Metabolic engineering to enhance the accumulation of bioactive flavonoids licochalcone A and echinatin in *Glycyrrhiza inflata* (Licorice) hairy roots

Zhigeng Wu1,2, Sanjay Kumar Singh3, Ruiqing Lyu3, Sitakanta Pattanaik3, Ying Wang1,2, Yongqing Li1,2, Ling Yuan1,3* and Yongliang Liu1,3*

1Key Laboratory of South China Agricultural Plant Molecular Analysis and Genetic Improvement and Guangdong Provincial Key Laboratory of Applied Botany, South China Botanical Garden, Chinese Academy of Sciences, Guangzhou, China, 2University of Chinese Academy of Sciences, Beijing, China, 3Department of Plant and Soil Sciences and Kentucky Tobacco Research and Development Center, University of Kentucky, Lexington, KY, United States

Echinatin and licochalcone A (LCA) are valuable chalcones preferentially accumulated in roots and rhizomes of licorice (*Glycyrrhiza inflata*). The licorice chalcones (licochalcones) are valued for their anti-inflammatory, antimicrobial, and antioxidant properties and have been widely used in cosmetic, pharmaceutical, and food industries. However, echinatin and LCA are accumulated in low quantities, and the biosynthesis and regulation of licochalcones have not been fully elucidated. In this study, we explored the potential of a R2R3-MYB transcription factor (TF) *AtMYB12*, a known regulator of flavonoid biosynthesis in *Arabidopsis*, for metabolic engineering of the bioactive flavonoids in *G. inflata* hairy roots. Overexpression of *AtMYB12* in the hairy roots greatly enhanced the production of total flavonoids (threefold), echinatin (twofold), and LCA (fivefold). RNA-seq analysis of *AtMYB12*-overexpressing hairy roots revealed that expression of phenylpropanoid/flavonoid pathway genes, such as *phenylalanine ammonia-lyase* (PAL), *chalcone synthase* (CHS), and *flavanone 3'-hydroxylase* (F3'H), is significantly induced compared to the control. Transient promoter activity assay indicated that *AtMYB12* activates the *GiCHS1* promoter in plant cells, and mutation to the MYB-binding motif in the *GiCHS1* promoter abolished activation. In addition, transcriptomic analysis revealed that *AtMYB12* overexpression reprograms carbohydrate metabolism likely to increase carbon flux into flavonoid biosynthesis. Further, *AtMYB12* activated the biotic defense pathways possibly by activating the salicylic acid and jasmonic acid signaling, as well as by upregulating WRKY TFs. The transcriptome of *AtMYB12*-overexpressing hairy roots serves as a valuable
Introduction

Glycyrrhiza species of the family Fabaceae, including Glycyrrhiza glabra L., Glycyrrhiza uralensis Fisch., and Glycyrrhiza inflata Bat., are valued greatly for their roots and rhizomes (licorice), which are widely used in cosmetics and herbal medicines (Zhang and Ye, 2009; Jiang et al., 2020). The bioactivity of licorice is mainly attributed to two groups of specialized metabolites; namely, triterpene saponins and flavonoids (Wang D. et al., 2020; Wang Z.-F. et al., 2020). Glycyrrhizin is the most abundant saponin in licorice and has long been recognized as a potent sweetening agent (Pandey and Ayangla, 2018). Glycyrrhizin also has been explored for anti-coronavirus properties in the current COVID-19 pandemic (Luo et al., 2020; Chrzanowski et al., 2021). The other major group of bioactive components present in licorice are flavonoids (Zhu et al., 2016; Cheng et al., 2021). The licorice flavonoids are known to possess anti-inflammatory, antioxidant, and antimicrobial properties (Wang Z.-F. et al., 2020; Husain et al., 2021). Among the different flavonoids, echinatin and licochalcone A (LCA) are predominantly present in G. inflata (Lin et al., 2017; Rizzato et al., 2017; Song et al., 2017). A high LCA cosmetic formulation reduces UV-induced erythema formation in human healthy volunteers possibly by modulation of dendritic cell activity (Kolbe et al., 2006). Because of its anti-inflammatory and antimicrobial properties, LCA has been used for the treatment of facial skin diseases such as acne and rosacea (Schoelermann et al., 2016; Yang et al., 2018). Therefore, there is a great demand of LCA in cosmetic industries (Nguyen et al., 2020; Cerulli et al., 2022). However, LCA is naturally accumulated at low levels in wild G. inflata, even less in cultivated G. inflata plants. Metabolic engineering is thus viewed as a rational alternative to increase LCA production.

Chalcones are a subgroup of polyphenol compounds that are synthesized through the phenylpropanoid pathway (Figure 1A). The precursor phenylalanine (Phe) is derived from the primary metabolic pathways, including glycolysis, the shikimate pathway, and Phe biosynthetic pathway (Tzin and Gallí, 2010). Chalcone synthase (CHS) is the first rate-limiting enzyme specific for flavonoid pathway (Saito et al., 2013). Regulation of the flavonoid biosynthesis has been extensively studied in numerous plant species including Arabidopsis thaliana (Saito et al., 2013). In Arabidopsis, three closely related MYB transcription factors (TFs), MYB11, MYB12, and MYB111, from subgroup 7 of the R2R3-MYB family, redundantly regulate the biosynthesis of flavonoids, especially flavonols (Mehrtens et al., 2005; Stracke et al., 2007). These MYBs bind to the promoters of key flavonoid biosynthetic pathway genes, such as CHS, to activate expression (Mehrtens et al., 2005; Stracke et al., 2007). The three MYBs exhibit distinct expression patterns, and AtMYB12 mainly controls flavonoid biosynthesis in Arabidopsis roots (Stracke et al., 2007). The regulatory role of AtMYB12 on flavonoid pathway has been further investigated through heterologous expression in tobacco leaves and tomato fruits (Luo et al., 2008; Pandey et al., 2015; Zhang et al., 2015). AtMYB12 induces the accumulation of flavonoids in tomato fruits by reprogramming the primary metabolism and directing the carbon flux toward flavonoid pathway (Zhang et al., 2015). Chlorogenic acid (CGA) is a subclass of polyphenols present in Solanaceous species (tomato and tobacco) and coffee, but not in Arabidopsis (Luo et al., 2008; Naved et al., 2018). In addition to flavonoids, ectopic expression of AtMYB12 in tobacco significantly increases CGA biosynthesis (Luo et al., 2008; Zhang et al., 2015). AtMYB12 also activates CGA biosynthetic genes in tomato fruits (Luo et al., 2008; Zhang et al., 2015). AtMYB12 overexpression in kale increases total flavonoid and phenolics in leaves (Lännenpää, 2014). These findings suggest that AtMYB12 is a potential candidate for metabolic engineering to induce flavonoids and flavonoid-derived metabolites in heterologous plant species.

As the biosynthetic pathway and gene regulation of licorice chalcones are not well elucidated, we aimed to explore the potential of AtMYB12 for metabolic engineering of licorice chalcones in G. inflata. We hypothesized that ectopic expression of AtMYB12 in G. inflata will lead to higher accumulation of licorice chalcones and identification of potential chalcone pathway genes. As protocol for the generation of stable transgenic lines is not established in G. inflata, we thus generated hairy roots overexpressing AtMYB12. Molecular and biochemical analyses of AtMYB12-overexpressing hairy roots showed higher expression of phenylpropanoid/flavonoid pathway genes, including GiCHS, and increased accumulation of flavonoids.
of total flavonoids and licorice-specific flavonoids, such as echinatin and LCA, confirming the regulatory roles of AtMYB12 on early flavonoid pathway genes in a heterogeneous plant species. In addition, RNA-seq data showed that the carbon flux was reprogrammed toward the flavonoid pathway. Our findings suggest that AtMYB12 is an effective regulator for engineering the production of licorice chalcones in G. inflata.

Materials and methods

Plant materials

Arabidopsis thaliana Col-0 accession was used for RNA isolation and AtMYB12 cloning. G. inflata seeds were provided by Gansu Jin You Kang Pharmaceutical Technology Co., Ltd., Lanzhou, China. G. inflata seeds were soaked in H$_2$SO$_4$ for 30 min, washed with water five times, then treated with 1% NaClO for 10 min, and washed with sterilized distilled water five times. Surface-sterilized seeds were germinated on Murashige and Skoog (MS) medium and kept in dark for 2 days before being transferred to light condition. Then, 8-day-old G. inflata seedlings were used for DNA, RNA isolation, transient gene expression, and generation of transgenic hairy roots.

Generation of transgenic AtMYB12 hairy roots

AtMYB12 was amplified from Arabidopsis cDNA and cloned into the pCAMBIA2301 vector containing CaMV35S promoter and rbcS terminator to generate pCAMBIA2301-AtMYB12. Primers used for cloning of AtMYB12 and other genes in this study are all listed in Supplementary Table 1. The empty vector (EV; pCAMBIA2301) and pCAMBIA2301-AtMYB12 plasmids were separately transformed into Agrobacterium rhizogenes R1000 by freeze–thaw method. The hypocotyl segments from 8-day-old G. inflata seedlings were submerged in the A. rhizogenes R1000 suspension for 30 min, blot-dried on sterile filter paper, and then placed on MS medium at 22°C in darkness. After co-cultivation for 2 days, the hypocotyl segments were transferred to MS medium supplemented with 400 mg/l cefotaxime. After 2–3 weeks of culture, hairy roots developed from hypocotyls and the rapidly growing hairy roots were excised and cultivated individually on solid MS medium supplemented with 400 mg/l cefotaxime and 100 mg/l kanamycin for 2 weeks at 25°C in dark. Rapidly growing root lines that showed kanamycin resistance were selected for further analysis. These hairy root lines were cultured in 125 ml flasks each containing 10 ml MS liquid medium on an orbital shaker at 100 rpm at 25°C. The hairy roots clones were routinely subcultured every 2 weeks and harvested after 2 months for RNA isolation and metabolite extraction.
cDNA synthesis and determination of transgenic status of the hairy roots

Total RNA was isolated from EV control and AtMYB12-overexpressing seedlings and hairy roots using the RNeasy Plant Mini Kit following the instructions of the manufacturer (QIAGEN, United States). Approximately 2 μg of total RNA was used for DNase I digestion. Synthesis of first-strand cDNA was performed using Superscript III reverse transcriptase (Invitrogen) in a total volume of 20 μl. To verify the transgenic status of AtMYB12-OX and EV control hairy root lines, gene-specific primers were used to PCR-amplify the rol B, rol C, vir C, and kanamycin-resistant (nptII) genes. PCR products were analyzed on a 1% ethidium bromide-stained agarose gel.

Determination of the contents of flavonoids in hairy roots

Total flavonoid contents were determined by sodium nitrite–aluminum nitrate colorimetric method using rutin as standard (Hao et al., 2018). The standards rutin, echinatin, and LCA were purchased from Biosynth Carbosynth, United States. The contents of echinatin and LCA were determined by LC-MS/MS.

Library construction and RNA sequencing

Three independent lines of both EV and AtMYB12-OX hairy roots were used for RNA-seq. Total RNA was isolated from hairy roots using the RNeasy Plant Mini Kit (QIAGEN, United States) following the instructions of the manufacturer. The RNA samples with RNA integrity number (RIN) 8 or above were used for library preparation and sequencing. The TruSeq RNA Sample Prep Kit (Illumina, United States) was used for making libraries according to the protocol of the manufacturer. Individually indexed libraries were combined at equal proportions and loaded onto a single lane of a flow cell. A 50-cycle single-end sequencing run was performed on the Illumina HiSeq2500 at the Duke Center for Genomic and Computational Biology.

Data processing, identification of differentially expressed genes, and gene ontology enrichment analysis

Raw Illumina sequence reads were processed as described previously (Singh et al., 2015). Read mapping was performed by Bowtie2 (Langmead and Salzberg, 2012) using an in-house-generated G. inflata transcriptome (unpublished data). Differential expression gene analysis was carried out using the DESeq2 Bioconductor package in R (Love et al., 2014). The differentially expressed genes (DEGs) were identified following two criteria: (i) fold change ≥ 2 and (ii) false discovery rate p-value correction of ≤ 0.05. Heatmaps were constructed using the Complex Heatmap (Gu et al., 2016) function in R through the Bioconductor package (R Core Team, 2022). Functional annotation of DEGs was performed with eggNOG 4.5 (Huerta-Cepas et al., 2016) database. Gene Ontology (GO) analysis of the enriched functional categories was performed using BiNGO (version 2.44) (Maere et al., 2005).

Reverse transcription quantitative PCR

Reverse transcription quantitative PCR (RT-qPCR) was used to measure transcripts levels of GiCHS genes. The GiActin gene was used as an internal control. Relative gene expression was measured as previously described (Liu et al., 2019). All qRT-PCRs were performed in triplicate and repeated twice. Primers used in qRT-PCR are listed in Supplementary Table 1.

Transient overexpression of AtMYB12 in Glycyrrhiza inflata seedlings

The EV and pCAMBIA2301-AtMYB12 were transformed into Agrobacterium tumefaciens GV3101 by freeze–thaw method and was plated on Luria–Bertani (LB) medium containing 100 μg ml⁻¹ kanamycin, 50 μg ml⁻¹ gentamicin, and 30 μg ml⁻¹ rifampicin. A single colony was transferred to 1 ml liquid LB medium containing the same antibiotics and incubated at 250 rpm and 28°C overnight. The overnight culture was diluted in 25 ml liquid LB medium and grown for 16 h at 250 rpm and 28°C. The cells were then centrifuged, and the pellet was resuspended in infiltration buffer (10 mM MgCl₂, 10 mM MES, 100 μM acetosyringone) to an OD₆₀₀ of 1.0, and incubated at 28°C for at least 3 h. Then, 8-day-old G. inflata seedlings were immersed in the infiltration solution under vacuum pressure for 1 h. After vacuum infiltration, seedlings were washed five times with sterile distilled water and laid on sterile wet filter papers in Petri dishes. After 5 days of incubation at room temperature, the transsected seedlings were collected for RNA isolation.

Cloning of the GiCHS1 promoter

Genomic DNA was extracted from G. inflata seedlings for promoter cloning. A forward primer (CHS1-pro-F) was designed based on the genomic sequence upstream of the coding region of G. uralensis homolog of GiCHS1. A reverse primer (GiCHS1-cds-R) was designed within the coding sequence of
et al., 2003). Based on the GiCHS1 promoter sequence, another pair of primers (GiCHS1-pro-F2 and GiCHS1-pro-R2) was designed for vector construction.

**Promoter activity assay in tobacco protoplasts**

Tobacco cell line described before (Pattanaik et al., 2010) was used for protoplast isolation and promoter activity assay. The effector plasmid was constructed by cloning AtMYB12 into a modified pBS vector under the control of the CaMV 35S promoter and rbcS terminator. The reporter plasmid was generated by cloning GiCHS1 promoter in a modified pUC vector containing the firefly luciferase (LUC) reporter and rbcS terminator. The MYB binding motif in GiCHS1 promoter was mutated by site-directed mutagenesis to generate mutant promoter. The GUS reporter driven by CaMV 35S promoter and rbcS terminator was used as an internal control in the protoplast assay. The reporter, effector, and internal control plasmids were electroporated into tobacco protoplasts in different combinations; luciferase and GUS activities in transfected protoplasts were measured as described previously (Pattanaik et al., 2010).

**Results**

**Ectopic expression of AtMYB12-induced total flavonoids and licorice-specific chalcones in Glycyrrhiza inflata hairy roots**

We generated transgenic hairy roots overexpressing AtMYB12 (AtMYB12-OX) aiming to increase flavonoid production. EV hairy root lines (EV-1, EV-2, and EV-3) served as control. Three independent AtMYB12-OX hairy root lines (AtMYB12-OX-1, AtMYB12-OX-2, and AtMYB12-OX-3) were selected for further analysis. The transgenic status of the independent EV and AtMYB12-OX hairy root lines was verified by PCR (Supplementary Figure 1). Total flavonoid contents of the three AtMYB12-OX lines were significantly higher (threefold) than those of the EV lines (Figures 1A,B). While echinatin and LCA in EV hairy root lines were approximately 18–24 and 25–31 ng mg⁻¹, respectively (Figures 1A,C), AtMYB12-OX lines showed a significant increase in the accumulation of echinatin (30–59 ng mg⁻¹; ~2.2-fold increase) and LCA (119–174 ng mg⁻¹; ~5.2-fold increase) (Figure 1C). The metabolic outcomes of AtMYB12 overexpression suggest that AtMYB12 is an effective gene for metabolic engineering of the licorice flavonoid pathway.

**AtMYB12-induced expression of phenylpropanoid/flavonoid pathway genes in Glycyrrhiza inflata hairy roots**

The metabolic outcomes of AtMYB12-OX hairy roots prompted us to generate and analyze the transcriptome data of EV and AtMYB12-OX lines. Sequencing of RNA libraries of EV and AtMYB12-OX lines generated a total of 1,110 million (M) clean reads. Each biological replicate was represented by an average of more than 170 M reads. On average, more than 70% of the total reads from EV and overexpression line libraries were successfully mapped to the G. inflata transcriptome (Supplementary Figure 2). Compared to the EV lines, 3,236 genes were differentially expressed in AtMYB12-OX lines, in which 1,722 genes were upregulated and 1,514 genes were downregulated (Supplementary Table 2). We particularly examined genes in the phenylpropanoid/flavonoid pathway. CHS is a key rate-limiting enzyme in flavonoid biosynthetic pathway (Zhang et al., 2017). Noticeably, 13 GiCHSs were identified among the DEGs, and 12 of them were upregulated in AtMYB12-OX hairy roots (Figure 2A and Table 1). In addition, we identified three G. inflata phenylalanine ammonia-lyase (PAL) and 9 flavanone 3'-hydroxylase (F3'H) genes among the DEGs, and all of them were induced in AtMYB12-OX hairy roots (Table 1). To verify the expression of selected DEGs in RNA-seq, we conducted RT-qPCR to measure the expression of two CHS genes, Gin33862 (hereafter designed as GiCHS1) and Gin35437, using independently isolated RNAs from the AtMYB12-OX hairy roots. The results confirmed the induction of both CHS genes in AtMYB12-OX hairy root lines (Figure 2B). These results suggest that the upregulation of GiPALS, GiCHSs, and GiF3'Hs likely leads to the enrichment of flavonoids in G. inflata hairy roots.

**GiCHS1 is highly expressed in roots**

LCA and echinatin are preferentially accumulated in G. inflata roots and rhizomes. We therefore analyzed the transcriptomes (SRA accession: PRJNA574093) of G. inflata leaves and roots, collected from two geographical locations in China (Guangzhou and Ningxia), to determine the tissue-specific expression of CHS. Among the 12 GiCHSs upregulated in AtMYB12-OX roots, expression of two GiCHSs was not detected in leaf and root transcriptomes. Among the other 10 GiCHSs, Gin33862 (GiCHS1) is preferentially expressed in G. inflata roots from both locations (Figure 2C). Two other GiCHSs, Gin35437 and Gin16453, showed increased expression only in the roots collected from Guangzhou (Figure 2C).
Transient overexpression of AtMYB12 in *Glycyrrhiza inflata* seedlings induced *GiCHS*s expression

To further verify the effect of AtMYB12 on LCA biosynthesis in *G. inflata*, we developed an Agrobacteria-mediated transient gene expression assay in *G. inflata* seedlings. Similar to that in the hairy roots, expression of *GiCHS1* and *Gin35437* was significantly induced by ectopic expression of AtMYB12 in *G. inflata* seedlings (Figures 3A,B). These results indicate that the heterologous AtMYB12 positively regulates flavonoid biosynthesis in *G. inflata* plants.

**AtMYB12 directly activates the *GiCHS1* promoter activity**

We next asked whether AtMYB12 directly activates the flavonoid pathway gene promoters in *G. inflata*. As *GiCHS1* expression is upregulated by AtMYB12 and highly expressed in roots, we cloned the *GiCHS1* promoter for activity assay. Due to the lack of genomic sequences for *G. inflata*, the *GiCHS1* promoter was cloned based on the *G. uralensis* genome sequences (Mochida et al., 2017) as *G. inflata* and *G. uralensis* are two closely related species. The amino acid sequence identity between *GiCHS1* and its *G. uralensis* homolog is 99%. The promoter of *GiCHS1* also shares high sequence identity (96%) with that of *G. uralensis CHS1* (Supplementary Figure 3). As shown in Figure 3D, transcriptional activity of the *GiCHS1* promoter (*GiCHS1-pro*) was significantly induced by AtMYB12, suggesting that AtMYB12 directly activates the *GiCHS1* promoter in plant cells. To further confirm the activation of the *GiCHS1* promoter by AtMYB12, we surveyed the promoter sequence for MYB recognition element (MRE) (A[A/C]CTACC) and identified a putative MRE (AACTACC) at -204 to -198 relative to ATG. This MRE is conserved among the *Arabidopsis CHS*, *CHI*, and FLS promoters and also present in the CHS promoters from other plants (Figure 3C). It is predicted to be targeted by R2R3 MYBs, including MYB11, MYB111, and...
Glucocorticoids (G. inflata) induced by AtMYB12 in three genes related to glycolysis and shikimate pathways were identified in DEGs. We observed that the shikimate pathway (Zhang et al., 2015). We speculated that this MRE promotes promoter activity assay showed that AtMYB12 is unable to directly bind to the MYB binding site in the AtMYB12 promoter is targeted by AtMYB12. MYB12 (Stracke et al., 2007). We speculated that this MRE (AACTACC) in the GiCHS1 promoter (GiCHS1m-pro) (Figure 3D). Results of promoter activity assay showed that AtMYB12 is unable to activate GiCHS1m-pro (Figure 3D), suggesting that AtMYB12 directly binds to the MYB binding site in the GiCHS1 promoter. 

RNA-seq revealed reprogramming of carbohydrate metabolism in AtMYB12-OX lines

Carbon resources of phenylpropanoids are derived from monosaccharides, such as glucose. The monosaccharides are directed to phenylpropanoid pathway through several primary pathways, including pentose phosphate pathway, glycolysis, and the shikimate pathway (Zhang et al., 2015). We observed that three genes related to glycolysis and shikimate pathways were induced by AtMYB12 in G. inflata hairy roots. Glucose also serves as a precursor of the polysaccharide cellulose, the major component of plant cell wall (Taylor, 2008; Yang et al., 2018). Further, GO enrichment analysis (Figure 4 and Supplementary Table 4) showed that several pathway genes related to cellulose synthesis and cell wall synthesis, including “cell wall biogenesis” and “cellulose metabolic process,” are downregulated in AtMYB12-OX lines. Cellulose production during cell wall biosynthesis has been shown to be dependent on cellulose synthase A (CESA). We identified seven G. inflata CESA genes in the DEGs that are downregulated in AtMYB12-OX lines (Table 2). These results indicate an increased carbon flux toward the phenylpropanoid pathway at the cost of cellulose synthesis in AtMYB12-OX hairy roots.

Table 1 Phenylpropanoid/flavonoid pathway genes identified in DEGs.

| Glycyrrhiza inflata gene ID | Log2 fold change | Arabidopsis homolog | Description |
|----------------------------|-----------------|---------------------|-------------|
| Gin05640                   | 1.618           | AT2G37040           | Phenylalanine | amonla-lyase (PAL) |
| Gin12084                   | 2.728           | AT2G37040           |             |
| Gin12083                   | 2.426           | AT3G10340           |             |
| Gin31596                   | -2.659          | AT5G13930           |             |
| Gin15004                   | 1.081           | AT5G13930           |             |
| Gin15005                   | 1.223           | AT5G13930           |             |
| Gin15006                   | 1.121           | AT5G13930           |             |
| Gin16453                   | 2.211           | AT5G13930           |             |
| Gin17304                   | 1.242           | AT5G13930           |             |
| Gin21634                   | 1.167           | AT5G13930           | Chalcone synthase (CHS) |
| Gin23193                   | 1.166           | AT5G13930           |             |
| Gin23194                   | 1.258           | AT5G13930           |             |
| Gin33862                   | 1.156           | AT5G13930           |             |
| Gin35437                   | 1.139           | AT5G13930           |             |
| Gin39577                   | 1.862           | AT5G13930           |             |
| Gin40074                   | 1.627           | AT5G13930           |             |
| Gin02931                   | 1.589           | AT5G07990           |             |
| Gin9638                    | 3.125           | AT5G07990           |             |
| Gin9639                    | 4.233           | AT5G07990           |             |
| Gin9641                    | 3.840           | AT5G07990           | Flavanone    |
| Gin13176                   | 4.344           | AT5G07990           | 3′-hydroxylase (F3′H) |
| Gin13177                   | 4.586           | AT5G07990           |             |
| Gin13178                   | 4.038           | AT5G07990           |             |
| Gin13179                   | 4.270           | AT5G07990           |             |
| Gin13180                   | 3.766           | AT5G07990           |             |

Table 3. GO enrichment analysis showed that, in AtMYB12-OX lines, most of the upregulated pathways are related to pathogen defense responses, including “defense response to bacterium,” “defense response to oomycetes,” “defense response to fungus,” and “innate immune response” (Figure 4). Salicylic acid (SA) and jasmonic acid (JA) are two important phytohormones that are particularly involved in pathogen defense (Yang et al., 2019; Chen et al., 2020). We observed that the SA and JA signaling pathways, including “response to salicylic acid,” “cellular response to salicylic acid stimulus,” and “response to jasmonic acid,” were activated (Figure 4 and Supplementary Table 4). In addition, a number of TF families were identified among the DEGs. In particular, members of the WRKY TFs are enriched in the DEGs (Figure 5). A growing body of research suggests that WRKY TFs are involved in pathogen resistance (Wani et al., 2021). Therefore, it is possible that AtMYB12-mediated defense responses are activated through SA and JA signaling, as well as the activation of WRKY TFs.

Pathogen defense response genes were activated in AtMYB12-OX lines

Discussion

TFs are ideal candidates for metabolic engineering because of their broad regulatory roles in metabolic pathways (Broun, 2004; Lu et al., 2016). Increasing evidence suggests that many TF functions and regulatory mechanisms are conserved across the species (Feller et al., 2011; Schlotenhofer and Yuan, 2015). For instance, ectopic expression of the maize bHLH TF Lc induces anthocyanin accumulation in tobacco and Arabidopsis (Lloyd et al., 1992). Similarly, expression of snapdragon R2R3 MYB Rosea1 and bHLH TF Delila, both driven by a fruit-specific promoter, induces anthocyanin accumulation in tomato fruit (Butelli et al., 2008). Ectopic expression of the Arabidopsis R2R3 MYB PAPI induces anthocyanin accumulation in tobacco.
FIGURE 3
Molecular analysis of *G. inflata* seedlings transiently overexpressing AtMYB12 and transactivation assay of the GiCHS1 promoter. (A) RT-PCR analysis showed AtMYB12 expression in *G. inflata* AtMYB12-OX seedlings but not in EV seedlings. The *G. inflata* actin gene (*GiActin*) served as internal control. (B) Relative expression of the two GiCHS genes in AtMYB12-OX seedlings was measured using qRT-PCR. GiActin was used as an internal control for normalization. Data are presented as the mean of three biological replicates ± SD. Asterisks indicate statistically significant differences compared with EV lines (***p* < 0.01, Student’s *t*-test). (C) Similar to the promoters of *Arabidopsis CHS*, *CHI*, and *FLS*, GiCHS1 and GuCHS1 promoters also contain the MYB recognition elements (MRE). Numbers by the ends of the DNA sequences, such as -130 at the right end of AtCHS promoter sequence, represent positions relative to translation start site. (D) The diagram in the right shows the MRE in the GiCHS1 promoter (GiCHS1-pro) and the mutated MRE sequence in the mutant promoter (GiCHS1m-pro). LUC, the reporter luciferase gene. Left panel shows transactivation of GiCHS1-pro and GiCHS1m-pro after infiltration of the promoter vector alone or in combination with the AtMYB12-expression vector into tobacco cells. Data presented as the mean of biological replicates ± SD (*n* = 3). Asterisks indicate statistically significant differences compared with EV lines (***p* < 0.01, Student’s *t*-test).
FIGURE 4
Significantly enriched GO terms in G. inflata AtMYB12-OX hairy roots. Gene Ontology (GO) analyses of differentially expressed genes (DEGs). Upregulated GO terms are colored in red while downregulated terms are in blue. Each circle represents one GO term. The circle size represents the number of genes in each GO category while the color represents the significance level. Description of the upregulated GO terms (from top to bottom): GO:0042742, defense response to bacterium; GO:0071446, cellular response to salicylic acid stimulus; GO:0044550, secondary metabolite biosynthetic process; GO:0009751, response to salicylic acid; GO:0009753, response to jasmonic acid; GO:0071229, cellular response to acid chemical; GO:0014070, response to organic cyclic compound; GO:0098542, defense response to other organism; GO:1900426, positive regulation of defense response to bacterium. Description of the downregulated GO terms (from top to bottom): GO:0006811, ion transport; GO:0030243, cellulose metabolic process; GO:0045229, external encapsulating structure organization; GO:0009834, plant-type secondary cell wall biogenesis; GO:1901348, positive regulation of secondary cell wall biogenesis; GO:0071669, plant-type cell wall organization or biogenesis; GO:0050832, defense response to fungus; GO:0071554, cell wall organization or biogenesis.

serves as a precursor for diverse sets of flavonoids (Dao et al., 2011). Our RNA-seq data showed that expression of 12 G. inflata CHSs is upregulated by AtMYB12 (Figure 2A and Table 1).

Agrobacterium-mediated transient transformation of whole seedlings has been used to study the regulation of metabolic pathways in different plant species, such as Catharanthus roseus (Liu et al., 2019; Mortensen et al., 2019). We transiently transformed G. inflata seedlings with Agrobacterium harboring AtMYB12 and measured the expression of selected CHSs. Similar to the hairy roots, expression of two selected GiCHSs was significantly induced in seedlings transformed with AtMYB12 (Figure 3B). We demonstrated that AtMYB12 activates the GiCHS1 promoter in plant cells by binding
TABLE 2  Cellulose synthase A (CESA) genes identified in DEGs.

| Glycyrrhiza inflata gene ID | Log2 fold change | Arabidopsis homolog | Description |
|---------------------------|------------------|---------------------|-------------|
| Gin23691                  | −1.032           | AT4G39350           | Cellulose synthase A2 (CESA2) |
| Gin14785                  | −1.563           | AT5G44030           | Cellulose synthase A4 (CESA4) |
| Gin34862                  | −1.408           | AT5G44030           | Cellulose synthase A4 (CESA4) |
| Gin05128                  | −1.259           | AT5G17420           | Cellulose synthase A7 (CESA7) |
| Gin32817                  | −1.666           | AT5G17420           | Cellulose synthase A7 (CESA7) |
| Gin06050                  | −3.691           | AT4G18780           | Cellulose synthase A8 (CESA8) |
| Gin19934                  | −1.230           | AT4G18780           | Cellulose synthase A8 (CESA8) |

In tomato fruits, other than increased accumulation of flavonoids, ectopic expression of AtMYB12 decreases the contents of carbon resources, including glucose and fructose (Zhang et al., 2015). Genes in the primary metabolic pathways were mostly induced by AtMYB12 (Zhang et al., 2015). Our results confirmed the induction of the genes in the primary pathways upstream of flavonoids (Supplementary Table 3). We also found that the genes related to cellulose synthesis were downregulated (Figure 4 and Table 2), suggesting that, in both tomato fruits (Zhang et al., 2015) and G. inflata hairy roots, AtMYB12 redirects carbon flux from other carbohydrate resources toward the flavonoid pathway. Therefore, we suggest that the functionally conserved nature of AtMYB12 makes it a promising candidate for metabolic engineering in other plant species.

AtMYB12 overexpression improves pathogen resistance in transgenic tobacco plants (Ding et al., 2021). In transgenic tobacco, AtMYB12 induces the production of flavonoid compounds, such as rutin, as well as reactive oxygen species, H₂O₂, and NO (Pandey et al., 2015; Ding et al., 2021). Similar to tobacco, our results revealed that the genes involved in pathogen resistance pathways (Figure 4), defense-associated plant hormone signaling (Figure 4), and WRKY TFs (Figure 5) were significantly enriched in AtMYB12-OX hairy roots. SA and JA play essential roles in plant defense against different pathogens (Yang et al., 2019; Chen et al., 2020). WRKY TFs are among the largest families of transcriptional regulators and contribute to various plant processes, including disease defense (Wani et al., 2021). Some WRKYs like AtWRKY33 could regulate SA/JA biosynthesis while others are regulated by SA/JA signaling (Birkenbihl et al., 2012; Wani et al., 2021). We speculate that AtMYB12 activated the SA/JA-WRKY network that contributes to the pathogen defense responses in G. inflata hairy roots.

Metabolic engineering offers an excellent approach for producing various bioactive, health-promoting phytochemicals.
in plants. This study underscores the importance of metabolic engineering for enhancing accumulation of valuable metabolites, such as licorice chalcones in *G. inflata*, through heterologous expression of a known flavonoid regulator. Metabolic pathways of non-model plants are relatively less studied. Transcriptomic and genomic resources will help unravel the biosynthetic pathways in non-model plants, such as *G. inflata*, and aid the bioengineering of bioactive compounds.

**Data availability statement**

The data presented in the study are deposited in the NCBI repository, accession number: PRJNA842240.

**Author contributions**

ZW and YLL performed the experiments, analyzed the data, and wrote the article. SS analyzed the RNA-seq data. RL, SP, YW, YQL, and LY evaluated the experiments and revised the article. YW, YQL, and LY initiated and supervised the project. All authors contributed to the article and approved the submitted version.

**Funding**

This study was partially supported by the National Key R&D Program of China (2019YFC1711100), by the grant nos. 201853000241001 and 201953000241005 from the Yunnan Tobacco Company, Harold R. Burton Endowed Professorship to LY and the Kentucky Tobacco Research and Development Center.

**Acknowledgments**

We thank Megan Combs (Department of Civil Engineering and Environmental Research Training Laboratories, University of Kentucky) for assistance on LC-MS/MS analyses.

**Conflict of interest**

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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**Supplementary material**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.932594/full#supplementary-material

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