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Flavanone-7-*O*-glucosyltransferase activity from *Petunia hybrida*

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Abstract

Citrus spp. are known for the accumulation of flavanone glycosides (e.g., naringin comprises up to 70% of the dry weight of very young grapefruit). In contrast, petunia utilizes relatively more naringenin for production of flavonol glycosides and anthocyanins. This investigation addressed whether or not petunia is capable of glucosylation of naringenin and if so, what are the characteristics of this flavanone glucosylating enzyme. Petunia leaf tissue contains some flavanone-7-*O*-glucosyltransferase (E.C. 2.4.1.185) activity, although at 90-fold lower levels than grapefruit leaves. This activity was partially purified 89-fold via ammonium sulfate fractionation followed by FPLC on Superose 12 and Mono Q yielding three chromatographically separate peaks of activity. The enzymes in the peak fractions glucosylated flavanone, flavonol, and flavone substrates. Enzymes in Mono Q peaks I and II were relatively more specific toward flavanone substrates and peak I was significantly more active. Enzyme activity was not effected by Ca^{2+}, Mg^{2+}, AMP, ADP, or ATP. The petunia enzyme was over 10,000 times more sensitive to UDP inhibition (Ki 0.89 μM) than the flavanone-specific 7GT in grapefruit. 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 (7GT) enzymes.

Keywords: *Petunia*; *Solanaceae*; *Antirrhinum*; *Scrophalariaceae*; Snapdragon; Flavonoid; Biosynthesis; Flavanone glucosyltransferase; Purification; Characterization; Naringenin; Prunin

1. Introduction

Flavonoids are a group of naturally occurring plant compounds that serve many functions. They effect plant interactions with microsymbionts (Romeo, Downum & Verpoorte, 1998), insect predators and pollinators (Dakora, 1995; Harborne, 1986; Stafford, 1997), and they function in pigmentation and UV protection (Brouillard, 1988; Harborne, 1994; Rozema, Staaij, Bjorn & Caldwell, 1997; van Tunen, Mur, Recourt, Gerats & Mol, 1988). While virtually all higher plants produce flavonoids, some of which are fairly ubiquitous, in many cases specific compounds are made and/or accumulated during plant growth and development. As a result, many plant families exhibit characteristic flavonoid profiles. For example, *Citrus* spp. are known for the accumulation of flavanone glycosides and the bitter flavanone diglycoside, naringin, can comprise 40–70% of the dry weight of very young grapefruit leaves and fruits (Jourdan, McIntosh & Mansell, 1985; Kesterson & Hendrickson, 1957). In contrast, petunia accumulates more anthocyanins and flavonol glycosides, and snapdragon contains anthocyanins, flavonol glycosides, and depending on variety, some flavanone glycosides.

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. The goal of this research was to elucidate potential mechanisms underlying differential accumulation of flavanone glycosides in petunia as compared to grapefruit. It was first necessary to establish flavanone-7-*O*-glucosyltransferase (7GT) activity in petunia and then to compare the petunia 7GT biochemical characteristics to those of the enzyme previously isolated and characterized from

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Table 1
[^{14}C]-glucose incorporated into prunin (pkatal)/g. fr. wt. tissue

	Tissue	Petunia	Snapdragon
A.	Young leaves	39	31
	Flower buds	22	16
B.	Sepals	11	12
	Flower minus sepals	0.37	0.30

grapefruit leaves (McIntosh, Latchinian & Mansell, 1990; McIntosh & Mansell, 1990), the only 7GT previously characterized. Relative levels of 7GT activity in petunia and grapefruit, in addition to characteristics of the enzymes, were evaluated in order to ascertain the potential contribution of 7GT properties and activity levels in the model plants to their respective flavanone glycoside accumulation patterns.

2. Results and discussion

2.1. Presence of 7GT activity in petunia

To assay flavanone-7-*O*-glucosyltransferase (7GT) activity in petunia and snapdragon, young leaves and flower buds were used as the enzyme source. Naringenin was used as the glucose acceptor and the production of prunin (naringenin 7-*O*-glucoside) was monitored. Results showed that leaves were a richer source of 7GT activity compared to whole flower buds (Table 1A). When flower buds were further examined to localize the presence of 7GT within the flower tissue, results revealed that over 96% of the 7GT activity was located in the sepal tissue (Table 1B). The occurrence of prunin has been previously reported in snapdragon, therefore it is not surprising that 7GT would be found in snapdragon tissues (Table 1). Finding 7GT activity in petunia tissues was somewhat unexpected since there has been no report of prunin being found in petunia to date.

Since both petunia (and snapdragon) are capable of glucosylating flavanones, why then are there such differences in the levels of flavanone glycosides found among them, and why do they differ in comparison to flavanone glycoside accumulation in grapefruit seedlings? It is possible that relative levels of 7GT activity are responsible for this differential accumulation. For example, young grapefruit leaves contain almost 90 times more 7GT activity per gram fresh weight of tissue (McIntosh & Mansell, 1990) than petunia leaves and over 100 times greater levels than snapdragon leaves. However, since the presence of flavanone glycosides has been reported in snapdragon, albeit at lower levels than in grapefruit, it is likely that there are additional factors involved such as variation in the biochemical characters or regulation of the 7GT enzymes from different sources. In order to determine the possible role of these factors, 7GT from petunia leaves was partially purified and characterized.

2.2. Purification of petunia 7GT

The protocol used for partial purification of petunia leaf 7GT is summarized in Table 2. Since earlier purification steps resulted in more than one EtOAc extractable glucoside, purification was calculated on the basis of incorporation into prunin (see McIntosh & Mansell, 1990). Crude extract was fractionated with ammonium sulfate and the fraction precipitated with 40–70% saturated ammonium sulfate was further chromatographed on a Superose 12 FPLC column. The 7GT activity eluted in a single peak from the Superose 12 column with a 6-fold increase in specific activity (Table 2). This sample glucosylated kaempferol (180%) and apigenin (60%) as well as the flavanone naringenin (Fig. 1), therefore the enzyme was further purified by chromatography on a Mono Q FPLC column.

Superose 12 fractions with the highest 7GT specific activities were pooled and applied to a Mono Q anion exchange column and eluted with a linear 0–500 mM NaCl gradient. This resulted in three peaks of 7GT activity (Fig. 2). Peak I eluted at a salt concentration of 0.18 M with an 89-fold increase in specific activity (SA) as compared to the crude extract (Table 2). There was a reproducible shoulder of activity eluting at 0.2 M salt which was designated peak II for further characterization (48-fold increase in SA), and peak III eluted with 0.35 M NaCl and a 31-fold increase in SA.

Three 7GT activity peaks were also obtained after purification of grapefruit leaf extract on Mono Q. The first peak, 7GT I, eluted at 0.14 M salt, 7GT II

Table 2
Petunia leaf 7GT purification. Data are from a representative purification. Reactions were co-chromatographed against authentic prunin as described in Section 4

Sample	Total units (pmol/min)	Total mg protein	Spec. act. (units/mg)	Purification
Crude	9139	22.0	407	1 ×
Salt fract.	2588	4.9	526	1 ×
Superose 12	432	0.18	2338	6 ×
Mono Q (I)	182	0.005	36400	89 ×

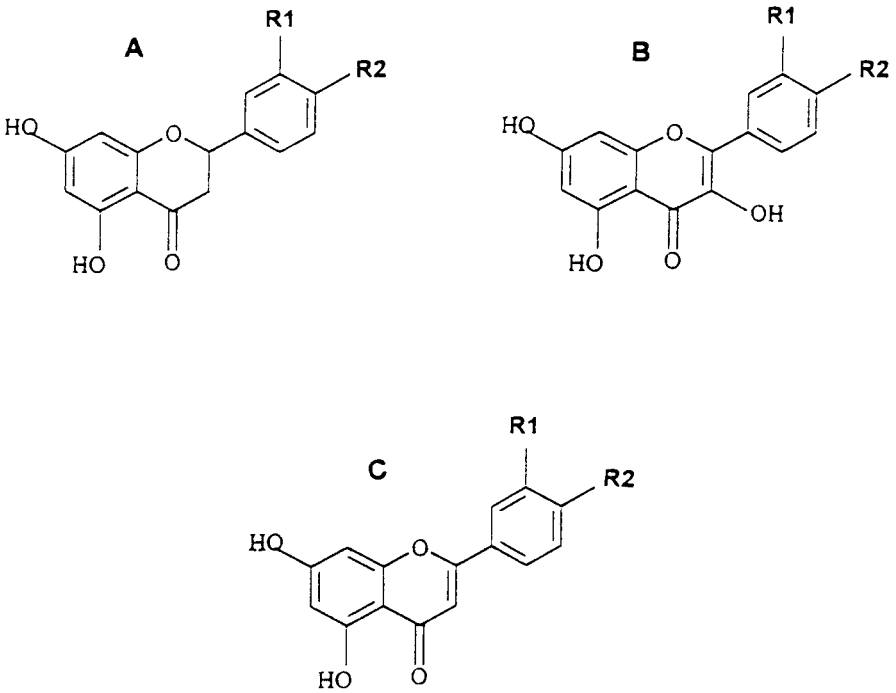

Fig. 1. Structures of flavonoid substrates. (A) flavanones; naringenin R1 = H, R2 = OH; hesperitin, R1 = OH, R2 = OCH_3; (B) flavonols; kaempferol R1 = H, R2 = OH; quercetin R1 = OH, R2 = OH; (C) flavones; apigenin, R1 = H, R2 = OH; luteolin, R1 = OH, R2 = OH.

eluted with 0.23 M salt, and 7GT III (flavanone-specific) eluted with 0.29 M salt (McIntosh et al., 1990). Comparison of Mono Q chromatographic results of petunia and grapefruit 7GT activity indicate that grapefruit 7GT I and the flavanone-specific 7GT III are relatively less anionic than petunia peak I and peak III, while the grapefruit GT II is relatively more anionic that petunia peak II. The apparent chemical differences in enzymes from these two sources suggest that biochemical properties of the 7GTs, rather than the absence or presence of 7GT activity, may contribute to the differential metabolism of naringenin in petunia and grapefruit. This idea was further tested by characterizing petunia 7GT and comparing properties to those of grapefruit 7GT.

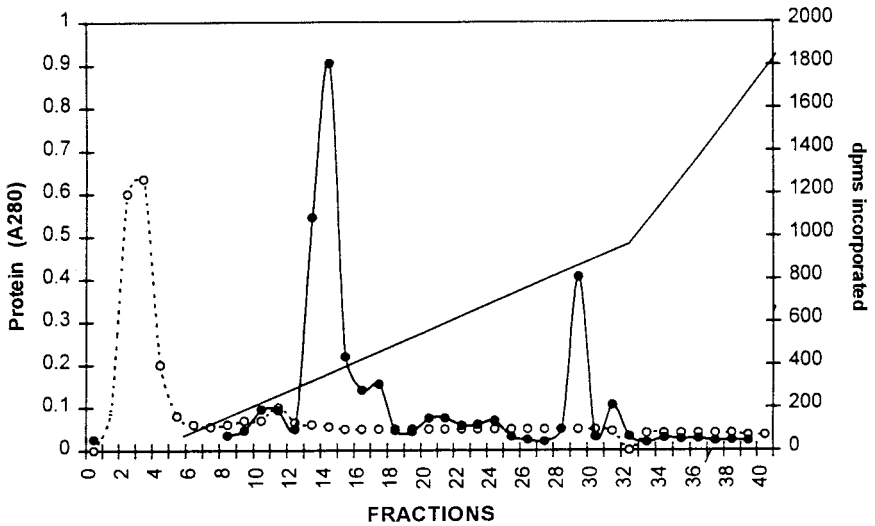

Fig. 2. Mono Q Elution Profile. Peak I = fraction 14; Peak II = fraction 17; and Peak III = fraction 29. The apparent peak at fraction 31 was not reproducible. – – o – – protein levels, —●— cpm incorporated into prunin, — — salt gradient.

Table 3
Properties of Mono Q-Purified Petunia Leaf 7GT. Peak III yield was too low for complete kinetic characterization[a]

Property	Peak I	Peak II	Peak III
Elution condition (mM NaCl)	175	200	330
Substrate specificity			
Flavanone			
Naringenin	100%	100%	100%
Hesperitin	70	nd	nd
Flavonol			
Kaempferol	50	46	70
Quercetin	42	nd	nd
Flavone			
Apigenin	49	45	95
Luteolin	52	nd	nd
Naringenin $_{app}K_m$	10 μM	27 μM	8 μM
UDP-glucose $_{app}K_m$	269 μM	74 μM	nd
UDP inhibition (Ki)	0.89 μM	0.95 μM	nd

[a] nd = not determined.

2.3. Properties

The optimum pH for assaying petunia 7GT activities was determined using the Superose-purified sample. The pH optimum was 7.5 with 50% maximal activity at pH 6.5 and 62% maximal activity at pH 9.0 (highest pH tested). All reactions were subsequently carried out at pH 7.5. This pH optimum is in the range of that obtained for grapefruit 7GT (McIntosh & Mansell 1990; McIntosh et al., 1990), as well as for many other flavonoid glucosyltransferases (e.g., Miller & Taylor, 1998; Steyns & van Brederode, 1986; Sutter, Ortmann & Grisebach, 1972).

Comparison of the petunia 7GT elution volume from Superose 12 to the elution volumes of standard proteins indicate the petunia enzyme has a Mr of $54{,}400 \pm 5100$ Da ($n = 5$). This is comparable to the monomeric 54,900 Da size of the flavanone-specific glucosyltransferase activity in grapefruit seedlings and is larger than either the flavonol and broader-spectrum flavonoid glucosyltransferase activities in grapefruit (49,000 Da) (McIntosh et al., 1990), or other flavonoid 7-*O*-glucosyltransferases in *Petroselinum hortense* (50,000 Da) (Sutter et al., 1972), *Silene pratensis* (45,000 Da) (Steyns & van Brederode, 1986), and *Cicer arietinum* (50,000 Da) (Koster & Barz, 1981).

The 7GT-containing Superose 12 peak also glucosylated flavone and flavonol substrates at 60% and 180% relative activity, respectively. After subsequent chromatography on Mono Q, the majority of flavonol glucosylating activity eluted in fractions 10–12 and, therefore, was separated from 7GT activities. Properties of the Mono Q peaks are summarized in Table 3. Peaks I and II were relatively more specific toward flavanone substrates while the more anionic peak III possessed broader aglycone specificity. Due to the similar characteristics of the 7GT activities in peaks I and II (with the exception of the UDPG $_{app}$Km), it is not clear at this time whether or not the 7GT activity in peak II represents a different form of the enzyme. While there were also three 7GT peaks obtained from a Mono Q column during isolation of the grapefruit enzyme, one of the grapefruit fractions showed absolute specificity toward flavanone aglycones (McIntosh et al., 1990).

Like grapefruit 7GT, the petunia enzymes were not effected by $CaCl_2$ or $MgCl_2$ (unlike Steyns & van Brederode, 1986). In addition, enzyme activity was neither stimulated nor inhibited by AMP, ADP, or ATP (data not shown) suggesting that the activities of the 7GTs themselves are not allosterically linked to energy charge. All fractions showed Michaelis-Menten kinetics with respect to naringenin and UDP-glucose (Table 3). Apparent Km's ($n = 3$) were similar to those found for the flavanone-specific 7GT isolated from grapefruit (naringenin $_{app}$Km = 62 μM, using naringenin as aglycone the UDPG $_{app}$Km = 51 μM; hesperetin $_{app}$Km = 124 μM, using hesperetin as aglycone the UDPG $_{app}$Km = 243 μM) (McIntosh et al., 1990). One of the more striking differences between petunia and grapefruit 7GTs was in sensitivity to UDP. Like all UDP-glucosyltransferases tested for UDP sensitivity to date, UDP is inhibitory. While UDP is a competitive inhibitor of petunia leaf 7GT (Table 3), the grapefruit enzyme is over 10,000 times less sensitive to the presence of UDP in the reaction (McIntosh & Mansell, 1990; McIntosh et al., 1990). The higher sensitivity of petunia 7GT to UDP may help account for the lack of flavanone glycoside accumulation in this plant, and the insensitivity of the grapefruit enzyme may help account for why grapefruit is capable of accumulating such high levels of flavanone glycosides. Future determination of the UDP kinetics of the 7GTs with other substrates may help elucidate this point.

3. Summary

Petunia leaves contain flavanone-7-*O*-glucosyltransferase activity. Since these plants are capable of glucosylation of flavanones, why then are there such differences in the levels of flavanone glycosides found in them, especially in comparison to flavanone glycoside accumulation in grapefruit seedlings? One potential contributing factor is the relative levels of 7GT activity found in these plants, with grapefruit leaves containing almost 90-fold higher levels than petunia (and over 100-fold higher levels than snapdragon). It is also likely that there are additional factors involved such as variation in the biochemical characteristics or regulation of the 7GT enzyme from different sources. For example, the petunia 7GT found in Mono Q peak I is relatively less specific for flavanone substrates, is

less anionic, and is over 10,000 times more sensitive to UDP inhibition than the grapefruit enzyme. The higher sensitivity of petunia 7GT to UDP may help account for why petunia does not accumulate flavanone glycosides, and the relative insensitivity of the grapefruit enzyme may help account for why grapefruit is capable of accumulating such high levels of flavanone glycosides.

Current and future work is focusing on the investigation of factors contributing to the developmentally-specific and tissue-specific utilization of naringenin during flavanone metabolism in petunia and grapefruit. This will be examined in greater detail when the responsible protein(s) are purified to apparent homogeneity. In addition, efforts are underway to clone the grapefruit 7GT and to obtain sequence information for analysis.

4. Experimental

4.1. Plant material

Seeds of *Petunia hybrida* (var. 'Celebrity Red Morn') and *Antirrhinum majus* (var. 'Lemon Rocket') were obtained from Park Seed (Greenwood, SC). Seeds were germinated in standard potting soil and grown under greenhouse conditions.

4.2. Chemicals

UDP-[^{14}C]glucose (261 mCi mmol^{-1}) was purchased from ICN (Irvine, CA). Unlabeled UDPG, NADPH, ATP, ADP, AMP, βME, polyvinylpolypyrrolidone (PVPP), quercetin, naringenin, and naringin were purchased from Sigma (St Louis, MO). Other flavonoids were obtained from our laboratory collection. Superose 12 HR 10/30, Mono Q HR 5/5 FPLC columns, and the FPLC system were obtained from Pharmacia (Uppsala, Sweden). Plastic-backed polyamide-6 and cellulose TLC plates were from JT Baker (Phillipsburg, NJ).

4.3. Buffers

The following buffers were used: (1) buffer A: 50 mM Tris-HCl pH 7.5 containing 14 mM βME; (2) buffer B: 0.2 M Tris-HCl pH 7.5 containing 42 mM βME and 5 mM EDTA. All buffers used with the FPLC system were filtered through a 0.2 micron millipore filter and degassed before use.

4.4. Preparation of enzyme extracts

All steps were carried out at 4°C unless noted otherwise. Designated tissues were ground to a fine powder using liquid N_2 and extracted with buffer B. The slurry was filtered through cheesecloth and the soluble portion stirred for 20 min with a weight of PVPP (previously equilibrated in buffer B) equal to that of the starting tissue. This homogenate was centrifuged for 15 min at 20,000 g and the pellet discarded. The supernatant was designated as the crude extract. Those proteins precipitating between 40 and 70% sat'd $(NH_4)_2SO_4$ (designated P2) were isolated from the crude extract, redissolved in a minimal volume of buffer A, applied to a Superose 12 FPLC column (25 ml) equilibrated with the same buffer, and eluted with buffer A at a flow rate of 0.4 ml min^{-1} (1.6 MPa). Fractions with the highest specific activity of enzyme were pooled and used for some characterization (e.g., pH optimum).

For further purification, the Superose 12 pooled fractions were applied to a Mono Q FPLC column (1 ml) previously equilibrated in buffer A and eluted with a shallow 0–0.5 M NaCl gradient followed by a steep 0.5–1.0 M NaCl gradient in buffer A at a flow rate of 0.7 ml min^{-1} (1.5 MPa). Samples were either assayed directly or concentrated and dialyzed using a Centricon-10 (Amicon) prior to analysis. This resulted in an 89× increase in the specific activity of the enzyme (peak I) as compared to the crude extract.

4.5. Glucosyltransferase assay and product identification

Glucosyl-transferase activity was assayed by measuring the incorporation of [^{14}C]glucose into flavonoid glycosides using a modification of the protocol in (McIntosh & Mansell, 1990; McIntosh et al., 1990). The standard assay mixture contained 50 nMol naringenin (or alternate aglycone) in 5 μl ethylene glycol monomethyl ether, 100 nmol UDPG (0.025 μCi) in 10 μl buffer A, and up to 0.6 mg protein in a total volume of 75 μl. The assay mixture was incubated at 30°C, and reactions were stopped by the addition of 15 μl 6 N HCl. The flavonoid glycosides were extracted with 250 μl ethyl acetate, dried, redissolved in methanol and chromatographed against standard compounds on Polyamide-6 TLC plates in acetone:$CHCl_3$:H_2O (100:25:6) (Barber, 1962). Flavonoid bands were located under UV light, cut from the plate, placed in a mini-vial containing 4.0 ml CytoScint (Fisher Scientific) and counted in a Beckman LS 3801 scintillation counter. Results showed that the reaction was linear over a 20–30 min time period. Therefore all reactions were incubated for 15 min unless otherwise noted.

For rapid monitoring of column fractions, the assays contained 50 nMol naringenin (or alternate aglycone) in 5 μl ethylene glycol monomethyl ether, 10 μl diluted UDP-[U-^{14}C]glucose (25,000 cpm) in buffer A, and 10–50 μl of sample in a total volume of 75 μl. The assay mixture was incubated for 15 min at 30°C and

reactions were stopped by the addition of 15 μl 6 N HCl. The flavonoid glycosides were extracted with 250 μl ethyl acetate and an aliquot of the organic phase was counted as previously described.

Reaction products were identified by co-chromatography with reference compounds on Polyamide-6 TLC plates in acetone:$CHCl_3$:H_2O (100:25:6) as well as on cellulose TLC plates in water. In addition, acetate derivatives of reaction products were prepared using Ac_2O and pyridine. Identification of derivatives was confirmed by co-chromatography with standard acetate derivatives in the systems described above.

4.6. Kinetic analysis

Kinetics for naringenin were determined by measuring the initial reaction velocities at varying concentrations of naringenin while maintaining the concentration of UDPG at 1.33 mM. Kinetics for UDPG were determined by measuring the initial reaction velocities at varying concentrations of UDPG maintaining the concentration of naringenin at 0.67 mM. Analyses of kinetic data were performed using Hyperbolic Regression Analysis (JS Easterby) World Wide Web freeware (www.liv.ac.uk/New/staff.html). Kinetic constants were determined using Hanes plots.

4.7. Molecular weight determination

The P2 fraction was applied to a Superose-12 HR 10/30 column which was previously calibrated with the following reference proteins:myosin (M_r 200,000), β-galactosidase (M_r 116,250), phosphorylase b (M_r 97,400), bovine serum albumin (M_r 66,200), ovalbumin (M_r 45,000), carbonic anhydrase (M_r 31,000), trypsin inhibitor (M_r 21,500), and lysozyme (M_r 14,400). The column was eluted with buffer A at a flow rate of 0.4 ml min^{-1} and 1.0 ml fractions collected and assayed for flavanone glucosyltransferase activity. The molecular weight of the enzyme was estimated from its elution volume from the column (Andrews, 1965).

4.8. Analytical procedures

Protein concentration was determined according to Bradford (1976) using bovine serum albumin as standard protein.

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