

Overexpression of the *Glycine max chalcone isomerase (GmCHI)* gene in transgenic *Talinum paniculatum* plants

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Abstract: *Talinum paniculatum* plants contain flavonoids and saponins, which have strong antioxidant properties and are used in the treatment of numerous diseases, such as inflammation, allergies, and gastric ulcers. Currently, there is no published research on the flavonoid content of *T. paniculatum* plants; however, it has been determined that the species in the genus *Talinum* have very low flavonoid content, and therefore the effective approach to enhance the flavonoid content in *T. paniculatum* plants is overexpression of the *Glycine max chalcone isomerase (GmCHI)* gene. In this study, we analyzed *GmCHI* gene overexpression, which has increased total flavonoid content in transgenic *T. paniculatum* lines. We have successfully transformed *T. paniculatum* plants with the *pCB301-GmCHI* vector and created 2 lines in generation T1 (T1-2.2 and T1-10) with a transgenic frequency of 0.27%. Recombinant CHI protein expressed in transgenic lines has a molecular weight of approximately 25 kDa, and the concentration of this protein in the two transgenic lines T1-2.2 and T1-10 was 4.29 $\mu\text{g mg}^{-1}$ and 6.14 $\mu\text{g mg}^{-1}$, respectively. Total flavonoid contents of the two transgenic lines T1-2.2 and T1-10 were 4.24 mg g^{-1} and 2.74 mg g^{-1} , respectively, which showed increases of 7.4-fold and 4.8-fold compared to that of the nontransgenic plants, respectively. Thus, overexpression of the *GmCHI* gene enabled *T. paniculatum* plants to improve their total flavonoid content. This work also confirmed the effectiveness of *Agrobacterium*-mediated plant transformation in enhancement of flavonoid accumulation in *T. paniculatum* plants.

Key words: *GmCHI* gene expression, chalcone isomerase, flavonoid, *Talinum paniculatum*, *Agrobacterium*-mediated transformation

1. Introduction

Flavonoids are groups of secondary metabolites that play a variety of significant roles in plants. They function as signal molecules in plant-microbe symbiosis (Ehrling et al., 2001) and are considered to play an important role during nodule meristem formation of the roots (Sundaravarathan et al., 2002). Many plant species produce flavonoids when they are infected by microorganisms (Park et al., 1999) and contaminated by toxic heavy metals (Kim et al., 1999). In addition, flavonoids also play important roles in human health. They are natural sources of antioxidants, which control many different human diseases (Kim et al., 2003). Several flavonoids such as catechin, apigenin, quercetin, naringenin, rutin, and venoruton are reported to have hepatoprotective activities (Tapas et al., 2008) that prevent the occurrence of age-related chronic diseases, such as cardiovascular disease or some types of cancer (Butelli et al., 2008; Luceri et al., 2008). Flavonoids are synthesized by the phenylpropanoid biosynthesis pathway, a fundamental secondary synthesis pathway in all higher

plants that converts the amino acid L-phenylalanine (L-Phe), or L-tyrosine (L-Tyr) in some cases (Rösler et al., 1997), into 4-coumaroyl CoA (or one thiol ester with the presence of a 4-hydroxycinnamate). These esters are used as precursors to synthesize major compounds, such as flavonoids, lignins, lignans, coumarins, furanocoumarins, and stilbenes (Thomas, 2010). Mutants affecting flavonoid synthesis were isolated in various plant species, such as corn (*Zea mays*), snapdragon (*Antirrhinum majus*), and petunia (*Petunia hybrida*), and as a result, many structural and regulatory flavonoid genes have been identified (Holton et al., 1993; Mol et al., 1998). Chalcone isomerase (CHI) is the key enzyme in flavanone biosynthesis that catalyzes the intramolecular cyclization of bicyclic chalcones (e.g., naringenin chalcone) into tricyclic (S)-flavanones (e.g., naringenin). The synthesis of naringenin, the first flavanone, directs the flavonoid pathway to the synthesis of other flavanones, flavones, flavonols, tannins, and anthocyanins (Weisshaar et al., 1998; Ehrling et al., 2001). *CHI* is divided into two types: type I isomerizes only 6'-hydroxychalcone

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to 5-hydroxyflavanone (2S-naringenin) and is found in most plants, including leguminous and nonleguminous plants, such as barley, *Arabidopsis*, and rice; type II, which is mainly found in leguminous plants, converts both 6'-deoxychalcone and 6'-hydroxychalcone to 5-deoxyflavanone and 5-hydroxyflavanone, respectively, from which 5-deoxyflavonoid subclasses, mainly isoflavone and flavone derivatives, are produced (Rieseberg et al., 1987; Norimoto et al., 2003). So far, there have been a number of research works that overexpressed the *CHI* gene in plants, such as expression of the *Sausurea medusa CHI* gene (Li et al., 2006) and *Paeonia suffruticosa CHI* gene (Lin et al., 2014) in tobacco plants, expression of the *Elaeagnus umbellata CHI1* gene in *Arabidopsis thaliana* (Kim et al., 2007), and overexpression of the *Petunia CHI* gene (Muir et al., 2001) and the onion *CHI* gene (Lim et al., 2017) in tomato. The flavonoid content in transgenic plants was shown to be increased many times compared to that in the wild type. Thus, enhancing *CHI* gene expression can be used as an effective method to increase the content of flavonoids in transgenic plants. *Talinum paniculatum* has been widely used in traditional medicine with reported antiinflammatory properties. *T. paniculatum* contains flavonoid, but its content is very low (0.897 mg g⁻¹ of fresh leaves in *Talinum triangulare* of the same genus) (Afolabi et al., 2014); thus, it is of great interest to improve its flavonoid content by overexpressing the *CHI* gene that encodes the key enzyme of flavonoid synthesis, and the *GmCHI* gene encoding chalcone isomerase, a key enzyme in metabolizing synthetic flavonoid in soybeans, was chosen.

In our previous studies, some genes related to drought tolerance from soybean plants were cloned and their expression was analyzed in tobacco plants (Dao et al., 2015; Lo et al., 2015). In this work, we present the results of overexpressing the *GmCHI* gene, which was isolated from soybean, in *T. paniculatum* plants to increase flavonoid content in transgenic *T. paniculatum* lines.

2. Materials and methods

2.1. Materials

T. paniculatum seeds were provided by the Seedling Center, Thai Nguyen Province, Vietnam. *Agrobacterium*

tumefaciens CV58 carrying the 35S-*CHI-cmyc* construct in a *pCB301* transgenic plasmid was provided by the Department of Modern Biology and Biological Education, Thai Nguyen University of Education. The *GmCHI* gene sequence in the *pCB301-CHI* vector was isolated from mRNA of the DT26 soybean cultivar and is 657 nucleotides in length, encoding 218 amino acids (Le et al., 2016; <https://www.ncbi.nlm.nih.gov/nucore/LT594994>). The *CaMV35S* promoter was isolated from *Cauliflower mosaic virus*. The nucleotide sequence of *Cmyc* encodes the peptide *cmyc*, which functions as an antigen to detect the target protein in western blot or ELISA. The nucleotide sequence of *KDEL* encodes the peptide *KDEL*. The abbreviation *KDEL* is formed by the corresponding letters of each amino acid: K - lysine, D - aspartic acid, E - glutamic acid, and L - leucine. *KDEL* is a target peptide sequence in the amino acid structure of a protein that prevents the protein from being secreted from the endoplasmic reticulum (ER) (Stornaiuolo et al., 2003). It has been reported that the addition of *KDEL* to the C terminal of vacuolar proteins, such as storage proteins, changes the intracellular localization of these fusion proteins to the ER (Herman et al., 1990; Wandelt et al., 1992). *LB*: Left bank; *RB*: right bank. The 35S-*CHI-cmyc* construct in the *pCB301* vector is shown in Figure 1.

2.2. Transferring the 35S-*CHI-cmyc* construct into *T. paniculatum* plants

Agrobacterium-mediated transformation via *T. paniculatum* cotyledonary nodes and regeneration and selection of transgenic *T. paniculatum* plants were performed following the method previously described by Olhoft et al. (2006). *T. paniculatum* seeds were surface-sterilized by soaking in 70% ethanol for 1 min and washed with distilled water, followed by rinsing with 60% bleach for approximately 10 min. The seeds were germinated on a Murashige and Skoog (1962) inorganic salt medium. The growth temperature and illumination protocol for *T. paniculatum* seeds is 25 ± 2 °C and 16/8 h light/dark, respectively. Cotyledons were harvested after 2 weeks of in vitro culture to be used as transformed materials. *A. tumefaciens* CV58 carrying the *pCB301-CHI* transgenic vector was cultured in liquid *LB* medium supplemented with kanamycin (50 mg L⁻¹) and rifamycin (50 mg L⁻¹) in a

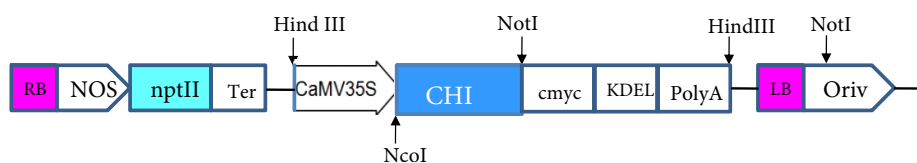


Figure 1. Diagram of *pCB301-CHI* transformation vector. *nptII*: Kanamycin resistance gene; *CaMV35S*: promoter 35S; *CHI*: *Glycine max chalcone isomerase* gene isolated from soybean plants; *cmyc*: nucleotide sequence coding the peptide *cmyc*; *KDEL*: nucleotide sequence coding the peptide *KDEL*. *LB*: left bank; *RB*: right bank.

shaking incubator at 28 °C at 150 rpm for 48 h.

2.3. Analysis of the presence and integration of the *CHI* transgene in transgenic plant genomes

Total DNA was extracted from the T0 generation transgenic leaves using a cetyltrimethylammonium bromide (CTAB) method (Shaghai-Marooif et al., 1984), and the total DNA was quality checked on a 0.8% agarose gel. The presence of the *CHI* gene in the transgenic plant genome was determined by PCR using specific primers *CHI-NcoI-F/CHI-NotI-R*, with the expected PCR product size of approximately 0.66 kb; PCR products were visualized on a 1% agarose gel.

CHI-NcoI-F: 5'

CATGCCATGGATGGCAACGATCAGCGCGGTT 3'

CHI-NotI-R: 5'

TTGCGGCCGCGACTATAATGCCGTGGCTC 3'

Southern blotting was performed to verify the integration of the *CHI* gene into transgenic plant genomes (Southern, 1975). Genomic DNA samples from PCR-positive transgenic plants were digested overnight by *NotI* at 37 °C. The digested genomic DNA was then separated on a 1% agarose gel and transferred to the cellulose membrane (blotting). The membrane was then washed with 2X SSC solution and hybridized with probe samples. The *GmCHI* gene fragment cloned by PCR with the specific primer pair *CHI-NcoI-F/CHI-NotI-R* was used to create a probe labeled with biotin-11-dUTP. Hybridization and detection were performed using a Biotin DecaLabel DNA Labeling Kit, following the manufacturer's instructions.

2.4. Analysis of recombinant CHI protein expression in transgenic plants by western blot and ELISA

Transgenic *T. paniculatum* plants with positive Southern blot results were subjected to western blot analysis to determine recombinant CHI protein expression. To extract total protein, 0.5 g of plant samples (roots, stems, leaves) were crushed in liquid nitrogen, dissolved in 1 mL of PBS with 0.05% Tween 20 (PBS-T), and then centrifuged at 13,000 rpm for 15 min. Proteins were denatured, run on 10% SDS-PAGE, and then transferred to nitrocellulose membranes using a Pierce G2 Fast Blotter (25 V, 1.3 mA for 20 min). Membranes were then blocked in blocking solution (5% skim milk in PBS-T) overnight and incubated with mouse anti-c-Myc monoclonal primary antibody (Santa Cruz Biotech, 1:700) for 3 h with shaking at room temperature, followed by 3 washes with PBS, and then incubated with an antimouse HRP-conjugated IgG secondary antibody for 1 h. The results were displayed using TMB (3,3',5,5'-tetramethyl benzidine) or DAB (3,3'-diaminobenzidine tetrahydrochloride) (Laemmli et al., 1970).

The recombinant CHI protein content in transgenic plants was determined by ELISA following the method of Sun et al. (2006). The recombinant CHI protein content

was calculated as $\mu\text{g mg}^{-1}$ total protein. The total protein extracted from each of the T1 generation plants was diluted to a concentration of 200 $\mu\text{g mL}^{-1}$, and 100 μL of each sample was added to individual wells in a microplate; each sample was run in triplicate. C-myc antibody, antimouse IgG antibody conjugated to horseradish peroxidase (HRP), and TMB solution were used to determine the recombinant CHI protein. C-myc-tagged ScFv protein was used as a positive control and to establish the standard curve.

2.5. Determination of total flavonoid content in *T. paniculatum* by absorption spectroscopy method

Flavonoid in the plant samples (roots, stems, leaves) was extracted with methanol, then reacted with aluminum chloride, and the absorbance of the test solution was measured at 415 nm, following the method of Kalita et al. (2013). A quercetin standard curve was constructed by analyzing the standard solutions, which had a concentration range from 10 $\mu\text{g mL}^{-1}$ to 100 $\mu\text{g mL}^{-1}$. *T. paniculatum* samples were crushed and mixed homogeneously. A total of 0.1–0.5 g of sample (equivalent to 10 mg quercetin) was sonicated for 30 min. Each sample comprised 0.5 mL of extract solution, 1.5 mL of methanol, 0.1 mL of aluminum chloride, 0.1 mL of potassium acetate solution, and 2.8 mL of distilled water, which was brought up to a total volume of 100 mL with methanol and mixed well. The absorbance was measured on a UV-Vis device (model UV2401, Shimadzu) at a wavelength of 415 nm. The result was calculated by the following formula:

$$C_m = \frac{A_m}{A_c} \cdot C_c \cdot k \frac{V}{m} \quad (\mu\text{g g}^{-1})$$

where C_m is the concentration of the sample ($\mu\text{g g}^{-1}$); C_c is the quercetin concentration in the standard solution ($\mu\text{g mL}^{-1}$); A_m and A_c are the absorbance of the sample and the standard solution; V is the final volume (mL); m is the sample weight (g); and k is the dilution factor.

3. Results

3.1. Transformation of *pCB301-CHI* vector and regeneration of transgenic *T. paniculatum*

The *pCB301-CHI* transformation vector was used to transform *T. paniculatum* through *A. tumefaciens* infection via cotyledonary nodes. *T. paniculatum* seeds (Figure 2A) were sterilized with 70% alcohol and then with bleach and germinated in GM medium (Figure 2B), and cotyledons were collected for transformation. Cotyledons were infected with *A. tumefaciens* carrying the *pCB301-CHI* transformation vector. In vitro regeneration of *T. paniculatum* in selection medium supplemented with antibiotics and growth of the transgenic plants in a greenhouse are shown in Figure 2.

From a total of 730 samples, 200 shoots were propagated in shoot elongation medium (SEM) supplemented with

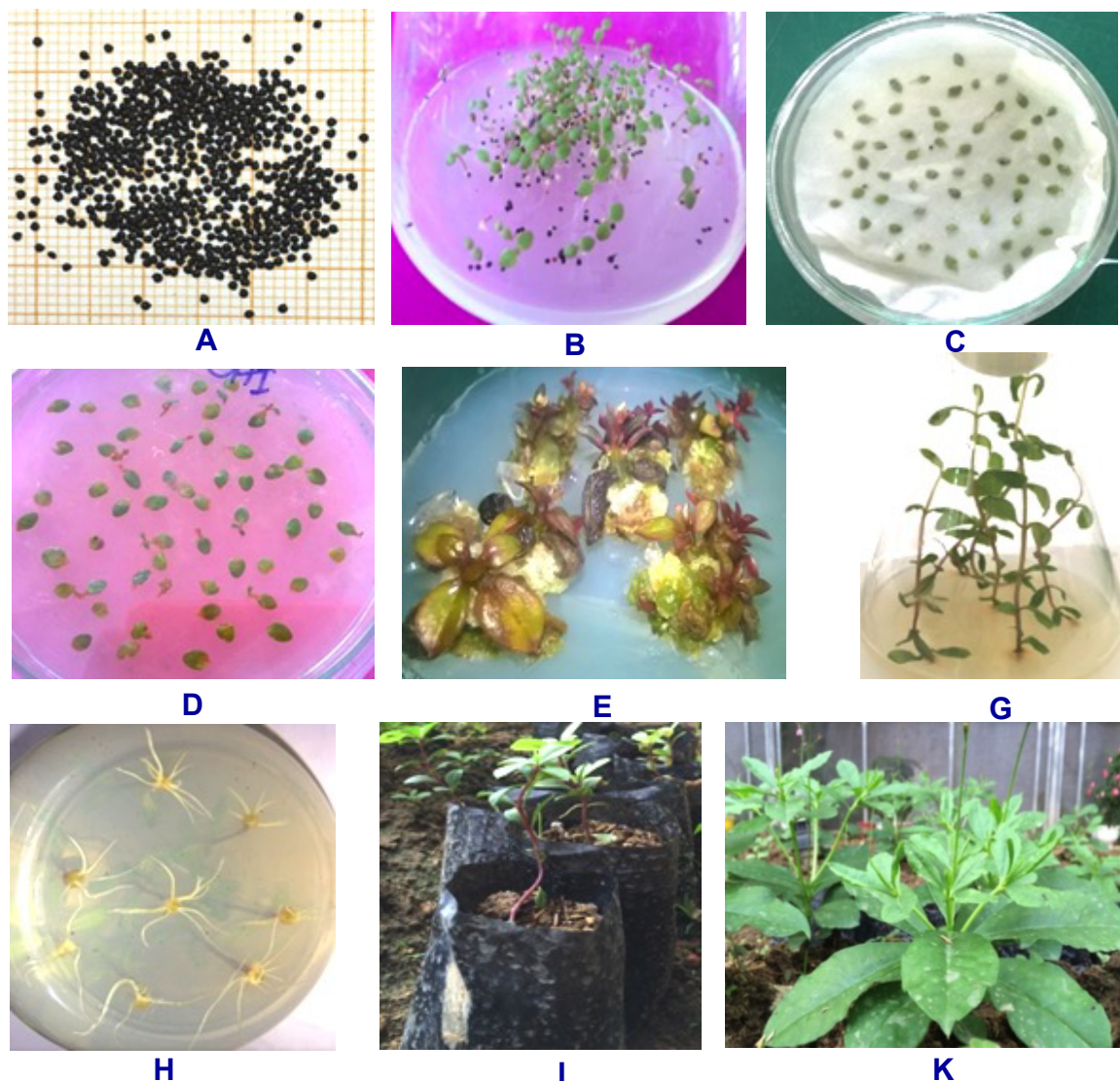


Figure 2. Transformation and regeneration of transgenic *T. paniculatum* plants. A: Seeds of *T. paniculatum*; B: germination in germination medium (GM); C: cocultivation in cocultivation medium (CCM) in the dark; D: inducing shoot generation; E: multiple shoot regeneration after 3 weeks; G, H: rooting and fully rooted plantlets in rooting medium (RM); I: seedling substratum; K: plants in greenhouse.

kanamycin and cefotaxime, and of these, 63 shoots were rooted and planted in substratum. Forty-three plantlets with fully developed root systems were transferred to the greenhouse, of which 28 plants belonging to 18 transgenic lines survived. Thus, from a total of 730 transformed samples, we obtained 18 *CHI* transgenic plant lines, accounting for 2.46% of the initial samples. In parallel with the *CHI* transgenic experiment, we cultivated two control batches called DC0 and DC1, in which DC0 contained nontransgenic samples cultured in medium supplemented with selection antibiotics and DC1 contained nontransgenic samples cultured in medium without selection antibiotics.

All samples in DC0 died as expected, while in DC1, 70 propagated shoots were formed, 40 plantlets with roots were grown in substratum, and 35 plants survived in the greenhouse. These 35 fully developed plants were used as controls for later experiments.

3.2. Verifying the integration of the *CHI* gene into *T. paniculatum* genomes

The expected size of the *CHI* transgene was 657 bp. The presence of the *CHI* transgene was verified by PCR using the specific primers *CHI-NcoI-F/CHI-NotI-R*. In 28 plants that we obtained after transformation and regeneration, 8 plants (T0-2.1, T0-2.2, T0-4, T0-7, T0-10, T0-12, T0-14,

and T0-16) belonging to 7 lines had PCR products with the approximate size of 0.66 kb, corresponding to the size of the *CHI* transgene (Figure 3). Thus, from 730 transformed samples, there were 7 lines in the T0 generation with positive PCR results. The transformation frequency of the *CHI* gene in this period was 0.96% ($7/730 = 0.96\%$).

Eight PCR-positive plants of 7 transgenic lines and wild-type plants were subjected to Southern blot analysis to determine whether the transgene was integrated into the transgenic plant genomes.

Figure 4 shows that DNA bands occurred in 6 transgenic plants belonging to 5 lines (T0-2.1, T0-2.2, T0-4, T0-7, T0-10, and T0-14), while there was no band in T0-12, T0-16, and wild-type plants. T0-7 showed 2 DNA bands corresponding to 2 copies, and the remaining lines, T0-2.1, T0-2.2, T0-4, T0-10, and T0-14, had only 1 copy. The transformation frequency of the *CHI* transgene at this stage was $5/730 = 0.68\%$. The growth and development of the transgenic plants with positive Southern blot hybridization results were evaluated, and their next generations were subjected to protein expression analysis.

3.3. Analysis of the recombinant CHI protein expression in the transgenic *T. paniculatum* lines in the T1 generation

All 6 transgenic plants grew and developed normally and could produce flowers and fruits. However, the seeds of

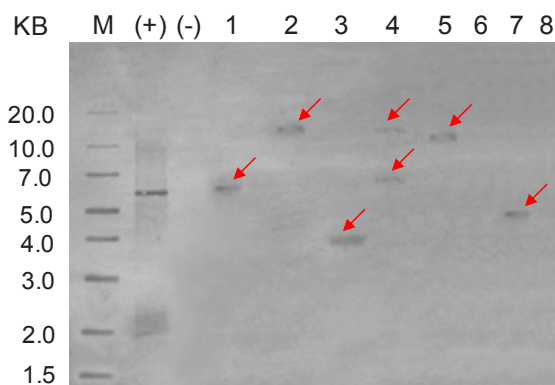


Figure 4. Integration of *CHI* gene into the *T. paniculatum* genome determined by Southern blot from PCR-positive transgenic *T. paniculatum* plants with probe segment *CHI* labeled with biotin. M: DNA ladder, 1 kb; (+): vector pCB301-*CHI*; (-): negative control (nontransgenic plants); 1: T0-2.1; 2: T0-2.2; 3: T0-4; 4: T0-7; 5: T0-10; 6: T0-12; 7: T0-14; 8: T0-16 were transgenic *T. paniculatum*-positive by PCR.

only 4 plants germinated and developed into T1 generation plants, namely T1-2.2, T1-4, T1-10, and T1-14. The leaves from these T1 generation transgenic lines were used to analyze the expression of recombinant CHI protein. Total protein extracted from the leaves of transgenic plants was denatured, run on 10% SDS-PAGE, and analyzed by western blot (Figure 5). As seen in Figure 5, we detected a band of approximately 25 kDa corresponding to the molecular weight of the recombinant CHI protein in 2 T1-generation lines, T1-2.2 and T1-10, while there was no band in the T1-4, T1-14, and wild-type lanes, suggesting that the *CHI* transgene was inherited from the T0 generation to the T1 generation in 2 transgenic lines, T1-2.2 and T1-10, and translated into recombinant CHI protein. Thus, the transformation frequency of the *CHI* gene in this period was 0.27% ($2/730$). The results of western blot analysis proved that recombinant CHI protein was expressed successfully in two transgenic *T. paniculatum* lines. In addition, these results demonstrated that the transgenic *CHI* genes were inherited through sexual reproduction from the T0 to the T1 generation and stably operated in two generations of transgenic *T. paniculatum* plants.

The contents of the recombinant CHI protein in two transgenic lines, T1-2.2 and T1-10, and wild-type plants were analyzed by ELISA (Figure 6). The recombinant CHI protein contents in T1-2.2 and T1-10 were $6.14 \mu\text{g mg}^{-1}$ and $4.29 \mu\text{g mg}^{-1}$, respectively. This result demonstrated that CHI protein was overexpressed in these two transgenic *T. paniculatum* lines.

3.4. Determination of total flavonoid content in the T1 generation transgenic *T. paniculatum* lines

Samples including leaves, stems, and roots from the two transgenic plants and wild-type plants were used to

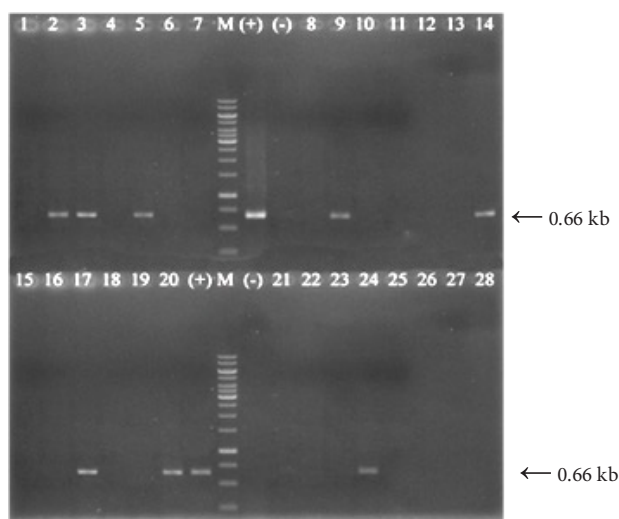


Figure 3. The presence of the *CHI* gene from transgenic *T. paniculatum* lines in the T0 generation identified by PCR using the specific primers *CHI-NcoI-F/CHI-NotI-R*. M: DNA marker, 1 kb; (+): positive control (plasmid pBT-*CHI*); (-): negative control (nontransgenic plants); 1: T0-1; 2: T0-2.1; 3: T0-2.2; 4: T0-3; 5: T0-4; 6: T0-5.1; 7: T0-5.2; 8: T0-6; 9: T0-7; 10: T0-8.1; 11: T0-8.2; 12: T0-9.1; 13: T0-9.2; 14: T0-10; 15: T0-11.1; 16: T0-11.2; 17: T0-12; 18: T0-13.1; 19: T0-13.2; 20: T0-14; 21: T0-15.1; 22: T0-15.2; 23: T0-15.3; 24: T0-16; 25: T0-17.1; 26: T0-17.2; 27: T0-18.1; 28: T0-18.2.

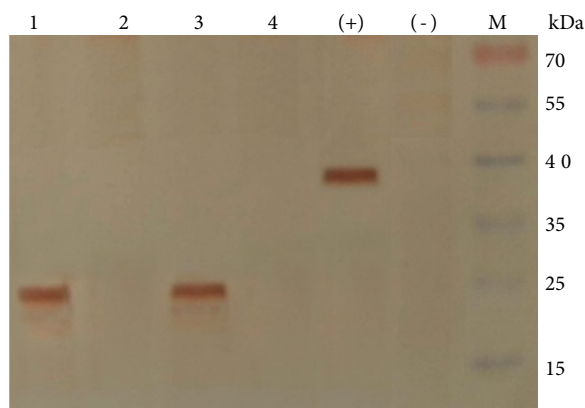


Figure 5. Western blot analysis for recombinant CHI protein expression in 4 transgenic *T. paniculatum* lines in the T1 generation and nontransgenic plants. M: Standard protein ladder (10–180 kDa); (-): protein of nontransgenic plants; (+): positive control protein; 1: T1-2.2; 2: T1-4; 3: T1-10; 4: T1-14: protein of 4 transgenic *T. paniculatum* lines positive by Southern blot. Primary antibody: mouse anti-c-Myc monoclonal; secondary antibody: antimouse IgG.

analyze the total flavonoid content (Table). T1-2.2 had the highest flavonoid content (approximately 4.24 mg g⁻¹), an increase of 743.86% compared to that of wild-type plants (approximately 0.57 mg g⁻¹). T1-10 had lower flavonoid content (approximately 2.74 mg g⁻¹), an increase of 480.70% compared to that of wild-type plants. These results illustrated that overexpression of the *CHI* gene in transgenic *T. paniculatum* lines T1-2.2 and T1-10 effectively increased the flavonoid content in transgenic plants.

4. Discussion

T. paniculatum contains flavonoid, which has strong antioxidant properties. Currently, there has not been any research work to determine the flavonoid content in *T. paniculatum*; however, it has been shown that there is a very low flavonoid level (approximately 0.897 mg g⁻¹ of fresh leaves) in *Talinum triangulare*, a species belonging to the same genus as *T. paniculatum* (Afolabi et al., 2014).

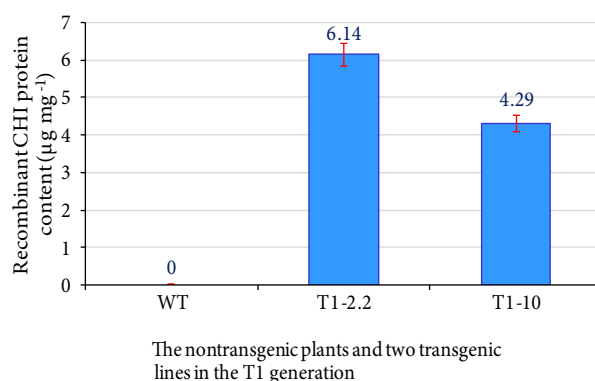


Figure 6. ELISA determined recombinant CHI protein content in the two transgenic *T. paniculatum* lines (T1-2.2 and T1-10) and nontransgenic plants (WT: wild type). Vertical bars represent standard error.

Until now, studies on improving the content of bioactive compounds including flavonoids in *T. paniculatum* have mainly focused on increasing the biomass of cells and hair roots. Zhao et al. (2009) proposed to select appropriate materials and concentrations of growth-promoting substances to maximize the formation of calli and buds, the rooting proportion, and the survival rate of seedlings in a greenhouse. Muhallilin et al. (2013) studied the effect of auxin-type plant growth regulators (IAA, NAA, IBA, and 2.4-D) at various concentrations (1 mg L⁻¹, 2 mg L⁻¹, and 3 mg L⁻¹) for root induction on leaf explants of *T. paniculatum*. Zhang et al. (2015) studied plant regeneration from protoplasts of *T. paniculatum* with different concentrations of NAA, 6-BAP, etc. to collect biomass. Manuhara et al. (2012) studied the effect of aeration and inoculum density on biomass and saponin content of *T. paniculatum* Gaertn. hairy roots in a balloon-type bubble bioreactor by transforming a leaf sample of *T. paniculatum* with *A. rhizogenes*. Thus, current research directions are mainly focused on cultivation in vitro to increase biomass, and there is no research work that establishes an effective gene transfer method to improve the content of bioactive

Table. Total flavonoid content of two transgenic *T. paniculatum* lines T1-2.2 and T1-10 and nontransgenic plants.

Samples	Total flavonoid content (mg g ⁻¹)	Increase compared to nontransgenic plants (%)
Nontransgenic plants	0.57 ± 0.12	100
T1-2.2	4.24 ± 0.15	743.86
T1-10	2.74 ± 0.19	480.70

Note: The symbol ± represents standard error.

compounds in *T. paniculatum*, including flavonoids. In this study, we overexpressed chalcone isomerase (CHI), a key enzyme in the flavonoid biosynthesis pathway, in order to increase the total flavonoid content in transgenic *T. paniculatum*. We isolated the *CHI* gene from soybean and transferred it into *T. paniculatum* via *A. tumefaciens* following a method previously described by Olhoft et al. (2006). The transformation frequency of the *CHI* transgene in the T0 generation based on Southern blot analysis was 0.68%. In the T1 generation, based on western blot analysis, the transformation frequency was 0.27%.

Previously, Li et al. (2006) transferred the *CHI* gene isolated from *Saussurea medusa* (Asteraceae) into transgenic tobacco plants, leading to an increase in the total flavonoid content 5-fold greater than that in nontransgenic plants. Kim et al. (2007) analyzed the function of *CHI* gene type I isolated from infected cells of root nodules of *Elaeagnus umbellata* and overexpressed it in an *Arabidopsis thaliana* transparent testa 5 (tt5) mutant. In the *Arabidopsis* tt5 mutant, the *CHI* gene was mutated, so there was no CHI enzyme synthesis and chalcone naringenin was not transformed into naringenin; as a result, the seed of the transgenic line was yellow due to lack of proanthocyanidin. However, when *CHI* was overexpressed in the *Arabidopsis* tt5 mutant, the seed color was recovered. In transgenic plants, naringenin is synthesized as it is in wild-type plants, while there is no naringenin in nontransgenic tt5 mutants. Lin et al. (2014) studied the function of the *CHI* gene in the flower-coloring mechanism in peony. The *CHI* gene isolated from peony (*Ps-CHI1*) is 924 bp in length, containing a 654-bp coding region, which encodes a peptide consisting of 217 amino acids with a molecular weight of 23.3 kDa. *Ps-CHI* expression is highest in the flower's petals, moderate in the calyx, low in the leaves and carpel, and lowest in the stamen. Transferring the *Ps-CHI1* gene into tobacco (*Nicotiana tabacum* L.) via *Agrobacterium* to obtain transgenic plants in the T1 generation caused a 3-fold increase in flavonoid and flavone contents compared to that in the wild type. Overexpression of the *CHI* gene isolated from

petunia in tomato (Muir et al., 2001) generated transgenic tomatoes with a flavonoid content 78-fold higher than that in the wild type. Lim et al. (2016) showed that tomato has very low anthocyanin content and that the overexpression of two transcription factors from *snapdragons*, Delila (Del) and Roseal (Ros1), in tomato generated transgenic tomato plants with purple color and increased anthocyanin content. However, the authors realized that the transcription factors Del/Ros1 (DR) in tomato could not regulate all the important endogenous genes related to the anthocyanin biosynthesis pathway, especially the *CHI* gene. The authors demonstrated that overexpression of the *DR* gene led to an 80- to 100-fold increase in anthocyanin in the fruit flesh and peel compared to that in the flesh and peel of the wild-type plants, while overexpression of the *CHI* gene isolated from onion together with the *DR* gene of tomato increased the anthocyanin content in the fruit flesh and peel by 260- to 400-fold compared to that in the nontransgenic plants. These studies confirmed the efficiency of transferring the *CHI* gene isolated from one species into another to increase the contents of flavones and isoflavones in transgenic plants.

In this study, we demonstrated that overexpression of the *CHI* gene isolated from soybean remarkably increased the total flavonoid content in transgenic *T. paniculatum* plants. We have successfully generated two transgenic lines, T1-2.2 and T1-10, that contain 4.24 mg g⁻¹ and 2.74 mg g⁻¹ flavonoid, respectively, which reflect increases of 7.4-fold and 4.8-fold, respectively, compared to that in wild-type plants. Moreover, the *CHI* transgene was inherited from the T0 to the T1 generation and stably expressed, suggesting that we have obtained two stable transgenic lines in which the transgenes would be passed through generations.

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