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Cover Page**

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Project Title: Study and manipulation of the flavonoid biosynthetic pathway in citrus for flavor engineering and seedless fruit.

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Keywords *not* appearing in the title and in order of importance. Avoid abbreviations.

Flavanone; Neohesperidoside; Naringin; Fruit flavor; Metabolic engineering

Abbreviations commonly used in the report, in alphabetical order: CHS- Chalcone synthase; CHI- Chalcone isomerase; 1,2RT- 1,2 Rhamnosyltransferas.

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Abstract

The proposal was aimed to identify and functionally characterize key genes/enzymes in the citrus flavanone neohesperidoside biosynthetic pathway and to use them as tools for metabolic engineering to decrease bitterness levels in grapefruit. The proposed section on fruit seediness was dropped as suggested by the reviewers of the proposal.

Citrus flavor and aroma is composed of complex combinations of soluble and volatile compounds. The former includes mainly sugars, acids and flavanones, a subgroup of flavonoids that includes bitter compounds responsible for the bitter flavor of grapefruit and pummelo. Bitter species contain mostly bitter flavanone neohesperidosides, while non-bitter species contain mostly tasteless flavanone rutinosides. Both flavanone versions are diglycosides consisting of a rhamnose-glucose oligosaccharide O-linked at position 7 to the flavanone skeleton. However, in the bitter neohesperidosides the rhamnose is attached at position 2 of the glucose moiety, while in the tasteless rutinosides the rhamnose is attached at position 6 of the glucose moiety. Thus, the position of the rhamnose moiety, determined by the specificity of the last enzymes in the pathway-rhamnosyltransferase (1,2 or 1,6 specificity), is the determinant of the bitter flavor. Flavanones, like all flavonoids are synthesized via one of the branches of the phenylpropanoid pathway; the first committed step is catalyzed by the enzyme Chalcone synthase (CHS) followed by Chalcone isomerase (CHI).

During the course of the work a key gene/enzyme in the biosynthesis of the bitter flavanones, a 1,2 rhamnosyltransferase (1,2RT), was functionally characterized using a transgenic cell-culture biotransformation system, confirming that this gene is a prime candidate for metabolic engineering of the pathway. This is the first direct functional evidence for the activity of a plant recombinant rhamnosyltransferase, the first confirmed rhamnosyltransferase gene with 1,2 specificity and the second confirmed rhamnosyltransferase gene altogether in plants. Additional genes of the flavanone pathway that were isolated during this work and are potential tools for metabolic engineering include (1) A putative 1,6 rhamnosyltransferase (1,6RT) from oranges, that is presumed to catalyze the biosynthesis of the tasteless flavanones. This gene is a prime candidate for use in future metabolic engineering for decreased bitterness and is currently being functionally characterized using the biotransformation system developed for characterizing rhamnosyltransferases. (2) A putative 7-O-glucosyltransferase presumed to catalyze the first glycosylation step of the flavanone aglycones.

Silencing of gene expression in grapefruit was attempted using three genes: (1) The "upstream" flavonoid biosynthesis genes CHS and CHI, by antisense and co-suppression; and (2) The "downstream" 1,2RT, by an RNAi approach. CHS and CHI silencing resulted in some plants with a dramatically decreased level of the bitter flavanone neohesperidoside naringin in leaves. We have yet to study the long-term effect of silencing these genes on tree physiology, and on the actual bitterness of fruit. The effect of 1,2RT silencing on naringin content in grapefruit has yet to be examined, but a slow growth phenotype for these plants was noted. We speculate that silencing of the final glycosylation step of the flavanones delays their evacuation to the vacuole, resulting in accumulation of flavanones in the cytoplasm, causing inhibitory effects on plant growth. This speculation is yet to be established at the product level. Future metabolic engineering experiments are planned with 1,6RT following functional characterization.

Achievements

Significance of main scientific achievements or innovations

1. We have shown that it is possible to apply metabolic engineering to affect flavonoid content in citrus by silencing key enzymes in the metabolic pathway. Silencing of the “upstream” enzymes CHS and CHI was found in some plants to significantly reduce naringin levels in leaves of grapefruit (Appendix B & C); The effect on fruit bitterness is yet to be determined when adult grapefruit trees are obtained. Also the overall physiological effect of reducing flavonoid content in the whole plant will need to be studied on the long run.
2. The gene putatively encoding the “downstream” key enzyme 1,2-rhamnosyltransferase, that was isolated prior to this research program, was functionally characterized in transgenic cell-cultures during this research and shown to catalyze the sought-after activity (Appendix A). This gene is the first functionally characterized recombinant rhamnosyltransferase in plants, and is the second identified rhamnosyltransferase in the plant kingdom (the previously characterized rhamnosyltransferase from petunia was found via a plant mutant). The results also establish a functional-assay system for characterizing rhamnosyltransferases, which are difficult to characterize due to the in-availability of the rhamnose donor, UDP-rhamnose, outside of the plant kingdom or commercially. Sequence similarity and phylogenetic analyses of the two RT encoding genes provides some insight for detection of additional plant RT genes involved in glycosylating flavonoid-glucosides (Appendix A).
3. Silencing the gene encoding 1,2-RT in grapefruit by an RNAi construct resulted in plants delayed in growth (Appendix C). Since the glycosylation steps enable the flavanones to be transported and stored in the vacuole, we speculate that the lack of sufficient rhamnosylation causes accumulation of flavanone-7-glucosides in the cytoplasm, resulting in inhibition of cell growth. This hypothesis needs to be established at the level of the flavanone compounds, and will be tested when plants are large enough to harvest several leaves. The implications are that for metabolic engineering of bitter flavanone-neohesperidoside content without affecting the overall level of flavonoids, and

without causing inhibitory effects, silencing may not be the correct approach. Instead, it may be necessary to transform grapefruit with a gene conferring 1,6RT activity, which would catalyze production of tasteless rutinoides at the expense of neohesperidosides in order to decrease bitterness. A putative 1,6RT encoding gene was isolated during this work (see below).

4. Screening for additional citrus rhamnosyltransferases relevant to the flavanone-glycoside biosynthetic pathway yielded a cDNA clone putatively encoding a for 1,6-rhamnosyltransferase (Appendix E). This clone, found to map phylogenetically with the only known rhamnosyltransferases (1,2-RT and petunia-RT), is currently being functionally characterized by the transgenic cell-culture biotransformation system developed. As previously mentioned, metabolic engineering for bitterness reduction in grapefruit would be expected to be optimal by overexpression of a 1,6 rhamnosyltransferase.

5. The screen for citrus flavonoid glycosyltransferase encoding genes yielded several additional genes including a putative 7-O-glucosyltransferase (Appendix E), that catalyzes the first glycosylation step of the flavanone biosynthetic pathway.

6. Substrate specificity of the enzyme 1,2RT was established (Appendix E) and found to include 7-glucosyl-flavanones and 7-glucosyl-flavones, but not 7-glucosyl-flavonols (Appendix F). The results suggest guidelines for predicting whether a given flavonoid may be a substrate. Results also suggest that plants producing diosmin (Such as *Lamiaceae*) may be good targets for producing the bitterness blocker neodiosmin via metabolic engineering (Appendix F).

7. The transgenic cell culture biotransformation system developed during this research as a tool for recombinant rhamnosyltransferase characterization was found to function as a cell-factory for production of high-value neohesperidin (a substrate for the sweetener NHDC) from orange peel waste hesperidin (Appendix D). In this work we were successful in showing the entire pathway from hesperidin to neohesperidin: (a) Extraction

of hesperidin from orange peels; (b) Hydrolysis of sugars by high temperature in mildly acidic conditions to obtain hesperetin-7-glucoside and hesperetin; (c) Biotransformation by cell-cultures transgenic for Cm1,2rt.

Agricultural and/or economic impacts of the research findings, if known

Several findings have potential economic impact on the long run:

1. Isolation of a gene putatively encoding 7-O-glucoside-1,6RT has strong potential to serve as the optimal tool for metabolic engineering for reduced grapefruit bitterness. If the activity is confirmed by functional analysis, the gene will be patented.
2. The gene 1,2RT has the potential to serve as a useful tool for the production of neodiosmin via metabolic engineering (in *Lamiaceae* plants for instance). Neodiosmin may have a market as a bitterness blocker for grapefruit juice (instead of de-bittering) and especially for bitter drugs. We plan to investigate the potential use of neodiosmin and to expand the existing patent on 1,2RT to include neodiosmin.
3. The results show that transgenic plants have been engineered to produce lower levels of the bitter flavanones. Once transgenic products become more acceptable, grapefruit with reduced bitterness have the potential to open new markets.

Details of cooperation

The project called for a combination of: (a) Gene isolation and functional analysis of recombinant enzyme; (b) Citrus transformation for gene silencing; (c) identification of flavonoid products. The combined expertise of the three groups resulted in a fruitful and synergistic collaboration, the results of which are now developing into publications (See Appendix).

List of Publications

Four manuscripts are in various stages of preparation; One is in an advanced draft stage (Appendix A), while others are at a preliminary stage.

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Citrus fruit bitter flavors: Isolation and functional characterization of the gene encoding a 1,2 rhamnosyltransferase, a key enzyme in the biosynthesis of the bitter flavonoids of citrus

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Introduction

Citrus fruit flavor and aroma is composed of complex combinations of soluble and volatile compounds. The former group comprises mainly sugars, acids and flavanones, a subgroup of flavonoids that includes bitter compounds responsible for the bitter flavor of grapefruit and pummelo (Horowitz and Gentili, 1961; Horowitz, 1986). Flavanones are the dominant group of flavonoids in citrus and beyond their effect on citrus flavor, have also been implicated as important dietary components with a role in maintaining healthy blood vessels and bones and as anti-cancer agents (Garg et al., 2001; Kim et al., 2001; Manthey et al., 2001; Manthey and Guthrie, 2002; Chiba et al., 2003). While some of the health-related effects may be due to the anti-oxidant characteristics of these compounds, other effects are not well understood at the mechanistic level. Citrus are the only edible fruits containing large quantities of flavanones and are therefore the major source of

flavanones in the human diet and the major source of these compounds for the food and para-pharmaceutical industries.

Bitterness is characteristic of some citrus species, such as grapefruit (*Citrus paradisi*) and pummelo (*Citrus maxima*), but is absent in other species such as oranges (*Citrus sinensis*), mandarins (*Citrus reticulata*) and lemons (*Citrus limon*). The “primary” bitterness caused by some flavanone-glycosides present in the fruit and the juice should not be confused with the “delayed” bitterness, which occurs only in the juice as a result of exposure of limonoids to atmospheric oxygen (Kita et al., 2000). Studies on the profile of flavonoids in various citrus species have established that the bitter species contain mostly flavanone neohesperidosides, which are bitter, while the non-bitter species contain mostly flavanone rutinosides, which are tasteless (Rousseff et al., 1987 and Figure 1). Both flavanone versions are diglycosides consisting of a rhamnose-glucose oligosaccharide O-linked at position 7 to the flavanone skeleton. However, in the bitter neohesperidosides the rhamnose is attached at position 2 of the glucose moiety, while in the tasteless rutinosides the rhamnose is attached at position 6 of the glucose moiety (Figure 1). Thus, the position of the rhamnose moiety is the determinant of the bitter flavor.

Flavanones, like all flavonoids, are synthesized via one of the branches of the phenylpropanoid pathway; the first committed step is catalyzed by the enzyme chalcone synthase followed by chalcone isomerase (see Figure 1). The flavanone naringenin, the dominant flavanone in grapefruit, undergoes two steps of glycosylation. The first step involves an O-linked glucosylation at position 7 of the flavanone catalyzed by a 7-O-glucosyltransferase (7GT). The second step involves two rhamnosyltransferases: Most of the naringenin-7-glucoside is metabolized in grapefruit into the bitter neohesperidoside naringin by a 1-2 rhamnosyltransferase (1,2RT), while the rest is metabolized into the tasteless rutinoside narirutin by a 1-6 rhamnosyltransferase (1,6RT) (Figure 1). In other citrus species the glycosylation process is essentially the same, but involves also other flavanones such as hesperetin in oranges. The ratio between the levels of flavanone-7-

neohesperidoside vs. flavanone-7-rutinoside end products, in any given citrus fruit species, determines the level of fruit bitterness.

Enzyme activities catalyzing citrus flavanone glycosylation (7GT, 1,2RT and 1,6RT) have been described (McIntosh et al., 1990a&b; Lewinsohn et al., 1989; Berhow and Smolensky, 1995), but only the 1,2RT enzyme has been purified to homogeneity (Bar-Peled et al., 1991) and none of the corresponding genes have been isolated up to now. Developmental studies on the accumulation of flavanone-glycosides in citrus and the corresponding glycosyltransferase enzyme activities show that flavanone-glycosides are synthesized in large quantities only in young tissue (including mainly leaves, flowers and fruit), and are later diluted in fruit to their final concentration during the process of development and ripening (Bar-Peled et al., 1993). It is not clear whether the flavanone-glycosides accumulating in fruit are synthesized solely in fruit or whether some flavanones synthesized in young leaves are eventually transported to the fruit (Berhow and Vandercook, 1991). Also, the biological role of these abundant compounds in fruit and other plant tissues is still unknown.

Glycosyltransferases involved in plant secondary metabolism are a large group of enzymes classified as family 1 UDP glycosyltransferases (Vogt and Jones, 2000; Coutinho and Henrissat 1999). Flavonoid glycosyltransferases, which belong to this family, have been studied in many species, and a growing number of genes have been isolated and functionally characterized (reviewed in Vogt and Jones, 2000). In addition, the Arabidopsis and Rice genomes as well as various EST projects have provided insight into the number of genes involved (Li et al., 2001), although most have not been assigned functions as yet. The emerging evolutionary picture is that of similarity of enzymes based on regiospecificity, rather than intraspecies specificity. However, the picture is still partial since the vast majority of the genes functionally characterized are glucosyltransferases and all are involved in direct O-linked glycosylation of the flavonoid skeleton. Therefore the data is still lacking on flavonoid glycosyltransferases that transfer sugars other than glucose, and on enzymes that specialize in glycosylation of the sugar moieties of flavonoid-glycosides.

In this work we describe the isolation and functional characterization of the gene *Cm1,2rt1*, which encodes a flavanone-7-O-glucoside-1,2-rhamnosyltransferase, the key enzyme directing biosynthesis of the bitter flavonoid compounds of citrus. Functional characterization of the recombinant enzyme was demonstrated by substrate biotransformation using a transgenic plant cell-culture line over-expressing the gene. Phylogenetic analysis of the flavonoid glycosyltransferase family places the gene *Cm1,2rt1* on a separate cluster together with the only other functionally characterized rhamnosyltransferase in the plant kingdom, thus suggesting a separate evolutionary branch of these glycosyltransferase encoding genes.

Results

Partial-peptide sequencing of citrus 1,2RT and isolation of the encoding gene *Cm1,2rt1*

The enzyme flavanone 7-O-glucoside-1,2 rhamnosyltransferase was purified to homogeneity as previously described (Bar-Peled et al., 1991) and subjected to partial proteolysis. Resulting peptides were purified and N-terminally sequenced (Table 1 in the materials and methods). Degenerate primers in sense and antisense orientations were designed based on peptide sequences (Table 1) and used in different combinations to isolate cDNA fragments via RT-PCR on mRNA isolated from young pummelo leaves. One cDNA fragment was confirmed as relevant based on the presence of an internal sequence corresponding to one of the partial peptide sequences. Specific primers designed based on the partial cDNA fragment were used to isolate a complete cDNA named *Cm1,2rt1* (Citrus maxima 1,2 rhamnosyltransferase; Figure 2) using 5' and 3' – RACE (Eyal et al., 1999; materials & methods). *Cm1,2rt1* was found to contain the conserved domain signature of UDP glycosyltransferases characteristic of family 1 glycosyltransferases (Coutinho and Henrissat 1999; Ross et al., 2001). When compared to sequences of characterized glycosyltransferases, *Cm1,2rt1* was found to be most similar to a petunia anthocyanidin rhamnosyltransferase (41% identity), the only previously characterized rhamnosyltransferase in the plant kingdom (Brugliera et al., 1994; Kroon et

al., 1994). Sequence analysis software (Target-P) projected localization of the encoded enzyme to the cytoplasm.

Recombinant 1,2RT activity is demonstrated in-vivo in transgenic tobacco cell-culture over-expressing Cm1,2rt.

Recombinant Cm1,2rt1 enzyme function was analyzed *in-vivo* by biotransformation of substrate in transgenic BY2 cell culture. The coding region of Cm1,2rt1 was cloned into a binary vector (PME504, see materials and methods) under the control of a CAMV-35S promoter. The resulting plasmid was used to transform tobacco BY2 cell suspension culture, to derive a transgenic cell line: BY2-1,2RT. Wild-type and transgenic BY2 cells were fed with the substrate naringenin-7-glucoside for 48 hours and flavonoids were extracted and analyzed by TLC. Separation of flavonoids by TLC shows that both wild type and transgenic cells contained a spot co-migrating with naringenin-7-glucoside as well as a slower unidentified spot (Figure 3a). However, only the transgenic cell line BY2-1,2RT contained a spot co-migrating with naringin. The putative-naringin spot was extracted from the silica-gel plate and confirmed to be naringin by LC-MS in comparison to standards (Figure 3b).

Analysis of the biotransformed flavonoid products was also performed by LC-MS (Figure 3c-e). Two flavanone peaks were detected in both wild type and transgenic lines and were identified based on retention time and mass-spectra to be: naringenin-glucoside (peak 1) and naringenin-diglucoside (peak 2). An additional peak (peak 3) was detected only in the transgenic line, BY2-1,2RT, and was identified as naringin (Figure 3c-e).

Transgenic tobacco cell-culture overexpressing Cm1,2rt1 performs a 2-step biotransformation of naringenin to naringin

Recent work (Taguchi et al., 2000a) has shown the presence of a broad substrate 7GT enzyme activity in tobacco cell culture. The spectrum of substrates was found to include coumarins and flavonols, and it was therefore reasonable to assume the enzyme would work on flavanones as well, which would allow a two step biotransformation of naringenin to naringin. To test this possibility, we repeated the biotransformation

experiment with naringenin as the fed substrate. Analysis of flavonoid products by TLC revealed that all the naringenin was converted into glycosides (Figure 4a). Both wild type and transgenic cell lines accumulated a spot co-migrating with naringenin-7-glucoside as well as a slower unidentified spot. Only the transgenic cell line BY2-1,2RT contained a spot co-migrating with naringin.

Similar results were obtained by analysis of the biotransformed flavonoid products using LC-MS (Figure 4b-d). Two flavanone peaks were detected in both wild type and transgenic lines and were identified based on retention time and mass-spectra to be: naringenin-glucoside (peak 1) and naringenin-diglucoside (peak 2). An additional peak (peak 3) was detected only in the transgenic line, BY2-1,2RT, and was identified as naringin (Figure 4b-d).

***Cm1,2rt1* maps phylogenetically to a new subgroup of the glycosyltransferase family**

We chose to study the phylogenetic relationship between *Cm1,2rt1* and other functionally characterized glycosyltransferases that have been shown to catalyze the glycosylation of flavonoids (including enzymes that naturally glycosylate other substrates). Phylogenetic analysis of the deduced amino-acid sequences of *Cm1,2rt1* and other flavonoid glycosyltransferases divides this family of enzymes into four clusters (Figure 5): (1) A cluster of enzymes catalyzing glucosylation at position 5 of substrate flavonoids (5GT cluster); (2) A cluster of enzymes catalyzing glycosylation at position 3 of substrate flavonoids (3GT and 3GaT cluster); (3) A cluster of enzymes catalyzing glucosylation at position 7 of substrate flavonoids (7GT cluster; some enzymes in this group maintain other site-specificities as well); (4) A cluster consisting of *Cm1,2rt1* and the only other known rhamnosyltransferase in plants, a petunia 1,6 rhamnosyltransferase.

Discussion

The enzyme encoded by *Cm1,2rt1* belongs structurally and functionally to subfamily 1 of the family of glycosyltransferases (Coutinho & Henrissat 1999, 2003). Studies correlating enzyme function and phylogenetic mapping reveal that regiospecificity is a primary characteristic of glycosyltransferases and therefore enzymes of distant species,

but of the same specificity, group together phylogenetically (Vogt and Jones, 2000; Jones and Vogt, 2001; Li et al., 2001; Ross et al., 2001; Figure 5). In contrast to regiospecificity, the correlation between transferase sugar-specificity and gene similarity has not been explored in depth since the vast majority of the flavonoid glycosyltransferase genes functionally characterized are glucosyltransferases. Two galactosyltransferases that were previously characterized (3GaT) appear to conform to the site specificity clusters of glucosyltransferases, and do not constitute a separate group by themselves (Figure 5). In contrast, the two characterized rhamnosyltransferases (Petunia: Brugliera et al., 1994; Kroon et al., 1994; and citrus: this work) appear to create a cluster of their own (Figure 5), but it would be difficult to determine at this stage whether the cluster represents rhamnosyltransferases or rather glycosyltransferases specializing in the glycosylation of sugar moieties of flavonoid glucosides. Both characterized rhamnosyltransferases catalyze the rhamnosylation of a glucose O-linked to the flavonoid skeleton, however the specificities are different. While the petunia enzyme functions on a 3-O-glucoside substrate and targets the rhamnose to position 6 of the glucose moiety, the citrus enzyme is specific for 7-O-glucoside substrates and targets the rhamnose to position 2 of the glucose moiety. Perhaps this major difference in specificity is reflected in the relatively low similarity between the two sequences, and the relatively “loose” cluster. Functional characterization of additional rhamnosyltransferases catalyzing glycosylation of the flavonoid skeleton or previously bound glucose moieties will be required to establish the precise functional significance of this “new” cluster.

While biotransformation of natural compounds using “wild-type” cell-cultures has been demonstrated for various species including citrus, biotransformation using transgenic cell cultures has rarely been used to study the function of recombinant enzymes (Cooper et al., 2002). Bacteria and yeast have usually been the systems of choice to demonstrate recombinant enzyme activity of plant genes, however, cell culture has some built-in advantages for the study of recombinant flavonoid rhamnosyltransferases: (1) The rhamnose source - UDP-rhamnose, which is not synthesized in yeast and bacteria and is not commercially available, is produced in plant cell cultures in sufficient quantities. (2) The *in-vivo* analysis approach allows for production of relatively large quantities of the

flavonoid-glycoside products, which facilitates non-radioactive analysis of product and identification by mass spectrometry. (3) The biotransformation products accumulate inside the cells (data not shown) and are most likely targeted to their natural location in the vacuole (Taguchi et al., 2000b), thus providing natural protection for the product until cells are harvested and extracted.

Analysis of flavonoid products using TLC and LC-MS was complementary in this work and provided a clear picture of the flavanone glycosylations occurring in the wild-type and transgenic cell-lines. TLC provided a rapid system to analyze different conditions to obtain a working biotransformation protocol, while LC-MS analysis contributed a more sound identification of the compounds. Accordingly, identification of naringin by co-migration using TLC was confirmed by LC-MS, thus establishing the activity of the recombinant enzyme encoded by *Cm1,2rt1* as a 1-2RT. The unidentified slow-migrating spot in the TLC (appearing in both wild-type and transgenic cell lines) was identified by LC-MS as a naringenin-diglucoside, which appears to result from endogenous wide-substrate specificity glucosyltransferases in the BY2 cell line (Taguchi et al., 2001).

Although rhamnosylation does occur in non-plant organisms such as bacteria, the rhamnose donor UDP-rhamnose has not been documented outside of the plant kingdom. Working on the speculation that a plant rhamnosyltransferase may be able to utilize the bacterial rhamnose donor dTDP-rhamnose, we overexpressed *Cm1,2rt1* in bacteria but were unable to detect any activity *in-vivo* (data not shown). It would be difficult, of-course, to determine whether the bacterial rhamnose donor was “at fault” or whether the pH and red-ox conditions prevailing in bacterial cells are not adequate for plant rhamnosyltransferase enzyme function. Therefore, the absence of UDP-rhamnose outside of the plant kingdom may limit the use of plant-originating rhamnosyltransferases to plants alone at this stage.

Finally, isolation of the gene encoding 1,2RT provides us with a tool to manipulate citrus flavor characteristics using a transgenic approach. Indeed experiments are well underway

to modulate grapefruit bitterness levels by metabolic engineering of the flavonoid biosynthetic pathway using the gene *Cm1,2rt1*.

Materials and methods

Plant material

Young leaves (up to 1 cm) were collected from pummelo trees (*Citrus maxima* cv. Chandler) and frozen at -80°C for the purpose of protein and mRNA purification. BY2 cell culture was maintained and transformed as previously described (Shaul et al., 1996).

Purification of 1,2 RT protein, proteolysis and protein sequence

1,2 rhamnosyltransferase was purified from pummelo leaves as described (Bar-Peled et al., 1991). Purified protein (10 µg) was reduced with 10 mM DTT and denatured with 1% SDS in 5 µl of 500mM Tris-HCl pH 7.5, for 20 minutes at 50°C. After cooling, 80 µl of distilled water and 10 µl of acetonitrile were added to the denatured protein, followed by the addition of 10 ng sequence grade protease (endoproteinase Asp-N; endoproteinase Lys-C; and trypsin; Roche Applied Science, Mannheim Germany). Samples were incubated for 5 hours at 37°C, and were then diluted with denaturing sample buffer before separation on 12.5% SDS-PAGE followed by electro-blotting to Immobilon-P 0.45 µm pore size PVDF membranes (Millipore, Bedford MA, USA). Membranes were stained with 0.1% coomassie brilliant blue (dissolved in 50% methanol) and destained with 30% methanol: 10% acetic-acid and then washed several times in distilled water. After air-drying, the bands of interest were cut and subjected to N-terminal amino acid sequencing. N-terminal sequencing was performed at the Biological Service Unit of the Weizmann Institute of Science using an Applied Biosystems 477A pulsed liquid protein sequencer. Peptide sequences that were obtained and the corresponding degenerate primers designed for gene isolation are detailed in Table 1.

Cleavage	Peptide sequence	Primers (5' -3')
Lys-C	INYFLHLTA	S: AAYTAYTTYCTICAYCTIACIGC

		AS: GCIGTIAGRTGIAGRAARTATT
Asp-N	YFPSLMGN	
Asp-N	EKMTIEEA	S: GARAARATGACIATHGARGARGC AS: GCYTCYTCRATIGTCATYTTYTC
Asp-N	IAAILFL	
Trypsin	VVDNGMGMVVPRDK	S: GAYAAYGGIATGGGIATGGT AS: ACCATICCCATIAARTTRTC

Isolation of the gene *Cm1,2rt1*

Total RNA was extracted from young pummelo leaves according to Logemann et al., 1987. mRNA was purified for RT-PCR applications by PolyAtract mRNA Isolation system III (Promega, WI, USA). Poly-A RNA (500ng) was reverse transcribed using Superscript II reverse transcriptase (BRL, Life Technologies, UK) and 10 pmoles of tailed oligo-(dT) primer (5'GTTTCCAGTCACGACGTTTTTTTTTTTTTTT). PCR was performed using different combinations of the degenerate primers (Table 1) and products were cloned and sequenced. A relevant product (obtained using primers Lys-C-S and Trypsin-AS) was identified by the presence of the internal sequence EKMTIEEA, which is identical to one of the Asp-N peptide sequences. Gene-specific primers based on the RT-PCR fragment sequence were used to isolate the complete cDNA by RACE techniques. The oligonucleotides 5' was used for 5'-RACE as described (Eyal et al., 1999). The gene-specific oligonucleotide was used in conjunction with tail oligonucleotide (5' GTTTCCAGTCACGACG) to obtain a 3'-RACE fragment.

Biotransformation of flavanones using transgenic BY2 cell culture

The coding sequence of *Cm1,2rt1* was cloned into a standard binary vector (PME504; Moshe Flaishman et al., unpublished) under the control of a CAMV-35S promoter, which was used to transform BY2 cell culture as previously described (Shaul et al., 1996). In order to initiate biotransformation studies, transgenic and wild-type cell cultures were diluted (400 µl culture into 20 ml new media) and grown in Erlenmeyer flasks for four days. Naringenin or naringenin-7-glucoside were dissolved in DMSO to a concentration

of 30 mM and added to the flasks to a final concentration of 0.15 mM. The cultures were grown for an additional 48 hours before harvest. Cell culture and media were boiled together for 15 minutes, cooled to room temperature and then extracted with water-saturated N-butanol. The butanol phase was transferred to a new tube and dried.

Analysis of flavanone-glycoside products by TLC

Biotransformation products were resuspended in DMSO and analyzed by TLC. 1-3 μ l samples were loaded on Silica gel 60 TLC plates (Merck, Darmstadt, Germany) and separated in solvent (N-butanol: methanol: ethylacetate: dichloromethane – 1:1:1:1) in a standard TLC chamber. Separated TLC plates were dried and fluorescence was developed by spraying with 1% AlCl_3 in methanol. Photographs were obtained using a digital camera (Nikon Coolpix 5000) while irradiating the TLC plate with medium-range UV (302 nm). Putative naringin spots, which appeared only in the transgenic line biotransformations, were scraped from the TLC plate, eluted in methanol, dried and finally analyzed by LC-MS (see below).

Analysis of flavanone-glycoside products by LC-MS

Sequence analysis and phylogenetic tree software tools

General sequence data manipulations and homology searches were conducted using CuraTools integrated bioinformatics tools (Curagen, CT, USA) and BLAST (REFF) Transit peptide analysis was conducted by Target P (Emanuelsson et al., 2000). Phylogenetic trees were obtained using BioEdit (Hall, 1999) and Treeview (Page, 1996) software.

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**Appendix A, Figure 1: Citrus
Flavanone Biosynthetic Pathway**

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Petunia-RT 1  MENEMKHSNDALHVVMFPFFAFGHISPFVQLANKLSSYGVKVSFFTASGNASRVKSMLNS
Citrus-RT 1  --MDTKHQ-DKPSILMLPWLAHGHIAPHLEIAKKLSQKNFHIYFCSTPNNLQSFGRNVEK

Petunia-RT 60  --APTTHIVPLTLP-HVEGLPPGAESTAELEPASAELLKVALDLMQPIKTLISHLKPHF
Citrus-RT 58  NFSSSIQLIELQLPNTFPPELPSQNQTTKNLPPHLIYTLVGAFEDAKPAFCNILETLKPTL

Petunia-RT 118 VLFDFAEWLPMANGLGIKTVYYSVVVALSTAFITCPARVLEPKKYPSEDMKKPPLGF
Citrus-RT 118 VMYDLFQPMAAEAYQYDIAAILFLPLSAVACSFL-----LHNIVNPSLKY
          *****

Petunia-RT 178 PQTSVTSVRTFEARDFLYVFKSFHNGPTLYDRIQSGLRG-CSAILAKTCSQMEGPYIKYV
Citrus-RT 164 PFFES-DYQDRESKNINYLHLTANGTLNKDRFLKAFELSCKFVFIKTSREIESKYLDYF
          *****

Petunia-RT 237 EAQFNKPVFSNRTRSSGRASGKLEEKWATWLNKFEGGTVIYCSFGSETFLTDQVKEIAL
Citrus-RT 223 PSLMGNEIIPVGPLIQEPTFKVDDTKIMDWLSQKEPRSVVYASFGSEYFPSTDEIHDAI
          *****

Petunia-RT 297 GLEQTGLPFFLVLNFPANVDVSAELNRALPEGFLERVK--DKGIHSGWVQQQNILAHSS
Citrus-RT 283 GLLLTEVNEIWAFLRHP--DEKMTIEEALQGFEEIERNNKGMIVQGWVPCAKILRHGS
          *****

Petunia-RT 355 VGCYVCHAGFSSVIEALVNDCCQVVMLPQKGDQILNAKLVSQDMEAGVEINRRDEDGYFGK
Citrus-RT 341 IGGFLSHCGWGSVVEGMVFGVPIIGVPMAYEQPSNAKVVV-DNGMGMVVPRDKINQRLGG
          ** *****

Petunia-RT 415 EDIKEAVEKVMVDVEKDPGKLIRENQKKWKEFLLNKDIQSKYIGNLVNEMTAMAKVSTT
Citrus-RT 400 EEVARVIKHVVLQEEA--KQIRRKANEISESMK--KIGDAQMSVVVEKLLQLVKKE-

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Appendix A, Figure 2: Alignment of plant rhamnosyltransferases. Deduced amino acid sequences are: Petunia 3-O-glucoside-1,6 rhamnosyltransferase (Petunia RT; Brugliera et al., 1994); Citrus 7-O-glucoside-1,2 rhamnosyltransferase (Citrus-RT; Cm1,2rt1).

Appendix A, Figure 3a: Biotransformation of naringenin-7-glucoside into naringin. Naringenin-7-glucoside was fed to BY2 cells and the resulting flavonoids were analyzed by TLC. Lanes are as follows: 1. naringenin-7-glucoside standard; 2. naringin standard; 3. Unfed BY2 cells transgenic for 1,2RT; 4. Naringenin-7-glucoside fed BY2 cells transgenic for 1,2RT; 5. Naringenin fed BY2 wild-type cells; 6. Unfed BY2 wild-type cells;

Sample	Retention time (min)	M/Z observed
Standard: Naringenin-7-glucoside	24.83	[M-H] ⁻ 433.0
Standard: Naringin	23.89	[M-H] ⁻ 579.2
Standard: Narirutin	22.54	[M-H] ⁻ 579.2
Putative Naringin TLC spot	23.69	[M-H] ⁻ 579.3

Appendix A, Figure 3b: Verification of naringin product by LC-MS. The TLC spot corresponding to naringin (see Figure 3a) was extracted and analyzed by LC-MS for retention time and mass spectrum in comparison to known standards.

Sample	Peak	Retention time (min)	M/Z observed	Identification
Standard: Naringenin-7-glucoside		24.83	[M-H] ⁻ 433.0	
Standard: Naringin		23.89	[M-H] ⁻ 579.2	
Standard: Narirutin		22.54	[M-H] ⁻ 579.2	
BY2 (control) biotransformation	1	24.54	[M-H] ⁻ 433.1	Naringenin-glucoside
	2	21.02	[M-H] ⁻ 595.3	Naringenin-diglucoside
BY2-1,2RT biotransformation	1	24.54	[M-H] ⁻ 433.3	Naringenin-glucoside
	3	23.71	[M-H] ⁻ 579.5	naringin
	2	20.94	[M-H] ⁻ 595.4	Naringenin-diglucoside

Appendix A, Figure 3c: Biotransformation experiment for naringenin-7-glucoside analyzed by TLC (See Figure 3a) was also analyzed by LC-MS. Biotransformation product LC-MS profiles were compared to known standards for identification.

Appendix A, Figure 4a: Biotransformation of naringenin into naringin. Naringenin was fed to BY2 cells and the resulting flavonoids were analyzed by TLC. Lanes are as follows: 1. naringenin standard; 2. naringenin-7-glucoside standard; 3. Naringin standard; 4. Unfed BY2 cells transgenic for 1,2RT; 5. naringenin fed BY2 cells transgenic for 1,2RT; 6. Naringenin fed BY2 wild-type cells; 7. Unfed BY2 wild-type cells; 8. Naringenin-7-glucoside standard; 9. Naringenin standard.

Sample	Peak	Retention time (min)	M/Z observed	Identification
Standard: Naringenin		35.95	[M-H] ⁻ 271.0	
Standard: Naringenin-7-glucoside		24.83	[M-H] ⁻ 433.0	
Standard: Naringin		23.89	[M-H] ⁻ 579.2	
Standard: Narirutin		22.54	[M-H] ⁻ 579.2	
BY2 (control) biotransformation	1	24.78	[M-H] ⁻ 433.1	Naringenin-glucoside
	2	21.12	[M-H] ⁻ 595.3	Naringenin-diglucoside
BY2-1,2RT biotransformation	1	24.67	[M-H] ⁻ 433.1	Naringenin-glucoside
	3	23.82	[M-H] ⁻ 579.4	Naringin
	2	21.03	[M-H] ⁻ 595.3	Naringenin-diglucoside

Appendix A, Figure 4b: Biotransformation experiment for naringenin analyzed by TLC (See Figure 4a) was also analyzed by LC-MS. Biotransformation product LC-MS profiles were compared to known standards for identification.

Appendix A, Figure 5: Phylogenetic map of functionally characterized flavonoid glycosyltransferase

Appendix B

Summary of transformation results and metabolically engineered plant results

1) Characterization of the family members of endogenous citrus CHS and CHI genes.

Information from our lab and others indicate that CHS and CHI both consist of small gene families. The particular gene used for transformation may differentially affect expression in different tissues, so it would be useful to know tissue expression patterns in nontransgenic plants.

CHS appears to have 3 family members in citrus. Two CHS genes, CitCHS1 (accession # AB009350) and CitCHS2 (accession # AB009351), have been isolated by others from *C. sinensis*. By using primers for conserved regions of the CHS genes from several plant species, we have been able to amplify 3 different partial CHS sequences from grapefruit genomic DNA. One of them is identical to the CHS gene previously isolated and the other two, although very similar to each other, are new. Southern blot analysis by using a CHI probe from grapefruit revealed that CHI might also have a small gene family. We are still working to obtain full length clones of these new grapefruit CHS and CHI genes. Gene-specific Taqman probes will then be designed in order to assess expression levels in different plant tissues using Real-Time PCR.

2) Transformation of grapefruit with cDNAs of structural genes from the flavonoid biosynthetic pathway, cloned in both the sense and antisense orientations, as well as in a construct that should produce a hairpin formation (RNAi).

Our aim was to alter gene expression in the flavonoid biosynthetic pathway using molecular methods. Gene expression can be modified by silencing the gene, via transformation with an antisense or a dsRNA-producing construct, causing low expression and sometimes no expression at all. Research by others during the course of the granting period has demonstrated that constructs producing self-complementary hairpin RNA (hpRNA) or double stranded RNA (dsRNA) silence target genes very efficiently.

Alternatively, one can attempt to increase the expression level via transformation with target genes as sense constructs. Surprisingly, however, the introduction of sense constructs may cause silencing of both the transgene and the endogenous target gene. Thus it is necessary to characterize a number of individual plants transformed with each construct to be tested.

One chalcone synthase (CHS) and one chalcone isomerase (CHI) cDNA were previously isolated in the Moore laboratory (by former PhD student D. Luth). Heterologous CHS and CHI probes were exploited to isolate the genes from a young leaf cDNA library of Marsh grapefruit. CHS and CHI sense and antisense constructs were cloned under the control of the CaMV 35S promoter for constitutive expression of the cDNAs in all plant tissues. The cDNAs were then cloned individually into the transformation vector pGA482GG. This vector plasmid provides efficient transformation of citrus when it is used with *Agrobacterium* strain EHA101. It has an NPTII gene for the selection of transformed segments resistant to kanamycin, a GUS gene to use as a scorable marker for transgenic plant tissue, and a gentamycin resistance gene to allow efficient selection of the plasmid in bacteria.

The 1,2 rhamnosyl transferase gene was isolated in the Eyal lab (see Appendix A). A construct of 1,2 RT was designed with either antisense alone or sense and antisense sequence in the same plasmid; the resulting RNA of the latter is expected to form a hairpin structure when the vector is transformed into the plant tissue. Therefore, it may be possible to compare these two potential methods for gene silencing. These two constructs were cloned into a transformation vector plasmid that does not contain a scorable marker gene. The *Agrobacterium*-mediated transformation protocol for citrus was already been established before the initiation of this project.

During the course of this research, numerous transformation experiments were done. For CHS and CHI, regenerated shoots were verified with histochemical analysis using the GUS assay prior to PCR analysis. The shoots produced with constructs that do not contain a scorable marker are directly analyzed by PCR. Growing the seedlings takes 1-2 months. Performing transformation experiments and recovering the transgenic shoots, rooting, and transferring to soil take 3-4 months. An additional period of 3-4 months is usually necessary for plants to become large enough to extract DNA for PCR analysis and Southern blot experiments.

Plants transformed with the construct CHS-S were usually not viable; they died when they were transferred to soil cups. Although some of the experiments were not very efficient, we were still able to obtain transgenic plants. Transformation experiments with 1,2 RT constructs are still in progress.

Table 1: *Agrobacterium*-mediated genetic transformation of *C. paradisi* with the constructs CHS-S (chalcone synthase sense), CHS-AS (chalcone synthase antisense), CHI-S (chalcone isomerase sense), and CHI-AS (chalcone isomerase antisense) cloned into the pGA482GG vector plasmid.

Construct	Total # of Segments	Segments with Shoots	Gus + Shoots	% of Gus+ Plants	PCR + Surviving Plants
CHS-S	14474	477	13	2.7	4
CHS-AS	15473	663	131	19.8	12
CHI-S	3866	345	106	30.7	22
CHI-AS	7052	569	179	31.5	20

Table 2: Preliminary results of *Agrobacterium*-mediated gene transformation of *Citrus paradisi* with 1,2 rhamnosyl transferase hairpin construct. pBINplus is the vector plasmid.

Construct	Total # of Segments	Total # of Shoots produced	# of NPTII plants	# of 1,2RT PCR plants	# of plants surviving will be screened by PCR
1,2 RT	9760	210	49	6	19

3) Characterization of the resulting transgenic plants for their morphology, transgene copy numbers, gene transcription levels and, in collaboration with Dr. M. Berhow, their production levels of flavanone neohesperidosides and other flavonoids.

Silencing would cause a decrease in expression levels of the target gene, in our case CHS, CHI, or 1,2 RT. This might alter the metabolic flux in the pathway. However, transfer of a sense gene construct might cause either co-suppression or overexpression of the transgene and endogenous gene. As a result of all these transformation experiments, we might see some altered physiology or morphology in the transgenic plants. Consequently, we are characterizing our confirmed transgenic plants for their transgene copy number via Southern blot analysis; flavonoid production levels by HPLC, in collaboration with Dr Berhow; and the transcription level of target steady-state mRNA by Real-Time RT PCR. Additionally, we will look for short RNAs in transgenic plants, which are shown to be the cause or result of silencing. These experiments are not completed and one or more additions to this report will be sent to BARD when they, or some portion of them, is finished. Plants will also be analyzed for their lignin content, response to environmental stress and resistance to pathogens.

Southern Blot Analysis: Southern blot analysis has been performed on some of the transgenic plants, but there are others still to be characterized. When the plants become large enough, DNA is extracted from them using DNAzol, which yields DNA of acceptable purity for

Southern blot analysis. For testing for presence and copy number of the CHS and CHI constructs, DNA is digested by *HpaI* restriction enzyme, which was chosen because it cuts only once in the transgene sequence. This produces border fragments so that we can estimate the copy number of the transgene. DNA probes are labeled with the Nonradioactive Dig-labeling method provided by Roche. Hybridization and detection are conducted as described in the Roche Molecular Biochemical DIG-labeling protocol. An example of this analysis is shown in Figure 1. Here the probe was GUS sequence labeled with Dig-UTP. We are finding that T-DNA copy number varies from one to many copies.

Figure 1. Southern blot analysis of some of the transgenic plants. Probe: GUS
 5 :CHI-AS; 20:CHI-AS; 38:CHI-S;40:CHI-S; 41:CHI-S; 32:CHS-AS; 36:CHS-AS; 37:CHS-AS; 56:CHS-S 74: (+)
 Control plant; C1: (-) Control plant

HPLC Analysis: Confirmed transgenic plants are being analyzed for their flavonoid levels through the kindly offered collaboration with Dr. M. Berhow. He already has the equipment for analysis of flavonoid compounds and the protocol that he applies for us. For this protocol, ground whole leaf samples are extracted with DMSO: MeOH or only MeOH at 50 C, 4 times with each extraction being at least 30 minutes. An aliquot is filtered for HPLC (Shimadzu 6A) analysis. Flavonoid concentration is calculated as mg/g for the leaf samples. We have preliminary HPLC analysis data (Figures 2 and 3) for some of the CHS and CHI transgenic plants. The plants that have altered flavonoid levels compared to control plants will be further evaluated. One surprising thing that we have observed in these first analyses is that in some of the antisense plants, naringin levels appear higher than in the control.

Figure 2. HPLC analysis of naringin levels in some of the chalcone synthase (CHS) transgenic lines in comparison to control plants.

Figure 3. HPLC analysis of naringin levels in some of the chalcone isomerase (CHI) transgenic lines in comparison to control plants, which were not transformed with a plasmid.

Determination of the Transcription Levels: A real-time RT-PCR technique, which is a quantitative and very sensitive method, has been developed to analyze steady state mRNA levels of the transgene and the endogenous gene(s).

P-1258

Manipulation of the Flavonoid Pathway in Citrus For Better Fruit Acceptability

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Abstract: Flavonoids are a widely distributed, diverse group of phytochemicals. They have an important role in protection of plants against UV light, in male fertility, pigment production, pollen growth, and plant-microbe signaling. They also have benefits for human health since they are antioxidant, anticancer, and protective against cardiovascular diseases^{1,2}. In citrus, flavanone disaccharides are the main group of flavonoids that accumulate, and rather than influencing color, may affect the taste of fruits. Specifically, flavanone neohesperidosides, for instance naringin, cause bitterness in fruit and fruit juice products. This bitterness of the fruit reduces the acceptability of the fresh fruit and juice products in commercial markets. Our main objective is to manipulate the production of flavanone neohesperidosides in citrus using molecular genetics and transformation techniques. As an initial step, we have isolated the cDNAs of chalcone synthase (CHS) and chalcone isomerase (CHI) genes. Sense and antisense constructs of these cDNAs were used to transform grapefruit seedlings, which also produce high levels of naringin, to suppress the expression of the target gene. To accomplish our goal we also transformed grapefruit with cDNA of a hairpin-forming construct of the 1-2 rhamnosyl transferase gene, which catalyzes the last biochemical step in the formation of naringin. The construct was designed to express dsRNA, which interferes with the target gene activity. Transgenic plants are being analyzed by HPLC for their flavonoid composition and are being characterized for their target gene copy numbers. Moreover, steady state RNA level of the target genes are being analyzed by using quantitative Real-Time reverse transcriptase PCR.

Figure 1. Proposed flavonoid biosynthetic pathway leading to flavanone glycosides³. If the rhamnose attaches to the 6 position in the glycoside, the resulting compound is tasteless; if it attaches at the 2 position the compound is very bitter.

Figure 2. Four transformation plasmids were made, chalcone synthase sense (CHS-S), chalcone isomerase sense (CHI-S), and their antisense (CHS-AS & CHI-AS) constructs. The paired system developed by Slightom⁴ (1991) was chosen that utilizes pUC18-exp17 and pGA482/GG plasmids. pUC18-exp17 contains an expression cassette that includes the CaMV 35S promoter, 5' translational enhancer from the cucumber mosaic virus, and a CaMV polyadenylation signal. The plasmid vector pGA482/GG contains right and left T-DNA borders. Between the borders there is a neomycin phosphotransferase gene (NPTII) to confer kanamycin resistance for plant selection, a polylinker site for inserting the pUC18 cassette and CaMV 35S driven GUS reporter gene. Outside the T-DNA borders there is a gentamycin (gen) resistance gene to maintain the plasmid in *Agrobacterium* cell lines and tetracycline (tet) resistance gene for maintenance in *E. coli* cell lines.

Construct	Total number of segments	Number of Segments with shoots	GUS + Shoots	Surviving PCR (+) Plants
CHS-S	14474	477	13	4
CHS-AS	16473	663	131	12
CHI-S	3866	345	106	22
CHI-AS	7052	569	179	20

Figure 3. *Agrobacterium*-mediated gene transformation results. Grapefruit (*Citrus paradisi* cv Duncan) epicotyl segments were transformed with the CHS and CHI genes according to the method described in Luth⁵ (1999). Plants that stained positive in the histochemical GUS assay were further analyzed by PCR using GUS and CHI or CHS gene primers to detect the presence of the transgene.

Figure 4. Southern blot analysis of selected transgenic (5:CHI-AS, 20:CHI-AS, 38:CHI-S, 40:CHI-S, 41:CHI-S, 32:CHS-AS, 36:CHS-AS, 37:CHS-AS, 56:CHS-S) and control plants (74 has GUS insert, C1 does not contain plasmid, pGA482: plasmid). To determine the copy number of the transgenes, 15 ug genomic DNA, digested by *Hpa*I, from each plant was loaded onto a 1% agarose gel, producing border fragments of varying length. The probe was 800bp of the GUS gene labeled with dig-UTP and hybridization was conducted at 42 °C overnight. The result showed that the transgene copy number varied from 1 to 4 per plant.

Figure 5. HPLC analysis of naringin levels in selected CHS-S/AS transgenic plants compared to naringin levels in control plants.

Figure 6. Steady state RNA levels of CHS gene in CHS-S/AS transgenic plants were analyzed by Real-Time RT-PCR and compared to CHS steady state RNA level in control plants using a logarithmic scale. The Taqman system was used to design the gene specific primers for quantitative analysis of steady state RNA levels and the $\Delta\Delta C_T$ method was chosen to calculate these levels. The logarithmic scale shows that CHS-S36 appears to have a low CHS steady state RNA level compared to the control.

Figure 7. HPLC analysis of naringin levels in selected CHI-S/AS transgenic plants compared to naringin levels in control plants.

Figure 8. Steady State RNA levels of the CHI gene in CHI-S/AS transgenic plants were analyzed by Real-Time RT-PCR and compared to CHI steady state RNA levels in control plants. The logarithmic scale shows that CHI-S38, CHI-40, and CHI-41 appear to have low CHI steady state RNA levels compared to the control.

Figure 9. The 1,2 Rhamnosyl transferase gene, recently cloned, was chosen for *Agrobacterium*-mediated gene transformation of grapefruit epicotyl segments. The rhamnosyl transferase (RT) construct was designed to produce dsRNA⁶ for effective interference of the endogenous gene. RT sense and antisense gene pieces were cloned together between the 35S CaMV promoter and terminator.

Construct	# of Segments	# of Shoots	# of NPTII + plants	# of RT 1,2 (gene) + plants
RT(1,2)hairpin	9760	210 (3%)	39 (19%)	4 (2%)

Figure 10. *Agrobacterium*-mediated gene transformation of grapefruit epicotyl segments with the rhamnosyl transferase hairpin construct. Since there is no reporter gene in this transformation vector, plant DNA was analyzed by PCR using 35S promoter (fwd) and RT specific (rev) primers to detect the presence of the transgene. Preliminary PCR results showed that only four transgenic plants were obtained with this construct.

Figure 11. 1,2 Rhamnosyl transferase transformed transgenic plants are growing more slowly than the control plant which does not have the plasmid in it.

Results

The bitter flavor of the flavonoid naringin can cause undesirable fruit and juice quality in grapefruit⁷. On the other hand, tasteless or sweet flavonoids can increase the pharmacological importance of fruits, perhaps yielding enhanced antioxidant or anti-tumor effects². The long term objective of this research is to decrease levels of naringin in fruits of *Citrus paradisi*, where levels are very high, by RNA interference. Additionally, we plan to increase the nonbitter flavonoids present in other citrus types by overexpression. First attempts do not specifically target naringin, but rather test whether altering gene expression of enzymes earlier in the pathway (Fig. 1) result in decreased or increased levels of naringin in transgenic plants. Although we eventually want to alter levels of rhamnosyl glycosides in fruit, the concept can be first tested in seedlings, which also produce these compounds and are easier to transform. In these first experiments, we obtained transgenic plants that appear to be producing less naringin than the control plants. We also obtained some transgenic plants that seem to be producing more naringin and some other flavonoid compounds than the control plants. All of our transgenic plants are being further analyzed for their steady state RNA level and their flavonoid levels.

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Appendix D, Figure 1: Biotransformation of hesperidin from citrus peels into neohesperidin. Hesperidin Was extracted from citrus peels and hydrolyzed in mildly acidic conditions in high temperature. The Resulting mix of hesperetin and hesperetin glucoside was was fed to BY2 cells and the resulting flavonoids were analyzed by TLC. Lanes are as follows: 1. Hydrolized hesperidin (hesperetin & hesperetin-glucoside; 2. Hesperidin standard; 3. neohesperidin standard; 4. Unfed BY2 cells transgenic for 1,2RT; 5. Hydrolized mix fed BY2 cells transgenic for 1,2RT; 6. Hydrolized mix fed BY2 wild-type cells; 7. Unfed BY2 wild-type cells; 8. neohesperidin standard; 9. Hesperidin standard; 10. Hydrolized mix.

Appendix E

Isolation of new genes putatively encoding enzymes (1,6RT and 7GT) in the flavanone biosynthetic pathway

The potential for metabolic engineering of the citrus flavanone biosynthetic pathway would be greatly enhanced if additional gene-tools are isolated. In particular, isolation of the following genes would be highly beneficial:

1. The gene encoding 1,6 rhamnosyltransferase (1,6RT) leads to the tasteless flavanone rutinosides and would be the ultimate candidate to decrease bitterness in grapefruit by overexpression. The enzyme 1,6RT would allow diversion of some flavanone-7-glucoside into tasteless compounds (instead of the bitter compounds produced by the dominant 1,2RT) without creating the negative effects of silencing the rhamnosylation step.

The prime target for isolation of the 1,6RT gene is *Citrus sinensis* (orange), since it is the only rhamnosyltransferase in this species.

2. The gene encoding 7-O-glucosyltransferase (7GT) is the initial glycosylation step and the determining step for flavanones. Silencing of this gene may allow diversion of naringenin into other flavonoids.

The gene encoding 7GT can be isolated from any of the citrus species.

Knowledge on conserved sequence elements among flavonoid glycosyltransferases was used to design degenerate primers for an exhaustive screen of *Citrus sinensis* flavonoid glycosyltransferases. A total of 9 new putative glycosyltransferase encoding genes were isolated and analyzed phylogenetically to determine the expected function:

- One gene encodes a putative 1,6RT (See Figure 1).
- One gene encodes a putative 7GT (See Figure 1)
- Two genes encode putative 5GT (not shown)
- Three genes encode putative 3GT (not shown)
- Two genes may not be associated with flavonoid biosynthesis.

Current work focuses on functionally characterizing the genes putatively encoding 1,6RT and 7GT.

Appendix E, Figure 1: Phylogenetic analysis of putative citrus 1,6RT and 7GT clones.
 Clusters are circled by dotted line. Citrus putative genes (not yet functionally analyzed) appear in bold.

Appendix F

Citrus 1,2 rhamnosyltransferase specificity and potential targets for metabolic engineering.

The transgenic biotransformation system developed during this work served to study 1,2RT substrate specificity and future potential targets for metabolic engineering. Flavonoid substrates tested are summarized in Table 1 and an example for biotransformation of the flavone diosmetin is shown in Figure 1.

Flavonoid name	Flavonoid subfamily	Glycosylation using transgenic biotransformation system	Rhamnosylation using transgenic biotransformation system
Naringenin	Flavanone	Yes	Yes
Hesperetin	Flavanone	Yes	Yes
Diosmetin	Flavone	Yes	Yes
Luteolin	Flavone	Yes	Yes
Quercetin	Flavonol	Yes	No

Table 1: Flavonoid aglycones analyzed for biotransformation using transgenic BY2 cell culture.

Conclusions

1. We concluded that flavanone-7-O-glucosides and flavone-7-O-glucosides are substrates for the enzyme 1,2RT, while flavonol-7-O-glucosides (represented by quercetin) are not substrates. The difference between flavones and flavonols is the hydroxyl at position 3 of ring-C, suggesting that there may not be any flexibility for modifications of ring-C. In contrast different modifications of ring B (as represented by naringenin vs. hesperetin and by diosmetin vs. luteolin) are accepted by the enzyme. The results suggest that the enzyme interacts mainly with rings A & C, and that ring B may not be part of the substrate recognition-interaction.
2. The flavanone diosmetin-7-glucoside is converted by 1,2RT into neodiosmin, a bitterness perception blocker which can potentially be added to bitter juices or to bitter drugs to relieve some of the bitterness. Neodiosmin is relatively scarce in nature, but its corresponding rutinoside, diosmin, is common in plants of the Lamiaceae (such as mint) which are used as sources for natural products for commercial applications. The results (See Figure 1) suggest that plants producing diosmin are potential targets for neodiosmin production via metabolic engineering using the gene encoding 1,2RT.

Appendix F, Figure 1: Biotransformation of diosmetin into neodiosmin. Diosmetin was fed to BY2 cells and the resulting flavonoids were analyzed by TLC. Lanes are as follows: 1. Diosmin standard; 2. Neodiosmin standard; 3-4. Unfed BY2 cells transgenic for 1,2RT; 5-6. Diosmetin fed BY2 cells transgenic for 1,2RT; 7-8. Diosmetin fed BY2 wild-type cells; 9-10. Unfed BY2 wild-type cells; 11. Diosmetin standard.