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ELEVATION OF THE FLAVONOID CONTENT IN GRAPEFRUIT BY INTRODUCING CHALCONE ISOMERASE GENE VIA BIOTECHNOLOGICAL METHODS

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Abstract

Flavonoids have numeruos biological and pharmacological effects shown by in vitro and in vivo studies. In addition to consumption of flavonoids by diet they are taken as tablets or dry extract capsules to have health benefits especially for their antioxidant and immunostimulant effects. The main goal of the study is to transfer flavonoid pathway genes to Citrus in order to increase the levels of characteristic flavonoid compounds. In this study the second gene, chalcone isomerase (CHI), from the flavonoid pathway was isolated and transferred succesfully to grapefruit plants via Agrobacterium mediated gene transfer method that is one of the biotechnological method. Transgenic plants, which were obtained have elevated flavonoid content compared to control plants.

Keywords: *Citrus flavonoids, chalcone isomerase, flavonoid pathway, transformation, gene transfer*

Greyfurt Flavonoid İçeriğinin Kalkon İzomeraz Geni'nin Biyoteknolojik Yöntemlerle Tanıtılmasıyla Artırılması

Flavonoitler gerek in vitro gerekse in vivo kanıtlanmış sayısız biyolojik ve farmakolojik aktiviteye sahiplerdir. Flavonoitler diyetle vücuda alınmaları dışında antioksidan ve immunostimulan etkilerinden dolayı tablet veya kuru ekstre kapsülleri şeklinde kullanılmaktadır. Çalışmanın temel amacı flavonoit yolağındaki genleri narenciye türlerine aktararak narenciye türlerine özgü flavonoit bileşiklerini artırmaktır. Bu çalışmada flavonoit yolağındaki ikinci gen, kalkon izomeraz biyoteknolojik metodlardan birisi olan Agrobacterium gen transfer yöntemi kullanılarak başarılı bir şekilde greyfurt'a transfer edildi. Kontrol bitkileriyle karşılaştırıldıklarında daha yüksek flavonoit miktarına sahip transgenik bitkiler elde edildi.

Anahtar Kelimeler: *Narenciye flavonoitleri, kalkon izomeraz, flavonoit yolağı, transformasyon, gen transferi*

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INTRODUCTION

Plants have been utilized to treat disease and disease symptoms for their biologically active phytochemicals. Flavonoids have variety of pharmacological effects including antioxidant, anticancer, anti-inflammatory, hepatoprotective and immunostimulant (1, 2, 3, 4). Moreover, studies revealed that flavonoid rich diet reduces cholesterol and improves conditions of cardiovascular system (5). Elevated demand on phytochemical remedies open the search for flavonoid sources for the mass production of flavonoid compounds to use in commercial preparations. For this purpose plants containing high levels of flavonoids were preferred for the mass extraction. There are limited plants, which produce desired levels of flavonoids. Collecting plants from wild that produce flavonoids, and utilizing them for direct extraction is easier in short term but it may cause to reduction of the origin even though it may put the plant in danger of extinction.

Metabolic engineering by means of transformation of target genes to produce desired product/products in plants is one of the biotechnological methods, which is in use for pharmaceutical applications (6). Developments in biochemistry and molecular biology open the door for gaining more information about the enzymes and genes of biosynthetic pathways of secondary metabolites.

Flavonoid pathway is one of the most studied biosynthetic pathways in plants (7). The early steps of the pathway are similar, and sequences of the genes are highly conserved among plants (Figure 1). The genes that function early in the pathway have been cloned and exploited to alter the phytochemicals in different plant species (6, 7, 8). The first step of the pathway is catalyzed by Chalcone synthase (CHS), which utilizes three molecules of malonyl CoA and one molecule of p-coumaryl CoA to produce naringenin chalcone. The second step is the formation of flavanone, in which flavanone occurs either spontaneously or with the help of chalcone isomerase (CHI) (9). From this point on, the genes that predominantly function in the pathway diverge depending upon the kinds of flavonoids that accumulate in plants. Putative flavonoid pathway in citrus was demonstrated in Figure 1 (10).

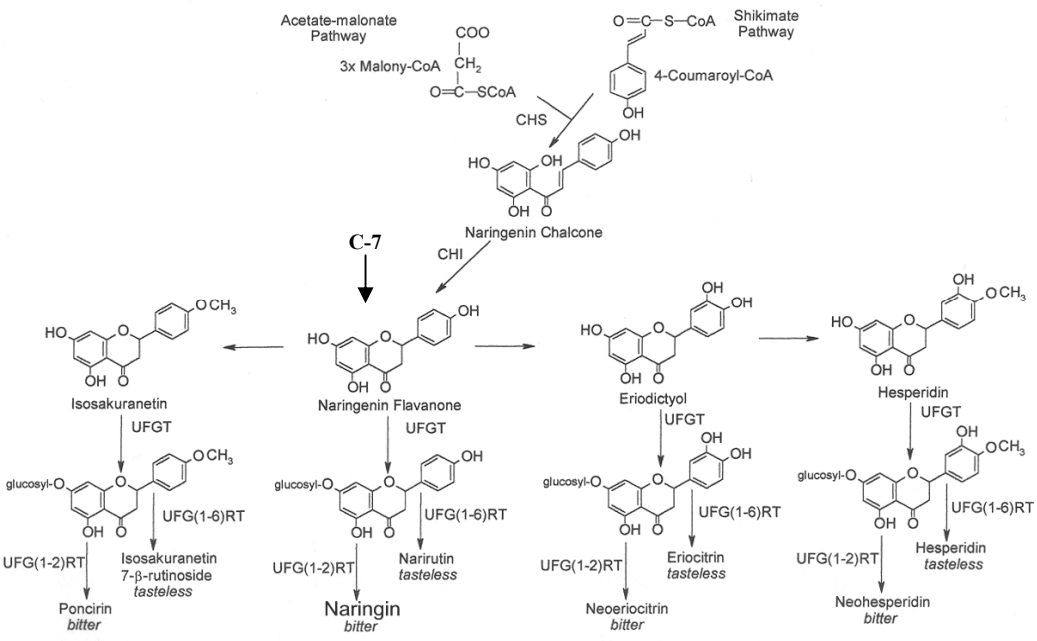


Figure 1. Proposed flavonoid biosynthetic pathway in citrus leading to flavanone glycosides (slightly modified from Rouseff (1987), CREC, University of Florida)

Flavanones are the abundant flavonoids in citrus (9). They tend to occur in glycoside form, in which a disaccharide is attached to the aglycone through the C-7 hydroxyl group. The disaccharide can be *ß*-rutinose, which is tasteless, or *ß*-neohesperidose, which is bitter (11). The main goal of the study is to increase flavonoid content in citrus species by transferring selected flavonoid pathway genes. First of all, CHS gene was transferred to grapefruit plants (data is not shown), unfortunately most of the plants died, only some of the transgenics survived (12). The aim of this study is to increase the flavonoid levels in citrus species by means of transferring CHI. Grapefruit has been chosen as a model plant, since the transformation protocol was developed in our laboratory (13).

EXPERIMENTAL

Previously, CHI cDNA was isolated from grapefruit (13). Sense and antisense constructs of the genes were transferred to grapefruit stem segments (13).

Agrobacterium strains and vector plasmids

Two transformation plasmids were utilized for our gene transformation experiments. Vector plasmids, CHI in both the sense (CHI-S) and antisense (CHI-AS) directions had been constructed in our laboratory (12). Additionally, vector plasmids pGA482/GG, containing only a GUS (beta-glucuronidase) reporter gene, exploited in transformation experiments for control purposes. This system was chosen because of its availability and verified effectiveness in citrus transformation experiments (14). *Agrobacterium* strain EHA101 was employed (14). EHA101 contains a disarmed pTiBo542 that is a hypervirulent tumor inducing plasmid, which has the vir components required for infection and integration into plant cell nuclei.

Plant material, transformation and regeneration

C. paradisi Macf. cv.Duncan (grapefruit) seeds were locally purchased, after the seed coats were removed they were sterilized in 70 % ethanol for 3 min, followed by 0.525 % sodium hypochloride for 10 min, then the seeds were rinsed with sterile water for 3 times (13). The seeds were placed individually into 15 x 20 cm tubes, which contained *in vitro* germination medium. The tube racks were kept in the dark for 4-6 weeks. Germinated-etiolated seedlings were employed in transformation experiments. The EHA101 *Agrobacterium* cultures that harbored pGA482/GG with the genes of interest were grown in YEP (yeast extract-peptone) medium with 60 mg/L gentamycin (Sigma Chemicals, St Louis, MO) and 50 mg/L kanamycin (Fisher Biotech Fairlawn, NJ) to an OD620= 0.6-1.0. Cultures were centrifuged at 3500 rpm for 5 min. The pellet was resuspended to a cell density of $5x10^8$ - $5x10^{10}$ in Murashige and Scoog (MS) medium with freshly made 100 µM acetosyringone added.

Etiolated seedlings were cut into 1 cm segments (13) immersed in *Agrobacterium* resuspended solution. Sixteen segments were placed horizontally onto a 100x15 mm petri plate that contained MSA medium (100 µM acetosyringone plus MS). The plates were kept in darkness for 2-3 days at 28^{o}C to allow *Agrobacterium* infection to take place, then segments were transferred to selection medium under 16h cool-white fluorescent light at 27-28 ^{o}C to induce shooting. The control segments were kept in MSBC media; its composition was the same as selection medium except without kanamycin. The selection medium (MSBCK) contains MS basal salts 4.33 g/L, (Phytotechnology Laboratories, Shawnee Mission, KS) benzyladenine (BA) 0.5 mg/L, kanamycin monosulfate 75 mg/L (Fisher Biotech Fair Lawn, NJ), and claforan 500 mg/L (Abbott Laboratories, Abbot Park, IL). The segments were transferred to fresh selection medium every 4 weeks. When regenerated shoots became 0.3 - 0.5 cm in length, they were tested with a histochemical GUS test (13). GUS-positive shoots were transferred to NAA

(α-naphthalene acetic acid, (Sigma Chemicals, St. Louise, MO) containing (5 mg/L) rooting medium. When the roots became at least 0.5 cm long, the rooted plants were transferred to soil cups containing ½ MS medium that was mixed with Metromix 300 soil (Scotts® Marysville, OH). The cups were covered with plastic wrap and kept in a growth chamber at 28-30 ^{o}C, 16h light/8h dark cycle. A few days later, small holes were opened in the wraps to decrease the extra humidity slowly. When the plantlets well established in soil, they were retested with a histochemical GUS assay. GUS positive plantlets were transferred to pots.

Histochemical GUS assay

A small section from the basal end of each regenerated shoot was cut and placed in a 96-well microtiter plate (Corning Incorporated Corning, NY 14831) containing 30 µl of x-gluc (1 mg/ml 5-bromo-4-chloro-3-indolyl-*β*-D-glucuronide in 0.1 M sodium phosphate buffer, pH 7.0 with Na_2EDTA) per well. The plate was incubated at 37 ^{o}C up to overnight. Then the tissue was washed and fixed with 50 µl 95% ethanol: glacial acetic acid (3:1 v/v). Thirty minutes later, blue sectors were scorable under the microscope.

PCR Analysis

DNA was extracted from the leaves of GUS positive plants by using the DNAzol®ES method (Molecular Research Center, Cincinnati, OH). Only 1 µl DNA extract was employed for each 50 µl PCR reaction. PCR reaction buffer, $MgCl_2$ and Taq polymerase were provided by Promega (Promega Madison, WI). The primer concentration was 1ng for each primer individually in a 50 µl PCR reaction unless otherwise changed for a specific reaction. For the analysis of CHI gene transferred plants, PCR was performed by using GUS forward 5'-TTGGGCAGGCCAGCGTATCGT -3' and reverse 5'– ATCACGCAGTTCAACGCTGAC -3' primers, which give a 400 bp PCR product (12). The cycles were as follows: 94 ^{o}C, 2 minutes, to denature the DNAs; then 29 cycles, 94 ^{o}C, 30 seconds; 55 ^{o}C, 30 seconds; 72 ^{o}C, 50 seconds; afterwards, 72 ^{o}C 5 minutes for final elongation. Additional PCR analysis was performed with CHI primers forward 5'- GGTCGAGAACGTCACTTTCAC-3'and reverse 5'-TTTCATCCTTGAAGACCTCAG -3'. The cycles were as follows: 94 ^{o}C, 2 minutes, to denature the DNAs; then 30 cycles, 94 ^{o}C, 30 seconds; 5 ^{o}C, 30 seconds; 72 ^{o}C, 50 seconds; afterwards, 72 ^{o}C 5 minutes for final elongation. PCR products (12 µl) were applied into a 2 % agarose gel (Gibco-BRL).

HPLC analysis

Leaf tissue of all of the surviving transgenic and control plants was collected at 4 different times of the year. Each time same size leaves were collected in the morning from 10.00 am to 11.30 am. Dried samples were crumbled in vials for further flavonoid extraction. Approximately 250 mg tissue was removed, weighed and extracted with 3 ml, 80% MeOH, 20% DMSO (15). The samples were sonicated at 50^{o}C for 15 minutes, allowed to stand at room temperature for 3 days, and then sonicated again for 5 minutes. The liquid was filtered through a 0.45 micron nylon filter (Alltech Associates, Deerfield, IL) into 1.5 ml auto injector vials. Samples were analyzed with a binary gradient on a Shimadzu (Columbia, MD) 10A system (SIL10A auto injector, two LC10AT pumps, CTO-10A column oven, SCL10A controller) with a Hewlett Packard 1040A diode-array detector (running under Chemstation A.02.05 software). Inertsil C18 reverse phase 4.6 mm x 250 mm column (5 micron, ODS 3, Varian, Torrance, CA) was employed. The initial column conditions were 20% 0.01 M phosphoric acid and 85% methanol at a flow rate of 1 ml/minute with the column oven set at 40 ^{o}C. The effluent was monitored at 285 nm. Two minutes after the initial conditions, the column was developed with a linear gradient to 100% methanol over 50 minutes. Standard curves were prepared from stock solutions of naringin for the flavanones and rhoifolin for flavones. Peaks were identified by

characteristic spectra and relative retention times from comparison to standards and/or LC-MS analysis.

Statistical Analysis

Mann-Whitney U statistical test was applied to CHI-AS vs. CHI-S, CHI-AS vs. total control, and CHI-S vs. total control HPLC data. In the statistical data $p<0.05$ was considered significant.

Southern Blot analysis

Transgenic plants were first identified with GUS histochemical tests then GUS and NPTII gene primers were used in PCR reactions to confirm that the plants were stably transformed. PCR positive plants were analyzed by Southern blot analysis to verify the transferred gene and to estimate e thtransgene copy number (12).

In pGA482GG, the transgene of interest was inserted between the NPTII and GUS genes. Therefore, NPTII (300 bp) (Neomycin phosphotransferase) antibiotic resistance gene for genetic selection of the transformed cells, and GUS (780 bp) probes were selected to estimate transgene numbers in CHI S/AS transgenic plants via Southern blot analysis. *Hpa*I was chosen as a restriction enzyme, because CHI cDNAs do not have a site for this enzyme and it cuts the T-DNA once between the GUS and NPTII genes. As a result, bands hybridizing with these two probes give an estimate of T-DNA copy numbers. Southern blot analysis was completed on a total of 15 CHI transgenic plants.

Probing the blots with both genes indicated that 1-3 copies of the transgenes were integrated into most of the transgenic plants. Copy numbers were not estimated for the transgenic plants 23, 60 and 61, because either the plant died too soon and/or the tissue was not adequate to extract DNA or the plant was chimeric for the GUS or NPTII gene and the probe did not find the target.

RESULTS AND DISCUSSION

Previously CHI cDNAs was isolated from Marsh grapefruit (*Citrus paradisi*) in our laboratory. Sense and antisense plasmid constructs were prepared from cDNAs of CHI to utilize in grapefruit transformation experiments. In order to transfer CHI gene constructs, seeds of Duncan grapefruit were peeled sterilized and planted into selected media (Figure 2, 1-4). Planted seeds were incubated in dark for 3-4 weeks to obtain etiolated seedlings which are easy to utilize for the transformation experiments (Figure 2, 4-5). Seedling segments were incubated in *Agrobacterium* suspensions (Figure 2, 6-7), which contain CHI sense or CHI antisense plasmid constructs separately, then, they were transferred to the selection medium, which contains kanamycin (Figure 2, 8). A small section of the shoots were tested by GUS histochemical test to observe whether they were transformed (Figure 2, 9-10). Only GUS positive shoots were transfered to root inducing media (Figure 2, 11). Rooted shoots were transferred to soil, when they grew larger, sequentually they were transferred to the bigger pots (Figure 2, 12-14). Same vector plasmid (pGA482GG) that does not contain the target gene was transferred to grapefruit to see the effects of the plasmid construct on the transformation efficiency. Only plasmid transferred plants were named as positive control. Additionally, grapefruit seedlings that were not transferred with a gene or a plasmid named negative controls. These seedlings were treated under the same tissue culture condition with the gene transferred plants. Segments and shoots on the stem segments were counted for each experiment (Table 1). Morphological features of the transgenics and the controls do not differ from each other, except the transformed plants looked healthier than the controls.

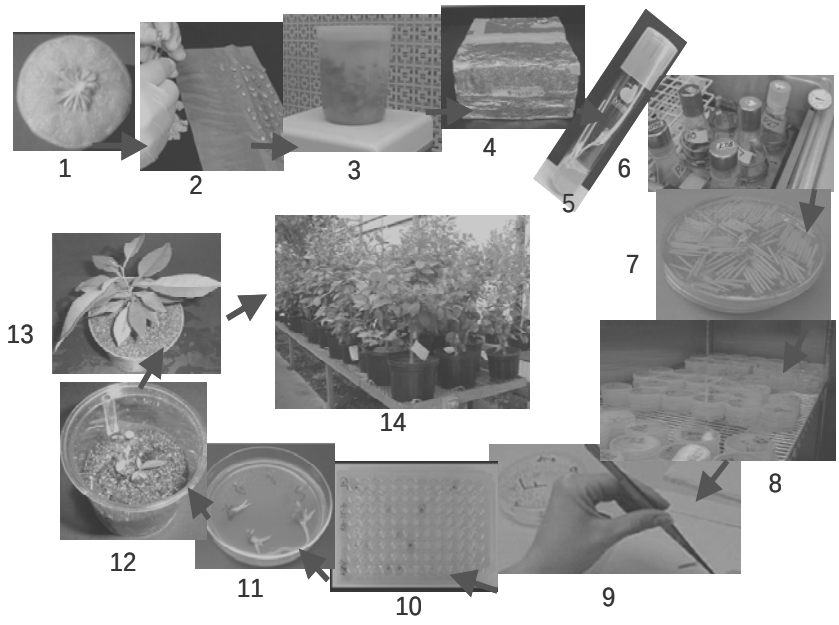


Figure 2. *Agrobacterium* mediated CHI-S/AS gene transformation process in grapefruit seedlings (12)

Transformation efficiency was shown in Table 1. Unfortunately, some transgenic shoots died before they were analyzed for their flavonoid content. The severe effect of the transgene or the weak features of the shoots might be the reason for their death. Transformation efficiency was estimated 43% for the positive control whereas it is estimated average of 29.12% for the total CHI constructs that might be due to the increase of the physical size of the plasmid vector with the gene. Addition of the gene to the vector plasmid might have decreased the efficiency or the detrimental feature of the gene. On the other hand transfromation efficiency of the CHI constructs (CHI-S, 27.49% and CHI-AS, 31.45%) might be considered as succesful compared to chalcone synthase (CHS) gene constructs (CHS-S 2.26% and CHS-AS 21.03%), which is the first gene in the flavonoid pathway (12).

Table 1. Constructed plasmids were utilized to transform grapefruit (*Citrus paradisi* cv. Duncan) seedlings. pGA482GG: Plants transformed with the plasmid without target genes (CHI or CHS). Negative control: Plants were not transformed with the plasmid.

Construct	Total number of segments	% Segments with shoots	Total number of shoots produced	Number of GUS positive shoots	% GUS positive shoots
CHI-S	3866	12.00	451	124	27.49
CHI-AS	7052	8.68	569	179	31.45
pGA482GG	4624	3.00	151	65	43.04
Negative control	192	99.00	394	0	0.00

In addition, grapefruit plants were transformed with several sequences from citrus tristeza closterovirus by using three different plasmid vectors (16). Average of 17% GUS positive shoots were obtained from these transformation experiments, which indicates the transformation efficiency of grapefruit as 17%. These results might indicate that even though transformation efficiency depends on the transferred gene, plant and the construct, citrus is not considered as an easy target for the transformation experiments.

Rooted plants were initially tested by GUS test to confirm that the gene was transferred to the plant. Plantlets, positive for the histochemical GUS test were further analysed by PCR to detect the presence of transgene (Figure 3). Forward and reverse primers were designed to amplify GUS and CHI transgene specific primers. Figure 3 represents the gene transferred plants shows a 420 bp band corresponding to the A) GUS and 400 bp band corresponding to the B) CHI transgene. Molecular weight marker was run with the samples to show the size of the bands. In figure 3-A), negative control (C) was applied into the gel to show that negative control plant does not have the transgene. Positive control plant (BL) was applied to show that they contain the transgene too. Plasmid was applied onto gel to show that the plasmid contain the transgene too. In figure 3-B) negative and positive control do not include the transgene CHI, therefore did not show a band corresponding to the CHI transgene. Plasmid pGA482GG does only have GUS but no CHI transgene.

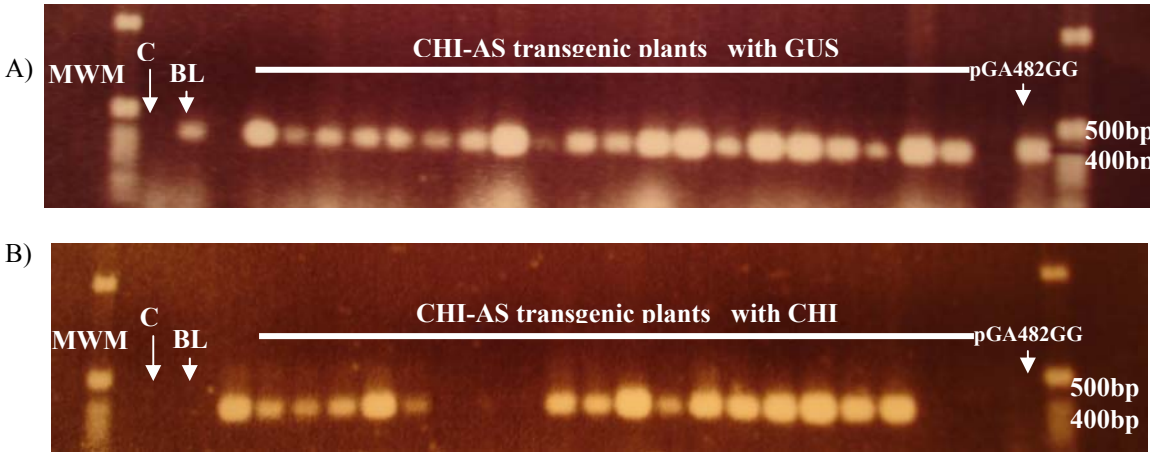


Figure 3. PCR Analysis of the chalcone isomerase antisense (CHI-AS) transgenic plants. MWM (molecular weight marker), C (negative control plant), BL (positive control plant), pGA482 (plasmid construct) A) with GUS primers, B) with CHI primers.

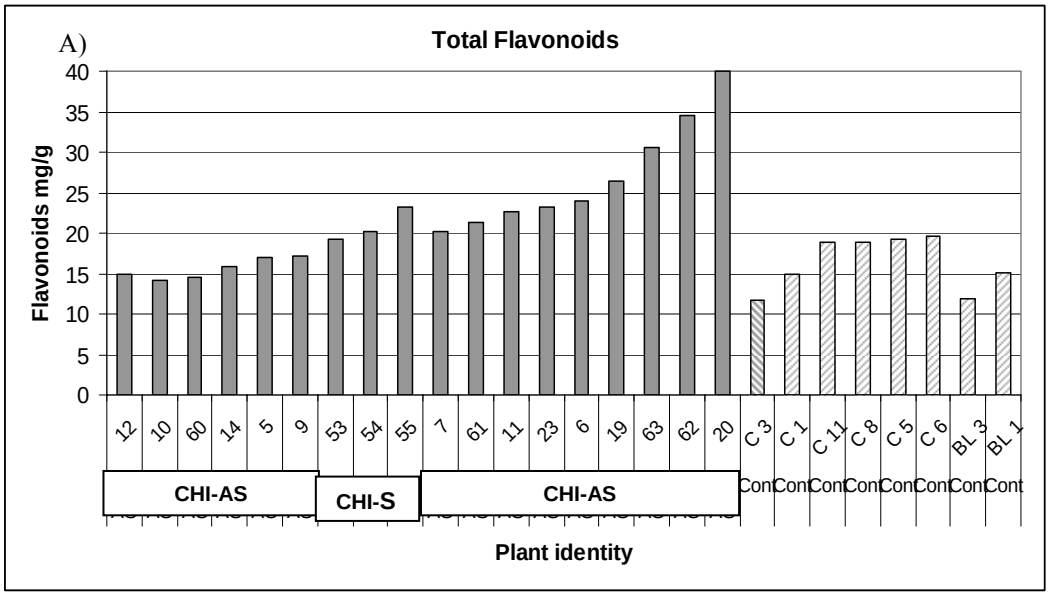
PCR positive plants were further analyzed by Southern blot analysis to observe the transgene and the transgene copy numbers. NPTII and GUS probes were used to estimate the CHI transgene copy numbers. CHI-S transgenic plant numbers 53, 54, 55 have 1 transgene copy number, CHI-AS transgenic plant numbers 6, 7, 9, 14, 62 have 1 transgene copy number, plant numbers 10 and 62 have 2 transgene copy numbers, and 5, 11, 12-13, 19, 20 have 3 transgene copy numbers. No correlation was observed between transgen copy number and the flavonoid content.

Regenerated plantlets were further analyzed by HPLC for their total flavonoid levels (Figure 4). Flavonoid contents (mg/g) of the transgenic grapefruit plants were compared to negative (C) and positive (BL) control plants.

Comparisons showed that most of the CHI antisense (CHI-AS) transgenic plants have higher flavonoid levels than the all control plants (Cont). On the other hand just a few CHI transgenic plants had higher flavonoid levels than the controls (Figure 4-A). For the statistical analysis Mann-Whitney U test was applied on HPLC data of transgenics and controls to demonstrate significance of the flavonoid contents (Figure 4-B).

Average flavonoid content of CHI-AS transgenic plants were compared to CHI-S transgenic plants, no significant difference ($p=0.9624$) was observed between them. Conversely, comparison of the average flavonoid contents of transgenics to controls showed significant difference ($p<0.05$). Consistent with the statistical analysis, CHI-AS transgenic plants had higher levels of flavonoids ($p=0.0094$) than the CHI-S transgenic plants ($p=0.033$) compared the total flavonoids of the controls.

Flavonoid pathway has been target of genetic engineering especially in flowering plants since the genes of the pathway are well known and the results could be observed (17). Flavonoid pathway genes were first utilized to change the flower color of petunia (18). Other ornamentals followed petunia. Afterward, the pathway was manipulated to obtain tomatoes producing high levels of quercetin and kaemferol flavonoids (19). CHI gene transfer elevated the flavonoid levels in the peel of tomato higher than in the flesh. Citrus plants, specifically grapefruits do not produce fruits before they become 5-7 years old. Therefore, flavonoid contents of the transgenic plants were analyzed in the leaves. According to the best of our knowledge this is the first paper showing that CHI gene transformation was able to increase the flavonoid levels in grapefruit leaves.



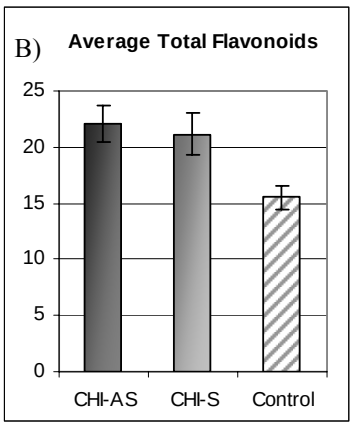


Figure 4. A) Comparison of chalcone isomerase antisense (CHI-AS), chalcone isomerase sense (CHI-S) transgenic grapefruit plants and control plants for their total flavonoid levels (mg/g) B) Statistical comparison of the average flavonoids (mg/g) of CHI-AS, CHI-S transgenics and total control plants.

CONCLUSION

Flavonoids are the largest group of secondary metabolites, which have numerous pharmacological effects, mostly, because of their antioxidant properties. Studies shown that flavonoid rich diet can improve and protect cardiovascular system, inhibit cancer proliferation and act as natural immunostimulant. Therefore, increasing flavonoid content in diet or consuming as preparations will help to improve the health conditions of individuals. In this study we demonstrated that flavonoid content can be increased in grapefruit by utilizing a flavonoid pathway gene, CHI. Interestingly, CHI-antisense construct transferred transgenic plants demonstrated higher flavonoid levels than that of CHI-sense construct transferred plants and the controls. In the pathway, catalyzes of chalcone isomerase step is unique, since this step can occur spontaneously too, without using the CHI enzyme for the ring closure and the production of Naringenin flavanone. This unexpected result might be due to the unique future of the CHI enzyme. Further studies should be conducted. The protocol should be considered as successful for CHI gene. Therefore, this might be applied to other citrus species to increase the flavonoid compounds in citrus for direct or commercial consumption.

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