

CtMYB1 regulate flavonoid biosynthesis in safflower flower by binding the CAACCA elements

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Abstract

Background

Safflower (*Carthamus tinctorius* L.) is a valuable crop known for its flowers, which are rich in flavonoids and are used for promoting blood circulation and preventing atherosclerosis. However, the molecular regulation of flavonoid biosynthesis in safflower is still poorly understood. In this study, we identified a *AtMYB12* homologous gene, *CtMYB1*, in safflower and characterized its sequence. The flower protoplast transient expression system and virus-induced gene silencing (VIGS) technique were established in safflower and we tested the role of *CtMYB1* in the regulation of flavonoid biosynthesis.

Results

Flower protoplast transient expression showed that flavonoid biosynthesis genes *CtC4H2*, *CtF3H4*, and *CtHCT12* were upregulated after transfection with *CtMYB1*. Meanwhile, VIGS showed that the transfected petals were lighter in color, and there was a decrease in the amount of the major component Hydroxysafflor yellow A (HSYA) compared to the control. Additionally, the interaction analysis by the use of Biacore system revealed that *CtMYB1* can bind to the CAACCA element of flavonoid biosynthesis genes promoters.

Conclusions

CtMYB1 can regulate flavonoid biosynthesis in safflower flower by binding the CAACCA elements of flavonoid biosynthesis related genes promoters, which shed light on the molecular regulation of flavonoid biosynthesis in safflower. The establishment of the flower protoplast expression system and VIGS in safflower provide a valuable tool for studying gene function, particularly those involved in the regulation and biosynthesis of active compounds of safflower.

1 Introduction

Safflower (*Carthamus tinctorius* L.) is an economically important crop grown worldwide, including in China where its flowers are used in traditional medicine. Flavonoids, including HSYA, naringenin, kaempferol, and quercetin, are the main active ingredients of safflower flowers and have been shown to improve blood circulation and prevent atherosclerosis. [1, 2] The composition and content of flavonoids have a direct impact on safflower quality, but the molecular regulation of their biosynthesis in safflower is not well understood.

The *MYB* transcription factor family is known to play a key role in the regulation of flavonoid biosynthesis in plants such as *Arabidopsis thaliana*, where the sixth family of *MYB* transcription factors is primarily involved [3]. The *AtMYB12* gene, as well as its homologues *AtMYB11* and *AtMYB111*, have been shown to regulate flavonoid biosynthesis in *Arabidopsis* [4, 5]. However, no *MYB* transcription factors involved in flavonoid biosynthesis have been reported in safflower.

The function of genes can be verified through stable or transient expression systems [6, 7]. The transient expression system, specifically protoplast expression, has several advantages including fast, cheap, and high-throughput analysis, as well as high transformation efficiency [8]. Protoplast expression systems have been

established in a variety of plants, such as *Arabidopsis* [9], cucumber [10], Rice [11], and other plants. At the same time, Virus-induced gene silencing (VIGS) technique based on tobacco rattle virus (TRV) as transient expression systems is also widely used for plant gene function verification, such as tobacco[12].

In this study, we cloned and characterized a *AtMYB12* orthologue, *CtMYB1*, in safflower. Using the safflower flower protoplast transient expression system and VIGS technique, we investigated the role of *CtMYB1* in the regulation of flavonoid biosynthesis. Our results shed light on the molecular regulation of flavonoid biosynthesis in safflower. And the establishment of flower protoplast expression system and VIGS technique in safflower are a valuable tool for studying gene function, particularly those involved in the regulation and biosynthesis of active compounds of safflower.

2 Materials and methods

2.1 Total RNA extraction and the cloning of *CtMYB1*

Safflower was grown at the Medicinal Botanical Garden of Chengdu University of Traditional Chinese Medicine. Total RNA was extracted using TRIzol reagent (TIANGEN, China). The purity of the obtained RNA was determined using a NanoPhotometer™ (N60 Touch) (IMPLEN, Germany). The first-strand cDNA was synthesized from total RNA using a cDNA reverse transcription kit (Takara, Dalian, China). A mixed cDNA library was created from roots, stems, leaves, and flowers. We analyzed the conserved domains of *MYB* transcription factors, and based on the conserved amino acid sequences, a pair of degenerate primers (5'-GRBTDMGRAARGGTCWTGGA-3' [Forward], 5'-GCWATHARDGACCAYCTRTT-3' [Reverse]) were designed and used to amplify the *MYB* core sequence from safflower using polymerase chain reaction (PCR). The PCR protocol consisted of an initial denaturation step at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 42°C for 30 seconds, extension at 72°C for 1 minute, and a final extension step at 72°C for 7 minutes. The PCR product was cloned into a pMD19-T vector (Takara, Dalian, China) and sequenced by Tsingke (Chengdu, China). The 3' and 5' end sequences of the core sequence were cloned using rapid amplification of cDNA ends (RACE), and the full-length cDNA of *CtMYB1* was finally cloned.

2.2 The isolation of flower protoplast in safflower

We carried out the isolation of flower protoplasts in safflower for the first time. The young flowers of safflower were cut into pieces measuring approximately 0.5 mm using sharp razors and were pretreated for 10 minutes in a dish containing 0.6 M mannitol. The chopped pieces were then transferred to an enzyme hydrolysis solution [0.6 M mannitol, 10 mM MES, 10 mM CaCl₂, 0.1% bovine serum albumin (BSA), 1.5% Cellulase RS (from Yakult, Japan) and 0.75% Macerozyme R-10 (Yakult, Japan)]. The solution was incubated in the dark at 25°C with shaking at 60 rpm for 3-4 hours. Before adding the CaCl₂ and BSA, the enzyme was activated by thermal treatment at 55°C for 10 minutes. The pieces were shaken three times during the process to ensure complete enzymolysis. After incubation, an equal volume of precooled W5 solution (154 mM NaCl, 125 mM CaCl₂, 5 mM KCl, 2 mM MES, pH 5.8) was added to the enzyme hydrolysis solution, and the mixture was shaken at 80 rpm for 4 minutes to stop the hydrolysis. The mixture was then filtered through a 40 µm nylon sieve, and the residue was washed three times with W5 solution. The filtrate was transferred to a 50-mL Eppendorf tube and horizontally centrifuged at 100 g for 3 minutes at room temperature using a Centrifuge Allegra X-30R (Beckman, Germany) to

collect the protoplasts. The supernatant was discarded, and the protoplasts were resuspended in 20 mL of precooled MMG solution (15 mM MgCl₂, 0.4 M mannitol, 4 mM MES, pH 5.7). The solution was then horizontally centrifuged at 100 g for 1 minute, and the supernatant was discarded. The protoplasts were resuspended in 3 mL of precooled MMG and kept on ice for 30 minutes. The upper liquid was discarded to obtain high quality protoplasts.

2.3 The transfection of flower protoplast in safflower

Protoplast transfection was performed based on our previous research [13] with some modifications. Firstly, 10 µg of plasmid DNA was added to a 2 mL centrifuge tube. Then, 100 µL of suspended protoplasts was added and gently mixed. 120 µL of PEG solution (40% PEG4000, 0.8 M mannitol, 100 mM CaCl₂) was then immediately added and gently mixed. The mixture was incubated at room temperature for 20 minutes. After incubation, the transfection mixture was gently diluted with 460 µL of W5 solution. The solution was then centrifuged horizontally at 100g for 3 minutes. The supernatant was discarded and the protoplasts were resuspended in 1 mL of MMG solution. The protoplasts were incubated in the dark at room temperature for 12 hours. Finally, the incubated protoplasts were centrifuged horizontally at 100 g for 3 minutes and used in subsequent experiments.

2.4 Construction of recombinant plasmids and subcellular localization of CtMYB1 protein

The PCR product of the gene *CtMYB1* was cloned into the plasmid *pMD19-T* (Takara, Dalian, China) following the instructions provided by the manufacturer. The *pMD19-T-CtMYB1* plasmid and *pET-32a (+)* were digested with the restriction enzyme BamH I and joined together using TA DNA ligase, resulting in the correct recombinant expression plasmid, which was named as *pET-32a (+)-CtMYB1*.

To express the gene *CtMYB1* in safflower flower protoplasts, the plasmid *pUbi::CtMYB1* was constructed. Primers were designed specifically for this purpose: the forward primer 5'-GCCATGGCTGATATCGGATCCATGATCCAAGATCAAGATCA-3' and the reverse primer 5'-ACGGAGCTCGAATTCGGATCCTTAATTAGTCACATTATAT-3', both of which contain the BamH I site (underlined). The fragment was then inserted into *pUbi::YFP* and replaced the YFP. High Fidelity PCR SuperMix (TransGen Biotech, Beijing, China) was used to amplify the plasmid under the following conditions: 98°C for 3 minutes, followed by 34 cycles of 98°C for 30 seconds, 62°C for 30 seconds, 72°C for 90 seconds, and a final extension of 72°C for 5 minutes.

The primers 5'-TTCCTGCAGCCCGGGGATCCATGATCCAAGATCAAGATCA-3' (forward) and 5'-ACTAGTATGGTGAGCGGATCCATTAGTCACATTATATATAC-3' (reverse), both of which contain the BamH I site (underlined), were used to construct the gene *CtMYB1-YFP*.

To determine the subcellular localization of the *CtMYB1* protein in safflower protoplasts, the fluorescence signal from YFP was detected using the Olympus FV1200 Confocal Laser Scanning Microscope (Olympus, Japan) with an excitation wavelength of 488 nm and a 50% capacity.

2.5 Vectors Construction for VIGS

pTRV1 and *pTRV2* are the two RNA strands of the TRV viral vectors commonly used in VIGS, and *pTRV2* contains multiple cloning sites within it. To silence the gene *CtMYB1* in safflower petals, the plasmid *pTRV2::CtMYB1* was constructed using the above safflower petal *CtMYB1* as a template. The forward primer is 5'-GTGAGTAAGGTTACCGAATTCATGATCCA AGATCAAGATCA-3' and the reverse primer is 5'-TGGAGGCCTTCTAGAGAGAATTCATTA GTCACATTATATATATAC-3', both of which contain the EcoR site (underlined). The 489 bp fragment was then inserted into the *pTRV2* using Exnase II enzyme. High-fidelity PCR Super Mix II (TransGen Biotech, Beijing, China) was used to amplify the plasmid under the following conditions: 98°C for 3 minutes, followed by 34 cycles: 98°C for 30 seconds, 58°C for 30 seconds, 72°C for 90 seconds, and a final extension at 72°C for 5 minutes.

2.6 Protoplasts RNA extraction and RT-PCR analysis

We use the MicroElute Total RNA kit (Omega Bio-Tek, USA) to extract protoplasts RNA. The purity of the RNA samples was assessed using a NanoDrop spectrophotometer (IMPLEN, Germany), and the A260/A280 ratios of all samples were found to be within the range of 1.9-2.1, indicating a pure RNA preparation. cDNA was synthesized from the purified RNA using the PrimeScript™ RT reagent Kit with gDNA Eraser (Takara, Dalian, China) following the standard protocol provided by the manufacturer.

Expression levels of selected genes were determined by real-time PCR (RT-PCR) after over-expression of the *CtMYB1* gene, using a CFX96™ Real-time System (Bio-Rad, Hercules, CA, USA). Three replicate samples were analyzed for each reaction. Specific primers were designed using the Primer Premier 5 (Table 1). The 25S rRNA gene from *Carthamus tinctorius* L. was used as the reference gene to normalize expression levels in each cDNA sample. The RT-PCR reaction mixture consisted of 1 µL of each forward and reverse primer, 1 µL of cDNA, 7 µL of double-distilled water, and 10 µL of SYBR Green II (Takara, Dalian, China), in a total volume of 20 µL. To detect expression levels of five genes involved in flavonoid biosynthesis in safflower flowers, RT-PCR conditions were as follows: an initial denaturation step at 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for 10 s, annealing at 58.5°C for 30 s, extension at 72°C for 30 s, and a final extension step of 72°C for 5 min.

Table 1. The primers for RT-PCR

Promoter name	PCR primer
<i>pCt25s</i>	F GGAGGTTGAGGGAAAAGGAG
	R GTGACCTCGTCACCCGTAGT
<i>pCtHCT5</i>	F AACCTTCCAGCCCTACGC
	R AATCCGACTCCGCCTCAA
<i>pCtHCT12</i>	F ATGGCATCTTTACAAATAACCG
	R CAACTCCTAGCGACACCC
<i>pCtC4H2</i>	F AAATCCAAGCGAAACTGA
	R GCAAACCTAACTGCCCCAC
<i>pCtF3H4</i>	F ATCTCAGAAGCCAGCAAA
	R GCCAAACCCTATGAAACA
<i>pCtOMT6</i>	F TTCTTTCTACCGCCCTTGC
	R TCGTAGCCGATGACTCCC

2.7 Establishment of VIGS system of safflower flowers

This method was mainly referred to the report in *S. cruentus* [14]. *pTRV1*, *pTRV2* and recombinant plasmid *pTRV2::CtMYB1* were transferred into *Agrobacterium C58C1*, and the transformed bacterium was cultured in 5 mL TY medium containing 100 mg/mL kanamycin at 37°C, shaken at 200 rpm until the OD₆₀₀ reaches 0.6~1.0, and then centrifuge at 4000×g for 10 minutes. Discard the supernatant and resuspend it with infiltration solution to OD₆₀₀=1.5~2.0 and make sure that the concentrations of each group are similar. The infiltrate *Agrobacterium* containing *pTRV1* was mixed with the infiltrates *Agrobacterium* containing *pTRV2* and *pTRV2::CtMYB1* 1:1 by volume, and left in the dark for 4-6 hours at room temperature. The newly picked safflower petals at the early flowering stage were immersed in equal amounts in different infiltration solutions, each group was placed under -0.10~-0.09 MPa air pressure at the same time after 5~6min treatment, the normal air pressure was slowly restored (about 2min), and the safflower was removed from the sample and rinsed with distilled water, and placed in equal amounts on sterile petri dishes (with a diameter of about 5ml) that were padded with sterile filter paper and about 5 ml of sterile water. The safflower was taken out and rinsed with distilled water, and equal amounts were placed in sterile petri dishes padded with sterile filter paper and added with about 5ml of sterile water, and then cultured in darkness for 3d at 5°C, and then counted as 0 d after taking out, and then cultured at 25°C for 6 d under the light, with water changed every day. The petals of 0d, 3d and 6d of light culture were photographed and recorded. Among them, safflower infiltrated with *Agrobacterium* transformed with *pTRV2::MYB1* was the experimental group (*pTRV2::MYB1*), safflower infiltrated with *Agrobacterium* transformed with empty vector *pTRV2* was the control group (CK), and safflower infiltrated with aseptic infiltration solution was the blank group (0).

2.8 HSYA determination

The content of Hydroxysafflor yellow A was determined according to our previous research [15]. After culturing safflower material, a portion of it was freeze-dried. Approximately 10 mg of the freeze-dried material was precisely

weighed and added to a centrifugal tube containing 2ml of a solution with small steel beads. The mixture was then ground using a milling machine, and the resulting powder was centrifuged at 9000 rpm for 5 minutes, causing it to settle at the bottom of the tube. Subsequently, about 1.25ml of 25% methanol was added, maintaining the same concentration as specified in the Pharmacopoeia. The mixture was subjected to ultrasonic treatment (using a power of 300W and a frequency of 50 kHz) for 40 minutes and then cooled. Afterwards, the powder was centrifuged once again at 9000 rpm for 5 minutes, and the supernatant was filtered through a 0.45µm filter membrane into an injection vial. The resulting sample was then injected following the aforementioned liquid phase conditions. The injector column used was the Eclipse XDB-C18, measuring 4.6 × 250mm with a 5µm particle size. The mobile phases used were as follows: Phase A consisted of a 0.7% phosphoric acid solution, Phase B was composed of methanol, and Phase C of acetonitrile. The three phases were eluted in an isocratic manner using a ratio of 72:26:2 respectively. The detection wavelength employed was 403 nm, and the injection temperature was room temperature. A volume of 10 µl was injected for analysis.

2.9 Recombinant protein expression and purification

The recombinant plasmid *pET-32a:: CtMYB1* was transformed into *E. coli* BL21 (DE3) (Tsingke Biotech Company, Chengdu, China). The transformed bacteria were grown in 5 mL LB medium containing 100 mg/mL of Ampicillin at 37°C with shaking at 200 rpm until the OD₆₀₀ reached 0.35-0.45. The expression of the *CtMYB1* fusion protein was then induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) at 200 rpm, 16°C, for 12 hours. The bacteria were centrifuged at 4°C and 12,000 rpm for 5 minutes, and the supernatant was discarded. The cell pellets were resuspended in 1 mL of water and centrifuged again at 12,000 rpm for 5 minutes. The pellet was then dissolved in precooled 1 mL of Lysis Buffer (50 mM HEPES, 500 mM NaCl, 5 mM imidazole, pH 7.0).

The sample was subjected to SDS-PAGE analysis by adding 10 µL of 5 × SDS loading sample buffer (Solarbio, Beijing, China) and boiling for 10 minutes, followed by centrifugation at 12,000 rpm for 5 minutes. The resulting supernatant was loaded onto a Tris-glycine gel in the 4-20% range and visualized using Coomassie Brilliant Blue G-250 staining.

To purify the soluble form of *CtMYB1*, BL21 (DE3) cells harboring *pET-32a:: CtMYB1* were grown in 1 L of LB medium with 100 mg/mL of Ampicillin at 16°C and 200 rpm for 12 hours. The cells were collected by centrifugation at 4°C and 12,000 rpm for 10 minutes, and the supernatant was discarded. The cells were resuspended in 50 mL of water, centrifuged, and resuspended in 30 mL of Lysis Buffer. The cells were lysed using a sonicator (SCIENTZ, Ningbo, China) on ice for 15 minutes at 30% duty. The soluble and insoluble fractions were separated by centrifugation at 4°C and 12,000 rpm for 45 minutes. The supernatant was filtered using a 0.45 µm filter, and the cellular debris were resuspended in 10 mL of water. 1 mL of avidin Ni-NTA was added to the supernatant and incubated at 4°C and 50 rpm for 1 hour. The final processed supernatant was applied to a column and eluted with increasing concentrations of imidazole (40, 80, and 160 mM). The eluted fractions were saved in Eppendorf tubes for SDS-PAGE analysis.

3 Results and Discussions

3.1 Cloning and sequence analysis of *CtMYB1*

In this study, a 1223-bp cDNA of the *CtMYB1* gene was cloned from safflower (Genbank KY554784) and sequence analysis revealed a 489-bp open reading frame (ORF) encoding a 162-amino acid protein with a molecular weight of 17878.15 and an isoelectric point of 4.82. The hydrophilicity of the *CtMYB1* protein was predicted using ProtScale and the gradient average hydrophobicity was found to be -0.307, indicating that the protein is hydrophilic. The prediction of the signal peptide cleavage site showed C, S, and Y scores of 0.117, 0.150, and 0.116, respectively. As all the scores were close to 0.1, it was deduced that the *CtMYB1* protein is non-secretory (Figure 1A).

We selected 9 transcription factors that have been reported to regulate flavonoid biosynthesis, including the maize flavonoid synthesis regulatory genes *P1* and *P2*, and the tomato flavonoid synthesis regulatory gene *SIMYB12*, for analysis. Protein sequence alignment analysis showed that all *MYB* transcription factors that regulate flavonoid biosynthesis are relatively conserved at the N-terminus, especially in the R2R3 domain, while they differ significantly in structure at the C-terminus. Despite the large structural differences at the C-terminus, two relatively conserved structural domains, SG7 (GRTxRSxMK) and SG7-2 ([W/x][L/x]LS), were still present at the C-terminus (Figure 1B). Studies have reported that the SG7 and SG7-2 domains are characteristic features of *MYB*-like transcription factors that regulate flavonoids [16, 17]. These findings indicate that the cloned *CtMYB1* we screened is likely to be involved in regulating flavonoid biosynthesis in safflower.

3.2 The establishment of flower protoplast in safflower

The use of protoplast transient expression systems has been crucial in plant research for studying gene functions, protein subcellular localization, and interaction assays. For instance, the mesophyll protoplast transient expression system of *Arabidopsis* is a highly efficient and important tool for gene function research [18, 19]. Similarly, the versatile and physiological protoplast system of rice has been demonstrated to be useful for research on light/chloroplast-related processes [20, 21]. However, heterologous systems may result in inaccurate findings due to the presence of a foreign genetic background. Thus, homologous transient expression systems are much more accurate for gene function research. The successful isolation of high-yield and viable protoplasts is critical for the protoplast transient expression system. Protoplasts freshly isolated from leaves have been shown to be a significant tool for gene function studies [22, 23], and a transient expression system using root protoplasts has also been established [24]. In contrast, the isolation of protoplasts from safflower flowers is challenging due to the structure of its corolla. Therefore, the proper selection of the explant is crucial for obtaining high-quality, viable protoplasts for subsequent experiments.

In this study, we compared the yield and quality of protoplasts isolated from corolla lobes and tubes. The steps for isolating and pretreating the safflower flower are outlined in Figure 2. We compared the yield and quality of protoplasts extracted from both the corolla lobes and tubes, and results revealed that a lower number of viable safflower protoplasts obtained from the lobes (Figure 3A) rather than the tubes (Figure 3B). The optimum explant for safflower protoplast isolation was determined to be young corolla tubes, as they produced a high number of transparent protoplasts after 4 hours of enzyme digestion. Our protoplast isolation protocol was suitable for subsequent research, yielding approximately 5×10^7 protoplast cells per corolla tube.

The viability and concentration of protoplasts are important factors in transfection efficiency. In this study, the transfection efficiency of safflower protoplasts was measured by detecting the yellow fluorescent protein (YFP)

signal. The stability of the transient expression vectors was verified as the YFP signal could be detected 18 hours after transformation (Figure 4). Our results showed that we achieved a high transfection efficiency.

3.3 Subcellular protein localization of CtMYB1 in safflower flower protoplasts

It should be noted that there have been no previous reports of characterizing genes using safflower protoplasts. To evaluate the suitability of the flower protoplast transient expression system for protein subcellular localization, we conducted an experiment. Our results showed that the fusion protein *CtMYB1-YFP* was expressed in safflower flower protoplasts and was localized in the nucleus (Figure 5A). To further demonstrate the versatility of this system, we used YFP markers for plasma membrane (MEM-MAR) and cytoplasm (CYT-MAR) localization that are conserved in our lab. After 18 hours of transfection, MEM-MAR was expressed in the plasma membrane, and CYT-MAR was expressed in the cytoplasm (Figure 5B and 5C). As a control for the subcellular localization studies, we used pUbi::YFP, which resulted in a brilliant green fluorescence observed throughout the cell (Figure 5D). The result suggest that safflower flower protoplasts can be used for characterizing genes.

3.4 CtMYB1 can upregulated flavonoid biosynthesis genes expressions

The goal of the study was to investigate the potential association between the *CtMYB1* gene and flavonoid biosynthesis in safflower. Ten genes related to flavonoid biosynthesis were selected based on transcriptome analysis. After transforming the *CtMYB1* gene into the safflower flower protoplasts, the RNA was isolated and RT-PCR was performed to measure the expression of the ten selected genes. The results showed that the expression of the genes *CtC4H2*, *CtF3H4*, and *CtHCT12* was upregulated by the *CtMYB1*, while the expression of *CtHCT5* and *CtOMT6* remained largely unchanged (Figure 6). *CtC4H2*, *CtF3H4*, and *CtHCT12* are important genes in safflower flavonoid biosynthesis. In our previous report, we found that the increased expression of these genes is associated with the content of flavonoids in safflower under MeJA treatment[25]. The result suggest that safflower flower protoplasts can be used for the expression analysis of flavonoid biosynthesis genes.

3.5 Silencing of CtMYB1 by the use of VIGS downregulated flavonoid biosynthesis

The color of safflower at the initial bloom stage changed from yellow to red after 6d of incubation by dipping. Compared with the control group (CK) transformed with the empty vector and the blank group (0) without *Agrobacterium*, the color change of safflower in the experimental group (*pTRV2::MYB1*) transformed with *pTRV2::MYB1* was small. The petal color of the experimental group (*pTRV2::MYB1*) was found to be significantly lighter than that of the control group (CK,0) by 6 d of incubation (Fig. 7A), which indicated that silencing *CtMYB1* will inhibit the change in safflower color. The content of HSYA was determined and result showed that the HSYA content of the experimental group was significantly lower than that of the control group (CK) and the blank group (0) (Fig. 7B). The VIGS results demonstrate that *CtMYB1* could regulates safflower flavonoid synthesis.

3.6 CtMYB1 can bind to the CAACCA element of flavonoids biosynthesis genes promoters

The Surface Plasmon Resonance (SPR) technology used by the Biacore system is capable of detecting real-time protein-protein and protein-nucleic acid interactions. In this study, the *CtMYB1* protein was produced by induction with 1 mM IPTG for 12 hours at 16°C. SDS-PAGE analysis showed that the *CtMYB1* fusion protein had a molecular weight of approximately 35 kDa, suggesting that it may exist as a dimer (Figure 8A). The Biacore system is user-friendly and has various applications, making it a useful research tool for detecting and measuring protein interactions [26-28].

In the Biacore assay, one of the interactants is immobilized on a sensor chip surface while the other is flowed over the surface in solution. The immobilized interactant is referred to as the ligand and the flowing interactant as the analyte. In this study, the Biacore T200 was used to measure protein-nucleic acid interactions. The *CtMYB1* protein was diluted to a final concentration of 50 µg/mL in sodium acetate solution (pH 4.0) and immobilized on a CM5 sensor chip by amine coupling to reach a density of 300 resonance units (RU). The immobilized *CtMYB1* was then used to capture core elements from the flavonoids biosynthesis genes promoter sequences. And the flavonoids biosynthesis genes in safflower used in this study were referred to our previous research [29] (Table 2). The data was recorded at 25°C using a running buffer of PBS-T (10 mM sodium phosphate, 150 mM NaCl, 0.005% Tween-20, pH 7.4) and 2% DMSO.

Table 2. MYB elements and the sequences of the promoters

MYB elements	Sequences of the promoters
TTAGGTT	AGTTAGGTTAAAGTTAGGTTAAAGTTAGGTTAA
CAGTTG	TCTCAGTTGAAATCTCAGTTGAAATCTCAGTTGAAA
TGGTTA	TTTTGGTTACAATTTTGGTTACAATTTTGGTTACAA
TAACCA	ACATAACCATTAACATAACCATTAACATAACCATTA
CTGTTG	TCACTGTTGGTGTCACTGTTGGTGTCACTGTTGGTG
TGGTTG	CGGTGGTTGTTACGGTGGTTGTTACGGTGGTTGTTA
TAACTG	CCCTAACTGTAACCCCTAACTGTAACCCCTAACTGTAA
CAGTTA	AAGCAGTTAACGAAGCAGTTAACGAAGCAGTTAACG
CACATG	ATACACATGCGTATACACATGCGTATACACATGCGT
CAATTG	GTGCAATTGGTTGTGCAATTGGTTGTGCAATTGGTT
AACCTAA	TGAACCTAATATGAACCTAATATGAACCTAATA
CAACAG	CTCCAACAGCGACTCCAACAGCGACTCCAACAGCGA
CATGTG	CGTCATGTGAGACGTCATGTGAGACGTCATGTGAGA
CATTTG	TCTCATTTGAACTCTCATTTGAACTCTCATTTGAAC
TAAGAGA	TATAAGAGACATATAAGAGACATATAAGAGACA
CAACTG	TGGCAACTGTGTTGGCAACTGTGTTGGCAACTGTGT
CCGTTG	CAGCCGTTGATGCAGCCGTTGATGCAGCCGTTGATG
CAAATG	GTTCAAATGAGAGTTCAAATGAGAGTTCAAATGAGA
CAACCA	TCTCAACCACCGTCTCAACCACCGTCTCAACCACCG
TAACCATTTTTTCTAT	AATAACCATTTTTTCTATTAAATAACCATTTTTTCTATTAAATAACCATTTTTTCTATTA
CAACGG	GCACAACGGGCAGCACAACGGGCAGCACAACGGGCA
TCTCTTA	TGTCTCTTATATGTCTCTTATATGTCTCTTATA
ATAGAAAAAATGGTTA	TAATAGAAAAAATGGTTATTTAATAGAAAAAATGGTTATTTAATAGAAAAAATGGTTATT

Six concentrations of synthetic promoters (0, 2, 4, 8, 16, 32 nM) were injected at a flow rate of 30 $\mu\text{L}/\text{min}$, with a 4-minute contact time and 5-minute dissociation time. A blank immobilization was performed on one of the sensor chips to correct the binding response. The results showed that the core element of *MYB*, CAACCA, could directly bind to the *CtMYB1* protein immobilized on the sensor chip surface. The binding concentration was 16 nM (Figure 8B). All in all, these results show that *CtMYB1* is a transcription factor that can regulates the biosynthesis of safflower flavonoids, and it is regulated by binding to ATATATAC element.

4 Conclusion

This study established an efficient method for flower protoplasts and VIGS and performing transient expression. The results showed that the *CtMYB1* gene is capable of regulating the expression of genes involved in flavonoid biosynthesis in safflower. Inhibiting the expression of *CtMYB1* can inhibit the color change of safflower flowers while significantly reducing the content of HSYA in the flowers. In addition, interaction analysis using the Biacore system indicated that *CtMYB1* regulate the synthesis of flavonoid components by binding to the CAACCA element of the flavonoid biosynthesis gene promoter. This study provides valuable insights into the molecular mechanisms of flavonoid biosynthesis in safflower, and the information gathered here will be valuable for future research in safflower.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

Sequence data that support the findings of this study have been deposited in the National Center for Biotechnology Information with the primary accession code KY554784.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Y.Z., J.W., Y.P. and C.C. finished the experiment and drafted the manuscript. J.P. and J.C. originated the work, led the discussions, provided helpful comments, and revised the manuscript. B.X., C.R. and Z.X. provided helpful comments and revised the manuscript. All authors have read and agreed to the published version of the manuscript.

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Figures

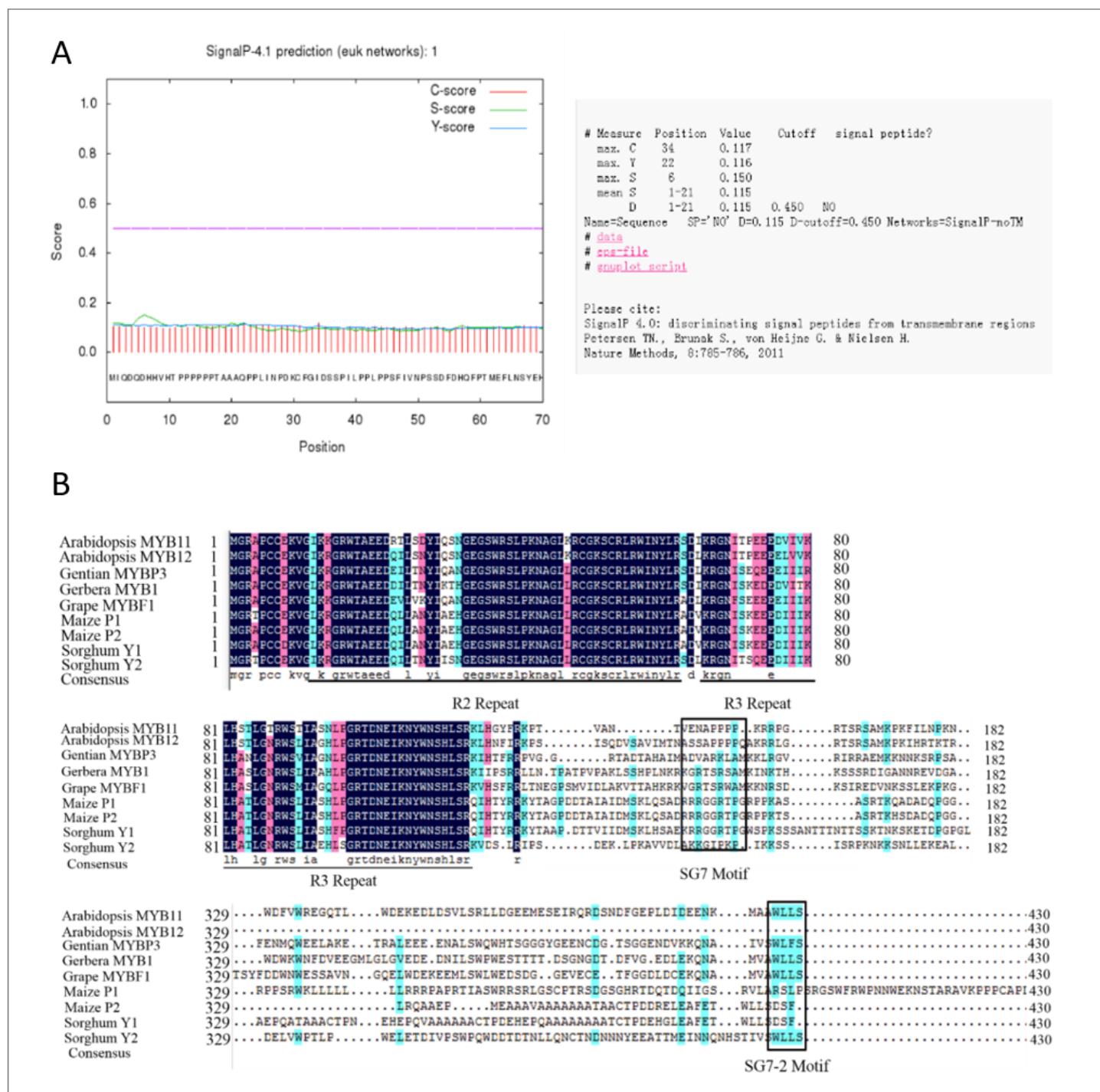


Figure 1

Sequence analysis of CtMYB1. (A) The predicted cleavage site of the signal peptide in CtMYB1. (B) The protein sequence alignment of MYB transcription factors, with R2 Repeat and R3 Repeat being typical domains of the R2R3 MYB transcription factor, and SG7 and SG7-2 being special domains involved in the regulation of flavonoid synthesis.

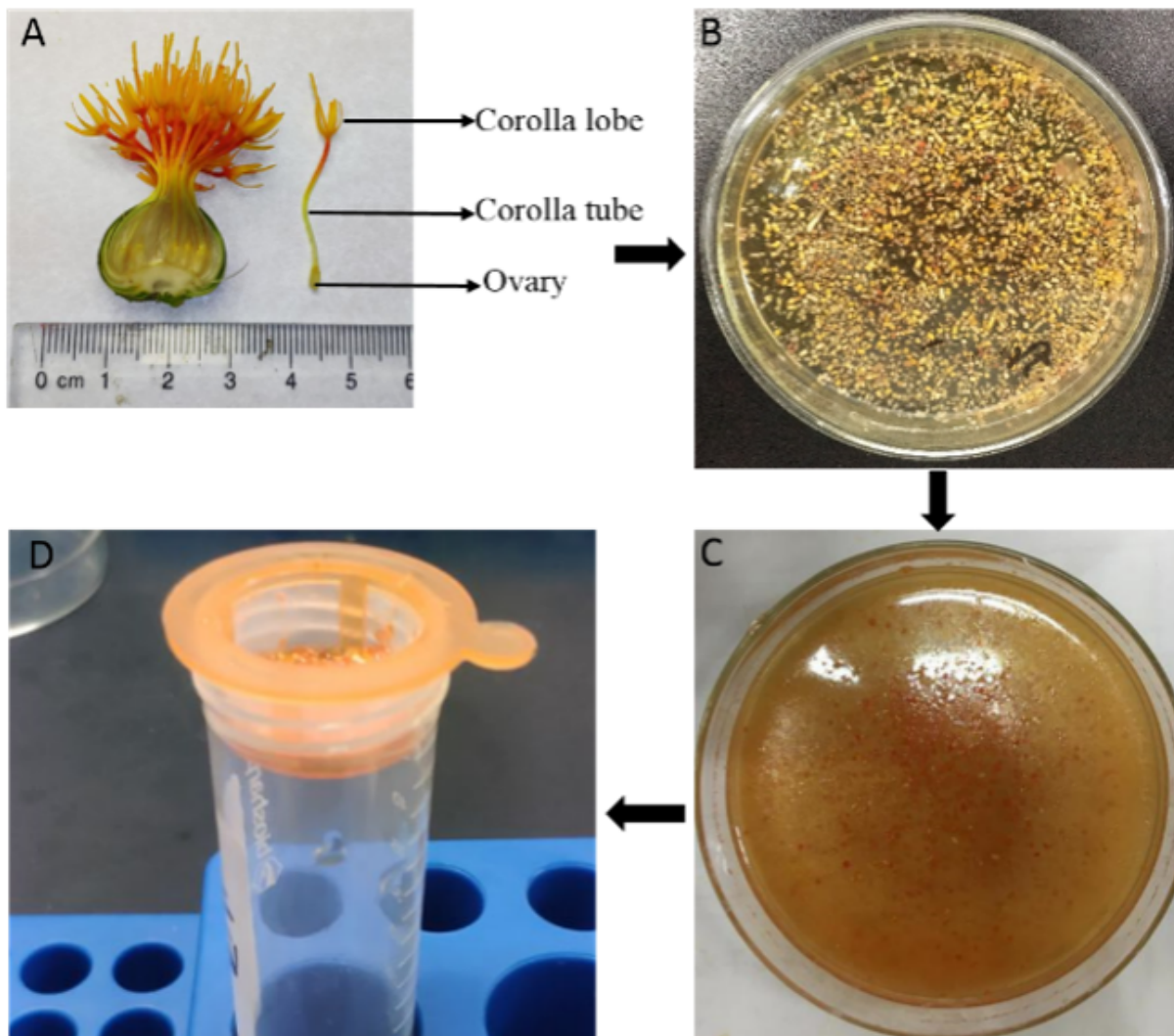


Figure 2

Overview of the separation and pretreatment of safflower flowers. (A) The separation of the corolla. (B) The gathering of the corolla in 0.6 M mannitol for osmotic treatment. C The enzymatic hydrolysis of the protoplasts. D The collection of the protoplasts.

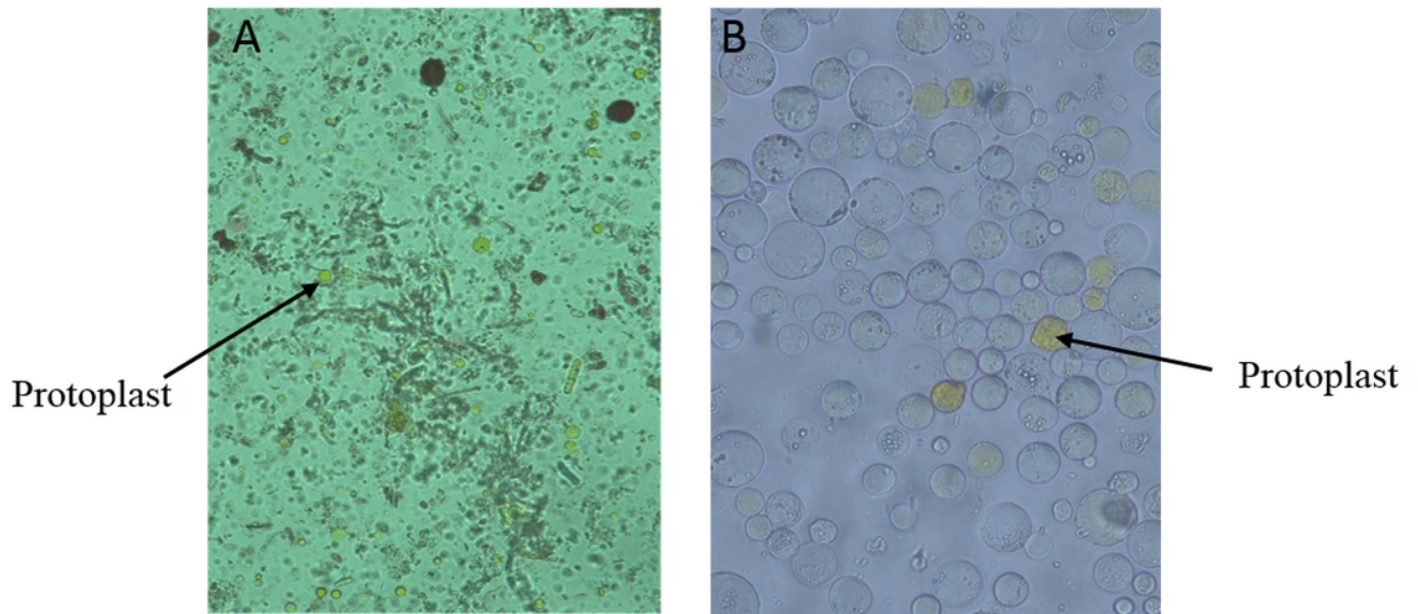


Figure 3

A comparison of the yield and quality of protoplasts extracted from both the corolla lobes and tubes. (A) Protoplasts isolated from the corolla lobe. (B) Protoplasts isolated from the corolla tube.

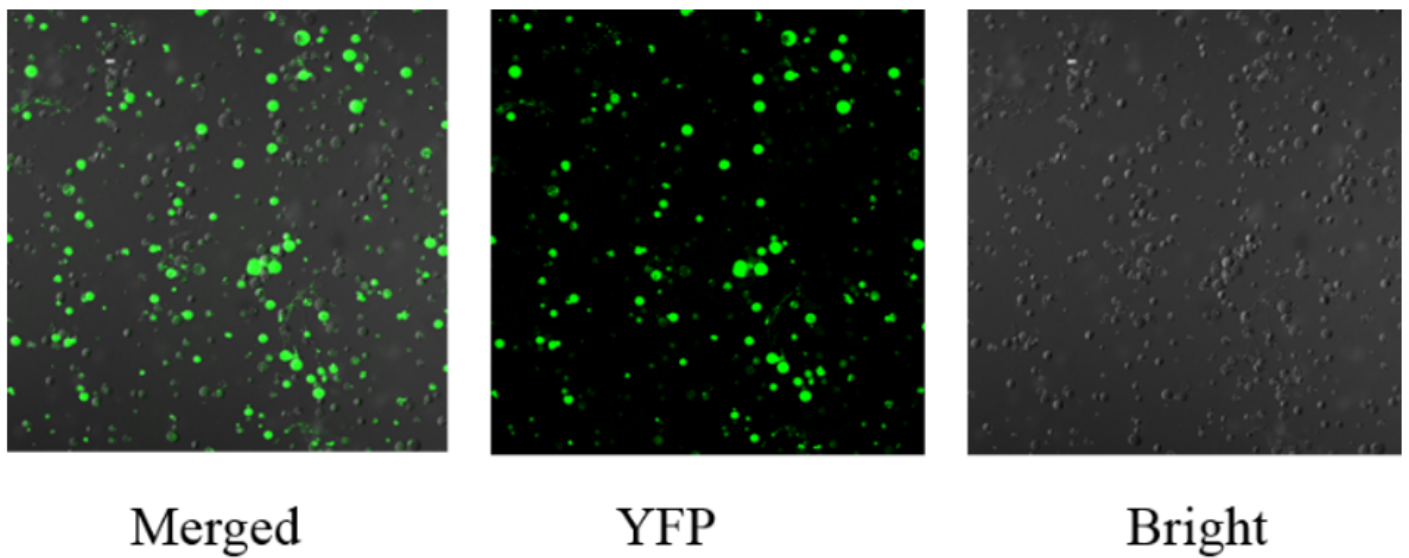


Figure 4

Protoplasts transfected with pUbi::YFP. The YFP signal is detected 18 hours after transformation.

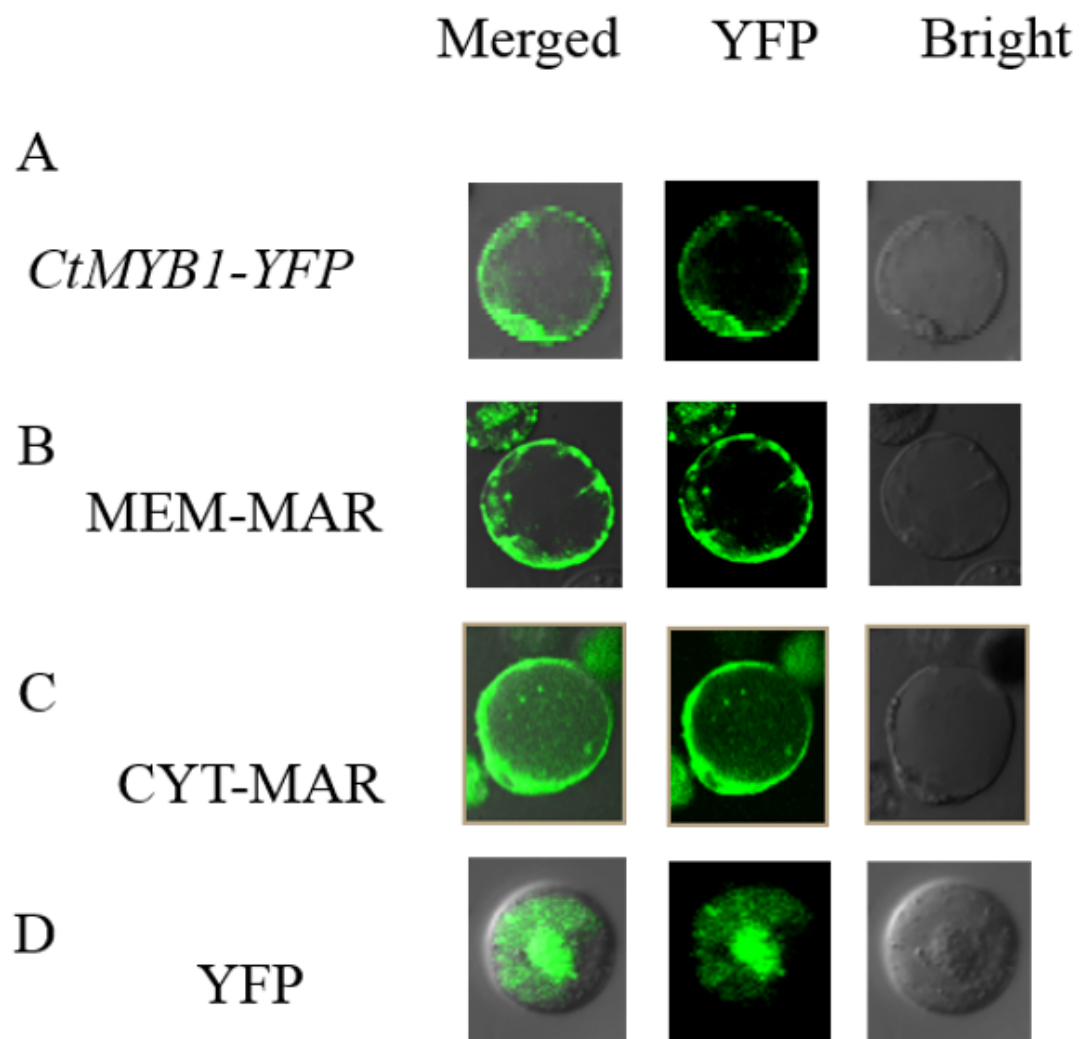


Figure 5

Subcellular localization in safflower flower protoplasts. (A) *CtMYB1-YFP* targeted to the cell nucleus. (B) A marker for membrane location. (C) A marker for cytoplasm location. D *pUbi::YFP* being used as the control, with YFP being expressed throughout the entire cell.

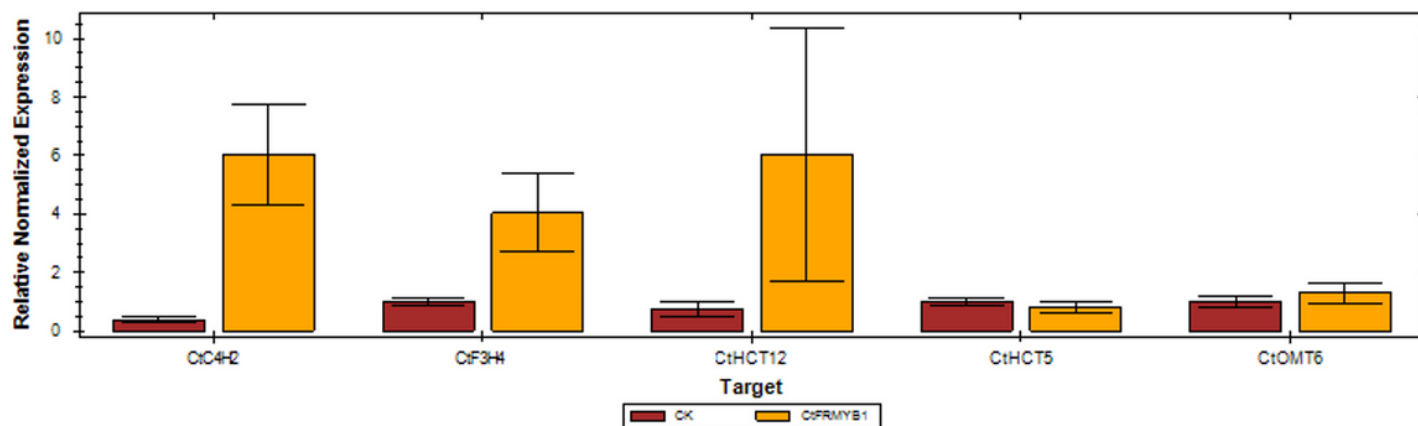


Figure 6

Expression analysis of genes involved in safflower flavonoid biosynthesis after infiltrated into safflower protoplasts. CtC4H2, CtF3H4, and CtHCT12 genes can be upregulated by CtMYB1.

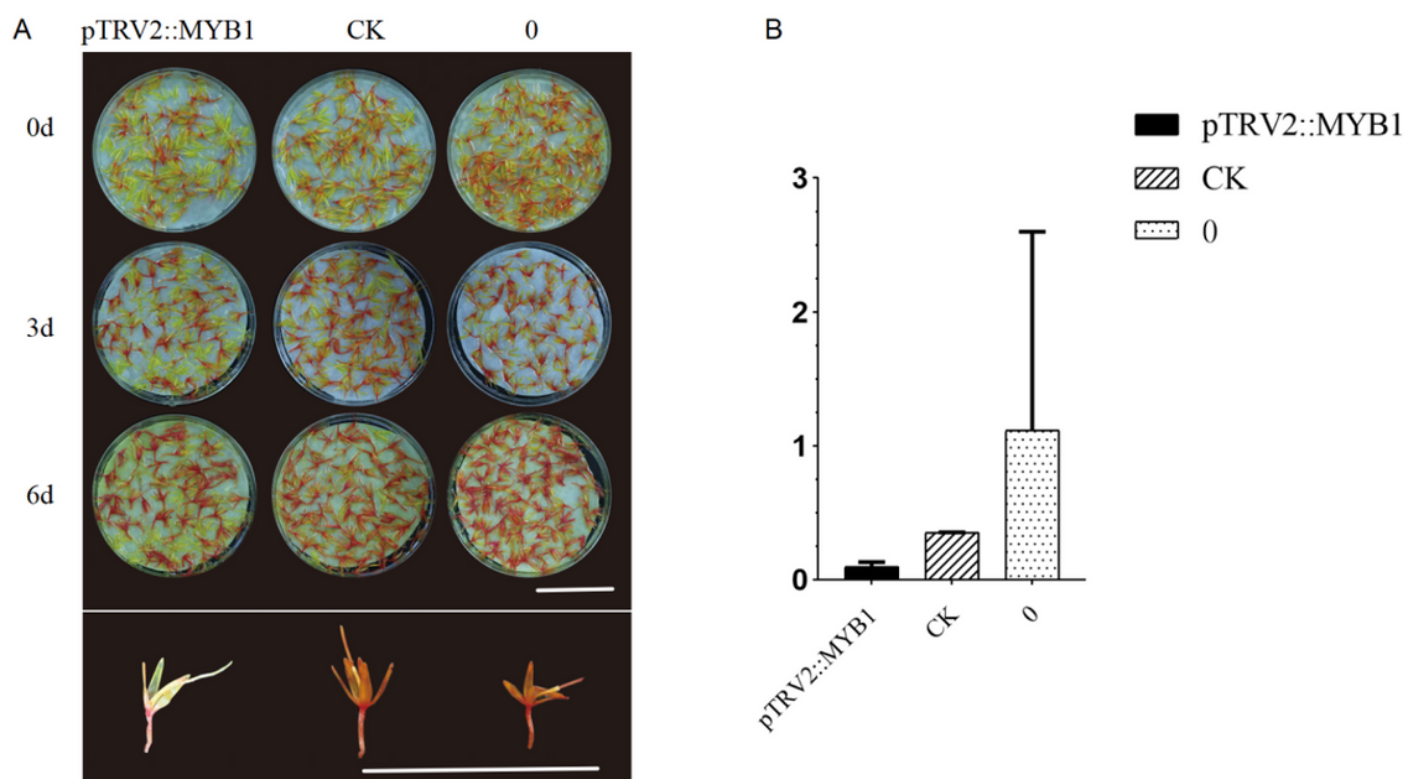


Figure 7

CtMYB1 can regulates flavonoid biosynthesis by the use of VIGS. (A) Vertical representation records the color change of Agrobacterium infiltration solution treated safflower after 0d, 3d and 6d. Horizontal representation records the color change of Agrobacterium infiltration solution-treated safflower transformed with pTRV2::MYB1, CK and (0) aseptic dip solution-treated safflower. Bar=5cm. (B) Relative content of HYSA in petals after Agrobacterium infiltration. pTRV2::MYB1, CK, and 0 were grouped as in A.

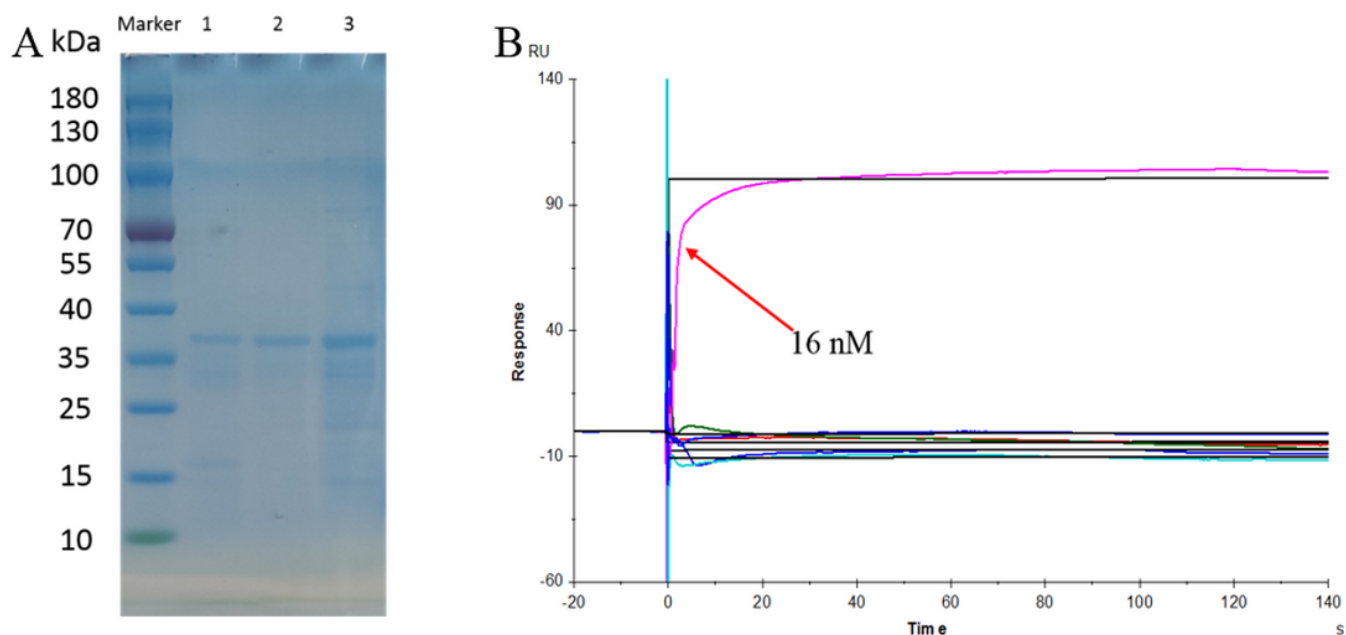


Figure 8

Binding analysis of CtMYB1 with DNA elements. (A) SDS-PAGE analysis of CtMYB1 fusion protein after purification by Ni-NTA Resin column, with 1-3 representing proteins eluted with imidazole of 160, 80, and 40 mM. (B) A representative sensorgram of fitted kinetic data from the kinetic analysis of nucleic acids binding to CtMYB1 that has been directly immobilized to Sensor Chip CM5 using amine coupling.

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