TÜBİTAK

Turkish Journal of Botany

http://journals.tubitak.gov.tr/botany/

Research Article

Turk J Bot
(2019) 43: 143-150
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doi:10.3906/bot-1809-25

Cloning and expression analysis of a new chalcone isomerase gene during flowering in safflower

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Received: 16.09.2018 • **Accepted/Published Online:** 17.12.2018 • **Final Version:** 07.03.2019

Abstract: Flowers of safflower (*Carthamus tinctorius* L.) have been widely used in traditional Chinese medicine because of their ability to improve cerebral blood flow. The major active ingredients in safflower are flavonoids. To date, few flavonoid biosynthesis genes have been cloned from this species. In our previous research, four isoform chalcone isomerase genes were screened. In this study, a new chalcone isomerase gene (designated as *CtCHI-N*) was cloned from a safflower cDNA library. A 696-bp cDNA for *CtCHI-N* was cloned, and its sequence was subsequently analyzed. The recombinant protein was successfully expressed in a prokaryotic expression system, and the expression of *CtCHI-N* in different tissues and at different stages of flowering was investigated. The results showed that *CtCHI-N* was expressed in a tissue-specific manner, and the greatest expression level occurred during early flowering. In addition, flavonoids at different stages of flowering were also measured, and the expression of *CtCHI-N* was found to correlate with flavonoid biosynthesis during flowering. *CtCHI-N* was successfully cloned in the experiment, and expression analysis revealed that *CtCHI-N* might influence flavonoid biosynthesis during flowering in safflower.

Key words: Safflower, chalcone isomerase, gene cloning, sequence analysis, flavonoid biosynthesis

1. Introduction

Safflower (*Carthamus tinctorius* L.) is a member of the Compositae or Asteraceae plant family and is an important economic crop in the world. Safflower seeds are rich in unsaturated fatty acids and the flowers are rich in flavonoids, both of which are very beneficial to human health. Safflower is grown worldwide, mainly for oil and pharmaceutical purposes.

Flavonoids are ubiquitous secondary metabolites that have various functions in plant physiology and ecology (Mol et al., 1998; Tian et al., 2008). Pharmacological studies have shown that flavonoids in safflower have several pharmacological effects, including cardioprotective (Jin et al., 2008), neuroprotective (Fan et al., 2009), and vasodilative effects, and exhibit antihypertensive (Asgarpanah and Kazemivash, 2013), antioxidative (Fan et al., 2014), antifibrotic (Li et al., 2015), anticoagulation, antithrombotic (Hong et al., 2015), and antiaging (Han et al., 2015) properties. Hydroxysafflor yellow A (HSYA) is the most widely studied chemical in safflower. HSYA can antagonize the binding of platelet-activating factor to its receptor (Zang et al., 2002), and HSYA is an effective treatment for focal cerebral ischemia (Wei et al., 2005; Zhu et al., 2005).

The main pathway of flavonoid biosynthesis is well understood, especially in Arabidopsis (Winkel-Shirley, 2002; Du et al., 2010). At the beginning of flavonoid biosynthesis, one molecule of 4-coumaroyl-CoA and three molecules of malonyl-CoA are combined into 4,2',4',6'-tetrahydroxychalcone by chalcone synthase (CHS, EC 2.3.1.74), which is the first committed enzyme in the pathway. This 4,2',4',6'-tetrahydroxychalcone intermediate is required for the biosynthesis of both quinochalcones and flavonoids; it can be hydroxylated or glycosylated, leading to the production of carthamone and HSYA. Chalcone isomerase (CHI, EC 5.5.1.6) subsequently converts 4,2',4',6'-tetrahydroxychalcone into naringenin. In one branch of the biosynthesis pathway in safflower, naringenin can be hydroxylated at the 6 position and glycosylated at the 5 position to produce 5,6,7,4'-tetrahydroxyflavanone-5-O-β-D-glucoside (neocarthamin), or it can be hydroxylated and glycosylated at the 6 and 7 positions to produce 5,6,7,4'-tetrahydroxyflavanone-6,7-di-O-β-D-glucoside. Additionally, naringenin can be converted

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by flavanone 3-hydroxylase (F3H, EC 1.14.11.9) into dihydrokaempferol, which can subsequently be converted into kaempferol; kaempferol can then be converted to quercetin (Huang et al., 2012). However, the molecular mechanisms of flavonoid biosynthesis in safflower have not yet been defined.

Flavonoids are major active ingredients in safflower. To date, for many other species, such as *Vitis vinifera* (Goto-Yamamoto et al., 2002) and *Chamaemelum nobile* (Wang et al., 2018), the isoform chalcone isomerase genes have been cloned and analyzed. However, few flavonoid biosynthesis genes have been cloned from safflower. In our previous research (Chen et al., 2018), four isoform chalcone isomerase genes were screened by third-generation sequencing platforms (PacBio RS II). In this investigation, we cloned a new chalcone isomerase gene (designated as *CtCHI-N*). Its sequence and the relationship between flavonoid biosynthesis and the expression of *CtCHI-N* in safflower were analyzed. Our results lay a foundation for further studies of the molecular mechanism of flavonoid biosynthesis in safflower.

2. Materials and methods

2.1. Plant material

Safflower plants, which were provided by the Sichuan Academy of Agricultural Sciences, were planted in the medical botanical garden of Wenjiang campus, Chengdu University of Traditional Chinese Medicine. The leaves, stems, roots, and flowers were subsequently collected. The total RNA from each sample was mixed for cDNA library construction. The flowers from the first day to the seventh day of flowering were also collected to analyze expression patterns and measure flavonoid contents. All samples were collected from the garden, immediately frozen in liquid nitrogen, and then stored at –80 °C.

2.2. Cloning *CtCHI-N*

A mixed cDNA library that included cDNA from leaves, stems, roots, and flowers was constructed. The total RNA was first isolated by TRIzol (Tiangen, China), after which first-strand cDNA was synthesized from the total RNA via a cDNA reverse transcription kit (Tiangen, China). The initial sequence of *CtCHI-N* was based on that from a previous report (Huang et al., 2012), and the sequence was analyzed by a BLAST query of the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The specific primer pair (*CtCHI-NF* and *CtCHI-NR*) was designed with Primer Premier 5.0 (Table 1). PCR was performed as follows: initial denaturation at 95 °C for 4 min; 34 cycles of 95 °C for 30 s, 56 °C for 30 s, and 72 °C for 2 min; and a final extension at 72 °C for 8 min. The PCR products were cloned into a *pMD19-T* vector (Tiangen, China) and sequenced by Tsingke (Chengdu, China).

2.3. Bioinformatics analysis of the sequence of *CtCHI-N*

The open reading frame (ORF) and the amino acid sequence were identified via ORF Finder (https://www.ncbi.nlm.nih.gov/orffinder/), and a phylogenetic tree was constructed via MEGA 5.0 software. The structure of CtCHI-N was deduced with online software (http://www.expasy.org/, http://bioinf.cs.uc.ac.uk/psipred, and http://swissmodel.expasy.org/interactive), and a web tool (http://psort.hgc.jp/) was used to predict the location of the proteins within the cells.

2.4. Expression analysis by real-time RT-PCR

The expression of *CtCHI-N* in different tissues and at different flower stages was determined via real-time RT-PCR. SYBR Green PCR Master Mix (Toyobo, Osaka, Japan) was used, and *Ct60S* (60S acidic ribosomal protein gene) served as the reference gene (Liu et al., 2016).

The gene-specific primers (*CHIF* and *CHIR*) and reference primers (*Ct60SF* and *Ct60SR*) used for real-time RT-PCR are listed in Table 1. Cycling involved an initial denaturation step at 95 °C for 30 s followed by 40 cycles of 95 °C for 5 s and 54 °C for 30 s. Each sample was repeated three times, and all reactions were performed on a Bio-Rad CFX manager system. The specificity of the PCR was verified by agarose gel electrophoresis.

2.5. Prokaryotic expression of recombinant proteins

pET-32 (Takara, Japan) was used for prokaryotic expression. On the basis of the ORF of the *CtCHI-N* gene, gene-specific primers were designed in accordance with restriction enzyme sites (*Hind*III and *Eco*RI); the primer sequences of *CHIRF* and *CHIRR* are listed in Table 1. *CtCHI-N* was cloned into *pET32*, which had same restriction enzyme sites using Solution I (Takara, Japan) at 16 °C for 3 h, after which the *pET32-CtCHI-N* recombinant plasmid was introduced into a *Rosetta* strain of *E. coli* (Takara, Japan) via the heat-shock method. A single colony of *Rosetta* cells harboring the expression plasmid was inoculated onto 37 °C LB medium that contained ampicillin (50 mg L^{-1}), and the cells were subsequently incubated with shaking (180 rpm) at 37 °C. When the optical density at 600 nm (OD 600) reached approximately 0.5, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added, after which the cells were further cultured at 25 °C for 4 h. Afterward, the cells were lysed in boiling water for 10 min and then centrifuged at 12,000 rpm for 10 min. The resulting supernatants and pellets were analyzed by SDS-PAGE.

2.6. Determination of flavonoid contents

The flavonoids (HSYA, quercetin, naringenin, and kaempferol) in the safflower samples were measured. To determine the HSYA content, 0.4 g of powdered flower tissue (collected during the first to seventh days of flowering) was dissolved in 50 mL of 25% methanol and then reacted by ultrasonic extraction for 40 min.

Table 1. *CtCHI-N* primers used in this study.

Primer	Sequence
CtCHI-NF	CCCCTCTCCACTGCATACTC
CtCHI-NR	TTTTAATACACATTGCGAAGGATGA
CHIF	AGCCTTTATCTTTGCTTGGACA
CHIR	TCATCTTCGGCTAACTCGGT
Ct60SF	CATCCATTATCCAACAATC
Ct60SR	AAGAGTAATCAGTCTCCA
CHIRF	CCGGAATTCATGGGATCAGAAATGGTG (*Eco*RI)
CHIRR	CCCAAGCTTTTATTTAGACAACTTCAA (*Hind*III)

The samples were then suitably diluted with methanol, filtered through a 0.45-μm filter membrane and analyzed by HPLC (Agilent 1200, USA). The HSYA content was analyzed with an Agilent C18 chromatographic column (4.6 mm × 250 mm, 5 μm) in conjunction with a mobile phase that consisted of methanol, acetonitrile, and 0.7% (v/v) phosphoric acid (26:2:72) for elution. The flow rate was 0.8 mL min^{-1}, the injection volume was 10 μL, and the UV detector was set such that λ = 407 nm.

To determine the quercetin, naringenin, and kaempferol contents, 1.0 g of powdered flower tissue was dissolved in 25 mL of methanol and then reacted by heat reflux extraction at 95 °C for 30 min followed by hydrochloric acid hydrolysis for 30 min. The sample solutions were suitably diluted with methanol, filtered through a 0.45 μm filter membrane, and analyzed by HPLC. The quercetin, naringenin, and kaempferol contents were analyzed via an Agilent C18 chromatographic column in conjunction with a mobile phase consisting of methanol and 0.4% (v/v) phosphoric acid for elution. The flow rate was 1.0 mL min^{-1}, the injection volume was 20 μL, and the UV detector was set such that λ = 360 nm and 254 nm. SPSS 21.0 was used to analyze the correlation between average content of flavonoids and *CtCHI-N* expression at different flowering stages.

3. Results

3.1. *CtCHI-N* cloning and sequence analysis

A 696-bp cDNA encoding *CtCHI-N* was cloned (Figure 1a); sequence analysis revealed that this cDNA contained a 633-bp ORF that encoded a protein of 210 amino acids in length. A BLAST query of the NCBI database revealed that *CtCHI-N* belongs to the chalcone superfamily. A phylogenetic tree was subsequently constructed, which is shown in Figure 1b. We also made a comparison between *CtCHI-N* and the other four isoforms (*CtCHI1*, *CtCHI2*, *CtCHI3*, and *CtCHI4*) reported in a previous study (Chen et al., 2018). Evolutionary tree analysis showed that *CtCHI-N* was a new chalcone isomerase gene (Figure 1c).

The molecular formula of CtCHI-N was determined to be $C_{1057}H_{1656}N_{256}O_{331}S_4$; the molecular weight was 23374.5 and the isoelectric point was 4.76. The hydrophobicity of CtCHI-N was predicted by Protscale: the highest hydrophobic peak was 1.367 and the highest hydrophilic peak was –3.300. The gradient average of hydropathicity (GRAVY) was –0.203; thus, we deduced that CtCHI-N was a hydrophilic protein. The predicted signal peptide cleavage site analysis yielded C, S, and Y scores of 0.110, 0.130, and 0.105, respectively. Because all these scores were close to 0.1, we deduced that CtCHI-N was a nonsecretory protein. In addition, subcellular localization prediction revealed that CtCHI-N had a 56.5% probability of being located in the cytoplasm and a 21.7% probability of being localized in the nucleus. CtCHI-N had 73 α-helices, 53 β-sheets, and 84 random coils and lacked a transmembrane helical structure (Figure 2a). The predicted tertiary structure of CtCHI-N is shown in Figure 2b.

3.2. Expression of *CtCHI-N* in different tissues and at different flowering stages

The expression of *CtCHI-N* in different tissues and at different flowering stages was measured. *CtCHI-N* showed tissue-specific expression mainly in the flowers and was expressed very little in the stems (Figure 3a). During flowering, the expression of *CtCHI-N* gradually increased from the first day to the fourth day but then decreased sharply from the fourth day to the seventh day. The greatest expression of *CtCHI-N* was observed on the fourth day of flowering (Figure 3b).

3.3. Prokaryotic expression of *CtCHI-N*

pET-32 was used for the prokaryotic expression of CtCHI-N. *CtCHI-N* was first cloned into *pMD19-T*. After digestion with *Eco*RI and *Hind*III, *CtCHI-N* was cloned into *pET-32*. The recombinant vector was subsequently confirmed by gel electrophoresis (Figure 4a). The strain containing *pET-32-CtCHI-N* was ultimately induced by IPTG for protein expression, and CtCHI-N was successfully expressed (Figure 4b).

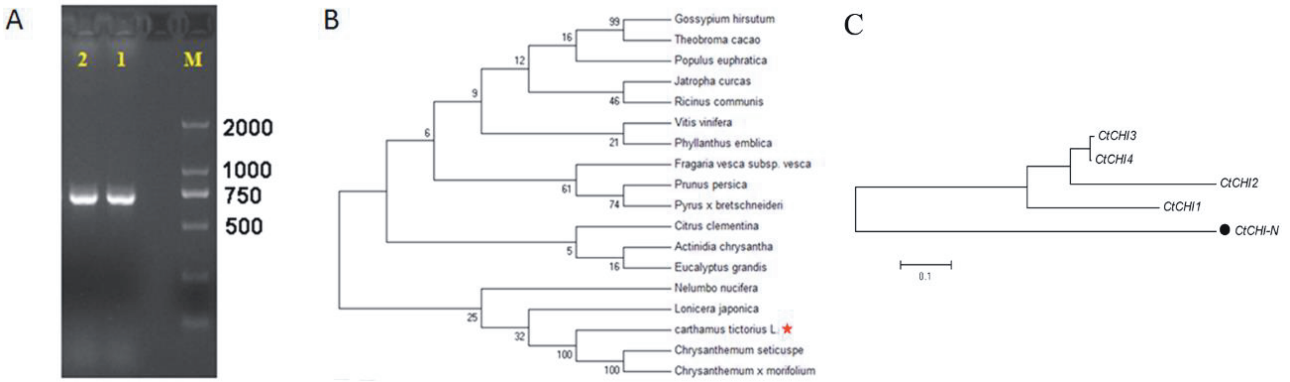

Figure 1. *CtCHI-N* cloning results and phylogenetic tree: a) *CtCHI-N* cloning, lines 1 and 2 are two replicates, and M represents a 2000-bp DNA marker; b) CHI phylogenetic tree of safflower and related species, safflower indicated with a red star; c) evolutionary analysis of the chalcone isomerase genes.

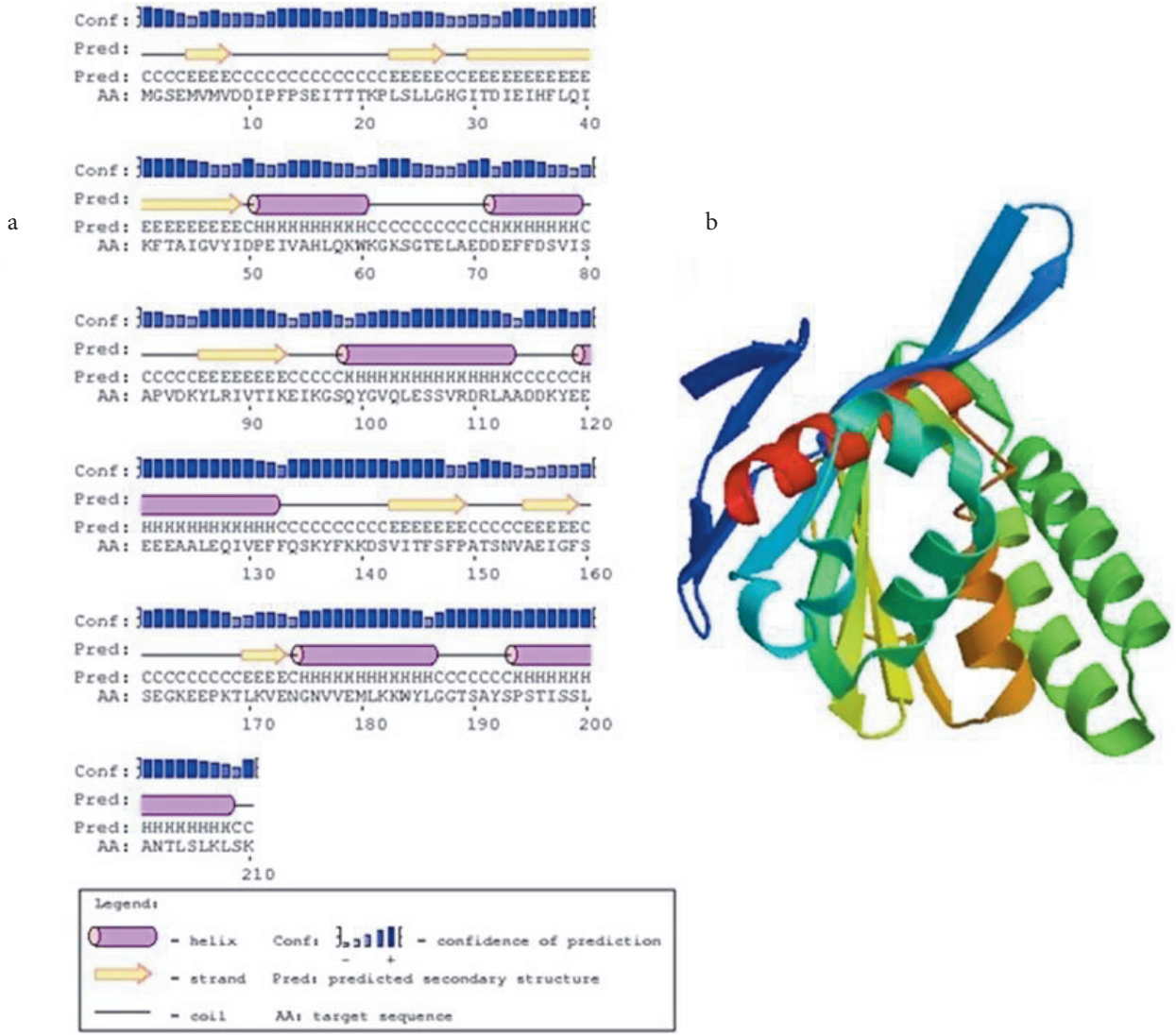

Figure 2. Protein structure prediction of CtCHI-N: a) secondary structure prediction of CtCHI-N; b) tertiary structure prediction of CtCHI-N.

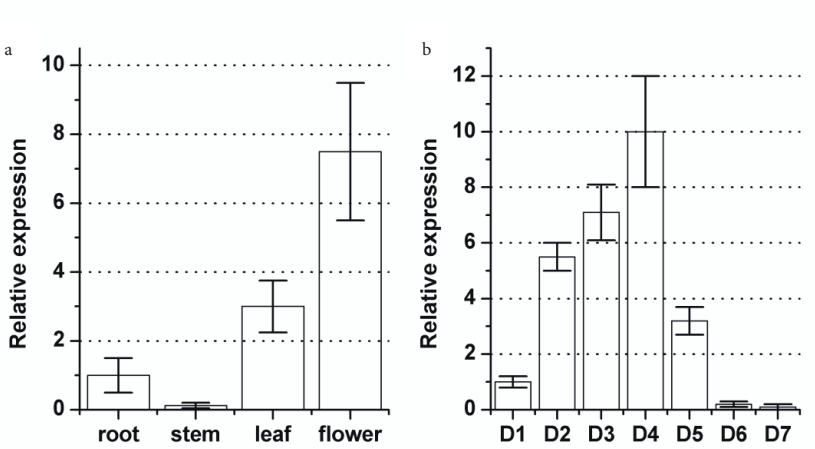

Figure 3. Expression of *CtCHI-N* in different tissues and stages of flowering: a) relative expression of *CtCHI-N* in different tissues (root, stem, leaf, flower); b) relative expression of *CtCHI-N* during different stages of flowering. D1–D7 represent flowers from the first to the seventh day of flowering. The bars are standard deviations of *CtCHI-N* relative expression in different tissues and stages, respectively.

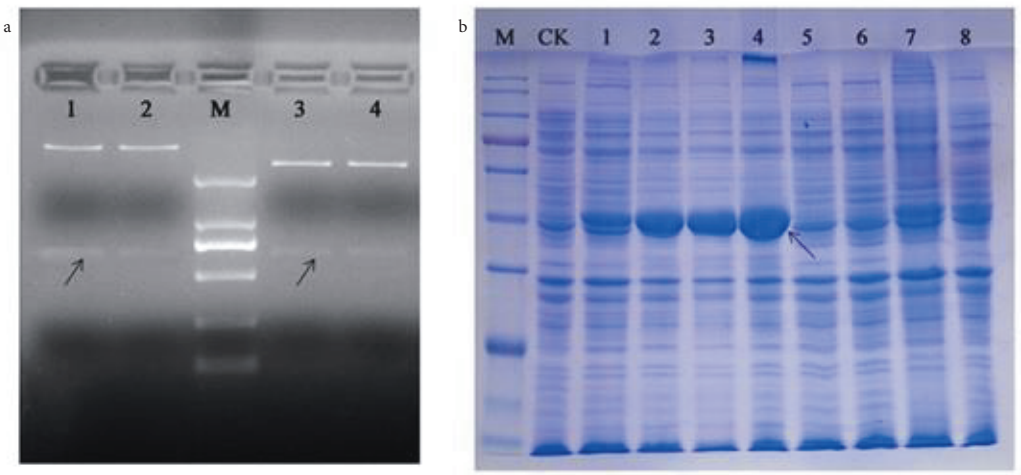

Figure 4. Construction of the recombinant vector and prokaryotic expression of CtCHI-N: a) construction of recombinant vector. Lines 1 and 2 are two replicates of the digestion of *pET-32-CtCHI-N*, and lines 3 and 4 are two replicates of the digestion of *pMD19-T-CtCHI-N*. The *CtCHI-N* fragment is indicated by an arrow, and M represents a 2000-bp DNA marker. b) Prokaryotic expression of CtCHI-N. CK represents the *Rosetta* strain without a recombinant plasmid. Lines 1 to 4 represent 1–4 h of IPTG induction, and lines 5 and 6 represent 1–4 h without IPTG induction. M represents a 170-kDa DNA marker.

3.4. Relationship between *CtCHI-N* expression and flavonoid biosynthesis during different flowering stages

The flavonoids were measured at different flowering stages (Table 2). The relationship between *CtCHI-N* expression and flavonoid biosynthesis during flowering was analyzed. *CtCHI-N* expression was detected during all stages of flowering; the expression gradually increased from the first day to the fourth day and peaked on the fourth day. Afterward, the expression sharply decreased during the next three days. Similarly, the HSYA content increased during the first four days but then decreased during the last three days (Figure 5a). In the other branch of the biosynthesis pathway, both quercetin and kaempferol peaked on the fourth day and again on the sixth day, and their maximum levels occurred on the sixth day. In contrast, naringenin displayed a single peak on the fourth day (Figure 5b). Overall, *CtCHI-N* expression was correlated with flavonoid biosynthesis.

Table 2. Percent composition of flavonoids in safflower flowers (n = 3).

Samples	D1	D2	D3	D4	D5	D6	D7
HSYA	3.420	3.558	3.730	3.840	3.648	2.963	2.234
Quercetin	0.026	0.034	0.043	0.053	0.051	0.140	0.043
Naringenin	0.032	0.040	0.056	0.074	0.061	0.058	0.020
Kaempferol	0.135	0.152	0.166	0.195	0.177	0.210	0.103

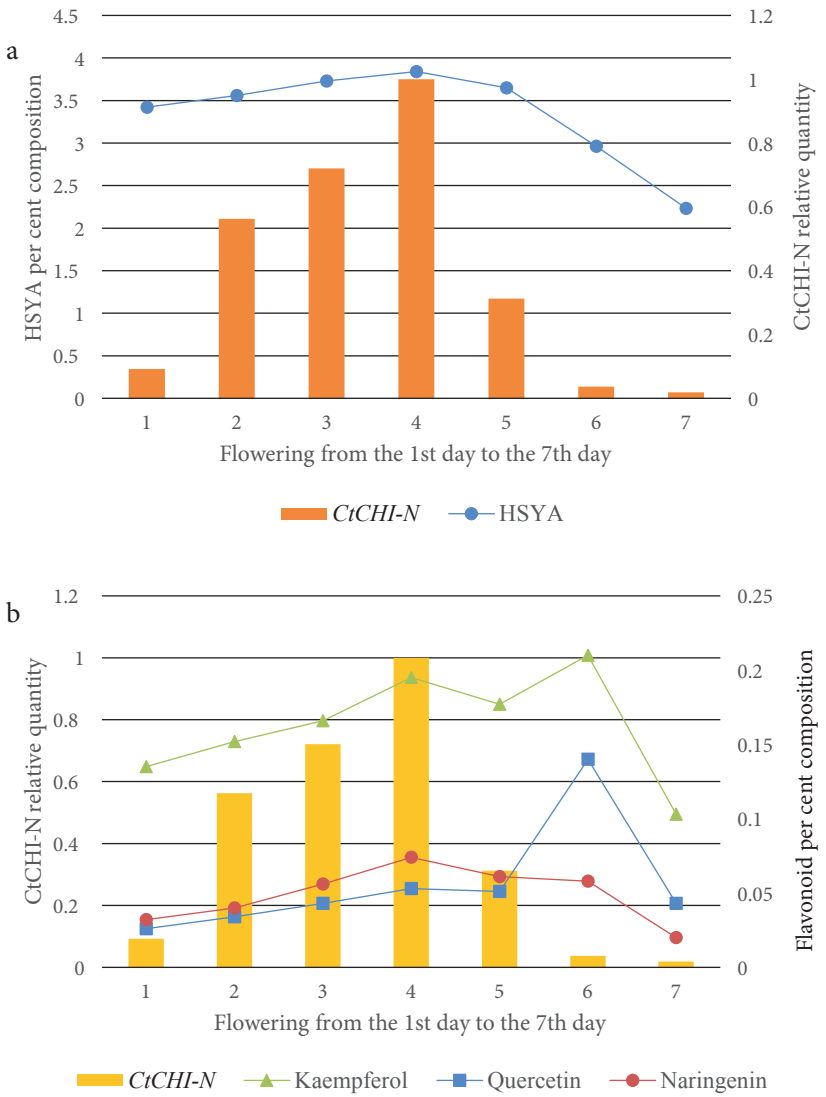

Figure 5. Relationship between *CtCHI-N* expression and flavonoid biosynthesis: a) HSYA accumulation and *CtCHI-N* expression; b) flavonoids accumulation and *CtCHI-N* expression.

4. Discussion

Safflower is a traditional medicinal plant in China, and the flavonoids produced by safflower plants are pharmaceutically active, especially HSYA. Via high-throughput transcriptome sequencing and analysis, many genes involved in flavonoid biosynthesis in safflower have been annotated (Huang et al., 2012; Li et al., 2012); however, only a few of these genes have been cloned and

analyzed. In our study, *CtCHI-N* was successfully cloned and expressed. In our previous research (Chen et al., 2018), four chalcone isomerase genes (*CtCHI1*, *CtCHI2*, *CtCHI3*, and *CtCHI4*) were screened, excluding *CtCHI-N*. The sequencing platform is different, and the depth of sequencing is different, which may induce different isoforms being screened.

In the phylogenetic tree analysis of the *CHI* gene, *CtCHI-N* had closer phylogenetic relationships with *Chrysanthemum seticuspe* and *Chrysanthemum* × *morifolium*; *CtCHI1*, *CtCHI2*, *CtCHI3*, and *CtCHI4* were more similar than *CtCHI-N*. Bioinformatic analysis revealed structural and physical properties of CtCHI-N. With the success of prokaryotic expression, these results were helpful for functional research of CHI in safflower.

Expression analysis revealed that *CtCHI-N* mRNA accumulated to maximum levels in the flowers, especially on the fourth day of flowering. This time corresponds precisely with the traditional harvest time for safflower flowers in China; the third or fourth day is when the flowers are at full blossom. *CtCHI-N* is a key enzyme involved in the flavonoid biosynthesis pathway of safflower. On the basis of the proposed metabolic pathway, *CtCHI-N* expression should be negatively correlated with HSYA biosynthesis. However, the high levels of *CtCHI-N* expression did not impede the biosynthesis of HSYA during the first four days of flowering. We propose that the initial substrates for flavonoid biosynthesis were abundant at the beginning of flowering. Although *CtCHI-N* expression will lead to increases in the compounds in other branches of the metabolic pathway, the HSYA content will also increase. The quercetin and kaempferol contents peaked on the fourth day of flowering, and their maximum values occurred on the sixth day after *CtCHI-N* expression sharply decreased, indicating that quercetin and kaempferol might be regulated not only by *CtCHI-N* but also by other genes, such as *F3H* or *FLS* (Huang et al., 2012). Naringenin biosynthesis exhibited the same trend as did the *CtCHI-N* expression, which indicated that the naringenin content was influenced mainly by *CtCHI-N* expression.

In summary, *CtCHI-N* was cloned and analyzed as a new chalcone isomerase gene. The recombinant protein was successfully expressed, and the expression analysis revealed that *CtCHI-N* was expressed in a tissue-specific manner, with the highest levels occurring during early flowering. The expression of *CtCHI-N* was strongly correlated with flavonoid biosynthesis during safflower flowering. Taken together, all of these results suggest that *CtCHI-N* might influence flavonoid biosynthesis during safflower flowering.

Acknowledgments

This project was supported by grants from the National Natural Science Foundation of China (81573544), the Sichuan Science and Technology Support Program (2014SZ0156), and the Chengdu University of Traditional Chinese Medicine Fund (ZRYY1610, ZRQN1647).

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