Cloning and Functional Characterization of a Chalcone Isomerase from *Trigonella foenum-graecum* L.

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Key words
- *Trigonella foenum-graecum* L.
- Leguminosae
- chalcone isomerase
- *Arabidopsis*
- *tt5*

Abstract

Flavonoids belong to a group of plant natural products with variable phenolic structures and play important roles in protection against biotic and abiotic stress. Fenugreek (*Trigonella foenum-graecum* L.) seeds and stems contain flavonol glycosides and isoflavone derivatives. Up to now, the molecular features of fenugreek flavonoid biosynthesis have not been characterized. Here we present cloning of a cDNA encoding a chalcone isomerase (namely *TFGCHI-1*) from the leaves of *T. foenum-graecum* which convert chalcones to flavanones *in vitro*. Transformation of *Arabidopsis* loss-of-function *tt5* (*chi*) mutant with a *TFGCHI-1* cDNA complemented *tt5* and produced higher levels of flavonol glycosides than wild-type Col-0.

Abbreviations

TFGCHI-1:	*Trigonella foenum-graecum* L. chalcone isomerase
tt5:	*transparent testa 5*
RACE:	rapid amplification of cDNA ends
LB:	lysogeny broth

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Introduction

Fenugreek (*Trigonella foenum-graecum* L.; Leguminosae) is an annual herbaceous aromatic leguminous, widely cultivated in Mediterranean countries and Asia [1]. In traditional Chinese medicine (TCM), the seeds have been prescribed as a tonic and for stomach disorders, and the whole aerial part of the plant is also used as a folk medicine for the treatment of renal diseases [2]. Phytochemical analysis showed that this plant contained flavonol glycosides, isoflavones, steroidal sapogenins, 4-hydroxyisoleucine, and the main impact odor compound sotolone (3-hydroxy-4,5-dimethyl-2(5H)-furanone) [3–10]. Flavonoids include flavonols, flavones, anthocyanins, isoflavones, and the polymeric proanthocyanidins (PAs, also called condensed tannins) [11]. Flavonoid biosynthesis genes are downstream to the phenylpropanoid pathway and can be categorized into two different groups: structural gene and regulatory gene. Structural genes (**Fig. 1**) include chalcone synthase (*CHS*), chalcone isomerase (*CHI*), flavonol-3-hydroxylase (*F3H*), flavonol-3′-hydroxylase (*F3′H*), flavonol synthase (*FLS*), isoflavone synthase (*IFS*), 2-hydroxyisoflavanone dehydratase (*HID*), dihydroflavonol reductase (*DFR*), and anthocyanidin synthase (*ANS*) [12]. Chalcone isomerase is one of the key enzymes in the flavonoid biosynthesis pathway catalyzing conversion of chalcones to flavanones which are intermediates of the subsequent flavonoid metabolism [13]. The first crystal structure of CHI was elucidated in *Medicago sativa* and provided insight into the enzyme architecture responsible for catalyzing a nearly diffusion controlled cyclization reaction that is primarily driven by entropy and induced fit [14]. It was also reported that introduction of the petunia *CHI* gene into tomato resulted in a 78-fold increase of peel flavonols in transgenic fruit [15]. As result of our effort on analyzing the flavonoid biosynthesis pathway in *Trigonella foenum-graecum* L., we cloned a key flavonoid structural gene *TFGCHI-1* and characterized it functionally by complementation of an *Arabidopsis tt5* mutant and by *in vitro* enzyme assay.

* These authors contributed equally to this work and are considered co-first authors.

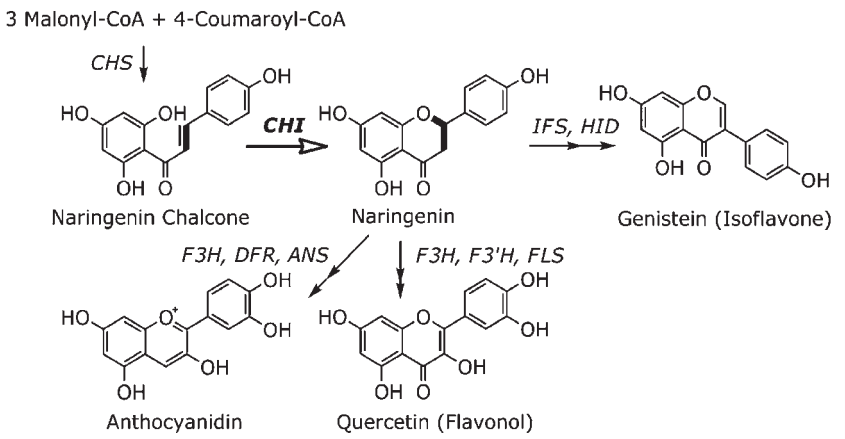

Fig. 1 Flavonoid biosynthesis pathway; CHS: chalcone synthase, CHI: chalcone isomerase, IFS: isoflavone synthase, HID: 2-hydroxyisoflavanone dehydratase, F3H: flavonol-3-hydroxylase, F3′H: flavonol-3′-hydroxylase, FLS: flavonol synthase, DFR: dihydroflavonol reductase, ANS: anthocyanidin synthase.

Materials and Methods

Chemicals, *E. coli* strains, plant materials, and plasmids

Narigenin chalcone, narigenin, and isoliquiritigenin were purchased from Sigma-Aldrich and Chromadex, respectively. All solvents used in this study were HPLC grade. 3′- and 5′-RACE kits were purchased from TAKARA. *Escherichia coli* strains DH10B and Bl-21 (DE3) (Invitrogen), expression vector pET28a (Novagen), and the sequence vector PGEM-T Easy (Promega) were used for cloning and overexpression of the *TFGCHI-1* genes. Seeds of *Trigonella foenum-graecum* L. (voucher no. XC-090718) were kindly provided and identified by Prof. Dr. Yi-nan Zheng, College of Chinese Medicinal Materials, Jilin Agricultural University. *Arabidopsis* seeds were purchased from ABRC (Department of Plant Cellular and Molecular Biology at The Ohio State University). *Arabidopsis* and *Trigonella foenum-graecum* L. seeds were cold-treated at 4 °C for 3 days and then allowed to germinate and grow in a greenhouse or on ½-strength MS medium containing 1% agar and 2% sucrose in a controlled growth cabinet (16-h light and 8-h dark cycle) at 20 °C.

Cloning of a full-length cDNA sequence from *Trigonella foenum-graecum* L.

A highly conserved region was identified by the comparisons of the *CHI* cDNA sequences from *Medicago sativa* (Accession No. M91079.1), *Pisum sativum* (U03433.2), and *Lotus japonicus* (AB054801.1). Based on this region, degenerated primers, 5′-CTCGGCGG(G/C/T)GC(A/T)GG(T/G)GAGAG-3′ (P1) and 5′-TGTGCCACACA(A/G)TTCTCCAT-3′ (P2), were designed to amplify an about 260 bp DNA fragment through RT-PCR using the cDNA from 22 d-fenugreek seedlings as the template. Based on the partial sequences obtained, the 5′ and 3′ rapid amplification of cDNA ends were performed to amplify the remaining cDNA regions of *TFGCHI-1* according to the manufacturer's instruction (TAKARA). The open reading frame (ORF) of *TFGCHI-1* was amplified using gene-specific primers 5′-CGCGGATCCATGGCAGCATCCATCACC-3′ and 5′-CGCGAGCTCTCAGTTTCCAATCTTGAAAGC-3′ and Taq DNA polymerase (Invitrogen). The PCR product was cloned into pGEM-T Easy Vector resulting in the plasmid pGEM-TFGCHI-1. The DNA sequence of the insert of pGEM-TFGCHI-1 was determined with an ABI3700 DNA sequencer using a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems).

Sequence alignment, phylogenetic analysis, and structural modeling of TFGCHI-1

Amino acid sequences were aligned using the AlignX program, part of the Vector NTI suite (Invitrogen), with default settings of parameters (gap opening penalty, 10; gap extension penalty, 0.05; gap separation penalty range, 8; identity for alignment delay, 40%) [16]. The phylogenetic tree was constructed using the neighbor-joining algorithm with MEGA version 4.0 [17] with 1000 bootstrap trials performed. For structural modeling, the sequences of the TFGCHI-1 protein were introduced into a protein structure prediction web server ESyPred3D (http://www.fundp.ac.be/sciences/biologie/urbm/bioinfo/esypred/) [18], using the crystallized *Medicago sativa* MsCHI-1 (1EYP) as a template. Structural simulations were conducted using ICM-pro v3.4 with a MMFF local and global Mol-Mechanics calculation module (MolSoft LLC).

Southern blot

For Southern blot analysis, genomic DNA was isolated from 10-day seedlings by the CTAB method [19], digested with Eco*RI*, Hind*III*, and Bam*HI* restriction enzymes and resolved on 1.2% agarose gels. Southern blot analysis was performed on nylon membranes according to Southern (1975) [20]. The *TFGCHI-1* coding region was radiolabeled with α-32P dCTP using Ready-to-Go DNA labeling beads (Amersham Pharmacia Biotech) and used as a probe. Hybridization was carried out at 55 °C for 2 h with 0.6 M NaCl, and blots were washed at high stringency (0.2 × SSC, 0.1% SDS).

Real-time QPCR

RNAs extracted from different growth stages, using a commercial RNAEasy mini kit (Qiagen), were used in quantitative real-time PCR reactions (Q-PCR) with gene-specific primers (TFGCHI-F: 5′-CCACCTGGTGCTTCTGTTTT-3′ and TFGCHI-R: 5′-CGTGCTCACCGATCATAGTC-3′). Q-PCR reactions were conducted using SuperScript III First-Strand Synthesis SuperMix, a Platinum SYBR Green qPCR SuperMix-UDG kit (Invitrogen), and the StepOnePlus Real-Time PCR System (Applied Biosystems) following the manufacturers' instructions and as described previously [21]. For gene-specific primers, gel electrophoresis and melting curve analyses were conducted to ensure a single PCR amplicon of the expected length and melting temperature. The level of each mRNA was calculated using the mean cycle threshold (Ct) value, and the dry seed mRNA level was designated as one. Data were analyzed us-

ing StepOne Software v2.0 (Applied Biosystems), and all results were shown as means of at least three independent RNA extractions with corresponding standard errors (SE).

Functional analysis of TFGCHI-1 *in vitro*

The ORF of *TFGCHI-1* from the vector pGEM-TFGCHI-1 was digested with Bam*HI* and Sac*I* and ligated into an *E. coli* expression vector pET28a, which led to the construct pET28a-TFGCHI-1. The empty vector pET28a and the construct pET28a-TFGCHI-1 were separately introduced into *E. coli* strain BL21 (DE3) (Novagen) using heat shock at 42 °C. Overnight cultures harboring pET28a or pET28a-TFGCHI-1 were used to inoculate 250 mL of LB liquid medium containing 50 µg/mL kanamycin and grown at 37 °C with shaking to OD_{600} of 0.6 followed by induction with 0.5 mM IPTG at 28 °C for 6 h. His-tagged TFGCHI-1 protein was purified at 4 °C using a His-Bind purification kit (Novagen) following the manufacturer's protocol. The concentration of purified protein was measured by NanoDrop® ND-1000 (NanoDrop Technologies, Inc.). The recombinant enzyme was visualized on 12.5% acrylamide gel stained with 0.25% Coomassie Blue. *In vitro* enzyme assay conditions were altered to include incubation at 30 °C for 5 min in 100 µL total volume containing 80 µL Tris-HCl buffer (100 mM, pH 7.6), 15 µL purified recombinant TFGCHI-1 protein (0.4 µg μL^{-1}), and 5 µL chalcones (100 µM) dissolved in methanol. Determinations of the K_m and the V_{max} for both naringenin chalcone and isoliquiritigenin were performed with varied substrate concentration ranges between 0.5 and 100 µM and calculated using a Lineweaver-Burk plot. The decrease of substrate A_{380} was monitored using a UV spectrophotometer (Thermo Scientific Instruments). The optimal pH was determined in 50 mM potassium phosphate at pH range 6.0 to 8.0 and 50 mM Tris-HCl at pH range 7.5 to 8.5 using naringenin chalcone as a substrate. The rate constant K_{uncat} for the spontaneous conversion of the respective chalcone to the corresponding flavanone was determined in 50 mM potassium phosphate buffer (pH 7.8) according to the method of Joseph and Joseph [22].

Complementation of *Arabidopsis tt5* by *TFGCHI-1*

The ORF of *TFGCHI-1* from the vector pGEM-TFGCHI-1 was digested with Bam*HI* and Sac*I* and ligated into a binary vector pBI121 under the control of the *35S* promoter, which led to the construct pBI121-TFGCHI-1. *Arabidopsis tt5* mutant that lacks the CHI activity was obtained from ABRC and was transformed with the *A. tumefaciens* GV3101 containing the binary construct pBI121-TFGCHI-1 by floral dipping [23] followed by selections with 100 µg/mL kanamycin and used for flavonoids analysis by HPLC.

HPLC UV/MS analyses of flavonoids

Flavonoids were extracted in triplicate batches from *Arabidopsis* seed and analyzed as described by Gao et al. [24] with minor modifications. Briefly, 100 mg of frozen seeds were ground in liquid nitrogen in a 20-mL Potter (Elvehjem), followed by grinding in 10 mL acetone/water (70 : 30; v/v) for 10 min. Following filtration, the pellet was re-extracted overnight at 4 °C in the dark, the two extracts were combined and evaporated at 35 °C under vacuum, and the dried extract was dissolved to 1 mg · mL^{-1} methanol/water (50 : 50; v/v). HPLC/UV analysis was conducted using a Hewlett Packard Agilent 1100 chromatograph, a G-7120 diode array detector, HP Chemstation ver. 8.01 software, and a Zorbax C_{18} column (150 × 4.6 mm, 5 µm ID) with a linear gradient of 20–45% for 5 min, constant 45% for 10 min, a linear gradient of 45–80% for 5 min, and finally a linear gradient of 80–100% for 10 min, followed by a 10-min washing with 100% methanol, a flow rate of 1.0 mL · min^{-1}, and an injection volume of 20 µL. Identical chromatography conditions were also used for LC-MS/MS using an 1100 HPLC and an "API QStar XL" pulsar hybrid system (Applied Biosystems).

Results and Discussion

Based on the known conserved features of *Medicago sativa* (M91079.1), *Pisum sativum* (U03433.2), and *Lotus japonicus* (AB054801.1) *CHI* genes, we used cDNA as a template for PCR, with degenerate primers (P1 + P2) to isolate a partial DNA fragment of the *CHI* gene from *Trigonella foenum-graecum* L. A 260-bp DNA fragment was obtained; then the complete sequence of this gene was determined by 5′- and 3′-RACE PCR based on the sequence of this partial DNA; it was named *TFGCHI-1*. The *TFGCHI-1* cDNA contains an open reading frame encoding a protein of 222 amino acids with a predicted molecular mass of 21 kDa. We performed multiple sequence alignment of TFGCHI-1 with other previously reported CHIs. As shown in **Fig. 2A**, phylogenetic analysis demonstrated that TFGCHI-1 can be grouped with type-II leguminous plants CHIs [25]. Motif scan results showed that the C-terminal portion of the protein includes a highly conserved chalcone-flavanone isomerase domain and shares more than 95% similarity in this region with MsCHI-1 from *Medicago sativa* (**Fig. 2C**) [26]. Three-dimensional structure was predicted based on the crystallized MsCHI-1 (**Fig. 2B**). The residues forming the active site were found conserved in TFGCHI-1, e.g., Thr-48, Tyr-106, Asn-113, and Thr-190. Thr-48 polarizes the ketone of the flavanone substrate, and Tyr-106 stabilizes a key catalytic water molecule. Asn-113 and Thr-190 orient the substrate at the active site and position the reactive 2′-oxyanion of the substrate in proximity to the *α,β*-unsaturated double bond for the intermolecular cyclization reaction [14].

When Eco*RI*, Hind*III*, and Bam*HI*-digested DNA samples were probed with *TFGCHI-1*, the number of the hybridization signals indicated that two copies of CHI existed in the *Trigonella foenum-graecum* L. genome (**Fig. 3A**). Temporal expression of *TFGCHI-1* in different tissues was detected by real-time PCR. *TFGCHI-1* transcript accumulated at a low level in the cotyledon, root, and leaves. The highest expression level was found in the developing silique and flower. The lowest level of *TFGCHI-1* mRNA was at the dry seed stage (**Fig. 3B**). This was consistent with the *Arabidopsis* chalcone isomerase (*TT5*) gene expression pattern during the different developing stages (data was extracted from www.arabidopsis.org).

CHI converts chalcones into the corresponding flavanones by selectively binding an ionized chalcone in a conformation conducive to ring closure in a diffusion controlled reaction, thus accelerating the stereochemically defined intramolecular cyclization reaction yielding a biologically active (*S*)-isomer [14]. To test the functionality of the TFGCHI-1, *E. coli* Bl21 was transformed with vector pET28a-TFGCHI-1. Cells containing TFGCHI-1 protein were easily lysed after growth (**Fig. 4A**, lane 3), and the recombinant protein was extracted and purified to yield a single distinct 28 kDa band after SDS-PAGE (**Fig. 4A**, lane 4). We used both 6′-hydroxychalcone and 6′-deoxychalcone as substrates to examine the enzyme activity. HPLC elution profiles of reaction products showed that TFGCHI-1 yielded naringenin from the incubation with naringenin chalcone as a substrate (**Fig. 4B** bottom).

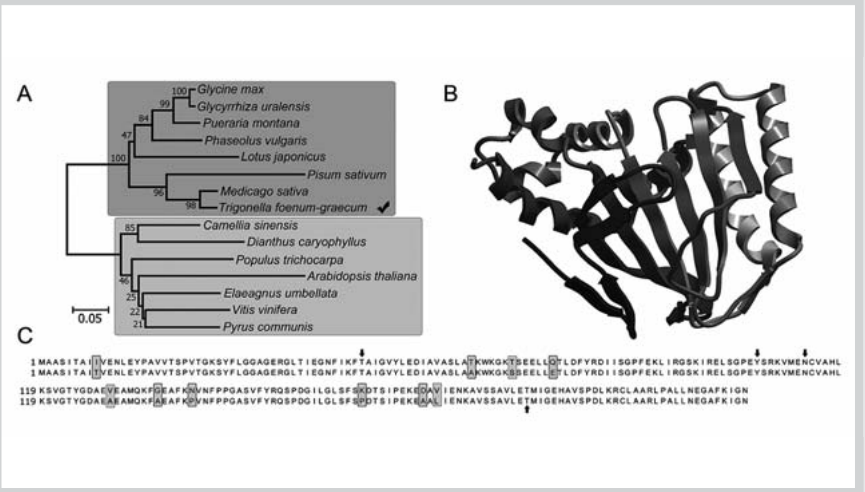

Fig. 2 Sequence information of TFGCHI-1. **A** Phylogenetic analysis of TFGCHI-1. Accession numbers of these proteins are as follows: *Glycine max*: Q93XE6; *Glycyrrhiza uralensis*: ABM66533; *Pueraria montana*: Q43056; *Phaseolus vulgaris*: P14298; *Lotus japonicus*: Q8H0F; *Pisum sativum*: P41089; *Medicago sativa*: P28013; *Camellia sinensis*: Q45QI7; *Dianthus caryophyllus*: Q43754; *Populus trichocarpa*: XP_002 315 258; *Arabidopsis thaliana*: NP_191 072; *Elaeagnus umbellate*: O6533; *Vitis vinifera*: P51117; *Pyrus communis*: A5HBK6. **B** Simulated three-dimensional structure of TFGCHI-1. Structure was predicted by ESyPred3D using the crystallized *Medicago sativa* MsCHI-1 (1EYP) as a template. **C** Alignment of TFGCHI-1 (top) with *Medicago sativa* MsCHI-1 (bottom). Amino acid differences were highlighted with grey boxes and binding sites were marked with arrows.

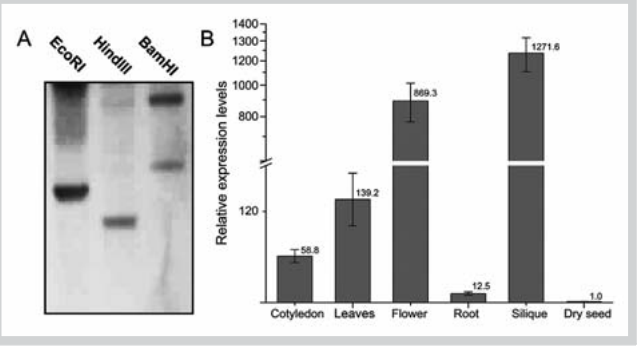

Fig. 3 Molecular analyses of *TFGCHI-1* in *Trigonella foenum-graecum* L. **A** Southern blot. Genomic DNA was digested with Eco*RI*, Hind*III*, and Bam*HI* restriction enzymes, and resolved on 1.2% agarose gels, then probed by α-32P dCTP labeled *TFGCHI-1*. **B** *TFGCHI-1* expression levels in different developing stages. Dry seed mRNA level was designated as one and all results were shown as means of at least three independent RNA extractions with corresponding standard errors (SE).

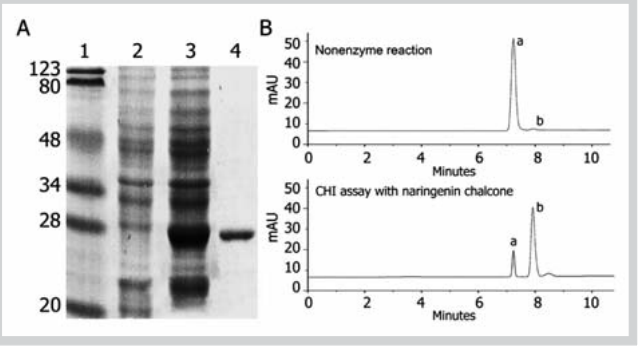

Fig. 4 Functional characterization of TFGCHI-1 *in vitro*. **A** SDS-PAGE analyses of TFGCHI-1 purified by Ni-NTA conjugation; lane 1, protein molecular weight marker; lane 2, supernatant of pET28a cell lysate; lane 3, supernatant of pET28a-TFGCHI-1 cell lysate; lane 4, purified TFGCHI-1 protein. **B** Representative HPLC profile of incubation of naringenin chalcone with (bottom) or without (top) purified TFGCHI-1 enzyme. Peak **a**: narigenin chalcone; peak **b**: narigenin.

The control reaction with naringenin chalcone using the same buffer without the TFGCHI-1 enzyme gave a peak of naringenin (**Fig. 4B**, top), which should be ascribed to the nonenzymatical conversion to naringenin. The temperature optimum for the isomerization of naringenin chalcone to naringenin as catalyzed by the purified enzyme was 36 °C; the pH optimal of naringenin chalcone and isoliquiritigenin were in the range of 7.5–8.0. Enzyme activity followed Michaelis–Menten kinetics. The K_m for naringenin chalcone and isoliquiritigenin were 67 and 26 µM, respectively. The V_{max} for naringenin chalcone and isoliquiritigenin were 1893 and 129.1 µmol·min^{-1}, respectively. The rate enhancement of the spontaneous cyclization by the purified chalcone isomerase (K_{cat}/K_{uncat}) were determined as 6.7×10^6 and 1.4×10^6 for naringenin chalcone and isoliquiritigenin, respectively (**Table 1**). Compared to the rate enhancement of approximately 10^6 brought about by enzymes from soy beans and alfalfa [22,25], TFGCHI-1 showed similar K_{cat}/K_{uncat} values for both naringenin chalcone and isoliquiritigenin, although the isomerization of 6′-deoxychalcone to 5-deoxyflavanone is rather slow in contrast to a rapid isomerization of 6′-hydroxychalcone into 5-hydroxyflavanone. Conversion of the flavanones from chalcones nonenzymatically under alkali conditions resulted in production of a racemic mixture of flavanones. Many flavonoid biosynthetic enzymes that are utilized in the production of other downstream flavonoids, such as flavonols and flavones, can only accept (2*S*)-forms. Although the chirality of narigenin produced by this recombinant strain was not determined with a chiral column, we suppose (2*S*)-narigenin was mainly produced by the action of TFGCHI-1 based on the previous results [14,27].

Table 1 Catalytic properties of chalcone isomerase from *T. foenum-graecum* regarding the different chalcone substrates

Substrate	V_{max} (µmol·min^{-1})	K_m (µM)	K_{uncat} (min^{-1})	K_{cat} (min^{-1})	Rate enhancement	pH optimal
Naringenin chalcone	1893	67	0.0554	371 200	6.7×10^6	7.5–8.0
Isoliquiritigenin	129.1	26	0.0096	13 600	1.4×10^6	7.5–8.0

To verify whether *TFGCHI-1* encodes a functional CHI in plants, we overexpressed it under the CaMV *35S* promoter in an *Arabidopsis tt5* mutant. RT-PCR analysis revealed that the transgene

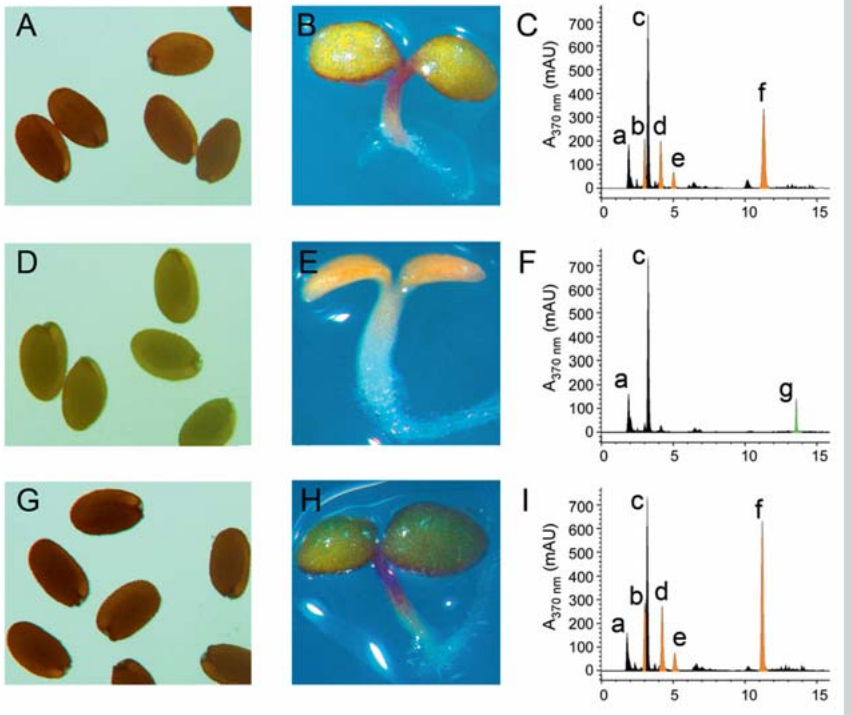

Fig. 5 Complementation of *Arabidopsis tt5* by *TFGCHI-1*. Mature seeds of **A** wild-type Col-0; **D** *tt5* mutant; **G** complementation of *tt5* by *TFGCHI-1*. Four-day seedling of **B** wild-type Col-0; **E** *tt5* mutant; **H** complementation of *tt5* by *TFGCHI-1*. Representative HPLC-UV ($\lambda_{370\,nm}$) chromatogram profiles of **C** wild-type Col-0; **F** *tt5* mutant; **I** complementation of *tt5* by *TFGCHI-1* seed methanol extracts. The peaks for flavonoids are indicated as: **b** quercetin-hexoside-rhamnoside; **d** quercetin-di-rhamnoside; **e** kaempferol-3,7-di-*O*-rhamnoside; **f** quercetin-3-*O*-rhamnoside; **g** narigenin chalcone. Peaks **a** and **c** are predicted to be sinapic acid derivatives based on MS and UV data.

was highly expressed (data not shown). *Arabidopsis* seed flavonols are present in both the testa and the embryo, while PAs accumulate only in the seed coat to protect the embryo and endosperm [12]. Quercetin-3-O-rhamnoside (Q3R), quercetin-di-rhamnoside (QDR), kaempferol-3,7-di-*O*-rhamnoside (KDR), and quercetin-hexoside-rhamnoside (QHR) are major flavonols in *Arabidopsis* seeds (**Fig. 5C**) [24]. The *tt5* mutant has yellow seeds, and no anthocyanins accumulated in the seedlings (**Fig. 5D–E**), which compared to wild-type brown seed with seedlings containing anthocyanins (**Fig. 5A–B**), due to the block in the flavonoid pathway caused by the mutation in CHI (**Fig. 1**) [12]. Seeds from the transgenic plants overexpressing *TFGCHI-1* were brown, and seedlings were found to accumulate anthocyanins, as in the wild type (**Fig. 5G–H**), indicating that *TFGCHI-1* complements the seed color and seedling anthocyanins accumulation phenotype of the *tt5* mutant. We analyzed the contents of the flavonoids in the wild type, *tt5* mutant, and *tt5* mutant overexpressing *TFGCHI-1* by HPLC UV/MS. Flavonol glycosides Q3R, QDR, KDR, and QHR were not detected in the *tt5* mutant, but this mutant accumulated narigenin chalcone (**Fig. 5F**), whereas in the transgenic line the levels of quercetin glycosides (peak b, d, and f in **Fig. 5I**) were higher than those in the wild type (**Fig. 5C**). This could be explained as we used virus *35S* rather than its native promoter, and this maybe caused the transcription level of *TFGCHI-1* to exceed the regular expression level of *Arabidopsis CHI* and led to the accumulation of a higher level of quercetin glycosides in the transgenic lines. This result was similar with the overexpression of the petunia *CHI-A* gene in the tomato fruit which resulted in a dramatic increase of flavonols (mainly rutin) in the peel at the expense of naringenin chalcone [15].

In this study we have provided molecular and biochemical evidence that *TFGCHI*-1 isolated from *Trigonella foenum-graecum* L. is a functional chalcone isomerase that can isomerize chalcone to produce flavonone. To our best knowledge, this is the first flavonoid biosynthesis gene characterized from *Trigonella foenum-graecum* L.

Acknowledgements

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