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# Transgenic Lettuce Expressing Chalcone Isomerase Gene of Chinese Cabbage Increased Levels of Flavonoids and Polyphenols

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**Abstract.** Flavonoid are large group of the polyphenolic compounds which are distinguished by an aromatic or phenolic ring structure and the phenolic compounds are induced by microbial infection, ultraviolet radiation, temperature and chemical stress. They are known for their antioxidant activity, anti-allergic, anti-inflammatory, anti-microbial and anti-cancer activities. In this study, changes in flavonoid content were investigated using heterologous chalcone isomerase (CHI) expression system. Also, phenolic compounds level was measured to examine the relation between flavonoids and phenols contents. Explants of lettuce (*Lactuca sativa* L.) were transformed with *Agrobacterium tumefaciens* LBA 4404 strain containing pFLH-CHI (derived from pPZP2Ha3) vector constructed with CHI gene from *Brassica rapa*. The putative transgenic plants were confirmed by genomic DNA PCR analysis. Also the transcription levels of the gene were analyzed by semi-quantitative RT-PCR with gene specific primers. The total flavonoid contents were increased at $T_0$ and $T_1$ generations over 1.4 and 4.0 fold, respectively. Total phenol contents also increased at $T_1$ generation. These results indicate that CHI gene plays an important role to regulate the accumulation of flavonoids and its component changes.

***Additional key words*:** *Brassica rapa*, *Lactuca sativa*, transformation

## Introduction

Lettuce (*Lactuca sativa*) is mostly consumed as a leafy vegetable of the family Acsteraceae. Lettuce requires light, sandy, humus rich, and moist soil for growth. It is cultivated both outside and in the greenhouse round the year (De Vries, 1997). The major nutritional components of lettuce are over 95% water, vitamin A, vitamin C, vitamin K, folate, and iron. Lettuce is an easy way to maintain a well-balanced diet (Jang et al., 2007). The six common cultivar groups of lettuce are Butterhead, Chinese lettuce, Crisphead, Looseleaf, Romaine, and Summer Crisp also called Batavian.

Polyphenols, one of the major groups of plant secondary metabolites, include hydroxybenzoic acids, hydroxycinnamic acids, lignans, stilbenes, and flavonoids (Manach et al., 2005). Flavonoids biosynthetic pathway is induced when most plant species are exposed to abiotic and biotic stresses such as wounding, UV light and pathogen attack. These stresses are known to play key roles in plant defense reaction mainly because of the synthesis and accumulation of protective compounds (Fofana et al., 2002). Flavonoids, the most ubiquitous polyphenolic compound group, are comprised of flavones, flavonols, anthocyanins, proanthocyanins and others (Manach, et al., 2005; Park et al., 2008). The basic structure of flavonoids allows a multitude of substitution patterns in the benzene rings A and B linked by various heterocyclic rings C (Hollman and Katan, 1999). Important roles of flavonoids include preventing degenerative diseases associated with oxidative stress and functioning as antiviral, anti-inflammatory, antihistamine, and antioxidants. Flavonoids also play a crucial role in fertility and sexual reproduction that exists in pollen and pistils of some plants, evidenced through petunia plants by Van der Meer et al. (1992). The petunia inhibited flavonoids production by antisense suppression of the gene encoding chalcone synthase (CHS), which is the first enzyme of the flavonoid biosynthesis pathway, and resulted not only in the inhibition of flower pigmentation but also in male sterility (Ylstra et al., 1992).

To regulate flavonoids biosynthetic pathway two groups of genes are needed. One group of genes is transcription factors that control the expression of the structural genes and the accumulation of the metabolites (Park et al., 2008). Another group is structural genes that encode the enzymes that directly take part in the biosynthetic reactions (Park et

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al., 2008). In the flavonoids biosynthesis pathway, the C15 compound, derived C6-C1 cyclization by CHS, is an aromatic ketone that forms the central core for a variety of important biological compounds, known as chalcones. Chalcones are then isomerized into flavanones, such as a naringenin containing three rings, by chalcone isomerase (CHI) (Leonard et al., 2006; Winkel-Shirly, 2001).

CHI (EC 5.5.1.6) stereospecifically directs and greatly catalyzes the cyclization of chalcone into flavanone in the cytoplasm of plant cells. This step can happen spontaneously: CHI can catalyze $10^7$-fold more efficiently (Li et al., 2006) to establish the flavonoid heterocyclic C-ring. In a recent report, artificial CHI suppression in transgenic tobacco plants showed a change of flavonoid components and colors, both in petals and pollen. This result also suggests that CHI plays a major part in the cyclization reaction from chalcone to flavanone, and that the cyclization reaction spontaneously reacts in tobacco plants (Nishihara et al., 2005). The importance of this gene on the flavonoid biosynthesis was proven through the research that CHI overexpressing tobacco plants produced up to five-fold total flavonoid over wild type tobacco and that antisense CHI transgenic tobacco plant accumulated smaller amounts of flavonoids (Li et al., 2006).

In this study, we overexpressed the CHI gene from Chinese cabbage in lettuce plants 'Chungchima' cultivar. Transcription levels were compared by semi-quantitative RT-PCR analysis. We showed that the CHI gene is functionally active in increasing accumulation level of flavonoids and total phenol in $T_1$ transgenic lettuce.

## Materials and Methods

### Plant Material

The cultivars used in this study were 'Chungchima' plants, supplied by Monsanto (Monsanto, USA). Seeds were surface-sterilized with 70% ethanol for 30 sec, followed by the submersion in a 1.0% solution of sodium hypochlorite for 10 min. After sterilization they were washed five times with sterile water and germinated on 1/2 strength MS medium (Murashige and Skoog, 1962) containing 1% (w/v) sucrose and solidified with 0.7% (w/v) agar. The pH of the medium was adjusted to 5.8 before agar addition and autoclaved at 121℃ for 15 min. The seeds are germinated under a 16-h light/8-h dark photoperiod at 25℃.

### Vector Construction

CHI cDNA of Chinese cabbage (*Brassica rapa* sub sp. Campestris, EU402416.1) was provided by National Academy of Agricultural Science in Rural Development Administration. The coding region of the gene was introduced into pFLH-CHI derived from pPZP2Ha3 (+) (Fuse et al., 2001). The complete coding sequence of CHI gene in the supplied vector was amplified with a specific forward primer attached on *Xba*I restriction site and a reverse primer designed to include an *Xho*I restriction site to allow subcloning as follows: forward: 5'- TCT AGA ATG TCT TCT TCC ACT GTC CG -3'; reverse: 5'- CTC GAG TCA GTT CTC TTT GGC CAG TTT -3'. After isolation of the cDNA with the enzyme site attached specific primers, the resulting PCR product was then cloned into the pGEM-T Easy vector (Promega, USA) and sequenced on both directions. The confirmed product was digested and the *Xba*I/*Xho*I fragment was cloned into multi cloning site of pPZP2Ha3 (+) between cauliflower mosaic virus (CaMV) 35S promoter and the NOS terminator, creating pFLH-CHI. These constructs were then introduced into the *Agrobacterium tumefaciens* LBA4404 strain using the electroporation method.

### Generation of the CHI Transgenic Lettuce

Transgenic lettuce plants were generated using the *Agrobacterium*-mediated transformation of cotyledons. The cotyledons were excised from the 6-day-old lettuce seedling and inoculated with *A. tumefaciens*. After inoculation, the cotyledons were cocultivated on MS salt medium solidified with 0.7% agar (Duchefa, the Netherlands), for 2 days at 25℃ in the dark. The cotyledon explants were then transferred to selection medium for shoot induction. These explants were cultured (seven per plate) on selection medium which was a MS basal medium supplemented with 10 mg·$L^{-1}$ hygromycin, 250 mg·$L^{-1}$ cefotaxime, 0.1 mg·$L^{-1}$ NAA, 0.5 mg·$L^{-1}$ BAP, and 10 mg·$L^{-1}$ cystein (Lee et al., 2004). The explants were subcultured every 4 weeks on the same medium. Shoots that regenerated from explants were rooted in 1/2 strength MS medium containing cefotaxime 250 mg·$L^{-1}$, hygromycin 5 mg·$L^{-1}$ and agar 7 g·$L^{-1}$. The transgenic plants were acclimated before transfer to the greenhouse.

### Genomic DNA PCR Analysis

Prior to semi-quantitative RT-PCR, insertion of T-DNAs was corroborated by genomic DNA PCR. Total genomic DNA was isolated from the leaf tissue of CHI transgenic plants using Exgene plant SV mini kit (GeneAll, Korea) following given instruction. PCR were performed with *TaKaRa LA Taq*™ (TAKARA BIO INC., Japan); 94℃ for 5 min followed by 30 cycles at 94℃ for 1 min, 52℃ for 1 min, 72℃ for 1 min, with a final extension at 72℃ for 10 min.

### Determination of Gene Transcript Levels by Semi-quantitative RT-PCR Analysis

The transcription levels of CHI gene were analyzed by semi-quantitative reverse transcription (RT)-PCR. Total RNA

was extracted from the transgenic and wild-type lettuce leaves using RiboEx (GeneAll, Korea), following the provided instructions. Single-strand cDNA synthesis of inserted CHI was carried out using 1 μg of total RNA, iScript™ cDNA synthesis Kit (Bio-rad, Canada) and oligo (dT) and random primer following the given direction. To estimate the transcript levels of inserted CHI in lettuce, we performed PCR using cDNA with the same gene specific primers as mentioned above. As control, 18S rRNA primers (forward: 5'- GGA TGG GTC GGC CGG TC -3'; reverse: 5'- CAG GCT GAG GTC TCG TTC -3') were used. The PCR reaction conditions for amplification were 94℃ for 5 min followed by 22 cycles at 94℃ for 1 min, 52℃ for 1 min, 72℃ for 1 min, with a final extension at 72℃ for 10 min. The PCR products were resolved on a 0.9% agarose gel, stained with ethidium bromide, and photographed.

### Screening of the T1 Generation CHI Transgenic Lettuce

$T_1$ generation of transgenic lettuce were seeded on a selection medium which was a MS basal medium supplemented with 12.5 $mg \cdot L^{-1}$ hygromycin, The transcription levels of selected $T_1$ generation lettuces were analyzed by semi-quantitative reverse transcription (RT)-PCR following the same methods mentioned above.

### Southern Hybridization Analysis

Southern hybridization was performed to confirm inserted T-DNA copy number into genomic DNA of the CHI transgenic plants. Total genomic DNA was isolated from $T_1$ generation leaf tissue of CHI transgenic plants using Exgene plant SV mini kit (GeneAll, Korea) following given instruction. The genomic DNA, digested with restriction enzymes, *Xba*I, for overnight at 37℃, was separated by electrophoresis and transferred to a Hybond™-N+-nylon membrane (Amersham, England). Introduced DNA was detected using a protocol of the Rediprime II DNA Labeling System (Amersham, England). The primers used for the CHI probe was amplified with the same gene specific primers as used above; 94℃ for 5 min followed by 30 cycles at 94℃ for 1 min, 52℃ for 1 min, 72℃ for 1 min, with a final extension at 72℃ for 10 min.

### Determination of Total Flavonoid and Total Phenol Contents

For extraction of flavonoids and phenols, the CHI transgenic lettuce $T_0$ and $T_1$ generation samples were ground with liquid nitrogen. The 10 mg of samples were extracted with 1.5 ml of 70% aqueous methanol and sonicated for an hour. To extract flavonoids, the extracts were centrifuged for 4 min and then filtered with syringe filter. To extract phenols, the extracts were centrifuged for 4 min and then filtered with syringe filter. 2 mL of 2% $Na_2CO_3$ were added to 200 μL of the extracts and reacted for 2 min; then, 50% Foling & Ciocalteu's Phenol Reagent (Sigma, USA) 100 μL was added and reacted for 30 min. Absorbance for total flavonoid was measured by UV/VIS spectrophotometer at 280 nm wave length on a Sinco S-4100 (Kyoto, Japan). As a standard, quercetin (Sigma, USA) was used. Absorbance was also measured at 750 nm wave length and (+)-catechin (Sigma, USA) was used for standard of total phenol.

## Results and Discussion

### Development of Transgenic Lettuce with the CHI Gene

To overexpress CHI gene, lettuce 'Chungchima' was transformed with pFLH-CHI T-DNA (Fig. 1). The cotyledons were excised from the 6-day-old lettuce seedling and inoculated with freshly grown *A. tumefaciens*. The explants were cultured on co-cultivation medium. After 2 days, the explants were transferred onto selection medium and then shoots were induced four to six weeks later. Subculture was performed every four weeks. Regenerated shoots were transferred to rooting media. The plantlets were acclimated to soil and grown in a glasshouse to mature plant. There were no significant differences detected on the CHI transgenic lettuce plants compared with wild type lettuce. The samples were collected and stored at -80℃, and later used for identification of transgenic plants. The introduced exogenous CHI gene was analyzed by genomic DNA PCR using gene specific primers. CHI transgenic plants showed 756 bp PCR products. Nine of the CHI transgenic plants (1, 2, 3, 4, 5, 6, 7, 9, and 10) were confirmed with PCR products (Fig. 2). CHI gene was not detected in wild type 'Chungchima' lettuce.

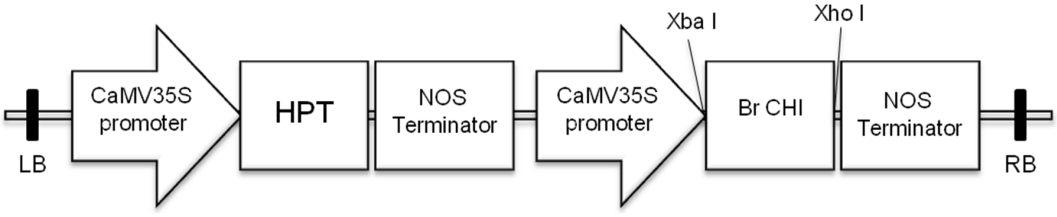


**Fig. 1.** Schematic drawing of the lettuce pFLH-CHI overexpression vector construction. Transgene expression with restriction enzyme site *Xba* I and *Xho* I was under control of the CaMV 35S promoter and terminated by nos terminator.

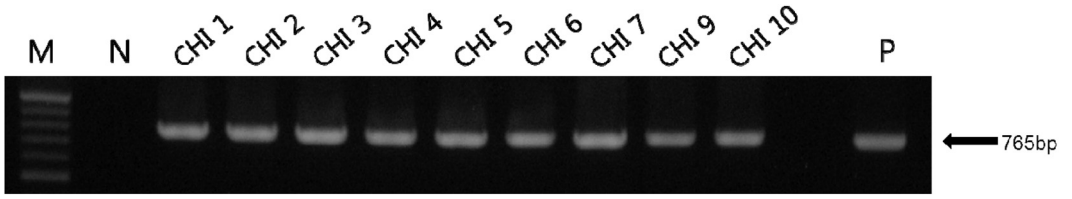


**Fig. 2.** Genomic DNA PCR analysis of CHI overexpression $T_0$ transgenic lettuce plants. M, Size marker (100 bp DNA ladder); N, 'Chungchima' lettuce; P, pFLH-CHI vector plasmid.

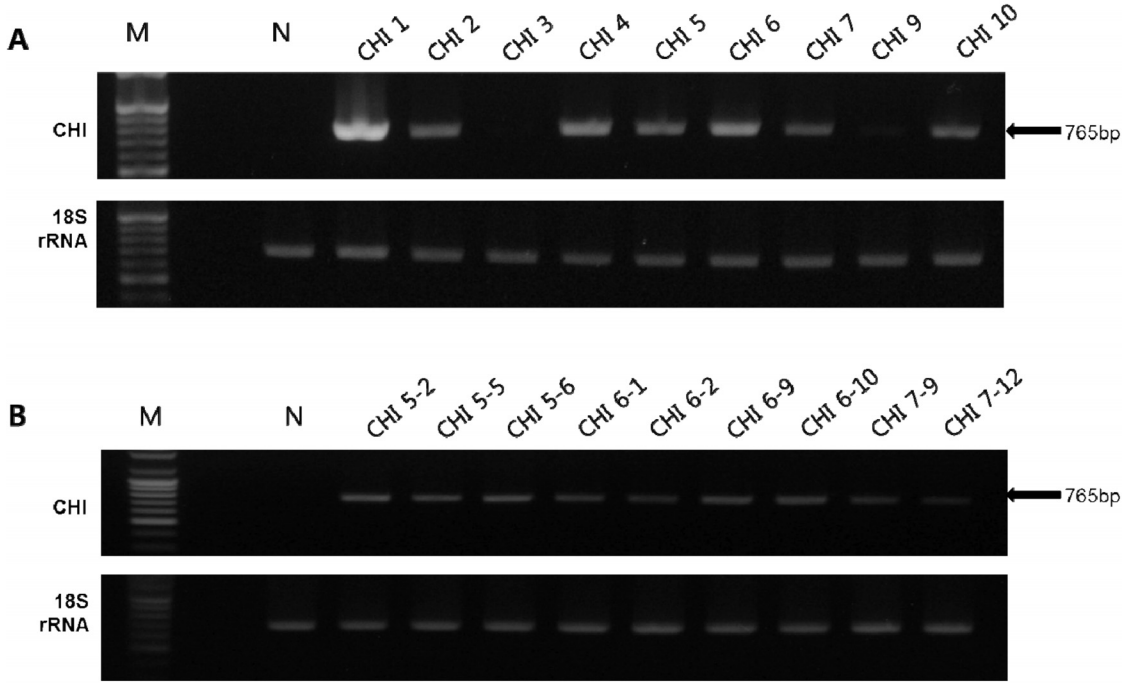


**Fig. 3.** Semi-quantitative RT-PCR analysis confirming the transcription of the CHI genes. A, Transcription level of $T_0$ generation. The CHI gene indicated in the transgenic lettuce lines and not in the CHI3, CHI 9 and wild-type (WT) plants; B, Transcription level of $T_1$ generation. 18S rRNA was used as the internal cDNA control. M, Size marker (100 bp DNA ladder); N, 'Chungchima' lettuce.

### Expression Analysis of the CHI Gene in Transgenic Lettuce Plants

To confirm the expression of the exogenous CHI gene in the transformed lettuce, total RNA was extracted from the leaves of four-week-old wild-type and transformed lettuce plants ($T_0$ generation) and analyzed by semi-quantitative RT-PCR analysis. Transgenic plants were confirmed by genomic DNA PCR and by subsequent RT-PCR with gene specific primers mentioned above.

Semi-quantitative RT-PCR analysis of $T_0$ generation showed that the exogenous CHI gene was expressed in CHI-1, CHI-2, CHI-4, CHI-5, CHI-6, CHI-7, and CHI-10 lines (Fig. 3A). 18S ribosomal RNA levels were used as normalization control. CHI-1, CHI-4, and CHI-6 showed especially high levels of gene expression. Semi-quantitative RT-PCR analysis of $T_1$ generation showed high expression level at CHI 5-2, CHI 5-5, CHI 5-6, CHI 6-9, and CHI 6-10 lines (Fig. 3B). Non-transcription of the CHI gene in lanes 3 and 9 could be

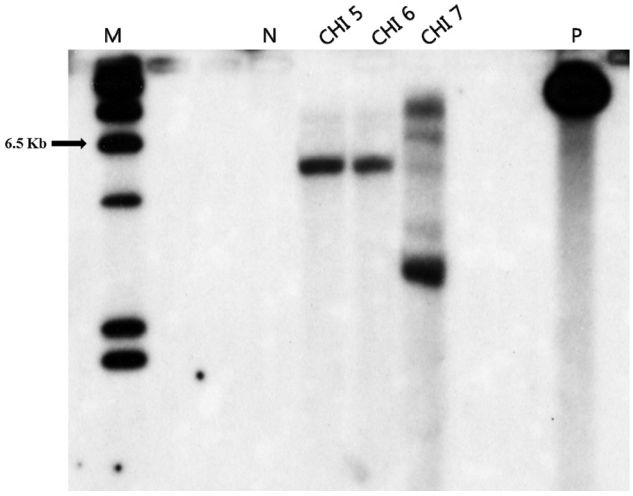


**Fig. 4.** Southern blot analysis of CHI overexpression transgenic plants. Genomic DNA was digested with *Xba*I and probed with [$^{32}$P]-labeled 756 bp fragment of CHI gene. M, Size marker (λ DNA/Hind III Marker); N, Non-transgenic lettuce; P, CHI positive control.

caused by silencing or because insertion site could be in the heterochromatin area (Avramova, 2002) or intron region (Vaucheret and Fagard, 2001). Also, it is possible that the integration of our binary vector is not intact; a portion of terminator and/or promoter may be missing (Kim et al., 2003). Southern blot analysis showed that non-transgenic lettuce did not detect CHI gene signal whereas transgenic lines did. We confirm that a single copy gene was inserted into CHI-5 and CHI-6 lines of $T_1$ generation (Fig. 4).

### Total Phenol and Total Flavonoid Contents Analysis

Total phenol and total flavonoid contents were analyzed by UV/Vis spectrophotometer (Sinco S-4100, Japan). Phenols were detected at 750 nm wave length and total flavonoids were detected at 280 nm wave length. 10 mg Fw of samples were used for the analysis and each line was measured three times. Total flavonoid contents of $T_0$ generation were increased at CHI-6 and CHI-7 by over 1.4 to 1.6 folds and flavonoid levels were increased in CHI-5 by over 1.3 folds. On the other side, total phenol contents decreased in CHI transgenic lines (Table 1). Furthermore, total flavonoid contents of $T_1$ generation were noticeably increased in CHI-5-2, CHI-5-5and CHI-6-10 lines by over 3.4 to 4 folds (Table 2). The phenol levels of $T_0$ generation were slightly decreased compared with the control (Table 1). However, the phenol contents of $T_1$ generation showed increasing tendency of up to 3.5 folds (Table 2), a trend similar to flavonoid contents increase.

In previous researches on total flavonoid contents of lettuce, the total flavonoid contents varied widely depending on their cultivars and the part of the samples. Aglycon, as one unit of flavonoid, was in the ranges of 0.3-229 $\mu g \cdot g^{-1}$ FW in varieties of lettuce (DuPont et al., 2000). Conjugated quercetin content also showed similar pattern. The whole lettuce of 'Round' lettuce var. Cortina, 'Green Salad Bowl', and 'LolloBionda' var. Cerieo showed 11 ± 0.5 $\mu g \cdot g^{-1}$ FW, 147 ± 5.2 $\mu g \cdot g^{-1}$ FW, and 94 ± 4.6 $\mu g \cdot g^{-1}$ FW in the conjugated quercetin content, respectively. Furthermore, the inner leaves of 'Marvel of Four Seasons' showed 22 ± 2.3 $\mu g \cdot g^{-1}$ FW and its outer leaves showed 911 ± 27 $\mu g \cdot g^{-1}$ FW (Crozier et al., 1997).

According to USDA database released in 2007, the concentration of quercetin used as a standard material of this research was 57.18 ± 0.52 $\mu g \cdot g^{-1}$ FW on green leaf (*Lactuca sativa* var. *crispa*), which is similar to 'Chungchima' (USDA, 2007).

Various earlier studies on CHI genes showed interesting

**Table 1.** Total flavonoids and total phenol contents of $T_0$ transgenic lettuce.

| Lines | Flavonoids ($\mu g \cdot g^{-1}$ Fw) | Contents variation ratio (%) | Phenols ($\mu g \cdot g^{-1}$ Fw) | Contents variation ratio (%) |
|---|---|---|---|---|
| WT | 58.33 ± 0.52[z]c[y] | 100.00 | 45.63 ± 1.23 ab | 100.00 |
| CHI4 | 63.80 ± 3.46 c | 109.37 | 41.88 ± 0.46 c | 91.78 |
| CHI5 | 80.43 ± 6.86 b | 137.89 | 45.57 ± 0.91 ab | 99.85 |
| CHI6 | 96.77 ± 5.19 a | 165.89 | 48.57 ± 1.06 a | 106.43 |
| CHI7 | 84.73 ± 5.92 ab | 145.25 | 43.03 ± 2.26 bc | 94.29 |

[z]Mean ± SD (n = 3).
[y]Mean separation within columns by Duncan's multiple range test at $P$ = 0.05.

**Table 2.** Total flavonoids and total phenol contents of $T_1$ transgenic lettuce.

| Lines | Flavonoids ($\mu g \cdot g^{-1}$ Fw) | Contents variation ratio (%) | Phenols ($\mu g \cdot g^{-1}$ Fw) | Contents variation ratio (%) |
|---|---|---|---|---|
| WT | 58.21 ± 4.662[z]i[y] | 100.00 | 57.07 ± 3.10 i | 100.00 |
| CHI5-2 | 199.97 ± 5.09 c | 343.53 | 165.63 ± 4.37 c | 290.22 |
| CHI5-5 | 205.10 ± 4.50 b | 352.34 | 175.95 ± 3.68 b | 308.29 |
| CHI5-6 | 172.43 ± 5.47 d | 296.22 | 85.52 ± 3.87 fg | 149.85 |
| CHI6-1 | 154.61 ± 4.05 e | 265.61 | 74.45 ± 2.92 h | 130.45 |
| CHI6-2 | 132.49 ± 4.17 f | 227.61 | 151.61 ± 3.48 d | 265.66 |
| CHI6-9 | 154.70 ± 4.36 e | 265.76 | 138.38 ± 2.71 e | 242.47 |
| CHI6-10 | 237.75 ± 5.93 a | 405.00 | 202.64 ± 4.22 a | 355.07 |
| CHI7-9 | 103.32 ± 5.67 g | 177.50 | 87.50 ± 2.87 f | 153.32 |
| CHI7-12 | 85.33 ± 4.75 h | 146.59 | 84.43 ± 3.02 g | 147.94 |

[z]Mean ± SD (n = 15).
[y]Mean separation within columns by Duncan's multiple range test at $P$ = 0.05.

results. Petunia CHI overexpression in tomato showed increase of flavonol contents of up to 78 fold in fruit peel (Muir et al., 2001). Also, overexpression of the *Saussurea medusa* CHI gene has been reported to increase total flavonoid contents up to 4 times in *S. involucrate* hairy root (Li et al., 2006). Petunia Po mutant lines, which are chiA gene inserted into popo lines isolated from Po, is a mutation which abolished chiA promoter activity by its regulatory region. As a result, the pollen color of Po mutant changed from yellow to white (Tunen et al., 1991). Inactivation of CHI caused by a frame shift, caused by a premature stop codon and a following single base-pair addition, results in the flavonoid pathway blocking and the accumulation offshoots of chalcone including a yellow pigment in gold color onion (Kim et al., 2004). CHI-suppression by RNA interference showed change of flavonoid components in flower petals and accumulated high levels of chalcone, showing a yellow color in pollen of tobacco plant (Nishihara et al., 2005). Transcription factors that control the expression of the structural genes and the accumulation of the metabolites (Park et al., 2008) were the interest subject in recent study. Transcription factor PAP1 regulates PAL, CHS, and dihydroflavonol 4-reductase (DFR) structural genes acting as activators (Borevitz et al., 2000). Furthermore, Arabidopsis PAP1 was expressed in canola; it showed increase of antioxidant capacity, cyanidin and pelargonidin levels, and guercetin and sinapic acid levels up to 4 fold, 50 fold and 5 fold, respectively (Li et al., 2010). As a repressor, overexpressed AtMYB60 gene in lettuce plants disturbs anthocyanin accumulation and plays a significant role in controlling anthocyanin biosynthesis during the transcription of the DFR gene (Park et al., 2008). Taken together, these results indicate that CHI gene has an important role in the flavonoid biosynthesis pathway in controlling the derivative accumulation.

In this study, CHI gene from Chinese cabbage was overexpressed in lettuce plants 'Chungchima' cultivar. Transcription levels were compared by semi-quantitative RT-PCR analysis. The total flavonoid accumulation of overexpressed CHI $T_1$ transgenic lines showed an increase of up to 4 fold whereas total phenol contents were increased up to 3.5 fold. To our knowledge, this is the first study in which flavonoid accumulation was confirmed in a lettuce plant cultivar overexpressed with Chinese cabbage CHI gene.

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