

The promoter sequence of (-)-limonene synthase in *Mentha Canadensis* and its strong activity in the glandular trichome and in the stomatal guard cells

Shumin Li

Institute of Botany Jiangsu Province and Chinese Academy of Sciences

Zhichao Xue

Institute of Botany Jiangsu Province and Chinese Academy of Sciences

Taolan Xiao

Institute of Botany Jiangsu Province and Chinese Academy of Sciences

Xiwu Qi

Institute of Botany Jiangsu Province and Chinese Academy of Sciences

Hailing Fang

Institute of Botany Jiangsu Province and Chinese Academy of Sciences

Li Li

Institute of Botany Jiangsu Province and Chinese Academy of Sciences

Yang Bai

Institute of Botany Jiangsu Province and Chinese Academy of Sciences

Dongmei Liu

Institute of Botany Jiangsu Province and Chinese Academy of Sciences

Qun Liu

Institute of Botany Jiangsu Province and Chinese Academy of Sciences

Zequn Chen

Institute of Botany Jiangsu Province and Chinese Academy of Sciences

Xu Yu

yuxu84@163.com

Institute of Botany Jiangsu Province and Chinese Academy of Sciences

Chengyuan Liang

Institute of Botany Jiangsu Province and Chinese Academy of Sciences

Research Article

Keywords: (-)-limonene synthase promoter, light regulation, tissue-specific, co-expression, transcription factors

Posted Date: June 13th, 2025

DOI: <https://doi.org/10.21203/rs.3.rs-6702558/v1>

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Version of Record: A version of this preprint was published at Acta Physiologiae Plantarum on January 16th, 2026. See the published version at <https://doi.org/10.1007/s11738-025-03880-8>.

Abstract

Mentha Species are well known for their useful essential oils. Limonene synthase is one of the key enzymes in the monoterpene biosynthesis pathway of mint. In this study, qRT-PCR analysis was conducted on various tissues and treatments of *Mentha canadensis* to reveal the limonene synthase (*McLS*) gene expression levels and expression response pattern. The results showed that *McLS* was highly expressed in young leaves, and induced by light, abscisic acid (ABA), methyl jasmonate (MeJA), NaCl, and mannitol treatments. The (-)-limonene synthase promoter (*proMcLS*) was isolated and its *cis* elements were analyzed. The upstream region of the (-)-limonene synthase gene contains several *cis*-acting elements, including the core *cis* elements of the TATA box and CAAT box, light-responsive motifs, ABA- and MeJA-responsive motifs, and guard cell-specific *cis* elements. Transcriptional fusion of the *proMcLS* to the *gusA* reporter gene was conducted in *N. tabacum* via *Agrobacterium*-mediated transformation. Transgenic T0 lines displayed β -glucuronidase histochemical staining activity in short glandular trichomes and the stigma of flowers. No signal was detected from tall glandular trichomes or stomatal guard cells, while T1 lines displayed β -glucuronidase activity in both short glandular trichomes and stomatal guard cells. The transcription factor families binding to the *McLS* promoter were predicted using PlantPAN 3.0, and transcription factors that were co-expressed with *McLS* in various light treatments were identified. These data describe a new tissue-specific transcriptional promoter that can be used for metabolic engineering of plants in the future.

Introduction

Mentha canadensis L. is a perennial plant belonging to Lamiaceae family. As an edible plant rich in essential oils, it is widely used in the food, cosmetics and hygiene product industries. As a traditional medicine that can be used to treat fever, cold, digestive issues, and throat inflammation, it is also used in the pharmaceutical industry (He et al., 2019). Menthol is the main bioactive monoterpene component of mint essential oil, with antimicrobial, antitumor, anesthetic, penetration-enhancing, and immunomodulating activities (Kamatou et al., 2013; Zhao et al., 2023).

Menthol is a cyclic monoterpene alcohol originating from the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway in plastids (Croteau et al., 2005). The biosynthesis pathway of peppermint oil monoterpenes has been well illustrated in previous studies, with eight enzymatic steps and nine enzymes that have been functionally characterized (Croteau et al., 2005). Homologous genes of these enzymes in cornmint (*M. canadensis*) were cloned and evaluated in our previous studies, including geranyl diphosphate synthase large subunit, geranyl diphosphate synthase small subunit, limonene synthase (*McLS*), (-)-limonene-3-hydroxylase, (-)-trans-isopiperitenol dehydrogenase, (-)-isopiperitenone reductase, (+)-pulegone reductase (*McPR*), menthofuran synthase, and menthol dehydrogenase (Yu et al., 2021). Among them, limonene synthase (*LS*) is the rate-limiting enzyme in menthol biosynthesis, responsible for catalyze the universal precursor geranyl diphosphate to limonene (Croteau and Gershenzone, 1994; Soheil et al., 2004). *LS* has been purified from the peppermint oil glands and characterized the structure and function (Rajaonarivony et al., 1992; Alonso et al., 1992). It is typical of angiosperm monoterpene

cyclases to localize to the plasmid and to require a divalent metal ion for catalysis in peppermint (Turner et al., 1999). To modify the metabolic engineering of monoterpene biosynthesis through LS expression, *M. spicata* limonene synthase gene was transformed into *M. piperita* and *M. arvensis*, the four transgenic lines of *M. piperita* exhibited increased total monoterpene contents while the two *M. arvensis* lines showed decreased total monoterpene contents (Diemer et al., 2001). Suppression *MsLS* through RNAi in *M. spicata* resulted transgenic lines showed significant reductions in limonene production (65–98%) but increases in sesquiterpenes (38%–96%), fatty acids (40–44%) (Li et al., 2020).

The biosynthesis and accumulation of monoterpenes are affected by environmental factors, including abiotic stressors, such as light, drought, temperature, and salts. Low light leads to the pulegone and piperitone accumulation and menthol reduction in mint leaves (Xu et al., 2021). Meanwhile, accumulation of menthol was observed in young leaves with long photoperiodic treatment (Bernard 1990). Enhanced essential oil yield from the shoots of *Mentha pulegium* under 50-mM NaCl stress was reported by Naioua et al. (2009). The levels of terpenoids also increased under stress in peppermint, with maximal accumulation observed under combined heat and drought stress (Alhaithloul et al., 2019). Although the impacts of various treatments on essential oil yield and composition have been investigated, the molecular mechanisms underlying these biological processes remain unknown.

The regulation of monoterpene biosynthesis is complex. The pathway fluxes are controlled at both the transcript and protein levels via feedback regulation (Vranová et al., 2013). At the transcript level, the patterns of *cis* elements located near the promoter and intronic regions determine the gene expression levels, while transcription factors (TFs) binding to the specific *cis* elements reveal further details of the molecular-level regulation of stress-responsive genes (Brown et al., 2007; Huang et al., 2016). Characterization of the expression patterns and fine regulation of genes using promoter/reporter constructs can provide useful information about their functions (Cinege et al., 2009). Although genes in the menthol biosynthesis pathway have been well characterized, the regulation of their transcription patterns and the recognition of *cis*-regulatory elements by transcription factors at target gene promoters have been scarcely reported.

Here, the expression patterns of *LS* genes under light, ABA, MeJA, NaCl, and mannitol treatments were investigated. The *McLS* promoter region was cloned using specific primers, and the main *cis*-acting regulatory elements were identified. We also produced tobacco lines transformed with the *McLS* promoter sequence fused to β -glucuronidase (GUS) to study tissue-specific promoter activation and fine regulation by developmental stage. TF-binding motifs and the co-expressed TFs with *McLS* gene under light treatment were also identified.

Materials and methods

Plant materials

The experimental mint (*Mentha canadensis* L.) plants were grown on the field in Institute of Botany, Jiangsu Province and Chinese Academy of Sciences. After disinfection, it is used for genetic transformation.

The seeds of tobacco variety 'K326' were preserved in the laboratory of the author. The seeds of 'K326' were sterilized with 75% ethanol for 1 min and then germinated on MS medium. Used for genetic transformation when the seedlings grow to about 8 cm in height.

The expression patterns of limonene synthase (LS) by quantitative realtime PCR

Plants were cultured in growth chamber under control condition at $1000 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, with the 14 h photoperiod and temperature maintained at 25°C/22°C. To study tissue expression patterns, different tissues of *M. canadensis*, including roots, stems, young leaves, old leaves, rhizomes, flowers and flower buds were harvested and collected. For darkness treatment, black paper bags was used to cover the whole plants for 24 h, then move the bags and exposure the plants to light 24 h. Leaves at the same developing stage were collection at 4h, 8h, 12h, 24h after dark/light treatment. For treatments of ABA, MeJA, NaCl and mannitol, 4-week-old *M. canadensis* were separately transferred into MS medium containing 100 μM ABA, and 200 μM MeJA, 150 mM NaCl and 300 mM mannitol for 0, 2, 4, 8, 12, and 24 h. Leaves at the same developing stage were collection at 4h, 8h, 12h, 24h. Three biological replicates were taken for each treatment. Samples were frozen in liquid nitrogen and extracted RNA (Promega).

The RNA samples were used for qRT-PCR. First-strand cDNA was synthesized with oligo (dT)18 and M-MLV reverse transcriptase (Promega). qRT-PCR analysis was carried out using the SYBR Universal qPCR Kit (Vazyme) on a qTOWER2.2 Real Time PCR Systems (Analytik, Jena, Germany), according to the manufacturer's instructions methods previously described (Qi, et al 2018). Quantification was performed using the $2^{-\Delta\Delta\text{CT}}$ method, and data were normalized to those of the actin gene transcript. Sequences of primers used are listed in additional file Supplementary table1. RT-PCR analysis was conducted with three technical replicates, and the data represent the means $\pm$ standard errors ($n = 3$)

Cloning and cis-element analysis of the McLS promoter

Genomic DNA was extracted from *Mentha canadensis*, amplified limonene synthase (McLS) promoters by gene-specific primers. Sequences of primers used are listed in additional file Supplementary table. PCR procedure was as follows: 95 °C for 5 min, 35 cycles of 95 °C for 30 s, 55°C for 30 s and 72 °C for 1min, 72°C for 5 min. PCR products were detected by 1% agarose gel electrophoresis, recovered using SanPrep Column DNA Gel Extraction Kit (Sangon) and ligated into pCE2 TA/Blunt-Zero vector by 5 min TA/Blunt-Zero Cloning Kit (Vazyme). The recombinant plasmid was transformed into Escherichia coli DH5 α (Tsingke), then kanamycin-resistant transformants were identified by sequencing analysis (Sangon).

McLS promoter sequences were analysed for the cis-acting elements using the PlantCARE database (<http://bioinformatics.psb.ugent.be/webtools/plantcare/>).

Generation of McLS promoter::reporter constructs in tobacco

The plant transformation vector pGC-GUS was used for transformation of *Nicotiana glauca* 'K326'. The LS promoter was amplified and constructed into pGC-GUS to generate ProMcLS::GUS vector. The recombinant vector was introduced into *Escherichia coli* DH5 α strain (Tsingke) and was grown in LB medium containing spectinomycin. The ProMcLS::GUS vector was purified and introduced into *Agrobacterium tumefaciens* strain EHA105 (Tsingke), transformants were selected by Spectinomycin and rifampicin and identified by sequencing.

Agrobacterium-mediated transformation method (Horsch et al. 1985) was used to generate transgenic tobacco lines. The leaves of half-month-old tobacco K326 sterile seedling were cut into a few pieces and immersed in *Agrobacterium* suspensions containing recombinant vector with an absorbance of 0.6 at 600nm for 15 minutes. Subsequently, the tobacco explants were cultured on co-cultivation medium for 4 d at 25°C in the dark. The explants were transferred to selection media containing 0.1 mg/L NAA, 1 mg/L BAP, 250mg/L Cefotaxime sodium and 100mg/L kanamycin. After the proliferation and regeneration, the plantlets were cultured in greenhouse. Finally, adventitious buds were transplanted onto the rooting medium which also contained Cefotaxime sodium and kanamycin. Kanamycin-resistant plants were obtained by selected and their DNA were extracted for PCR detection of specific primers pCambia1305.1 vector with CaMV35S promoter transcribed GUS gene expression was used as positive control.

Histochemical GUS staining

Tissue samples were collected from transgenic lines and wild type lines. GUS assays were performed according to Jefferson (Jefferson et al 1987). The samples were placed in GUS staining solution (50 mM sodium phosphate, pH7.0, 0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide, 0.5 mg/ml 5-bromo-4 chloro-3-indolyl- β -D-glucuronide (X-Gluc), 0.1% Triton X-100 and 20% methanol) incubated overnight at 37°C. After staining, the chlorophyll was removed by decolorization with ethanol series (30%, 50%, 70%, 85%, 95% and 100%), each for 1 h. The tissue samples were then observed under the stereomicroscope, and the tissues stained blue has GUS expression activity.

Quantitative determination of GUS activity

Tissues samples 100 mg of wild-type and transgenic tobacco were collected and rapidly frozen with liquid nitrogen. The samples were ground with liquid nitrogen and vortexed with 1 ml GUS extraction buffer (50 mM sodium phosphate, pH 7.0, 10 mM EDTA, pH 8.0, 10 mM β -mercaptoethanol, 0.1% Triton X-100). After centrifuged 10 min at 15,000 rpm, the supernatant was collected. Each sample 150 μ l was added to 150 μ l 4-methylumbelliferone (4-MU) substrate, and the reaction was carried out at 37 °C for 60 min. After the reaction, 900 μ l termination solution (0.2M Na₂CO₃) was added to terminate the reaction, and the fluorescence value of (4-MU) generated by the reaction was measured using the enzyme-labeled

instrument with an excitation/ emission wavelength of 365/456 nm. The amount of 4-MU was determined from a standard curve, and GUS activity was calculated as 4-MU/min/μg protein. Protein concentration in supernatant was determined by measuring absorbance at 595nm using Bradford method with bovine serum albumin (BSA) as a standard (Bradford 1976).

Identification of TF-binding motifs and *McLS* co-expressed TF analysis with light treatment

The TF binding sites in the promoter for *McLS* was scanned using PlantPAN 3.0 (Chow et al. 2019). Co-expression network of *McLS* and differentially expressed TF genes in leaves of *Mentha Canadensis* with 24 h darkness treatment and 24 h recovery light treatment was performed using spearman method with corrected p-value < 0.001 and correlation coefficient threshold > 0.8. The transcriptome sequencing methods and the main results were reported (Yu et al., 2021). Raw sequencing reads could be found in the SRA database Available online: <https://www.ncbi.nlm.nih.gov/Traces/study/?acc=PRJNA724910> (accessed on 28 April 2021)

Accession numbers for promoter sequence

The promoter sequences for (-)-limonene synthase (LS) of *Mentha canadensis* are available in Genbank with accession number OR428312.

Results

Expression patterns of LS revealed with quantitative real-time polymerase chain reaction

Menthol is the most abundant chemical compound among the essential oil compounds of *Mentha canadensis*. LS is a major rate-limiting enzyme in the menthol synthase pathway. The transcript levels of *McLS* were detected in various tissues using quantitative polymerase chain reaction (Fig. 1A). The results showed that the *McLS* gene was expressed at the highest levels in young leaves, followed by flower buds, flowers, mature leaves, stems, rhizomes, and roots in descending order (Fig. 1B). *McLS* expression levels in leaves were also examined under light, abscisic acid (ABA), NaCl, and mannitol treatments. Expression levels of *McLS* were downregulated with 8-h darkness treatment and significantly upregulated after exposure to light (Fig. 1C). Gene expression in leaves was markedly induced with 4 and 8 hours of ABA and NaCl treatment, increasing by almost 7-fold after 8-h ABA treatment (Fig. 1D, F). With MeJA and mannitol treatment, the expression levels of *McLS* peaked at 12 hours after treatment (Fig. 1E, G). These results suggest that *McLS* expression is regulated by light condition, ABA, MeJA, NaCl, and mannitol stress, and was most intensely linked to ABA among these treatments.

Cloning and sequence analysis of the *McLS* promoter

To investigate the possible regulatory mechanism of the *McLS* gene, a pair of specific primers was designed with reference to the genome of *Mentha longifolia* and used for cloning of the nucleotide sequence of the upstream promoter region of the *McLS* gene. Using the PlantCARE database, the main *cis*-acting regulatory elements located in the – 988-bp region upstream of the initiation codon of *McLS* were identified. The identified *cis*-acting motifs were categorized into conserved, phytohormone, and abiotic stress-responsive motifs (Table 1 and Fig. 2). The conserved motifs included the core *cis* elements of the TATA box and CAAT box. The nearest TATA box motif, ATATAA, was detected at – 79 bp upstream of the translational start codon ATG. Additionally, the transcriptional enhancer motif TAATAATT was identified at – 1033 bp upstream of the ATG codon. Furthermore, the promoters contained several motifs related to phytohormones. Two ACGT-containing ABA-responsive element (ABRE) motifs and MeJA-responsive motifs were identified. The abiotic stress-responsive motifs included the low temperature-responsive motif LTR (1, on the positive DNA strand), wound-responsive WUN motif (1, on the negative DNA strand), and both MYB (2, both on the positive DNA strand) and MYC (1, on the positive DNA strand) binding sites. The most widely distributed motifs were four light-responsive G-box motifs at two locations (– 107 and – 703 bp upstream of the ATG on the negative DNA strand), the AE-BOX at – 887 bp upstream of the ATG on the positive DNA strand, BOX-4 at two locations (– 131 and – 259 bp upstream of the ATG, both on the negative DNA strand) and chs-CMA1a at – 353 bp upstream of the ATG codon on the negative DNA strand. The high abundance of light-response motifs in the promoter of *McLS* suggests that *McLS* transcript expression may be regulated by light condition. The presence of two ABA-responsive ABRE motifs may also explain the reported transcriptional regulation by ABA.

Spatiotemporal expression patterns of proMcLS in tobacco

Tissue expression profiles of the *McLS* gene were analyzed in transgenic tobacco lines. The isolated promoter regions of *McLS* were fused to *GUS* gene-coding sequences, resulting in the expression vector *Pro_{McLS}-GUS*. The fusion expression vector was transformed into tobacco 'K326' via the *Agrobacterium*-mediated leaf disk transformation method. Stable, independent *Pro_{McLS}-GUS* transgenic lines were generated and identified. GUS activity was monitored in the T0 and T1 lines via GUS staining. Wild-type tobacco was used as the negative control, and 35S promoter fusion GUS-transformed tobacco was used as the positive control (Fig. 3). Histochemical GUS-activity analysis indicated that the *McLS* promoter is highly active in the head cells of short glandular trichomes on the buds and young leaves of the generated T0 lines, while no staining was detected in the tall glandular trichomes (Fig. 4A, C). The same pattern was observed among stem glandular trichomes (Fig. 4B). Lower levels of promoter activity were identified in other tissues, including the stigma (S1 Fig. 1). Moreover, GUS expression was detectable in the glandular trichomes of petals (S1 Fig. 1). To confirm these results, T1 seedlings were generated and their GUS activity was monitored. Interestingly, T1 tobacco seedlings displayed GUS activity in the stomatal guard cells of leaves, which was not detected in T0 leaves (Fig. 4D). Leaves at the immature stage exhibited stronger GUS activity than mature leaves. Decreasing GUS activity was detected from the top (P1) leaves to the bottom (P6) leaves based on quantitative measurement (S1 Fig. 2, 3). Some of the

short glandular trichomes and stomatal guard cells of the bottom leaves had no GUS activity (S1 Fig. 2). These results suggest that the developmental stage of certain organs regulates the McLS expression level.

Identification of TF-binding motifs using PlantPAN 3.0 software and McLS co-expressed TF analysis with light treatment

Promoter analysis of McLS using PlantPAN 3.0 software resulted in the identification of 498 *cis* elements belonging to various TF families. The promoter region was enriched in TF-binding sites (TFBSs) belonging to the TF families of AT-hook, bZIP, MYB-related, MYB, DNA binding with one finger (DOF), AP2_ERF, AP2_B3, and HSF. The largest number of TFBSs was found for HD-ZIP, while only a single TFBS was found in each of the VOZ and CSD TF families. The distribution of *cis* elements was analyzed by dividing the McLS promoter region into three regions designated region 1 (– 1 to – 200 bp), region 2 (– 200 to – 500 bp), and region 3 (– 500 to – 1041 bp). The highest abundance of TFBSs was observed in region 3, while DOF-family binding sites were mostly distributed in region 1, and MYB-family binding sites were mostly distributed in region 2 (S1 Fig. 4).

High-throughput RNA sequencing data were generated for screening of expression profiles under the conditions of 24-h darkness treatment and a recovery light period of 24 h. To identify correlations of differentially expressed TFs with the McLS gene, Pearson's correlations were calculated. Gene sets were identified as significantly co-expressed using criteria of correlation > 0.8 and P-value < 0.001. A total of 167 differentially expressed TFs were co-expressed with the McLS gene, among which 39 TFs had positive correlations, including McKAN4, McSCL3, McMYB3R4, McNAC022 and others (S2). Interestingly, all of these 39 co-expressed TFs overlapped with those containing binding sites in the LS promoter region. The known key TF genes involved in hormone signaling pathway were also co-expressed with McLS, such as ethylene-related ERF, WIN and GA-related SCL6 (DELLA) genes. These TFs could be the candidate genes which would release molecular mechanisms of transcriptional regulation of monoterpene synthesis.

Discussion

During the stages of growth and development, essential oil biosynthesis is regulated by various abiotic and biotic factors, including light, water, temperature, salts, and disease. With low-light treatment, essential oil and menthol production were dramatically reduced in *Mentha Canadensis* (Yu et al., 2021). Burbott AJ and Loomis WD found that the total content of monoterpenes in *Mentha piperita* increased under long-day conditions (Burbott and Loomis 1967). Salt and osmotic stresses also affect the essential oil yields of *Mentha* species (Najoua et al., 2009; Xu et al., 2015; Denys et al., 1990). In *Mentha*, essential oil is synthesized via the MEP pathway (Croteau et al., 2005). Regulation of MEP pathway gene expression occurs mainly at the transcriptional level, regulated by both developmental and environmental cues as well as pathway feedback signals. In *Arabidopsis thaliana*, MEP pathway genes are expressed in all plant organs and at all developmental stages (Vranová et al., 2013). Some MEP

pathway genes are also expressed in roots, particularly AtDXS, because roots produce ABA, strigolactones, and phytochromes via the MEP pathway (Xing et al., 2010). LS is one of the key enzymes in the monoterpene biosynthesis pathway of mint. In our research, the McLS gene is expressed mainly in young leaves and barely in roots contrast to the MEP pathway gene expression patterns in *A. thaliana*., transcription levels of the McLS gene were also regulated by light, ABA, MeJA, NaCl, and mannitol stresses. Qamar (Qamar et al., 2022) noted that peppermint *MpLS* promoters included ABA-responsive, light-responsive, pathogen-associated, and salt-induced elements. Similarly, using PlantCARE analysis, we identified several ABRE motifs involved in the ABA, AE-box, G-box, and Box 4 motifs that are involved in light responses, as well as the Me-JARE-box motif, which is involved in the MeJA pathway. The response element sequences identified in the *McLS* gene promoter region may provide clues into its regulation mechanism.

As the location of chemical compounds synthesis, glandular trichomes have been developed into an integrative model system for investigating the interplay among plant cell differentiation processes and chemical compound synthesis pathways (Schuurink and Tissier, 2020). Several trichome promoters have been reported. A tobacco trichome-specific P450 gene (CYP71D16) showed expression specific to glandular trichomes based on a promoter–GUS fusion study (Wang et al., 2002), and some transcription factors that play important roles in secondary-metabolite biosynthesis have trichome-specific expression (Liu et al. 2021). In our case, the 988-bp fragment of the McLS promoter has strong expression activity in the head cells of short glandular trichome (SGTs) but no expression activity in either the head cells or basal cells of tall glandular trichomes (TGSTs). Ultrastructural studies of the subcellular structures of *N. tabacum* SGTs and TGSTs were reported (Akers et al., 1978; Uzelac et al., 2017;) indicate that SGTs have approximately four cells separated by large, specifically oriented intracellular spaces containing plastids but not chloroplasts, which are present in TGSTs, along with substantial amounts of OsO₄-stained material. The morphology and production of SGTs in *N. tabacum* are similar to those of mint peltate trichomes, which contain eight secretory cells and plastids as well as OsO₄-stained material (Turner et al., 2000). Other studies have revealed SGT-specific genes, such as T-phylloplanin, with promoters that are activated only in SGTs (Shepherd et al., 2005). By contrast, the NtLTP1 and NtMALD1 promoters were identified as long glandular trichome-specific promoters (Choi et al., 2012; Pottier et al., 2020). Qamar et al. (Qamar et al., 2022) identified the LS promoter in peppermint and reported that T0 transgenic tobacco showed GUS activity in leaf trichome glands and stalk cells. Their results could not confirm whether any GUS signal arose from SGTs. A possible reason for these differing results is that longer versions of the same promoter may have different expression activities. In the present study, we found that reporter gene expression was not constant, but instead was higher at the early stage of leaf development and lower in mature leaves. Quantitative determination showed that GUS activity is much higher in the top leaves than the bottom leaves on a plant. These results are consistent with the McLS gene expression results, which showed the highest expression levels in young leaves.

The 988-bp mint LS promoter, which directs GUS gene activity in guard cells, contains four copies of the guard cell-specific 5'-TAAAG-3' *cis* element, which is related to target sites for a novel class of zinc finger transcription factors, the DOF proteins (Yanagisawa S, 2004; Zou et al., 2023). Qamar et al. (Qamar et al., 2022) reported that the peppermint LS promoter also contains this guard cell-specific *cis* element, but their investigation showed that the LS promoter of peppermint is a trichome-specific promoter. The TAAAG motifs found in the guard cell-specific promoter are embedded within the perfectly conserved 10-bp element TTCTTAAAGC, which has also been reported in the KST1 and KAT1 promoter (Kelly et al., 2017; Nakamura et al., 1995). Other guard cell-specific promoters for AtMYB60 (Rusconi et al., 2013) contain only 5-bp elements of (T/A)AAAG that are similar to those in the mint McLS promoter. Addition of the guard cell-specific 5'-TAAAG-3' *cis* element, as well as the *cis*-acting elements 5'-CACGAGA-3' and 5'-CACATGTTTCCC-3', is necessary for guard cell-specific expression of the genes SCAP1 and HT1 (Moriwaki et al., 2022).

Monoterpenoids such as crocusatin M have been isolated and identified from dried stigmas of *Crocus sativus* (Fang QW et al 2022). Studies of monoterpenoid compounds isolated from stigmas of *Mentha* species and their structural identification have been limited. In our research, the *McLS* promoter was active in the flower stigma, indicating that monoterpenoid synthesis may occur in the mint stigma.

The predicted TFBSs suggest that *McLS* expression may be subject to complex transcriptional regulation. Furthermore, the MYB, MYC, WRKY, and AP2/ERF transcription factors can bind directly the *McLS* promoter regulatory region. The KAN4 gene has been reported to positively regulate flavonoid and proanthocyanidin biosynthesis in *Arabidopsis*, but no role in the regulation of essential oil biosynthesis has been revealed (Gao et al., 2010). In this study, McKAN4 had a binding site in the *McLS* promoter and was strongly co-expressed with *McLS*, demonstrating the potential role of its new regulatory function. DOF is a classic protein in the zinc finger superfamily. Previous studies have revealed that maize DOF1 regulates leaf development in a light-dependent manner (Yanagisawa and Sheen, 1998). The DOF transcription factor SCAP1 was shown to participate in stomatal guard cell differentiation and maturation in *Arabidopsis*, and DOF proteins have been reported to play roles in plant metabolic regulatory networks (Negi et al., 2013). The only DOF protein found in *Chlamydomonas reinhardtii* is involved in the regulation of fatty acid metabolism (Ibáñez-Salazar et al., 2014). Overexpression of soybean GmDOF4 increases seed lipid content. *Arabidopsis* AtDOF4.2 regulates phenylpropane metabolism in various tissues and under various stresses (Skirycz et al., 2007). In our research, expression of the predicted McDOF4.2 was significantly correlated with that of McLS, indicating that McDOF4.2 is also a light-dependent TF. Whether this TF functions in regulating stomatal guard cell differentiation and maturation or essential oil biosynthesis requires further genetic study.

Together, these results provide practical information to elucidate the possible transcriptional regulation network of essential oil biosynthesis as well as the regulation of the key enzyme LS by TFs. In-depth and detailed studies involving the generation of overexpression and knockdown lines will provide further evidence and insights into the developmental and environmental regulatory mechanisms occurring in *Mentha* species.

Declarations

Author contribution statement XY, XWQ, HLF, LL and BY designed the experiments and analyzed the data. SML, ZCX, TLX, DML, LQ and ZQC performed experiments. XY and CYL wrote the manuscript.

Acknowledgments The work was supported by the National Natural Science Foundation of China (31970353) and the National Natural Science Foundation of China (32200302).

Data availability All data generated or analysed during this study are included in this article [and its supplementary information files] and nucleotide sequence information is available in GeneBank database [<http://www.ncbi.nlm.nih.gov>] under accession numbers mentioned in materials and methods.

Conflict of interest The authors report no conflicts of interest in this work and have nothing to disclose.

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Table

Table 1 not available with this version

Figures

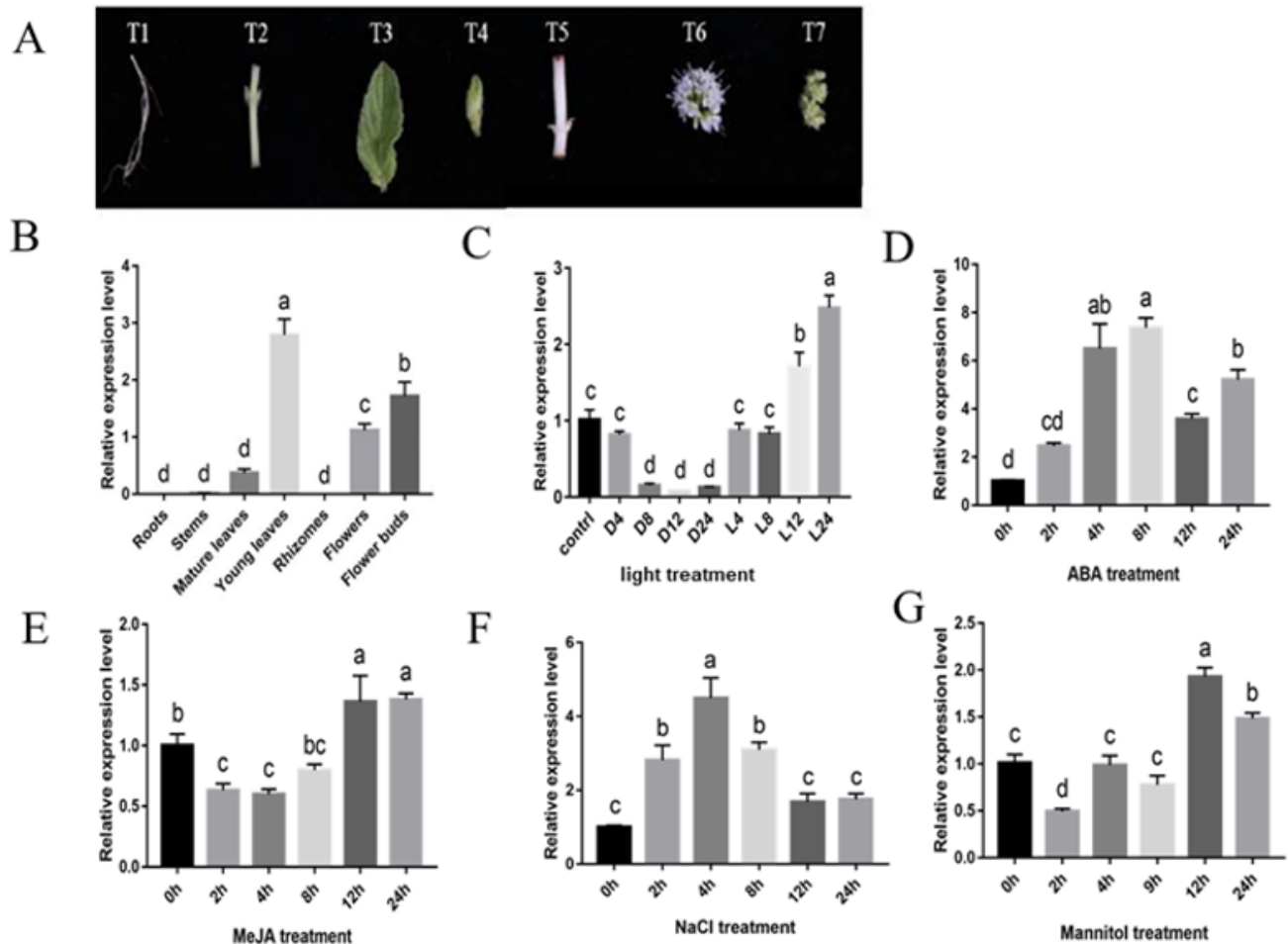


Figure 1

Expression pattern of *McLS* in plants. (A) The different tissues used for total RNA extraction, including Root (T1), Stem (T2), Mature leaf (T3), Young Leaf (T4) and Rhizomes (T5) Flowers (T6) Flower buds(T7). (B) Expression levels of *McLS* in different tissue (C) Time-course expression levels of *McLS* in leaves after light treatment, D represent darkness treatment, L represent expose to light. (D) Time-course expression levels of *McLS* in leaves under treatment with 100 μ M ABA. (E) Time-course expression levels of *McLS* in leaves under treatment with 150 mM NaCl. (F) Time-course expression levels of *McLS* in leaves under treatment with 300 mM mannitol (G) Time-course expression levels of *McLS* in leaves and roots under treatment with 200 μ M MeJA. For each treatment, *McActin* was used as the reference gene, and expression values are relative to that of 0 h, and data represented means $\pm$ SE of three replicates. Different letters indicate significant differences at $P < 0.05$, as determined by one-way analysis of variance (ANOVA) with Tukey's post-test.

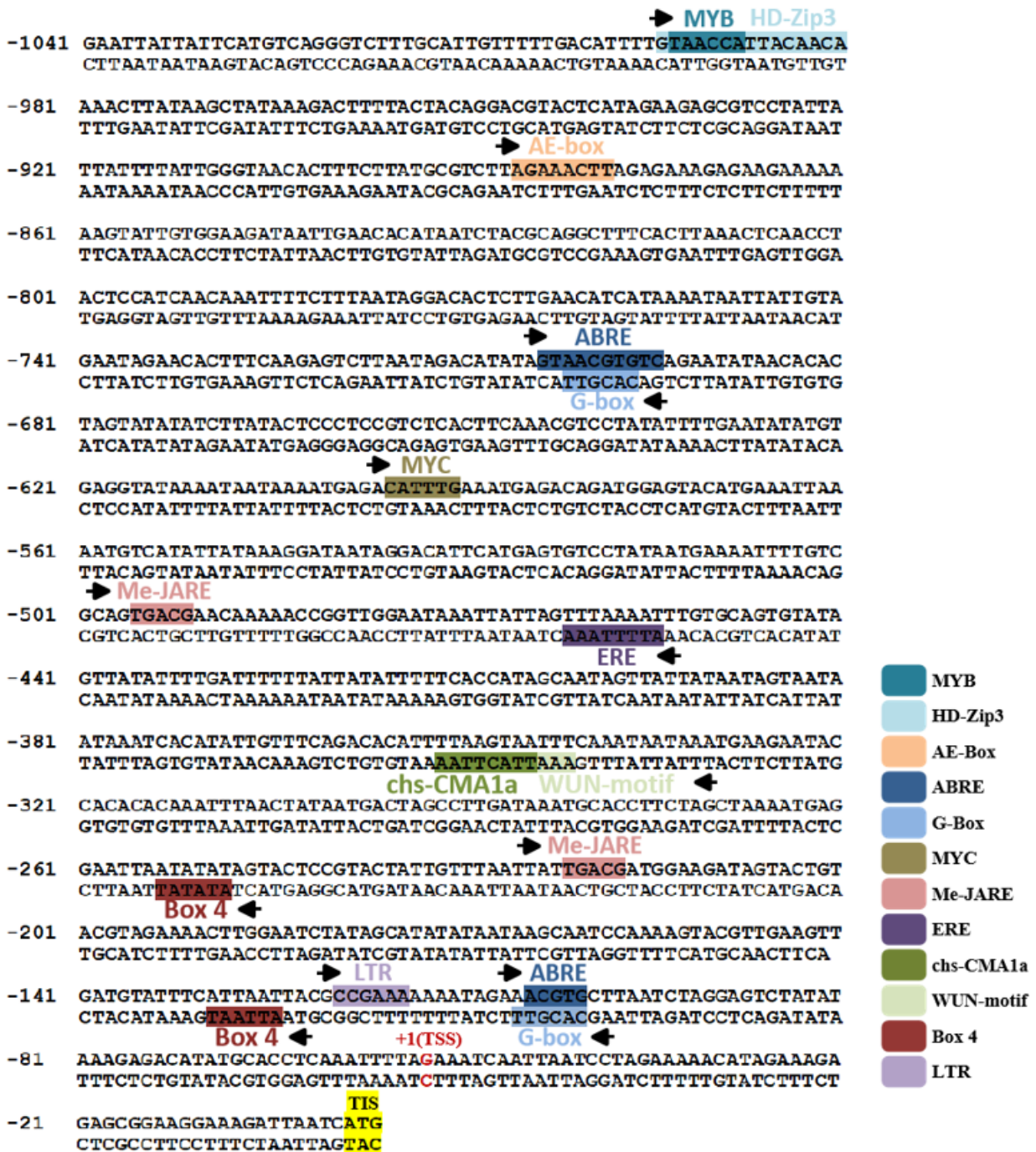


Figure 2

Upstream sequence of the McLS gene denoting the cis-elements predicted by the PlantCARE databases. The transcription start site is defined as +1. The MYB, HD-ZIP3, AE-box, MYC, ERE, chs-CMA1a, BOX4, LTR, G-box, ABRE, AT-rich elements, WUN-motif and TGACG-motif were marked with different color.

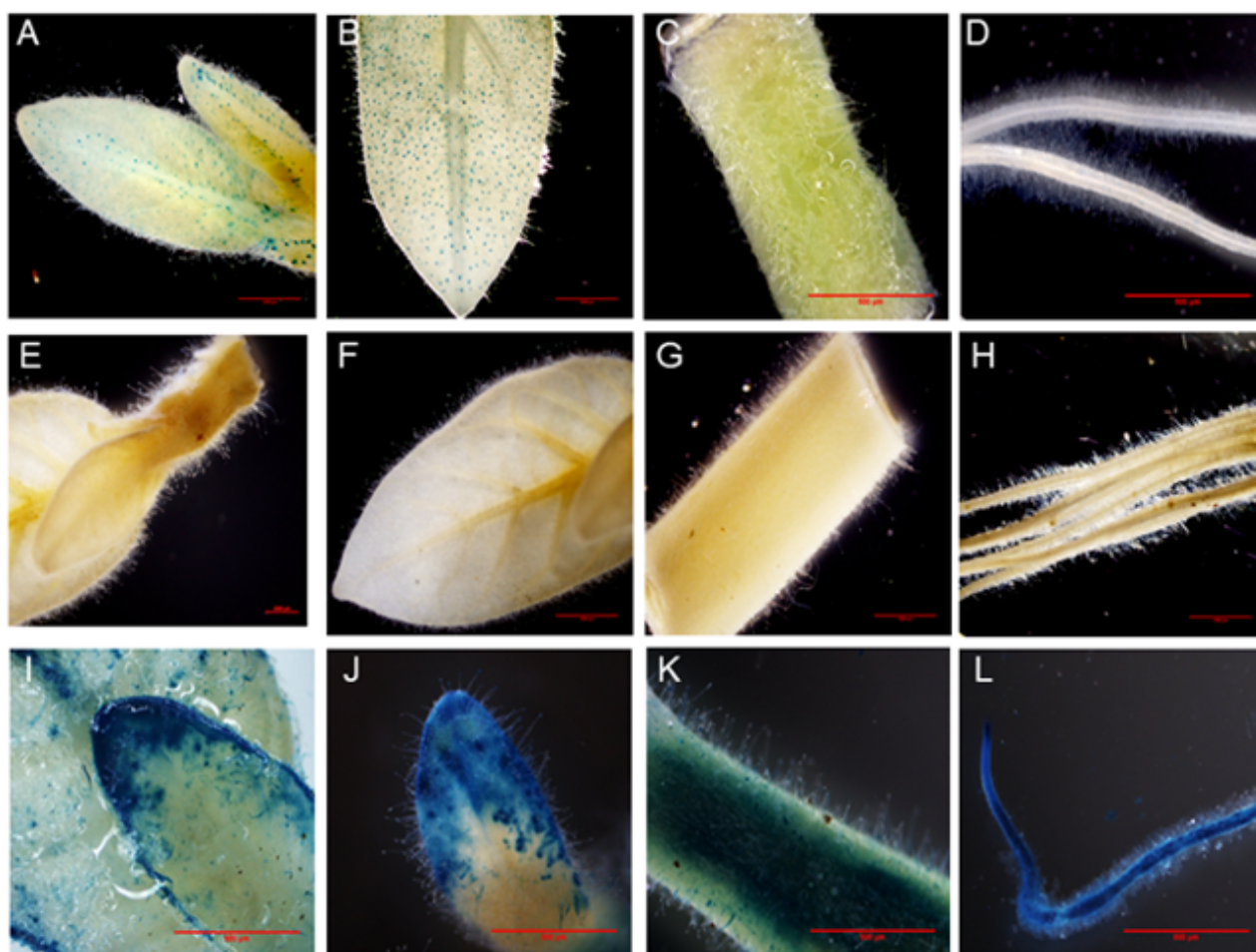


Figure 3

Histochemical distribution of ProMcLS-GUS activity in tobacco. A-D Histochemical staining of GUS in T0 transgenic tobacco shoot, leaf, stem and root. F-H Histochemical staining of GUS in T0 negative control vector transgenic tobacco. I-L Histochemical staining of GUS in T0 positive control of CaMV35S:GUS vector transgenic tobacco.

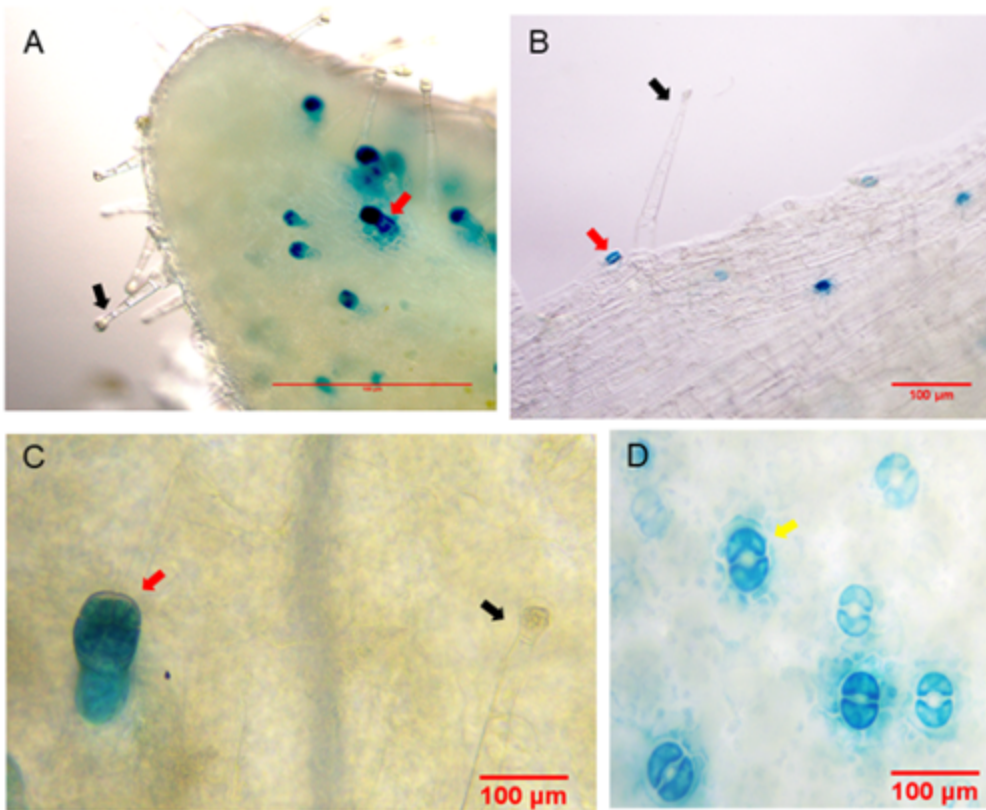


Figure 4

Histochemical distribution of ProMcLS-GUS activity in tobacco Positive GUS activity detected in short glandular trichomes and stomatal guard cell of T1 transgenic tobacco leaves, no staining was detected in the tall glandular trichomes. The same results were observed in the stem glandular, red arrow indicates high GUS expression in glandular trichomes; black arrow indicates no GUS expression in long glandular; yellow arrow indicates high GUS expression in stomatal guard cell.

Supplementary Files

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