flower-2	24,844,633	7,438,750,734	42.58%	94.81%	49,689,266
Flower-3	21,680,946	6,491,325,066	42.59%	95.08%	43,361,892
Fruit-1	20,698,862	6,196,316,816	42.78%	95.10%	41,397,724
Fruit-2	19,288,560	5,774,477,710	42.13%	94.98%	38,577,120
Fruit-3	21,871,094	6,547,980,308	42.22%	94.86%	43,742,188
Leaf-1	22,519,097	6,743,921,392	42.33%	94.69%	45,038,194
Leaf-2	23,490,539	7,034,719,032	42.21%	94.92%	46,981,078
Leaf-3	20,035,207	5,998,901,816	42.07%	94.78%	40,070,414
PR-1	19,990,642	5,984,977,532	42.96%	94.25%	39,981,284
PR-2	21,249,355	6,359,057,558	42.94%	94.91%	42,498,710
PR-3	20,825,144	6,233,208,310	42.87%	94.63%	41,650,288
Stem-1	21,476,136	6,423,857,126	42.19%	95.02%	42,952,272
Stem-2	20,458,651	6,125,644,130	42.01%	94.69%	40,917,302
Stem-3	21,037,941	6,296,989,810	42.03%	94.74%	42,075,882

3.2. The Identification of Genes Involved in the Biosynthesis of TAs

Since the genes involved in the TAs biosynthesis of *A. belladonna* have all been identified [6], we performed a homologous gene search in *A. luridus* by using a combination of the BLAST search and HMM methods, with the functional tested sequences in *A. belladonna* as a reference. We manually corrected the faulty automatic annotation and classified all homologous genes into 13 enzyme families. The amino acid sequences of TAs biosynthesis genes identified in *A. luridus* and those in *A. belladonna* are highly conserved, with identity values exceeding 90% (Table 2; Figure 1). The conservation of these genes across species within the same lineage likely reflect their shared evolutionary history and functional importance in TAs biosynthesis.

Table 2. Amino acid sequence identity of TAs biosynthesis genes from *A. belladonna* and *A. luridus*.

Name	Identity (%) *A. luridus* vs. *A. belladonna*
ODC	97.67%
PMT	97.34%
MPO	90.35%
PYKS	97.19%
CYP82M3	97.30%
TRI	90.48%
AT4	97.16%
PPAR	91.84%
UGT1	93.28%
LS	92.65%
CYP80F1	95.47%
HDH	98.63%
H6H	90.41%

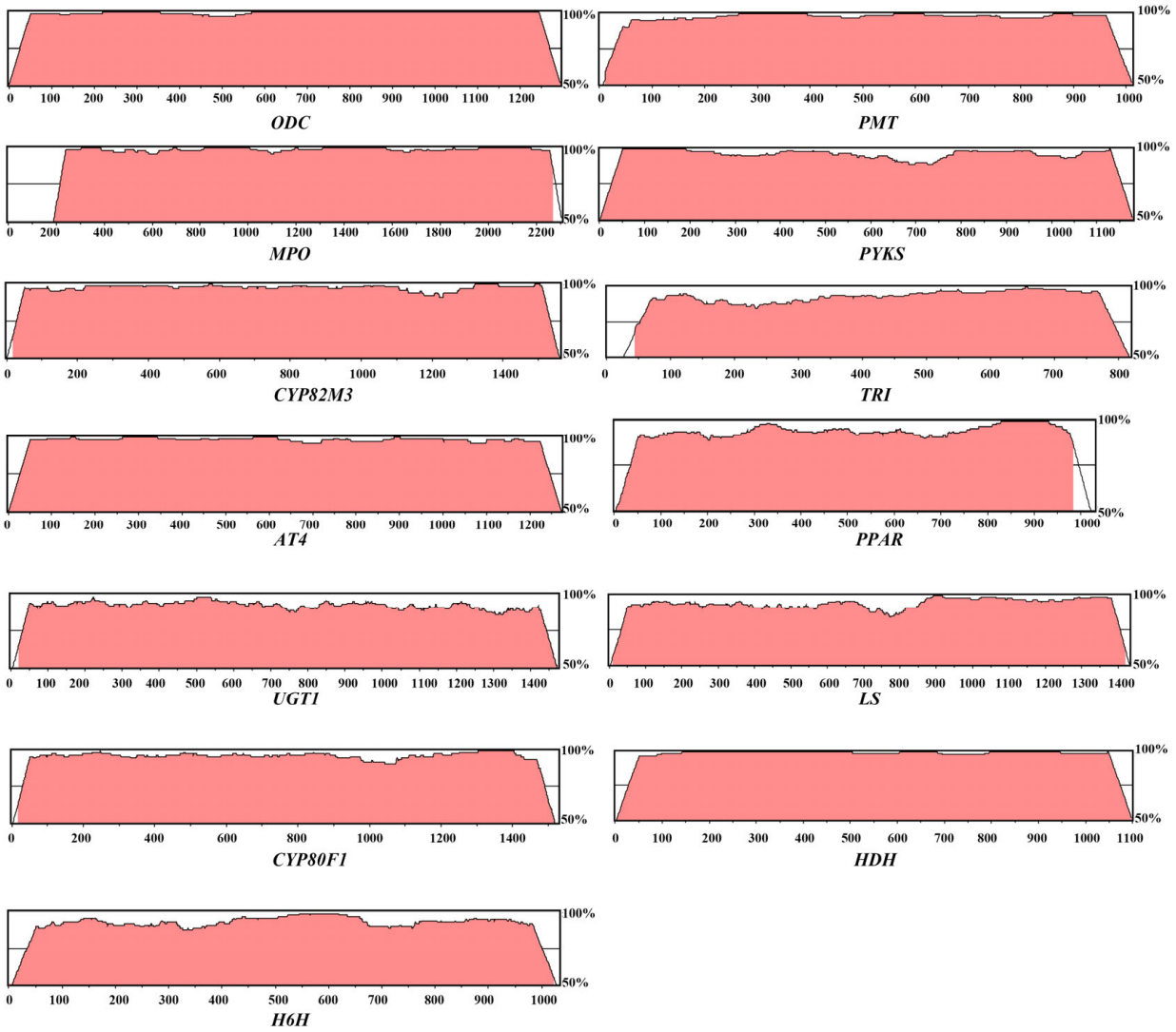

Figure 1. VISTA [38] sequence conservation plot between TAs biosynthesis genes from *A. luridus* using *A. belladonna* as reference.

3.3. Analysis of Physical and Chemical Properties and Subcellular Locations

The 13 candidate TAs biosynthesis genes of *A. luridus* were analyzed, as shown in Table 3. The length of the coding DNA sequence (CDS) ranged from 801 bp (*AlTRI*) to 2118 bp (*AlMPO*), and the predicted proteins ranged from 266 to 705 amino acids (AA) in length. The prediction of subcellular localization showed that AlODC, AlAT4, and AlLS were located in the plasma membrane, and the other ten proteins were located in the cytoplasm.

Table 3. Physical and chemical properties and subcellular locations of candidate TAs biosynthesis genes of *A. luridus*.

Gene Name	Length of CDS	Num. of AA	MW (Da)	pI	Subcellular Location
AlODC	1293	430	46,526.93	5.61	Plasma Membrane
AlPMT	1017	338	37,176.54	5.73	Cytoplasmic
AlMPO	2118	705	79,090.01	6.38	Cytoplasmic
AlPYKS	1179	392	43,261.08	7.15	Cytoplasmic
AlCYP82M3	1560	519	59,463.58	6.69	Cytoplasmic
AlTRI	801	266	28,652.85	6.45	Cytoplasmic
AlAT4	1272	423	47,057.53	5.89	Plasma Membrane
AlPPAR	1029	342	38,254.44	5.92	Cytoplasmic
AlUGT1	1476	491	54,927.14	5.54	Cytoplasmic
AlLS	1431	476	53,931.69	6.07	Plasma Membrane
AlCYP80F1	1521	506	57,674.2	7.19	Cytoplasmic
AlHDH	1098	365	38,951.97	5.26	Cytoplasmic
AlH6H	1035	344	38,883.49	5.02	Cytoplasmic

3.4. Expression Analysis Based on RNA-Seq

The accumulation of secondary metabolites was usually tissue-/organ-specific. As reported, the TAs biosynthesis gene identified at each biosynthesis step of *A. belladonna* and *D. stramonium* was highly expressed in the roots [6]. To examine the tissue expression pattern, transcriptome data of the secondary root (SR), primary root (PR), stem, leaf, flower, and fruit of *A. luridus* were used for the expression analysis. The results (Figure 2) show that all 13 candidate TAs biosynthesis genes from *A. luridus* were highly or specifically expressed in the root, especially in the secondary root. This was consistent with the report that TAs were synthesized in the roots of medicinal plants from Solanaceae [39].

3.5. RT-PCR Validation

To verify the expression profiles characterized by transcriptome data, candidate TAs biosynthesis genes were subjected to a RT-PCR analysis in different tissues. The results (Figure 3) show that all 13 candidate genes (*AlODC*, *AlPMT*, *AlMPO*, *AlPYKS*, *AlCYP82M3*, *AlTRI*, *AlAT4*, *AlPPAR*, *AlUGT1*, *AlLS*, *AlCYP80F1*, *AlHDH*, and *AlH6H*) for TAs biosynthesis were highly expressed in the roots (PR and SR) and lowly expressed in the stems, leaves, flowers, and fruits, and the expression trend was intensively consistent with the transcriptome data, indicating that the tissue expression pattern analysis is reliable.

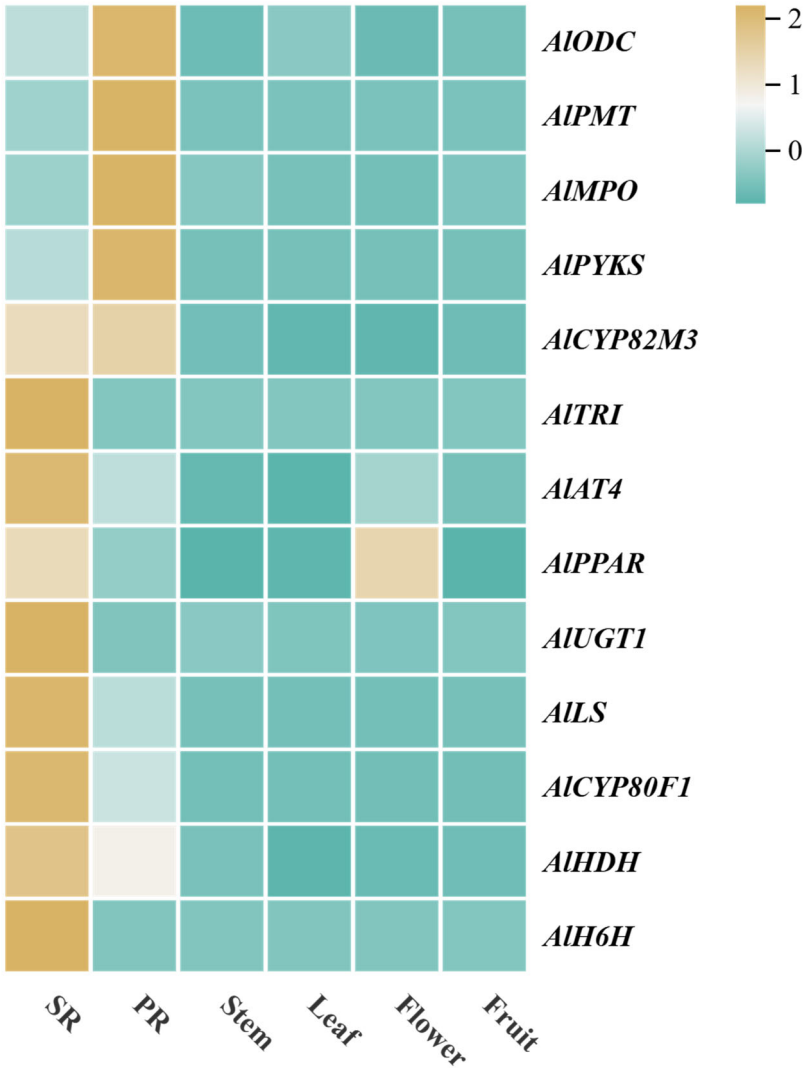

Figure 2. Tissue expression patterns of candidate TAs biosynthesis genes of *A. luridus* based on RNA-Seq. SR: secondary root; PR: primary root.

3.6. De Novo Synthesis of TAs in N. benthamiana

To verify that the 13 candidate genes are responsible for the biosynthesis of TAs, we established a transient expression system in the leaves of *N. benthamiana*, a species that does not naturally produce TAs, utilizing the pEAQ vector system, which enables high-level expression of recombinant proteins [37]. Leaves infiltrated with *Agrobacterium* were used to analyze the content of TAs by LC-MS. In the LC-MS detection system, the retention times (RTs) of the hyoscyamine standard, anisodamine standard, and scopolamine standard were 6.99 min, 6.25 min, and 6.10 min, respectively, and the corresponding m/z values were 290.1757, 306.1706, and 304.1548, respectively.

The results (Figure 4) show that hyoscyamine, anisodamine, and scopolamine could be detected when 13 candidate TAs biosynthesis genes of *A. luridus* were co-expressed in *N. benthamiana* cells, while neither hyoscyamine nor scopolamine was detected in the vector controls. Based on the results of transient co-expression, the biosynthetic pathway of TAs in *A. luridus* was mapped (Figure 5). Therefore, we could draw a conclusion that the TAs biosynthesis genes identified in *A. luridus* were validated to be involved in the biosynthesis of TAs, and the de novo synthesis of TAs was realized in *N. benthamiana* for the first time.

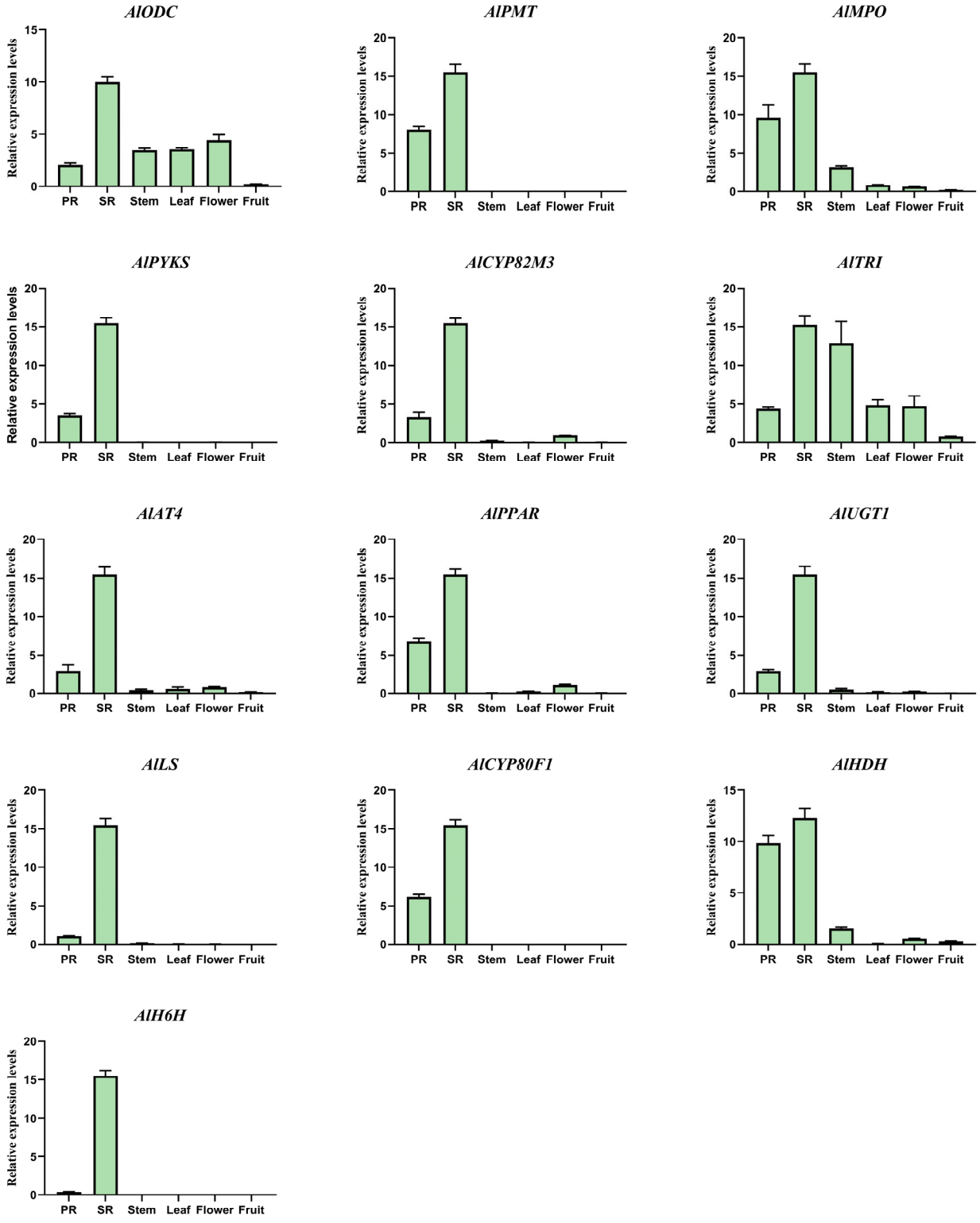

Figure 3. RT-PCR validation of candidate TAs biosynthesis genes of *A. luridus* in different tissues. PR: primary root; SR: secondary root.

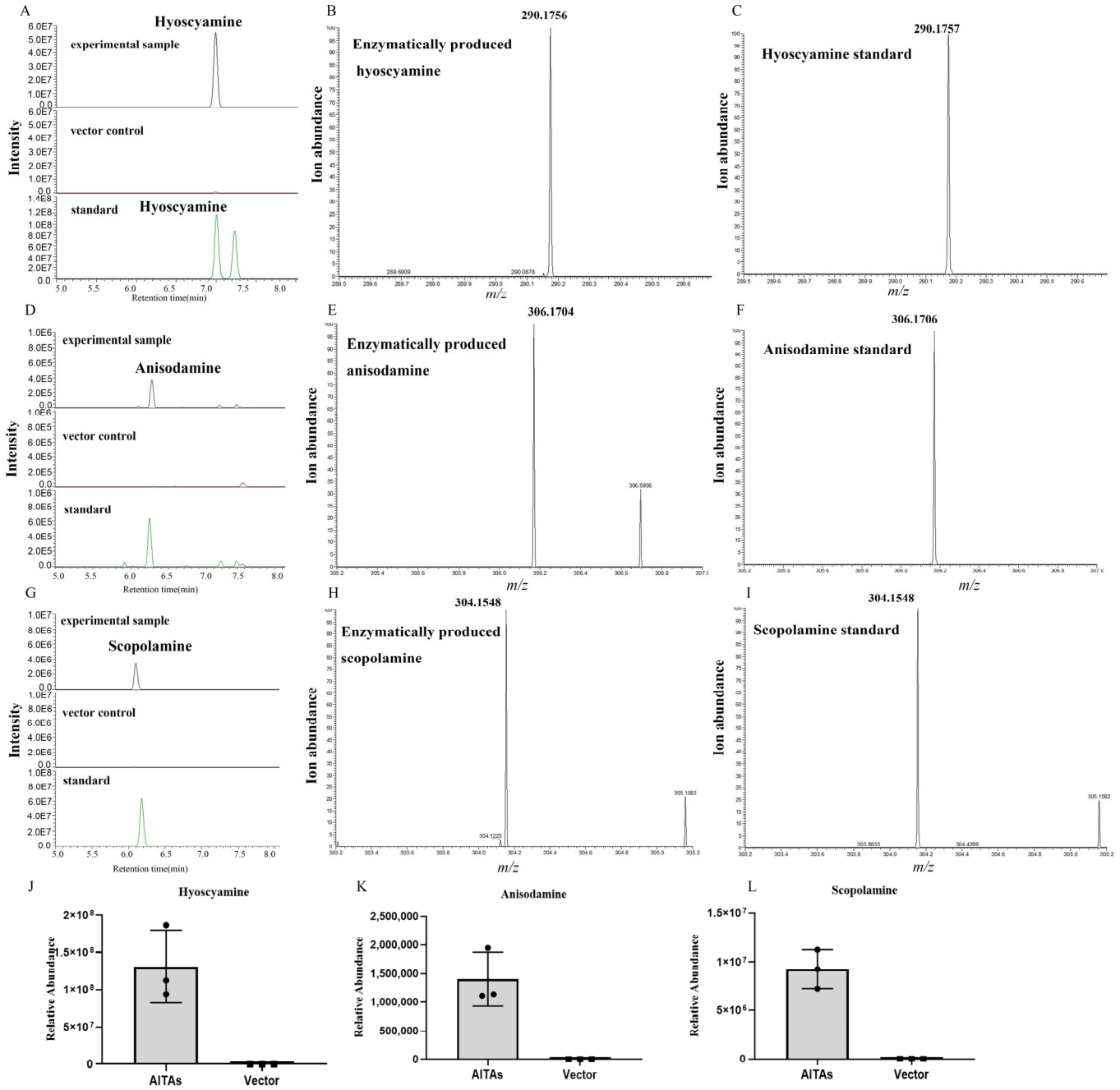

Figure 4. Detection of hyoscyamine, anisodamine, and scopolamine in leaf extract by LC-MS. (**A**,**D**,**G**) Extracted ion chromatograms (EICs) for hyoscyamine, anisodamine, and scopolamine, respectively, produced by enzymes co-expressed in *N. benthamiana* leaves, compared with corresponding authentic standard. Vector control was used as negative control. (**B**,**C**) MS data of hyoscyamine produced by enzymes co-expressed in *N. benthamiana* leaves (**B**) compared with fragmentation of hyoscyamine standard (**C**). (**E**,**F**) MS data of anisodamine produced by enzymes co-expressed in *N. benthamiana* leaves (**E**) compared with fragmentation of anisodamine standard (**F**). (**H**,**I**) MS data of scopolamine produced by enzymes co-expressed in *N. benthamiana* leaves (**H**) compared with fragmentation of scopolamine standard (**I**). (**J**–**L**) Relative abundance of LC-MS ion for hyoscyamine (**J**), anisodamine (**K**), and scopolamine (**L**) produced in *N. benthamiana* within three biologically independent samples.

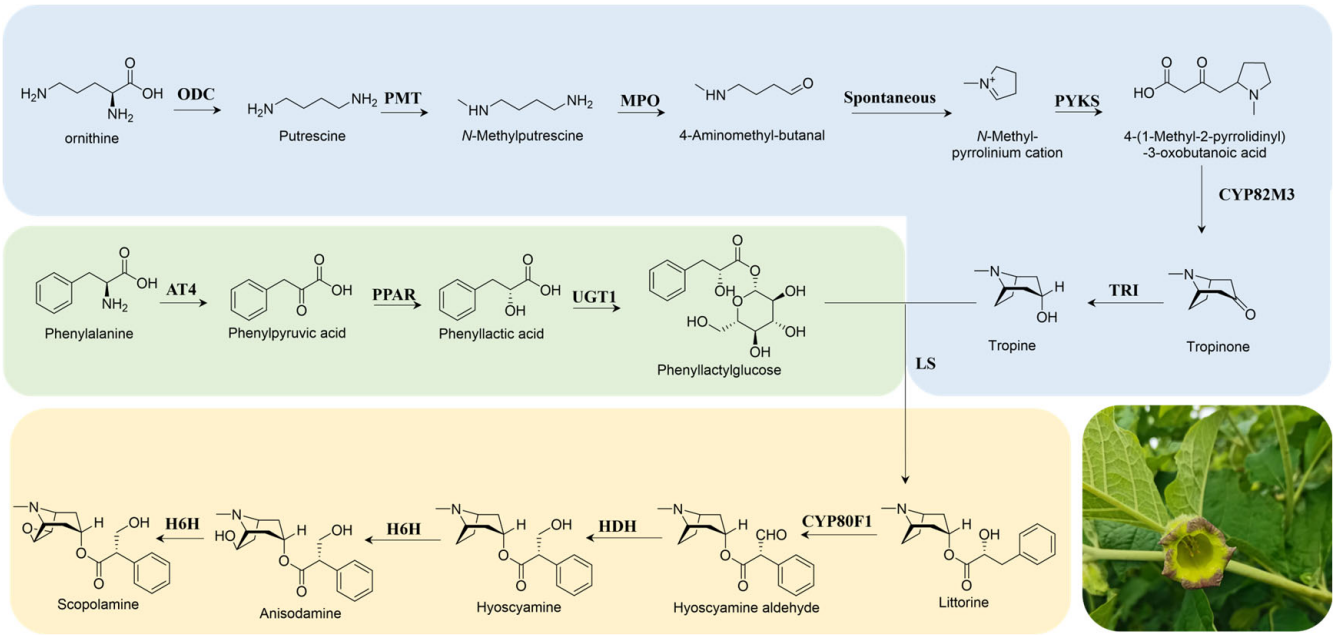

Figure 5. The biosynthetic pathway of TAs in *A. luridus*. ODC, ornithine decarboxylase; PMT, putrescine *N*-methyltransferase; MPO, *N*-methylputrescine oxidase; PYKS, polyketide type III synthase; CYP82M3, tropinone synthase; TRI, tropinone reductase I; AT4, phenylalanine aminotransferase; PPAR, phenylpyruvic acid reductase; UGT1, phenyllactate UDP-glycosyltransferase; LS, littorine synthase; CYP80F1, littorine mutase; HDH, hyoscyamine dehydrogenase; H6H, hyoscyamine 6*β*-hydroxylase.

4. Discussion

Pharmaceutical TAs, including scopolamine and hyoscyamine, are derived primarily from Solanaceae species and have been found in more than ten species, such as *A. belladonna*, *D. stramonium*, *B. arborea*, *Hyoscyamus niger*, *A. luridus*, *Hyoscyamus muticus*, *A. acutangulus*, etc. [4]. Since the scopolamine biosynthetic pathway was fully analyzed in 2021 [5], breakthroughs have been obtained on related research in recent years. The de novo production of hyoscyamine and scopolamine was achieved in yeast [17]. In 2023 and 2024, the whole genomes of six TAs-producing Solanaceae species, including *A. belladonna*, *D. stramonium*, *A. tanguticus*, *B. arborea*, *M. caulescens*, and *A. acutangulus*, were sequenced, assembled, and annotated. Additionally, the evolution of tropane alkaloid biosynthesis was thoroughly studied [6,7,19,20]. The biosynthetic pathway of TAs has existed since the origin of Solanaceae, and in subsequent diversification and evolution, the pseudogenization or multiple loss of TAs biosynthesis genes resulted in the inability to synthesize TAs [6,7]. Transcription factor AaWRKR11 from *A. acutangulus* was successively reported, which can transcriptionally activate the expression of *H6H* and therefore regulate TAs biosynthesis [20]. Recently, the identification of a mitochondrion-localized BAHD acyltransferase from *A. belladonna*, 3*β*-tigloyloxytropane synthase (TS), which catalyzes 3*β*-tropanol and tigloyl-CoA to form 3*β*-tigloyloxytropane, enriched the knowledge of TAs biosynthesis [40].

With the identification of *AbTRI* and *AbMPO*, all genes involved in scopolamine biosynthesis have been identified in *A. belladonna* [6]. In this study, we used the functional identified TAs biosynthesis gene from *A. belladonna* as a reference and conducted homologous gene research in the transcriptome data of different tissues of *A. luridus*. The biosynthesis genes of TAs identified in *A. luridus* were highly conserved in terms of sequence similarity and gene expression patterns compared to those of *A. belladonna*. The results of our study indicate that the two species may share the same biosynthetic pathway

for TAs biosynthesis. It is further suggested that the TAs biosynthetic pathway is conserved across TAs-producing plant species.

The metabolic engineering of TAs has been extensively reported and reviewed in detail by Srivastava et al. [2]. The overexpression of *NtPMT* in hairy root cultures of *Datura metel* and *Scopolia parviflora* could increase the contents of hyoscyamine and scopolamine in transgenic hair roots [41,42]. When *DsTRI* was overexpressed in hairy root cultures of *A. belladonna*, both hyoscyamine and scopolamine production were improved [43]. Yun et al. overexpressed the *HnH6H* in *A. belladonna* plants, which greatly promoted the transformation of hyoscyamine into scopolamine, resulting in almost all scopolamine in the leaves of the transgenic plants, with the highest content of scopolamine exceeding 1% dry weight [44]. The scopolamine and anisodamine contents were remarkably elevated in the root cultures of *S. lurida* overexpressing *SlH6H/HnH6H* [1]. Previously, *A. luridus* was reported with a high yield of TAs, especially hyoscyamine [1]. The functional identification of the TAs biosynthesis genes of *A. luridus* facilitates the screening of enzymes with high catalytic activity. However, there are still a lot of interesting studies worth conducting, such as studies to determine the in vitro enzyme activity of identified genes, the identification of rate-limiting steps, etc.

Transient protein expression in a heterologous system is very useful in many areas of research. Tobacco, as a plant expression system, has the advantages of large leaves, easy injection, easy sampling, etc., and is widely used in the study of protein interaction, transcription regulation, subcellular localization, etc. Wang et al. achieved the de novo synthesis of cocaine by transiently expressing *EnCYP81AN15* and *EnMT4*, as well as four identified functional enzymes (EnPKS1, EnATR, EnMecgoR, and EnCS15), in *N. benthamiana* [28]. Similarly, the simultaneous transient expression of *AbPMT*, *AbMPO*, *AbPYKS*, and *AbCYP82M3* in *N. benthamiana* can detect the production of tropinone [11]. In this study, we employed a similar approach to verify the functions of identified genes. This marks the systematic and comprehensive elucidation of the biosynthetic pathway of TAs within *A. luridus*.

The heterologous synthesis of tropane alkaloids and their precursors in other organisms has been reported. The de novo synthesis of tropine, hyoscyamine, and scopolamine in yeast and the de novo synthesis of cocaine in *N. benthamiana* have both been achieved [17,28,45]. Littorine can be synthesized by co-expressing *AbUGT1* and *AbLS* in *N. benthamiana* leaves when tropine and phenyllactate are added [15]. In this study, a total of 13 TAs biosynthesis genes identified in *A. luridus* were co-expressed in *N. benthamiana*, and scopolamine was detected in leaf extract, indicating that these 13 genes were involved in the biosynthesis of TAs in *A. luridus*. The results of our study not only systematically elucidate the tropane alkaloid biosynthetic pathway of *A. luridus*, but also show the realization of the de novo synthesis of TAs in *N. benthamiana* for the first time. This study provides new ideas for the identification of TAs biosynthetic pathways in other TAs-producing plants and provides more functional genes for the efficient production of TAs by metabolic engineering or synthetic biotechnology.

5. Conclusions

Based on homologous gene retrieval, genes identified in *A. luridus* were highly conserved in terms of sequence similarity and gene expression patterns compared to those from *A. belladonna*, and they were confirmed to be involved in TAs biosynthesis by co-expression in *N. benthamiana*. The functional identification of the TAs biosynthesis genes of *A. luridus* facilitates the screening of enzymes with high catalytic activity.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/agronomy14112460/s1, Table S1: Primers used in this study. Table S2: The CDS of TAs biosynthesis genes identified in *A. luridus*.

Author Contributions: Formal Analysis, Investigation, and Validation, M.W.; Conceptualization, Funding Acquisition, Project Administration, Resources, and Supervision, Z.L.; Writing—Original

Draft, M.W. and Z.L.; Writing—Review and Editing, J.Z., F.Q. and F.Z. All authors have read and agreed to the published version of the manuscript.

Funding: This work was financially supported by the NSFC project (Grant No. 32101224), the National Key Research and Development Program of China (Grant No. 2022YFD1201600), the Fourth National Survey of Traditional Chinese Medicine Resources, Chinese or Tibet Medicinal Resources Investigation in Tibet Autonomous Region (State Administration of Chinese Traditional Medicine 20191217-540124 and 20200501-542329), the Doctoral Research Innovation Project of Chongqing (CYB22115), and the Experimental Technology Research Project of Southwest University (SYJ2024028).

Data Availability Statement: The original contributions presented in this study are included in the article/Supplementary Materials; further inquiries can be directed to the corresponding author(s). Raw data of RNA-seq is available at National Genomics Data Center (https://ngdc.cncb.ac.cn/?lang=zh, BioProject: PRJCA031299).

Acknowledgments: We thank Xiaozhong Lan, Min Chen, and Chunxian Yang for critically reviewing the study proposal. We thank Lingjiang Zeng for helping with the LC-MS analysis and Wentang Chen for helping with data collection.

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Lan, X.; Zeng, J.; Liu, K.; Zhang, F.; Bai, G.; Chen, M.; Liao, Z.; Huang, L. Comparison of two hyoscyamine 6β-hydroxylases in engineering scopolamine biosynthesis in root cultures of *Scopolia lurida*. *Biochem. Biophys. Res. Commun.* **2018**, *497*, 25–31. [CrossRef] [PubMed]
2. Srivastava, V.; Mehrotra, S.; Mishra, D.S. *Tropane Alkaloids Pathways, Potential and Biotechnological Applications: Pathways, Potential and Biotechnological Applications*; Springer: Berlin/Heidelberg, Germany, 2021. [CrossRef]
3. Ullrich, S.F.; Hagels, H.; Kayser, O. Scopolamine: A journey from the field to clinics. *Phytochem. Rev.* **2017**, *16*, 333–353. [CrossRef]
4. Kohnen-Johannsen, K.L.; Kayser, O. Tropane Alkaloids: Chemistry, Pharmacology, Biosynthesis and Production. *Molecules* **2019**, *24*, 796. [CrossRef]
5. Huang, J.P.; Wang, Y.J.; Tian, T.; Wang, L.; Yan, Y.; Huang, S.X. Tropane alkaloid biosynthesis: A centennial review. *Nat. Prod. Rep.* **2021**, *38*, 1634–1658. [CrossRef]
6. Zhang, F.; Qiu, F.; Zeng, J.; Xu, Z.; Tang, Y.; Zhao, T.; Gou, Y.; Su, F.; Wang, S.; Sun, X.; et al. Revealing evolution of tropane alkaloid biosynthesis by analyzing two genomes in the Solanaceae family. *Nat. Commun.* **2023**, *14*, 1446. [CrossRef] [PubMed]
7. Yang, J.; Wu, Y.; Zhang, P.; Ma, J.; Yao, Y.J.; Ma, Y.L.; Zhang, L.; Yang, Y.; Zhao, C.; Wu, J.; et al. Multiple independent losses of the biosynthetic pathway for two tropane alkaloids in the Solanaceae family. *Nat. Commun.* **2023**, *14*, 8457. [CrossRef]
8. Michael, A.J.; Furze, J.M.; Rhodes, M.J.; Burtin, D. Molecular cloning and functional identification of a plant ornithine decarboxylase cDNA. *Biochem. J.* **1996**, *314 Pt 1*, 241–248. [CrossRef] [PubMed]
9. Suzuki, K.; Yamada, Y.; Hashimoto, T. Expression of *Atropa belladonna* putrescine *N*-methyltransferase gene in root pericycle. *Plant Cell Physiol.* **1999**, *40*, 289–297. [CrossRef]
10. Huang, J.P.; Fang, C.; Ma, X.; Wang, L.; Yang, J.; Luo, J.; Yan, Y.; Zhang, Y.; Huang, S.X. Tropane alkaloids biosynthesis involves an unusual type III polyketide synthase and non-enzymatic condensation. *Nat. Commun.* **2019**, *10*, 4036. [CrossRef]
11. Bedewitz, M.A.; Jones, A.D.; D'Auria, J.C.; Barry, C.S. Tropinone synthesis via an atypical polyketide synthase and P450-mediated cyclization. *Nat. Commun.* **2018**, *9*, 5281. [CrossRef]
12. Nakajima, K.; Hashimoto, T.; Yamada, Y. Two tropinone reductases with different stereospecificities are short-chain dehydrogenases evolved from a common ancestor. *Proc. Natl. Acad. Sci. USA* **1993**, *90*, 9591–9595. [CrossRef] [PubMed]
13. Bedewitz, M.A.; Góngora-Castillo, E.; Uebler, J.B.; Gonzales-Vigil, E.; Wiegert-Rininger, K.E.; Childs, K.L.; Hamilton, J.P.; Vaillancourt, B.; Yeo, Y.S.; Chappell, J.; et al. A root-expressed L-phenylalanine:4-hydroxyphenylpyruvate aminotransferase is required for tropane alkaloid biosynthesis in *Atropa belladonna*. *Plant Cell* **2014**, *26*, 3745–3762. [CrossRef] [PubMed]
14. Qiu, F.; Yang, C.; Yuan, L.; Xiang, D.; Lan, X.; Chen, M.; Liao, Z. A Phenylpyruvic Acid Reductase Is Required for Biosynthesis of Tropane Alkaloids. *Org. Lett.* **2018**, *20*, 7807–7810. [CrossRef]
15. Qiu, F.; Zeng, J.; Wang, J.; Huang, J.P.; Zhou, W.; Yang, C.; Lan, X.; Chen, M.; Huang, S.X.; Kai, G.; et al. Functional genomics analysis reveals two novel genes required for littorine biosynthesis. *New Phytol.* **2020**, *225*, 1906–1914. [CrossRef]
16. Li, R.; Reed, D.W.; Liu, E.; Nowak, J.; Pelcher, L.E.; Page, J.E.; Covello, P.S. Functional genomic analysis of alkaloid biosynthesis in *Hyoscyamus niger* reveals a cytochrome P450 involved in littorine rearrangement. *Chem. Biol.* **2006**, *13*, 513–520. [CrossRef] [PubMed]
17. Srinivasan, P.; Smolke, C.D. Biosynthesis of medicinal tropane alkaloids in yeast. *Nature* **2020**, *585*, 614–619. [CrossRef]
18. Hashimoto, T.; Matsuda, J.; Yamada, Y. Two-step epoxidation of hyoscyamine to scopolamine is catalyzed by bifunctional hyoscyamine 6 beta-hydroxylase. *FEBS Lett.* **1993**, *329*, 35–39. [CrossRef]
19. Wang, Y.J.; Tain, T.; Yu, J.Y.; Li, J.; Xu, B.; Chen, J.; D'Auria, J.C.; Huang, J.P.; Huang, S.X. Genomic and structural basis for evolution of tropane alkaloid biosynthesis. *Proc. Natl. Acad. Sci. USA* **2023**, *120*, e2302448120. [CrossRef] [PubMed]

20. Zhou, W.; Wang, C.; Hao, X.; Chen, F.; Huang, Q.; Liu, T.; Xu, J.; Guo, S.; Liao, B.; Liu, Z.; et al. A chromosome-level genome assembly of anesthetic drug-producing *Anisodus acutangulus* provides insights into its evolution and the biosynthesis of tropane alkaloids. *Plant Commun.* **2024**, *5*, 100680. [CrossRef]
21. Golubova, D.; Tansley, C.; Su, H.; Patron, N.J. Engineering *Nicotiana benthamiana* as a platform for natural product biosynthesis. *Curr. Opin. Plant Biol.* **2024**, *81*, 102611. [CrossRef]
22. Zhang, Y.; Wiese, L.; Fang, H.; Alseekh, S.; Perez de Souza, L.; Scossa, F.; Molloy, J.; Christmann, M.; Fernie, A.R. Synthetic biology identifies the minimal gene set required for paclitaxel biosynthesis in a plant chassis. *Mol. Plant* **2023**, *16*, 1951–1961. [CrossRef] [PubMed]
23. Reed, J.; Orme, A.; El-Demerdash, A.; Owen, C.; Martin, L.B.B.; Misra, R.C.; Kikuchi, S.; Rejzek, M.; Martin, A.C.; Harkess, A.; et al. Elucidation of the pathway for biosynthesis of saponin adjuvants from the soapbark tree. *Science* **2023**, *379*, 1252–1264. [CrossRef]
24. Martin, L.B.B.; Kikuchi, S.; Rejzek, M.; Owen, C.; Reed, J.; Orme, A.; Misra, R.C.; El-Demerdash, A.; Hill, L.; Hodgson, H.; et al. Complete biosynthesis of the potent vaccine adjuvant QS-21. *Nat. Chem. Biol.* **2024**, *20*, 493–502. [CrossRef]
25. Berman, P.; de Haro, L.A.; Jozwiak, A.; Panda, S.; Pinkas, Z.; Dong, Y.; Cveticanin, J.; Barbole, R.; Livne, R.; Scherf, T.; et al. Parallel evolution of cannabinoid biosynthesis. *Nat. Plants* **2023**, *9*, 817–831. [CrossRef] [PubMed]
26. Pan, Z.; Bajsa-Hirschel, J.; Vaughn, J.N.; Rimando, A.M.; Baerson, S.R.; Duke, S.O. In vivo assembly of the sorgoleone biosynthetic pathway and its impact on agroinfiltrated leaves of *Nicotiana benthamiana*. *New Phytol.* **2021**, *230*, 683–697. [CrossRef] [PubMed]
27. Kruse, L.H.; Sunstrum, F.G.; Garcia, D.; López Pérez, G.; Jancsik, S.; Bohlmann, J.; Irmisch, S. Improved production of the antidiabetic metabolite montbretin A in *Nicotiana benthamiana*: Discovery, characterization, and use of Crocosmia shikimate shunt genes. *Plant J.* **2024**, *117*, 766–785. [CrossRef]
28. Wang, Y.J.; Huang, J.P.; Tian, T.; Yan, Y.; Chen, Y.; Yang, J.; Chen, J.; Gu, Y.C.; Huang, S.X. Discovery and Engineering of the Cocaine Biosynthetic Pathway. *J. Am. Chem. Soc.* **2022**, *144*, 22000–22007. [CrossRef]
29. Hong, B.; Grzech, D.; Caputi, L.; Sonawane, P.; López, C.E.R.; Kamileen, M.O.; Hernández Lozada, N.J.; Grabe, V.; O'Connor, S.E. Biosynthesis of strychnine. *Nature* **2022**, *607*, 617–622. [CrossRef]
30. Florean, M.; Luck, K.; Hong, B.; Nakamura, Y.; O'Connor, S.E.; Köllner, T.G. Reinventing metabolic pathways: Independent evolution of benzoxazinoids in flowering plants. *Proc. Natl. Acad. Sci. USA* **2023**, *120*, e2307981120. [CrossRef]
31. Nett, R.S.; Lau, W.; Sattely, E.S. Discovery and engineering of colchicine alkaloid biosynthesis. *Nature* **2020**, *584*, 148–153. [CrossRef]
32. Yin, X.; Liu, J.; Kou, C.; Lu, J.; Zhang, H.; Song, W.; Li, Y.; Xue, Z.; Hua, X. Deciphering the network of cholesterol biosynthesis in Paris polyphylla laid a base for efficient diosgenin production in plant chassis. *Metab. Eng.* **2023**, *76*, 232–246. [CrossRef] [PubMed]
33. Lv, Y.; Zhu, J.; Huang, S.; Xing, X.; Zhou, S.; Yao, H.; Yang, Z.; Liu, L.; Huang, S.; Miao, Y.; et al. Metabolome profiling and transcriptome analysis filling the early crucial missing steps of piperine biosynthesis in *Piper nigrum* L. *Plant J.* **2024**, *117*, 107–120. [CrossRef] [PubMed]
34. Chen, C.; Chen, H.; Zhang, Y.; Thomas, H.R.; Frank, M.H.; He, Y.; Xia, R. TBtools: An Integrative Toolkit Developed for Interactive Analyses of Big Biological Data. *Mol. Plant* **2020**, *13*, 1194–1202. [CrossRef] [PubMed]
35. Leete, E.; Marion, L.; Spenser, I.D. Biogenesis of hyoscyamine. *Nature* **1954**, *174*, 650–651. [CrossRef] [PubMed]
36. Horton, P.; Park, K.J.; Obayashi, T.; Fujita, N.; Harada, H.; Adams-Collier, C.J.; Nakai, K. WoLF PSORT: Protein localization predictor. *Nucleic Acids Res.* **2007**, *35*, W585–W587. [CrossRef]
37. Sainsbury, F.; Thuenemann, E.C.; Lomonossoff, G.P. pEAQ: Versatile expression vectors for easy and quick transient expression of heterologous proteins in plants. *Plant Biotechnol. J.* **2009**, *7*, 682–693. [CrossRef]
38. Frazer, K.A.; Pachter, L.; Poliakov, A.; Rubin, E.M.; Dubchak, I. VISTA: Computational tools for comparative genomics. *Nucleic Acids Res.* **2004**, *32*, W273–W279. [CrossRef]
39. Facchini, P.J. Alkaloid Biosynthesis in Plants: Biochemistry, Cell Biology, Molecular Regulation, and Metabolic Engineering Applications. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **2001**, *52*, 29–66. [CrossRef]
40. Zeng, J.; Liu, X.; Dong, Z.; Zhang, F.; Qiu, F.; Zhong, M.; Zhao, T.; Yang, C.; Zeng, L.; Lan, X.; et al. Discovering a mitochondrion-localized BAHD acyltransferase involved in calystegine biosynthesis and engineering the production of 3β-tigloyloxytropane. *Nat. Commun.* **2024**, *15*, 3623. [CrossRef]
41. Moyano, E.; Jouhikainen, K.; Tammela, P.; Palazón, J.; Cusidó, R.M.; Piñol, M.T.; Teeri, T.H.; Oksman-Caldentey, K.M. Effect of pmt gene overexpression on tropane alkaloid production in transformed root cultures of *Datura metel* and *Hyoscyamus muticus*. *J. Exp. Bot.* **2003**, *54*, 203–211. [CrossRef]
42. Lee, O.-S.; Kang, Y.-M.; Jung, H.-Y.; Min, J.-Y.; Kang, S.-M.; Karigar, C.S.; Prasad, D.T.; Bahk, J.-D.; Choi, M.-S. Enhanced production of tropane alkaloids in *Scopolia parviflora* by introducing the *PMT* (putrescine N-methyltransferase) gene. *In Vitro Cell. Dev.-Plant* **2005**, *41*, 167–172. [CrossRef]
43. Richter, U.; Rothe, G.; Fabian, A.K.; Rahfeld, B.; Dräger, B. Overexpression of tropinone reductases alters alkaloid composition in *Atropa belladonna* root cultures. *J. Exp. Bot.* **2005**, *56*, 645–652. [CrossRef] [PubMed]

44. Yun, D.J.; Hashimoto, T.; Yamada, Y. Metabolic engineering of medicinal plants: Transgenic *Atropa belladonna* with an improved alkaloid composition. *Proc. Natl. Acad. Sci. USA* **1992**, *89*, 11799–11803. [CrossRef] [PubMed]
45. Srinivasan, P.; Smolke, C.D. Engineering a microbial biosynthesis platform for de novo production of tropane alkaloids. *Nat. Commun.* **2019**, *10*, 3634. [CrossRef] [PubMed]