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Abstract
Pyrethrins constitute a class of terpene derivatives with high insecticidal activity and are mainly synthesized in the capitula of the horticulturally important plant, Tanacetum cinerariifolium. Treatment of T. cinerariifolium with methyl jasmonate (MeJA) in the field induces pyrethrin biosynthesis, but the mechanism linking MeJA with pyrethrin biosynthesis remains unclear. In this study, we explored the transcription factors involved in regulating MeJA-induced pyrethrin biosynthesis. A single spray application of MeJA to T. cinerariifolium leaves rapidly upregulated the expression of most known pyrethrin biosynthesis genes and subsequently increased the total pyrethrin content in the leaf. A continuous 2-week MeJA treatment resulted in enhanced pyrethrin content and increased trichome density. TcMYC2, a key gene in jasmonate signaling, was screened at the transcriptome after MeJA treatment. Further transient overexpression and viral-induced gene-silencing experiments demonstrated that TcMYC2 positively promoted pyrethrin biosynthesis. Collectively, the results reveal a novel molecular mechanism for MeJA-induced pyrethrin biosynthesis in T. cinerariifolium involving TcMYC2.

Introduction
Tanacetum cinerariifolium is an economically important ornamental plant valued as a natural source of pyrethrins, a group of highly effective insecticidal secondary metabolites [1]. Natural pyrethrins have been widely used as an environmentally friendly insecticide, with low mammalian toxicity and no known insect immunity [2]. Consequently, T. cinerariifolium is cultivated in several countries, including Kenya, Tanzania, Uganda, Rwanda, China, and Australia [3], as a commercially important source of natural pesticides.

Natural pyrethrins comprise six types of monoterpene esters. The esterification involves an acid moiety (pyrethric acid and chrysanthemic acid) and an alcohol moiety (pyrethrolone, cinerolone, and jasomolone). The monoterpene acid moiety is derived from the methylerythritol-4-phosphate pathway and is exclusive to T. cinerariifolium and closely related species [4]. The first committed step is catalyzed by chrysanthemol synthase (CHS) [5, 6], which catalyzes the condensation of two dimethylallyl diphosphate (DMAPP) molecules to produce trans-chrysanthemol. Subsequently, trans-chrysanthemol is oxidized by alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) to produce trans-chrysanthenonic acid [7]. Simultaneously, trans-chrysanthemal or trans-chrysanthenonic acid is converted into pyrethric acid by chrysanthemol 10-hydroxylase (CHH) and 10-carboxychrysanthenic acid 10-methyltransferase [8]. Finally, GDSL lipase-like protein (GLIP) esterifies the acid and alcohol moieties into pyrethrins [9].

Synthesis of the alcohol moiety is at least partially shared with that of jasmonic acid (JA), a plant hormone that regulates the synthesis of plant secondary metabolites [10, 11]. Enzymes involved in steps of JA biosynthesis, including lipoxygenase (LOX), allene oxide synthase (AOS), allene oxide cyclase (AOC), and oxophytodienoic acid reductase, have been well established [10]. In the downstream pathway, jasmonate hydroxylase (JMH) converts jasmonate to jasmonolone, and pyrethrolone synthase (PYS) catalyzes jasmonolone to pyrethrolone [12, 13].

The pyrethrin content differs among organs in T. cinerariifolium. The pyrethrin content is ~1%–2% in the capitulum and 0.1% (dry weight) in leaves [14]. In industrial production, leaves are usually discarded and cause waste of feedstock. However, the content of pyrethrins in unexpanded leaves is relatively higher than in mature leaves and even comparable to that of capitula [15]. Mechanical damage may rapidly increase the pyrethrin content in leaves and lead to accumulation of JAs [16]. Therefore, there is potential to increase the pyrethrin content in leaves for commercial extraction and JAs might be crucial to promoting pyrethrin...
production. Methyl jasmonate (MeJA) regulates the biosynthesis of certain secondary metabolites in plants via transcription factors (TFs), such as phenolic acid and tanshinone in Salvia miltiorrhiza [17], artemisinin in Artemisia annua [18, 19], and flavor volatiles in Chrysanthemum indicum [20]. Typically, TFs positively or negatively regulate metabolite contents by directly binding to a downstream secondary metabolite biosynthesis target gene [21, 22]. MYC family proteins are master regulators of the JA signaling pathway [23]. Many MYC TFs respond to the MeJA signal and play critical roles in regulating terpenoid biosynthesis in diverse plant species [24, 25]. For instance, the JA-responsive TF AaMYC2 positively regulates agarwood sesquiterpene synthesis by binding to the sesquiterpene synthase 1 (ASS1) promoter [26]. AaMYC2 directly binds to the G-box motif in the promoters of CYP71AV1 and DBR2 to promote JA-mediated artemisinin biosynthesis [24]. In Taxis chinensis, TcMYC2a regulates taxol biosynthesis either directly or via ERF TFs depending on JA signaling [27].

In T. cinerariifolium, MeJA treatment and additional stimuli, such as mechanical wounding and volatile organic compounds, induce pyrethrin synthesis [16, 28, 29]. However, no TFs involved in the MeJA-mediated pyrethrin biosynthesis regulatory network have been reported in T. cinerariifolium. In this study, we found that short MeJA treatment applied to T. cinerariifolium leaves transiently induced the expression of pyrethrin biosynthetic genes and raised the pyrethrin content, and a continuous MeJA treatment even increased the trichome density, which further led to increasing pyrethrins content. Based on transcriptome analysis, we discovered a MeJA responsive TcMYC2 TF which could directly bind to the promoter regions of key pyrethrins biosynthetic genes. Further transient overexpression and virus-induced gene silencing (VIGS) of TcMYC2 in T. cinerariifolium leaves confirmed that TcMYC2 was a positive factor involved in regulating pyrethrin biosynthesis.

Results

Exogenous MeJA upregulates pyrethrin biosynthesis gene expression

Pyrethrins are esters of an acid moiety and an alcohol moiety derived from two pathways: an irregular monoterpene biosynthesis pathway (specific to T. cinerariifolium) and the JA biosynthesis pathway (Fig. 1a). To investigate the mechanism of MeJA-induced pyrethrin biosynthesis, the transcriptome of T. cinerariifolium leaves was analyzed at 0 (control), 2, 4, 6, 8, 10, 12, and 24 h after a single spray application of MeJA, and differentially expressed genes (DEGs) associated with pyrethrin synthesis were identified. Transcriptome assembly and analysis details are presented in Figs S1 and S2, see online supplementary material. Expression levels of TcLOX (KC441523.1), TcAOS (MK139710.1), and TcAOC (MH397471.1), which function upstream of JA, rapidly increased at 2 h and were maintained at a higher level compared with the control (Fig. 1b). The monoterpene pathway genes TcCHS (JX913536.1), TcADH (MF497444.1), TcALDH (MF497445.1), TcCHH (KC441525.1), and TcMT (MK139710.1) were strongly upregulated within 2 h but thereafter were dramatically downregulated to levels much lower than those of the control (Fig. 1b). We speculated that although the short MeJA treatment could transiently and sharply induce the pyrethrins biosynthesis genes, the expression of these genes, especially which involved the synthesis of the acid moiety unique to T. cinerariifolium, was finally downregulated. Moreover, the expression of genes involved in the final steps of pyrethrin biosynthesis, including TcMHI (MG189994.1), TcPYS (MG874680.1), and TcGLIP (JN418994.1), was decreased in response to MeJA treatment, and we speculate this may be due to feedback regulation to maintain cell homeostasis (Fig. 1b). The expression patterns of these genes was confirmed by real-time quantitative reverse-transcription PCR (qRT-PCR) (Fig. 1b).

Exogenous MeJA increases pyrethrin content in leaves

Methyl jasmonate stimulates overaccumulation of many secondary metabolites in plants [11]. Therefore, gas chromatography–mass spectrometry (GC–MS) analysis was conducted to detect pyrethrin accumulation in leaves of MeJA-treated plants (Fig. 1c). Contents of pyrethrin compounds, especially pyrethrin I and pyrethrin II, were significantly increased at 6 h after MeJA treatment, consistent with the timing of pyrethrin biosynthesis gene expression. At subsequent time points, the pyrethrin contents declined to levels lower than those at 0 h consistent with the gene expression profiles (Fig. 1d). Hence, we concluded that MeJA could promote pyrethrins production in response to a transient upregulation of pyrethrins biosynthetic genes, however, it did not maintain the higher pyrethrins content over time by a transient MeJA induction.

TcMYC2 is a potential regulator of MeJA-induced pyrethrin biosynthesis

To examine the role of JA pathway genes in pyrethrin biosynthesis, based on the FPKM expression matrix of 28,552 DEGs, a weighted gene coexpression network analysis (WGCNA) module was constructed. The 33 modules were obtained with a shear value of 0.2 (Fig. S3). The Cyan module (2320 genes) and Skyblue1 (211 genes) module were strongly correlated with pyrethrin synthesis. KEGG and GO enrichment analysis confirmed the genes were involved in secondary metabolite biosynthesis (Fig. S4). To explore the key regulatory genes, a candidate hub gene network was visualized with Cytoscape. Among the hub genes showing high connectivity, TcMYC2 was detected (Fig. S4).

To identify the TFs activated by MeJA that potentially regulated pyrethrin biosynthesis, the promoter sequences of TcCHS, TcALDH, TcAOC, and TcGLIP were cloned and putative TF-binding sites in the promoter sequences was analysed using PlantCARE and PlantPAN3.0 (Fig. 2a). TcMYC2-responsive elements were detected in the TcALDH, TcAOC, and TcGLIP promoters. In addition, E-box and G-box motifs, the binding sites of bHLH TFs, were identified in the promoter of each gene. Fifty-five bHLH TFs with FPKM >1 were identified with PlantTFDB (Fig. S3). To obtain candidate MeJA-regulated bHLHs regulating pyrethrin biosynthesis, a heatmap was constructed using the RNA-seq data for pyrethrin biosynthesis genes obtained from the MeJA treatment and different development stages (Fig. 2b). Based on Pearson distance measurement and Ward clustering algorithm, the candidate unigene66762 showed the most similar expression pattern to TcCHS or TcAOC. Phylogenetic analysis of bHLHs showed that unigene66762 belonged to the MYC2-like subclade (Figs S5 and S6, see online supplementary material) and contained a conserved MYC domain (Fig. 2c). Thus, we named unigene66762 as TcMYC2 and selected it for further analysis.

Expression profiles and subcellular localization of TcMYC2

To validate MeJA-mediated induction of TcMYC2 expression, qRT-PCR was used to analyse TcMYC2 expression. The TcMYC2 transcript abundance strongly increased at 2 h, and was maintained at a high level after prolonged MeJA exposure (4, 6, and 8 h), until returning to the original level at 24 h (Fig. 3a).
Figure 1. Pyrethrin biosynthesis gene expression and pyrethrin content in Tanacetum cinerariifolium leaves in response to short-term MeJA treatment. 

**a** Pyrethrin biosynthesis pathway. Heatmaps indicate the gene expression profile at different time points after MeJA treatment. Different colours represent the log10 FPKM value. Solid arrows indicate known steps in the pathway. Dotted arrows indicate steps not yet elucidated. The solid box encloses the six major pyrethrins. Abbreviations: ADH2, alcohol dehydrogenase2; ALDH, aldehyde dehydrogenase; CHH, chrysanthemol 10-hydroxylase; CHS, chrysanthemol synthase; GLIP, GDSL lipase-like protein; JMH, jasmolone hydroxylase; MT, 10-carboxychrysanthemic acid 10-methyltransferase; PYS, pyrethrolone synthase.

**b** qRT-PCR analysis of pyrethrin biosynthesis genes. Blue bars are the fold change in average fragments per kilobase of transcript per million mapped reads (FPKM). Green bars are the qRT-PCR results normalized with the $2^{-\Delta\Delta Ct}$ method using GADPH as an internal reference. The experiment was performed three times with three biological replicates. Error bars represent mean ± SD.

**c** GC–MS chromatograms of leaves treated for 0, 6, 12, and 24 h. The relative pyrethrin content was determined using tetradecane as an internal standard, with three biological replicates.

**d** Relative contents of total pyrethrins. The y-axis represents peak area intensity. Error bars represent the SD of the mean of three replicates. Different lower-case letters indicate a significant difference ($P < 0.05$) from one-way ANOVA followed by a post-hoc Tukey test.

Results were consistent with the expression pattern of TcAOC in MeJA-treated leaves (Fig. 1b). To investigate potential similarities in expression patterns between TcMYC2 and other pyrethrin biosynthesis genes, the relative expression levels of TcMYC2 were analysed in different tissues and flowers at different developmental stages. The TcMYC2 expression level was lowest in leaves, low in the receptacle and pedicel, and highest in ray florets and disc florets (Fig. 3b). The expression profiles of TcMYC2
Figure 2. Bioinformatic analysis of pyrethrin biosynthesis gene promoters and expression profile of bHLH and pyrethrin biosynthesis genes. 

a. Cis-regulatory elements in promoters of key pyrethrin biosynthesis genes. 
b. Heat maps of coexpressed genes based on RNA-Seq analyses (at time points after MeJA treatment of the leaves and at four flower developmental stages). 
c. Alignment of amino acid sequences for TcMYC2 and homologous proteins. Artemisia annua MYC2 (AKO62850.1); Chrysanthemum indicum MYC2 (QED22045.1); Arabidopsis thaliana MYC2 (NP_174541.1). The conserved MYC domains are indicated by lines below sequences. Black line, bHLH_MYC_N domain; blue line, HLH domain.

at different stages of flowering (Fig. 3c) were similar to those of other pyrethrin biosynthesis genes, including TcCHS, TcALDH, and TcGLIP, which are highly expressed at the first two stages of flowering. These results suggest that TcMYC2 is involved in the regulation of pyrethrins biosynthesis. Thus, we speculated that TcMYC2 co-regulates pyrethrin production by interacting with pyrethrin biosynthetic gene promoters.

To determine the subcellular localization of TcMYC2, the coding sequence of TcMYC2 was fused in-frame to the green fluorescent protein (GFP) under the control of the Cauliflower mosaic virus (CaMV) 35S promoter. When TcMYC2-GFP was transiently expressed in Nicotiana benthamiana leaves, strong fluorescence was localized to the nucleus. The GFP signal co-localized with the RFP nuclear localization signal (NLS; Fig. 3d). These results indicate that TcMYC2 is a nuclear-localized protein according to its potential role as a TF.

TcMYC2 activates and directly binds to TcCHS, TcAOC, and TcGLIP promoters

Given that binding sites of bHLH TFs were predicted in the TcCHS, TcALDH, TcAOC, and TcGLIP promoter sequences, and the TcMYC2 expression pattern was similar to that of four pyrethrin biosyn-
thesis genes, yeast one-hybrid (Y1H) assays were conducted to test the hypothesis that TcMYC2 regulates the expression of these genes by directly binding to the corresponding promoter. The promoter of each gene was cloned and inserted into the pHis2.1 vector to generate reporter constructs. TcMYC2 was fused to the GAL4 activation domain to generate the effector construct pGADT7-TcMYC2 (Fig. 4a). The reporter and effector constructs were co-transformed into yeast cells. Yeast grew on SD/−Leu/−Trp/−His medium only when pGADT7-TcMYC2 was co-transformed with pHis2.1-TcCHS, pHis2.1-TcAOC, or pHis2.1-TcGLIP but not with pHis2.1, suggesting that TcMYC2 can directly bind to the TcCHS, TcAOC, and TcGLIP promoters (Fig. 4b). The direct binding motif in the promoters was verified by electrophoretic mobility shift assays (EMSA). The shifted band was separately detected in the presence of both TcMYC2 and a labeled probe containing the G-box in the TcAOC promoter or E-box in the TcCHS and TcGLIP promoters (Fig. 4c). The intensity of the shifted band decreased with addition of 50-fold concentrations of cold competitor (unlabeled probe) and recovered with addition of mutant competitor probe.

To verify the interaction of TcMYC2 with the TcCHS, TcAOC, and TcGLIP promoters in vivo, transient dual-luciferase (dual-LUC) assays were performed. Reporter constructs (TcCHS/TcAOC/TcGLIPpro:LUC) and effector constructs (35S:TcMYC2) were transiently coexpressed in N. benthamiana leaves (Fig. 4d). Strong LUC activity was observed in leaves co-transformed with TcCHS/TcAOC/TcGLIPpro:LUC and 35S-TcMYC2. No signal was detected in leaves transformed with TcCHS/TcAOC/TcGLIPpro:LUC or the empty vector (Fig. 4e). Collectively, we demonstrated that TcMYC2 directly activates these gene promoters and regulates pyrethrins biosynthesis in T. cinerariifolium.

Stable transformation of TcMYC2 in hairy roots
To verify the function of stably overexpressed TcMYC2, TcMYC2 was inserted in the pBI121 vector, and the recombinant vector was transferred to Agrobacterium rhizogenes via Agrobacterium rhizogenes to obtain stable transgenic hairy roots. After 30 d (Fig. 5a), positive transgenic hairy roots were selected via PCR amplification of rolB and 35 s-TcMYC2 (Fig. 5b). We first checked the gene expression of TcMYC2 and its target genes TcCHS, TcAOC, and TcGLIP in transgenic hairy root. As expected, TcAOC and TcGLIP expression was significantly upregulated, suggesting that TcMYC2 may positively regulate its target genes (Fig. 5c). However, the C value of TcCHS was close to 40 and was barely expressed in hairy roots, owing to the lack of trichome tissue in which TcCHS is specifically expressed [30]. The GC–MS analysis revealed that no pyrethrins were produced in hairy roots except for (E)-β-farnesene (Ef), which shares the substrate DMAPP with pyrethrins. Thus, TcCHS expression in the trichome was indispensable for de novo pyrethrin biosynthesis even though the substrate was
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Figure 4. TcMYC2 directly binds to and activates the promoters of TcCHS, TcAOC, and TcGLIP. a Schematic diagram of Y1H vectors. Reporters: the promoter of TcCHS/TcAOC/TcGLIP was inserted into the pHis2.1 vector. Effector: TcMYC2 was inserted into the pGADT7 vector. b Y1H assay showing TcMYC2 binding to TcCHS/TcAOC/TcGLIP promoter fragments. Growth on SD/−Leu/−Trp and SD/−Leu/−Trp/−His medium in a 10-fold dilution series. pHis2.1-pGADT7-TcMYC2 was used as a negative control, pHis2.1-p53/pGADT7-p53 was used as a positive control. c EMSA of interaction between TcMYC2 and the promoters of TcCHS/TcAOC/TcGLIP. The probe was designed from a fragment of the promoter containing E-box or G-box motifs. Purified His-tagged TcMYC2 protein solution was incubated with 10 μM FAM-labeled probe. Non-labeled specific cold probes and mutated cold probes at 50-fold dilution were used for the competition test. The shifted bands are indicated with an arrow. d Schematic diagram of dual-luciferase (LUC) vectors. Effector: TcMYC2 was inserted into the pGreen-62-SK vector driven by the CAMV 35S promoter. Reporters: the promoters of TcCHS/TcAOC/TcGLIP were inserted into the pGreen II-0800-LUC vector and drove LUC RNA translation. e Transient expression assays showing TcMYC2 binding to the TcCHS/TcAOC/TcGLIP promoters and promotion of LUC expression. (a) pGreen-II 62-SK/pGreen II 0800-LUC-Promoter (CHS, GLIP, AOC). (b) pGreen-II 62-SK. (c) pGreen-II 62-SK-MYC2/pGreen II 0800-LUC-Promoter (CHS, GLIP, AOC).

Transient overexpression of TcMYC2 upregulates pyrethrin biosynthesis genes and pyrethrin accumulation

The role of TcMYC2 in regulating pyrethrin biosynthesis in T. cinerariifolium was investigated. Given that stable transformation of T. cinerariifolium is not available and to mimic short-term MeJA-induced upregulation of pyrethrin biosynthesis, we transiently overexpressed TcMYC2 in T. cinerariifolium leaves, and determined the change in expression of pyrethrin biosynthesis genes by qRT-PCR at 2, 4, and 6 d post transformation. Compared with mock plants transformed with blank pGreenII62-SK, TcMYC2 transcript abundance was significantly increased in transformed plants, peaking at 4 d, and thereafter declined; however, at 6 d post transformation, TcMYC2 transcript abundance in transformed plants remained higher than that in the mock plants (Fig. 6a). Expression of TcCHS and TcAOC was markedly elevated in TcMYC2-overexpressing plants, peaking at 4 d, which was 11.7- to 55.7-fold higher than that in the mock plants (Fig. 6a). Expression of TcGLIP increased by 2.2-fold at 2 d compared with the control, and thereafter decreased to levels lower than the control (Fig. 6a). Significant differences were detected in the total pyrethrin contents between TcMYC2-overexpressing and mock leaves (Fig. 6bc). These supplied. No pyrethrins were detected in stable transformed green callus, which further supported this hypothesis (Fig. 5d).

Thus, based on stable transformation in hairy roots, TcMYC2 positively upregulated its target genes TcAOC and TcGLIP in T. cinerariifolium.
results demonstrated that overexpression of TcMYC2 enhanced pyrethrin accumulation.

**Transient silencing of TcMYC2 decreases pyrethrin biosynthesis gene expression and pyrethrin accumulation**

A viral-induced gene silencing (VIGS) assay was conducted to confirm the indispensable role of TcMYC2 in regulating pyrethrin biosynthesis. Compared with the mock (empty pTRV2 vector), TcMYC2 expression in T. cinerariifolium leaves was significantly reduced by ~93% in silenced plants (Fig. 6d). Downregulation of TcMYC2 expression resulted in a significant decrease (~1.2- to 2.5-fold) in the expression level of TcCHS, TcAOC, and TcGLIP (Fig. 6d). The total pyrethrin content was also reduced in TcMYC2-silenced T. cinerariifolium leaves (Fig. 6). These results suggest that TcMYC2 is an important positive regulator of pyrethrin biosynthesis, which acts by directly activating the pyrethrins biosynthetic genes.

**Continuous MeJA application increases trichome density and pyrethrin content**

Phytohormones promote the formation of leaf trichomes in plants [31, 32]. In T. cinerariifolium, the ovary epidermis has a high density of glandular trichomes in which pyrethrin precursors are produced [33]. However, the trichome number on leaves is limited. To examine the effect of MeJA treatment on trichome development in T. cinerariifolium, seedlings at the four-leaf stage were subjected to continuous MeJA treatment for 2 weeks. A significant (1.6-fold) increase in trichome density was observed on MeJA-treated leaves compared with mock leaves (Fig. 7a and b), suggesting that prolonged application of MeJA promotes the initiation of glandular trichomes. Given that the short MeJA treatment only transiently enhanced the pyrethrin content in the first few hours, we performed high-performance liquid chromatography (HPLC) analysis to detect pyrethrins in samples treated with MeJA for 2 weeks. Compared with mock plants, the pyrethrin I and pyrethrin II contents were significantly increased in MeJA-treated plants by
Figure 6. Transient overexpression and TRV-VIGS treatment of TcMYC2 in *Tanacetum cinerariifolium* leaves. **a** Pyrethrin biosynthesis gene expression in *T. cinerariifolium* leaves at 2, 4, and 6 d after transient overexpression of TcMYC2. Mock: transient overexpression of the empty vector; TcMYC2-OE: transient overexpression of pGreen-62-SK-TcMYC2. The fold change in relative expression level was normalized to the mock expression. **b** Representative HPLC chromatogram of total pyrethrins extracted from leaves after transient TcMYC2 overexpression. CK: transient overexpression of the empty vector; TcMYC2-OE: transient overexpression of pGreen-62-SK-TcMYC2. **c** Pyrethrin content in *T. cinerariifolium* leaves after transient TcMYC2 overexpression at 4 d. The ordinate represents the intensity peak area detected by HPLC; the abscissa represents the six major pyrethrin compounds. **d** Pyrethrin biosynthesis gene expression in *T. cinerariifolium* leaves after TRV-VIGS treatment of TcMYC2 at 14 d. Mock: VIGS empty pTRV2 vector; pTRV2-TcMYC2: TcMYC2 silenced leaves. The fold change in relative expression level was normalized to the mock expression. **e** Representative HPLC chromatogram of total pyrethrins extracted from TRV-VIGS treatment leaves. CK: VIGS empty pTRV2 vector; pTRV2-TcMYC2: TcMYC2 silenced leaves. **f** Pyrethrin content in *T. cinerariifolium* leaves after TRV-VIGS treatment of TcMYC2. The ordinate represents the intensity peak area detected by HPLC; the abscissa represents the six major pyrethrin compounds. Values are the mean ± SD of three biological replicates. *P < 0.05, **P < 0.01 (two-tailed Student’s t-test).

14% at 2 d post treatment, in contrast to the rapid decline in pyrethrin content at 8 h after short-term MeJA treatment (Fig. 7c). Thus, prolonged application of MeJA positively regulated glandular trichome development and pyrethrin accumulation in leaves.

Discussion

Jasmonates play an important role in controlling secondary metabolite synthesis through a network of TFs that regulate metabolite biosynthesis genes [11]. In this study, transient short-term MeJA treatment of *T. cinerariifolium* leaves strongly induced expression of upstream genes in the pyrethrin biosynthesis pathway, although the expression levels quickly declined. A transient increase in pyrethrin accumulation resulted, which lasted for only several hours and thereafter declined. The pyrethrin biosynthetic pathway is long and complex. Most pyrethrin biosynthesis genes and precursors are expressed in and exported from trichomes to synthesize pyrethrins, which mainly accumulate in the secretory

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**Figure 6.** a) Relative expression of pyrethrin biosynthesis genes in *T. cinerariifolium* leaves at 2, 4, and 6 d after transient overexpression of TcMYC2. b) Representative HPLC chromatogram of total pyrethrins extracted from leaves after transient TcMYC2 overexpression. c) Pyrethrin content in *T. cinerariifolium* leaves after transient TcMYC2 overexpression at 4 d. d) Pyrethrin biosynthesis gene expression in *T. cinerariifolium* leaves after TRV-VIGS treatment of TcMYC2 at 14 d. e) Representative HPLC chromatogram of total pyrethrins extracted from TRV-VIGS treatment leaves. f) Pyrethrin content in *T. cinerariifolium* leaves after TRV-VIGS treatment of TcMYC2. Values are the mean ± SD of three biological replicates. *P < 0.05, **P < 0.01 (two-tailed Student’s t-test).
Figure 7. Prolonged MeJA treatment regulates trichomes density and pyrethrin content of Tanacetum cinerariifolium leaves. a Scanning electron micrographs of T. cinerariifolium leaves. Mock: seedlings treated with 0.8% ethanol; MeJA: seedlings treated with 300 μM MeJA dissolved in 0.8% ethanol. MeJA was applied every 2 d for 14 d. b Trichome number on T. cinerariifolium leaves. Bars and error bars indicate the mean ± SD from six biological replicates. c Relative content of total pyrethrins. Bars and error bars indicate the mean ± SD from four biological replicates. *P < 0.05, **P < 0.01 (Student’s paired t-test).

cavity of the ovary wall to protect the seed [33]. Mature leaves contain no corresponding storage structures and the sudden excessive accumulation of pyrethrins might disturb the cellular homeostasis. This was shown previously in T. cinerariifolium with overexpression of TcCHS driven by the Chrysanthemum RUBISCO promoter. Overexpression of TcCHS increased the pyrethrin content but led to a significant decrease in chlorophyll content [34]. A pyrethrin content of 0.05% or 0.001% still exerts good control of pyrethrin-sensitive herbivorous insects [35, 36]. The average pyrethrin content in leaves is ~0.1% (dry weight), which is still toxic to pyrethrin-sensitive insects in leaves without external stimuli. We presume that it is not necessarily energetically costly to respond to JAs to increase the pyrethrin content.

Continuous MeJA treatment of seedlings at the four-leaf stage for 2 weeks increased the glandular trichome density and pyrethrin accumulation in the leaves. We speculate that under long-term MeJA exposure, seedlings gradually adapt to exogenous hormone signals, which results in morphological changes that enable increased pyrethrin production. These changes have been reported in other Asteraceae species, such as Asteraceae annua, which showed upregulation of the artemisinin content in leaves together with trichome development on the leaf surface [37]. Similarly, a significant correlation between the monoterpene and sesquiterpene content and gland density on leaves in chrysanthemum has been reported [38].

MYC, a member of the bHLH superfamily that harbors the conserved HLH domain, can bind to the G-box or E-box motifs in the promoters of target genes to regulate their involvement in secondary metabolite biosynthesis [39]. As expected, TcMYC2 could bind to the TcCHS, TcAOC, and TcGLIP promoters and
positively regulate expression of downstream genes in vitro. Plants transiently silenced for TcMYC2 expression showed significant downregulation of pyrethrin biosynthesis genes and dramatically reduced pyrethrin contents (Fig 6d and e). Thus, TcMYC2 positively regulated pyrethrin biosynthesis in T. cinerariifolium leaves. In JA-treated Arabidopsis plants, MYC2 positively regulates the expression of flavonoid biosynthesis genes [40]. MYC2 orthologs of tobacco regulate nicotine biosynthesis [41]. These MYC2 proteins show the strongest binding activity to the G-box followed by the 5′-CACGTG-3′ sequence. Thus, similar mechanisms operate in the regulation of the biosynthesis of various secondary metabolites, indicating that MYC2-like genes have conserved functions in these species [42]. MYCs generally exhibit functional redundancy and weakening of the function of one MYC can be compensated by other MYCs. In Arabidopsis, MYC5 has functions redundant with other MYCs, which participate in root growth inhibition, leaf senescence, and plant defense against insect attack and pathogen infection regulated by JA [43]. In Nicotiana tabacum, NtMYC2a coordinates with NtMYC2b to positively regulate nicotine biosynthesis [44].

TcMYC2 showed strong activity on pyrethrin biosynthesis genes in vitro and a strong decrease in pyrethrin content in VIGS plants, gene expression and pyrethrin content in leaves with transient overexpression of TcMYC2 showed only a slight and noncontinuous increase. In other studies, MYC-mediated continuous activation of JA responses inhibit growth, and are potentially harmful or fatal [45]. For example, overexpression of TmMYC2a and TmMYC2b results in downregulation of triptolide biosynthesis in hairy roots of Tripterygium wilfordii [46]. A possible reason is that TcMYC2 has multiple functions and may activate other negative transcription factors simultaneously. For instance, MYC2 directly binds to the promoter of JAZ repressors, which restrain JA response [47, 48]. Plants overexpressing MYC2 constitutively resynthesize JAZ repressors in response to JA stimulation to close activation of the pathway in a controlled manner [48].

To further explore the function of TcMYC2, stable transformation of hairy roots with TcMYC2 was conducted. The target genes TcAOC and TcGLIP were significantly upregulated, whereas TcCHS transcripts were not detected, and no pyrethrins were produced either with incubation with substrates. TcCHS is almost exclusively expressed in glands, but no pyrethrins and glands are produced by hairy roots of T. cinerariifolium [49]. Furthermore, tissue differentiation is a prerequisite for production of certain secondary metabolites [50]. Typical examples include some Hypericum species that yield hypericin, but their hairy roots are devoid of hypericin [51]. To clarify whether glands are essential for pyrethrin synthesis, the function of TcMYC2 requires further study in other tissues and experimental systems.

In summary, this study demonstrates that MeJA induces TcMYC2 expression in T. cinerariifolium and subsequently activates the expression of pyrethrin biosynthesis genes by binding to their promoters. Prolonged exposure of T. cinerariifolium seedlings to MeJA slightly enhances production of glandular trichomes, and consequently pyrethrin accumulation, but only in immature leaves (Fig 8). This study reports the first of likely several TFs that are indispensable to promoting the expression of pyrethrin biosynthesis genes and pyrethrin accumulation. Elucidation of the full spectrum of relevant TFs may provide a novel means of enhancing pyrethrin yield through metabolic engineering of T. cinerariifolium.

Materials and methods

Plant materials
T. cinerariifolium ‘W99’ was used in this study because it is easy rooting, grows rapidly, and has high pyrethrin content. T. cinerariifolium is a self-incompatible, highly heterogenous species [52], and individual genotypes have a unique pyrethrin content. One-month-old uniformly sized ‘W99’ seedlings grown in vitro on half-strength Murashige and Skoog medium in large airtight containers of identical size were used for MeJA treatment. The seedlings were grown at 25°C under a 16 h/8 h (light/dark) photoperiod.

Pyrethrum flower heads and leaves at different developmental stages (S1–S6 [3] and L1–L2 [15], respectively) as well as flower tissues, were collected in triplicate, immediately frozen in liquid nitrogen, and stored at −80°C until use.

MeJA treatment
For short-term MeJA treatment, 1-month-old ‘W99’ seedlings grown in vitro were sprayed with 5 mL of 2 mM MeJA dissolved in 0.8% ethanol. Leaves were collected in triplicate after 0 (control), 2, 4, 6, 8, 12, and 24 h. The samples were immediately frozen in liquid nitrogen and stored at −80°C until use.

For long-term MeJA treatment, ‘W99’ seedlings at the four-leaf stage were sprayed with 5 mL of 300 μM MeJA dissolved in 0.8% ethanol, and the roots were irrigated with 5 mL every 2 d for a total of 2 weeks. Plants sprayed with 0.8% ethanol served as a mock control. Subsequently, leaves were collected and oven-dried at 50°C for 48 h to constant dry weight.

RNA extraction, transcriptome assembly, and qRT-PCR
Total RNA was extracted from the samples using the standard phenol-chloroform extraction method. Twenty-four RNA-seq libraries were constructed and sequenced by a commercial sequencing service provider (Benagen, Wuhan, China). After filtering out low-quality reads and adapter sequences using fastp v0.18.0 software [53], the clean reads were de novo assembled using Trinity v2.6.6 software [54]. The assembly quality was evaluated using N50 and BUSCO v3.0.2 software [55]. Sequences were annotated with Diamond v0.9.24 using the nucleotide sequence (NT), nonredundant protein (NR), Clusters of Orthologous groups of proteins (COG), Kyoto Encyclopedia of Genes and
Genomes (KEGG), Swiss-Prot, and Gene Ontology (GO) databases. Gene expression levels were calculated using the RNA-Seq by Expectation–Maximization v1.3.1 software [56] and normalized to the FPKM values [57]. The DEGs were identified using DESeq2 software [58] with the following thresholds: log2 fold change > 1; \( p_{\text{adj}} < 0.05 \). GO and KEGG enrichment analyses of the DEGs were performed using ClusterProfiler [59]. A WGCNA (\( \gamma = 0.703 \)) analysis was performed to identify modules of highly correlated genes [60]. KEGG and GO annotation of the modules was performed with KOBASE v3.0 software [61]. The protein association network was searched using the STRING v11.5 database (http://string-db.org/).

The qRT-PCR assay was performed on the Roche LightCycler® 96 Real-time PCR System (Roche, Basel, Switzerland), in accordance with the manufacturer’s instructions, using SYBR Premix Ex Taq II (Takara, Kusatsu, Japan) and sequence-specific primers described previously [12]. Other primers were based on the transcriptome and checked using TBtools [64]. Primers used for qRT-PCR are listed in Table S1 (see online supplementary material). Three biological replicates were performed for each gene, with two technical repeats per biological replicate. 18S RRNA was used as the internal reference gene. Relative gene expression was calculated using the 2\(^{-\Delta \Delta CT}\) method [65]. Student’s two-tailed t-test was used to determine statistical significance at the 5% and 1% significance levels.

**Transcription factor and TcMYC2 identification**

PlantTFDB 5.0 was used to predict all TFs [66]. Protein domains were identified using the SMART online tool [67]. After filtering out weakly expressed genes and proteins lacking conserved domains, all bHLH TFs were visualized. The FPKM values of genes from the MeJA treatment and flower stage transcriptome were used to perform a cluster analysis. Heatmaps were generated using TBtools [64].

The open reading frame of TcMYC2 was cloned from T. cinerariafolium cDNA using sequence-specific primers (Table S1, see online supplementary material). Sequences of T. cinerariafolium homologous to Arabidopsis genes were identified by a BLAST search against The Arabidopsis Information Resource Database, and to genes of other plant species by a BLAST search against The Arabidopsis Information Resource database. The AtMYC proteins were scanned using HmmerSearch v3.0 software [68]. TcMYC2 and AtMYC family coding sequences were aligned using mafft v7.158 software [69]. A maximum-likelihood phylogenetic tree was constructed using IQtree2 software [70], using the best-fit model (JTT + F + I + G4) selected by ModelFinder [71] and with 1000 bootstrap replications.

**Cloning and bioinformatic analysis of gene promoters**

The promoter sequences of TcCHS, TcALDH, TcAOS, and TcGLIP were mined from the draft genome sequence of T. cinerariafolium [72]. The associated cis-elements and their biological functions were determined using PlantCARE [73], and the cis-regulatory elements (E-box, G-box, and G-box-like) were identified using PlantPAN3.0 [74].

**Y1H assay**

The full-length TcMYC2 cDNA was cloned into the pGADT7 vector using NdeI and XhoI restriction endonucleases. The promoter fragments of TcCHS, TcGLIP, TcALDH, and TcAOC were cloned into the EcoRI- and MluI-digested pHis2.1 vector. The resultant pGADT7::TcMYC2 plasmid was co-transformed into yeast strain Y187 together with pHis2.1::TcCHS, pHis2.1::TcGLIP, pHis2.1::TcAOC, or pHis2.1::TcALDH using the Super Yeast Transformation Kit (Coolaber, Beijing, China). The transformed yeast cells were selected on DDO (SD/−Leu/−Try) medium and interactions were tested on TDO (SD/−Leu/−Try/−His) medium at 30°C for 3 d.

**Dual-LUC transient expression assay**

To generate reporter constructs, promoter fragments of downstream genes (TcCHS, TcGLIP, TcALDH, and TcAOC) were cloned into the HindIII-linearized pGreenII0800-LUC vector. The TcMYC2 coding sequence was cloned into the HindIII-linearized pGreenII62-SK vector under the control of the CaMV 35S promoter. The resultant vectors were transiently coexpressed in N. benthamiana leaves. Luminescence was detected using the LB 985 Nightshade system (Berthold, Bad Wildbad, Germany). Introduction of the pGreen-62-SK empty vector and of pGreen-62-SK/pGreenII-0800-LUC::proTcCHS/TcGLIP/TcAOC constructs served as a negative control.

**EMSA**

The open reading frame without the terminator codon of TcMYC2 was inserted in the pET6HN-C vector protein, fused in the N-terminal frame of 6 × His. The vector pET6HN-C::TcMYC2-6 × His was transformed into Escherichia coli strain Rosetta (DE3). For induction of the recombinant protein, 0.5 mM IPTG was used, and the cultures were incubated at 18°C for 16 h. Then, Ni-NTA was used to purify the recombinant proteins. For EMSA, the promoter fragments of TcCHS, TcAOC, and TcGLIP contained E-box or G-box cis-elements. FAM-labeled probes and the same unlabeled DNA fragments, cis-element mutant DNA fragments were used as competitors in the assay. The EMSA assays were performed as described previously [75]. FAM-labeled DNA was detected by the chemiluminescence method.

**Subcellular localization of TcMYC2**

The coding sequence of TcMYC2 was amplified by PCR using sequence-specific primers containing the Sual and Km restriction sites at their ends (Table S1, see online supplementary material). The PCR product was cloned into pSuper1300-GFP, derived sequence-specific primers containing the CaMV 35S promoter. The resultant plasmid was introduced into Agrobacterium tumefaciens strain GV3101, and then co-infiltrated into N. benthamiana leaves together with the RFP-NLS plasmid (nuclear marker) following methods described previously [76]. The empty vector was used as a control. After 72 h of weak light exposure, fluorescence signals were observed with a confocal laser scanning microscope (Leica TCS-SP8; Leica, Wetzlar, Germany).

**TcMYC2 stable transformation of hairy roots**

The full-length TcMYC2 coding sequence was inserted into pBI121 vector digested with BamHI and SacI using homologous recombination, and transferred into A. rhizogenes strain MSU440. The resultant plasmid was transformed into Agrobacterium tumefaciens strain GV3101, and then co-infiltrated into N. benthamiana leaves together with the RFP-NLS plasmid (nuclear marker) following methods described previously [76]. The empty vector was used as a control. After 30 d using the CTAB method. Successful transfer of the transgene-positive cells was confirmed by PCR. The primers for rolB of the A. rhizogenes Ri plasmid were designed in accordance with a previous study [77]. Transgenic hairy roots were determined by qRT-PCR and GC–MS analysis.
Transient overexpression of TcMYC2 in T. cinerariifolium leaves

The full-length TcMYC2 coding sequence was cloned into the HindIII-linearized pGreenI62-SK vector downstream of the CaMV 35S promoter using gene-specific primers (Table S1, see online supplementary material). The pGreenI62-SK: TcMYC2 vector was co-transformed together with the helper plasmid pSoup19 into A. tumefaciens strain GV3101. Leaves of T. cinerariifolium were placed in a 500 mL beaker containing A. tumefaciens suspension. The leaves were infiltrated under vacuum of 0.23 atm for 5 min. The agroinfiltrated leaves were dried with filter paper and stored in a Petri dish lined with a moisture-retaining filter paper at the bottom, as described previously [78]. After 2, 4, and 6 d of incubation, the agroinfiltrated leaf samples were used for qRT-PCR, and the 4 d samples were used for HPLC analysis.

VIGS assay

Tobacco rattle virus (TRV)-based vectors, pTRV1 and pTRV2, were used for the VIGS assay. A 256 bp fragment complementary to the TcMYC2 coding sequence was designed, searched in the local transcriptome library using siRNA Finder [79], and cloned into the BamHI-linearized pTRV2 vector by homologous recombination using gene-specific primers (Table S1, see online supplementary material). The pTRV2:TcMYC2 and pTRV1 plasmids were transformed into chemically active GV3101 cells using the liquid nitrogen freeze–thaw method. The VIGS assay was conducted as described previously [80] with three biological replicates. Empty vectors were used as controls. After 14 d, the samples were subjected to qRT-PCR and HPLC analyses.

Measurement of pyrethrin content by GC–MS

Leaf samples exposed to short-term MeJA treatment were ground into a fine powder in liquid nitrogen. Powdered tissue (0.5 g) was transferred to a tube containing 2 mL methyl tert–butyl ether (MTBE), with 0.01 ng/mL tetradecane as an internal standard. The tube was vortexed for 3 min at maximum speed and incubated at 24°C with rotation of 50 rpm for 2 h. The sample was dried using Na2SO4 and filtered through a 0.22-μm mesh filter. The pyrethrin content was measured using a GC/MS-QP2010Ultra apparatus (Shimadzu Corporation, Kyoto, Japan) equipped with a HP-5 MS column. Three biological replicates were performed for each sample. The GC process was performed as described previously [81]. Commercial pyrethrin extract (Sigma-Aldrich, St Louis, MI, USA) was used as the standard.

Pyrethrin content measurement by HPLC

The transiently transformed samples and samples exposed to long-term MeJA treatment were oven-dried at 50°C for 48 h to constant dry weight. The dried leaves were milled into a fine powder and dissolved in n-hexane at a concentration of 1 g per 6 mL in glass tubes with screwcaps. After vortexing for 30 s, the samples were extracted in an ultrasound water bath for 10 min, then vortexed again for 30 s, as described previously [74]. The samples were filtered through a 0.22-μm mesh filter. The pyrethrin content was measured by HPLC, with three biological replicates, as described previously [6]. Commercial pyrethrin extract (Sigma-Aldrich, St Louis, MI, USA) was used as the standard.

Analysis of the effect of MeJA on trichome density

Seedlings at the four-leaf stage were sprayed with 300 μM MeJA every 2 d for 2 weeks. The mock control was treated with 0.8% ethanol. The sixth leaf, which emerged after MeJA treatment, was excised and prepared for scanning electron microscopy as described previously [82]. The trichome number was determined from the micrographs of six biological replicates. Statistical significance was determined by Student’s t-test at P < 0.05.

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Author contributions

T.Z., H.H., J-W.L., and C-Y.W. planned and designed research; T.Z., J-W.L., Z-Z.X., L.Z., Q.Y., and H.H. performed research; T.Z., J-W.L., and H.H. analysed data; H.H., T.Z., J-W.L., and C-Y.W. wrote the paper; H.H., T.Z., J-W.L., J-J.L., J-L., Z-L.C., M.A.J and C-Y.W. revised the paper.

Data availability

All transcriptome raw data can be downloaded from NCBI (BioProjects: PRJNA723714, https://www.ncbi.nlm.nih.gov/sra/PRJNA723714).

Conflict of interests

The authors declare that they have no conflict of interest.

Supplementary data

Supplementary data is available at Horticulture Research online.

References

1. Casida JE, Quistad GB. Pyrethrum Flowers: Production, Chemistry, Toxicology, and Uses. Oxford: Oxford University Press; 1995.
2. Katsuda Y. Development of and future prospects for pyrethroid chemistry. Pest Sci. 1999;55:775–82.
3. Ramirez AM. Pyrethrum secondary metabolism: biosynthesis, localization and ecology of defence compounds. Vol. Ph.D. Thesis, Wageningen University, 2013.
4. lybrand DB, Xu H, Last RL et al. How plants synthesize pyrethrins: safe and biodegradable insecticides. Trends Plant Sci. 2020;25:1240–51.
5. Rivera SB, Swedlund BD, King GJ et al. Chrysanthemyl diphosphate synthase: isolation of the gene and characterization of the recombinant non-head-to-tail monoterpene synthase from chrysanthemum cinerariaefolium. P Natl Acad Sci USA. 2001;98:4373–8.
6. Hu H, Li J, Delatte T et al. Modification of chrysanthemum odour and taste with chrysanthemol synthase induces strong dual resistance against cotton aphids. Plant Biotechnol J. 2018;16:1434–45.
7. Xu H, Moghe GD, Wiegt-Rininger K et al. Coexpression analysis identifies two oxidoreductases involved in the biosynthesis of
the monoterpene acid moiety of natural pyrethrin insecticides in *Tanacetum cinerariifolium*. *Plant Physiol.* 2018;176:524–37.

8. Xu H, Li W, Schilmiller AL et al. Pyrethric acid of natural pyrethrin insecticide: complete pathway elucidation and reconstitution in *Nicotiana benthamiana*. *New Phytol*. 2019;223:751–65.

9. Kikuta Y, Ueda H, Takahashi M et al. Identification and characterization of a GDSL lipase-like protein that catalyzes the ester-forming reaction for pyrethrin biosynthesis in *Tanacetum cinerariifolium*: a new target for plant protection. *Plant J.* 2012;71:183–93.

10. Ghorbel M, Brini F, Sharma A et al. Role of jasmonic acid in plants: the molecular point of view. *Plant Cell Rep.* 2021;40:1471–94.

11. Wasternack C, Strnad M. Jasmonates are signals in the biosynthesis of secondary metabolites — pathways, transcription factors and applied aspects — a brief review. *New Biotechnol*. 2019;48:1–11.

12. Li W, Zhou F, Pichersky E. Jasmonic hydroxylase, a key enzyme in the synthesis of the alcohol moiety of pyrethrin insecticides. *Plant Physiol.* 2018;177:1498–509.

13. Li W, Lybrand DB, Zhou F et al. Pyrethrin biosynthesis: the cytochrome P450 oxidoreductase CYP82Q3 converts jasmonolide to pyrethronine. *Plant Physiol.* 2019;181:934–44.

14. Yang T, Stoopen G, Wiegers G et al. Pyrethrins protect pyrethrum leaves against attack by Western flower Thrips. *J Chem Ecol*. 2012;38:370–7.

15. Zito SW, Zieg RG, Staba EJ. Distribution of pyrethrins in oil glands and leaf tissue of *chrysanthemum cinerariaefolium*. *Planta Med.* 1983;47:205–7.

16. Baldwin IT, Karb MJ, Callahan P. Foliar and floral pyrethrins of *chrysanthemum cinerariaefolium* are not induced by leaf damage. *J Chem Ecol.* 1993;19:2081–7.

17. Li L, Wang D, Zhou L et al. JA-responsive transcription factor SmMYB97 promotes phenolic acid and tannin accumulation in salvia miltiorrhiza. *J Agr Food Chem.* 2020;68:14850–62.

18. Fu X, Peng G, Hassan H et al. *AaWRKY9* contributes to light- and jasmonate-mediated to regulate the biosynthesis of artemisinin in *Artemisia annua*. *New Phytol.* 2021;231:1858–74.

19. Ma Y-N, Xu DB, Yan X et al. Jasmonate- and abscisic acid-activated *AaGSw1-AaTCP15/AaORA* transcriptional cascade promotes artemisinin biosynthesis in *Artemisia annua*. *Plant Biotechnol J.* 2021;19:1412–28.

20. Gao WJ, Meng Q, Luo H et al. Transcriptional responses for biosynthesis of flavor volatiles in methyl jasmonate-treated *Chrysanthemum indicum* var. aromaticum leaves. *Ind Crop Prod.* 2020;147:112254.

21. An C, Sheng L, du X et al. Overexpression of *CmMYB15* provides chrysanthemum resistance to aphids by regulating the biosynthesis of lignin. *Hortic Res.* 2019;6:84.

22. Zhang W, Gao T, Li P et al. *Chrysanthemum CmWRKY53* negatively regulates the resistance of *chrysanthemum to the aphid Macrosiphoniella sanborni*. *Hortic Res.* 2020;7:109.

23. Bai JF, Wang YK, Guo LP et al. Genomic identification and characterization of MYC family genes in wheat (*Triticum aestivum*). *BMC Genomics*. 2019;20:1032.

24. Shen Q, Lu X, Yan T et al. The jasmonate-responsive *AaMYC2* transcription factor positively regulates artemisinin biosynthesis in *Artemisia annua*. *New Phytol.* 2016;210:1269–81.

25. Hong GJ, Xue XY, Yao YB et al. *Arabidopsis MYC2* interacts with DELLAs proteins in regulating sesquiterpene synthase gene expression. *Plant Cell*. 2012;24:2635–48.

26. Xu YH, Liao YC, Lv FF et al. Transcription factor *AaMYC2* controls the Jasmonate-responsive expression of *ASS1* regulating sesquiterpene biosynthesis in *Aquilaria sinensis* (lour). *Gigl. Plant Cell Physiol.* 2017;58:1924–33.

27. Zhang M, Jia X, Chen Y et al. *TcMYC2a*, a basic helix loop helix transcription factor, transduces jas-signals and regulates taxol biosynthesis in *Taxus chinensis*. *Front Plant Sci.* 2018;9:863.

28. Dabiri M, Majdi M, Bahramnejad B. Partial sequence isolation of DXS and AOS genes and gene expression analysis of terpenoids and pyrethrin biosynthetic pathway of *chrysanthemum cinerariaefolium* under abiotic elicitation. *Acta Physiol Plant*. 2020;42:30.

29. Kikuta Y, Ueda H, Nakayama K et al. Specific regulation of pyrethrin biosynthesis in *chrysanthemum cinerariaefolium* by a blend of volatiles emitted from artificially damaged conspecific plants. *Plant Cell Physiol.* 2011;52:588–94.

30. Sultana S, Hu H, Gao L et al. Molecular cloning and characterization of the trichome specific chrysanthemyl diphenylphosphate/chrysanthemol synthase promoter from *Tanacetum cinerariifolium*. *Sci Hortic.* 2015;185:193–9.

31. Maes L, van Nieuwerburgh FCW, Zhang Y et al. Dissection of the phytohormonal regulation of trichome formation and biosynthesis of the antimalarial compound artemisinin in *Artemisia annua* plants. *New Phytol.* 2011;189:176–89.

32. Boughton AJ, Hoover K, Felton GW. Methyl jasmonate application induces increased densities of glandular trichomes on tomato. *J Chem Ecol.* 2005;31:2211–6.

33. Ramirez AM, Stoopen G, Menzel TR et al. Bidirectional secretions from glandular trichomes of pyrethrum enable immunization of seedlings. *Plant Cell*. 2012;24:4252–65.

34. Li J, Xu Z, Zeng T et al. Overexpression of *TcCHS* increases Pyrethrin content when using a genotype-independent transformation system in pyrethrum (Tanacetum cinerariifolium). *Plant Theory*. 2022;11:1575.

35. Andreev R, Kutinkova H, Baltas K. Non-chemical control of some important pests of sweet cherry. *Journal of Plant Protection Research*. 2008;48:503–8.

36. Shukla S, Tiwari SK. The influence of pyrethrum extract on the developmental stages of the rice-moth, *Corcyra cephalonica* Stanion (Lepidoptera: Pyralidae). *Egypt J Biol.* 2012;14:57–62.

37. Xie L, Yan T, Li L et al. An HD-ZIP-MYB complex regulates glandular secretory trichome initiation in *Artemisia annua*. *New Phytol.* 2021;231:2050–64.

38. Guan Y, Chen S, Chen F et al. Exploring the relationship between Trichome and Terpene chemistry in *chrysanthemum*. *Plant Theory*. 2022;11:1410.

39. Hao Y, Zong X, Ren P et al. Basic helix-loop-helix (bHLH) transcription factors regulate a wide range of functions in *Arabidopsis*. *Int J Mol Sci*. 2021;22:7152.

40. Dombrecht B, Xue GP, Sprague SJ et al. The influence of pyrethrum extract on the developmental stages of the rice-moth, *Corcyra cephalonica* Stanion (Lepidoptera: Pyralidae). *Acta Physiol Plant*. 2013;35:109.

41. Todd AT, Liu E, Polvi SL et al. A functional genomics screen identifies diverse transcription factors that regulate alkaloid biosynthesis in *Nicotiana benthamiana*. *Plant J.* 2010;62:589–600.

42. Shoji T, Hashimoto T. Tobacco *MYC2* regulates jasmonate-inducible nicotine biosynthesis genes directly and by way of the NtC2-locus ERF genes. *Plant Cell Physiol.* 2011;52:1117–30.

43. Song S, Huang H, Wang J et al. *MYC5* is involved in jasmonate-regulated plant growth, leaf senescence and defense responses. *Plant Cell Physiol.* 2017;58:1752–63.

44. Zhang HB, Bokowiec MT, Rushton PJ et al. Tobacco transcription factors NtMYC2a and NtMYC2b form nuclear complexes with the nta1-z1 repressor and regulate multiple jasmonate-inducible steps in nicotine biosynthesis. *Mol Plant*. 2012;5:73–84.
45. Chico JM, Lechner E, Fernandez-Barbero G et al. CUL3BPM E3 ubiquitin ligases regulate MYC2, MYC3, and MYC4 stability and JA responses. Proc Natl Acad Sci USA. 2020;117:6205–15.

46. Huo YB, Zhang J, Zhang B et al. MYC2 transcription factors TwMYC2a and TwMYC2b negatively regulate Triptolide biosynthesis in Tripterygium wilfordii hairy roots. Plants. 2021;10:679.

47. Figueroa P, Browse J. The Arabidopsis JAZ2 promoter contains a G-box and thymidine-rich module that are necessary and sufficient for jasmonate-dependent activation by MYC transcription factors and repression by JAZ proteins. Plant Cell Physiol. 2012;53:330–43.

48. Chini A, Fonseca S, Fernández G et al. The JAZ family of repressors is the missing link in jasmonate signalling. Nature. 2007;448:666–71.

49. Li JW, Zeng T, Xu ZZ et al. Ribosome-mediated CRISPR/Cas9 gene editing in pyrethrum (Tanacetum cinerariifolium) hairy roots using a RNA polymerase II-dependent promoter. Plant Methods. 2022;18:32.

50. Ramawat KG. An introduction to the process of cell, tissue, and organ differentiation, and production of secondary metabolites. In: Ramawat KG (ed). Plant Cell and Tissue Differentiation and Secondary Metabolites. Springer International Publishing, Cham, 2021, pp. 1–22.

51. Nigutová K, Kusari S, Sezgin S et al. Chemometric evaluation of hypericin and related phytochemicals in 17 in vitro cultured hypericum species, hairy root cultures and hairy root-derived transgenic plants. J Pharm Pharmacol. 2019;71:46–57.

52. Brewer JG. Incompatibility relationship in pyrethrum (chrysantheum cinerariifolium var. Euphytica). 1974;23:45–7.

53. Chen SF, Zhou YQ, Chen YR et al. Fastp: an ultra-fast all-in-one FASTQ preprocessor. Bioinformatics. 2018;34:i884–90.

54. Grabherr MG, Haas BJ, Yassour M et al. Full-length transcriptome assembly from RNA-Seq data without a reference genome. Nat Biotechnol. 2011;29:644–52.

55. Simão FA, Waterhouse RM, Ioannidis P et al. BUSCO: assessing genome assembly and annotation completeness with single-copy orthologs. Bioinformatics. 2015;31:3210–2.

56. Li B, Dewey CN. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. BMC Bioinformatics. 2011;12:16:323.

57. Trapnell C, Williams BA, Pertea G et al. Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. Nat Biotechnol. 2010;28:511–5.

58. Anders S, Huber W. Differential expression analysis for sequence count data. Genome Biol. 2010;11:R106.

59. Yu G, Wang LG, Han Y et al. Cluster profiler: an R package for comparing biological themes among gene clusters. OMICS J Integr Biol. 2012;16:284–7.

60. Zhang B, Horvath S. A general framework for weighted gene co-expression network analysis. Stat Appl Genet Mol Biol. 2005;4:Article17.

61. Bu D, Luo H, Huo P et al. KOBAS-i: intelligent prioritization and exploratory visualization of biological functions for gene enrichment analysis. Nucleic Acids Res. 2021;49:W317–25.

62. Chin CH, Chen SH, Wu HH et al. cytoHubba: identifying hub objects and sub-networks from complex interactome. BMC Syst Biol. 2014;8:S11.

63. Shannon P, Markiel A, Ozier O et al. Cytoscape: a software environment for integrated models of biomolecular interaction networks. Genome Res. 2003;13:2498–504.