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Juvenile-Specific Localization and Accumulation of a Rhamnosyltransferase and Its Bitter Flavonoid in Foliage, Flowers, and Young Citrus Fruits[1]

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1-2-Rhamnosyltransferase catalyzes the production of disaccharide-flavonoids that accumulate to 75% of dry weight. Vast energy is expended in a short time span to produce these flavonoids. The highest rhamnosyltransferase activities and immunodetected concentrations were observed in early development of *Citrus grandis* (pummelo), coinciding with up to 13% of fresh weight as naringin. The concentration of naringin in leaves, petals, receptacles, filaments, albedo, and flavedo drops drastically during development and correlates directly with a decrease in the activity and amounts of 1-2-rhamnosyltransferase. Anthers had minute rhamnosyltransferase activities and low concentrations of naringin. Conversely, high 1-2-rhamnosyltransferase activity and naringin concentrations appeared in both young and mature ovaries, as well as in young fruits. The total amounts of naringin in mature leaves decreased without detectable in vitro degradation of naringin in leaves. There was still a net accumulation of naringin in the albedo and flavedo of older fruit even though these tissues had only traces of 1-2-rhamnosyltransferase. Traces of enzyme synthesis in fruits, or import of the product from leaves, may explain the net accumulation of naringin in growing fruits. Unlike the late-expressed genes for glycosyltransferases in anthocyanin biosynthesis, the rhamnosyltransferases from *Citrus* are active only in juvenile stages of development.

Plant flavonoids are either constitutively synthesized or made in response to diverse environmental stimuli (cf. Stafford and Ibrahim, 1992). Specific flavonoids play a role in protection against UV light (Li et al., 1993), defense against pathogens (Lamb et al., 1989), and induction of *nod* genes of *Rhizobium* (Fisher and Long, 1992). Some pigments such as anthocyanins are also flavonoids (Ebel and Hahlbrock, 1982).

Most secondary metabolites, including flavonoid pigments and their glycosides, accumulate when tissues and organs have finished growth (Kamsteeg et al., 1980; Kleinehollenhorst et al., 1982). In contrast, juvenile, rapidly growing grapefruit and pummelo (*Citrus grandis*) accumulate large amounts of the extremely bitter flavanone-glycosides naringin and neohesperidin (Horowitz, 1986). These flavonoids can reach up to 75% of the dry weight of young, approximately 1-cm diameter fruits (Kesterson and Hendrickson, 1953), but their physiological roles are not yet clear. These *Citrus* spp. are thus interesting models for juvenile-specific promotion and cessation of metabolic pathways.

The pathway of biosynthesis of bitter flavanone-glycosides in *Citrus* has recently been elucidated (Fig. 1) and found to be similar to the well-documented pathway in other species (cf. Ebel and Hahlbrock, 1982; Stafford, 1990), despite earlier, alternative suggestions (Raymond and Maier, 1977). *Citrus* leaves contain all of the activities necessary to synthesize naringen, as outlined in Figure 1. The last enzyme in the pathway, a 1-2-rhamnosyltransferase (UDP-rhamnose: flavanone-7-*O*-glucoside-2″-*O*-rhamnosyltransferase) has been purified to homogeneity by our group (Bar-Peled et al., 1991).

Naringenin can be converted in some *Citrus* species to hesperetin, possibly by a 3′-hydroxylase and a 4′-*O*-meth-

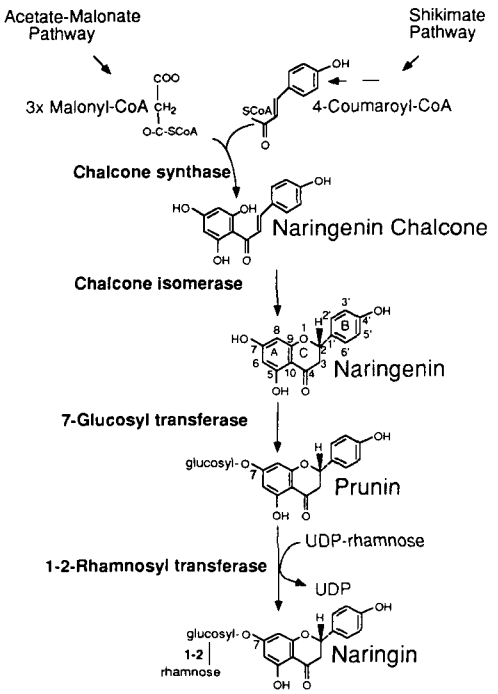

Figure 1. The biosynthesis of naringin in *Citrus* (based on Lewinsohn et al., 1989b; McIntosh et al., 1990; Bar-Peled et al., 1991).

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yltransferase. Hesperetin is then glucosylated to hesperitin-7-*O*-glucoside (Lewinsohn et al., 1989b; McIntosh et al., 1990) and is finally rhamnosylated to neohesperidin by the same rhamnosyltransferase, producing naringin (Lewinsohn et al., 1989b; Bar-Peled et al., 1991).

The accumulation and distribution of the major flavanone-glycoside products have been investigated during different stages of growth of grapefruit and sour orange without parallel studies of the enzymes involved. Flavanone-glycosides were found in almost all plant parts, but rapidly accumulated in young, developing *Citrus* leaves (Jourdan et al., 1985; Berhow and Vandercook, 1989, 1991; Castillo et al., 1992). The specific parameters controlling synthesis and localization of flavanone-glycosides in *Citrus* could not be studied until the terminal enzyme in the pathway itself was purified and an antibody was raised against it. Antibodies are necessary to detect enzyme protein when the enzyme (or pro-enzyme) is inactive. The purified monomeric (approximately 50 kD) rhamnosyltransferase from young pummelo leaves (Bar-Peled et al., 1991) was used to raise a specific antibody to this 1–2-rhamnosyltransferase (M. Bar-Peled, R. Fluhr, and J. Gressel, unpublished results). We set out to ascertain when this enzyme is present or limiting during development and to evaluate if it could be responsible for the levels of product at each stage. The amounts of enzyme and its specific activity are reported, along with the levels of the end product naringin, during *Citrus* leaf, flower, and fruit development for specific juvenile stages.

MATERIALS AND METHODS

Plant Materials

The *Citrus* species and hybrids used in this study were: *Citrus grandis* Osbeck cv Goliat (pummelo), *Citrus aurantium* L. (sour orange), *Citrus paradisi* Macf. cv Duncan (grapefruit), *Citrus paradisi* × *Poncirus trifoliata* (Swingle citrumelo), *Citrus paradisi* × *Citrus grandis* (oroblanco), *Citrus mitis* Blanco (calamondin). They were obtained from local sources. Some plants were growing in commercial groves and some were cultivated in the greenhouse. All plant materials were harvested and immediately analyzed or frozen at −70°C until use. Callus cultures derived from nucellus tissue of grapefruit similar to those described by Gavish et al. (1992) were kindly supplied by Dr. Aliza Vardi.

Protein Extraction and Rhamnosyltransferase Assay

Five to 18 leaves, or pieces of similar size of tissue at each stage of growth, were combined and frozen. One-gram samples were ground under liquid nitrogen to a fine powder in a mortar and pestle. Sodium ascorbate (100 mg), polyvinylpolypyrrolidone (100 mg), and acid-washed sand (200 mg) were added, and the proteins were then extracted by further grinding with 4 mL of an extraction buffer containing 90 mM Tris-HCl (pH 7.5), 10% glycerol (v/v), 1 mM Na_2-EDTA, and 0.2 mM PMSF. The homogenates were centrifuged twice at 30,000*g* for 20 min at 4°C, and the supernatants were stored at −70°C. Protein concentrations were determined with Coomassie brilliant blue using BSA as the standard (Bradford, 1976). 1–2-Rhamnosyltransferase activity (from 40 μg of total soluble proteins) was assayed by detecting the isotope-labeled rhamnose moiety transferred from UDP-[^{14}C]rhamnose onto the flavanone-glucoside acceptor prunin, as described previously (Bar-Peled et al., 1991). The incubation time was 60 min at 37°C unless otherwise stated, when activities were high enough that substrate became limiting (Bar-Peled et al., 1991).

Antibody Preparation, Electrophoresis, and Immunoblotting

The purified 1–2-rhamnosyltransferase (approximately 200 μg in 3 mL) eluted from a Mono-Q column (Bar-Peled et al., 1991) was denatured with SDS and used to generate a polyclonal antibody in rabbits (M. Bar-Peled, R. Fluhr, and J. Gressel, unpublished results). Crude extracts of total soluble protein (40 μg) and purified 0.5 to 120 ng of 1–2-rhamnosyltransferase used for calibration were separated by electrophoresis on 12.5% acrylamide SDS-PAGE using a modified Laemmli system as described previously (Bar-Peled et al., 1991). Molecular mass markers were: BSA (67 kD), ovalbumin (43 kD), chymotrypsinogen A (25 kD), and Cyt *c* (12.5 kD) (Sigma); they were run with each SDS-PAGE as indicated on the figures. After electrophoresis, proteins were electroblotted onto 0.22-μm pore size nitrocellulose membranes using a semi-dry electroblotting buffer system (Kyhse-Anderson, 1984) for 35 min at a constant current of 3 mA/cm^2 of gel. Proteins were visualized after electroblotting by staining the membranes with 0.2% Ponceau S dissolved in 0.5% acetic acid for 2 min followed by 5 min of destaining in distilled water. Blots were then incubated for 2 h in blocking solution containing both TBS-T (50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 0.1% [v/v] Tween-20) and low-fat powdered milk reconstituted to 5% (w/v). Blots were incubated for 2 h at room temperature with anti-1–2-rhamnosyltransferase serum diluted 1:5000 in blocking solution. After the membranes were washed in TBS-T, blots were incubated for 1 h at room temperature with goat anti-rabbit immunoglobulin G conjugated to horseradish peroxidase (Sigma), diluted 1:5000 in blocking solution. Blots were washed four times for 5 min each with TBS-T. Each membrane was placed in a plastic tray containing 10 mL of Enhanced Chemiluminescence (ECL, Amersham) detection solutions for 1 min. Immunoreactive, chemiluminescent bands were visualized by exposing the blots to Kodak X-Omat film. Autoradiographs of the western blots were scanned by densitometry. Four or five pure rhamnosyltransferase standards (0.5–120 ng) were run simultaneously on each gel and they plotted linearly. The blot areas of the rhamnosyltransferase from tissue samples were measured and interpolated.

Flavonoid Extraction and HPLC Analysis

Five to 12 leaves or pieces of similar size of tissue at each stage of growth were combined and frozen. One-half gram of each sample was placed in a 15-mL round-bottom polypropylene centrifuge tube and then ground to a fine powder under liquid nitrogen using a glass homogenizer. The flavonoids were then extracted while grinding with 2.5 mL of methanol:dimethyl sulfoxide (1:1, v/v). After 20 min of shak-

ing at 37°C, the homogenates were transferred to 1.5-mL tubes and centrifuged at room temperature for 10 min at 12,000*g*. The supernatants were filtered through disposable 0.45-μm (pore size) polytetrafluoroethylene membranes prior to injection onto the HPLC column. These filters do not retain any of the UV-absorbing materials or flavonoid standards.

Flavonoids were separated on an Adsorbosphere C_{18} reverse-phase HPLC column (4.6 × 250 mm, 5 μm average particle size) using an isocratic solvent system (Castillo et al., 1992) of water:acetonitrile:methanol:glacial acetic acid (15:2:2:1, v/v). Crude flavonoid extracts (2–15 μL) or flavonoid standards (dissolved in methanol:dimethyl sulfoxide, 1:1 [v/v]) were injected via a 100-μL loop at a flow rate of 1 mL/min. The flavonoids were isocratically eluted at the same flow rate and detected by monitoring the A_{283} of the column effluent. The relative amount in each peak was determined with a BarSpec integrator attached to the Milton-Roy HPLC system. The retention times were: naringin (13.6 min); prunin (15.1); hesperidin (16.5); neohesperidin (19.7); hesperitin-7-*O*-glucoside (20.8); naringenin (46.2); and hesperetin (68.2 min). The quantity of naringin in crude extracts was determined by a linear regression equation derived from 10 standard samples of naringin over a range of 1 to 20 μg.

In Vitro Assay for Naringin Degradation

The crude protein extracts from pummelo leaves at different stages of growth were also used for a degradation assay. Degradation activity was assayed by detecting the loss of naringin and its possible conversion to prunin and naringenin in two different buffers. The reaction mixture (total volume of 200 μL) contained either buffer I, 50 mM Tris-HCl, pH 7.5; or buffer II, 50 mM sodium acetate, pH 4.5; 200 μM naringin (from a stock solution of 2 mM dissolved in dimethyl sulfoxide); and 100 μg of protein extract from pummelo leaves. Degradation by 1 unit of naringinase from *Penicillium* (Sigma) was additionally used as a model for one type of degradation. The enzymatic reaction was terminated after 1 to 6 h of incubation at 37°C by the addition of 400 μL of ethyl acetate. The mixture was centrifuged for 5 min at 12,000*g*. The flavonoid products in the upper organic phase were removed, lyophilized, dissolved in 20 μL of methanol:dimethyl sulfoxide (1:1, v/v), and then injected into the HPLC apparatus. Identification and quantification of the decrease in naringin and the increase in its glycoside and aglycone were measured by reverse-phase HPLC and by TLC (Bar-Peled et al., 1991).

RESULTS

Disappearance of Rhamnosyltransferase during Leaf Maturation

We analyzed protein extracts for both the presence and activity of rhamnosyltransferase in leaves at varying stages of growth to ascertain whether the lower concentrations of naringin found in mature tissues are due to dilution, turnover, and/or regulation of the 1–2-rhamnosyltransferase. The greatest concentrations of rhamnosyltransferase (Fig. 2A) and the highest specific activities (Fig. 2B) were observed in 0.5- to 3-cm-long, young, developing leaves (Fig. 2). A parallel progressive decline in the concentration and the activity of

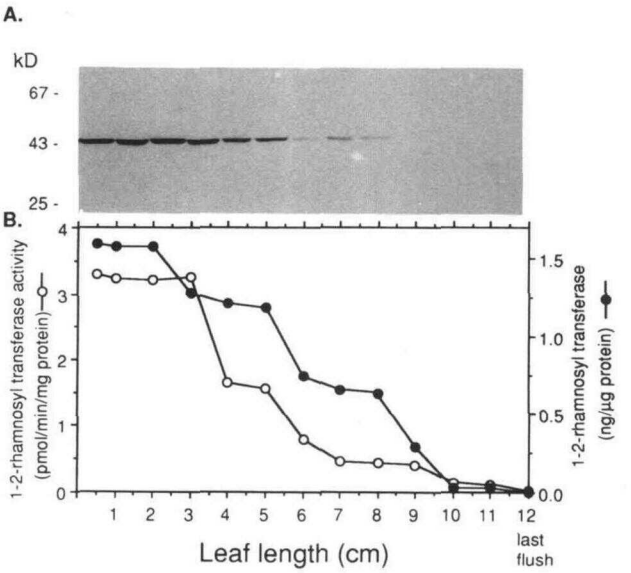

Figure 2. Disappearance of rhamnosyltransferase during the development of pummelo leaves. Proteins extracted from 16 pooled leaves at each stage of growth were analyzed for 1–2-rhamnosyltransferase either by immunoblotting with anti-1–2-rhamnosyltransferase serum (A) or quantitative assays of activity and enzyme concentration (B). All leaves (0.5–11 cm long) were actively growing, light-green leaves, except for the dark-green, older leaves from the previous leaf flush. O, Activity. The enzyme reaction was terminated after 30 instead of 60 min for the youngest leaves (up to 4 cm long) due to the possibility of having the radioactive substrate become limiting (Bar-Peled et al., 1991). Data are the mean of two to three replicates and the values within treatments varied by no more than 10%. ●, Enzyme concentration. The average quantitative data obtained by densitometry compared with pure rhamnosyltransferase standards from nine similar experiments are shown.

the enzyme was observed in extracts made from 3- to 8-cm-long leaves, followed by a sharp decline to almost 0 in fully grown yet light-green leaves (9–11 cm long). Neither enzyme activity nor protein was observed in extracts from mature, dark-green leaves from the previous leaf flush. Similar patterns were obtained from pummelo leaves grown in the greenhouse or in the grove at different periods during the year.

We could not detect the enzyme in the emerging cotyledons of pummelo seedlings grown in darkness or in the light (data not shown). Indeed, naringin was shown previously not to accumulate in cotyledons of grapefruit (Jourdan et al., 1985). The appearance of 1–2-rhamnosyltransferase in etiolated and green seedlings begins with the initiation of true leaves, and the enzyme disappears as the leaves mature (data not shown), as described for older plants.

The above finding that rhamnosyltransferase from pummelo is expressed only in juvenile stages of true leaf development was substantiated when examining other *Citrus* spp. that accumulate bitter flavonoids. 1–2-Rhamnosyltransferase was found in young leaves but not in mature leaves of other *Citrus* spp. (Fig. 3). The molecular masses of the enzyme from all six species are similar (Fig. 3).

A mixing experiment was performed to ascertain whether

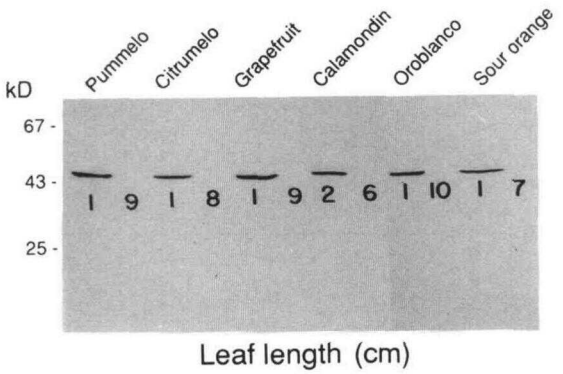

Figure 3. High levels of 1–2-rhamnosyltransferase in young but not in mature leaves of *Citrus* spp. Proteins extracted from actively growing young (1–2 cm long) and from expanded, light-green, mature leaves (6–10 cm long) of different *Citrus* spp. Proteins were separated by SDS-PAGE, electroblotted, and reacted with anti-1–2-rhamnosyltransferase serum. Typical results from one of six similar experiments are shown. The numbers under the bands refer to the length in cm of the tested leaves.

extracts of mature leaves lacked the enzyme or contained an inhibitor precluding accurate assay. Adding equal amounts of extracts from 12-cm-long mature leaves that had no detectable activity did not suppress the activity of 1–2-rhamnosyltransferase from extracts of 2-cm-long leaves (3.15 ± 0.1 pmol min^{-1} mg^{-1} [SD], alone, versus 3.10 ± 0.2 pmol min^{-1} mg^{-1} mixed). This suggests that mature leaves do not contain inhibitors of 1–2-rhamnosyltransferase activity; the activity is just not present.

Rapid Synthesis and Accumulation of Bitter Flavonoids in Young Pummelo Leaves

Flavonoids were extracted from leaves to ascertain whether the decrease in the rhamnosyltransferase activity during leaf growth coincided with the decreasing amounts of its product, naringin. One major and several very minor 283-nm-absorbing peaks were detected in flavonoid extracts of young leaves (Fig. 4A). The major compound contained over 80% of the A_{283} absorption of material eluted from the HPLC column. It was identified as naringin based on the retention time of a standard in this and another HPLC running system, as well as on TLC (the latter two according to Bar-Peled et al., 1991). Because prunin, the monoglucoside, has almost the same retention time as naringin, we assayed for its presence by TLC and by the more time-consuming, second HPLC method. No traces of prunin were discerned at the various times checked (data not shown). The highest concentrations of naringin (approximately 100 μmol naringin/g fresh weight) and the most rapid accumulations were found in 0.5- to 4-cm-long actively growing leaves (Fig. 4B). The amounts of naringin per leaf increased only slightly as the leaves expanded from 5 to 11 cm in length and matured (Fig. 4B). Mature, dark-green leaves from the previous leaf flush contained 30% less naringin per leaf than similarly sized, newly expanded leaves and only 5% the amount per fresh weight of 0.5- to 4-cm-long leaves. Thus, the production of naringin appears to occur during early development in the 0.5- to 5-cm-long leaves and is correlated with the highest specific activity of the rhamnosyltransferase (Figs. 2 and 4B). It has already been shown that young *Citrus* leaves readily incorporate radioactive Phe into naringin, whereas older leaves do not (Maier and Hasegawa, 1970; Berhow and Vandercook, 1991).

Is Naringin Degraded or Exported from Fully Expanded Leaves as They Mature?

There was no accumulation of the possible degradation products of naringin (prunin and naringenin) in the fully expanded, mature, dark-green leaves, which could account for the net decrease in naringin content during maturation. The same extracts used for the 1–2-rhamnosyltransferase assay were used to analyze protein extracts from leaves for their ability to perform in vitro deglycosylation or degradation of naringin. There was no loss of naringin caused by protein extracts from young, fully expanded, and mature leaves, nor were the deglycosylated products or unknown metabolites seen in the HPLC profiles or on TLC (data not shown). Naringinase activity, such as that in the commercial *Penicillium* enzyme that hydrolyzes naringin to prunin and narin-

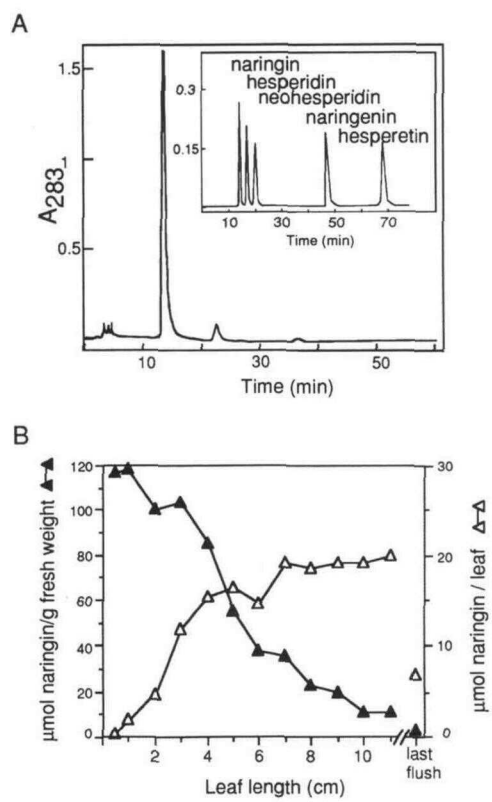

Figure 4. Changes in naringin content in developing pummelo leaves. A, Typical separation of flavonoids extracted from pummelo leaves on reverse-phase HPLC. Inset, Separation of flavonoid standards on the column. B, Leaves 0.5 to 11 cm long were light green and actively growing; 12-cm-long leaves from the previous leaf flush were dark green (1.72 μmol = 1 mg of naringin).

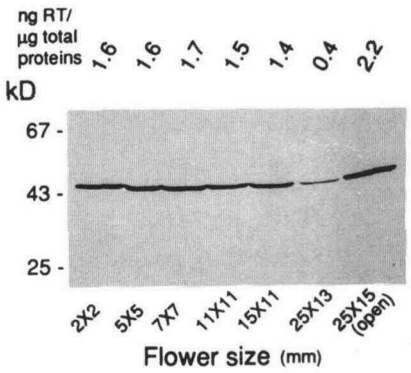

Figure 5. Decline of rhamnosyltransferase (RT) during the development of pummelo flowers and appearance after fertilization. Proteins were extracted from seven pooled flowers at each stage of growth and size (length × diameter). Proteins were separated, electroblotted, reacted with antibody, and quantified. All flower buds were closed except for the open, fertilized flowers, which were collected just at the time of petal drop. Some of the flowers were without petals, increasing the relative amount of ovary tissue. The figure displays results from one of three similar experiments.

genin, was not found in mature leaves. Because flavonoid-glycosides are more water soluble than their aglycones, one cannot exclude the aqueous transport of the bitter naringin to other parts of the plant, as has been found with other glycosides of secondary metabolites (cf. Croteau et al., 1987). Naringin has been found in *Citrus* stems and roots, which do not synthesize naringin (Jourdan et al., 1985; Berhow and Vandercook, 1991; Castillo et al., 1992). Thus, we assayed for both rhamnosyltransferase activity and the protein in the roots and stems of pummelo. The enzyme was not detected in either manner in either tissue (data not shown).

All Floral Parts Except Anthers Have High 1–2-Rhamnosyltransferase Activity

Unopened, juvenile flower buds up to 15 mm in length and 11 mm in diameter had a 4 times higher concentration of 1–2-rhamnosyltransferase than larger, unopened flower buds that had reached 25 mm in length and 13 mm in diameter (Fig. 5). The abrupt drop in concentration may be attributed to the larger proportion of petals compared with the other closed flower parts in larger buds. A high concentration of rhamnosyltransferase was again observed in fertilized, open flowers at the time of petal drop (Fig. 5). This increase in enzyme may result from the loss of the petals, leaving the ovary as the main organ.

To test this hypothesis based on the above results, two sizes of closed pummelo flower buds were selected for further tissue analysis: (a) young flower buds having green petals, greenish-clear short filaments, and greenish-clear anthers, and (b) preanthesis flower buds having white petals, fully developed yellow anthers, and long white filaments. Flower parts of 18 flowers at each stage of growth were combined for analyses. Enzyme reactions for young and preanthesis ovaries and young stigmas were terminated after 30 instead of 60 min to preclude substrate limitation. The highest specific activities and contents of 1–2-rhamnosyltransferase were found in the ovary, stigma, petal, receptacle, and sepal tissues (Table I). The concentration of the enzyme and its specific activity in the anthers was very low. A similar pattern was observed in the preanthesis flower buds, with the ovaries having the highest specific activity of 1–2-rhamnosyltransferase. (Table I).

The specific activity of the rhamnosyltransferase and the amounts of the protein detected by the antibody are in positive correlation with the amounts of flavonoids extracted from different parts of the closed flower buds (Table II). Flower parts from 16 pooled flowers at each stage of growth were separated, and the typical results from one of two similar experiments determining naringin content are shown in Table II. The highest concentration of naringin (13% of fresh weight) was observed in the ovary of small flower buds, and the concentration remained high during further development of the ovary and stigma. The lowest concentrations were found in undeveloped and in mature yellow anthers. The rapid synthesis and accumulation of naringin in young sepals and petals resembles the pattern observed in the young

Table I. *Decline of rhamnosyltransferase activity and concentration during the development of pummelo flowers*

Data are ± SD.

Tissue	Young Flower Bud[a]		Preanthesis Flower Bud[b]	
	Enzyme specific activity	Enzyme protein	Enzyme specific activity	Enzyme protein
	pmol min^{-1} µg^{-1}	*ng/µg protein*	*pmol min^{-1} µg^{-1}*	*ng µg^{-1} protein*
Calyx	1.4 ± 0.1	0.9 ± 0.06	0.1	0.04
Receptacle	1.2	0.4	nd[c]	nd
Petal	3.0 ± 0.2	1.3 ± 0.04	0.9 ± 0.05	0.4
Filament	ts[d]	ts	0.7	0.2
Anther	0.2	0.05 ± 0.01	<0.1	0.0
Stigma	3.1	1.7	1.5	0.6
Ovary	3.6 ± 0.2	2.1 ± 0.19	3.1 ± 0.1	1.3 ± 0.15

[a] 7 × 7 (length × diameter) mm unopened flower buds. [b] 24 × 13 mm unopened flower buds. [c] nd, Not determined. [d] ts, Filaments are too small to obtain enough material for analysis.

Table II. *Changes in naringin content of developing flower buds in different tissues of pummelo flowers*

Data are ± SD.

Tissue	Young Flower Bud[a]		Preanthesis Flower Bud[b]	
	μmol/g fresh tissue	*μmol/tissue*	*μmol/g fresh tissue*	*μmol/tissue*
Sepal	34.2 ± 2.6	2.2	22.5	4.9
Petal	53.1	1.9	13.3 ± 2	5.8
Filament	ts[c]	ts	31.7	1.4
Anther	13.5	0.4	4.9 ± 0.7	0.7
Stigma	nd[d]	nd	110.5 ± 16	8.4
Ovary	228.3 ± 21	3.42	124.9 ± 9	7.4

[a] 6 × 7 (length × diameter) mm unopened flower buds. [b] 22 × 11 mm unopened flower buds. [c] ts, Filaments are too small to obtain enough for measurement. [d] nd, Not determined.

leaves. The only juvenile tissue lacking the enzyme and its product are the anthers. Ovaries retain enzyme and product through their maturation and subsequent development into juvenile fruit.

High 1–2-Rhamnosyltransferase Activity in Young Fruit

The concentrations of 1–2-rhamnosyltransferase in tiny fruits (up to 1.5 g) were similar to the concentrations in mature ovaries prior to fertilization (compare quantitative data in Figs. 5 and 6). Due to the difficulty of separating the fruit parts of smaller fruits, we selected two larger sizes of fruits—growing fruits (<200 g) and full-size fruits (>500 g)—for further tissue analyses. Fruit parts from two fruits at each stage of growth and size were separated and proteins were extracted from each part. The proteins were analyzed for 1–2-rhamnosyltransferase protein as well as for enzyme specific activity. The full-size unripe fruits, but not the young, growing fruits, had visible seeds. Typical results from one of two similar experiments are shown in Table III. In growing fruit, the highest activity of rhamnosyltransferase was found in the membrane that covers the juice sacs, followed by the juice sacs, albedo, and flavedo. The concentrations of 1–2-rhamnosyltransferase and the levels of enzyme activity in growing fruits were low compared with young leaves (Fig. 2). We could find only traces of rhamnosyltransferase activity in the membrane, but none in the seeds, flavedo, and albedo of mature, yellow-green, full-size fruits (Table III).

Accumulation of Naringin in Older Fruit Parts

The synthesis and rapid accumulation of naringin in fruits is primarily associated with growth and development (Fig. 7). Naringin was more than 85% of the total flavanones in the albedo and flavedo, as it was in pummelo flowers and leaves and as it was previously found to be in grapefruit (Shaw et al., 1991). Higher concentrations of naringin (approximately twice the amounts in small, growing leaves) were observed in the flavedo and albedo of tiny fruits, followed by a sharp, approximately 50% decrease in fruits that reached approximately 600 mg (Fig. 7). The concentration of naringin in tiny and small fruits (0.6 to approximately 17 g) is very high and constant, with about 100 μmol naringin/g fresh weight of albedo and flavedo, suggesting continual synthesis that matches the increase in fresh weight. Later, there is a sharp and then progressive decline in naringin concentration during fruit maturation, leading to 9 and 4 μmol naringin/g fresh weight of albedo and flavedo in approximately 300-g fruits (Fig. 7). A log-log plot shows how the concentration in the albedo and flavedo decrease in parallel (Fig. 7, inset). Still, the total amounts of naringin per flavedo and albedo of the fruit increased during fruit maturation. There was a total of 684 mg of naringin in the albedo of 77-g fruits and 1344 mg in 320-g fruits.

The rapid increase in naringin content to approximately 30% of fruit weight during early stages of fruit development is most probably due to the synthesis catalyzed by rhamnosyltransferase within the fruit itself. However, with further maturation of the fruit, the amounts of 1–2-rhamnosyltransferase are very low (Table III), and yet there is the net accumulation of naringin. The traces of remaining enzyme present might have catalyzed the production of the additional naringin during the months of fruit growth, but transport from other parts of the fruit or from the older leaves (where there was a net loss) cannot be excluded.

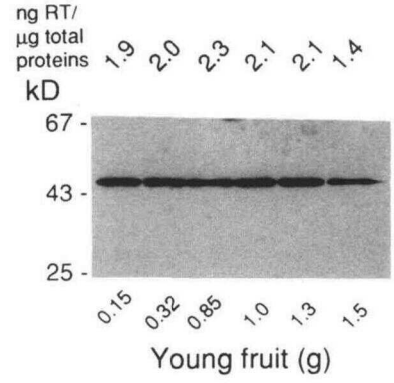

Figure 6. High levels of 1–2-rhamnosyltransferase (RT) in young, developing fruits after anthesis. Proteins were extracted from eight pooled fruits at each stage of growth and weight. The fruits were collected until approximately 3 weeks after anthesis. Proteins were separated, electroblotted, reacted with antibody, and quantified as in Figure 2. Typical results from one of two similar experiments are shown and should be compared with data for older fruits (Table III).

Table III. *Decline of rhamnosyltransferase during the development of pummelo fruits*
Data are ± SD.

Tissue	Growing Fruit[a]		Full-Size Fruit[b]	
	Enzyme specific activity	Enzyme protein	Enzyme specific activity	Enzyme protein
	pmol min^{-1} μg^{-1}	*ng μg^{-1} protein*	*pmol min^{-1} μg^{-1}*	*ng μg^{-1} protein*
Flavedo	0.5 ± 0.01	0.2 ± 0.01	<0.1	0
Albedo	0.8	0.3	<0.1	0
Membrane	1.1 ± 0.04	0.5 ± 0.02	0.2	0.01
Juice sac	0.7	0.2	<0.1	0
Seed			<0.1	0

[a] Average weight 150 g. [b] Average weight 727 g.

DISCUSSION

The anti-1–2-rhamnosyltransferase serum raised against the pure enzyme from pummelo young leaves did not cross-react with 1–6-rhamnosyltransferase from orange and mandarin (M. Bar-Peled, R. Fluhr, and J. Gressel, unpublished results). The antibody, however, did cross-react with the enzyme from other naringin-producing species (Fig. 3). The polyclonal antibody recognizes most peptide pieces of the enzyme after proteolysis (M. Bar-Peled, R. Fluhr, and J. Gressel, unpublished results), and would thus likely react with possible inactive forms of the enzyme. Activity and antibody assays gave parallel results throughout this study, suggesting that there are probably no inactive forms of the enzyme.

The activity and concentration of 1–2-rhamnosyltransferase are highest during early stages of development of leaves, flowers, and fruits but not of other organs. The timing coincides with the highest concentrations of its product, naringin (Figs. 2–4 and Tables I-III). The enzyme level decreases until it is no longer detectable during maturation. Thus, rhamnosyltransferase expression appears to be under developmentally specific regulation.

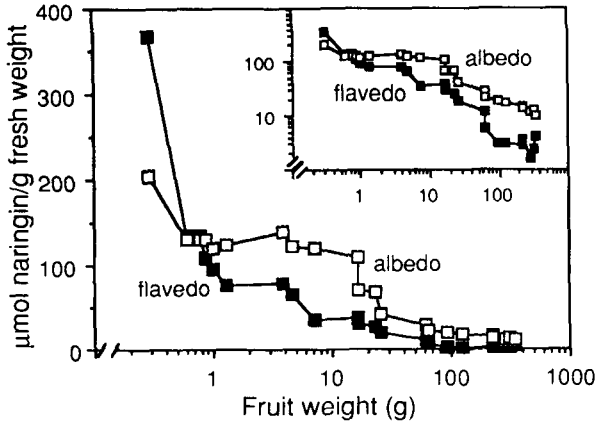

Figure 7. Changes in naringin content in the albedo and flavedo of developing pummelo fruits. Fruit parts from two fruits at each stage of growth and weight were separated. Flavonoids from each of the combined parts were extracted and chromatographed on a reverse-phase HPLC column. Typical results from one of three similar experiments are shown. The inset shows the similarity of slopes when data are plotted log-log.

One might expect that flavonoids would be found in exponential phase tissue cultures, because they are continuously juvenile. Flavonoids were not detected in undifferentiated grapefruit calli nor in cell suspensions; however, these same cultures catalyzed the glucosylation of exogenous naringenin to prunin (Lewinsohn et al., 1986). We could not detect 1–2-rhamnosyltransferase in undifferentiated grapefruit calli with the antibody (data not shown). Therefore, one can presume that the expression of the glucosyltransferase gene is controlled separately from the rhamnosyltransferase gene. Recently, sour orange cell cultures were reported to accumulate small amounts of neohesperidin and naringin (Del Rio et al., 1992), whereas these were not synthesized in other *Citrus* cell cultures (Barthe et al., 1987; Lewinsohn et al., 1989a).

It is tempting to speculate that in *Citrus* there is also a regulatory mechanism during leaf development that coordinates the regulation of the genes encoding earlier enzymes in the flavonoid pathway (members of the Phe ammonia lyase and chalcone synthase gene families, chalcone isomerase and glucosyltransferase). Indeed, there is high chalcone synthase activity in young *Citrus* leaves (Lewinsohn et al., 1989b). Furthermore, there was a higher specific activity of 7-*O*-glucosyltransferase in young leaves of calamondin and grapefruit than in slightly more expanded leaves (Lewinsohn et al., 1989b; McIntosh et al., 1990).

We have analyzed the N-terminal amino acid sequence of several peptides generated from the 1–2-rhamnosyltransferase and constructed a cDNA library from young pummelo leaves. Several clones from this library expressed peptides recognized by the antibody for the 1–2-rhamnosyltransferase (M. Bar-Peled, R. Fluhr, and J. Gressel, unpublished results). This should lead to the isolation of the gene and elucidation of whether it has noncoding regions controlling developmental regulation. The coding region of the gene inserted in an inverse orientation to generate antisense RNA should allow generation of plants producing less bitter flavonoids. Thus, both the coding regions of the gene for this enzyme and the noncoding regions regulating juvenile activities should have interesting implications and applications for further research.

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