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AabHLH112, a bHLH transcription factor, positively regulates sesquiterpenes biosynthesis in *Artemisia annua*

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The bHLH transcription factors play important roles in the regulation of plant growth, development, and secondary metabolism. β -Caryophyllene, *epi*-cedrol, and β -farnesene, three kinds of sesquiterpenes mainly found in plants, are widely used as spice in the food industry and biological pesticides in agricultural production. Furthermore, they also have a significant value in the pharmaceutical industry. However, there is currently a lack of knowledge on the function of bHLH family TFs in β -caryophyllene, *epi*-cedrol, and β -farnesene biosynthesis. Here, we found that AabHLH112 transcription factor had a novel function to positively regulate β -caryophyllene, *epi*-cedrol, and β -farnesene biosynthesis in *Artemisia annua*. Exogenous MeJA enhanced the expression of AabHLH112 and genes of β -caryophyllene synthase (*CPS*), *epi*-cedrol synthase (*ECS*), and β -farnesene synthase (*BFS*), as well as sesquiterpenes content. Dual-LUC assay showed the activation of Aa*CPS*, Aa*ECS*, and Aa*BFS* promoters were enhanced by AabHLH112. Yeast one-hybrid assay showed AabHLH112 could bind to the G-box (CANNTG) *cis*-element in promoters of both Aa*CPS* and Aa*ECS*. In addition, overexpression of AabHLH112 in *A. annua* significantly elevated the expression levels of Aa*CPS*, Aa*ECS*, and Aa*BFS* as well as the contents of β -caryophyllene, *epi*-cedrol, and β -farnesene, while suppressing AabHLH112 expression by RNAi reduced the expression of the three genes and the contents of the three sesquiterpenes. These results suggested that AabHLH112 is a positive regulator of β -caryophyllene, *epi*-cedrol, and β -farnesene biosynthesis in *A. annua*.

KEYWORDS

Artemisia annua, AabHLH112, β -caryophyllene, *epi*-cedrol, β -farnesene, metabolic regulation

Introduction

Artemisia annua, a traditional Chinese medicinal plant, not only produces the well-known artemisinin, but also produces a number of sesquiterpenes that play an important role in plants' stress resistance, communication, and growth regulation (Chadwick et al., 2013). β -caryophyllene, *epi*-cedrol, and β -farnesene, three kinds of sesquiterpenes, are used as spice in the food industry and biological pesticides in agricultural production (Yu X. et al., 2012; Cheng et al., 2022). Besides, they also have a significant value in the pharmaceutical industry (Cheng et al., 2022). β -Caryophyllene was found to have good curative effect on colitis (Bento et al., 2011), cerebral ischemia (Chang et al., 2013), diabetes (Basha and Sankaranarayanan, 2014), anxiety and depression (Bahi et al., 2014), liver fibrosis (Mahmoud et al., 2014), and osteoarthritis (Rufino et al., 2015). In cancer studies, β -caryophyllene demonstrated synergy with the chemotherapy drug paclitaxel on human tumor cell lines, and alone it stimulates apoptosis and suppresses tumor growth (Legault and Pichette, 2007). *epi*-Cedrol is widely used in pharmaceutical industry for its sedative, anti-inflammatory, and cytotoxic activities (Zhang et al., 2016; Luo et al., 2019). β -farnesene, an important acyclic and volatile sesquiterpenes acting as the main component of the aphid alarm pheromones for many pest aphids, can be used to create new biological pesticides (Wang et al., 2014). Because of their pharmacological activity and commercial value, numerous studies have focused on improving the production of sesquiterpenes in *A. annua*.

β -Caryophyllene, *epi*-cedrol, and β -farnesene are biosynthesized via both the cytoplasmic mevalonate (MVA) pathway and plastidial methylerythritol phosphate (MEP) pathway (Navale et al., 2019). Plant cells use one molecule of dimethylallyl diphosphate (DMAPP) and two molecules of isopentenyl diphosphate (IPP) to produce farnesyl diphosphate (FPP) in a reaction catalyzed by FPP synthase (FPS) (Navale et al., 2019). Then the sesquiterpene synthase takes FPP as the substrate to synthesize a variety of sesquiterpenes (Figure 1).

Transcription factors (TFs) are sequence-specific DNA-binding proteins which interact with the promoter regions of target genes and regulate their transcription (Yang et al., 2012). It is a useful tool to improve the production of secondary metabolites in pharmaceutically important plants, such as flavonoids or alkaloids (Gantet and Memelink, 2002). Recently, a number of AP2/ERF, WRKY, and bHLH transcription factors have been found to have global regulatory functions in pharmaceutical terpenoids biosynthesis (Lu et al., 2016; Li et al., 2017). In *A. annua*, overexpression of *AaERF1* and *AaERF2* significantly promoted the transcription of *ADS* and *CYP71AV1*, resulting in an increase of artemisinin content (Yu Z. et al., 2012). Overexpression of *SmERF1L1* significantly increased tanshinones production in transgenic *Salvia miltiorrhiza* hairy roots (Huang et al., 2019). In addition, overexpression of *SmWRKY1* significantly elevated

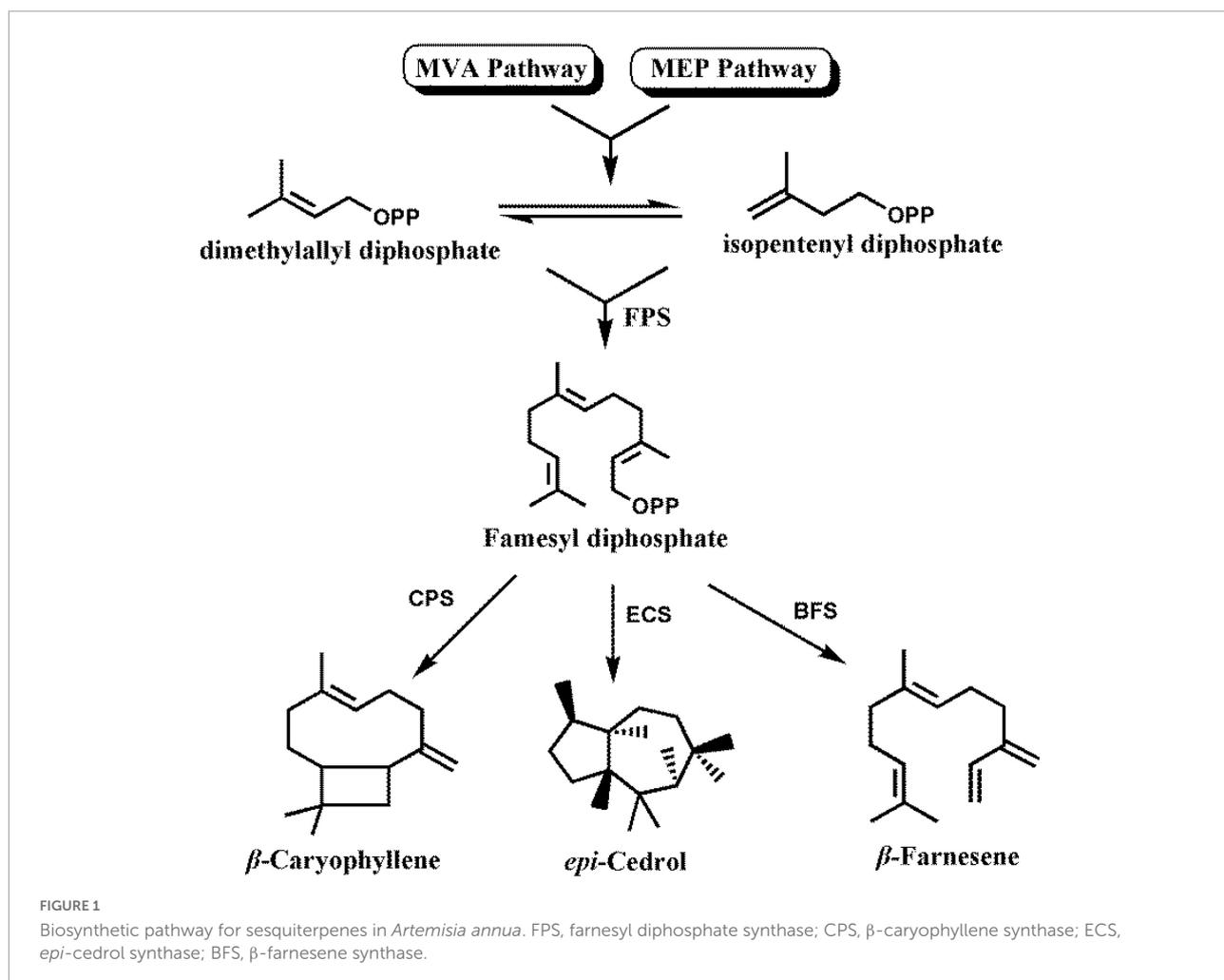
the transcription of *SmDXS* and *SmDXR*, increasing tanshinone production in *S. miltiorrhiza* (Cao et al., 2018).

The bHLH family is the second largest transcription factor family in plants, which plays an important role in the regulation of pharmaceutical terpenoids biosynthesis (Ji et al., 2014). It was reported that *SmbHHLH3* acts as a transcription repressor for both phenolic acids and tanshinones biosynthesis in *S. miltiorrhiza* hairy roots (Zhang et al., 2020). In *Arabidopsis*, AtMYC2, a JA-responsive TF, can enhance sesquiterpenes biosynthesis by binding to the promoters of *TPS11* and *TPS21* (Hong et al., 2012). Chuang et al. (2018) reported that overexpression of *PbbHHLH4* markedly increased terpenes content in *Phalaenopsis*. JA-responsive *SlMYC1* differentially regulates monoterpene and sesquiterpenes biosynthesis in tomato, which positively regulates monoterpene biosynthesis in leaf and stem but negatively regulates sesquiterpenes biosynthesis in stem (Xu et al., 2018). *CrMYC2* directly activates the expression of ORCA genes through binding to the G-box in its promoter, and promotes vinblastine and vincristine biosynthesis in *Catharanthus roseus* (Zhang et al., 2011). *BpMYC4* and *BpbHHLH9* from *Betula platyphylla* Suk are involved in regulating the biosynthesis of triterpenes (Yin et al., 2017). Furthermore, studies also showed that bHLH TFs could act as negative regulators in terpenes biosynthesis, including the biosynthesis of the diterpene paclitaxel and monoterpene indole alkaloid in *Taxus cuspidata* (Lenka et al., 2015) and *Catharanthus roseus* (Patra et al., 2018), respectively. One hundred and twenty two putative bHLH TFs were found in *A. annua* by genome-wide identification (Xiang et al., 2019) and only three bHLH TFs—*AabHHLH1*, *AabHHLH12*, and *AaMYC2*, respectively, have been reported to play regulatory roles in artemisinin biosynthesis (Ji et al., 2014; Shen et al., 2016; Xiang et al., 2019). Now, although our knowledge of the regulation of artemisinin biosynthesis is increasing, little is known about the regulation of non-artemisinin sesquiterpenes biosynthesis in *A. annua*. In this study, we found that the *AabHHLH12* has a novel function in improving the accumulation of β -caryophyllene, *epi*-cedrol, and β -farnesene in *A. annua*, and revealed the mechanism of *AabHHLH12*-mediated upregulation of sesquiterpenes biosynthesis. Our results suggested that this transcription factor could be a useful tool to improve sesquiterpenes production in *A. annua*.

Materials and methods

Plant material and growth conditions

Seeds were harvested from wild-type *A. annua* grown in the experimental field of Southwest University (Chongqing, China) for this study. These seeds were surface-sterilized with 20% sodium hypochlorite solution for 20 min, and then washed three times with sterile water. Subsequently, seeds were germinated on



1/2 MS solid medium at $23 \pm 2^\circ\text{C}$ under a light period of 16-h-light/8-h-dark. All seedlings were grown in pots with organic substrates in an artificial climate room at $23 \pm 2^\circ\text{C}$ under a light period of 16-h-light/8-h-dark. *Nicotiana benthamiana* seeds were sown directly on solid and their growth conditions are consistent with *A. annua*. *Nicotiana benthamiana* plants grown for 30 days were used for dual-luciferase assays.

Exogenous methyl jasmonate treatment and sample collection

One-month old wild-type *A. annua* plants were treated according to the report (Xiang et al., 2015) with modifications. 300 μM exogenous MeJA solutions containing 0.4% ethanol was sprayed on the plant surfaces. Then the leaves of *A. annua* plants at 0, 3, 6, 12, and 24 h after exogenous MeJA treatment were collected respectively and then put in liquid nitrogen immediately for RNA extraction and determination of sesquiterpenes contents.

Artemisia annua transformation

The coding sequence of *AabHLH112* was amplified and inserted into the pHB binary plasmid vector through *Bam*HI and *Pst*I sites to construct the plant overexpression vector, pHB-AabHLH112. Besides, the 352bp fragment from the *AabHLH112* was amplified and inserted into the intermediate pHANNIBAL vector, and then the expression cassette was recombined into the pBin19 plasmid for constructing the *AabHLH112*-RNAi vector. pHB-AabHLH112 and pBin19-RNAi-AabHLH112 vectors were transferred into *A. tumefaciens* strain EHA105 to form engineering strains. These strains were grown on YEP solid medium containing related antibiotics for 48 h. Subsequently, positive monoclonal strain was inoculated into YEP liquid medium containing related antibiotics to culture $\text{OD}_{600} = 0.5\sim 0.6$. The supernatant was discarded after centrifugation, and then resuspended in the 1/2 MS liquid to $\text{OD}_{600} = 0.3\sim 0.5$. Then the culture was shaken at 200 rpm/ 28°C for 30 min. Cultured engineered strains were used to transform *A. annua* via *Agrobacterium*-mediated

transformation as described previously (Xiang et al., 2019). After that, the transformed seedlings were transplanted into pots with organic substrates and cultured in an artificial climate room at $23 \pm 2^\circ\text{C}$ under a light period of 16 h-light/8 h-dark.

Quantitative real-time polymerase chain reaction

Quantitative real-time PCR was performed to analyze genes' expression in this study. Total RNA of root, stem, leaf and flower was extracted using the total plant RNA Extract Kit (Tiangen, China), and then reversely transcribed into cDNA using Fast King RT Kit (Tiangen, China). Subsequently, the cDNA was used as the template for detecting the expression levels of *AabHLH112* and sesquiterpene synthase genes *AaCPS*, *AaECS*, and *AaBFS* by qPCR experiment. The qPCR amplification conditions were 95°C for 3 min, followed by 40 cycles of 95°C for 15 s, 55°C for 20 s, and 72°C for 20 s. β -actin of *A. annua* was used as the reference gene in this study (Ma et al., 2018), and the relative expression levels were calculated using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001). In addition, the expression levels of sesquiterpene-related genes were detected in *AabHLH112*-overexpressing and RNAi-*AabHLH112* transgenic *A. annua* using the above method. The primer sequences in qPCR experiment were listed in **Supplementary Table 1**.

Plant sesquiterpenes extraction and gas chromatography-mass spectrometry analysis

The sesquiterpenes of *A. annua* leaves were analyzed by gas chromatography-mass spectrometry (GC-MS) as described before with some modifications (Fu et al., 2017). The fresh leaves 1–8 from the main stem of 3 months old *AabHLH112*-overexpressing transgenic and wild-type *A. annua* plants were harvested, frozen immediately in liquid nitrogen, and then freeze-dried for 72 h at -80°C . 100 mg leaf powder was soaked in 4 mL *n*-hexane in 15 mL centrifuge tube with 35 μL trans-farnesol (80 $\mu\text{g}/\text{mL}$) as the internal standard, and ultrasonically extracted for 45 min at 28°C in an ultrasonic processor (KQ-500DE; Kunshan Ultrasonic Instrument Co. Ltd., Kunshan, China). Plant extract was centrifuged at 1,000 rpm for 10 min. The supernatant was filtered through 0.22 μm pore size filters and then analyzed by gas chromatography-mass spectrometry (GCMS-QP2010 Ultra; Shimadzu) with the temperature program: initial temperature of 70°C (1 min hold), increase to 160°C at $10^\circ\text{C}/\text{min}$, ramp to 240°C at $5^\circ\text{C}/\text{min}$, and finally increase to 280°C at $20^\circ\text{C}/\text{min}$ (17 min hold). Helium was used as a carrier gas and 1 μL sample was injected in split mode; split rate, 2:1; ion source temperature, 230°C ; ionization voltage,

70 eV with scanning from m/z 33 to 500. Qualitative analysis of all compounds was fulfilled by comparing with NIST (National Institute of Standards and Technology) database and Wiley libraries. The relative contents of sesquiterpenes were calculated by comparing peak areas with that of the internal standards. Trans-farnesol was purchased from Sigma-Aldrich in this study.

Cloning and analysis of the promoter regions of sesquiterpene synthase genes

To isolate the promoter fragments of sesquiterpene synthase genes (*AaCPS*, *AaECS*, and *AaBFS*), the total DNA of *A. annua* leaves was extracted as the template using cetyltrimethylammonium bromide (CTAB) method. All primer sequences were designed according to the genome of *A. annua*. To avoid non-specific amplification, forward and reverse primer sequences are located in their promoter and ORF regions, respectively. These promoter fragments were obtained by nested PCR using the Pro Taq DNA polymerase (Aikerui, Changsha, China) according to the manufacturer's instructions. Agarose gel electrophoresis was used to detect PCR products. Subsequently, these fragments were inserted into the pJET2.1 vector (Thermo Fisher Scientific, Waltham, MA, United States) and sequenced respectively. The *cis*-elements of promoter region in *AaCPS*, *AaECS*, and *AaBFS* were predicted using plantCARE online website.¹ The promoter sequences of sesquiterpene synthase genes were shown in **Supplementary Data Sheet 1**. Primers were listed in **Supplementary Table 1**.

Dual-luciferase assay

Dual-LUC assays were performed using the methods reported previously (Xiang et al., 2019). The promoter sequences of *AaCPS* (OP056317), *AaECS* (OP056318), and *AaBFS* (OP056319) genes were inserted into pGreenII 0800-LUC plasmid to generate pAaCPS:LUC, pAaECS:LUC, and pAaBFS:LUC constructs as the reporter vector, respectively. Subsequently, these reporter vectors were transferred into *A. tumefaciens* strain GV3101 together with the pSoup plasmid. The *AabHLH112* (MG872820) was inserted into the pHB plasmid driven by CaMV 35S promoter as the effector vector and transferred into *A. tumefaciens* strain GV3101. Meanwhile, the pHB-YFP plasmid was transferred into GV3101 as a negative control. All engineering and control strains were inoculated into YEP liquid selective medium and cultured overnight at 28°C . The agrobacterium cells were

¹ <http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>

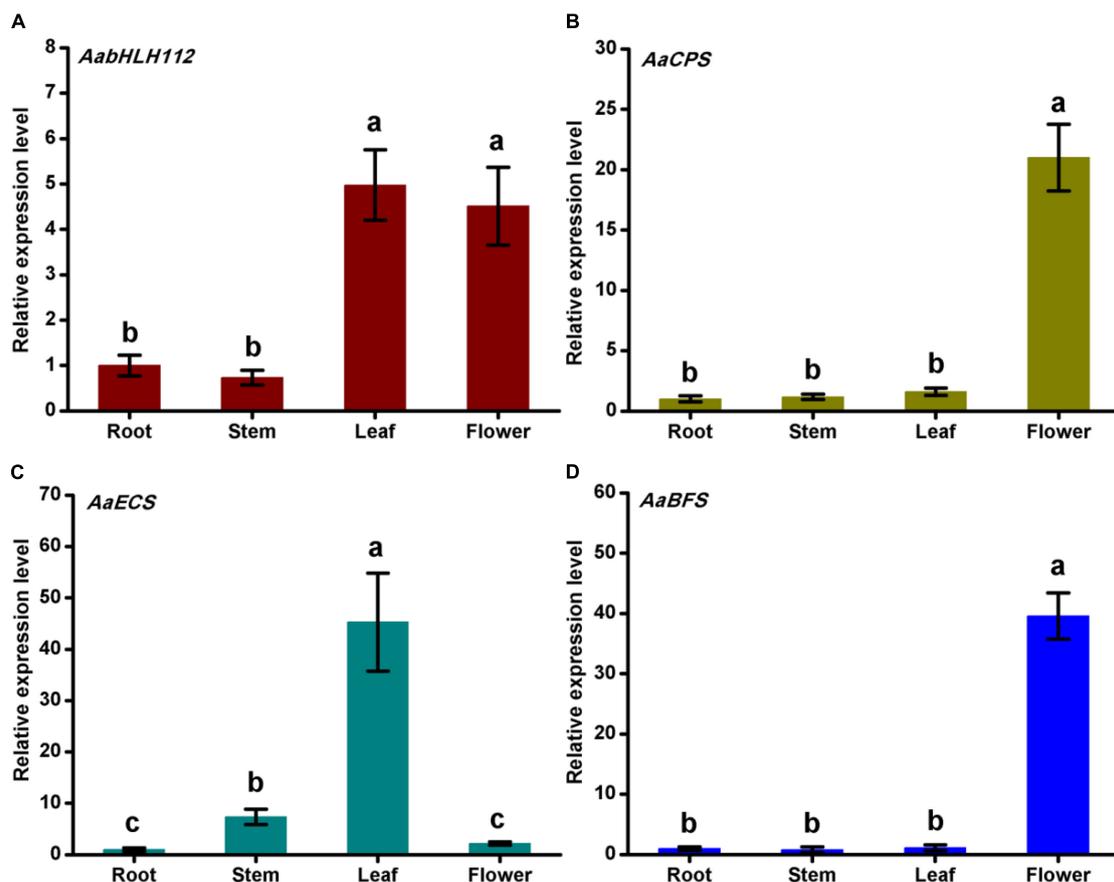


FIGURE 2

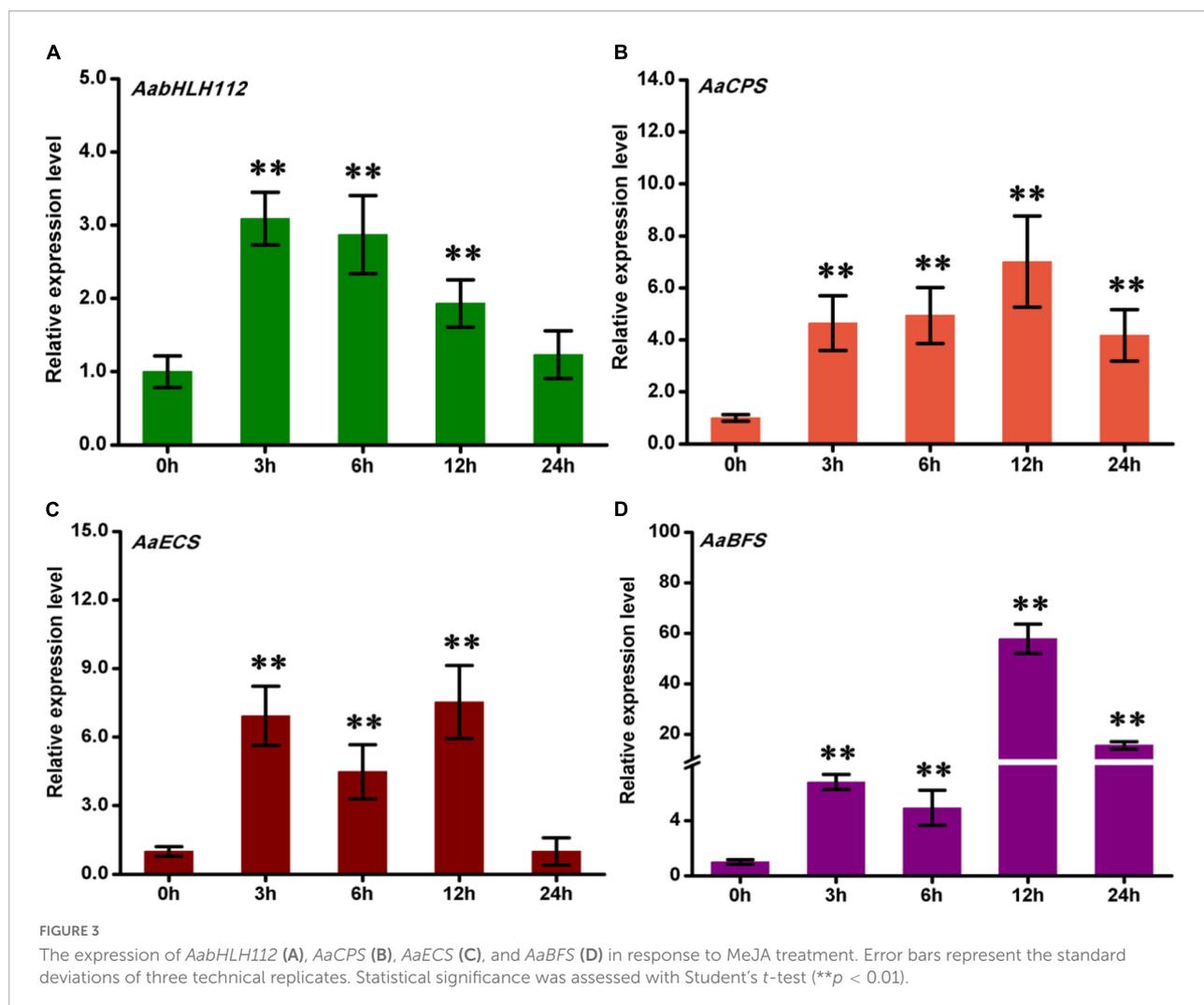
Genes expression levels of *AabHLH112* (A), *AaCPS* (B), *AaECS* (C), and *AaBFS* (D) in root, stem, leaf, and flower of *Artemisia annua*. Bars are means \pm SD from three independent biological replicates. One-way ANOVA was tested for significant differences among the means (indicated by different letters at $p < 0.01$).

collected by centrifuge at 4,500 rpm for 10 min and resuspended in the MS liquid to $OD_{600} = 0.6 \pm 0.05$. The acetosyringone (As, 100 mM, 1:500, v:v) and 2-(*N*-morpholino) ethanesulfonic acid [MES, 0.5 M (pH = 5.7), 1:50, v:v] were added to the resuspension and then were injected into tobacco leaves after being placed for 4 h at room temperature. Tobacco plants injected with agrobacterium cells were exposed to weak light for 48–72 h. Infiltration and detection were performed as described previously (Ma et al., 2018). All experiments were repeated five times for each combination. Primers are listed in [Supplementary Table 1](#).

Yeast one-hybrid assay

To investigate how *AabHLH112* regulates the expression of *AaCPS*, *AaECS*, and *AaBFS*, yeast one-hybrid assays were fulfilled as described previously (Xiang et al., 2019). The coding sequence of *AabHLH112* was amplified and

inserted into pB42AD plasmid with the GAL4 activation domain (AD) through *EcoRI* and *XhoI* sites to generate pB42AD-*AabHLH112* constructs as the prey. The 45~55 bp fragments containing one G-box motifs from *AaCPS*, *AaECS*, and *AaBFS* promoters, named pAaCPS-G1 (–884~–840), pAaCPS-G2 (–838~–794), pAaCPS-G3 (–806~–762), pAaCPS-G4 (–430~–386), pAaECS-G1 (–1228~–1178), pAaECS-G2 (–915~–866), pAaECS-G3 (–385~–336), pAaBFS-G1 (–1045~–996), pAaBFS-G2 (–985~–935), pAaBFS-G3 (–66~–15) and the mutant of G-box were inserted into pLacZ plasmid through *KpnI* and *XhoI* sites as the bait, respectively. The pB42AD-*AabHLH112* plasmid was co-transformed into yeast strain EGY48 with each individually bait constructs, respectively. The yeast cells were grown on SD-Ura-Trp selective medium for 48 h at 30°C. All independent yeast cells were shifted into SD-Ura-Trp liquid medium and cultured overnight at 30°C, and then these cells were collected by microcentrifugation and resuspended in 100 μ L sterile water. Resuspended cells were grown on SD/-Ura-Trp medium



with 5-Bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal) for 24–48 h at 30°C. The empty pB42AD and pLacZ plasmids were used as the negative control. Five independent biological replicates were implemented for each experiment in this study. The primers are listed in [Supplementary Table 1](#).

Results

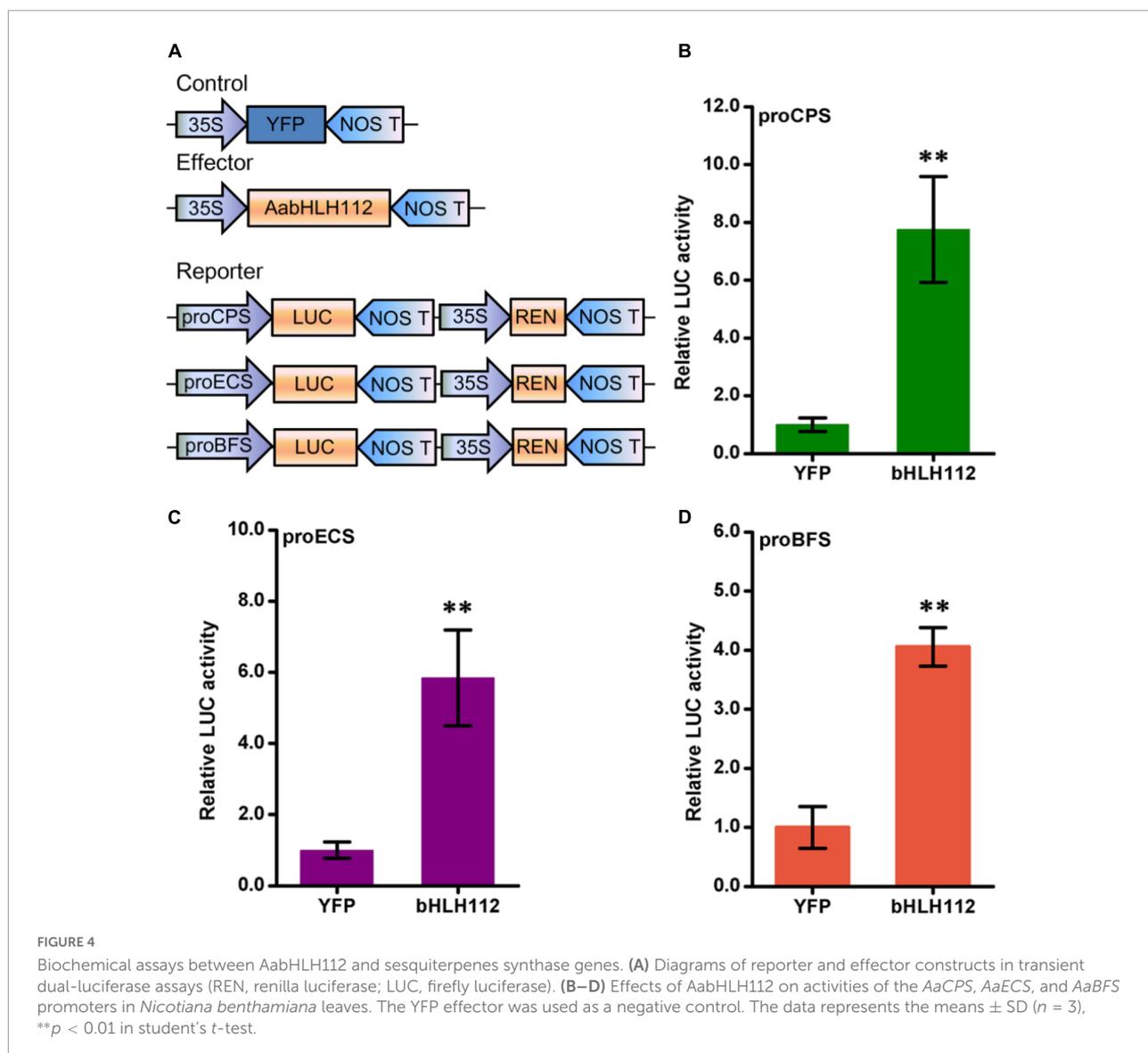
Tissues expression patterns of *AabHLH112* and three sesquiterpene synthase genes

To study the tissue expression patterns of *AabHLH112* and sesquiterpene biosynthesis genes, we measured their expression in various *A. annua* tissues including roots, stems, leaves, and flowers. *AabHLH112* was highly expressed in flowers and leaves, and lowly expressed in roots and stems ([Figure 2A](#)).

AaCPS and *AaBFS* genes' expression has a tissue-specific mode, with a high expression in flowers and low expression in leaves, stems, and roots ([Figures 2B,D](#)). By comparison, *AaECS* was highly expressed in leaves and lowly expressed in roots and flowers ([Figure 2C](#)). Together, *AabHLH112*, *AaCPS*, *AaECS*, and *AaBFS* were expressed in all of the detected tissues.

Methyl jasmonate treatment induced the expression of *AabHLH112* and three sesquiterpene synthase genes

The phytohormone JA plays a critical role in plant stress resistance and secondary metabolism ([Wasternack and Song, 2017](#); [Kianersi et al., 2021](#)). To investigate the response of *AabHLH112*, *AaCPS*, *AaECS*, and *AaBFS* to MeJA treatment, wild type *A. annua* plants were treated with exogenous MeJA, and all the genes' expression was

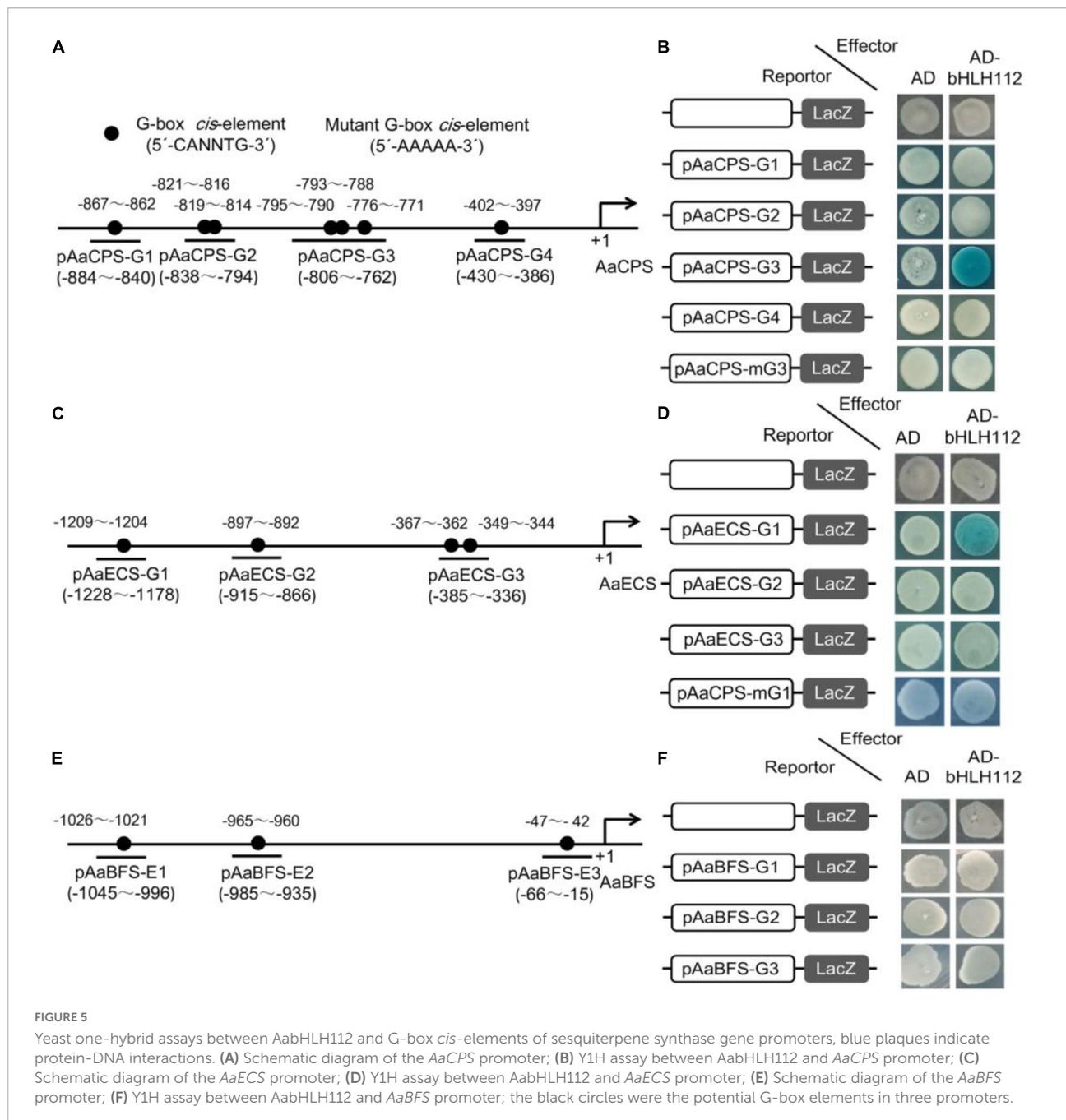


quantified by qPCR. The expression of *AabHLH112* was induced by MeJA, rapidly increased and peaked after 3 h of treatment, followed by a gradual decrease (Figure 3A). This result indicated that *AabHLH112* is involved in MeJA response in *A. annua*, as in the case with the *Arabidopsis* homologous *AtbHLH33* (Li et al., 2020). Several studies have shown that the expression of many genes involved in terpenes biosynthesis is significantly increased under JA treatment. Here, we found the expression levels of *AaECS*, *AaCPS*, and *AaBFS* were significantly upregulated under exogenous MeJA treatment (Figures 3B–D); besides, the accumulation of β -caryophyllene, *epi*-cedrol, and β -farnesene, direct products of the three enzymes, also showed an upward trend after MeJA treatment (Supplementary Figure 1). These results suggest that MeJA can induce the expression of these sesquiterpenes synthase genes, thus enhancing the

biosynthesis of β -caryophyllene, *epi*-cedrol and β -farnesene in *A. annua*.

AabHLH112 activates the expression of the *AaECS*, *AaCPS*, and *AaBFS*

Dual-LUC assays can be used to study the transcriptional regulation of transcription factors on target genes at the transcriptional level (Ma et al., 2018). The coding sequence of *AabHLH112* was inserted into the plant overexpression vector pHB as an effector construct, and the promoter sequences of *AaCPS* (Supplementary Figure 2), *AaECS* (Supplementary Figure 3), and *AaBFS* (Supplementary Figure 4) genes were inserted into pGreenII 0800-LUC plasmid to generate pAaCPS:LUC, pAaECS:LUC, and pAaBFS:LUC constructs as



the reporter vector, respectively. Meanwhile, the pHB-YFP (yellow fluorescent protein) plasmid was used as a negative control (Figure 4A). The effector and reporter constructs were transiently expressed in *N. benthamiana* leaves using *Agrobacterium tumefaciens*-mediated co-infiltration. The dual-LUC assay results showed that the promoter activity of the above sesquiterpene synthase genes *AaCPS*, *AaECS*, and *AaBFS* was significantly increased, with the LUC/REN value increased by 7.75, 5.58, and 4.05-folds than the YFP control (Figures 4B–D), respectively.

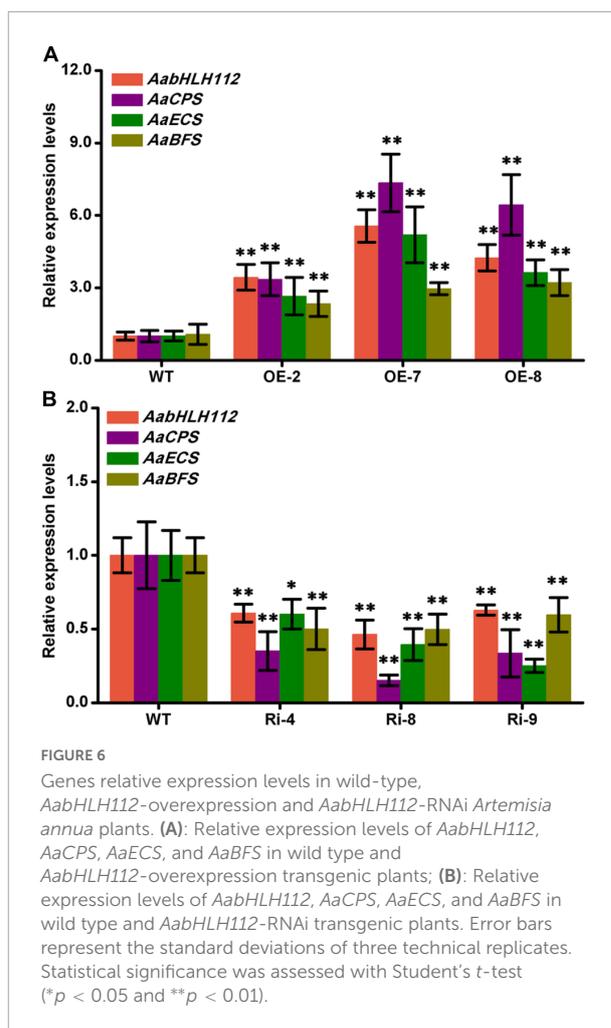
AabHLH112 bind to G-box *cis*-elements in the promoters of *AaCPS* and *AaECS*

The core DNA sequence motif recognized by bHLH proteins is a consensus hexanucleotide sequence known as the G-box (5-CANNTG-3). It was previously reported that AaMYC2 can specifically bind to the G-box *cis*-elements in the promoters of key enzyme genes of artemisinin biosynthesis, including *AaCYP71AV1* and *AaDBR2* (Shen et al., 2016).

Recently, Xiang et al. (2019) reported that AabHLH112 can directly bind to G-box in the *AaERF1* promoter and promote artemisinin biosynthesis. The above dual-LUC results showed that AabHLH112 could activate the transcription of *AaCPS*, *AaECS*, and *AaBFS*. In order to further investigate how AabHLH112 regulates the transcription of these sesquiterpene synthase genes, the promoter sequences of *AaCPS*, *AaECS*, and *AaBFS* were analysed by PlantCARE online website, and multiple G-box motifs were found in each promoter (Figures 5A,C,E). Following that, yeast one-hybrid assay was used to evaluate whether AabHLH112 can directly bind to the G-box motifs in these promoters. 45 50 bp fragments containing G-box motifs from each promoter were inserted into pLacZ plasmid as the bait, and *AabHLH112* was inserted into pB42AD plasmid with the GAL4 activation domain to generate pB42AD-*AabHLH112* constructs as the prey. The results indicated that AabHLH112 can directly bind to pAaCPS-G3 and pAaECS-G1 fragments containing the G-box in *AaCPS* and *AaECS* promoters (Figures 5B,D), but not the pAaCPS1-G1, pAaCPS-G2, pAaCPS-G4, pAaECS-G2, pAaECS-G3, and pAaBFS-G1, pAaBFS-G2, pAaBFS-G3 fragments. When the G-box *cis*-elements in the pAaCPS-G3 and pAaECS-G1 fragments were mutated to the 5'-AAAAA-3' sequences resulting in pAaCPS-mG3 and pAaECS-mG1, respectively, the binding signals of AabHLH112 and pAaCPS-mG3 and pAaECS-mG1 disappeared (Figures 5B,D). These results demonstrate that AabHLH112 enhances *AaCPS* and *AaECS* transcription by directly binding to the specific G-box *cis*-elements in their promoters.

Generation of *AabHLH112*-overexpression and RNAi transgenic *Artemisia annua*

To better explore the function of *AabHLH112* in sesquiterpenes biosynthesis in *A. annua*, *AabHLH112*-overexpressing and *AabHLH112*-RNAi transgenic *A. annua* plants were generated (Supplementary Figure 5). Primers designed according to the sequences of rubisco gene (*rbc*), *AabHLH112* and marker gene (hygromycin-resistant) were used to determine the regeneration plants by genomic PCR. The 877bp fragment of the hygromycin-resistant gene and coding sequence of *AabHLH112* with partial *rbc* terminator (1383bp) were specifically amplified from the transformed plants and the positive control (pHB-*AabHLH112*), while these specific fragments could not be amplified from wild-type plants (Supplementary Figures 5B,C). Besides, *AabHLH112*-RNAi transgenic plants were also generated and detected by genomic PCR. Specific detection primers were designed according to the sequences of *AabHLH112*, *OCS* terminator and *NptII* (kanamycin) genes. As shown in Supplementary Figures 5D,E, the partial *AabHLH112* sequence and *NptII* gene were detected in the *AabHLH112*-RNAi transgenic plants.



AabHLH112 positively regulates the expression of *AaCPS*, *AaECS*, and *AaBFS* in *Artemisia annua*

The expression level of *AabHLH112* in transgenic *A. annua* lines was determined by qPCR. Compared with the wild type (WT) *A. annua*, *AabHLH112* presented higher expression level in *AabHLH112*-overexpression lines of OE-2, OE-7, and OE-8 with 3.44-fold, 5.56-fold, and 4.24-fold higher, respectively (Figure 6A). Meanwhile, the expression levels of *AaCPS*, *AaECS*, and *AaBFS* in the three lines (OE-2, OE-7, and OE-8) were approximately 3.35-7.34 fold, 2.65-9.15 fold, and 2.24-2.55 fold higher compared to the WT respectively (Figure 6A). On the contrary, The expression level of *AabHLH112* were markedly decreased in *AabHLH112*-RNAi lines of Ri-4, Ri-8, and Ri-9 (Figure 6B), and the expression of *AaCPS*, *AaECS*, and *AaBFS* was also significantly reduced (Figure 6B) in the three *AabHLH112*-RNAi lines compared to WT plants. These results suggest that *AabHLH112* positively regulates the expression of *AaCPS*, *AaECS* and *AaBFS* in *A. annua*.

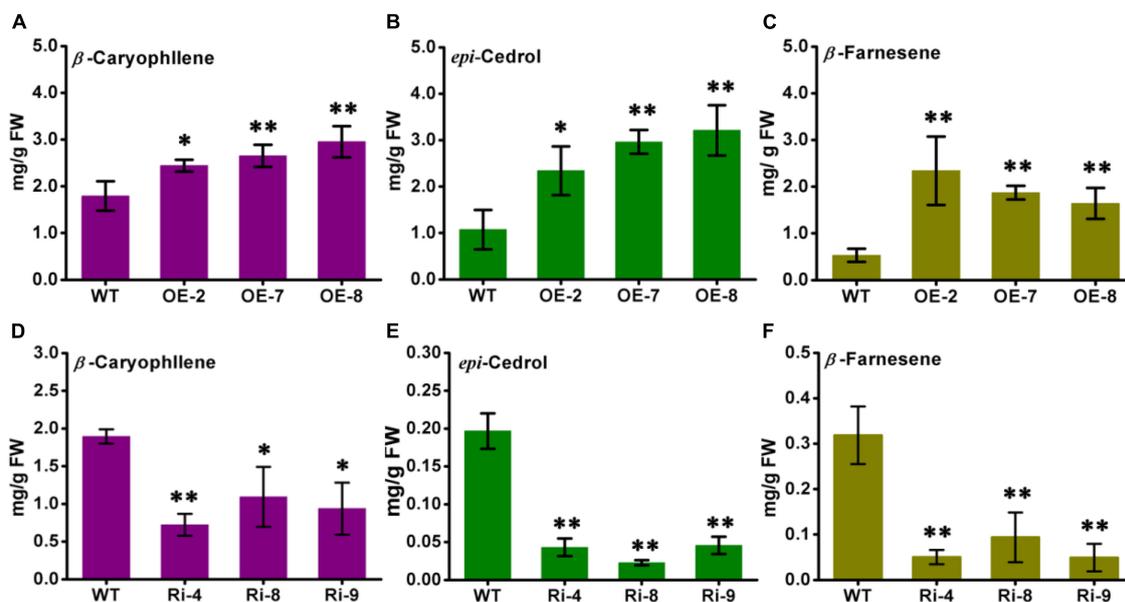


FIGURE 7

The contents of β -caryophyllene, *epi*-cedrol, and β -farnesene in wild-type, *AabHLH112*-overexpression, and *AabHLH112*-RNAi *Artemisia annua* plants. (A) β -caryophyllene, (B) *epi*-cedrol, and (C) β -farnesene in *AabHLH112*-overexpressing lines, WT, wild type; OE-2, OE-7 and OE-8 are independent lines of *AabHLH112*-overexpressing lines; (D) β -caryophyllene, (E) *epi*-cedrol and (F) β -farnesene in *AabHLH112*-RNAi lines, WT, wild type; Ri-4, Ri-8, and Ri-9 are independent lines of *AabHLH112*-RNAi lines, the data represents the means \pm SD ($n = 3$), * $p < 0.05$, ** $p < 0.01$ in student's *t*-test.

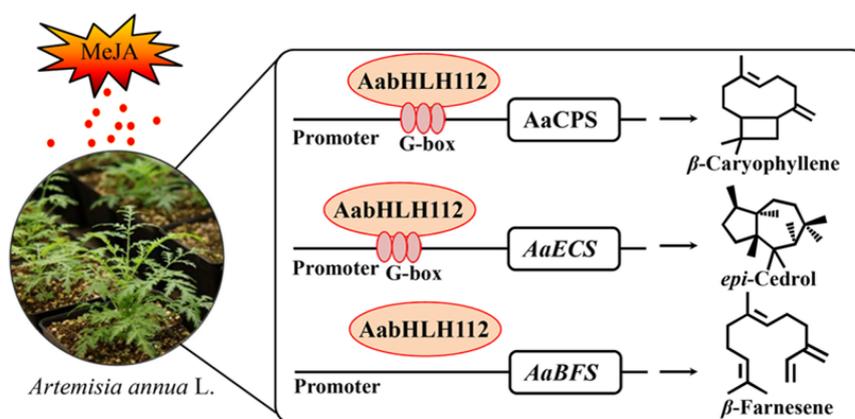


FIGURE 8

A simplified model for the regulation of sesquiterpenes biosynthesis by *AabHLH112* in *Artemisia annua*.

AabHLH112 positively regulates β -caryophyllene, *epi*-cedrol, and β -farnesene biosynthesis in *Artemisia annua*

The contents of β -caryophyllene, *epi*-cedrol, and β -farnesene in the *AabHLH112*-overexpression and RNAi transgenic lines were detected by the GC-MS method. The wild type *A. annua* produced 1.87 mg/g FW (fresh weight) of

β -caryophyllene, 1.07 mg/g FW of *epi*-cedrol and 0.53 mg/g FW of β -farnesene, while the three independent *AabHLH112*-overexpression lines (OE-2, OE-7, and OE-8) exhibited significant increase in sesquiterpenes contents. OE-2, OE-7, and OE-8 contained β -caryophyllene at levels of 2.34 mg/g FW, 2.45 mg/g FW, and 2.95 mg/g FW, respectively, representing 25.13, 31.01, and 57.75% increases in content compared with WT plants (Figure 7A). Compared to WT plants, the content of *epi*-cedrol was increased by 118, 176, and 200% in the three

AabHLH112-overexpression lines respectively (Figure 7B); and the content of β -farnesene was increased by 341, 253, and 201% in the three *AabHLH112*-overexpression lines (Figure 7C). In the *AabHLH112*-RNAi lines of Ri-4, Ri-8 and Ri-9, the contents of β -caryophyllene, *epi*-cedrol and β -farnesene were significantly reduced, compared to that in WT plants. Ri-4, Ri-8, and Ri-9 lines contained β -caryophyllene at levels of 0.73 mg/g FW, 1.09 mg/g FW, and 0.94 mg/g FW respectively, representing 61.91, 42.28, and 50.54% reduction in content compared with WT plants (Figure 7D). *Epi*-cedrol content was reduced by 78.12, 88.45, and 76.82% in Ri-4, Ri-8 and Ri-9 lines respectively, relative to that in WT plants (Figure 7E). β -farnesene content in Ri-4, Ri-8, and Ri-9 lines was reduced by 84.27, 70.55, and 84.58% respectively, compared with that in WT plants (Figure 7F). These results showed that *AabHLH112* is a positive regulator of β -caryophyllene, *epi*-cedrol, and β -farnesene biosynthesis in *A. annua*.

Discussion

Artemisia annua is a traditional Chinese medicinal plant, not only producing the well-known drug artemisinin, but also producing other important sesquiterpenes of medicinal value, such as β -caryophyllene and *epi*-cedrol. The bHLH TFs are the second largest transcription factor family in the plants, which play an important role in plant growth, development, and secondary metabolism. Previous research revealed that bHLH TFs play an important part in the regulation of terpenoids biosynthesis. In tomato, overexpression of *CubHLH1* resulted in a significant increase in carotenoid content (Endo et al., 2016). MYC, one subgroup of bHLH family, plays an important role in JA, ABA, and GA signaling and is involved in the regulation of terpenoids biosynthesis in plants (Hong et al., 2012; Du et al., 2017). In *A. thaliana*, *AtMYC2* could directly bind to promoters of the sesquiterpene synthase genes *TPS21* and *TPS11* and activate their expression, thus increasing sesquiterpenes biosynthesis (Hong et al., 2012). *AaMYC2* overexpression could activate the transcription of *CYP71AV1* and *DBR2*, resulting in an increase of artemisinin content in *A. annua* (Shen et al., 2016). Besides, *AabHLH1* positively regulated biosynthesis of artemisinin via activating the promoters of *ADS* and *CYP71AV1* (Ji et al., 2014). In this study, we found that *AbHLH112* positively regulates the biosynthesis of β -caryophyllene, *epi*-cedrol, and β -farnesene in *A. annua* besides upregulating artemisinin biosynthesis.

The gene expression was synchronous with its product. In this study, we measured the expression levels of *AabHLH112*, *AaCPS*, *AaECS*, and *AaBFS* in roots, stems, leaves and flowers. And we found that *AabHLH112* has the highest expression in leaves and flowers and the lowest expression in stems and roots, which has a similar expression pattern with *AaADS* involved in artemisinin biosynthesis (Pu et al., 2013). *AaCPS* and *AaBFS*,

involved in β -caryophyllene and β -farnesene biosynthesis respectively, have a tissue-specific expression pattern, with the highest expression in flowers and the lowest expression in roots, stems and leaves. By contrast, *AaECS* is highly expressed in leaves and lowly expressed in roots and flowers, showing a tissue difference in the expression of these genes.

Methyl jasmonate (MeJA) is one of the most potent elicitors that can induce overaccumulation of many natural products in plants, such as artemisinin, tanshinones, vinblastine, and so on (Xiang et al., 2015; Shi et al., 2016; Zhang et al., 2018). Later studies showed that exogenous MeJA activated the transcription of some genes leading to the products' overaccumulation (Wang et al., 2020). In this study, the 1-month-old *A. annua* seedlings were treated with MeJA for up to 24 h and the four genes expression were quantified by qPCR. The results showed that the genes detected had a higher expression after the MeJA treatment, indicating that exogenous MeJA treatment can induce the expression of sesquiterpene biosynthesis genes in *A. annua*. In addition, we found that the expression level of *AabHLH112* was induced as early as 3 h after the treatment, and then began to decline, while the highest expression levels of *AaECS* and *AaCPS* were detected at 12 h after MeJA treatment. This result suggested that these sesquiterpenes synthase genes might be regulated by *AabHLH112*. To further explore the function of *AabHLH112* in the regulation of sesquiterpene biosynthesis genes expression, dual-LUC assay was taken to determine the activation of *AaCPS*, *AaECS*, and *AaBFS* promoters by *AabHLH112*. The results indicated *AabHLH112* can significantly enhance the transcriptional activity of *AaCPS*, *AaECS*, and *AaBFS* promoters in *N. benthamiana* leaves (Figures 4B–D).

It is known that G-box is a putative recognition site for bHLH TFs. We have reported in a previous study that *AabHLH112* can bind to the G-box in the *AaERF1* promoter and enhanced its transcription to upregulate artemisinin biosynthesis (Xiang et al., 2019). In this study, yeast one-hybrid assay (Y1H) showed that *AabHLH112* can directly bind to pAaCPS-G3 and pAaECS-G1 fragments containing the G-box in the promoters of *AaCPS* and *AaECS*, but not bind to pAaCPS1-G1, pAaCPS-G2, pAaCPS-G4, pAaECS-G2, pAaECS-G3, and pAaBFS-G1, pAaBFS-G2, pAaBFS-G3 fragments (Figures 5B,D,F). Based on these results, we estimated that the flanking sequence of G-box *cis*-elements may affect the recognition of *AabHLH112* protein. In addition, there are three G-box *cis*-elements in the pAaCPS-G3 fragment and they are very close to each other. So, a profound study should be carried out to analyze which G-box is the real recognition site by *AabHLH112*. Interestingly, *AabHLH112* positively regulated *AaBFS* expression to increase β -farnesene accumulation, but Y1H result showed there was no direct interaction between *AabHLH112* and *AaBFS* promoter, suggesting that *AabHLH112* upregulated β -farnesene biosynthesis through an indirect pathway, which deserves further exploration.

Taken together, our study indicated that AabHLH112, which is induced by MeJA, positively regulates the expression of *AaCPS*, *AaECS*, and *AaBFS* through directly binding to their promoters or an indirect pathway, thereby enhancing the biosynthesis of β -caryophyllene, *epi*-cedrol, and β -farnesene in *Artemisia annua* (Figure 8). Our study revealed the mechanism of AabHLH112-mediated upregulation of sesquiterpenes biosynthesis, and provided a potentially useful transcription factor that could be used to improve sesquiterpenes production in *A. annua*.

Data availability statement

The original contributions presented in this study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author/s.

Author contributions

LX, ZL, and YT conceived and coordinated the study and wrote the manuscript. LX performed the gene cloning, vector construction, and plant transformation work. GS, MY, and MW did the gene expression, luciferase analysis, and Y1H experiments. GS, XL, and PH helped with the metabolites analysis by GC-MS. All authors reviewed the results and approved the final version of the manuscript.

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References

- Bahi, A., Mansouri, S., Memari, E., Ameri, M., Nurulain, S., and Ojha, S. (2014). β -Caryophyllene, a CB2 receptor agonist produces multiple behavioral changes relevant to anxiety and depression in mice. *Physiol. Behav.* 135, 119–124. doi: 10.1016/j.physbeh.2014.06.003
- Basha, R., and Sankaranarayanan, C. (2014). β -Caryophyllene, a natural sesquiterpene, modulates carbohydrate metabolism in streptozotocin-induced diabetic rats. *Acta Histochem.* 116, 1469–1479. doi: 10.1016/j.acthis.2014.10.001
- Bento, A., Marcon, R., Dutra, R., Claudino, R., Cola, M., Leite, D., et al. (2011). β -Caryophyllene inhibits dextran sulfate sodium-induced colitis in mice through CB2 receptor activation and PPAR γ pathway. *Am. J. Pathol.* 178, 1153–1166. doi: 10.1016/j.ajpath.2010.11.052
- Cao, W., Wang, Y., Shi, M., Hao, X., Zhao, W., Wang, Y., et al. (2018). Transcription factor SmWRKY1 positively promotes the biosynthesis of tanshinones in *Salvia miltiorrhiza*. *Front. Plant Sci.* 9:554. doi: 10.3389/fpls.2018.00554
- Chadwick, M., Trewin, H., Gawthrop, F., and Wagstaff, G. (2013). Sesquiterpenoids lactones: benefits to plants and people. *Int. J. Mol. Sci.* 14, 12780–12805. doi: 10.3390/ijms140612780
- Chang, H., Kim, J., Lee, J., Kim, W., and Chun, H. (2013). Protective effect of β -caryophyllene, a natural bicyclic sesquiterpene, against cerebral ischemic injury. *J. Med. Food* 16, 471–480. doi: 10.1089/jmf.2012.2283
- Cheng, T., Zhang, K., Guo, J., Yang, Q., Li, T., Xiao, M., et al. (2022). Highly efficient biosynthesis of β -caryophyllene with a new sesquiterpene synthase from tobacco. *Biotechnol. Biofuels Bioproducts* 15:39. doi: 10.1186/s13068-022-02136-8
- Chuang, Y., Hung, Y., Tsai, W., Chen, W., and Chen, H. (2018). PbbHLH4 regulates floral monoterpene biosynthesis in *Phalaenopsis* orchids. *J. Exp. Bot.* 69, 4363–4377. doi: 10.1093/jxb/ery246
- Du, M., Zhao, J., Tzeng, D., Liu, Y., Deng, W., Ynag, T., et al. (2017). MYC2 orchestrates a hierarchical transcriptional cascade that regulates jasmonate-mediated plant immunity in tomato. *Plant Cell* 29, 1883–1906. doi: 10.1105/tpc.16.00953
- Endo, T., Fujii, H., Sugiyama, A., Nakano, M., Nakajima, N., and Ikoma, Y. (2016). Overexpression of a citrus basic helix-loop-helix transcription factor (CubHLH1), which is homologous to Arabidopsis activation-tagged bri1 suppressor 1 interacting factor genes, modulates carotenoid metabolism in transgenic tomato. *Plant Sci.* 243, 35–48. doi: 10.1016/j.plantsci.2015.11.005

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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.2022.973591/full#supplementary-material>

- Fu, X., Shi, P., He, Q., Shen, Q., Tang, Y., Pan, Q., et al. (2017). AaPDR3, a PDR transporter 3, is involved in sesquiterpene β -caryophyllene transport in *Artemisia annua*. *Front. Plant Sci.* 8:723. doi: 10.3389/fpls.2017.00723
- Gantet, P., and Memelink, J. (2002). Transcription factors: tools to engineer the production of pharmacologically active plant metabolite. *Trends Pharmacol. Sci.* 23, 563–569. doi: 10.1016/s0165-6147(02)02098-9
- Hong, G., Xue, X., Mao, Y., Wang, L., and Chen, X. (2012). Arabidopsis MYC2 interacts with DELLA proteins in regulating sesquiterpene synthase gene expression. *Plant Cell* 24, 2635–2648. doi: 10.1105/tpc.112.098749
- Huang, Q., Sun, M., Yuan, T., Wang, Y., Shi, M., and Lu, S. (2019). The AP2/ERF transcription factor SmERF1L1 regulates the biosynthesis of tanshinones and phenolic acids in *Salvia miltiorrhiza*. *Food Chem.* 274, 368–375. doi: 10.1016/j.foodchem.2018.08.119
- Ji, Y., Xia, J., Shen, Y., Ma, D., Li, Z., and Pu, G. (2014). Cloning and characterization of AabHLH1, a bHLH transcription factor that positively regulates artemisinin biosynthesis in *Artemisia annua*. *Plant Cell Physiol.* 55, 1592–1604. doi: 10.1093/pcp/pcu090
- Kianersi, F., Pour-Aboughadareh, A., Majidi, M., and Poczai, P. (2021). Effect of methyl jasmonate on thymol, carvacrol, phytochemical accumulation, and expression of key genes involved in thymol/carvacrol biosynthetic pathway in some iranian thyme species. *Int. J. Mol. Sci.* 22:11124. doi: 10.3390/ijms222011124
- Legault, J., and Pichette, A. (2007). Potentiating effect of β -caryophyllene on anticancer activity of α -humulene, isocaryophyllene and paclitaxel. *J. Pharmacy Pharmacol.* 59, 1643–1647. doi: 10.1211/jpp.59.12.0005
- Lenka, S., Nims, N., Vongpaseuth, K., Boshar, R., Roberts, S., and Walker, E. (2015). Jasmonate-responsive expression of paclitaxel biosynthesis genes in *Taxus cuspidata* cultured cells is negatively regulated by the bHLH transcription factors TcJAMYC1, TcJAMYC2, and TcJAMYC4. *Front. Plant Sci.* 6:115. doi: 10.3389/fpls.2015.00115
- Li, X., Xu, Y., Shen, S., Yin, X., Klee, H., Zhang, B., et al. (2017). Transcription factor CitERF71 activates the terpene synthase gene CitTPS16 involved in the synthesis of E-geraniol in sweet orange fruit. *J. Exp. Bot.* 68, 4929–4938. doi: 10.1093/jxb/erx316
- Li, Y., Li, L., Ding, W., Li, H., Shi, T., Yang, X., et al. (2020). Genome-wide identification of osmanthus fragrans bHLH transcription factors and their expression analysis in response to abiotic stress. *Environ. Exp. Bot.* 172:103990.
- Livak, K. J., and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2^{-Delta Delta C(T)} Method. *Methods* 25, 402–408. doi: 10.1006/meth.2001.1262
- Lu, X., Tang, K., and Li, P. (2016). Plant metabolic engineering strategies for the production of pharmaceutical terpenoids. *Front. Plant Sci.* 7:1647. doi: 10.3389/fpls.2016.01647
- Luo, F., Ling, Y., Li, Den, Tang, T., Liu, Y. C., Liu, Y., et al. (2019). Characterization of a sesquiterpene cyclase from the glandular trichomes of *Leucoscepterum canum* for sole production of cedrol in *Escherichia coli* and *Nicotiana benthamiana*. *Phytochemistry* 162, 121–128. doi: 10.1016/j.phytochem.2019.03.009
- Ma, Y., Xu, D., Li, L., Zhang, F., Fu, X., Shen, Q., et al. (2018). Jasmonate promotes artemisinin biosynthesis by activating the TCP14-ORA complex in *Artemisia annua*. *Sci. Adv.* 4:eaas9357. doi: 10.1126/sciadv.aas9357
- Mahmoud, M., Swefy, S., Hasan, R., and Ibrahim, A. (2014). Role of cannabinoid receptors in hepatic fibrosis and apoptosis associated with bile duct ligation in rats. *Eur. J. Pharmacol.* 742, 118–124. doi: 10.1016/j.ejphar.2014.08.021
- Navale, G., Sharma, P., Said, M., Ramkumar, S., Dharné, M., Thulasiram, H., et al. (2019). Enhancing epi-cedrol production in *Escherichia coli* by fusion expression of farnesyl pyrophosphate synthase and epi-cedrol synthase. *Eng. Life Sci.* 19, 606–616. doi: 10.1002/elsc.201900103
- Patra, B., Pattanaik, S., Schluttenhofer, C., and Yuan, L. (2018). A network of jasmonate-responsive bHLH factors modulate monoterpenoid indole alkaloid biosynthesis in *Catharanthus roseus*. *New Phytol.* 4, 1566–1581. doi: 10.1111/nph.14910
- Pu, G., Ma, D., Wang, H., Ye, H., and Liu, B. (2013). Expression and localization of amorpho-4,11-diene synthase in *Artemisia annua* L. *Plant Mol. Biol. Rep.* 31, 32–37. doi: 10.1007/s11105-012-0472-0
- Rufino, A., Ribeiro, M., Sousa, C., Judas, F., Salgueiro, L., Carlos Cavaleiro, C., et al. (2015). Evaluation of the anti-inflammatory, anti-catabolic and pro-anabolic effects of E-caryophyllene, myrcene and limonene in a cell model of osteoarthritis. *Eur. J. Pharmacol.* 750, 141–150. doi: 10.1016/j.ejphar.2015.01.018
- Shen, Q., Lu, X., Yan, T., Fu, X., Lv, Z., Zhang, F., et al. (2016). The jasmonate-responsive AaMYC2 transcription factor positively regulates artemisinin biosynthesis in *Artemisia annua*. *New Phytol.* 210, 1269–1281. doi: 10.1111/nph.13874
- Shi, M., Zhou, W., Zhang, J., Huang, S., Wang, H., and Kai, G. (2016). Methyl jasmonate induction of tanshinone biosynthesis in *Salvia miltiorrhiza* hairy roots is mediated by JASMONATE ZIM-DOMAIN repressor proteins. *Sci. Rep.* 15:20919. doi: 10.1038/srep20919
- Wang, G., Yu, D., Fan, J., Wang, C., and Xia, L. (2014). Expressing an (E)- β -farnesene synthase in the chloroplast of tobacco affects the preference of green peach aphid and its parasitoid. *Plant Biol. J. Integr.* 57, 770–782. doi: 10.1111/jipb.12319
- Wang, J., Song, L., Gong, X., Xu, J., and Li, M. (2020). Functions of jasmonic acid in plant regulation and response to abiotic stress. *Int. J. Mol. Sci.* 21:1446. doi: 10.3390/ijms21041446
- Wasternack, C., and Song, S. (2017). Jasmonates: biosynthesis, metabolism, and signaling by proteins activating and repressing transcription. *J. Exp. Bot.* 68, 1303–1321. doi: 10.1093/jxb/erw443
- Xiang, L., Jian, D., Zhang, F., Yang, C., Bai, G., Lan, X., et al. (2019). The cold-induced transcription factor bHLH112 promotes artemisinin biosynthesis indirectly via ERF1 in *Artemisia annua*. *J. Exp. Bot.* 70, 4835–4848. doi: 10.1093/jxb/erz220
- Xiang, L., Zhu, S., Zhao, T., Zhang, M., Liu, W., Chen, M., et al. (2015). Enhancement of artemisinin content and relative expression of genes of artemisinin biosynthesis in *Artemisia annua* by exogenous MeJA treatment. *Plant Growth Regul.* 75, 435–441. doi: 10.1007/s10725-014-0004-z
- Xu, J., Herwijnen, Z. O., Dräger, D., Sui, C., Haring, M., and Schuurink, R. (2018). SIMYC1 regulates type VI glandular trichome formation and terpene biosynthesis in tomato glandular cells. *Plant Cell* 30, 2988–3005. doi: 10.1105/tpc.18.00571
- Yang, C., Fang, X., Wu, X., Mao, Y., Wang, L., and Chen, X. Y. (2012). Transcriptional regulation of plant secondary metabolism. *J. Integr. Plant Biol.* 54, 703–712. doi: 10.1111/j.1744-7909.2012.01161.x
- Yin, J., Li, X., Zhan, Y., Li, Y., Qu, Z., Sun, L., et al. (2017). Cloning and expression of BpMYC4 and BpbHLH9 genes and the role of BpbHLH9 in triterpenoid synthesis in birch. *BMC Plant Biol.* 17:214. doi: 10.1186/s12870-017-1150-z
- Yu, X., Pickett, J., Ma, Y., Bruce, T., Napier, J., Huw, D., et al. (2012). Metabolic engineering of plant-derived (E)- β -farnesene synthase genes for a novel type of aphid-resistant genetically modified crop plants. *J. Integr. Plant Biol.* 54, 282–299. doi: 10.1111/j.1744-7909.2012.01107.x
- Yu, Z., Li, J., Yang, C., Hu, W., Wang, L., and Chen, X. (2012). The jasmonate-responsive AP2/ERF transcription factors AaERF1 and AaERF2 positively regulate artemisinin biosynthesis in *Artemisia annua* L. *Mol. Plant* 5, 353–365. doi: 10.1093/mp/sss087
- Zhang, C., Xing, B., Yang, D., Ren, M., Guo, H., Yang, S., et al. (2020). SmbHLH3 acts as a transcription repressor for both phenolic acids and tanshinone biosynthesis in *Salvia miltiorrhiza* hairy roots. *Phytochemistry*. 169:112183. doi: 10.1016/j.phytochem.2019.112183
- Zhang, H., Hedhili, S., Montiel, G., Zhang, Y., Chatel, G., Pré, M., et al. (2011). The basic helix-loop-helix transcription factor CrMYC2 controls the jasmonate-responsive expression of the ORCA genes that regulate alkaloid biosynthesis in *Catharanthus roseus*. *Plant J.* 67, 61–71. doi: 10.1111/j.1365-313X.2011.04575.x
- Zhang, X., Liu, J., Liu, Y., Wang, Y., Abozeid, A., Yu, Z., et al. (2018). Metabolomics Analysis reveals that ethylene and methyl jasmonate regulate different branch pathways to promote the accumulation of terpenoid indole alkaloids in *Catharanthus roseus*. *J. Nat. Products* 81, 335–342. doi: 10.1021/acs.jnatprod.7b00782
- Zhang, Y., Han, L., Chen, S., Guan, J., Qu, F. Z., Zhao, Y. Q., et al. (2016). Hair growth promoting activity of cedrol isolated from the leaves of *Platycladus orientalis*. *Biomed. Pharmacother.* 83, 641–647. doi: 10.1016/j.biopha.2016.07.022