CpMYB14, a novel R2R3-MYB transcription factor, interacts with Cp1-SST to negatively regulate inulin-type fructan accumulation and modulates plant growth in Codonopsis pilosula

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Abstract

Codonopsis polysaccharides (CPPs), the primary bioactive constituents of the edible Chinese medicinal plant *Codonopsis pilosula* (Franch.) Nannf., are mainly composed of inulin-type fructans. The biosynthesis of these fructans is catalyzed by Cp1-SST (sucrose:sucrose 1-fructosyltransferase), a key enzyme whose gene expression can be regulated by MYB transcription factors. However, the regulatory mechanism of *Cp1-SST* remained unclear. Here, two MYB cis-elements were found in *Cp1-SST* promoter which exhibited transcriptional activation activity and responded to cold stress. subsequently a R2R3-MYB transcription factor, CpMYB14, was isolated and functionally characterized. CpMYB14 was most responsive to cold, temperature difference, iron overload stress and was localized in the nucleus. Silencing of *CpMYB14* resulted in a significant upregulation of *Cp1-SST* expression and inulin-type fructan content but a decreased expression of the genes associated with CPPs hydrolysis metabolism, including *Cp1-FEH*, *Cp6-FEH*, *CpSuSy* and *CpNI*. Concurrently, the plant stem height and root length were markedly increased in *CpMYB14* gene-silenced lines. Oppositely, overexpression of *CpMYB14* significantly decreased the *Cp1-SST* expression in *C. pilosula* but with significantly increased *Cp1-FEH*, *Cp6-FEH* and *CpSuSy* expression. Yeast one-hybrid and chromatin immunoprecipitation assays confirmed that CpMYB14 directly binds to the promoter of *Cp1-SST*. Therefore, CpMYB14 is a negative regulator of *Cp1-SST* and inulin-type fructan biosynthesis. These findings provide a theoretical foundation for the molecular breeding aimed at developing high-quality varieties of *C. pilosula*.

1. Introduction

Codonopsis pilosula (Franch.) Nannf. is a perennial herb whose root is utilized both as the traditional Chinese medicinal material known as Codonopsis Radix (CR) and as a functional food ingredient. The main bioactive constituents of CR are Codonopsis polysaccharides (CPPs), which exhibit a range of notable biological activities including immunomodulation, anti-tumor, prebiotic, antifatigue, antiviral, and antioxidative effects[1, 2]. Recent studies further shows that CPPs can alleviate diet-induced metabolic disorders; for instance, they help reduce hepatic lipid accumulation by modulating related metabolic pathways [3]. Interestingly, most CPPs belong to the inulin-type fructans (ITFs) [4]. ITFs have long been used as sweeteners in diabetic foods and as dietary fiber sources [5, 6]. Specifically, ITFs derived from CR have been shown to promote gastrointestinal functions, such as anti-gastric, immunoregulation, and intestinal microbial regulation activities [1, 2]. Within the plant itself, fructans play a dual role: they act not only as reserve carbohydrates but also as important regulatory molecules that aid in adapting to environmental stress [7].

ITFs metabolism involves several key enzymes. In plants, sucrose 1-fructosyltransferase (1-SST) acts as the gateway enzyme for ITFs biosynthesis, catalyzing the transfer of a fructosyl unit between sucrose molecules to yield 1-kestose, the foundational building block of ITFs [8]. Plant fructan exohydrolases (FEHs) are evolutionarily derived from ancestral cell wall invertases (CW-INVs) [9]. Various types of plant FEHs have been cloned and identified from species such as chicory, wheat, and perennial ryegrass. Among them, fructan 1-exohydrolase (1-FEH) specifically hydrolyzes the β-(2→1)-linked fructosyl group

at the terminal of ITFs or mixed-type fructans [10], while fructan 6-exohydrolase (6-FEH) cleaves the β-(2→6) fructosyl group at the terminal of levan or mixed-type fructans[11]. Previous study has demonstrated that Cp1-SST which synthesizes fructooligosaccharides with different degrees of polymerization is the key enzyme for ITFs biosynthesis in *C. pilosula*. Furthermore, its overexpression enhances cold tolerance by modulating fructan accumulation [12].

Transcription factors (TFs), which play an important role in plant stress response and metabolic engineering, can coordinately regulate the expression of genes across multiple biosynthetic pathways. In the Asteraceae and Poaceae families, several TFs, primarily from the MYB and DOF families, have been identified as regulators of 1-SST expression [13–15]. Notably, CiMYB17, a stress-induced R2R3-MYB transcription factor in *Cichorium intybus*, directly activates 1-SST transcription by binding to the conserved DTT HGGt motif in the 1-SST promoter [13]. Beyond 1-SST, CiMYB17 also coregulates the downstream genes involved in fructan synthesis (*1-FFT*) and hydrolysis (*1-FEH*), establishing a bidirectional "synthesis-degradation" network. Genomic analysis further reveals that fructan-active enzymes (FAZYs) such as 1-SST and 1-FFT are spatially clustered with transcription factor genes like *MYB17*, *MYB3*, and *MYB5* in chicory, providing a structural basis for their coordinated regulation [13]. In barley, Overexpression of a sucrose-induced wheat MYB transcription factor, *TaMYB13-1*, was demonstrated to activate the promoter functions of sucrose:sucrose 1-fructosyltransferase (1-SST) and sucrose:fructan 6-fructosyltransferase (6-SFT) in transient transactivation experiments, thereby increasing fructan levels in both leaves and stems[14].

The accumulation of bioactive constituents in medicinal plants is influenced by the special ecological environment factors of the genuine production areas, and moderate environmental stress can promote the biosynthesis of the bioactive constituents[16]. Lu Dangshen, a genuine medicinal material of CR from Shanxi Province, contains higher levels of CPPs compared with materials from other producing areas. It is primarily cultivated in high-altitude regions characterized by a cold and semiarid climate and iron-rich soils. In plants, transcription factors play a central role in abiotic stress responses, where they can either activate or repress the expression of downstream target genes [17]. Under stress conditions, plants activate internal signaling cascades to modulate gene expression, thereby adjusting the production of secondary metabolites and enhancing adaptation to environmental challenges[18, 19]. However, the transcriptional regulatory mechanism of the key enzyme gene *Cp1-SST* for ITFs synthesis remains unclear in *C. pilosula*.

Here, the *CpMYB14* gene was characterized based on the cis-element of the *Cp1-SST* promoter to investigate its function by gene expression analysis, subcellular localization, virus-induced gene silencing (VIGS), and overexpression genetic transformation. The interaction between CpMYB14 and the *Cp1-SST* gene promoter was confirmed in vivo and in vitro via yeast one-hybrid (Y1H) and chromatin immunoprecipitation (ChIP) technologies. The primary objective of this research is to elucidate the regulatory role of the transcription factor CpMYB14 in ITFs synthesis in *C. pilosula*, thereby establishing a theoretical basis for understanding the genetic mechanisms underlying the genuineness of Lu Dangshen.

2. Materials and methods

2.1 Plant materials and growth conditions

C. pilosula plants were grown in a field in Lingchuan, Shanxi, China (1,522 m altitude, 35°47' N, 113°24' E) for two years under good agricultural practices. Different tissues of the plants and the roots at different growth stages were used for gene expression analysis. The flower buds, stems, leaves, and roots were collected at the bloom stage and the roots were collected at the seedling, bloom, fruiting and harvest stages for gene expression analysis. The samples were immediately frozen in liquid nitrogen and stored at -80°C for library preparation.

To analyze gene expression under stress, the seeds of *C. pilosula* were germinated in petri dishes and subsequently grown in plastic trays containing steam-sterilized growing medium at 25℃ with a 16 h light/8 h dark cycle. Four weeks after germination, the seedlings were transferred to individual pots containing an equal amount of dried soil and maintained under the same climatic conditions for 3 d. Thereafter, drought stress was applied by irrigation with 12% PEG6000, while cold stress was imposed by transferring plants to 4℃. Root samples were collected at 0, 4, 8, 12, 24 and 48 h after each treatment. Plants irrigated with water and grown at 25℃ were used as the controls for drought and cold stress, respectively. For large day-and-night temperature-difference stress, plants were subjected to 22℃/4℃ (day/night) and the roots were collected at 0, 6, 12, 18, and 24 d after treatment. Plants cultured at 22℃/16℃ (day/night) were used as the controls. Iron deficiency and iron overload stress were applied by culturing seedlings in liquid 1/2MS medium with 0 μM EDTA-Fe and 150 μM EDTA-Fe, respectively. Plants treated with 50 μM EDTA-Fe were used as the iron-sufficient control. All collected root samples were stored at -80℃ for further analysis.

2.2 Gene *Expression analysis*

RNA extraction and real-time polymerase chain reaction (RT-PCR) were performed as described by Ji et al. [12]. The primer sequences are shown in Table 1.

2.3 Cloning and analysis of Cp1-SST promoter and GUS staining

The upstream region of *Cp1-SST* was amplified via Tail-PCR as described by Ji et al. [20]. To generate the *Cp1-SST* promoter-GUS construct, the 5´-flanking DNA of the Cp1-SST coding region was amplified with p*1-SST* F and p*1-SST* R. The 750-bp PCR fragment was cloned into the pCAMBIA1381 vector. The construct was introduced into tobacco leaves via transient transformation. Histochemical staining for GUS activity in transgenic plants was performed using the GUS stain kit (Takara, Japan). Plants transformed with pCAMBIA1381 were used as a parallel negative control.

2.4 Subcellular Localization

The open reading frame (ORF) of *CpMYB14* was recombined into the pCAMBIA3301-eGFP vector using seamless cloning (TransGen, Beijing, China) to generate plasmid pCAMBIA3301::*CpMYB14-eGFP*. The plasmid was used for transient transformation of tobacco (*N. benthamiana*) leaf epidermal cells. A confocal microscope was used to visualize green fluorescent protein (GFP) in transformed protoplasts after 48 h of incubation.

2.5 Virus-induced gene silencing of CpMYB14 in C. pilosula

The 213-bp fragment of the *CpMYB14* cDNA (vCpMYB14) was cloned from *C. pilosula* and inserted into the pTRV2 vector to yield pTRV2::*CpMYB14* for *CpMYB14* gene silencing. The pTRV1 and the pTRV2 vectors with or without VCpMYB14 were transformed into the *Agrobacterium* strain, GV3101. The *C. pilosula* plants inoculated with pTRV2 empty vector were used as vector control (VCK). Subsequently, the Agrobacterium transformants were co-injected into the *C. polosula* roots as described by Ji et al. [12].

2.6 Overexpression of CpMYB14 in C. pilosula

The entire coding sequence of *CpMYB14* was ligated into pCAMBIA1381-35S vector by seamless cloning (TransGen, Beijing, China) to construct 35S::*CpMYB14*. Recombinant vector was used to transform *C. pilosula* with the stem as the explant as described Ji et al. [12]. The transgenic calli inoculated with the pCAMBIA1381-35S empty vector were used as the controls (35S::).

2.7 Yeast one-hybrid assay

The binding assay utilizing yeast one-hybrid system (Clontech) was performed according to the manual provided by the manufacture. The ORF sequence of *CpMYB14* were constructed into the pGADT7 vector. The *Cp1-SST* promoter sequences (from − 567bp to -750bp, relative to translation start) were inserted into a pAbAi plasmid. Subsequently, the recombinant plasmids were co-introduced into yeast strain Y1H. The transformants were cultured and selected on SD/-Ura/-Leu solid medium supplemented with 700 ng/μL AbA for 72 h. The empty vectors pAbAi and pGADT7 were used as negative controls.

2.8 Chromatin immunoprecipitation (ChIP)-qPCR Assay

The transgenic *C. pilosula* calli 35S::*CpMYB14*-eGFP were applied to ChIP analysis. Chromatin immunoprecipitation was conducted according to Pierce Magnetic ChIP Kit (Thermo Scientific, Waltham, USA). An anti-GFP antibody (Engibody, Shanghai, China) was used for ChIP qPCR. The immunoprecipitated samples were used as template for qPCR analysis. The primers were designed according to the sequence containing MYB recognition sites in the *Cp1-SST* promoter (Table 1). The samples without anti-GFP were used as the control.

2.9 High Performance Gel Permeation Chromatography (HPGPC) Analysis of ITFs content

The ITFs content was measured by high-performance gel-permeation chromatography (HPGPC) analysis [21].

3. Results

3.1 Cis-acting elements analysis of the Cp1-SST promoter in C. pilosula

Cp1-SST is the key enzyme gene involved in the synthesis of ITFs in *C. pilosula* [12]. In our previous study, the expression of *Cp1-SST* was found to be induced by cold stress [12]. To explain why its expression is induced by cold stress and identify the transcription factors interacting with *Cp1-SST*, the promoter region of *Cp1-SST* was analyzed via Tail-PCR. The *Cp1-SST* promoter was found to contain various cis-elements, including three MYB binding sites (Fig. 1A), three light responsive elements, one MeJA-responsiveness element, one dehydration-responsive element, one gibberellin-responsive element, one low-temperature response element and one auxin-responsive element (Table S1).

To examine if the transcription activity of the *Cp1-SST* promoter is induced by cold stress, a GUS reporter gene was fused downstream from the promoter. The resulting p*1-SST*::GUS construct was then genetically transformed into the tobacco leaves. GUS staining demonstrated that cold stress treatment noticeably decreased the GUS activity (Fig. 1B), which indicated that p*Cp1-SST* had transcription activation activity and negatively responded to cold stress.

3.2 Isolation and characterization of CpMYB14

To screen for transcription factors interacting with *Cp1-SST* based on cis-acting elements in its promoter, three MYB transcription factors were obtained from the *C. pilosula* transcriptome database [22]. Gene expression pattern analysis revealed that the gene expression level of *MYB32* was significantly higher at the seedling, bloom, and fruiting stages compared to the harvest stage, while *PHL11* gene expression showed no significant differences at different developmental stages. The expression of *MYB14* was extremely significantly higher at the seedling stage than at other stages. Notably, the expression trends of *MYB32* and *MYB14* were opposite to the changes in Codonopsis polysaccharides (CPPs) content at different developmental stages (Fig. 2). Tissue-specific expression analysis indicated that the expression of *MYB14* in *C. pilosula* roots was significantly higher than in stems, leaves, and flowers, consistent with the significantly higher CPPs content observed in roots compared to other tissues.

Gene sequence analysis revealed that CpMYB14 contained an 879bp ORF encoding 292 amino acids. The results of the BLAST-Protein (BLASTP) online (http://www.ncbi.nlm.gov/blast) showed that CpMYB14 contained two characteristic SANT (SWI3, ADA2, N-CoR and TFIIIB) domains, confirming its classification within the R2R3-MYB transcription factor family. Phylogenetic tree analysis indicated that CpMYB14 had the closest evolutionary relationships to CiMYB14 (Fig. 3).

3.3 Expression patterns under different environmental stress of CpMYB14

The accumulation of secondary metabolites in medicinal plants is influenced by the specific ecological factors in their genuine producing regions, and moderate environmental stress can enhance this process [16]. To investigate the stress-responsive behavior of *CpMYB14*, the expression pattern of *CpMYB14* was analyzed in the roots of *C. pilosula* seedlings subjected to different stress treatments.

Under drought stress, expression of the *CpMYB14* gene increased sharply after 24 h, whereas it remained relatively stable in the control group. Interestingly, under cold stress, the expression pattern of the *CpMYB14* gene was opposite to that of the control, suggesting its potential involvement in cold stress response. Furthermore, temperature difference stress also triggered upregulation of *CpMYB14* expression in *C. pilosula* roots. Notably, the overall expression level of *CpMYB14* under temperature difference stress was significantly higher compared with the control. Under iron deficiency stress, the expression of *CpMYB14* in both the control and treatment group showed a fluctuating trend, characterized by an initial increase, followed by a decline, and then a subsequent rise. Notably, at 48 h after treatment, the expression level of *CpMYB14* in the treated plants was significantly higher than that in the control group. Under iron overload stress, the *CpMYB14* expression sharply increased and peaked at 8 h and 48 h after treatment, while the CK maintained a consistently low and stable expression level throughout the experimental period (Fig. 4A). All these finding indicated that the expression of CpMYB14 could respond to cold, temperature difference and iron overload stress, especially the cold stress.

3.4 Subcellular localization of CpMYB14

To identify the subcellular location of CpMYB14 *in vivo*, the coding sequence of *CpMYB14* was fused to the 5' end of the GFP reporter gene driven by the cauliflower mosaic virus (CaMV) 35S promoter. The *CpMYB14-eGFP* fused expression vector was used to carry out a transient expression assay in *A. thaliana* protoplasts. CpMYB14 was identified to be localized to the nucleus (Fig. 4B), whereas GFP controls were distributed evenly throughout the cell.

3.5 CpMYB14 Gene silencing promotes Cp1-SST gene expression, plant growth and ITFs accumulation in C. pilosula

To further investigate the function of *CpMYB14*, the virus-induced transient gene silencing vector pTRV2-*CpMYB14* was constructed for infection of the *C. pilosula* roots. Expression levels of *CpMYB14* were significantly reduced in the gene silenced plants (VCpMYB14) compared to the non-silenced plants (VCK), confirming the effectiveness of *CpMYB14* silencing (Fig. 5C).

Comparative morphological analysis revealed striking phenotypic changes in the *CpMYB14*-silenced plants (Fig. 5A). Their plant height was significantly greater than that of VCK. Consistently, the root

length was also markedly increased following *CpMYB14* gene silencing (Fig. 5B).

To further investigate the effect of *CpMYB14* on inulin-fructan biosynthesis in *C. pilosula*, the expression levels of the genes related to ITFs biosynthesis were detected using qRT-PCR. The results showed that the expression of *Cp1-SST* was markedly elevated in the *CpMYB14*-silenced plants. However, a significant down-regulation was observed in the expression of *Cp1-FEH* (Fructan 1-Exohydrolase) and *Cp6-FEH* (Fructan 6-Exohydrolase), the key enzyme genes involved in ITFs hydrolysis. Similarly, the expression of *CpNI* (Neutral invertase) related to sucrose hydrolysis significantly decreased (Fig. 5C). These inverse expression patterns indicated that *CpMYB14* gene silencing inhibited the expression of ITFs hydrolysis-related genes in *C. pilosula*. Notably, the ITFs content (the peak at 31 min) in the *CpMYB14*-silenced roots was significantly upregulated (Fig. 5D). Collectively, the results implied that *CpMYB14* might negatively regulated the expression of *Cp1-SST* and ITFs accumulation.

3.6 Overexpression of CpMYB14 inhibits the expression of Cp1-SST and promotes the expression of the genes related to ITFs hydrolysis in C. pilosula

To further confirm the effect of CpMYB14 on ITFs biosynthesis, we also conducted *CpMYB14*-overexpression vector for induction of callus lines of *C. pilosula*. The positive transgenic lines were detected with genomic PCR. We further used GUS staining by GUS stain kit (Solarbio, China) to confirm the positive transgenic lines which could be stained blue (Fig. 6A). qRT-PCR results also showed that *CpMYB14* gene was successfully overexpressed in *C. pilosula* calli.

Overexpression of *CpMYB14* resulted in the significantly reduction of *Cp1-SST* expression. In addition, the expression level of *CpSuSy* and the key enzyme genes involved in inulin-fructan hydrolysis, *Cp1-FEH* and *Cp6-FEH*, significantly increased in *CpMYB14*-overexpressed *C. pilosula* calli (Fig. 6A). The results were consistently with *CpMYB14* gene silencing and indicated that expression of *CpMYB14* negatively regulated the expression of *Cp1-SST*, but positively regulated the expression of the genes related to inulin-fructan hydrolysis.

HPGPC was used to analyze the CPPs profile characteristics and detect ITFs concentrations in the *CpMYB14*-overexpressed calli. The results showed that the content of the large-molecular-weight polysaccharides (the peak at 17–29 min) in the overexpressed calli was significantly lower than that in the control. No ITFs (the peak at 31 min) was detected (Fig. 6B), likely because the calli lack chloroplasts, the organelle where Cp1-SST is localized [12]. Therefore, CpMYB14 is a key transcription factor in negatively regulating CPPs accumulation probably through regulating the expression of *Cp1-SST*.

3.7 CpMYB14 binds to the promoter of Cp1-SST in C. pilosula

To determine whether CpMYB14 actually binds to the *Cp1-SST* promoter to activate the gene transcription, yeast one-hybrid (Y1H) and chromatin immunoprecipitation (ChIP-PCR) assays were

performed. For the Y1H assays, the *Cp1-SST* promoter sequence containing MYB elements was fused to the pAbAi vector, and the full sequence of *CpMYB14* was fused to the activation domain AD. When fused p*1-SST*-pAbAi was co-expressed with CpMYB14-pGADT7 in yeast, the strain was able to grow on an SD/-Leu/AbA700 plate. No growth was observed in the negative control expression in which the CpMYB14 transcription factor was lacked (Fig. 7A). These results provided *in vitro* evidence for the specific binding of CpMYB14 to the *Cp1-SST* promoter.

To verify the specific *in vivo* binding of CpMYB14 to *Cp1-SST* promoter, ChIP-PCR assays were conducted using pCAMBIA3301::*CpMYB14*-eGFP and pCAMBIA3301::eGFP transgenic *C. pilosula* calli, respectively. The MYB element-containing promoter regions of *Cp1-SST* were enriched by ChIP in the pCAMBIA3301::*CpMYB14*-eGFP transgenic calli compared to the pCAMBIA3301::eGFP control (Fig. 7B). It showed that CpMYB14 specifically bound to the MYB element on the promoter of *Cp1-SST*.

4. Discussion

Inulin-type fructans (ITFs), the primary constituents of *Codonopsis* polysaccharides (CPPs), serve as the key bioactive compounds mediating the intestinal protective and immunomodulatory effects of CR [23]. The expression of *Cp1-SST*, a crucial enzyme gene in ITFs biosynthesis in *C. pilosula*, is known to be induced by cold stress, yet the underlying regulatory mechanism remains unclear. In this study, we analyzed the promoter cis-elements of *Cp1-SST* and, based on the presence of MYB-binding motifs, identified the transcription factor *CpMYB14*. Functional characterization revealed that CpMYB14 responds to abiotic stresses such as low temperature, interacts with *Cp1-SST*, and acts as a negative regulator of ITF accumulation in *C. pilosula*.

Recent studies have confirmed that various soluble polysaccharides, including ITFs, play significant roles in plant stress responses[24]. Previous work has shown that overexpression of *Cp1-SST* in tobacco plants which naturally lack fructans effectively promotes fructan synthesis and enhances cold stress tolerance [12]. In the present study, transgenic tobacco plants carrying the p*1-SST*::GUS plasmid were subjected to cold stress and analyzed using GUS staining. The results revealed a marked reduction in GUS activity over the course of cold treatment, indicating that the *Cp1-SST* promoter responds negatively to low temperature by downregulating downstream gene expression. This observation further corroborates earlier findings on the stress-responsive regulation of the *Cp1-SST* gene.

The MYB transcription factors family is well recognized for its critical role in regulating plant growth, development, and abiotic stress responses [25]. MYB proteins are classified into four groups according to the number of conserved MYB repeats in their sequences: 1R-MYB (containing one repeat), R2R3-MYB (two repeats), 3R-MYB (three repeats), and 4R-MYB (four repeats) [26]. Notably, R2R3-MYB proteins, the largest subgroup within the MYB family, have been shown to play a key role in mediating plant adaptation to drought and low-temperature stress [27]. CpMYB14 with two SANT domains, was characterized as an R2R3-MYB transcription factor in this study [28, 29]. Subcellular localization confirmed that CpMYB14 localizes to the nucleus, which aligns with reports on other R2R3-MYB transcription factors such as

SmMYB88 in eggplant (*Solanum melongena*) and VvMYB14 in grapevine (*Vitis vinifera*) [30, 31]. Gene expression patterns under multiple stress conditions indicated that *CpMYB14* is primarily induced by cold and iron overload stress. Previous studies have demonstrated that overexpression of the strawberry *FvMYB44* gene in *Arabidopsis thaliana* improves cold tolerance in transgenic plants [32]. Similarly, overexpression of *VaMyb14* in *A. thaliana* upregulated antioxidant enzyme activity and enhanced cold resistance compared with wild-type plants [33]. While iron is an essential micronutrient for plants, the role of MYB transcription factors in iron overload stress responses remains largely unexplored. In the present study, we observed a pronounced upregulation of *CpMYB14* under high-iron treatment (150 μM EDTA-Fe), in contrast to the control, which showed no significant change. These findings suggest that CpMYB14 is capable of actively responding to iron overload stress.

Previous studies have reported that in radish (*Raphanus sativus*) taproots, the transcription factor RsMYB90 is cold-inducible, directly binds to low-temperature-responsive elements within the *RsCOR78* promoter, and activates its expression to enhance cold tolerance [34]. Here, we observed that the *Cp1-SST* promoter was down-regulated under low-temperature stress, consistent with the presence of low-temperature-responsive *cis*-element. Furthermore, the expression pattern of *CpMYB14* under cold stress was inversely correlated with that of *Cp1-SST*, and the *Cp1-SST* promoter harbors multiple MYB-binding sites [12]. CiMYB17 binds to the consensus DNA-motif DTTHGGT and activate 1-SST transcription in Cichorium intybus [13]. TaMYB13 binds to a (A/G/T)TT(A/T/C)GGT core sequence in the promoters of wheat Ta1-SST and markedly enhance the expression of 1-SST and 6-SFT promoter-driven reporter genes in wheat [35]. The interaction between CpMYB14 and p*Cp1-SST* was verified in vitro and in vivo through Y1H and CHIP experiments, respectively. Unlike other MYB transcription factors that positively regulate the expression of 1-SST, gene silencing and overexpression analysis demonstrated that CpMYB14 negatively regulates the expression of *Cp1-SST*.

After silencing and overexpressing the *CpMYB14* gene, the expression of the key enzyme gene *Cp1-SST* related to ITFs synthesis in *C. polosula* was negatively correlated with the expression of *CpMYB14*, and also negatively correlated with the expression of the fructan hydrolysis-related gene *CpFEHs* and the sucrose hydrolysis-related genes sucrose synthase and acid invertase. These results indicated that the *Cp1-SST* gene expression was negatively correlated with the expression of *CpFEHs*, *CpSuSy* and *CpNI*, which is consistent with the previous research results [12].

In this study, overexpression of *CpMYB14* in *C. pilosula* significantly downregulated the *Cp1-SST* expression, yet no ITFs were detected via HPGPC analysis. This is likely attributable to the chloroplast localization of the Cp1-SST protein, coupled with the extremely low chloroplast content in callus, which together impede fructan accumulation[12]. Nonetheless, the diminished Cp1-SST activity still impaired the accumulation of other polysaccharides in *C. pilosula*. Conversely, silencing of *CpMYB14* markedly increased the *Cp1-SST* expression and ITFs content, consistent with prior observations that Cp1-SST catalyze the biosynthesis of fructooligosaccharides with different degrees of polymerization[12]. However, the silenced lines displayed significantly enhanced root length and plant height, a phenotype opposite to the stunted growth previously reported in tobacco overexpression of *Cp1-SST*. This

phenotypic divergence may arise from the high *Cp1-SST* expression in silenced lines, which could deplete its substrate, sucrose, while concurrently reducing the activity of sucrose-hydrolyzing enzymes such as NI and SuSy. Consequently, the typical pathway for carbon skeleton provision via sucrose hydrolysis may be disrupted. Given the complexity of plant carbon metabolism, alternative compensatory pathways might be activated [36–38]. Additionally, some MYB transcription factors are recognized as growth repressors in plants. The binding of MYB73/77 to UVR8 inhibits shoot elongation and lateral root growth by reducing expression of IAA19 in Arabidopsis [39]. Plants overexpression of *OsMYB91* showed reduced plant growth [40]. Overexpression of *StMYB66* resulted in shorter plant height[41]. Therefore, it can be inferred that the CpMYB14 transcription factor may be a core growth inhibitory factor. Its down-regulation directly relieves the inhibitory effect on plant growth-promoting genes.

Conclusions

In summary, the *Cp1-SST* promoter contained three MYB recognition sites and negatively regulated downstream gene expression under cold stress. *CpMYB14* could respond to cold, temperature difference and iron overload stress. Express patterns indicated that CpMYB14 was located to the nucleus and shared the closest evolutionary relationships with CiMYB14. Functionally analysis revealed that CpMYB14 interacted with the *Cp1-SST* promoter, negatively regulating the *Cp1-SST* gene expression while positively regulating the genes related to fructan hydrolysis including *FEHs* and *SuSy*, thereby negatively regulating ITFs accumulation. Meanwhile, CpMYB14 expression could affect the plant height and root length. These findings provide a foundation for elucidating the regulatory mechanism underlying CR quality formation mediated by ITFs, with potential economic value.

Declarations

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Table 1

Table 1 is available in the supplementary files section

Figures

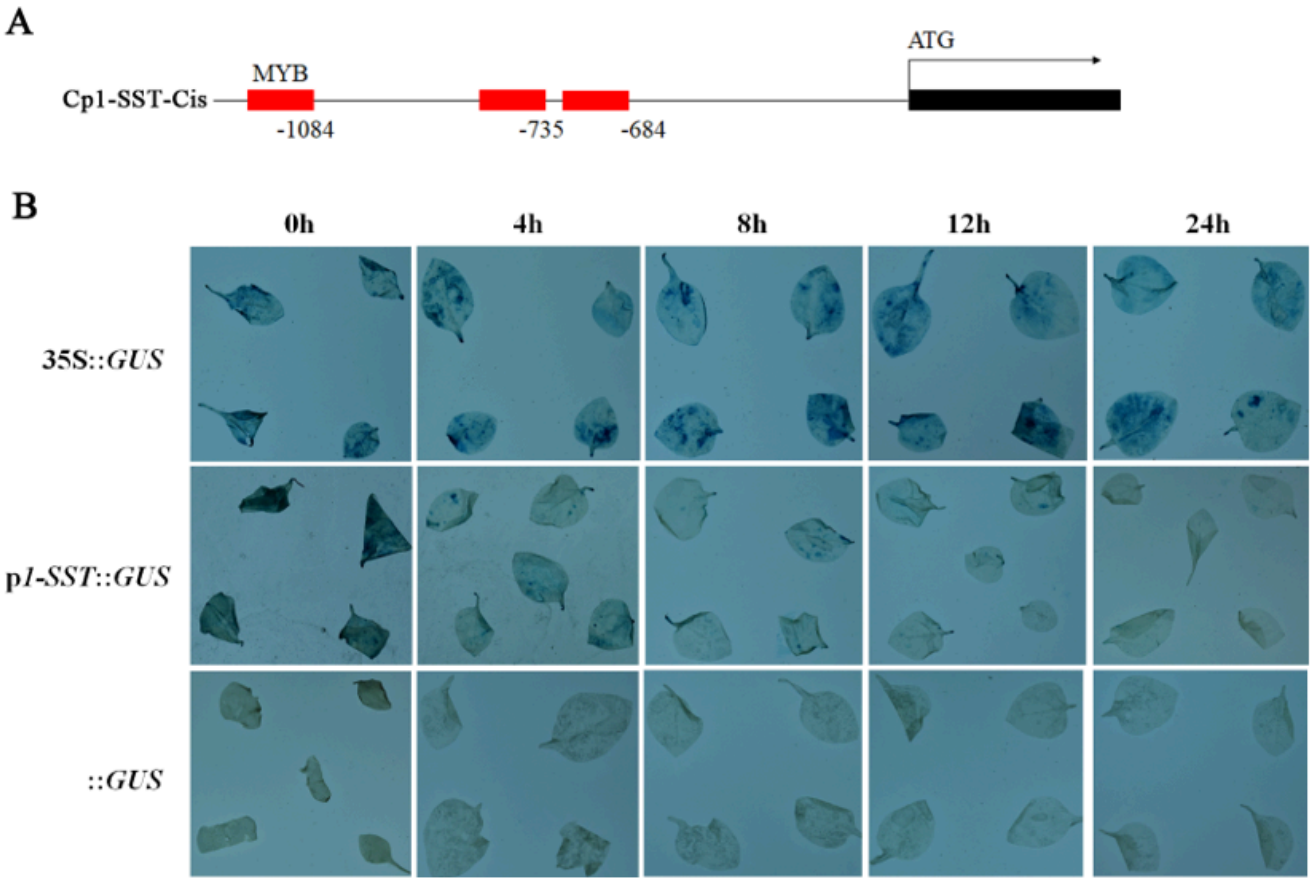

Figure 1

Schematic diagram and transcriptional activate activity of the *Cp1-SST* promoter. (A) Schematic diagram showing the cis-element of the *Cp1-SST*promoter (PlantCARE; http://bioinformatics.psb.ugent.be/webtools/plantcare/html). Red box indicated MYB recognizition sites in the *Cp1-SST* promoter. (B) β-Glucuronidase (GUS) staining of transgenic tobacco leaves transformed with the p*1-SST*::*GUS* plasmid at different time of treatment with 4°C cold stress.

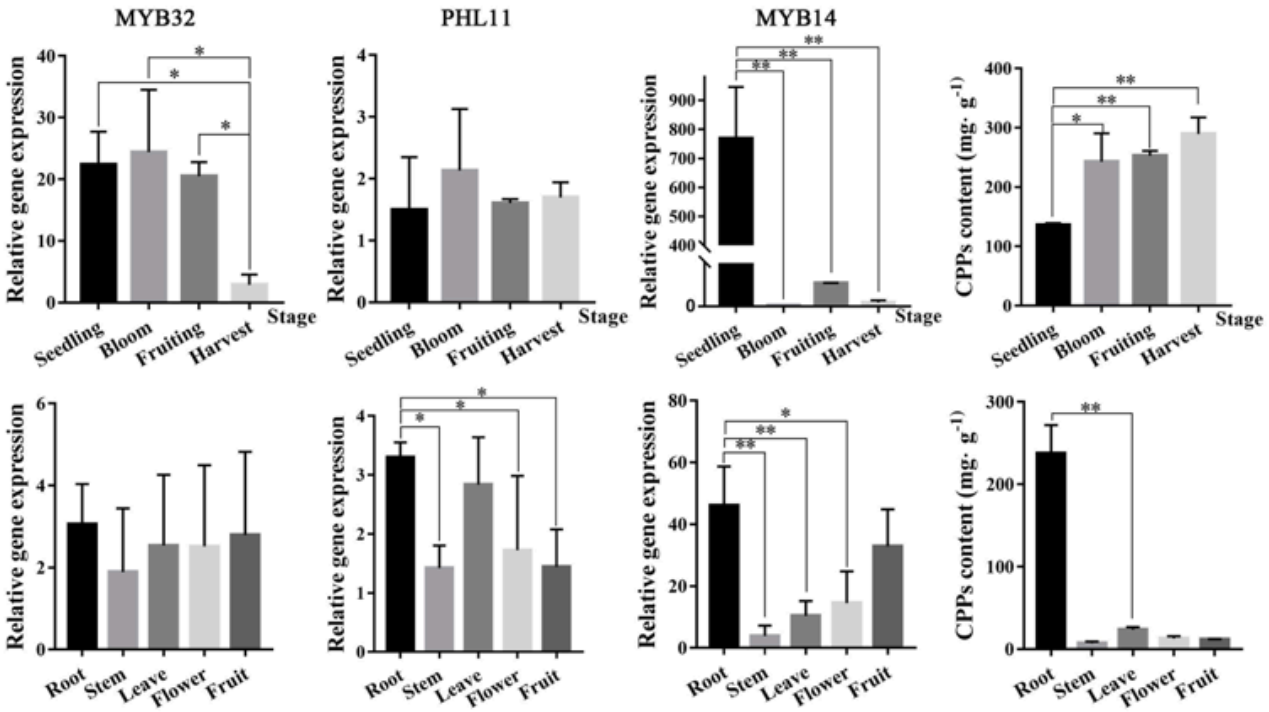

Figure 2

Spatial and temporal expression profile of *MYB*stranscription factor and CPPs content in *C. pilosula*. Statistical significance was assessed with Student's t test (**: $P < 0.01$; *: $P < 0.05$).

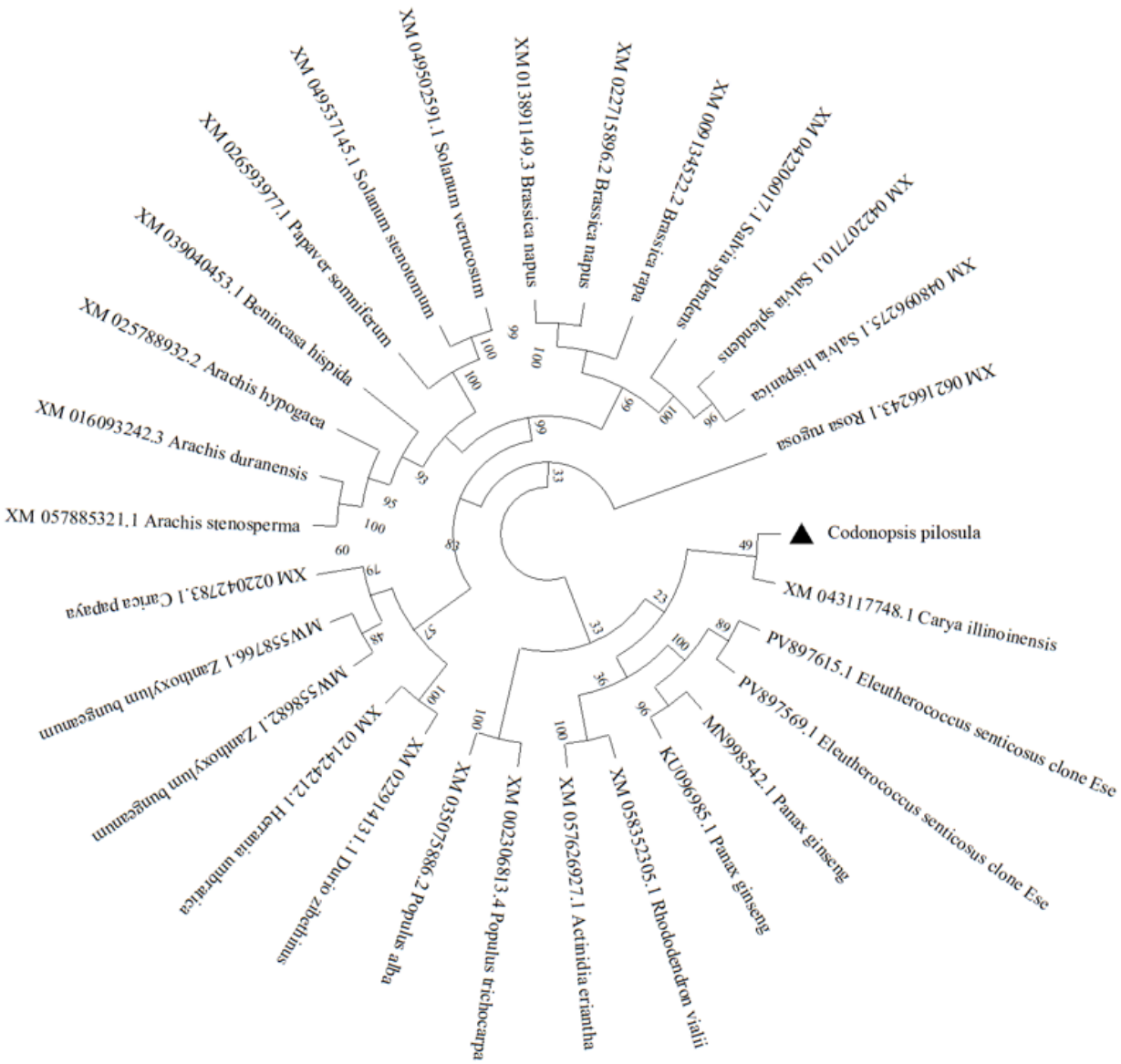

Figure 3

Phylogenetic tree of MYB14 in different species. The tree was constructed using Neighbor-joining method based on the complete amino acid sequences by MEGA 7.

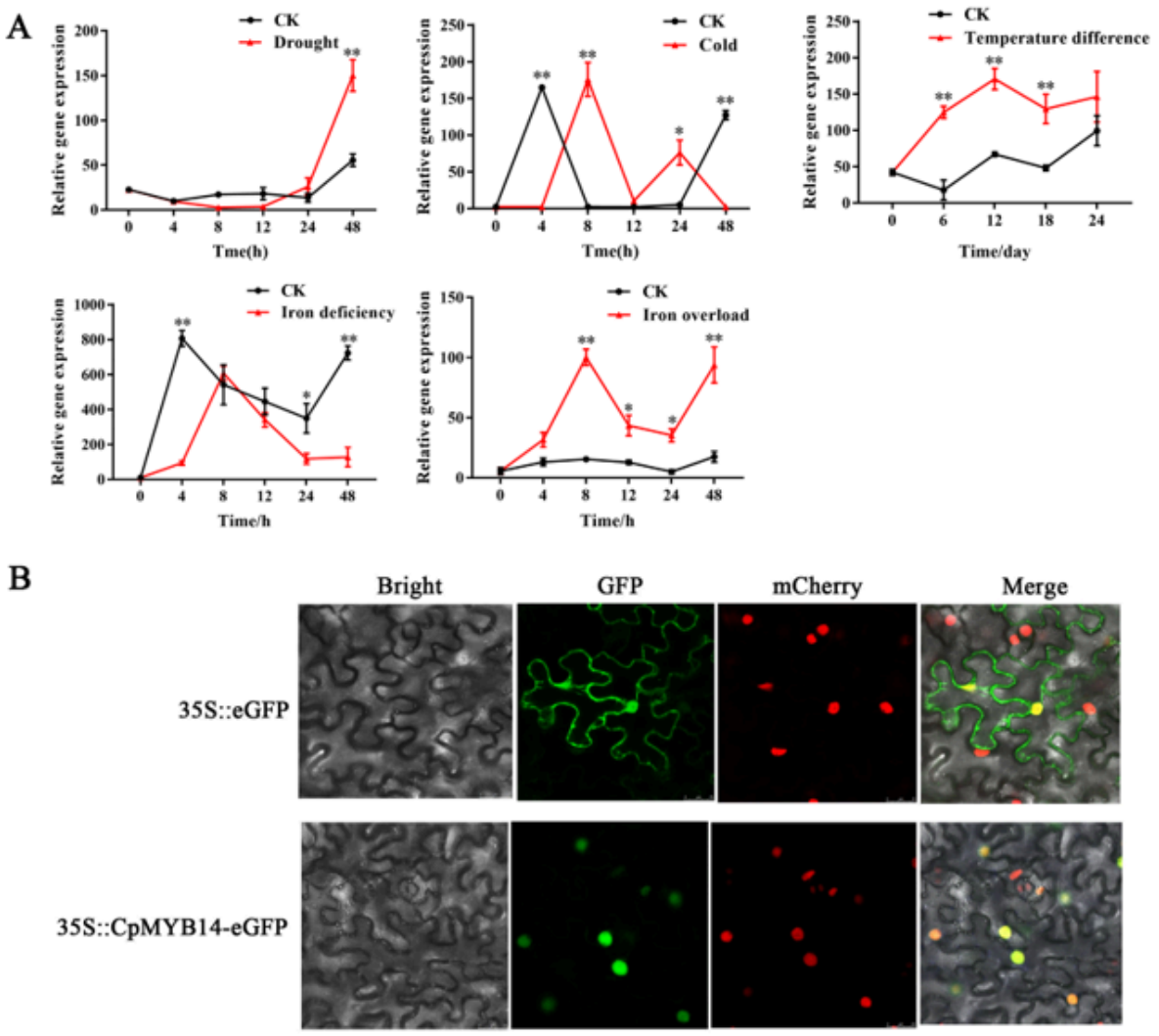

Figure 4

Expression patterns under different environmental stress and subcellular localization of CpMYB14. (A) Gene expression of *CpMYB14* in the roots of *C. pilosula*subjected to different environmental stress. Statistical significance was assessed with Student's t test (**: $P < 0.01$; *: $P < 0.05$). (B) Nuclear localization of CpMYB14 proteins in tobacco (*Nicotiana benthamiana*) leaves. Tobacco leaf cells were transiently transformed with constructs containing *CpMYB14*-eGFP, or eGFP (control) under the control of the 35S promoter.

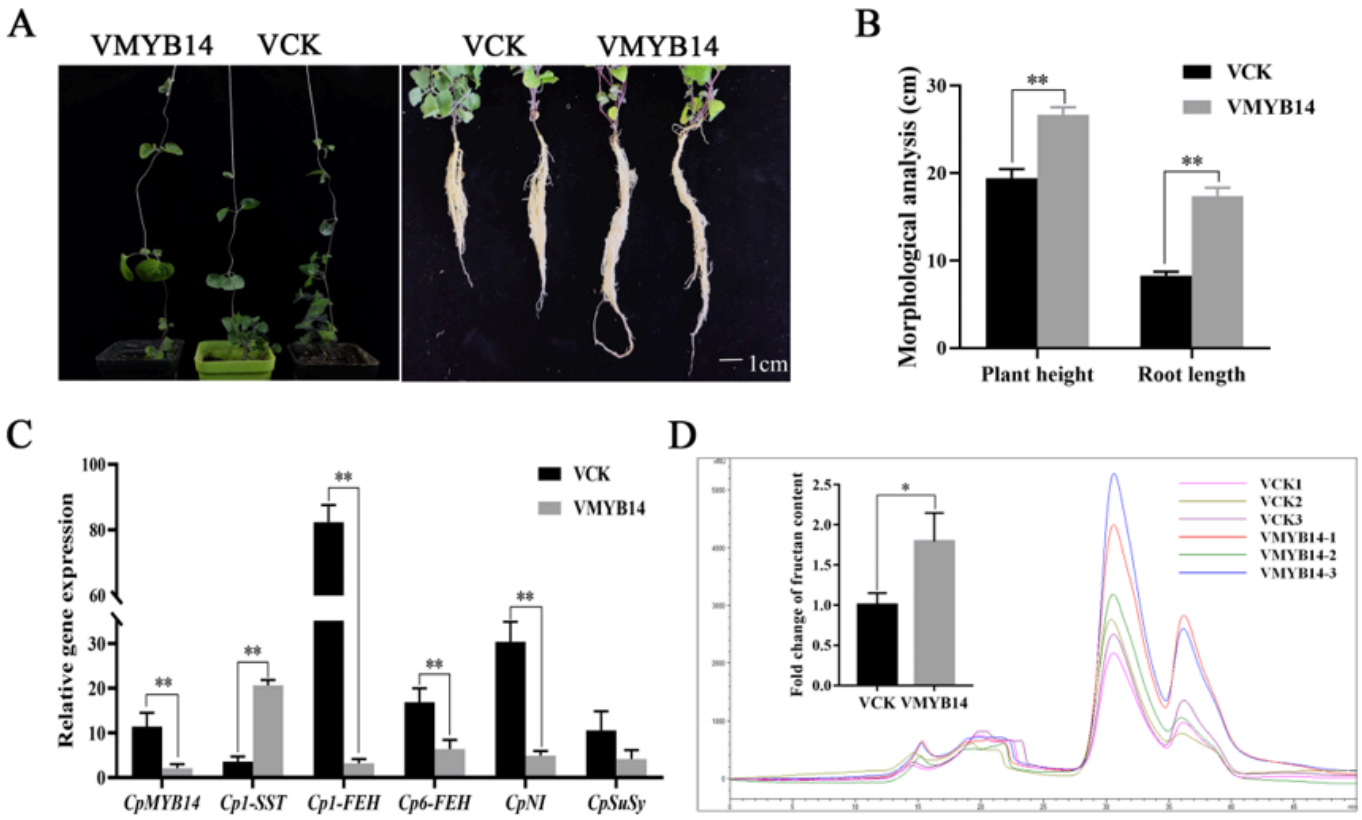

Figure 5

Gene silencing of *CpMYB14*by VIGS in *C. pilosula*. (A) Seedling phenotype of pTRV2::*CpMYB14* (VMYB14) and pTRV2:: (VCK) transgenic *C. pilosula* plants. (B) Morphological analysis of the transgenic plants. (C) Expression analysis of the genes related to ITFs metabolism in the transgenic plants. (D) HPGPC analysis of CPPs for the determination of ITFs content in the transgenic plants. Statistical significance was assessed with Student's t test (**: $P < 0.01$; *: $P < 0.05$).

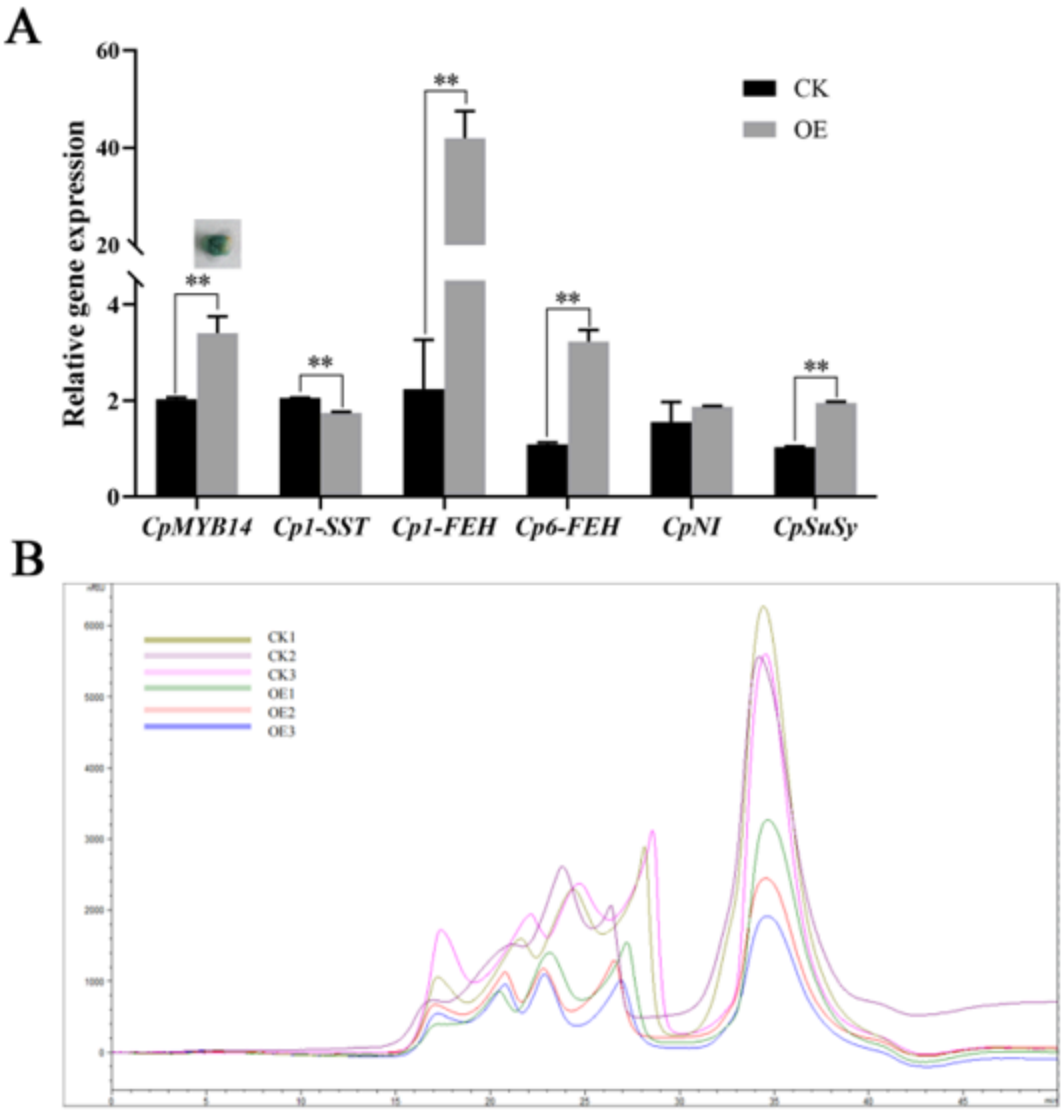

Figure 6

Overexpression of *CpMYB14* in *C. pilosula* calli. (A) Expression analysis of the genes related to ITFs metabolism in the 35S::*CpMYB14* -GUS (OE) and 35S::GUS (CK) transgenic *C. pilosula* calli. (B) HPGPC analysis of CPPs for the determination of ITFs content in the transgenic calli. Statistical significance was assessed with Student's t test (**: $P < 0.01$; *: $P < 0.05$).

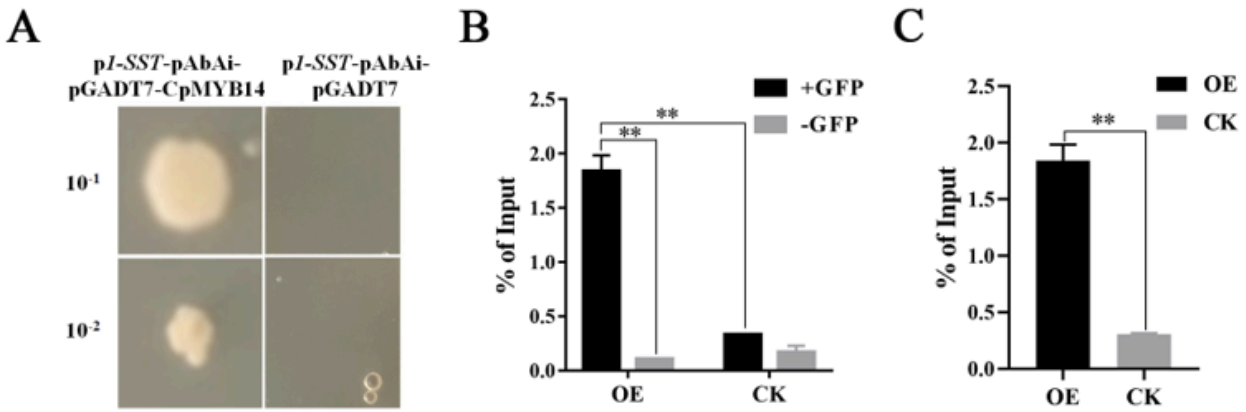

Figure 7

The transcription factor CpMYB14 binds to the MYB element of the *Cp1-SST*promoter. (A) Yeast one-hybrid assay showed that the promoter of *Cp1-SST*was fused to the pAbAi vector, and CpMYB14 was fused to activation domain AD. (B) ChIP-qPCR showed the DNA specific enrichment with GFP-antibody or without GFP antibody in 35S::*CpMYB14*-eGFP (OE) and 35S::-eGFP (CK) transgenic *C. pilosul* calli. (C) ChIP-qPCR showed the DNA specific enrichment in 35S::*CpMYB14*-eGFP (OE) and 35S::-eGFP (CK) transgenic *C. pilosul* calli. Statistical significance was assessed with Student's t test (**: $P < 0.01$; *: $P < 0.05$).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Table1.docx