Cloning and Functional Analysis of TcALDH and TcGLIP Gene Promoters in Pyrethrum: Post-print

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Abstract

Natural pyrethrins are green plant-derived bioinsecticides extracted from pyrethrum (Tanacetum cinerariifolium). Aldehyde dehydrogenase (TcALDH) and GDSL lipase (TcGLIP) are key rate-limiting enzymes in the pyrethrin biosynthetic pathway. To investigate the functions of TcALDH and TcGLIP genes, this study cloned the promoters of these genes from pyrethrum clone 'W99' , and analyzed their regulatory elements, promoter activity, hormone-inducible specificity, and tissue specificity through bioinformatics analysis, histochemical staining (GUS staining), luciferase reporter assays, and exogenous plant hormone treatment experiments. The results demonstrated that: (1) The cloned promoter sequences of TcALDH and TcGLIP were 2,848 bp and 1,343 bp, respectively, both containing multiple cis-acting elements associated with stress response and hormone signaling. (2) Plant expression vectors harboring promoter-luciferase fusions were constructed, and fluorescence imaging in tobacco leaves revealed that the TcALDH promoter exhibited hormone-inducible specificity for methyl jasmonate (MeJA) and abscisic acid (ABA). (3) Treatment of pyrethrum 'W99' tissue culture seedlings with MeJA and ABA demonstrated that TcALDH expression was up-regulated by ABA within 12 h, whereas MeJA induction resulted in an initial increase followed by a subsequent decrease; TcGLIP expression was down-regulated by both ABA and MeJA treatments. (4) Plant expression vectors harboring promoter-GUS fusions were constructed and transformed into tobacco; GUS activity staining of transgenic leaves revealed that the TcALDH promoter was active in tobacco leaf glands, glandular trichome heads, and mesophyll, while the TcGLIP promoter was active exclusively in mesophyll cells. In summary, the promoters of TcALDH and TcGLIP exhibit tissue specificity, and the TcALDH promoter displays hormone-inducible characteristics in response to methyl jasmonate and abscisic acid. This study provides novel insights into

the regulatory mechanisms by which TcALDH and TcGLIP genes participate in pyrethrin synthesis in pyrethrum.

Full Text

Cloning and Functional Analysis of TcALDH and TcGLIP Gene Promoters from Pyrethrum

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Abstract

Natural pyrethrins are green botanical insecticides extracted from pyrethrum (*Tanacetum cinerariifolium*). Aldehyde dehydrogenase (TcALDH) and GDSL lipase (TcGLIP) are key rate-limiting enzymes in the pyrethrin biosynthetic pathway. To investigate the functions of TcALDH and TcGLIP genes, this study cloned the promoters of these genes from pyrethrum clone 'W99'. Through bioinformatics analysis, histochemical staining (GUS staining), luciferase reporter assays, and exogenous plant hormone treatments, we analyzed the regulatory elements, promoter activity, hormone-induced specificity, and tissue specificity of these promoters. The results showed: (1) The cloned promoter sequences of TcALDH and TcGLIP were 2,848 bp and 1,343 bp, respectively, both containing multiple cis-acting elements related to stress response and hormone signaling. (2) Plant expression vectors fusing the promoters with luciferase were constructed, and fluorescence imaging in tobacco leaves revealed that the TcALDH promoter exhibited hormone induction specificity for methyl jasmonate (MeJA) and abscisic acid (ABA). (3) Treatment of pyrethrum 'W99'plantlets with MeJA and ABA showed that TcALDH expression was upregulated by ABA within 12 h, while under MeJA induction it first increased then decreased; TcGLIP expression was downregulated by both ABA and MeJA. (4) Plant expression vectors fusing TcALDH and TcGLIP promoters with the GUS gene were constructed and transformed into tobacco. GUS activity staining of transgenic leaves showed that the TcALDH promoter was expressed in tobacco leaf glands, glandular trichome heads, and mesophyll, while the TcGLIP promoter was expressed only in tobacco mesophyll cells. In conclusion, the promoters of TcALDH and TcGLIP exhibit tissue specificity, and the TcALDH promoter possesses MeJA and ABA hormone induction characteristics. This study provides new insights into the regulatory mechanisms of TcALDH and TcGLIP genes involved in pyrethrin synthesis.

Keywords: *Tanacetum cinerariifolium*, TcALDH, TcGLIP, promoter, functional analysis

Introduction

Pyrethrum (*Tanacetum cinerariifolium*), a perennial Asteraceae plant, has been used for centuries to extract the green botanical insecticide pyrethrin (Lybrand et al., 2020). Pyrethrins extracted from flower heads have characteristics such as rapid insecticidal action, easy degradation without accumulation, low toxicity to mammals, and suitability for sensitive populations, making them widely used in organic agriculture and household pest control (Nelson, 1974). The flower heads also release large amounts of the volatile terpene (E)-beta-farnesene (EβF), which can attract ladybugs and repel aphids in the field (Li et al., 2019; Li et al., 2021), while simultaneously attracting numerous pollinators such as hoverflies (Zeng et al., 2021; Zeng et al., 2021). Therefore, pyrethrum is also used as an intercropping crop and has extensive applications in Yunnan, China (Zhou et al., 2022). The global demand for pyrethrins is substantial, as they serve as natural botanical insecticides to avoid excessive use of chemically synthesized insecticides (Suraweera et al., 2017). How to increase pyrethrin content has always been a hot spot and focus in both the pyrethrum industry and basic research.

Pyrethrins in plants are formed through esterification of monoterpene carboxylic acid moieties (chrysanthemic acid and pyrethric acid) with keto-alcohol moieties (pyrethrolone, jasmolone, and cinerolone) (Staudinger & Ruzicka 1924; Mossa et al., 2018). The acid precursors are derived from the plastidial methylerythritol 4-phosphate (MEP) pathway of terpenoid metabolism (Lybrand et al., 2020), while the keto-alcohol precursors originate from the jasmonate (JAs) pathway (Matsuda et al., 2005). In the monoterpene synthesis model of pyrethrins, two molecules of dimethylallyl pyrophosphate (DMAPP) are sequentially catalyzed in glandular trichomes by chrysanthemyl diphosphate synthase (CDS), alcohol dehydrogenase 2 (ADH2), and aldehyde dehydrogenase 1 (ALDH1) to form the precursor molecule chrysanthemic acid. Subsequently, chrysanthemic acid is transported to the subepidermal tissues beneath the glands, where it is finally catalyzed by GDSL lipase (GLIP) in the seed pericarp to form pyrethrins, which are then absorbed by the embryo and transferred to seedling tissues (Kikuta et al., 2012; Ramirez et al., 2012; Xu et al., 2018; Lybrand et al., 2020; Li et al., 2022a). TcALDH participates in the final catalytic reaction of the pyrethric acid moiety to synthesize chrysanthemic acid CoA, which then forms an ester bond with the keto-alcohol under the catalysis of TcGLIP to produce the final product pyrethrin (Kikuta et al., 2012; Wang et al., 2022). Thus, TcALDH and TcGLIP are key rate-limiting enzyme genes in pyrethrin synthesis. Currently, numerous ALDH and GLIP gene promoters have been cloned from Arabidopsis and cotton (Hou & Bartels, 2015; Guo et al., 2017; Ma et al., 2018; Yang et al., 2021), and ALDH gene promoters have also been reported in sorghum, soybean, and *Artemisia annua*. However, only one promoter of the gland-specific chrysanthemyl diphosphate synthase gene TcCHS has been isolated from pyrethrum (Sultana et al., 2015). Therefore, studying the promoters of other pyrethrin synthase genes is a prerequisite for elucidating the regulatory mechanisms of

pyrethrin synthesis.

Jasmonic acid and its derivatives not only participate as reaction substrates in the biosynthesis of the keto-alcohol moiety of pyrethrins but also regulate pyrethrin synthesis as defense stress hormones (Matsuda et al., 2005; Li et al., 2018). Our research group previously performed transcriptome data analysis of white pyrethrum leaves and flowers under methyl jasmonate treatment, annotating many genes related to pyrethrin synthesis and metabolism. Based on this, the present study cloned the promoter sequences of TcALDH and TcGLIP genes from pyrethrum clone 'W99' , analyzed their regulatory elements, promoter activity, hormone-induced specificity, and tissue specificity, further revealing the role of plant hormones in the synthesis mechanism and metabolic regulation of pyrethrins, thereby providing a theoretical basis for breeding excellent pyrethrum varieties and increasing pyrethrin content.

Materials and Methods

1.1 Experimental Materials

Leaves of pyrethrum clone 'W99' were obtained from the tissue culture room at Huazhong Agricultural University. *Escherichia coli* DH5α and *Agrobacterium tumefaciens* strains GV3101 and EHA105 were purchased from Shanghai Weidi Biotechnology Co., Ltd. *Nicotiana benthamiana* used for transformation was grown in a tissue culture room at 25 °C under a 16 h light/8 h dark photoperiod.

1.2 Promoter Cloning

Genomic DNA (gDNA) was extracted from pyrethrum leaves using the RaPure Plant DNA Mini Kit (Magen, China) and used as a template. Based on the pyrethrum genome (Yamashiro et al., 2019), specific primers F-ALDH-pro and R-ALDH-ORF were designed for the TcALDH gene promoter, and F-GLIP-pro and R-GLIP-ORF for the TcGLIP gene promoter (Table 1). Amplification was performed using High-Fidelity Master Mix (MCLAB, China). The amplified products were ligated into the pBLUE-T vector (ZOMANBIO, China), transformed into *E. coli*, and single colonies were selected for sequencing. The obtained fragments were verified against the pyrethrum genome. Regulatory elements in the promoters were predicted using PlantCARE software.

1.3 Vector Construction

Using the correctly sequenced plasmids as templates, amplification was performed with primers containing homologous recombination fragments: Luc-aldh-F, Luc-aldh-R, Luc-glip-F, and Luc-glip-R (Table 1). The PCR products were recovered and ligated into the HindIII-digested pGreenII 0800-LUC vector, transformed into *E. coli*, and single colonies were selected for sequencing. The correctly sequenced vectors were transformed into GV3101.

Using the correctly sequenced plasmids as templates, amplification was performed with primers containing homologous recombination fragments: F-aldh121pro, R-aldh121pro, F-glip121pro, and R-glip121pro (Table 1). The PCR products were recovered and ligated into the HindIII and BamHI-digested PBI121 vector, transformed into *E. coli*, and single colonies were selected for sequencing. The correctly sequenced vectors were transformed into EHA105 *Agrobacterium*.

1.4 LUC Transient Expression and Fluorescence Imaging in Tobacco

Agrobacterium colonies containing the recombinant pGreenII 0800-LUC vector were inoculated into YEP liquid medium and cultured to $OD_{600} = 0.5$. After centrifugation at 5,000 $r \cdot min^{-1}$ for 7 min, the pellets were resuspended in MES infiltration buffer and adjusted to $OD_{600} = 1.0$. Two- to three-week-old *N. benthamiana* plants were used. *Agrobacterium* suspension was infiltrated into tobacco leaves using a needleless syringe, with the left half of each leaf infiltrated with *Agrobacterium* containing the empty pGreenII 0800-LUC vector and the right half with *Agrobacterium* containing the recombinant pGreenII 0800-LUC vector. The infiltrated tobacco plants were cultured in a growth chamber for 2 days, then sprayed with 50 $mol \cdot L^{-1}$ abscisic acid (ABA) (concentration reference: Hu et al., 2021) or 100 $mol \cdot L^{-1}$ methyl jasmonate (MeJA) (concentration reference: Chen et al., 2021), with water-sprayed plants serving as controls. After 12 h, the Luciferase Assay Substrate and Luciferase Assay Buffer II from the Dual-Luciferase Reporter Assay Kit were mixed and applied to the infiltrated regions of tobacco leaves. Fluorescence was then observed using the LB 985 Nightshade system (Berthold, Bad Wildbad, Germany).

1.5 Analysis of TcALDH and TcGLIP Gene Expression in Pyrethrum Under Different Hormone Treatments

MeJA treatment: Tissue-cultured pyrethrum 'W99' plantlets subcultured for one month were used as experimental material. The plantlets were sprayed with 5 mL of 2 $mmol \cdot L^{-1}$ MeJA solution (concentration reference: Buraphaka & Putalun, 2020), the caps were tightened, and after culturing for 0, 4, and 12 h, the leaves were rapidly cooled in liquid nitrogen and stored at -80 °C.

ABA treatment: Tissue-cultured pyrethrum 'W99' plantlets subcultured for one month were used as experimental material. The plantlets were sprayed with 5 mL of 1 $mmol \cdot L^{-1}$ ABA solution (concentration reference), the caps were tightened, and after culturing for 0, 4, and 12 h, the leaves were rapidly cooled in liquid nitrogen and stored at -80 °C.

Gene expression levels after the two hormone treatments were analyzed using real-time qPCR. RNA was extracted using the Ultrapure RNA Kit (CWBIO, China), and cDNA was synthesized using the EasyScript One-Step gDNA Removal and cDNA Synthesis SuperMix (TRANS, China). Fluorescence quantification was performed on the Applied Biosystems 7500 platform

using 2×Sybr Green qPCR Mix (Aidlab, Beijing, China). Primers used for qPCR were TcALDH-RT-F, TcALDH-RT-R, TcGLIP-RT-F, TcGLIP-RT-R, TcGAPDH-F, and TcGAPDH-R (Table 1) (Ramirez et al., 2012).

1.6 Tobacco Genetic Transformation Screening and GUS Expression

Agrobacterium colonies containing the pTcGLIP-GUS plasmid were inoculated into YEP liquid medium and cultured to $OD_{600} = 0.5$. After centrifugation at 5,000 $r \cdot min^{-1}$ for 7 min, the pellets were resuspended in liquid MS medium and adjusted to $OD_{600} = 0.5$. Tobacco leaves were cut into 0.5 cm × 0.5 cm pieces, immersed in the *Agrobacterium* suspension for 10 min, blotted dry on filter paper, and placed on tobacco co-cultivation medium consisting of MS basal medium (Murashige & Skoog basic medium, MS) + 2.25 $mg \cdot L^{-1}$ 6-benzylaminopurine (6-BA) + 0.3 $mg \cdot L^{-1}$ 1-naphthylacetic acid (NAA). After culturing in darkness for 2 days, the leaves were transferred to selection medium consisting of MS + 2.25 $mg \cdot L^{-1}$ 6-BA + 0.3 $mg \cdot L^{-1}$ NAA + 400 $mg \cdot L^{-1}$ cefotaxime (Cef) + 50 $mg \cdot L^{-1}$ kanamycin (Kan), subcultured every 2 weeks. When resistant shoots grew to 1 cm, they were excised and placed on MS medium containing 400 $mg \cdot L^{-1}$ Cef and 50 $mg \cdot L^{-1}$ Kan for continued culture.

DNA was extracted from kanamycin-resistant *N. benthamiana* leaves using the HiPure Plant DNA Mini Kit (Magen, China). Using this DNA as template, pTcALDH-GUS transgenic tobacco was verified with F-aldh121pro as the forward primer and TcALDH promoter-specific primer R-aldh-pro280 as the reverse primer; pTcGLIP-GUS transgenic tobacco was verified with F-glip121pro as the forward primer and R-GUS-T as the reverse primer. The transformation strain served as positive control and wild-type tobacco as negative control. PCR amplification was performed using 2×Taq Master Mix (Novoprotein, China).

Positive tobacco plants subcultured for one month were stained using the GUS Staining Kit (Coolaber, China) following the manufacturer' s protocol. After staining, the leaves were decolorized with 70% ethanol until completely chlorophyll-free, and staining patterns were observed under a stereomicroscope. Three independent transgenic lines were stained for each promoter.

Table 1 Primers used in the experiments

Primer	Sequence (5 to 3)
F-GLIP-pro	AAACTAGAAGCAAAGATCATCGTACT
R-GLIP-ORF	TTAACATGGGTGTTGATGTGGT
F-ALDH-pro	CCCCTCTATAGAAAGATAATTTAATTC
R-ALDH-ORF	GAACTTGATGTCATAAGCTAA
F-glip121pro	GACCATGATTACGCCAAGCTTGAAAAACTAGAAGCAAAGATCATCGT
R-glip121pro	GGACTGACCACCCGGGGATCCAGCTTATATGTGCTCAGACAAGAGGT
F-aldh121pro	GACCATGATTACGCCAAGCTTCCCCTCTATAGAAAGATAATTTAATTCTTG
R-aldh121pro	GGACTGACCACCCGGGGATCCTTTTCTCCTCTCTCTCTCTTTTTTTAATT

Primer	Sequence (5 to 3)
R-GUS-T	TGGCCTGCCCAACCTTTCG
R-aldh-pro280	GGCGACGGTAGGAACTCAA
F-npt ii-orf	ATGATTGAACAAGATGGATTGCACGC
R-npt ii-orf	TCAGAAGAACTCGTCAAGAAGGCG
F-Reverse-npt ii	TCCTGTCAAACACTGATAG
R-Reverse-npt ii	AGGATATATTGGCGGGTAAACC
TcALDH-RT-F	CATTCCGCTACTTTGCTGGTGC
TcALDH-RT-R	TCCAAGGAATGATGTGTCCAACTAC
TcGLIP-RT-F	GCCGGGAATGCGAGCAAAACAAC
TcGLIP-RT-R	CGCTCTCGCCTTCCTTAAAACCATA
TcGAPDH-F	AAGGAGGAATCTGAAGGAAAGCTG
TcGAPDH-R	GTTGTTGTTCAAAGCGATTCCAGC

Results

2.1 Cloning of TcALDH and TcGLIP Promoters

Using extracted pyrethrum genomic DNA as template, the TcALDH gene promoter upstream of the ATG start codon was cloned using specific primers F-ALDH-pro and R-ALDH-ORF, yielding a 2,848 bp promoter sequence. The TcGLIP promoter was amplified using primers F-GLIP-pro and R-GLIP-ORF, producing a 1,343 bp fragment (Figure 1 [Figure 1: see original paper]). These were designated pTcALDH and pTcGLIP, respectively.

M. DL 2,000 marker; 1. pTcALDH fragment; 2. pTcGLIP fragment.

Figure 1 PCR amplification of pTcALDH (A) and pTcGLIP (B)

2.2 Analysis of pTcALDH and pTcGLIP Regulatory Elements

The cis-acting elements of pTcALDH and pTcGLIP were analyzed using PlantCARE software. The results showed that a total of 210 elements of 43 types were detected in the 2,848 bp region of pTcALDH sequence, and 116 elements of 30 types were detected in the 1,343 bp region of pTcGLIP sequence. Both pTcALDH and pTcGLIP contained multiple core promoter elements (TATA-box) and enhancer elements (CAAT-box) as basic characteristic elements, in addition to many elements related to hormone response (methyl jasmonate, salicylic acid, auxin, abscisic acid, gibberellin, etc.), stress response (wounding, low temperature, drought, etc.), and light response (Tables 2 and 3). Both promoter sequences contained methyl jasmonate-responsive elements (TGACG-motif and CGTCA-motif) and ABA-responsive elements (ABRE).

Table 2 Part of cis-acting regulatory elements of pTcALDH

Class	Core sequence	Number	Predictive function
Transcriptional regulation			
TATA-box			Core promoter element around -30 of transcription start
CAAT-box			Common cis-acting element in promoter and enhancer regions
Hormone response			
TGA-element	AACGAC		Auxin-responsive element
TGACG-motif	TGACG		cis-acting regulatory element involved in the MeJA-responsiveness
CGTCA-motif	CGTCA		cis-acting regulatory element involved in the MeJA-responsiveness
ABRE	GCAACGTGTC		cis-acting element involved in the abscisic acid responsiveness
MYCATRD22	CACATG		cis-acting element involved in gibberellin-responsiveness
TATC-box	TATCCCA		cis-acting element involved in salicylic acid responsiveness
TCA-element	CCATCTTTTT		MYB binding site involved in drought-inducibility
Stress response			
W-box	TTGACC		Trauma response
CCGAAA			cis-acting element involved in low-temperature responsiveness
TC-rich repeats	ATTCTCTAAC		cis-acting element involved in defense and stress responsiveness
AAACCA			cis-acting regulatory element essential for the anaerobic induction

Class	Core sequence	Number	Predictive function
Light response			
AE-box	AGAAACA/TA/T		Part of a module for light response
Box 4	ATTAAT		Part of a conserved DNA module involved in light responsiveness
chs-CMA1a	TTACTTAA		Part of a light responsive element
chs-CMA2a	TCACTTGA		Light responsive element
GATA-motif	GATAGGA		Light responsive element
Box II	TGGTAATAA		Light responsive element
I-box	atGATAAGGTC		cis-acting regulatory element involved in light responsiveness
GA-motif	ATAGATAA		cis-acting regulatory element involved in light responsiveness
GT1-motif	GGTTAAT		cis-acting regulatory element involved in light responsiveness
G-Box	CACGTT		cis-acting regulatory element involved in light responsiveness
G-box	CACGAC		cis-acting regulatory element involved in light responsiveness

Table 3 Part of cis-acting regulatory elements of pTcGLIP

Class	Core sequence	Predictive function
Transcriptional regulation		
TATA-box		Core promoter element around -30 of transcription start
CAAT-box		Common cis-acting element in promoter and enhancer regions
Hormone response		

Class	Core sequence	Predictive function
CGTCA-motif	CGTCA	cis-acting regulatory element involved in the MeJA-responsiveness
TGACG-motif	TGACG	cis-acting element involved in the abscisic acid responsiveness
MYCATRD22	GCCGCGTGGC	Trauma response
Stress response		
W-box	CACATG	cis-acting element involved in low-temperature responsiveness
CCGAAA	TTGACC	cis-acting element involved in defense and stress responsiveness
TC-rich repeats	ATTCTCTAAC	MYB binding site involved in drought-inducibility
I-box	CAACTG	Part of a module for light response
Light response		
G-box	gGATAAGGTG	cis-acting regulatory element involved in light responsiveness
GT1-motif	TACGTG	Light responsive element
	GGGCGG	Light responsive element
	GGTTAA	Light responsive element

2.3 Analysis of pTcALDH and pTcGLIP Activity and Hormone Inducibility

Agrobacterium containing pTcALDH-LUC and pTcGLIP-LUC expression vectors were infiltrated into tobacco leaves. Fluorescence imaging results showed that the fluorescence intensity at the infiltration sites of *Agrobacterium* containing pTcALDH-LUC and pTcGLIP-LUC vectors was higher than that of the empty vector control, indicating that pTcALDH and pTcGLIP could drive LUC gene expression and possessed promoter activity. Leaves infiltrated with *Agrobacterium* containing the pTcALDH-LUC expression vector showed significantly higher fluorescence intensity after ABA and MeJA hormone treatment compared to the water control, demonstrating that pTcALDH had ABA and MeJA hormone induction characteristics (Figure 2 [Figure 2: see original paper]). In contrast, leaves infiltrated with *Agrobacterium* containing the pTcGLIP-LUC expression vector showed lower fluorescence intensity after ABA and MeJA treatment compared to the water control (Figure 2).

A. 1 mmol · L^{-1} ABA treatment; B. 2 mmol · L^{-1} MeJA treatment.

Figure 2 Firefly luminescence imaging of pTcALDH-LUC and pTcGLIP-LUC in transgenic tobacco under different hormone treatments

2.4 Analysis of TcALDH and TcGLIP Gene Expression in Pyrethrum Leaves Under Different Hormone Treatments

Pyrethrum plantlets were treated with ABA and MeJA, and the transcript levels of TcALDH and TcGLIP genes were detected. The results showed that MeJA and ABA treatment could significantly affect the expression of TcALDH and TcGLIP genes in pyrethrum. TcALDH expression was upregulated by ABA induction and first increased then decreased under MeJA induction; TcGLIP expression was downregulated by both ABA and MeJA (Figure 3 [Figure 3: see original paper]).

A. Expression analysis of TcALDH under ABA treatment; B. Expression analysis of TcALDH under MeJA treatment; C. Expression analysis of TcGLIP under ABA treatment; D. Expression analysis of TcGLIP under MeJA treatment. Different letters indicate significant differences ($P < 0.05$).

Figure 3 Expression analysis of TcALDH and TcGLIP in *Tanacetum cinerariifolium* leaves under different treatments

2.5 Tissue Specificity Analysis of pTcALDH and pTcGLIP

The TcALDH and TcGLIP promoter regions were fused with the GUS reporter gene and transformed into tobacco, yielding four independent pTcALDH-GUS transgenic lines and five independent pTcGLIP-GUS transgenic lines. GUS staining of these transgenic lines showed that no blue color was observed in any tissue of wild-type plants. In pTcALDH-GUS transgenic tobacco, obvious blue coloration appeared in the glandular trichome heads of leaves, while no obvious tissue-specific staining was observed in pTcGLIP-GUS transgenic tobacco leaves (Figure 4 [Figure 4: see original paper]).

Figure 4 GUS staining of transgenic and wild-type *Nicotiana benthamiana*

Discussion and Conclusion

In this study, the promoter sequences of TcALDH and TcGLIP were obtained through PCR sequencing verification using the pyrethrum genome. Both pTcALDH and pTcGLIP sequences contained multiple core promoter elements (TATA-box) and enhancer elements (CAAT-box) as basic characteristic elements, indicating that these gene promoters possess typical promoter functions. In addition, the pTcALDH and pTcGLIP regions also contained hormone-responsive, stress-responsive, and light-responsive elements. Promoter sequence analysis showed that both pTcALDH and pTcGLIP contained CGTCA motifs involved in MeJA response and ABRE motifs, which explains why MeJA can promote pyrethrin synthesis. Similarly, MBS elements, which are MYB binding sites involved in drought induction, were found in both pTcALDH and pTcGLIP, and interestingly, previous studies have shown that pyrethrin yield is affected by drought (Suraweera et al., 2017). In addition to TcALDH and TcGLIP genes in pyrethrum responding to mechanical damage to increase pyrethrin content

(Kikuta et al., 2012), the AaALDH gene in *Artemisia annua*, a close relative of pyrethrum, is also upregulated by wounding treatment (Wang, 2016), which may be related to the wound-responsive element W-box. In summary, the pTcALDH and pTcGLIP sequences contain elements that can be bound by transcription factors such as MYB, MYC, BZIP, and WRKY, such as MBS, RBRE, G-box, and W-box (Yang et al., 2020; Fu et al., 2021). Therefore, we speculate that the expression of these two genes may be regulated by these transcription factors. Recent studies have confirmed that both TcMYC2 and TcMYB8 in white pyrethrum can increase pyrethrin content in leaves by upregulating TcGLIP gene expression (Zhou et al., 2022; Zeng et al., 2022).

Plant secondary metabolite synthesis is regulated by plant hormones such as jasmonic acid, abscisic acid, and salicylic acid (Lv et al., 2017). As a key gene in pyrethrin synthesis, the TcALDH promoter has ABA and MeJA hormone induction characteristics. The expression level of TcALDH was upregulated after ABA treatment and first increased significantly then decreased under MeJA induction. This is basically consistent with previous research results that TcALDH and TcGLIP gene expression in pyrethrum is affected by MeJA (Li et al., 2018), further proving that methyl jasmonate can regulate pyrethrin synthesis. In *Artemisia annua*, a close relative of pyrethrum, the expression level of the AaALDH1 gene also increased significantly when treated with MeJA (Wang, 2016). This indicates that MeJA also regulates artemisinin synthesis. In contrast to the TcALDH treatment results, the TcGLIP promoter does not have MeJA and ABA induction specificity, and gene expression levels decreased significantly after treatment. This is basically consistent with previous reports that Arabidopsis NtGLIP1 is not regulated by jasmonic acid, as NtGLIP1 is only a salicylic acid (SA)-responsive secreted protein (Oh et al., 2005). However, NtGLIP2 can respond to JA and resist biotic stress by responding to auxin (Lee et al., 2009). ALDH gene promoters from Arabidopsis, cotton, and pyrethrum can all respond to ABA. In addition, pyrethrum pTcALDH can respond to MeJA; while Arabidopsis and cotton GLIP gene promoters can respond to ethylene (ET), and the Arabidopsis NtGLIP gene promoter has auxin induction characteristics, which have not been reported in pyrethrum (Kirch et al., 2005; Hou & Bartels, 2015; Guo et al., 2017; Hu, 2019). This indicates that genes involved in metabolic synthesis usually have their own hormone induction specificity and regulate secondary metabolite synthesis by responding to one or multiple plant hormones, playing a role in plant biotic or abiotic stress.

Numerous studies have shown that MeJA can promote the accumulation of secondary metabolites in plants (Wasternack et al., 2019). Short-term MeJA treatment can promote transient upregulation of pyrethrin synthesis genes but cannot maintain high pyrethrin content; conversely, the expression levels of some JA pathway synthase genes and the TcGLIP gene are downregulated (Zeng et al., 2022). Pyrethrin precursors are mainly synthesized in glands, transported outside the cell to form the final product, and stored in the ovary of flower heads (Wang et al., 2021). Flower heads have abundant special structures such as secretory ducts/cavities or extracellular spaces (Ramirez et al., 2012). However,

no similar structures or spaces for storing pyrethrins have been found in leaves. Pyrethrum hairy roots also contain only extremely low levels of pyrethrins (Li et al., 2022b). This suggests that pyrethrin biosynthesis may be subject to strong negative feedback to avoid excessive accumulation of pyrethrins. This negative feedback regulatory mechanism in plants may reduce pyrethrin content by inhibiting GLIP enzyme activity.

In addition, GUS tissue staining results showed that TcALDH is mainly expressed in glands, while TcGLIP is expressed only in subepidermal parenchyma cells. These results are basically consistent with previous studies, where TcALDH expressed in glands is used to synthesize chrysanthemic acid, and TcGLIP expressed in parenchyma cells is used to synthesize pyrethrins (Kikuta et al., 2012; Ramirez et al., 2012; Xu et al., 2018). The *Artemisia annua* ALDH1 gene is expressed only in glandular trichomes of mature rosette leaves (Wang, 2016). However, the results of this experiment differ slightly from previous predictions. This study shows that parenchyma cells may also have the ability to express TcALDH, and the TcALDH gene is not specifically expressed in glands. In Arabidopsis, NtGLIP1 and other NtGLIPs are also localized in the cell wall or extracellular space (Oh et al., 2005; Lee et al., 2009). Subcellular localization studies of Arabidopsis NtALDH and NtGLIP show that NtALDH proteins are mostly secreted in plastids and cytoplasm, while NtGLIP proteins are usually distributed in the cell wall or extracellular space (Oh et al., 2005; Lee et al., 2009; Hou & Bartels, 2015). Current reports have only analyzed the genomic data and promoter elements of cotton GhALDH and GhGLIP, with no reports on spatial expression (Guo et al., 2017; Ma et al., 2018).

This study analyzed the regulatory elements, promoter activity, hormone induction specificity, and tissue specificity of pTcALDH and pTcGLIP. Among them, pTcALDH has MeJA and ABA hormone induction specificity, and these two hormones affect the expression levels of pyrethrin synthesis genes. TcALDH is mainly localized in glands, while TcGLIP is mainly localized extracellularly, which again confirms that chrysanthemic acid is synthesized in glands while pyrethrins are synthesized extracellularly. This study can provide a theoretical reference for further exploring the expression regulatory mechanisms of TcALDH and TcGLIP genes in pyrethrum.

References

BURAPHAKA H, PUTALUN W, 2020. Stimulation of health-promoting triterpenoids accumulation in *Centella asiatica* (L.) Urban leaves triggered by postharvest application of methyl jasmonate and salicylic acid elicitors [J]. *Ind Crops Products*, 146: 112171.

CHEN YQ, XU G, LINGHU QQ, 2021. Cloning and functional analysis of *Jatropha curcas* JcGAST1 gene promoter [J]. *Plant Physiol J*, 57(11): 2145-2154.

FU XQ, PENG BW, HASSANI D, et al., 2021. AaWRKY9 contributes to light-

and jasmonate-mediated to regulate the biosynthesis of artemisinin in *Artemisia annua* [J]. *New Phytol*, 231(5): 1858-1874.

GUO XL, WANG YY, LU HJ, et al., 2017. Genome-wide characterization and expression analysis of the aldehyde dehydrogenase (ALDH) gene superfamily under abiotic stresses in cotton [J]. *Gene*, 628: 230-245.

HOU QC, BARTELS D, 2015. Comparative study of the aldehyde dehydrogenase (ALDH) gene superfamily in the glycophyte *Arabidopsis thaliana* and Eutrema halophytes [J]. *Ann Bot*, 115(3): 465-479.

HU HM, CAI WH, MU KB, et al., 2021. Study on the heterologous expression of GmCDPK SK5 gene in soybean ABA signaling pathway [J]. *Soybean Sci*, 40(6): 737-747.

HU YG, 2019. Preliminary analysis of ALDH7 and ALDH12 gene functions in cotton under salt and drought stress [D]. Kaifeng: Henan University.

KIKUTA Y, UEDA H, TAKAHASHI M, et al., 2012. 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 [J]. *Plant J*, 71(2): 183-193.

KIRCH HH, SCHLINGENSIEPEN S, KOTCHONI S, et al, 2005. Detailed expression analysis of selected genes of the aldehyde dehydrogenase (ALDH) gene superfamily in *Arabidopsis thaliana* [J]. *Plant Mol Biol*, 57: 315-332.

LEE DS, KIM BK, KWON SJ, et al., 2009. *Arabidopsis* GDSL lipase 2 plays a role in pathogen defense via negative regulation of auxin signaling [J]. *Biochem Biophys Res Commun*, 379(4): 1038-1042.

LI JW, XU ZZ, ZENG T, et al., 2022a. Overexpression of TcCHS increases pyrethrin content transformation system in pyrethrum (*Tanacetum cinerariifolium*) [J]. *Plants (Basel)*, 11(12): 1575.

LI JW, ZENG T, XU ZZ, et al., 2022b. Ribozyme-mediated CRISPR/Cas9 gene editing in pyrethrum (*Tanacetum cinerariifolium*) hairy roots using a RNA polymerase II-dependent promoter [J]. *Plant Methods*, 18(1): 32.

LI JJ, HU H, MAO J, et al., 2019. Defense of pyrethrum flowers: repelling herbivores and recruiting carnivores by producing aphid alarm pheromone [J]. *New Phytol*, 223(3): 1607-1620.

LI JJ, HU H, CHEN Y, et al., 2021. Tissue specificity of (E)-β-farnesene and germacrene D accumulation in pyrethrum flowers [J]. *Phytochemistry*, 187: 112768.

LI W, ZHOU F, PICHERSKY E, 2018. Jasmone hydroxylase, a key enzyme in the synthesis of the alcohol moiety of pyrethrin insecticides [J]. *Plant Physiol*, 177(4): 1498-1509.

LV ZY, ZHANG L, TANG KX, 2017. New insights into artemisinin regulation [J]. *Plant Signal Behav*, 12(10): e1366398.

LYBRAND DB, XU HY, LAST RL, et al., 2020. How plants synthesize pyrethrins: safe and biodegradable insecticides [J]. *Trends Plant Sci*, 25(12): 1240-1251.

MA R, YUAN HL, AN J, et al., 2018. A *Gossypium hirsutum* GDSL lipase/hydrolase gene (GhGLIP) appears to be involved in promoting seed growth in *Arabidopsis* [J]. *PLoS ONE*, 13(4): e0195556.

MATSUDA K, KIKUTA Y, HABA A, et al., 2005. Biosynthesis of pyrethrin I in seedlings of *Chrysanthemum cinerariaefolium* [J]. *Phytochemistry*, 66(13): 1529-1535.

MOSSA AH, MOHAFRASH SMM, CHANDRASEKARAN N, 2018. Safety of natural insecticides: toxic effects on experimental animals [J]. *Biomed Res Int*, 2018(1): 1-17.

NELSON RH, 1974. Pyrethrum: The Natural Insecticide [J]. *Bull Esa*, 20(3): 16.

OH IS, PARK AR, BAE MS, et al., 2005. Secretome analysis reveals an *Arabidopsis* lipase involved in defense against *Alternaria brassicicola* [J]. *Plant Cell*, 17(10): 2832-2847.

RAMIREZ AM, STOOPEN G, MENZEL TR, et al., 2012. Bidirectional secretions from glandular trichomes of pyrethrum enable immunization of seedlings [J]. *Plant Cell*, 24(10): 4252-4265.

STAUDINGER H, RUZICKA L, 1924. Insektentötende Stoffe IV. Konstitution des Tetrahydro-pyrethrons [J]. *Helvetica Chimica Acta*, 7(1): 236-244.

SULTANA S, HU H, GAO L, et al., 2015. Molecular cloning and characterization of the trichome from *Tanacetum* synthase promoter specific chrysanthemyl diphosphate/chrysanthemol *cinerariifolium* [J]. *Sci Hort*, 185: 193-199.

SURAWEERA DD, GROOM T, TAYLOR PWJ, et al., 2017. Dynamics of flower, achene and trichome development governs the accumulation of pyrethrins in pyrethrum (*Tanacetum cinerariifolium*) under irrigated and dryland conditions [J]. *Indust Crops Prod*, 109: 123-133.

WANG HY, 2016. Molecular cloning and characterization of the promoter of aldehyde dehydrogenase gene from *Artemisia annua* L. [D]. Chongqing: Southwest University.

WASTERNACK C, STRNAD M, 2019. Jasmonates are signals in the biosynthesis of secondary metabolites—Pathways, transcription factors and applied aspects—A brief review [J]. *New Biotechnology*, 48: 1-11.

WANG Y, WEN J, LIU L, et al., 2022. Engineering of tomato type VI glandular trichomes for trans-chrysanthemic acid biosynthesis, the acid moiety of natural pyrethrin insecticides [J]. *Metab Eng*, 72: 188-199.

WANG FJ, XU HY, YAN JB, et al., 2021. Biosynthesis and application of pyrethrins: a natural pesticide from plants [J]. *Synth Biol J*, 2(5): 753-761.

XU HY, MOGHE GD, WIEGERT-RININGER K, et al., 2018. Coexpression analysis identifies two oxidoreductases involved in the biosynthesis of the monoterpene acid moiety of natural pyrethrin insecticides in *Tanacetum cinerariifolium* [J]. *Plant Physiol*, 176(1): 524-537.

YAMASHIRO T, SHIRAISHI A, SATAKE H, et al., 2019. Draft genome of *Tanacetum cinerariifolium*, the natural source of mosquito coil [J]. *Sci Rep*, 9(1): 18249.

YANG Y, YU TF, MA J, et al., 2020. The soybean bZIP transcription factor gene GmbZIP2 confers drought and salt resistances in transgenic plants [J]. *Int J Mol Sci*, 21(2): 670.

YANG YN, KIM Y, KIM H, et al., 2021. The transcription factor ORA59 exhibits dual DNA binding specificity that differentially regulates ethylene- and jasmonic acid-induced genes in plant immunity [J]. *Plant Physiol*, 187(4): 2763-2784.

ZENG T, LI JW, XU ZZ, et al., 2022. TcMYC2 regulates pyrethrin biosynthesis in *Tanacetum cinerariifolium* [J]. *Hortic Res*, 9: uhac178.

ZENG T, LI JW, ZHOU L, et al., 2021. Transcriptional responses and GCMS analysis for the biosynthesis of pyrethrins and volatile terpenes in *Tanacetum coccineum* [J]. *Int J Mol Sci*, 22(23): 13005.

ZENG T, LI JW, ZHOU L, et al, 2021. Advances in the mutualistic and antagonistic interactions between flower colors and the pollinators of ornamental plants [J]. *Acta Hortic Sin*, 48(10): 2001-2017.

ZHOU L, LI JW, ZENG T, et al., 2022. TcMYB8, a R3-MYB transcription factor, positively regulates pyrethrin biosynthesis in *Tanacetum cinerariifolium* [J]. *Int J Mol Sci*, 23(20): 12186.

ZHOU L, ZENG T, LI JJ, et al., 2022. The preliminary investigation on the interplanting patterns and agronomic effects of *Tanacetum cinerariifolium* and horticultural plants [J]. *J Guizhou Norm Univ (Nat Sci Ed)*, 40(3): 18-26.

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