Research

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Rapid report

Functional genomics analysis reveals two novel genes required for littorine biosynthesis

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Summary

- Some medicinal plants of the Solanaceae produce pharmaceutical tropane alkaloids (TAs), such as hyoscyamine and scopolamine. Littorine is a key biosynthetic intermediate in the hyoscyamine and scopolamine biosynthetic pathways. However, the mechanism underlying littorine formation from the precursors phenyllactate and tropine is not completely understood.
- Here, we report the elucidation of littorine biosynthesis through a functional genomics approach and functional identification of two novel biosynthesis genes that encode phenyllactate UDP-glycosyltransferase (UGT1) and littorine synthase (LS).
- *UGT1* and *LS* are highly and specifically expressed in *Atropa belladonna* secondary roots. Suppression of either *UGT1* or *LS* disrupted the biosynthesis of littorine and its TA derivatives (hyoscyamine and scopolamine). Purified His-tagged UGT1 catalysed phenyllactate glycosylation to form phenyllactylglucose. *UGT1* and *LS* co-expression in tobacco leaves led to littorine synthesis if tropine and phenyllactate were added.
- This identification of UGT1 and LS provides the missing link in littorine biosynthesis. The results pave the way for producing hyoscyamine and scopolamine for medical use by metabolic engineering or synthetic biology.

Introduction

Solanaceous medicinal plants, including *Atropa belladonna*, *Datura stramonium*, and *Hyoscyamus niger*, have been used to treat various disorders since ancient times (Kohnen-Johannsen & Kayser, 2019), due to the anticholinergic activity of tropane alkaloids (TAs) – hyoscyamine and scopolamine – in these plants (Humphrey & O'Hagan, 2001). Accordingly, there is a longstanding interest in elucidating the TA biosynthetic pathway. Most enzyme-catalysed reactions associated with TA biosynthesis have been characterized, including those related to putrescine *N*-methyltransferase (PMT), *N*-methylputrescine oxidase (MPO), type III polyketide synthase (PYKS), tropinone synthase (CYP82M3), tropine-forming reductase (TRI), aromatic amino acid aminotransferase (AT4), phenylpyruvic acid reductase (PPAR), littorine mutase (CYP80F1), and hyoscyamine 6β-hydroxylase (H6H) (Li *et al.*, 2006, 2012; Katoh *et al.*, 2007; Bedewitz *et al.*, 2014, 2018; Qiang *et al.*, 2016; Geng *et al.*, 2018; Qiu *et al.*, 2018) (Fig. 1). Nevertheless, the enzymatic reactions responsible for littorine and hyoscyamine formation remain to be unveiled.

Littorine is a TA that is a key intermediate in hyoscyamine and scopolamine biosynthesis (Hashimoto *et al.*, 1993; Robins *et al.*,

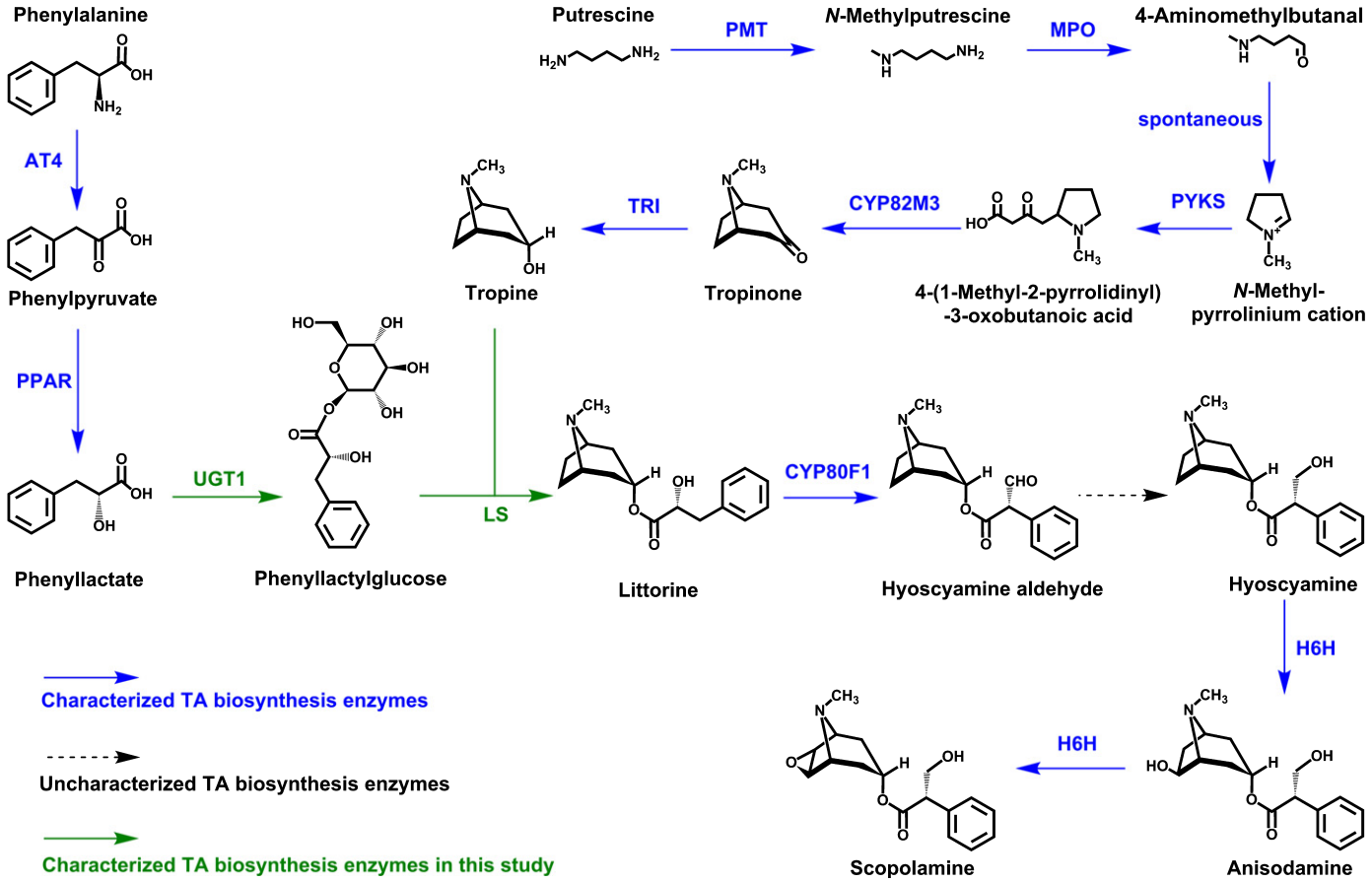

Fig. 1 Biosynthetic pathway of tropane alkaloids (TAs) in solanaceous plants. PMT, putrescine *N*-methyltransferase; MPO, *N*-methylputrescine oxidase; PYKS, type III polyketide synthase; CYP82M3, tropinone synthase; TRI, tropinone reductase I; AT4, aromatic amino acid aminotransferase 4; PPAR, phenylpyruvic acid reductase; UGT1, phenyllactate UDP-glycosyltransferase; LS, littorine synthase; CYP80F1, littorine mutase; H6H, hyscyamine 6β-hydroxylase.

1994a). It is produced by esterification between tropine and phenyllactate (Robins *et al.*, 1994b). Esterification between tropine (the acyl acceptor) and phenyllactate (the acyl donor) is unlikely because the compounds are very stable. In littorine biosynthesis, tropine is the acyl acceptor, and phenyllactate in energy-rich or activated form is the acyl donor. Generally, acyl donors in organisms are energy-rich acyl-CoA thioesters or 1-O-β-glucose esters (Bontpart *et al.*, 2015). Acyl-CoA thioesters can be produced by CoA-ligase using specific substrates and CoA. For example, benzoyl-CoA is used as acyl donor in cocaine biosynthesis mediated by a BAHD acyltransferase (Schmidt *et al.*, 2015). Moreover, 1-O-β-glucose esters can be produced by UDP-glycosyltransferase (UGT) using specific substrates and UDP-glucose (Schulenburg *et al.*, 2016; Zhao *et al.*, 2016). Previous research identified *A. belladonna* enzymes responsible for producing acetylpseudotropine, tigloylpseudotropine, and phenylacetylpseudotropine, but not for acetyltropine or phenylacetyltropine (Robins *et al.*, 1994c), suggesting that BAHD acyltransferases, taking CoA activated compounds, participate in acyl modification of pseudotropine but not tropine. Thus, glycosylated phenyllactate (phenyllactylglucose) might be the acyl donor for tropine, and tropine and phenyllactylglucose might condense to littorine when catalysed by serine carboxypeptidase (SCP)-like acyltransferases (SCPL-ATs), which catalyse esterification between specific compounds and 1-O-β-glucose esters (Li & Steffens, 2000).

Materials and Methods

Bioinformatics analysis

Hidden Markov Model (HMM) protein domain profiles for UGTs (PF00201) and SCP and SCPL-ATs (PF00450) were used to screen for candidates using the HMM search program against the *Atropa belladonna* transcriptomes released by Michigan State University (http://medicinalplantgenomics.msu.edu/index.shtml). The FPKM values of UGT/SCP unigenes and all the previously characterized TA biosynthesis genes were used to generate digital tissue profiles, based on hierarchical cluster analysis (Eisen *et al.*, 1998) in MULTIPLE EXPERIMENT VIEWER (MeV) software (Saeed *et al.*, 2003). The neighbour-joining phylogenetic trees were generated using CLUSTALX algorithm in MEGA7 (Kumar *et al.*, 2016), in which all *Arabidopsis* UGTs and the characterized plant SCPL-ATs were used as references.

UGT1 and *LS* cloning

cDNAs were synthesized from mRNAs isolated from secondary roots of *A. belladonna*, using PrimeScript Kit (TaKaRa, Beijing, China). The cDNA sequences were isolated using the fUGT1 and

rUGT1 primer pair for *UGT1* and the fLS and rLS primer pair for *LS* (Supporting Information Table S1). PCR was performed using HyPerFUsion™ DNA Polymerase (APExBIO Technology, Houston, TX, USA). The PCR products were subcloned into a pJET1.2 vector (ThermoFisher Scientific, Waltham, MA, USA) and sequenced.

Gene expression analysis

UGT1 and *LS* tissue profiles (with at least three biological replicates) were analysed by qPCR, with *PGK* as the internal reference gene (Li *et al.*, 2014). Secondary roots, primary roots, stems, and leaves of 4-month-old *A. belladonna* plants were harvested and used for RNA isolation and cDNA synthesis using kits from Tiangen Biotech (Beijing, China). SYBR qPCR Mix was purchased from Novoprotein (Shanghai, China). qPCR was performed using an IQ5 System (Bio-Rad, Hercules, CA, USA), which was also used to assess *UGT1* and *LS* expression in gene-silenced seedlings and RNAi root cultures. All the primers used in this study are listed in Table S1. The efficiencies of the primers used for qPCR analysis were assessed using LinRegPCR (Ramakers *et al.*, 2003) (Table S1), and their specifies were evaluated using melting temperature (Fig. S1).

Virus-induced gene silencing (VIGS)

A 495-bp *UGT1* fragment and a 582-bp *LS* fragment was independently inserted into the tobacco rattle virus (TRV) vector (pTRV2), using the restriction enzymes, *Xho*I and *Kpn*I, to produce pTRV2-UGT1 and pTRV2-LS vectors. These vectors were separately introduced into *Agrobacterium tumefaciens* GV3101. *UGT1*/*LS* silencing was performed in young *A. belladonna* seedlings as previously described (Bedewitz *et al.*, 2018). After 28 d, underground parts were collected for the earlier-mentioned gene expression analysis, and aboveground parts were used for hyoscyamine detection as previously described (Lan *et al.*, 2018; Qiu *et al.*, 2018).

RNAi gene suppression in root cultures

A 495-bp *UGT1*-RNAi fragment and a 486-bp *LS*-RNAi fragment was inserted into pBin19 to produce pBin19-UGT1 and pBin19-LS RNAi vectors, respectively. The methods to construct pBin19-UGT1 and pBin19-LS RNAi vectors were in detail described in the Supporting Information (Figs S2, S3). These vectors were separately introduced into *Agrobacterium tumefaciens* C58C1 harbouring pRiA4 (Moyano *et al.*, 2002; Zhang *et al.*, 2004). *UGT1*-RNAi, *LS*-RNAi and control root cultures were established and cultured as previously described (Yang *et al.*, 2011). Genes of interest, *rolB*, *rolC*, *NPTII*, 35S::UGT1, and 35S::LS, were detected using PCR as previously described (Qiu *et al.*, 2018). *UGT1* and *LS* expression was analysed using qPCR as described earlier. For gene expression and metabolite analysis, root lines cultured in liquid Murashige and Skoog (MS) medium without antibiotics for 30 d were used (Qiu *et al.*, 2018).

Metabolite quantification in *A. belladonna* root cultures

Quantification of phenyllactate, littorine, hyoscyamine, and scopolamine was performed as previously described (Bedewitz *et al.*, 2014, 2018) with several modifications. Control, *UGT1*-RNAi and *LS*-RNAi root cultures were lyophilized and ground, with 25 mg, then used for the extraction (Qiu *et al.*, 2018).

Littorine, hyoscyamine, and scopolamine (injection volume, 2 μl) were quantified using a Waters UPLC-Xevo TQD mass spectrometer with an Acquity UPLC BEH C18 column (2.1 mm × 50 mm, 1.7 μm). The samples were separated using binary gradient elution with 0.1% formic acid in water (A) and acetonitrile (B). The flow rate was 0.4 ml min^{-1} and oven temperature was 40°C. The elution procedures are shown in Table S2. Measurements were performed using electron spray ionization (ESI) in positive ion mode and optimized multiple reaction monitoring (MRM) transitions (Table S3). Instrument parameters: source voltage, 3.0 kV; source temperature, 500°C; gas flow, 1000 l h^{-1}; cone, 50 l h^{-1}.

Phenyllactate (injection volume, 8 μl) was quantified using the earlier ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) system (and the same mobile phases and instrument parameters) but with a different column (Symmetry C18 Column, 2.1 mm × 100 mm, 3.5 μm). The elution procedures are shown in Table S4. Measurements were performed using ESI in negative ion mode and optimized MRM transitions (Table S5).

Additionally, 100 mg lyophilized and ground root powder was prepared for quantification of tropine, which was extracted and analysed as previously reported (Richter *et al.*, 2005). A GCMS-QP2010 system (Shimadzu, Kyoto, Japan) with an Rtx-5 column (30 m × 0.25 mm, 0.25 μm) was used. Parameters: ion source temperature, 230°C; helium carrier gas, 1.05 ml min^{-1}. Temperature programme: began at 65°C, 10°C min^{-1} up to 150°C, held 2 min, 10°C min^{-1} up to 280°C, and held 10 min. The injection was performed in the splitless mode and the inlet temperature was 250°C and volume was 9 μl.

Purification of His-tagged UGT1 and enzymatic assay

The UGT1 coding sequence (CDS) was inserted into pET28a using the restriction enzymes *Bam*HI and *Hind*III. His-tagged UGT1 was produced in engineered *Escherichia coli*. The cultures were induced and harvested under the exact same conditions as previously described (Qiu *et al.*, 2018). His-tagged UGT1 was purified using HisPur Ni-NTA Resin (ThermoFisher Scientific) as previously described (Qiu *et al.*, 2018). After purification, fresh proteins were immediately used for an enzymatic assay. The reaction system (200 μl) consisted of 10 μg purified UGT1, 1 mM phenyllactate, 5 mM UDP-glucose, 5 mM $MnCl_2$, Tris-HCl (pH 7.2). Boiled UGT1 was used as the control. After 120-min reaction at 30°C, the supernatant (1 μl) of reaction mixture was analysed using a quadrupole time-of-flight (QTOF) mass spectrometer (Bruker Impact II, Karlsruhe, Germany). The samples were separated by Symmetry C18 column (2.1 mm × 100 mm, 3.5 μm) using binary gradient elution, and the elution procedures are shown

in Table S6. The compounds were detected using ESI full scan in negative ion mode and instrument parameters were set as: capillary voltage, 3.0 kV; temperature of dry heater, 180°C; nebulizer pressure, 0.4 Bar; the flowing rate of dry gas, 4.0 l min^{-1}.

Western blotting

The *LS* CDS fused with C-terminal HA-tag was inserted into pEAQ-HT (Zhao *et al.*, 2016) to generate a vector for transient expression in tobacco leaf cells. Genetic transformation and western blotting were performed as previously described (Zhang *et al.*, 2015), using the HA antibody.

Functional identification of LS by reconstructing littorine biosynthesis in tobacco cells

Full-length CDSs of *YFP* (control), *UGT1,* and *LS* were respectively cloned into pEAQ-HT using the restriction enzymes *Age*I and *Xho*I to generate pEAQ-YFP, pEAQ-UGT1 and pEAQ-LS, and independently transformed into *Agrobacterium* GV3101. *Nicotiana benthamiana* leaves were infiltrated with bacteria and metabolites were extracted as previously described (Lau & Sattely, 2015) with slight modification involving using 1 mM tropine and 1 mM phenyllactate in infiltration solution. Metabolites were detected using an LC-30AD UPLC system (Shimadzu) with an AB Sciex Triple Quad 5500 mass spectrometer detector. The samples were separated using a Symmetry C18 Column (2.1 mm × 100 mm, 3.5 μm) at 40°C and binary gradient elution involving 0.1% formic acid in water (A) and acetonitrile (B). The flow rate was 0.4 ml min^{-1}. The elution procedures are shown in Table S7. Measurements were performed using ESI in positive ion mode and optimized MRM transitions (Table S8). Instrument parameters: ion spray voltage, 5.5 kV; source temperature, 500°C; curtain gas, 40 psi; ion source gas, 50 psi.

Results and Discussion

To find the candidate genes (*UGT1* and *LS*) involved in littorine biosynthesis, bioinformatic analysis was performed. Using the UGT conserved domain (PF00201) to search *A. belladonna* transcriptomes, 102 unigenes were identified (Table S9). Using the conserved domain (PF00450) in SCPL-ATs and SCP (which both belong to the α/β hydrolase superfamily) to search *A. belladonna* transcriptomes, 33 unigenes were obtained (Table S9). TAs are synthesized in secondary roots, in which known TA biosynthesis genes are specifically or highly expressed (Suzuki *et al.*, 1999; Bedewitz *et al.*, 2014, 2018; Qiu *et al.*, 2018). Seven of the 102 UGT genes and two of the 33 SCP genes showed similar tissue expression profiles to the characterized TA biosynthesis genes (Figs S4, S5). Two of the UGT genes were not expressed in sterile seedlings where TAs were detected, suggesting that they might not participate in TA biosynthesis. The other five UGTs were phylogenetically analysed with all the *Arabidopsis* UGTs (Fig. S6). The aba_locus_19485 unigene clustered with two *Arabidopsis* UGTs, AT4G15480.1 and AT3G21560.1, which catalyse C1-glycosylation of phenylpropanoids with similar structures (Lim *et al.*, 2001). Phenyllactate has a similar structure to phenylpropanoid substrates used by these *Arabidopsis* UGTs. Thus, aba_locus_19485 was hypothesized to be phenyllactate UDP-glycosyltransferase (designated *UGT1*). Of the two SCP genes, aba_locus_17884 (designated littorine synthase, *LS*) was expressed in sterile seedlings, while aba_locus_128152 was not, so the former is the sole candidate SCPL-AT leading to littorine production. Phylogenetic analysis showed that LS was clustered with SCPL-ATs (Fig. S7), which condense acyl acceptors and phenylpropanoid glucose esters (acyl donors) via esterification (Lehfeldt *et al.*, 2000; Shirley & Chapple, 2003; Noda *et al.*, 2006; Fraser *et al.*, 2007; Weier *et al.*, 2008), suggesting that LS might have similar functions to the characterized SCPL-ATs. The sequences of *UGT1* and *LS* were cloned from cDNAs synthesized from mRNAs of secondary roots of *A. belladonna*, and their sequences were deposited in GenBank with accession numbers MN256145 and MN256146, respectively. Further analysis of digital gene expression confirmed that *UGT1* and *LS* had tissue profiles similar to known TA biosynthesis genes (Fig. 2a). The tissue profiles were validated using qPCR (Fig. 2b,c), indicating that they were highly or specifically expressed in *A. belladonna* secondary roots.

Several novel TA biosynthesis genes (*AT4*, *PYKS*, and *CYP82M3*) have recently been identified using virus-induced gene silencing (VIGS) in *A. belladonna* (Bedewitz *et al.*, 2018). Generally, when a TA biosynthesis gene is silenced, TA production greatly decreases (Bedewitz *et al.*, 2018). In this study, *UGT1* and *LS* were individually silenced in *A. belladonna* seedlings to investigate their roles in TA biosynthesis. The *UGT1*/*LS* transcript levels greatly decreased in the roots of gene-silenced seedlings (Fig. 2d,e). Hyoscyamine, the dominant alkaloid in *A. belladonna* (Xia *et al.*, 2016), is often used as a marker alkaloid. Hyoscyamine production greatly decreased in gene-silenced plants (Fig. 2f), indicating that UGT1 and LS participate in hyoscyamine biosynthesis.

To confirm the roles of *UGT1* and *LS* in TA biosynthesis, transgenic *A. belladonna* root cultures, in which *UGT1* and *LS* were separately suppressed using RNAi, were established (Fig. S8). The *UGT1*/*LS* expression in RNAi root cultures greatly decreased (Fig. 3a). The TA production (littorine, hyoscyamine, and scopolamine) was much lower in RNAi lines than in controls; trace amounts of the three TAs were detected in two *LS*-RNAi lines (ILS-1 and ILS-5). The littorine content was 234.00 μg g^{-1} dry weight (DW) in controls, and 16.65–50.31 μg g^{-1} DW in *UGT1*-RNAi root cultures (Fig. 3b), equivalent to 7.1–21.5% of the littorine content in controls. *LS* suppression decreased the littorine content more significantly, to 0.09–4.12 μg g^{-1} DW (Fig. 3b), equivalent to 0.04–1.76% of the littorine content in controls. Thus, RNAi suppression of either *UGT1* or *LS* disrupted littorine production. Due to the reduced supply of littorine (the intermediate compound), hyoscyamine and scopolamine production was also markedly decreased in *UGT1*-RNAi and *LS*-RNAi root cultures. In control root cultures, the levels of these compounds were 3.01 mg g^{-1} DW and 0.83 mg g^{-1} DW, respectively (Fig. 3c, d). *UGT1* suppression led to levels of 0.11–0.43 and 0.05–0.21 mg g^{-1} DW, respectively (Fig. 3c,d), while *LS* silencing led to

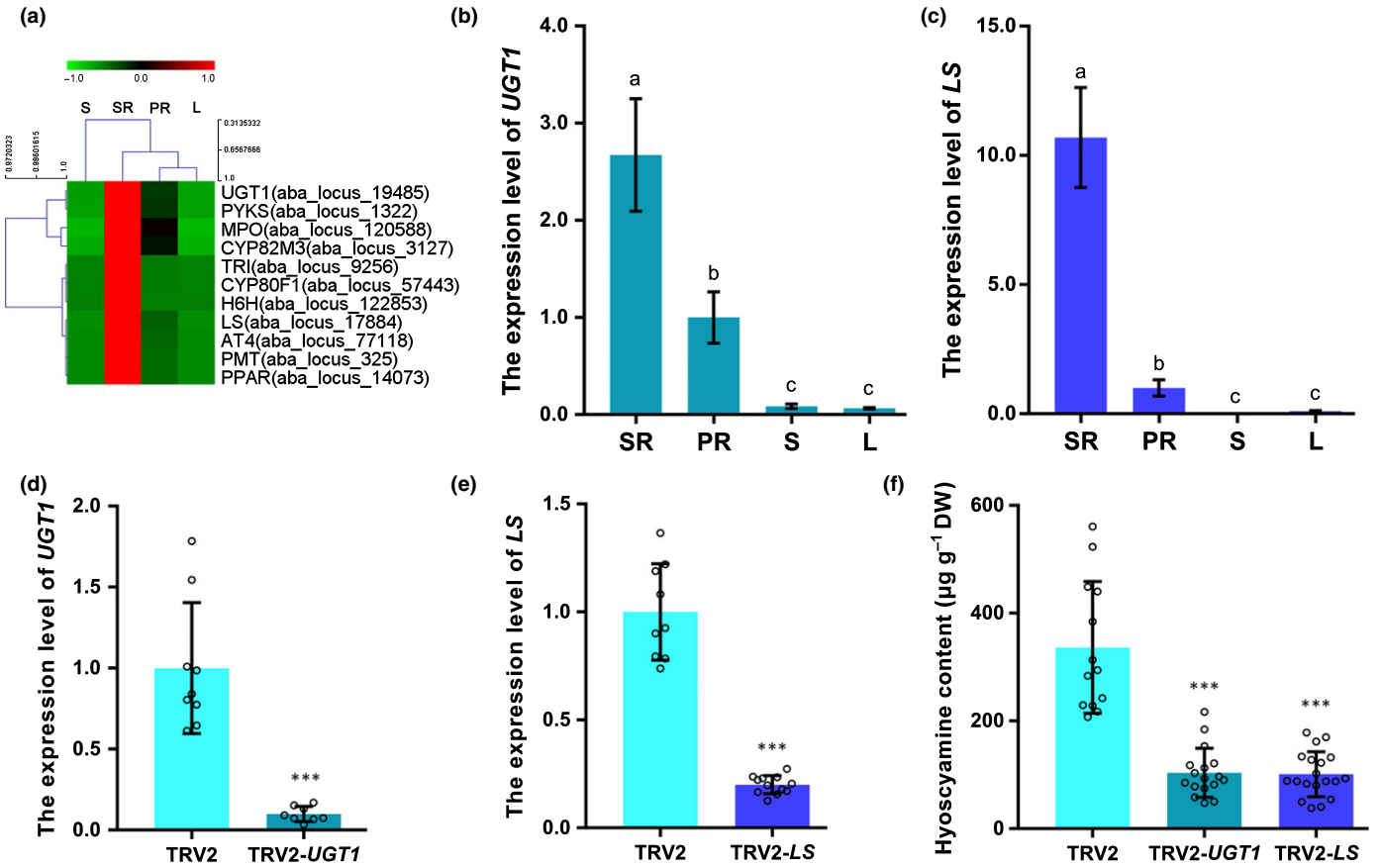

Fig. 2 Tissue profiles of tropane alkaloid (TA) biosynthesis genes and the virus-induced gene silencing (VIGS) of *UGT1*/*LS* in *Atropa belladonna* seedlings. (a) Digital gene expression of TA biosynthesis genes in different *A. belladonna* organs. Tissue profile of (b) *UGT1* and (c) *LS* in *A. belladonna*. Different letters on columns indicate significant difference at the level of $P < 0.05$ given by Duncan's test. SR, secondary root; PR, primary root; S, stem; L, leaf. (d) *UGT1* expression in *A. belladonna* roots. TRV2, control; TRV2-UGT1, *UGT1*-silenced plant. ***, $P < 0.001$ (given by the *t*-test). (e) *LS* expression in *A. belladonna* roots. TRV2, control; TRV2-LS, *LS*-silenced plant. ***, $P < 0.001$ (given by the *t*-test). (f) Hyoscyamine production. TRV2, control; TRV2-UGT1, *UGT1*-silenced plant; TRV2-LS, *LS*-silenced plant. Data are presented as mean ± SD. ***, $P < 0.001$ (given by the *t*-test).

levels of 0.009–0.11 and 0.002–0.029 mg g^{-1} DW, respectively (Fig. 3c,d). This further confirmed that the two genes are indeed involved in TA biosynthesis.

Suppression of biosynthesis genes usually causes accumulation of certain precursors for target metabolites at higher levels. Previously, when *AbPYKS* and *AbCYP82M3* were individually suppressed, the accumulation of their substrates (*N*-methyl-Δ^1-pyrrolinium and 4-(1-methyl-2-pyrrolidinyl)-3-oxobutanoic acid, respectively) was significantly elevated (Bedewitz *et al.*, 2018). Thus, phenyllactate and tropine accumulation was also analysed in *A. belladonna* root cultures. *UGT1* or *LS* suppression significantly increased tropine accumulation in root cultures presumably due to less tropine being metabolized into littorine (Fig. 3e). *LS* suppression also significantly increased phenyllactate accumulation, but *UGT1* suppression did not (Fig. 3f). Many UGTs catalyse the glycosylation of substrates with similar structures (Lim *et al.*, 2004; Torrens-Spence *et al.*, 2018; Sun *et al.*, 2018). UGT1 in this study may not be the only UGT to produce phenyllactylglucose from phenyllactate, as other uncharacterized *A. belladonna* UGTs may also produce phenyllactylglucose in a small amount for littorine biosynthesis. Another possibility is that the less efficient reduction of TA seen with the *UGT1*-RNAi constructs (compared to the *LS*-RNAi constructs) is not enough to cause the accumulation of phenyllactate. Similarly, *AbPPAR* suppression dramatically reduced its direct product (phenyllactate), and TA biosynthesis was substantially disrupted, but the accumulation of the AbPPAR substrate (phenylpyruvate) was unaltered (Qiu *et al.*, 2018).

To identify catalytic activity, N-terminal His-tagged UGT1 was produced in *Escherichia coli* and then purified for enzymatic assays. On the sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) gel, there was a single protein band with a molecular weight (MW) of 58.1 kDa, consistent with its predicted MW (Fig. 4a). When phenyllactate and UDP-glucose were both provided to the UGT1 enzyme in the reaction system, a new product, identified as phenyllactoylglucose (*m/z* 327.1088), was formed (Fig. 4c,d), as indicated by liquid chromatography mass spectrometry (LC-MS). The negative control (boiled UGT1) did not produce phenyllactylglucose (Fig. 4b). These data indicated that UGT1 catalyses phenyllactylglucose formation using phenyllactate and UDP-glucose. The UGT1 kinetics were not analysed due to lack of commercially authentic phenyllactylglucose. Due to low production, not enough UGT1-produced phenyllactylglucose

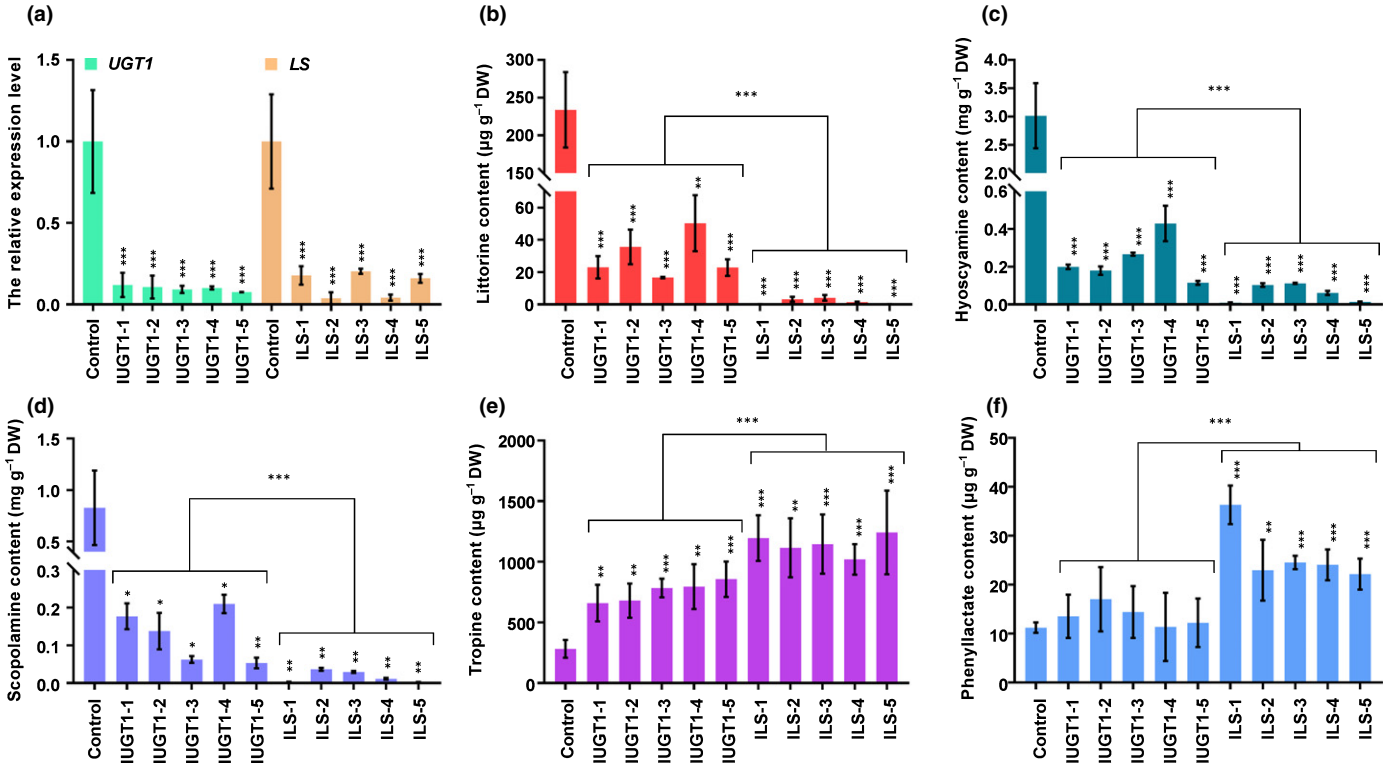

Fig. 3 Effects of *UGT1*/*LS* suppression on the biosynthesis of tropane alkaloids (TAs) and accumulation of their precursors in *Atropa belladonna* root cultures. (a) *UGT1* and *LS* expression. Level of (b) littorine, (c) hyoscyamine, (d) scopolamine, (e) tropine, and (f) phenyllactate. Control, root cultures with expression of an empty pBin19 vector; IUGT1, root cultures with UGT1 suppression using RNAi; ILS, root cultures with LS suppression using RNAi. The bars on columns indicate SD ($n \geq 3$). Data are presented as mean ± SD. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (given by the *t*-test).

could be isolated for an LS enzymatic assay, which was a major reason for subsequently identifying the function of LS using an *in planta* transient expression method.

Recombinant SCPL-ATs purified from *Escherichia coli* usually lose their catalytic activities due to lack of post-translational modification (Shirley & Chapple, 2003; Stehle *et al.*, 2008; Weier *et al.*, 2008). Notably, SCPL-ATs might be processed into different subunits, but the processing mechanism is completely unknown. To assess whether LS is processed into subunits *in planta*, the C-terminal HA-tagged *LS* gene in frame was transiently expressed in tobacco leaves. Western blotting generated two bands from *LS*-expressing tobacco leaf cells, while no bands were detected in control tobacco leaf cells. One band indicated a MW of 55.1 kDa, which was consistent with the predicted MW of HA-tagged LS, and the other band indicated a 36 kDa MW, much smaller than the predicted MW of HA-tagged LS (Fig. 4e). This indicated that LS is post-translationally processed in plant cells. Previously, both raw and processed SCPL-ATs were detected after heterologous SCPL-AT expression (Li & Steffens, 2000; Mugford *et al.*, 2009). However, only processed SCPL-ATs were detected in native plants with SCPL-ATs. For example, only processed oat SCPL-AT (SAD7) was detected in oat root cells, but both raw and processed SAD7 were detected in tobacco cells with heterologous SAD7 expression (Mugford *et al.*, 2009). As LS was processed into subunits, a transient expression method was used to confirm the function of LS. TAs are not produced in tobacco, which has been exploited to identify TA biosynthesis genes (Bedewitz *et al.*, 2018).

When *UGT1* and *LS* were co-expressed in tobacco cells in combination with the addition of tropine and phenyllactate, littorine was detected at a retention time of 5.45 min (Fig. 4g), consistent with authentic littorine (Fig. 4f). When *LS* only was expressed in tobacco leaf cells, littorine was also detected, but in trace amounts (Fig. 4h), suggesting that unknown UGT enzymes in tobacco might convert phenyllactate to its glycosylated ester. Generally, many UGTs can accept compounds with similar structures (Cui *et al.*, 2016; Wang *et al.*, 2019). Notably, the littorine level was approximately 10-fold higher in tobacco leaf cells with *UGT1* and *LS* co-expression than in cells with *LS* expression. Tobacco leaf cells expressing the *yellow fluorescent protein* (*YFP*) control (Fig. 4i) or *UGT1* did not produce littorine (Fig. 4j). These data indicated that LS was responsible for littorine production. In the biosynthesis of cocaine, the representative tropane alkaloid of coca tree, a BAHD acyltransferase condensed methylecgonine and CoA activated benzoic acid to produce cocaine via esterification in *Erythoxylum coca* (Schmidt *et al.*, 2015). The difference between cocaine biosynthesis and littorine biosynthesis is in line with the proposition that TA biosynthesis evolved independently in the Erythroxylaceae and Solanaceae (Jirschitzka *et al.*, 2012).

In summary, two novel genes required for littorine biosynthesis were identified. One is UGT1, which catalyses phenyllactate and UDP-glucose ligation to produce phenyllactylglucose, and the

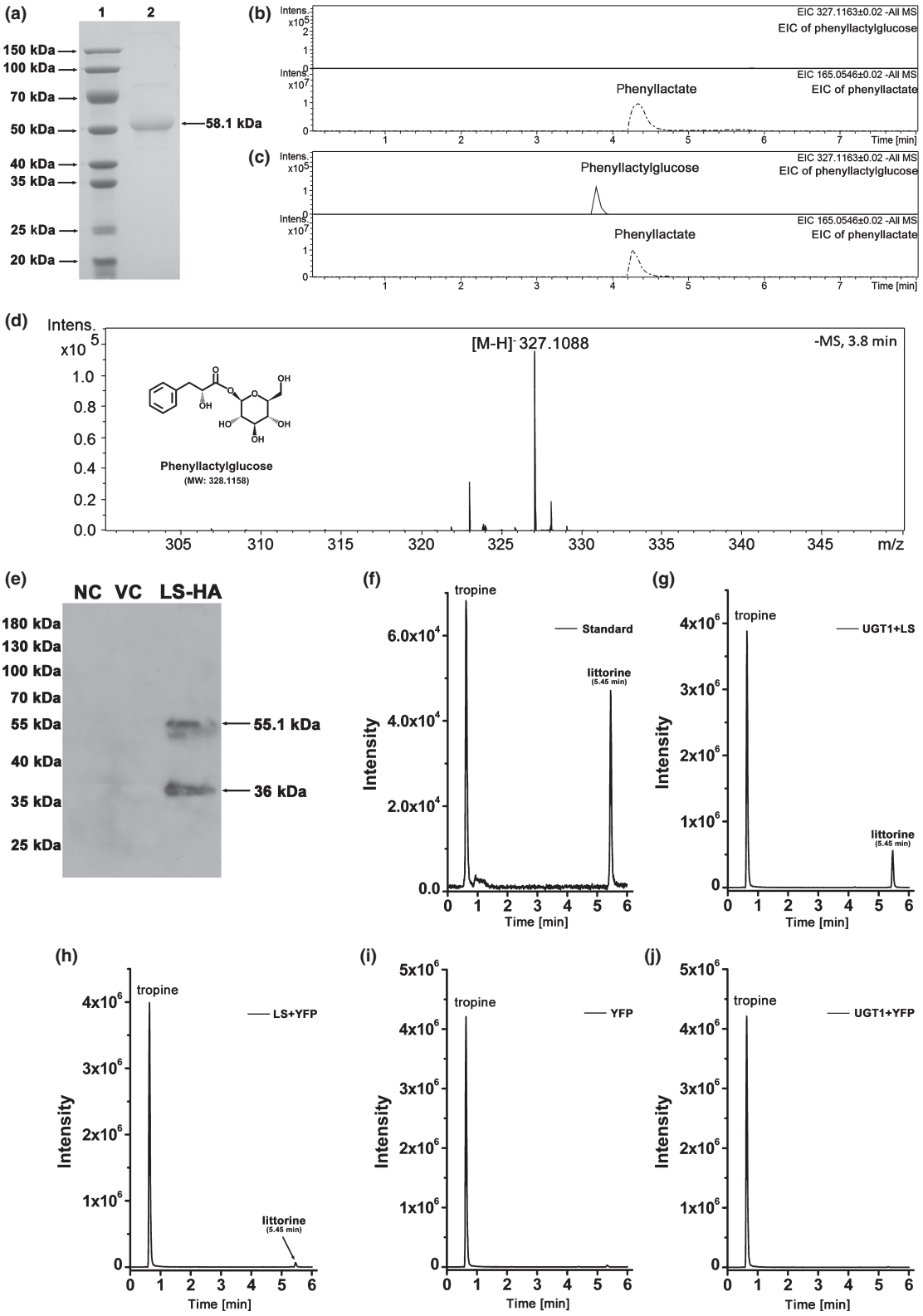

Fig. 4 Functional identification of UGT1 and LS. (a) Purified His-tagged UGT1. 1, protein marker; 2, purified His-tagged UGT1 protein. (b) Extracted-ion chromatogram (EIC) trace of control (boiled) UGT1-mediated reaction system. (c) EIC trace of UGT1-mediated reaction system. (d) 3.80-min MS data extracted from (c). (e) Detection of LS-HA heterologously produced in tobacco cells. NC, negative control; VC, vector control; LS-HA, expression of LS fused with an HA tag at its C-terminal in tobacco leaf cells. (f–j) Representative MRM traces obtained using UPLC-MS/MS and tobacco leaf cells. (f) Tropine and littorine standards. (g) Tobacco leaf cells co-expressing UGT1 and LS. (h) Tobacco leaf cells expressing LS and YFP. (i) YFP control. (j) Tobacco leaf cells expressing UGT1 and YFP.

other is littorine synthase, which condenses phenyllactylglucose and tropine via esterification to form littorine. This study provides valuable genes for producing TAs by synthetic biology or metabolic engineering.

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Author contributions

FQ, ZL and S-XH conceived and designed the study. FQ, JZ and JW performed gene cloning, expression analysis, genetic transformation and enzymatic assays. J-PH, WZ, XL and MC analysed the metabolites. CY managed the plant materials. ZL, S-XH and GK analysed the data and wrote the article.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 The melting curves of qPCR products specific to UGT1 and LS.

Fig. S2 The diagram of UGT1-RNAi construct.

Fig. S3 The diagram of LS-RNAi construct.

Fig. S4 Digital gene expression of UGTs and TA biosynthesis genes.

Fig. S5 Digital gene expression of SCPs and TA biosynthesis genes.

Fig. S6 Phylogenetic analysis of glycosyltransferases.

Fig. S7 Phylogenetic analysis of SCPL-ATs.

Fig. S8 Establishment of *Atropa belladonna* root cultures and PCR detection.

Table S1 The primers used in this study.

Table S2 UPLC mobile phase gradients for analysing alkaloids.

Table S3 Optimized MRM parameters for analysing alkaloids.

Table S4 UPLC mobile phase gradients for analysing phenyllactate.

Table S5 Optimized MRM parameters for analysing phenyllactate.

Table S6 UPLC mobile phase gradients for analysing phenyllactylglucose.

Table S7 UPLC mobile phase gradients for analysing littorine.

Table S8 Optimized MRM parameters for analysing littorine.

Table S9 Locus IDs of UGTs and SCPs from *Atropa belladonna*.

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