Genome-Wide Identification and Expression Analysis of Flavonoid 3'-Hydroxylase Gene Family in *Carthamus Tinctorius* L.

Hoàng Việt Nguyễn Quốc (✉ harryken090495@gmail.com)

Jilin Agricultural University <https://orcid.org/0000-0001-7820-2735>

Kong Jie

Jilin Agricultural University

Naveed Ahmad

Jilin Agricultural University

Yang Zhuoda

Jilin Agricultural University

Wang Nan

Jilin Agricultural University

Ma Xintong

Jilin Agricultural University

Zhang Xin Yue

Jilin Agricultural University

Liu Xiuming

Jilin Agricultural University

Yao Na

Jilin Agricultural University

Jin Libo

Jilin Agricultural University

Research Article

Keywords: Flavonoid 3'-hydroxylase (F3'H), safflower, expression analysis, subcellular localization

Posted Date: June 8th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-575863/v1>

License: This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Objective

Through experiments and bioinformatic analysis clearly demonstrate considerable information about the genetic evolution of the flavonoid 3'-hydroxylase (F3'H) gene in Safflower and in plants.

Results

Here, we performed genome wide survey of safflower genome and identified a total of 22 CtF3'H enzyme encoding genes. Phylogenetic analyses revealed the classifications of these CtF3'Hs into nine subgroups demonstrating their evolutionary relationship. The distribution of the conserved protein motifs, and cis-regulatory units of CtF3'Hs indicated essential structure-to-function components leading to the final function of protein or its interactions. Furthermore, the results of digital expression analysis and the qRT-PCR pattern of 22 putative CtF3'H genes during different flowering stages suggested their requisite roles in safflower petal pigmentation. In addition, the fusions construct of plant expression vector pCAMBIA1302-GFP-CtF3'H5 in onion epidermal cells verified the subcellular localization of CtF3'H5 to the plasma membrane. Subsequently, the prokaryotic expression and western blot hybridization of CtF3'H5 resulted in a stable 50.3kD target protein. These results partly demonstrate the influence of F3'Hs on plants.

Conclusions

In this study, the results of digital expression and qRT-PCR analysis of 22 putative CtF3'H genes in different flowering stages indicate their essential role in safflower petal pigmentation. clearly demonstrates significant information on the genetic evolution of important enzyme-coding genes and will provide a pathway for future functional studies of F3'Hs in safflower as well as in plants.

Introduction

Safflower (*Carthamus tinctorius* L.) is widely known as one of the important medicinal plants worldwide. Safflower contains various active ingredients, including flavonoids, quinolones, alkaloids, and safflower polysaccharides (Ambreen et al. 2018). Safflower can exert various biological effects, including anti-oxidant, anti-inflammatory (Alaiye et al. 2020), and antibacterial activities (Cho et al. 2017). It has been reported that safflower is beneficial for improving acute cerebral infarction and ischemic stroke (Wang et al. 2019). Among flavonoids, Safflower Yellow is the main active ingredient of safflower, and it has a variety of pharmacological effects such as dilating blood vessels, protecting myocardial ischemia, and antioxidant activity. Flavonoids are an important class of natural products (Cerqueira et al. 2021); In particular, they belong to the polyphenolic group of plant metabolites, which are widely found in fruits, vegetables, and some beverages. The biochemical effects as well as antioxidant abilities of flavonoids are directly related to various health diseases such as cancer, alzheimer's disease (AD) (Du et al. 2021), atherosclerosis, etc. They are also sometimes act as strong inhibitors of different group of enzymes, for

example xanthine oxyase (XO)(Lin et al. 2021), cyclo-oxyase (COX), lipoxygenase, and 3-kinase phosphoinositide (Panche et al. 2016).

The flavonoid 3'-hydroxylase (F3'H) is the core enzyme of flavonoid metabolic pathway which belongs to the superfamily of cytochrome P450 (Choudhary et al. 2016). F3'H hydroxylation of position 3 'of the B ring of flavonoids (Guo et al. 2019) directs the production of cyanide red pigments. The F3'H gene that encodes CYPs has been cloned and well investigated in a variety of plant species such as hybrid Petunia (De Palma et al. 2014), and Arabidopsis thaliana (Gao et al. 2020). Furthermore, it was also suggested that the precursors molecules of flavonols are usually hydroxylated using 3'-hydroxylation via the overexpression of SbF3'H in sorghum. Similarly, the incredible loss of glycine max fuzz was achieved by viral-mediated silence of the F3'H gene (Nagamatsu et al. 2009). Moreover, the results of ectopic expression level of the MdF3'H identified from *Malus × domestica* stimulated the accumulation pattern of anthocyanin (Y et al. 2010). Nonetheless, the biosynthetic pathway of flavonoids in the safflower is still unknown. It is reported in the literature that the late branches of the flavonoid biosynthetic pathway produce flavanols, flavonols, or anthocyanins, Therefore, it is important to fully understand and investigate the evolution and regulation of flavonoid 3'-hydroxylase (F3'H) enzyme encoding genes which is of great significance for the downstream regulation of flavonoid content in safflower(Ren et al. 2021).

The purpose of the present study was aimed to present a complete overview of the genome wide identification of CtF3'H enzyme encoding subgene family in safflower and further study the evolutionary classification from their ancestors. This study also investigates several structural and functional dynamics of CtF3'H subfamily using multiple in silico predictions and molecular techniques. This researsh work in general highlights the groundwork for the identification of candidate *CtF3'H* genes for future molecular breeding programs to produce new safflower varieties with rich anthocyanin content.

Materials And Methods

Plant Materials, vectors and strains

The safflower Jihong early-maturing variety was used experimental source in this study. The seeds were purchased from Xinjiang Honghuayuan Technology Co., Ltd. and planted in the experimental station of the Jilin Agricultural University. The petals of safflower bud, initial, flowering and fade stages were used for expression analysis. Agrobacterium tumefaciens strain EHA105, E. coli BL21, E. coli DH5α cells, prokaryotic expression vector (PET28a+CtCYP81E8), subcellular localization vector pCAMBIA1302-CtCYP81E8-GFP) were constructed and stored in a refrigerator at -80 ° C with 75% glycerol until the next use.

Identification of CtF3'H in safflower

The genome of JH1 variety of Carthamus tinctorius was extracted from the online project of Jilin Agricultural University (PRJNA399628; filed on August 23, 2017) in the NCBI (<https://www.ncbi.nlm.nih.gov>). BioEdit software was used to BLAST the putative *CtF3'H* in the genome

of safflower. The identified *CtF3'H* genes were further validated on the Pfam database (<http://pfam.xfam.org>) and the identified accessions were assembled for further analysis. The molecular weight (MW) and isoelectric point (pI) of each CtF3'H protein was calculated using the ExPASy program (<http://www.baoasy.org/>). The amino acid sequence of each protein was aligned using DNAMAN software (Version 7; Lynnon Corporation, Quebec, Canada), using the default parameters.

Phylogenetic Analysis

The amino acid sequences of 22 CF3'H Proteins extracted from safflower genome were aligned with the help of Clustal W (2.0) package. Similarly, 136 members of the Arabidopsis F3'H family were extracted from the Arabidopsis Information Resources for comparative analysis. A neighbour-joining evolutionary tree was constructed from these 22 safflower CF3'H Proteins and 136 Arabidopsis F3'H proteins using 1000 bootstrap method in the MEGA 5 software version 4.1 (<http://www.megasoftware.net/>) (Schrage et al. 2018). The organization and divergence of the CF3'H Proteins in safflower with the Arabidopsis F3'H was clustered together in various clans following their structural and functional similarities which classified them into different subfamilies.

Motif Elicitation and Promoter Analysis

The conserved protein motifs of the CtF3'H proteins were determined by uploading 22 amino acid sequences into the MEME web server's online tool (<http://meme.nbcr.net/meme/cgi-bin> version/meme.cgi). The parameters are optimized as follows: zero or one, the rate of occurrence of a pattern on each sequence; 10 BP, module width range; and three other broad ranges of patterns were identified, and finally 10 motifs were selected. All different parameters follow with default values. In the same way, the distribution of the all conserved cis-elements of the promoter sequences of safflower *CtF3'H* genes were extensively investigated by exploiting the PlantCARE software available at (<https://sogo.dna.affrc.go.jp/>).

Transcriptomic profiling and qRT-PCR analysis

The digital expression analysis of identified *CtF3'H* genes in safflower was conducted using the RNA-seq metadata in four different flowering stages, root, seed, and stem tissues respectively. A combined heatmap was generated from the kilobase model of exon model per million mapped read (RPKM) values in each tissue under investigation. Furthermore, the expression level of each CtF3'H transcript was validated with qRT-PCR analysis using four different flowering stages of safflower. For this purpose, the primer pairs for these selected 22 *CtF3'H* genes were synthesized from the genomic data. The total RNA extraction was carried out from each flower tissue with the help of TRIzol reagent. The synthesis of cDNA templates were produced using the reverse transcriptase enzyme.

All qRT-PCR reactions were carried out with SYBR® Premix Ex Taq™ (TliRNaseH Plus) from TaKaRa Biotechnology Co., Ltd. (Dalian, China) using a real-time PCR device (Biosystems 7500). Following the manufacturer's protocol, a 20 µL of PCR mixture was prepared including 1.0 µL cDNA, 0.4 µM each primer (F/R), 0.4 ROX dye, 10 µL main mixture, and RNase free water 7.8 µL. The internal reference gene 18s ribosomal RNA of safflower was used as control. The relative expression level for each transcript was

measured according to $2^{-\Delta\Delta Ct}$ method (Min et al. 2020). The primer details are listed in (Supplementary Table S1).

Gene cloning and subcellular localization of CtF3'H5

The flower petals of the safflower were stored at -80°C and then ground in liquid nitrogen and then subject to total RNA extraction with TRIzol reagent. RNA concentrations were determined by NanoDrop 2000 based on OD₂₆₀ / 280 values and checked by electrophoresis on 1% agarose gel. Following the manufacturer's instructions, reverse transcription was carried out to synthesize the first strand cDNA templates for PCR amplification. The gene specific primers (Supplementary Table S2) were designed according to the genomic sequence of *CtF3'H5*. The PCR reaction conditions were as follow: 98°C for 10 seconds, 98°C for 10 seconds, 65°C for 15 seconds, 72°C for 2 minutes, 35 cycles; 72°C for 10 minutes. The amplified PCR products of *CtF3'H5* gene were detected on 1% agarose gel electrophoresis and the results were recovered using a gel recovery kit and then ligated to pEASY-T1 Simple vector, and then transformed into the competent cells of bacteria. The positive colonies of pEASY-T1- *CtF3'H5* were selected on LB agar plates and the results was confirmed with half colony PCR method using gene specific primers. After PCR confirmation, the plasmids was extracted from the positive colonies and further confirmed with double restriction enzyme digestion, and then sent to Shanghai Biotech Engineering Services Co., Ltd for sequencing. Similarly, using the *CtF3'H5* T vector plasmid, the full length cDNA sequence of *CtF3'H5* was amplified using a new primer pairs (Table S2) containing BglII, and NcoI dual restriction sites, and the target band was subcloned into a new plant expression vector pCAMBIA1302-GFP-35S for the investigation of subcellular localization. The recombinant plasmid was confirmed initially with double restriction digestion using BglII and NcoI enzymes and then verified by sequencing. The recombinant plasmid (pCAMBIA1302-GFP-*CtF3'H5*) was then transformed into agrobacterium EHA105 competent cells using heat and shock method and the positive bacterial strains were selected on YEP agar medium. The transient transformation system was established using the agrobacterium mediated infection of the onion epidermal cell under controlled conditions. The Agrobacterium-infected onion epidermal cells containing the recombinant plasmid (pCAMBIA1302-GFP-*CtF3'H5*), were plated on the solid medium of 1/2 MS, placed in dark culture medium at 28°C for 18 hours and then the results of the GFP expression was analyzed by scanning confocal laser microscopy.

Prokaryotic expression and Western Blot

The full-length cDNA sequence of *CtF3'H5* was amplified using Pfu DNA polymerase (Takara) with the help of a newly designed set of primers (Table S2) with an added Hind III and XhoI restriction sites. The complete fragment of the amplified *CtF3'H5* gene was cut and recovered, mixed with the prokaryotic expression vector pET-28a⁺ in a 4:1 ratio, and then ligation was performed into the same digested empty pET28a + vector using T4 DNA ligase enzyme overnight at 16°C for 16 h. The ligated product was transferred to the DH5 α strain and recombinant plasmid cells were selected on kanamycin-resistant LB plate, and the positive clones were confirmed by PCR following double digestion system sequencing. The successfully constructed expression plasmid was named pET-28a-*CtF3'H5*, and the correctly sequenced plasmid was transformed into the competent BL21 cell. The positive clones were identified and cultured

at a speed of 180 r/min at 37°C and 28°C respectively in 10ml of LB liquid medium containing 50 µg/mL of ampicillin. When the absorbance at 600nm (A600) reaches 0.8, 1 mol/L, the induction with IPTG was carried out using different concentration (0.2,0.4,0.5,0.6,0.7,0.8) at different time periods including 2 h, 4 h, 5 h, 6 h, and 8 h respectively. The soluble protein extract of *CtF3'H5* was detected on 12% SDS-PAGE, and then the target bands were stained with coomassie brilliant blue dye. The proteins obtained were further subjected to PVDF membrane for 2 hours, and the membrane was washed for 5 min using Tris buffer saline Tween (TBST) and then blocked with the blocking buffer (TBST buffer containing 5% skim milk powder) for 1 hour. After performing the incubation with primary antibody at room temperature, we discarded the blocking buffer. The incubation was allowed at 4°C overnight followed by thorough washing with TBST 5 times for 5 min each time. The addition of the second antibody onto the membrane was enabled for 2 hours incubation following by washing 5 times with TBST for 5 min each time. The antibody binding complex was analyzed with the help of ECL chemiluminescence and this experiment was repeated three times.

Statistical analysis

All the experiments were conducted three times. Data were shown as the mean $\pm$ standard deviation of three parallel experiments (N = 3) with the standard deviation always less than 5%.

Results

Identification and Physicochemical Properties of the Safflower *CtF3'H* Genes

To localize all putative *CtF3'H* genes in safflower, we performed a series of electronic searches and Pfam ID scan for safflower genome database. The F3'H orthologes were also screened out from TAIR database for comparative analysis. A total of 22 candidate *CtF3'H* genes were identified in safflower genome and was subjected to blast for validation. The physico-chemical properties of *CtF3'H* proteins were observed including the protein length ranging from 128aa to 518aa, the molecular weight ranges from 14.84 kDa (*CtF3'H22*) to 58.6 kDa (*CtF3'H23*), with the average is 40.75 kDa. The isoelectric point values ranged from 4.94 (*CtF3'H7*) to 9.49 (*CtF3'H37*) with an average of 7.14. Similarly, the analysis of grand average of hydropathicity (GRAVY) showed that the majority of *CtF3'H* proteins were hydrophilic, only *CtF3'H37* with GRAVY 0.88 expressed hydrophilic. (Supplementary Table 1)

Phylogenetic Analysis of *CtF3'H* family with Arabidopsis P450

The protein-encoding *CtF3'Hs* with Arabidopsis AtP450s were assembled in a phylogenetic tree which is mainly divided into two main branches: type A and non-A. These two branches are clustered into nine subclasses including, clan71, clan75, clan81, clan76, clan78, clan72, clan97, clan77, clan51 and clan79 (Fig. 1). The detailed overview of phylogenetic analysis showed that clan71, clan72, clan77, clan79 and

clan 81, clans51 are clans that do not contain the *CtF3'H* gene family, whereas the other three clans unevenly contained members of *CtF3'H* gene family. All putative *CtF3'H* genes were identified as non-A type of Cytochrome P450s which is further classified into 22 subcategories. On the other hand, the subfamily CYP71 is the largest A-class (48.18%) however, our analysis showed that it does not contain any genes of *CtF3'H*. The organization of clan 75 indicated the assembly of various other AtCYP450 grouped with 14 putative *CtF3'H* genes. Similarly, clan 76 also showed a clear association between 8 AtCYP450 members and 10 *CtF3'H* genes of safflower. However, the distribution of genes at clan 78 showed 7 members of AtCYP450 grouped with 2 *CtF3'H* genes, but exceptionally, these 2 *CtF3'H* genes originated directly from the root of the tree located at the same branch as clan 78 revealing their ancestral divergence points.

The distribution of conserved protein motifs and alignment of CtF3'H Proteins

The organization and distribution of the conserved protein motifs of CtF3'H protein were extensively investigated and screened with MEME online tool version 5.1.1. The output results of the 22 putative CtF3'H protein sequence were combined into a phylogenetic tree. We found two highly conserved protein motifs expressed as red and blue masses, correspondingly (Fig. 2). The location and distribution pattern also revealed that most of the conserved motifs were localized to C terminus of CtF3'H proteins. The subfamily members such as *CtF3'H9* and *CtF3'H37* do not showed considerable conservation of the investigated protein motifs. Similarly, the composition of motifs conservation in *CtF3'H27* described incomplete pattern. Notably, in most cases, the putative proteins of safflower *CtF3'H* demonstrated the two conserved regions which usually linked next to each other; but, in the case of *CtF3'H26*, these two conserved regions were found separated from each other. On the contrary side, some proteins indicated inadequate conservation modules such as *CtF3'H24* and *CtF3'H22*.

In addition, we also performed multiple sequence alignment of all putative 22 CtF3'H proteins to determine their sequence homology (Fig. 3). The highly conserved homology within the same subfamily members was shown with red and blue color. The similarity of amino acid conservation pattern confirms the previous results of MEME scores obtained from CtF3'H proteins. The corresponding locations of each CtF3'H protein pattern were found conserved, however, motif 1 and motif 2 were found scattered among all CtF3'H proteins. Meanwhile, the occurrence of motif 3 is present in all subtypes except *CtF3'H1* and *CtF3'H2*. Similarly, motif 4 is not present in *CtF3'H7* and part of CtF3'H8 whereas motif 5 was found in some proteins such as *CtF3'H1*, 3, 4, 5, and 10. The presence of motif 6 is found in *CtF3'H1,3,4,6* while motif 8 was only screened for the proteins of *CtF3'H8*, 9 and 10. Altogether, the distribution and conservation of signature protein motifs as well as the progressive alignment network revealed significant evolutionary and sequential features of CtF3'H subfamily in safflower.

Organization of cis-regulatory units of CtF3'H promoters

To identify different variants of gene promoter cis-regulatory elements, we extensively analyzed the 2kb upstream to the ATG starting site of *CtF3'H* genes using the PlantCARE's software. The abundantly

annotated cis-elements within the promoter sequence of most of the *CtF3'H* genes contained factors related to light reactions for example, 19/22 genes *CtF3'H* contain the G-Box cis-element, suggesting that signals induction might play crucial roles during the transcription activation and regulation of *CtF3'H* genes in safflower (Fig. 4). Moreover, our analysis revealed the presence of MYB and MYC responsive elements which are actively implicated during flavonoid biosynthesis. In addition, the occurrence of cis-regulatory units conserved in other major plant reactions have also been studied within the promoters of different *CtF3'H* genes, such as drought (MBS), and abscisic acid reaction (ABRE). Convincingly, the presence of such types of cis-acting elements in safflower *CtF3'H* genes suggested positive hallmarks during plant secondary metabolism and various stress related and hormonal induced pathways.

Expression analysis of CtF3'H subfamily genes

We conducted a meta-analysis of the data sets of 22 *CtF3'H* genes showing that the digital expression of all putative *CtF3'H* genes exhibit a differential expression pattern through different developmental stages of safflower (Fig. 5A). The developmental stages include both the vegetative stages as well as the reproductive stages. Some *CtF3'H* genes (*CtF3'H5,20,32*) seem to be expressed at all stages with strong expression specified in shoot tissues. However, some of the *CtF3'H* genes including (*CtF3'H27, 1, 2, 13*) were abundantly expressed in the root tissue/organs and also through different growth stages of the seeds. Furthermore, the expression level of *CtF3'H33, 20, 37, 28, 34* showed highest expression in the intermediate seed maturation stage, besides (*CtF3'H23, 24, 33*). Specifically, the abundance of *CtF3'H22* appears only in the seeds development stages. In addition, to confirm the expression level of these putative *CtF3'H* genes and their correlation the during flower petals development experimentally, we conducted the qRT-PCR analysis of 22 putative *CtF3'H* genes previously obtained from safflower genome using four different flowering stages (Fig. 5B). The highest expression level was observed for *CtF3'H23* at the fading stage of flower development. The relative expression level of *CtF3'H5* was uniformly detected during all four stages of flowering. The transcript abundance of almost all putative *CtF3'H* genes demonstrated a differential pattern of transcription with slight differences e.g. highest expression in the flowering period, and descending in the initial, bud, and fading stages. Our qRT-PCR results showed that the fold change values of the *CtF3'H* genes were in accordance with the prediction of the digital expression presented earlier. The expression assay of *CtF3'H* genes during flower development in safflower suggested a variable transcription regulation pattern highlighting their decisive roles during petal development in safflower.

Cloning and Subcellular Localization of CtF3'H5

The coding region of *CtF3'H5* target gene encoding 455 amino acids was cloned from safflower using Taq polymerase enzyme and the result was confirmed on 1% agarose gel electrophoresis and sanger sequencing. The sequencing results show that the obtained sequence is consistent with the expected result of the *CtF3'H5* gene, indicating that the gene has been successfully cloned. (Fig. 6A). The protein sequence of *CtF3'H5* was subjected to multiple amino acid sequence alignments with various other F3'H proteins obtained from 14 different species including callistephus chinensis, camellia sinensis, dahlia pinnata and centaurea cyanus etc (Fig. 6B). It was found that the amino acid sequence of *CtF3'H5*

contained the common conserved "GGEK" sequence at positions 434 to 437, which is the specific to F3'H gene. The next conserved sequence "LPPGP" starting at position 39 which is the main conserved domain of cytochrome P450 was also found confirming the authenticity of P450 system. The 3D structure prediction of CtF3'H5 target protein was performed using the SWISS-MODEL online software (Fig. 6C) (Lu et al. 2018) by translating the genomic sequence of *CtF3'H5* into amino with the help of Expasy ProtParam 4.0 online tool. The theoretical molecular weight was observed as 50.03 kD with the average total hydrophobic value – 0,035 suggesting that *CtF3'H5* is a hydrophilic protein.

In addition, the subcellular localization prediction for *CtF3'H5* was carried out by exploiting the online webserver of ProtComp version 9.0, indicated the predominant signals to the plasma membrane. However, to confirm the experimental status of the subcellular localization of our target CtF3'H5 protein, we therefore, investigated the transient expression system using the fusion vector of pCambia1302 and *CtF3'H5* constructing pCambia1302-*CtF3'H5*-GFP vector. The aforesaid recombinant vector was transformed into onion epidermal cells by agrobacterium mediated infection system under controlled conditions. Our findings marginally demonstrated that the GFP signals were largely appeared within the plasma membrane section as compare to other parts of the cell (Figs. 7D-F). However, on the other hand, the GFP signals in the control vector indicated a dispersed pattern throughout the cell (Fig. 7A-C).

Prokaryotic expression and Western Blot analysis of CtF3'H5

The quantitative realtime expression analysis of *CtF3'H5* was investigated in different flowering stages of safflower as shown in (Fig. 8A). The results showed that *CtF3'H5* exhibits highest expression at the flowering stage of safflower followed by the initial stage. However, the remaining stages of flowering demonstrated lower expression level. These findings suggested the correlation of *CtF3'H5* with red and yellow color connotation in the safflower petals which is morphologically appeared during the flowering and initial stages. In the next phase, we cloned the full length *CtF3'H5* gene and ligated the final product into the expression cassette of pET28a⁺ prokaryotic expression vector. The construction of the recombinant vector (pET28a⁺-*CtF3'H5*) was confirmed with double restriction digestion system (Fig. 8B&C) and further accuracy was confirmed with sequencing. In the follow up experiments, the heterologously expressed CtF3'H5 protein was induced by treating the recombinant bacterial cells of BL21 strain carrying the (pET28a⁺-*CtF3'H5*) vector with different concentrations of IPTG for different time periods. After the induction, the target protein product was confirmed on coomassie blue staining SDS-PAGE which resulted in the expected band size of 50.3 Kda however, it was observed that the use of different IPTG concentrations did not influence the expression level of the protein in bacterial system (Fig. 8D). Similarly, the denaturation of the heterologously expressed CtF3'H5 protein at different IPTG concentrations was performed using western blot hybridization method. As described (Fig. 8E), we observed a unique band of the same size (50.3 Kda), however, a variable expression level was found at different IPTG concentration. These results stringly suggested that the target CtF3'H5 protein is stably expressed in the bacterial system but its expression level could be marginally influenced when induced with different concentrations of IPTG.

Discussion

The flavonoid metabolism is one of the important secondary metabolic pathways in higher plants (Dermauw et al. 2020). In plants, F3'H plays an important role in determining flower color, seed pods and the color of stems and leaves. The ratio of F3'H to F3'5'H determines the color level of grapes (D and D 2011). In tea tree, catechin, a metabolite in the flavonoid pathway, may help the body improve its ability to fight cancer, antioxidants, mutants, tumors and cardiovascular disease. Flavonoid 3'-hydroxylase is a key enzyme in the flavonoid metabolism pathway, which can catalyze the hydroxylation of position 3 of the B-ring of naringenin and dihydro kaempferol. Naringenin and dihydro kaempferol are oxidized to form a series of important intermediates in the flavonoid pathway. The structural stability and antioxidant function of these intermediates are closely related to the F3'H enzyme (Wei et al. 2015) identified naringenin as the optimal substrate for CsF3'H in enzyme activity experiments (Lv et al. 2017; Wang et al. 2014). In this study, we presented a brief overview of the genome wide identification and several structural and functional characteristics of CtF3'H subgene family in safflower. We have combined 110 Arabidopsis P450 genes and 22 identified *CtF3'H* genes from the safflower genome to study their phylogenetic relationship resulted in two main classes A-type and non-A type (Fig. 1). Further classifications were grouped into nine clans of the phylogenetic tree, which was found consistent with the previous studies of the plant P450 family (Barvkar et al. 2012). Based on the evolutionary pattern, the *CtF3'H5* gene family in safflower was recognized in the CYP clan 75,76,78 respectively. In previous studies on the F3'H monocot plants, they were also clearly classified with two separate states. CYP78 is one of the P450 families involved in flavonoid biosynthesis.

Flavonoid biosynthesis are tightly regulated by different spatial and time signals that can limit the accumulation of these compounds in plants (Musiol-Kroll et al. 2019). A number of studies have demonstrated the role of F3'H gene in regulating plant metabolism and changes in flavonoid composition in the color of flower petals (Ueyama et al. 2002). Hence, the transcription regulation of F3'H gene may lead to significant amount of understanding of the flavonoid pathway in plants. For this purpose, we extensively conducted the expression analysis of all identified *CtF3'H* genes identified in the safflower genome at different flowering stage (Fig. 5). Our findings confirmed significant differences during the transcription of *CtF3'H* genes at different flowering stages in safflower reaching to its peak at flowering stage. Initially when the expression pattern was downregulated at bud stages where the pigment content is lower. However, the assumption was established on the basis of initial flowering and flowering stages, which is highest because the color of the petals appeared as red and yellow. Nonetheless, the expression level of few *CtF3'H* genes was not significant and quite faint including *CtF3'H1*, *CtF3'H2* suggesting the synergetic effect of *CtF3'H* mutation which limit some steps in flavonoid biosynthesis (Y et al. 2019). The results of the *CtF3'H5* transcript showed a uniformly expression scheme in all four stages and the highest in the flowering period, this result was also found consistent with the digital expression prediction in the heatmap (Fig. 5).

We further characterized a putative *CtF3'H5* by cloning the full length cDNA sequence of this gene and performed multiple functional analyses to confirm its integrity with respect to flower pigmentation in

safflower (Fig. 6). A variety of conserved amino acid sequences suggested that *CtF3'H5* is highly conserved as in other plant species (M et al. 2014). The subcellular localization study is probably one of the essential links to estimate the final function of a target protein (Naqvi et al. 2016). Hence, we experimentally investigated the localization of the CtF3'H5 target protein using the onion epidermal cells, through *Agrobacterium* mediated transformation by transiently expressing the fusion construct of pCAMBIA1302-*CtF3'H5* containing the GFP cassettes (Fig. 7). The result suggested that the target protein of *CtF3'H5* was detected in the plasma membrane of the onion cell epithelial cells, disagreeing with the previous result of (Chen et al. 2017), who reported the F3'H gene on *Brassica napus* was localized into endoplasmic reticulum. This differences may occur due to the chromosomal position of the gene which is varies in safflower as compare to *Brassica napus*, however, the function of this protein was generally ascribed to flavonoid biosynthesis.

The enzymatic activity of Cytochrome P450 system exhibits a unique oxidative and reductive pattern. Nevertheless, to comprehend the vast substrate specificity of complex P450 proteins, the establishment of stable and cost effective expression system is yet a challenging task. The benefit of bacterial P450 expression systems has been covered by (Zelasko et al. 2013) presenting a fast and accurate expression system. In this study, we demonstrated a competent and stable expression system to produce a heterologously expressed CtF3'H5 protein by exploiting the prokaryotic expression system of bacteria (Fig. 8D). Our results identified an expected target protein of *CtF3'H5* expressin by a competent strain of bacteria (BI21) comprising a 50.3 kDa size of band size in the SDS PAGE analysis. The level of CtF3'H5 target protein induction with IPTG indicated a stable production of the target protein however, with the use of different IPTG concentrations could possibly affect the expression of our target protein (Wei and Chen 2018), as described in our western blot hybridization analysis (Fig. 8E). Our findings from these analyses provide the foundation for screening highly expressive putative F3'H genes and identifying their related functions in plants which will be helpful to explain the underline rationale of this exceptionally important class of gene family.

Declarations

Author's contributions

Experimental work and data analysis was conducted by NH, KJ and NA. Software and formal analysis were carried out by YZ, MX and ZX. Statistical analysis was performed by LX,WN, LJ and JL. The project was designed and supervised by LX, YN. The manuscript draft was prepared by NA. All authors read and approved the final manuscript.

Acknowledgments

This work was funded by grants from the National Natural Science Foundation of China (No. 31771868, 31501366), the Science and Technology Development Project of Jilin Province (No. 20190201172JC, 20190201175JC, 20150623024TC-11), Student innovation and entrepreneurship training program of Jilin Agricultural University.

Conflicts of Interest

The authors declare no conflict of interest

References

- Alaiye A, Kaya E, Pnarbal MZ, Harmanc N & Cingi C (2020) An Experimental Comparison of the Analgesic and Anti-Inflammatory Effects of Safflower Oil, Benzydamine HCl, and Naproxen Sodium. *Journal of Medicinal Food* 23(8)
- Ambreen H, Kumar S, Kumar A, Agarwal M, Jagannath A & Goel S (2018) *Carthamus tinctorius* Association Mapping for Important Agronomic Traits in Safflower (L.) Core Collection Using Microsatellite Markers. *Frontiers in plant science* 9:402 doi:10.3389/fpls.2018.00402
- Barvkar VT, Pardeshi VC, Kale SM, Kadoo NY & Gupta VS (2012) Phylogenomic analysis of UDP glycosyltransferase 1 multigene family in *Linum usitatissimum* identified genes with varied expression patterns. *Bmc Genomics* 13(1):175-175
- Cerqueira A, Santana I, Araújo J, Lima H, Batatinha M, Branco A, Santos Junior M & Botura M (2021) in vitro Homology modeling, docking, molecular dynamics and studies to identify acetylcholinesterase inhibitors. *Journal of biomolecular structure & dynamics*:1-11 doi:10.1080/07391102.2021.1889666
- Chen K, Liu H, Lou Q & Liu Y (2017) *Muscari armeniacum* Ectopic Expression of the Grape Hyacinth () R2R3-MYB Transcription Factor Gene, , Induces Anthocyanin Accumulation in Tobacco. *Frontiers in plant science* 8:965 doi:10.3389/fpls.2017.00965
- Cho H, Kim O, Lee Y, Kang L, Nguyen C, Ishihara A & Kim H (2017) Feruloylserotonin inhibits hydrogen peroxide-induced melanogenesis and apoptosis in B16F10 and SK-Mel-2 melanoma cells. *Biochemical and biophysical research communications* 491(4):973-979 doi:10.1016/j.bbrc.2017.07.158
- Choudhary D, Pandey A, Adhikary S, Ahmad N & Trivedi R (2016) Genetically engineered flavonol enriched tomato fruit modulates chondrogenesis to increase bone length in growing animals. *Scientific Reports* 6:21668
- D N & D W-R (2011) A P450-centric view of plant evolution. *The Plant journal : for cell and molecular biology* 66(1):194-211 doi:10.1111/j.1365-313X.2011.04529.x
- De Palma M, Fratianni F, Nazzaro F & Tucci M (2014) Isolation and functional characterization of a novel gene coding for flavonoid 3'-hydroxylase from globe artichoke. *Biologia Plantarum* 58(3):445-455
- Dermauw W, Van Leeuwen T & Feyereisen R (2020) Diversity and evolution of the P450 family in arthropods. *Insect biochemistry and molecular biology* 127:103490 doi:10.1016/j.ibmb.2020.103490

- Du C, Hou J, Wang C, Zhang M, Zheng Y, Yang G & Hu Y (2021) Effects of safflower yellow on cholesterol levels in serum and brain tissue of APP/PS1 mice. *Metabolic brain disease* 36(4):557-569 doi:10.1007/s11011-021-00680-0
- Gao S, Xu X, Zeng W, Xu S, Lyv Y, Feng Y, Kai G, Zhou J & Chen J (2020) SEfficient Biosynthesis of (2)-Eriodictyol from (2)-Naringenin in through a Combination of Promoter Adjustment and Directed Evolution. *ACS synthetic biology* 9(12):3288-3297 doi:10.1021/acssynbio.0c00346
- Guo L, Gao L, Ma X, Guo F, Ruan H, Bao Y, Xia T & Wang Y (2019) Functional analysis of flavonoid 3'-hydroxylase and flavonoid 3',5'-hydroxylases from tea plant (*Camellia sinensis*), involved in the B-ring hydroxylation of flavonoids. *Gene* 717:144046 doi:10.1016/j.gene.2019.144046
- Lin K, Qin Z, Qu C, Chen X, Jiang Q, Li M, Zheng Q & Li D (2021) Hydroxyl safflower yellow B combined with doxorubicin inhibits the proliferation of human breast cancer MCF-7 cells. *Oncology letters* 21(5):426 doi:10.3892/ol.2021.12687
- Lu R, Yuan T, Wang Y, Zhang T, Yuan Y, Wu D, Zhou M, He Z, Lu Y, Chen Y, Fan J, Liang J & Cheng Y (2018) Spontaneous severe hypercholesterolemia and atherosclerosis lesions in rabbits with deficiency of low-density lipoprotein receptor (LDLR) on exon 7. *EBioMedicine* 36:29-38 doi:10.1016/j.ebiom.2018.09.020
- Lv GY, Guo XG, Xie LP, Xie CG, Zhang XH, Yuan Y, Lei X, Tang YY, Pan XL & Guo AG (2017) Molecular Characterization, Gene Evolution, and Expression Analysis of the Fructose-1, 6-bisphosphate Aldolase (FBA) Gene Family in Wheat (*Triticum aestivum* L.). *Frontiers in Plant Science* 8
- M B, S B, A W, K A, G S, T S, F K, T GC, M B, L B & T S (2014) SWISS-MODEL: modelling protein tertiary and quaternary structure using evolutionary information. *Nucleic acids research* 42:W252-8 doi:10.1093/nar/gku340
- Min F, Sun H, Wang B, Ahmad N & Li H (2020) Hepatoprotective effects of hydroxysafflor yellow A in D-galactose-treated aging mice. *European Journal of Pharmacology*:173214
- Musiol-Kroll EM, Tocchetti A, Sosio M & Stegmann E (2019) Challenges and advances in genetic manipulation of filamentous actinomycetes – the remarkable producers of specialized metabolites. *Natural Product Reports* 36(12)
- Nagamatsu A, Masuta C, Matsuura H, Kitamura K, Abe J & Kanazawa A (2009) Down-regulation of flavonoid 3'-hydroxylase gene expression by virus-induced gene silencing in soybean reveals the presence of a threshold mRNA level associated with pigmentation in pubescence. *Journal of Plant Physiology* 166(1):0-39
- Naqvi AAT, Anjum F, Khan FI, Islam A & Hassan MI (2016) Sequence Analysis of Hypothetical Proteins from *Helicobacter pylori* 26695 to Identify Potential Virulence Factors. *Genomics Informatics* 14(3)
- Panche, Diwan & Chandra (2016) Flavonoids: an overview. *Journal of Nutritional Science*

- Ren T, Zheng P, Zhang K, Liao J, Xiong F, Shen Q, Ma Y, Fang W & Zhu X (2021) Effects of GABA on the polyphenol accumulation and antioxidant activities in tea plants (*Camellia sinensis* L.) under heat-stress conditions. *Plant physiology and biochemistry* : PPB 159:363-371 doi:10.1016/j.plaphy.2021.01.003
- Schrago C, Aguiar B & Mello B (2018) Comparative evaluation of maximum parsimony and Bayesian phylogenetic reconstruction using empirical morphological data. *Journal of evolutionary biology* 31(10):1477-1484 doi:10.1111/jeb.13344
- Ueyama Y, Suzuki KI, Fukuchi-Mizutani M, Fukui Y & Tanaka Y (2002) Molecular and biochemical characterization of torenia flavonoid 3'-hydroxylase and flavone synthase II and modification of flower color by modulating the expression of these genes. *Plant Science* 163(2):253-263
- Wang X, Xu Y, Chu C, Li H, Mi J, Wen Z, Zhang S, Wang Q & Quan S (2019) Effect of safflower yellow on early type II diabetic nephropathy: a systematic review and meta-analysis of randomized controlled trials. *Journal of Pediatric Endocrinology & Metabolism Jpem* 32(7):653
- Wang Y-S, Xu Y-J, Gao L-P, Yu O, Wang X-Z, He X-J, Jiang X-L, Liu Y-J & Xia T (2014) Functional analysis of Flavonoid 3',5'-hydroxylase from Tea plant (*Camellia sinensis*): critical role in the accumulation of catechins. *Bmc Plant Biology* 14(1):347
- Wei K & Chen H (2018) Global identification, structural analysis and expression characterization of cytochrome P450 monooxygenase superfamily in rice. *BMC genomics* 19(1):35
- Wei K, Wang L, Zhang C, Wu L, Li H, Zhang F & Cheng H (2015) Transcriptome Analysis Reveals Key Flavonoid 3'-Hydroxylase and Flavonoid 3',5'-Hydroxylase Genes in Affecting the Ratio of Dihydroxylated to Trihydroxylated Catechins in *Camellia sinensis*. *PloS one* 10(9):e0137925 doi:10.1371/journal.pone.0137925
- Y H, S V, RE S-G, S R-M, D Z, AV L & SS K (2010) Ectopic expression of apple F3'H genes contributes to anthocyanin accumulation in the *Arabidopsis* tt7 mutant grown under nitrogen stress. *Plant physiology* 153(2):806-20 doi:10.1104/pp.109.152801
- Y J, B L, Y Z, X Z, Y X & C L (2019) Evolutionary dynamic analyses on monocot flavonoid 3'-hydroxylase gene family reveal evidence of plant-environment interaction. *BMC plant biology* 19(1):347 doi:10.1186/s12870-019-1947-z
- Zelasko S, Palaria A & Das A (2013) Optimizations to achieve high-level expression of cytochrome P450 proteins using *Escherichia coli* expression systems. *Protein expression and purification* 92(1):77-87

Figures

Phylogenetic Analysis of 22 CtF3'H and 110 CYP450 from Arabidopsis Thaliana Phylogenetic tree is constructed by using MEGA-X with 1000 bootstrap replicates: Different lineages are represented by different colors. The abbreviations used for different plant protein chains are as follows: At - Arabidopsis thaliana) ● (Ct - Carthamus tinctorius)

Figure 2

The conserved protein motifs in CtF3'H protein obtained from safflower genome. The online webserver of MEME was exploited to identify the conserved patterns of CtF3'H genes.

Figure 3

The analysis of multiple sequence alignment of CtF3'H proteins in safflower . The color boxes indicate 70 and 50% of the amino acid identity.

Figure 4

The graphical illustrations of key cis-regulatory units found in the promoter region of CtF3'H genes from safflower.

Figure 5

(A) Heat map representing the digital expression profile CtF3'H genes. The map shows the development time and expression of CtF3'H in each development stage of Safflower. (B) Expression profiles of 22 putative CtF3'H genes in different flowering stages of safflower were determined by qRT-PCR analysis. Different background colors represent different periods of the safflower CtF3'H gene. Expression analysis

Figure 7

The GFP fluorescence pattern of CtF3'H5 under SEM. (A–C) The induction of empty vector 35S:GFP in onion cells. (D–F) The transient expression of pCAMBIA-CtF3'H5–GFP vector using the onion epidermal cells. Scar bar = 75 µm.

Figure 8

Expression analysis, heterologous expression and western blot hybridization of CtF3'H5 target protein from safflower (A) The realtime quantitative expression level of CtF3'H5 in four different flower developmental stages in safflower (B-C) Cloning and confirmation of PET28a+ CtF3'H5 recombinant plasmid using double restriction digestion system. (D) SDS-PAGE analysis of heterologously expressed CtF3'H5 protein under various concentration of 4 IPTG (E) Western Blot analysis of CtF3'H5 protein. The different concentrations of IPTG used in this experiment were as follow: 0.2 IPTG; 0.4 IPTG, 0.5 IPTG, 0.6 IPTG, 0.7 IPTG, 0.8 IPTG.

Supplementary Files

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

- [Supplementarymaterial.docx](#)