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Flavanone 3-hydroxylase expression in *Citrus paradisi* and *Petunia hybrida* seedlings

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Dedicated to the memory of Professor Jeffrey B. Harborne

Abstract

Petunia hybrida and *Citrus paradisi* have significantly different flavonoid accumulation patterns. *Petunia* sp. tend to accumulate flavonol glycosides and anthocyanins while *Citrus paradisi* is known for its accumulation of flavanone diglycosides. One possible point of regulation of flavanone metabolism is flavanone 3-hydroxylase (F3H) expression. To test whether this is a key factor in the different flavanone usage by *Petunia hybrida* and *Citrus paradisi*, F3H mRNA expression in seedlings of different developmental stages was measured using semi-quantitative RT-PCR. Primers were designed to conserved regions of F3H and used to amplify an approximately 350 bp segment for quantitation by PhosphorImaging. Primary leaves of 32 day old grapefruit seedlings and a grapefruit flower bud had the highest levels of F3H mRNA expression. Petunia seedlings had much lower levels of F3H mRNA expression relative to grapefruit. The highest expression in petunia was in primary leaves and roots of 65 day old seedlings. These results indicate that preferential use of naringenin for production of high levels of flavanone glycosides in young grapefruit leaves cannot be attributed to decreased F3H mRNA expression.

Keywords: *Citrus paradisi*; Rutaceae; *Petunia hybrida*; Solanaceae; Grapefruit; Developmental expression; Flavonoid; Flavanone 3-hydroxylase; F3H

1. Introduction

Flavonoids are a group of C-15 phenolic compounds which comprise one of the most abundant groups, as well as the most structurally and functionally diverse group, of secondary metabolites in plants. Flavonoids serve a wide variety of roles: UV protectants in leaves (Werner et al., 1998); an important cue in pollen development (Waser and Price, 1983) and pollen germination (Mo et al., 1992; Vogt and Taylor, 1995; Vogt et al., 1995); phytoalexins giving resistance to pathogenic microbial attack (Lamb et al., 1989; Mori et al., 1987); inducing *nod* genes of rhizobia for nodule formation of roots (Koes et al., 1994; Romeo et al., 1998; Stafford, 1997, and refs. therein); and as defense agents against predation and pathogens (Dakora, 1995 and references therein). It has been also well established that some flavonoids, such as anthocyanins, are significant factors in floral coloration and can serve as pollinator cues (Koes et al., 1994, and refs. therein).

Biosynthesis of the C15 flavonoid skeleton has been well-studied and reviewed (Heller and Forkmann, 1994; Stafford, 1990). Commonly, flavonoids are further modified by the addition of substituent groups such as methyl groups, aromatic acyl groups, and/or sugar moieties (Heller and Forkmann, 1994; Holton and Cornish, 1995; and Refs. therein). For example, naringin (**1**), a bitter tasting flavanone diglycoside, accounts for up to 40–70% of the dry weight of very young green fruit and leaves of grapefruit plants. (Jourdan et al., 1985; McIntosh and Mansell, 1990). In comparison, relatively little of the later flavonoids, such as dihydroflavonols and flavonols, are produced in these tissues

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(McIntosh and Mansell, 1990). Alternately, the flavanone aglycone (e.g., **2**) can serve as an intermediate in reactions that form other types of flavonoids such as dihydroflavonols **3** (by the action of F3H), isoflavones (e.g. **4**), or flavones (e.g. **5**) (Fig. 1). In plants such as petunia, flavonoid accumulation in wild type plants favors flavonol and anthocyanin glycoside accumulation as well as some dihydroflavonol glycosides (Harborne, 1988, 1994, and refs. therein). There is no report of flavanone accumulation in any tissue of petunia, although there are a couple of reports from *Lycopersicum*, another member of the Solanaceae (Harborne, 1988, 1994, and refs. therein). This suggests that biosynthetic output in petunia may be directed more toward other types of flavonoids as the brilliant anthocyanin-pigmented flowers would indicate. Partially opened petunia flowers, which are reported to have the highest levels of anthocyanins, also have elevated F3H activity levels (Froemel et al., 1985). Thus, distribution patterns of flavonoid products widely exist among different plant species, sometimes even within tissues of the same plant.

Plants with different flavonoid accumulation patterns can be used to study regulation of the flavanone metabolic branch point and to elucidate potential mechanisms controlling the "metabolic fate" of naringenin (**2**). When looking at regulation of a pathway (or a branch point), many factors must be considered. These include (but are not limited to) biochemical regulation of enzyme activity by factors such as substrate availability, competition for substrate, enzyme affinities, presence of inhibitors and/or activators, microcosm pH, association with other enzymes, reversible covalent modification, the number of enzyme molecules present, and compartmentation. Interrelated with some of these aspects are genetic factors such as presence/absence of genes and regulation of expression.

In order to elucidate potential mechanisms underlying differential accumulation of flavanone glycosides in petunia as compared to grapefruit, we first determined whether both model plants contain enzyme activities capable of flavanone glucosylation. We have isolated and characterized a flavanone-7-*O*-glucosyltransferase (also had some activity with flavonol substrates) from petunia (Durren and McIntosh, 1999) and a flavanone-specific 7-*O*-glucosyltransferase (7GT) from very young grapefruit leaves, that is able to glucosylate **2** to form **7** (McIntosh and Mansell, 1990; McIntosh et al., 1990). Grapefruit 7GT was present at much higher levels and was over 10,000 times less sensitive to UDP inhibition as compared to the petunia enzyme. These and other results suggest that different flavonoid accumulation patterns in these two plants may be partially due to the different relative levels and biochemical properties of their flavanone glucosylating enzymes. This does not rule out contributions from other potential types of

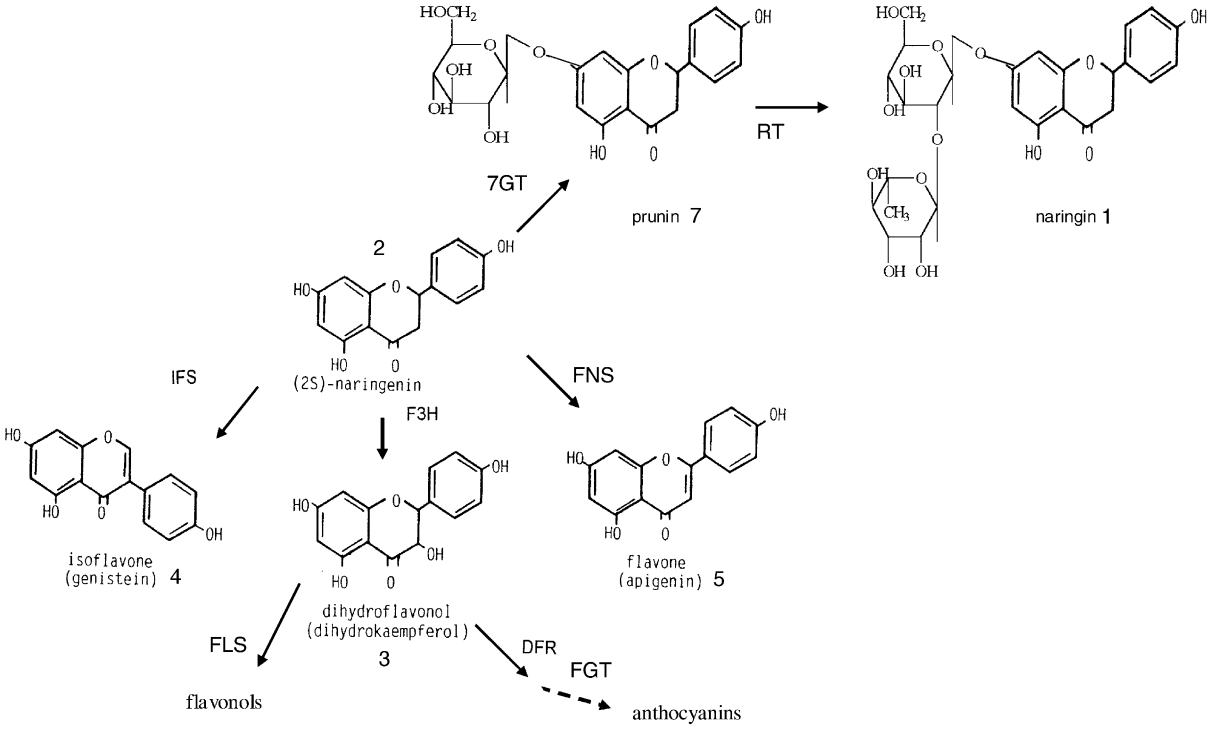

Fig. 1. Flavanone branch point showing possible biosynthetic fates of naringenin (**2**). IFS = isoflavone synthase, F3H = flavanone 3-hydroxytransferase, FLS = flavonol synthase system, FNS = flavone synthase, DFR = dihydroflavonol reductase, FGT = "flavonoid"-3-*O*-glucosyltransferase, 7GT = flavanone-7-*O*-glucosyltransferase, RT = rhamnosyltransferase.

regulation of the flavanone branch point. Indeed, there are many examples of regulation of the flavonoid pathway at the transcriptional level (Dooner et al., 1991; Gong et al., 1997; Holton and Cornish, 1995; Koes et al., 1994; Martin et al., 2001; Quattrocchio et al., 1998, and refs. therein).

Flavanone-3-hydroxylase (F3H) is one of the "core" enzymes acting at the flavanone branchpoint (Fig. 1). In a stereospecific reaction, F3H catalyzes the 3 β-hydroxylation of 2*S*-flavanones to form 2*R*,3*R*-dihydroflavonols such as dihydrokaempferol (**3**). The dihydroflavonol product can serve as an intermediate for the biosynthesis of flavonols and anthocyanidins (Holton and Cornish, 1995, and refs. therein).

Several studies have been conducted on transcriptional regulation of F3H expression. The *Delila* gene of snapdragon coordinately controls F3H expression with downstream "anthocyanin-specific" genes (type A) such as dihydroflavonol reductase (Dooner et al., 1991; Martin et al., 1991), and the F3H gene can be independently expressed (type F) such as in petunia (Quattrochio et al., 1993) or coordinately controlled with the upstream genes for chalcone isomerase and chalcone synthase by regulatory genes such as *ttg* in *Arabidopsis* (Pelletier and Shirley, 1996). Further evidence for differential control of F3H expression has been provided by complementation studies that show that these regulatory genes are highly similar across species and may be functionally equivalent; however, *cis*-acting sequences within the target genes may be divergent (Quattrocchio et al., 1998).

Developmentally regulated, tissue-specific accumulation of particular flavonoids is well documented (e.g., Jourdan et al., 1985; Barthe et al., 1987; Dooner et al., 1991, and refs. therein). However, very little comprehensive work has been performed to quantitate relative F3H mRNA expression in different plant tissues of various developmental stages, or to determine if differential expression of this gene may contribute to the metabolic fate of naringenin (**2**). We set out to address whether differential F3H mRNA expression contributes to the preferential accumulation of flavanone glycosides in grapefruit. Specific primer pairs for grapefruit and petunia F3H were constructed and used to quantitate F3H mRNA levels in different tissues at different developmental stages in grapefruit and petunia seedlings. Relative levels were evaluated in order to ascertain the potential contribution of F3H expression in the model plants to their respective flavanone glycoside accumulation patterns.

2. Results and discussion

Investigations of control of flux of metabolites through the flavanone branch point (Fig. 1) have addressed two potential levels of control. The contribution of biochemical regulation of naringenin (**2**) metabolism in different plants due to differential biochemical properties of their respective glucosyltransferases was tested, and results support that regulation can occur at this level (Durren and McIntosh, 1999). This does not preclude involvement of other control factors that may regulate competing processes. While developmentally regulated, tissue-specific accumulation of particular flavonoids is well documented, very little comprehensive work has been performed to quantitate relative F3H mRNA expression in different plant tissues of various developmental stages or to evaluate how this may impact the metabolic fate of naringenin (**2**). We used quantitative RT-PCR to determine levels of F3H mRNA expression in petunia and grapefruit seedling tissues during development in order to determine whether the preferential use of naringenin (**2**) for production of flavanone diglycosides in grapefruit seedlings can be even partially attributable to lower relative levels of F3H mRNA expression.

On the basis of conserved nucleotides between F3H sequences of *Petunia hybrida* and *Citrus sinensis*, primer pairs were designed to amplify an approximately 350 bp region of citrus and petunia F3H cDNA. The design of the grapefruit primers relied heavily upon the sequence of *C. sinensis* F3H (Accession No. ABO11795) nucleotides 578 through 914 (Fig. 2). The petunia primer set was based on *P. hybrida* F3H (Accession No. X60512) nucleotides 617 through 949 (Fig. 2).

When the amplified F3H sequences of petunia and grapefruit were compared, there was an overall homology of 84%. In addition, there was only a single nucleotide substitution between the grapefruit and *C. sinensis* sequences in this region. This substitution is at position 651nt within the *C. sinensis* F3H sequence (Fig. 2).

A Northern Blot was performed to verify detection of F3H transcript in grapefruit tissues using the randomly primed grapefruit partial F3H cDNA as a probe against 11.0 μg of citrus total RNA. A single band, approximately 1.6 kb in size, was detected in the grapefruit lane (data not shown). Further verification that the sequence amplified by the primers was F3H was carried out by additional PCR out to the 3′ end using the SMART®RACE system. Comparison of the inferred amino acid sequences of *C. sinensis* F3H with that of the 3′RACE *Citrus paradisi* F3H partial clone (Fig. 3) showed that, in this region, there were only 2 amino acids that differed between *C. sinensis* and *C. paradisi*. This confirms that the amplification product obtained during RT-PCR was a partial F3H clone. Comparisons of amino acid sequences of other plant F3H's with the *Citrus* sequences (Fig. 3) show that, while the primary structure of this protein is well-conserved between genera, it appears to be even more highly conserved

between the two *Citrus* species (at least at the carboxy terminus).

To establish the linear range of RT-PCR amplification of F3H for grapefruit and petunia, cDNA from young petunia and grapefruit secondary leaf tissue was PCR amplified with γ-^{32}P ATP end-labeled F3H primers (Fig. 4). PCR reactions were removed from the thermal controller at every odd numbered cycle between cycles 15–35. The reaction products were then resolved on a 6.0% polyacrylamide gel and quantified with use of a Phosphorimager. The linear range of F3H amplification in citrus was between cycles 23 and 29, and in petunia the linear range of F3H amplification was between cycles 27 and 31 (Fig. 4). The same procedure was used to determine the limits of linear amplification of 18S rRNA internal standard primers, which was between 15 and 20 cycles (Fig. 4).

Roots as well as primary (first leaf set to emerge), secondary, and tertiary leaves of petunia and grapefruit seedlings were used for the F3H quantitative RT-PCR assays. The tissues were collected from 7, 18, 28, and 65 day old petunia plants (stages 1–4, respectively) and from 17, 25, 32, and 51 day old grapefruit plants (stages 1–4, respectively) grown in the greenhouse. F3H mRNA expression was normalized to 18S rRNA expression. The highest relative level of F3H mRNA expression in vegetative tissues was in the primary leaves of stage 3 grapefruit seedlings (Fig. 5). With the exception of stage 1 stems, F3H mRNA expression was highest in grapefruit leaves, in general. Relative expression in grapefruit

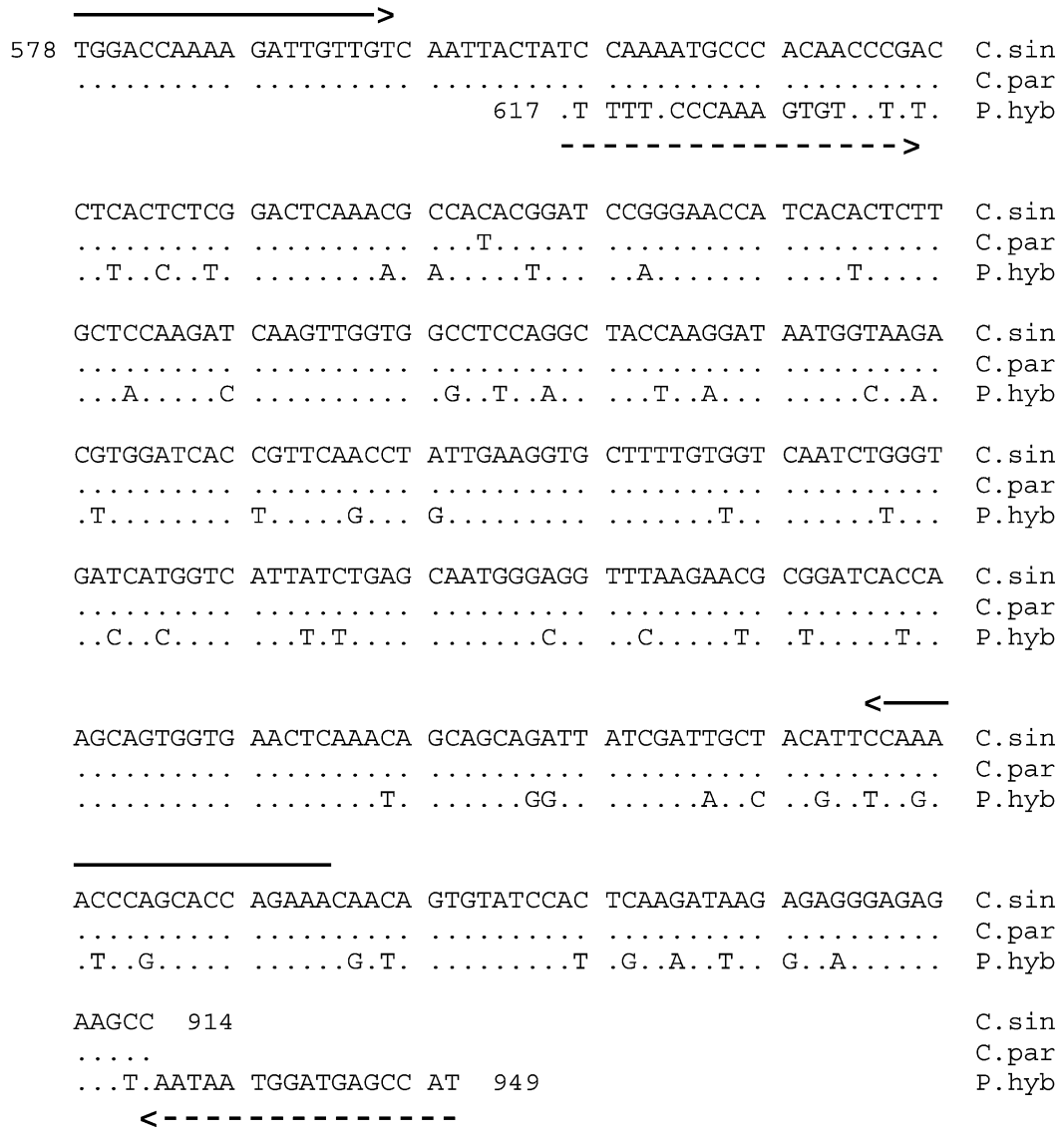

Fig. 2. Alignments of *Citrus sinensis* (C.sin), *Citrus paradisi* (C.par), and *Petunia hybrida* (P.hyb) F3H cDNA sequences. The arrows denote primer sequences; dashed arrows for petunia primers and black arrows for grapefruit primers. The (.) identifies identical bases.

roots was highest during stage 1 and declined to undetectable levels in stage 4 roots (Fig. 5). The highest relative level of F3H mRNA expression in petunia vegetative tissues was found in stage 4 primary leaves, followed by stage 3 and stage 4 root tissue (Fig. 5). It is important to note that grapefruit expressed F3H at significantly higher levels than petunia.

Preliminary experiments were conducted to determine F3H copy number in grapefruit to determine if higher levels of F3H mRNA expression could be due to measuring expression of more than one gene in the RT-PCR experiments. Results were consistent with what would be seen if F3H was present in a single copy (data not shown). This finding correlated well with other non-polyploid plants characterized which also contain a single F3H gene copy (Pelletier and Shirley, 1996, and refs. therein).

In order to verify that the higher levels of F3H mRNA expression detected in grapefruit tissues was due to actual mRNA levels rather than the petunia primer having a lower amplification efficiency, relative amplification efficiencies of both the petunia and grapefruit

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1   ---PRVTPSTLTALAEEKTLQTSFIRDEDERPKVAYNQFSNEIPIISLEGIDDETGKRAE  P.hyb
1   --AP----G...E..G.SK.NSK.V...........E..D.......A....VD...GE  M.inc
1   MVA--EK.K...S.EGDDK.NSN.V...........E...D......A...G.--..G.  D.car
1   MAP---M.TS.LS...QNS.LQT.............E..HD..V...A..HGD--D.V.  A.maj
1   MAPVSNETFLP.EAWG.A..RP..V..........HDR..DAV.L...H...GA--R..Q  H.vul
1   MAA.----IS.-KWE.HSLHENK.VF........P..T......V...A...G--CR...  C.sin

59  ICDKIVKACEDWGVFQVVDHGVDAEVISQMTTFAKEFFALPPEEKLRFDMSGGKKGGFIV  P.hyb
55  ..RE..E...N..I.........TSLVAD..RL.RD.........................  M.inc
57  ..R...E......I........GDDL.AD..RL.R......A...................  D.car
56  ..R...A...E..I.G....SI.LKIVRE..DM.R....M.AG..................  A.maj
59  .R.RVAA......I...I..G...DL.AD..RL.R......A.D...Y............  H.vul
54  ...E.........I.........TKLL.D..GL.RD..H..TQ.......T.........  C.sin

115 SSHLQGEVVQDWREIVTYF-SYPTRARDYSRWPDKPEGWIAVTQKYSEKLMELACKLLDV  P.hyb
114 .......A...........-...VRN........J.Q..AK..EE......G......E.  M.inc
116 ..................-....NS...T..........K..EE..N...T...T..G.  D.car
119 .......I..N...M....S...IKT..........A..K.A.ET...E..K.NS...E.  A.maj
119 .......A...........-...VKA...G...E..A..C..VER...R..G.S.N.MG.  H.vul
117 .......A...........-...IKA.....E..K.NE.R...EE..KV..G......E.  C.sin

178 LSEAMGLEKEALTKACVDMDQKVVVNFYPKCPEPDLTLGLKRHTDPGTITLLLQDQVGGL  P.hyb
174 ..........S..N........I...Y.....Q...........................  M.inc
176 ........L.............I.........Q...........................  D.car
176 .....E...D..SN..L.F.......F.T...Q...........................  A.maj
178 ........T...A.............F..R..Q.....F................L....  H.vul
173 ............................Y.....Q...........................  C.sin
                                ....Q...........................  C.par

238 QATKDNGKTWITVQPVEGAFVVNLGDHGHFLSNGRFKNADHQAVVNSNSSRLSIATFQNP  P.hyb
234 ...R.D.N.....................................................  M.inc
236 .....G..........P.............................EC............  D.car
236 .V...G.........................Y..................C...........  A.maj
238 .....G..N......IS.............M................GE............  H.vul
233 .....G.S.....K.I....G........Y..................ST............  C.sin
    ...............I.............Y...............................  C.par

298 APEAIVYPLKIR-EGEKSIMDEPITFAEMYRRKMSKDLELARLKKQAKEQQLQAEVAAEK  P.hyb
294 ....T.....V.-....A..E.........K...GR.........L...EHNHK.A.KPL  M.inc
296 S.D.T....A..-...N..........DL.....A..I....H.RL...EMPFK.LDEA.  D.car
296 ..D.I.....NQQ...E.........T.......G......KNGLKKLAKEKLQ.EELEK  A.maj
298 ..D.R.W..AV.-...EP.LE.....T.......ER..D..KR..Q..D.LM.QQLQLQQ  H.vul
293 ....T......N-....PVLGEP.P.F..................L.N.KKQYSQK.KLD  C.sin
    ....T....K..-....PVLGEP.P.F..................L.N.KHQDSQK.KLD  C.par

    AKPIQQILA  C.sin
    AKPIQQILA  C.par
```

Fig. 3. Amino acid alignments of *Petunia hybrida* (P.hyb; X60512) *Matthiola incana* (M.inc; X72594), *Dianthus caryophyllus* (D.car; X72592); *Antirrhinum majus* (A.maj; S60876); *Hordeum vulgare* (H.vul; X58138), *Citrus sinensis* (C.sin; ABO11795), and *Citrus paradisi* (C.par) F3H. (.) identifies identical amino acids.

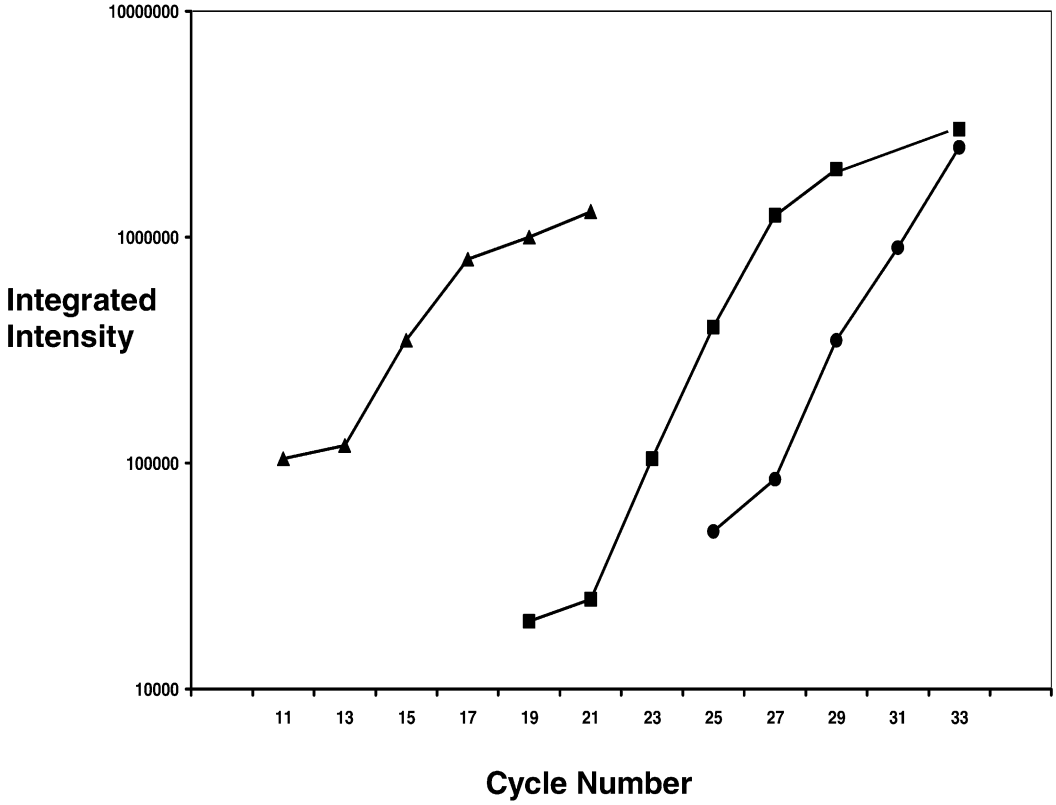

Fig. 4. RT-PCR amplification of F3H mRNA in *Citrus paradisi* (■), *Petunia hybrida* (●), and 18S rRNA (▲); cycle number versus amplimer intensity (integrated). Results from a duplicate experiment showed the same trends.

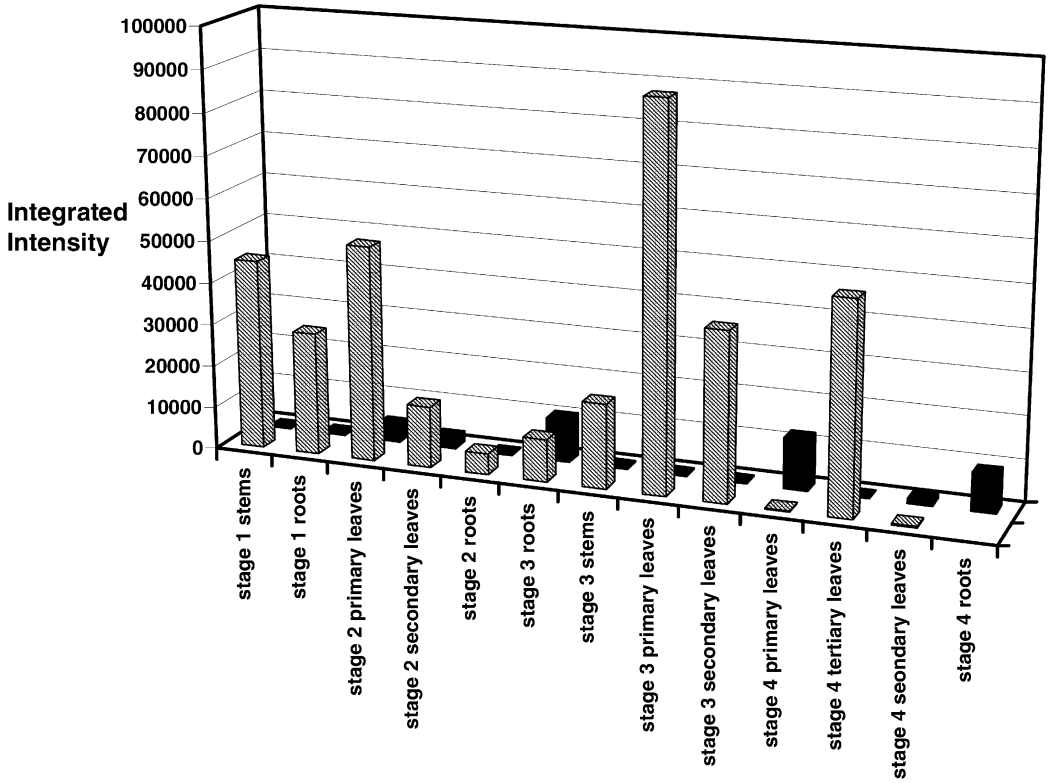

Fig. 5. Quantitative RT-PCR. Mean relative RT-PCR amplification of grapefruit (grey bars) and petunia tissues (black bars) at four different developmental stages ($n=2$). Results are presented grouping all tissues within plants in order of increasing age/stage of development. Values represent F3H expression for each plant species normalized to their respective 18S rRNA expression levels.

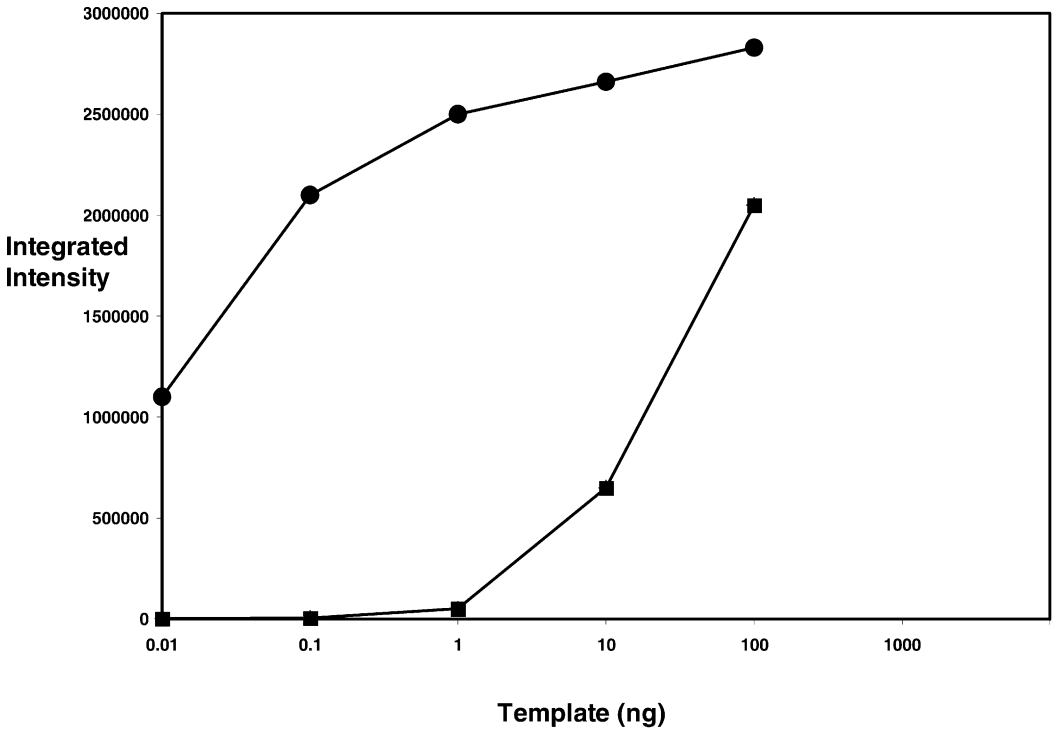

Fig. 6. RT-PCR amplification efficiencies of the grapefruit (■) and petunia (●) F3H primers; ng of template versus integrated intensity.

F3H primer sets were determined. This was done by using serial dilutions of the PCR templates ranging from 0.01–100 ng in a PCR experiment and levels of products quantified as described. Results indicated that the petunia F3H primers were at least 2.3-fold more efficient at F3H PCR amplification than the grapefruit F3H primers (Fig. 6). Therefore, higher relative levels of F3H mRNA expression seen in grapefruit tissues is not due to more efficient amplification by the grapefruit primer pair.

Production of high levels of the bitter flavanone diglycoside naringin (**1**) in very young grapefruit tissues clearly demonstrates that earlier reactions in the flavonoid biosynthetic pathway catalyzed by chalcone synthase and chalcone isomerase (as well as 7GT) are fully functional. Our results show that the preferential utilization of flavanones for naringin (**1**) biosynthesis (rather than flavonol accumulation) is not due to lack of expression of F3H mRNA. This supports the idea that F3H may not direct flux further into flavonoid metabolism, at least not in grapefruit tissues. It is, of course, possible that F3H enzyme expression is post-transcriptionally regulated. In future studies, F3H protein expression and enzyme activity will be ascertained in order to address this possibility. Other potential factors such as differential association of competing enzymes into "metabolons" (Winkel-Shirley, 1999, and refs. therein) also need to be evaluated.

It is clear that the biochemical differences between the flavanone ("flavonoid") 7-*O*-glucosyltransferase in petunia and the highly active flavanone-specific 7GT in grapefruit (Durren and McIntosh, 1999) may have a major impact on the preferential use of naringenin (**2**) for production of flavanone diglycosides in grapefruit seedlings. These differences include relative levels of activity, substrate preferences and affinities, and different responses to potential inhibitors such as UDP. Current efforts are focusing on further evaluation of factors contributing to the tissue-specific and developmentally regulated utilization of naringenin (**2**) during flavanone metabolism in grapefruit. Efforts are underway to clone grapefruit 7GT for structure/function analyses.

3. Experimental

3.1. General experimental procedures

Sources of molecular biology materials: RNeasy RNA purification kits (Qiagen); RQ1 RNase-Free DNAse I, DNAse I buffer, Recombinant RNasin Ribonuclease Inhibitor, RT buffer, Reverse Transcriptase (MMLV), Wizard PCR purification kit, Wizard MidiPrep kit, and T_4 Kinase (Promega); RETROscript random Decamers, NorthernMax™ kit, DECAprime II, and QuantumRNA 18S primers (Ambion); DTT and F3H primers (petunia and grapefruit) (GIBCO BRL); Amplitaq gold, dNTP's, and PCR reagents (Perkin Elmer); pCR®2.1 vector for T-A cloning (Invitrogen); T4 DNA ligase (New England Biolabs);

Epicurian Coli XL-1 Blue MRF' supercompetent cells (Stratagene); Biotech Hybond-N^+ Bond nylon membrane (Amersham); 100×Denhardt's and 40% acrylamide (acryl:bis-acryl = 19:1) (Ameresco); sheared Herring sperm DNA and DEPC (Sigma); SMART®RACE cDNA Amplification kit (Clontech); RPI-Bio Safe II scintillation fluid (Labindustries, Inc.); γ-^{32}P ATP (specific activity 7000 μCi/mmol, ICN); deionized formamide, 20× SSC, and other chemicals were molecular biology grade (Fisher).

The following equipment was used: PTC-100™ Programmable Thermal Controller (MJ Research, Inc.); UV Crosslinker FB-UVXL-1000 (Fisher); Hybridiser HB-1D oven (Techne); Beckman scintillation counter; PhosphorImaging Screen (Kodak); band intensities were quantified using a Molecular Imager® Fx phosphorimager (Biorad).

3.2. Plant sources

Seeds of *P. hybrida* (Celebrity Red Morn variety) were obtained from Park Seed. *C. paradisi* (Duncan variety) seeds were obtained from the Citrus Budwood Registry (Lake Alfred, FL). Seedlings were grown under greenhouse conditions.

3.3. RNA isolation

The experimental approach was to assay F3H RNA levels within various tissues at different developmental stages of grapefruit and petunia plants by semi-quantitative RT-PCR. Roots, flowers, and primary, secondary, and tertiary leaves were collected from 7, 18, 28, 65 day old petunia seedlings and 17, 25, 32, 51 day old grapefruit seedlings. Total RNA was extracted from approximately 100 mg of fresh plant tissue, spectrophotometrically quantitated, and stored as an ethanol precipitate (−80 °C).

3.4. Synthesis of cDNA

Prior to reverse transcription, RNA (2.0 μg) for each sample was recovered from the ethanol precipitate and resuspended into dH_2O (GIBCO). The RNA samples were treated with 6U of RQ1 RNase-Free DNAse I, 1 × DNAseI buffer, 40U Recombinant RNasin Ribonuclease Inhibitor, and 2 mM DTT for 1 h at 37 °C and reactions terminated by heating 20 min, 65 °C), after which one tenth volume of 3 M sodium acetate (pH 5.3) was added followed by phenol:CHISAM extraction and EtOH precipitation.

For reverse transcription reactions, the treated RNA was recovered from the EtOH precipitate and resuspended into PCR quality water. RETROscript random Decamers (320 ng) were added and reactions incubated at 70 °C for 10 min. Reactions were quenched on ice to anneal the primers and aliquotted into two U.V. treated 0.5 ml thin-walled microcentrifuge tubes for RT(+) and RT(−) reactions. A 1 X reaction mix was set up as follows: 1×RT buffer, 10 mM DTT, and 200 μM each dNTP were added to each annealed primer/RNA sample and vortexed. Reactions were incubated on a 42 °C heat block for two minutes after which 200 μg of Reverse transcriptase was added to each RT(+) reaction and PCR H_2O was added to each RT(−) reaction; final reaction volume for each was 20 μl. Reactions were incubated 90 min at 42 °C, followed by 15 min at 70 °C. Once reactions were complete, the newly synthesized cDNAs were diluted 10-fold in PCR grade H_2O and stored at −20 °C (Schoborg et al., 1994).

3.5. Primer design and verification of amplification products

On the basis of conserved regions between *Citrus sinensis* (accession No. ABO11795) F3H cDNA sequences and other F3H sequences, the following new 19-mer primers were designed: forward primer (cF3H578) TGG ACC AAA AGA TTG TTG T ; reverse primer (cF3H914) GGC TTC TCT CCC TCT CTT A. To amplify F3H from *P. hybrida*, the following primers were designed based on the petunia F3H sequence (Genbank Accession No. X60512): forward primer (pFHT617) TTT TTA CCC AAA GTG TCC TG ; reverse primer (pFHT949) ATG GGC TCA TCC ATT ATT G.

These primers PCR amplify approximately 355 and 325 base pairs of the *C. paradisi* and *P. hybrida* F3H cDNA respectively using the following conditions (25 μl reaction): 1× Buffer, 1.5 mM Mg^{2+} (final concentration), 2.0 μM cF3H519, 2.0 μM cF3H914, 12.5 mM each dNTPs, 2.0 μl of cDNA template, and 1.25 U of Amplitaq gold. "Hotstart" PCR was performed with the following conditions: step 1, 94 °C for 9 min; step 2, 94 °C for 30 s; step 3, 54.5 °C for 45 s; step 4, 72 °C for 2.5 min; step 5, repeat steps 2–4 for 49 cycles; step 6, 72 °C for 5 min; step 7, 4.0 °C overnight. Sizes of the grapefruit and petunia F3H RT(+)-PCR products were verified by running an aliquot of each on an agarose gel; the remainder of the PCR products were purified using a Wizard PCR purification kit.

Identities of the F3H PCR products were further verified by direct sequencing (Univ. Georgia) followed by Genbank Blast homology analysis against the published sequences [Advanced Blast Search analysis (Entrez)].

3.6. Cloning of PCR products

The remaining 150 ng citrus F3H PCR product was T-A cloned into a pCR®2.1 vector (Invitrogen) as follows (10 μl reaction): 25 μg pCR®2.1 vector, 400 U T4 DNA ligase, and 1 × ligation buffer. The ligation reaction was incubated at 14 °C for 17–19 h and

subsequently transformed into Epicurian Coli XL-1 Blue MRF' supercompetent cells. Positive clones were blue-white selected on LB/amp agar plates with IPTG/X-gal. Mini preps of selected clones were screened by EcoRI diagnostic restriction digests which cleave out the 355 bp insert. Large scale preparation of DNA from several clones was performed using the WIZARD MIDIPREP kit. Identity of putative clones was verified by sequencing.

Further verification of grapefruit partial F3H clone identity was done using RT-PCR to obtain additional sequence information toward the 3′ end. Total RNA was isolated as previously described and cDNA was synthesized using the SMART®RACE cDNA Amplification kit and the grapefruit F3H forward primer as the gene specific primer. The single amplified band was cloned and sequenced as previously described.

3.7. Labeling of primers for quantitative RT-PCR

PCR primers and CIAP-treated 100 bp markers were end-labeled with γ-^{32}P ATP. To 1.0 nmol of the respective primer pair [18S (Ambion), cF3H, or pFHT] or 2.0 μg CIAP-treated 100 bp markers, the following were added (60 μl final reaction in dH_2O): 1.0 μl of T_4 Kinase (10 U/μl), 1.0 μl of γ-^{32}P ATP (7000 μCi/mmol), and 6.0 μl of 10× kinase buffer. Reactions were incubated at 37 °C for 1 h. Each reaction was then brought up to 100 μl with dH_2O, split into two aliquots, and pipetted onto prepared ProbeQuant G-50 columns. Labeled DNA was eluted from the column by centrifugation at 3000 rpm for 2 min.

3.8. Quantitative RT-PCR

PCR reactions were set up as described as above with the exception of using a final concentration of 1.6 μM of each "cold" F3H primer and 0.64 μM of each labeled F3H primer pair. To establish the linear range of RT-PCR amplification of both petunia and citrus F3H with the appropriate primer sets, replicate PCR reactions were removed from the thermal cycler at odd numbered cycles between 15 and 35 and quenched on ice.

Once each RT-PCR reaction had been terminated, the radio-labeled PCR products were electrophoresed on a 6.0% non-denaturing polyacrylamide gel (13 ×15 cm × 0.75 mm gels, composed of: 15 ml 10 × TBE, 40% acrylamide, 120 μl 10% ammonium persulfate, and 16 μl TEMED). Approximately 5–10 μl of the PCR reaction was loaded with an equal volume of 10 × TBE loading buffer. Gels were run in 1 × TBE running buffer at 100 V for ~6 h with a circulating coolant at 10 °C to avoid heat denaturation. Gels were dried, exposed to a PhosphorImaging Screen, and band intensities quantified using a Molecular Imager® Fx phosphorimager (Biorad). Grapefruit RT-PCR assays were repeated in triplicate from independent mRNA extractions.

3.9. Amplification efficiency of primers

Product from grapefruit and petunia F3H RT-PCR reactions was purified and then quantified spectrophotometrically. To further assure the diluted PCR products were present in equal amounts, 5 μl of each was run out on a 1.0% TBE gel. Step-wise serial dilutions of the PCR templates ranging from 0.01 to 100 ng were used in a PCR experiment and levels of products quantified as described above.

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