UDP-D-Glucose: Flavonol 3-O- and 7-O-Glucosyl Transferases from Young Leaves of *Paederia scandens* var. *mairei*

Nariyuki Ishikura, Zhi-qing Yang, and Susumu Teramoto

Department of Biological Science, Faculty of Science, Kumamoto University, Kurokami, Kumamoto 860, Japan

Z. Naturforsch. **48c,** 563–569 (1993); received February 17/April 15, 1993

Rubiaceae, *Paederia,* Flavonol O-Glucosylation, O-Glucosyl Transferases

Two different O-glucosyl transferases (F3GT and F7GT) catalyzing the transfer of D-glucose from UDP-D-glucose to the 3 and 7 positions of flavonol, respectively, were isolated from the young leaves of *Paederia scandens* var. *mairei*. F3GT and F7GT, which were recovered in about a 1:1 ratio in the activity, were purified by about 140- and 136-fold, respectively, by precipitation with ammonium sulfate followed by ion exchange chromatography and chromatofocusing. F3GT and F7GT both had a pH optimum of 7.5 in Tris-HCl buffer, and an M_r of 43 kDa. Neither F3GT nor F7GT had a Mg^{2+} requirement. Both were inhibited by each 1 mM of Zn^{2+}, Cu^{2+}, N-ethylmaleimide and *p*-chloromercuribenzoate, and both were stimulated by 14 mM 2-ME. F3GT and F7GT were different from each other in having an isoelectric point (p*I*) at pH 5.12 and 4.50, respectively. F3GT mediated the transfer of D-glucose exclusively to the 3-hydroxyl group of kaempferol and some flavonols, but neither the 7-O-glucosides nor the 3-O-glucosides of their flavonols were able to accept D-glucose. On the other hand, F7GT mediated the transfer of D-glucose exclusively to the 7-hydroxyl group of kaempferol and some flavonols, and in addition, the 3-O-glucosides of kaempferol and quercetin were able to accept D-glucose though less efficiently. Consequently, the possibility of sequential steps of 3-O- and then 7-O-glucosylations of flavonols to give the 3,7-di-O-glucoside was discussed.

Introduction

A number of flavonoid glycosides found in nature suggests the presence of a great range of O-glycosyl transferases with varying substrate specificities [1, 2], and glycosylation seems to occur subsequent to all other substitutions and modifications of the flavonoid ring structure [3]. The most commonly found flavonol glycosides are the 3-O-glucosides, which are produced by the action of a specific F3GT that uses UDP-D-glucose as the sugar donor. The enzyme catalyzing this reaction has been shown by Sutter and Grisebach [4] using cell suspension cultures of parsley. According to them, the 7-O-glycosides and 3,7-di-O-glycosides of flavonols in parsley are formed by sequential glycosylation steps, *i.e.,* the first reaction of this sequence is catalyzed by UDP-D-glucose: flavone/flavonol 7-O-glucosyl transferase and the second glucosylation of flavonols is catalyzed by F3GT which has completely been separated from the former. In the previous paper [5], we reported that the leaves of *Paederia scandens* var. *mairei* contain nine flavonol glycosides, which consist of the 3-O-, 7-O- and 3,7-di-O-glycosides of kaempferol and quercetin. Therefore, the occurrence of F3GT and F7GT in *Paederia* leaves seemed highly likely. So we attempted to detect both enzymes, characterize them and carefully compare their nature with those of the enzymes of parsley, which have been reported by Sutter and Grisebach [4]. In this paper the isolation, partial purification and characterization of F3GT and F7GT in the leaves of *P. scandens* var. *mairei* are reported so as to clarify their role in the glycosylation steps of flavonols.

Abbreviations: DIECA, diethylammonium diethyldithiocarbamate; DMSO, dimethyl sulfoxide; F3GT, UDP-D-glucose:flavonol 3-O-glucosyl transferase; F7GT, UDP-D-glucose:flavonol 7-O-glucosyl transferase; 2-ME, 2-mercaptoethanol; TLC, thin layer chromatography.

Reprint requests to Prof. Dr. N. Ishikura.

Verlag der Zeitschrift für Naturforschung, D-72072 Tübingen
0939–5075/93/0700–0563 $ 01.30/0

Materials and Methods

Plant materials

Fresh young leaves of *Paederia scandens* (Lour.) Merrill var. *mairei* (Leveille) Hara (Rubiaceae) were collected in suburbs of Kumamoto from early April to early May. Leaves collected were

weighed, frozen in liquid nitrogen and stored at −80 °C.

Labeled compounds and buffers

UDP-D-(U-^{14}C)glucose (10.9 GBq/mmol) and ADP-D-(U-^{14}C)glucose (7.4 GBq/mmol) were obtained from Amersham International plc, Buckinghamshire, England. GDP-D-(U-^{14}C)glucose (8.7 GBq/mmol) was obtained from ICN Biochemicals, Inc., Irvine, California, U.S.A.

The following buffers were used: A, 0.2 M Tris-HCl, pH 7.5, containing 14 mM 2-ME, 5 mM EDTA and 10 mM DIECA; B, 20 mM Tris-HCl, pH 7.5, containing 14 mM 2-ME and 10% glycerol; C, the same as buffer B except that it contained no 2-ME; D, 50 mM Tris-HCl, pH 7.5, containing 14 mM 2-ME and 10% glycerol; E, 25 mM imidazole-HCl, pH 7.4, containing 14 mM 2-ME and 10% glycerol; and F, Polybuffer 74-HCl/H_2O (1:8), pH 4.0, containing 14 mM 2-ME and 10% glycerol.

Extraction and partial purification of enzymes

From about 60 g of *Paederia* leaves protein was extracted with buffer A as previously described [6]. The protein was fractionated with solid ammonium sulfate and the protein fraction which precipitated between 45% and 75% salt saturation was collected by centrifugation. The protein pellet was suspended in the minimum amount of buffer B and desalted by passage through a column of Sephadex G-25 (18 mm i.d. × 260 mm) which had previously been equilibrated with buffer B. The desalted solution of enzyme was purified by FPLC (Pharmacia) as follows: 10 ml aliquot of the enzyme solution was chromatographed on a Mono Q™ HR 5/5 column, which had previously been equilibrated with buffer D. Proteins were eluted with a linear gradient of 0 to 300 mM KCl in buffer D at a flow rate of 0.5 ml/min (pressure of 2.5 MPa) and one ml fractions were collected and assayed for F3GT and F7GT using substrates kaempferol and quercetin. The active fractions were pooled and subjected to chromatofocusing on a Mono P™ HR 5/20 column which had previously been equilibrated with buffer E. Elution of the bound proteins was performed with 40 ml of buffer F which generated a linear gradient between pH 7.0 and 4.0. The flow rate was 0.5 ml/min (pressure of 3.0 MPa) and one ml fractions were collected and assayed for F3GT and F7GT activities. In order to remove the Polybuffer, the protein was precipitated with ammonium sulfate and subsequently desalted on Sephadex G-25 in buffer B.

Assay of enzymatic activity

The standard enzyme assays were performed as described previously [6] in buffer B using UDP-D-(U-^{14}C)glucose as glucosyl donor, and the incubation time was 60 min.

Chromatographic identification of reaction products

The identification of reaction products was effected by TLC and autoradiography as described previously [6] using the authentic samples. Several solvent systems including 15% acetic acid (15% HOAc) and *n*-butanol–acetic acid–H_2O (BAW) were used for TLC.

Estimation of protein and determination of molecular weight

Protein concentrations and the molecular weights of F3GT and F7GT were determined as described earlier [7].

Results

Separation and purification of enzymes F3GT and F7GT

Crude preparation of enzyme from the young leaves of *P. scandens* var. *mairei* catalyzed the glucosylation of the 3- and 7-positions of kaempferol in the presence of UDP-D-(U-^{14}C)glucose as glucosyl donor. The enzymes tentatively designated F3GT and F7GT were partially purified by precipitation in 45–75% saturated ammonium sulfate and subsequent chromatography on a Mono Q™ column (Fig. 1). The reaction products of 3-O- and 7-O-glucosylations were identified as the 3-O-glucoside, the 7-O-glucoside and the 3,7-di-O-glucoside of kaempferol by TLC and autoradiography. On TLC, kaempferol 3-O-glucoside and kaempferol 3,7-di-O-glucoside, R_F values of which were 0.41 and 0.65 in 15% HOAc and 0.72 and 0.28 in BAW, respectively, appeared deep purple in UV light (254 nm) and turned yellow in UV light with NH_3. Kaempferol 7-O-glucoside, R_F val-

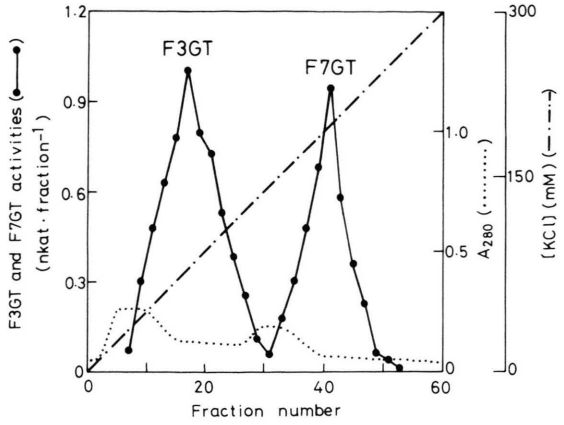

Fig. 1. Profile of elution of the activities of O-glucosyl transferases F3GT and F7GT from a Mono Q™ column. The enzymes from a column of Sephadex G-25 were applied to a column which had previously been equilibrated with buffer D and they were eluted with a linear gradient of 0 to 300 mM KCl in the same buffer. Fractions of 1.0 ml were collected. The fractions were assayed with kaempferol for both F3GT and F7GT.

ues of which were 0.12 in 15% HOAc and 0.60 in BAW, appeared bright yellow in UV light without NH_3, which is in accordance with the previous report of Ishikura and Yang [6].

F3GT and F7GT were recovered in two different fractions at a ratio of about 1:1 in the activity after chromatography on a Mono Q™ column. Activities of both F3GT and F7GT with either kaempferol or quercetin as substrate were recovered within the same fraction. Further purification of both F3GT and F7GT by chromatofocusing on a Mono P™ resulted in increases in specific activity of 139.5- and 135.5-fold, respectively, as compared with the activity of the crude extract, when kaempferol was used as substrate (Table I). The peaks of F3GT and F7GT activities appeared in the range of pH 5.12 and 4.50, respectively, corresponding to each apparent p*I*. Moreover, F3GT and F7GT activities were recovered at a ratio of about 1:1 within the same protein peak when the protein fraction obtained by precipitation in 45–75% saturated ammonium sulfate was chromatographed on a column Sephadex G-100.

Enzyme properties

The pH optima for both F3GT and F7GT activities, as determined in different buffers, were found to be 7.5 in Tris-HCl and histidine-HCl buffers, and 8.0 in imidazole-HCl buffer (Fig. 2). At the optimum pH, the rates of reactions catalyzed by F3GT and F7GT in Tris-HCl buffer were linear for at least 60 min and also the rates were proportional to the amount of protein in the assays. Storage of the enzymes F3GT and F7GT, which were eluted from a column of Sephadex G-25 after 45–75% ammonium sulfate fractionation, in buffer B at −20 °C resulted in a 15% loss

Table I. Partial purification of the O-glucosyl transferases F3GT and F7GT from young leaves of *P. scandens* var. *mairei*[a].

Purification step	Total protein [mg]	Specific activity [nkat · mg protein^{-1}]	Purification (-fold)	Recovery (%)
Crude extract[b]	160.6	0.20	1	100
Dowex 1 × 2[b,c]	131.2	0.24	1.2	98
Sephadex G-25 after 45–75% $(NH_4)_2SO_4$[b]	30.7	0.52	2.6	50
Ion-exchange chromatography[d]				
F3GT	2.1	4.87	24.4	32
F7GT	1.3	5.38	26.9	22
Chromatofocusing[e]				
F3GT	0.2	27.90	139.5	17
F7GT	0.1	27.09	135.5	8

[a] 2-ME was routinely added to the protein preparations to avoid rapid enzyme denaturation.
[b] Total activity of O-glucosyl transferases including F3GT and F7GT.
[c] Treatment with Dowex 1 × 2 which had previously been equilibrated with buffer A.
[d] On Mono Q™ HR 5/5 column.
[e] On Mono P™ HR 5/20 column.

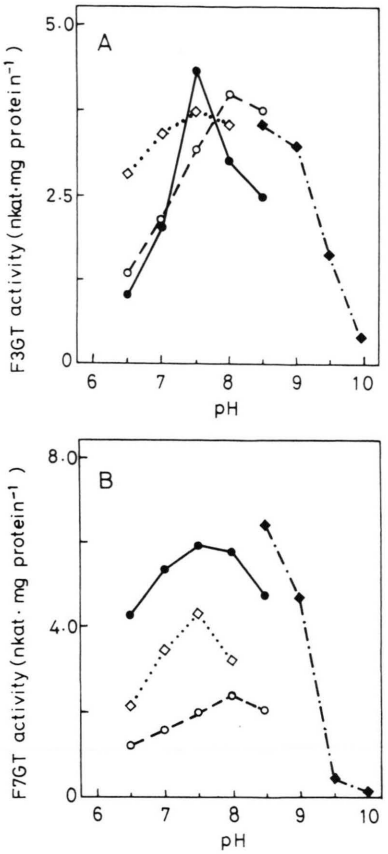

Fig. 2. The pH optima of F3GT (A) and F7GT (B) activities with kaempferol as substrate in 0.2 M buffers of Tris-HCl (−●−), imidazole-HCl (− −○− −), histidine-HCl (·····◇·····) and glycine-HCl (−·−◆−·−).

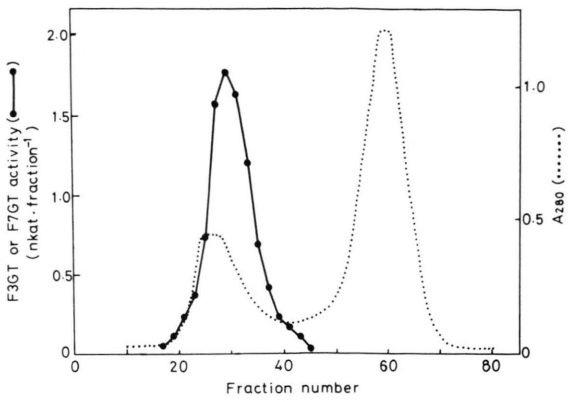

Fig. 3. Profile of elution of the activities of O-glucosyl transferases F3GT and F7GT from a column of Sephadex G-100 (20 mm i.d. × 490 mm) with kaempferol as substrate. The protein fraction obtained by precipitation with 45–75% ammonium sulfate was applied to a column which had been equilibrated with buffer B, and 3 ml fractions were collected and assayed for enzyme activities. The column was calibrated with the reference proteins (*cf.* Fig. 4).

of activity after two days. After eight days storage under the same conditions, about 40% of the original activities of both enzymes remained.

The M_rs of F3GT and F7GT, which had an isoelectric point at pH 5.12 and 4.50, respectively, were almost the same and were estimated to be about 43 kDa by column chromatography on Sephadex G-100 (Fig. 3 and 4). F3GT and F7GT had respective K_m values of 1.14 μM and 7.69 μM for kaempferol, and 13.33 μM and 15.39 μM for UDP-D-glucose with kaempferol as substrate. Although various sugar nucleotides, UDP-D-glucose, ADP-D-glucose and GDP-D-glucose, were tested as glucosyl donors, the specificity of both F3GT and F7GT for the sugar donor is quite distinct in that they accept from only UDP-D-glucose, which is in agreement with the previous results [7]. Table II lists the relative acceptor specificities for 3-O- and 7-O-glucosylations of a number of flavonols and their glycosides, and some phenols. Kaempferol proved to be the best substrate for both F3GT and F7GT, which mediated the transfer of D-glucose exclusively to the 3- and 7-hydroxyl groups of flavonols, respectively. Quercetin, isorhamnetin, rhamnetin and myricetin could also function as substrate for F3GT, but no

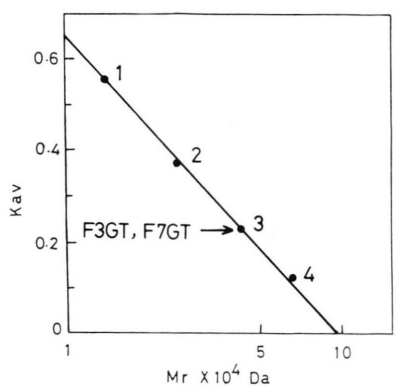

Fig. 4. Estimation of the molecular weight of O-glucosyl transferases F3GT and F7GT by elution from a column of Sephadex G-100 which had previously been calibrated with the reference proteins indicated as 1 (ribonuclease A, 13.7 kDa), 2 (chymotrypsinogen A, 25 kDa), 3 (ovalbumin, 43 kDa) and 4 (albumin, 67 kDa).

Table II. Acceptor specificity of the O-glucosyl transferases F3GT and F7GT.

Substrate[a]	Relative activity[b] [%] F3GT	F7GT
Kaempferol	100	100
Kaempferol 3,4′-dimethyl ether	4	30
Kaempferol 5,7,4′-trimethyl ether	4	9
Isorhamnetin (5,7,4′-trihydroxy-3′-methoxyflavonol)	71	88
Rhamnetin (5,3′,4′-trihydroxy-7-methoxyflavonol)	70	1
Quercetin	76	45
Myricetin	43	26
Astragalin (kaempferol 3-O-glucoside)	6	17
Kaempferol 3-O-rhamnosylglucoside	4	11
Kaempferol 7-O-glucoside	4	2
Isoquercitrin (quercetin 3-O-glucoside)	5	32
Rutin (quercetin 3-O-rhamnosylglucoside)	4	21
Quercimeritrin (quercetin 7-O-glucoside)	1	3
Hydroquinone	7	10
Aescretin	2	10
Scopoletin	3	8
p-Coumaric acid	2	9
Caffeic acid	3	14

[a] Final concentration of all substrates in the reaction mixture is 15 μM.
[b] The enzymes F3GT and F7GT used were the fractions after chromatofocusing on a Mono P™ column. Kaempferol was used as substrate for both assays of F3GT and F7GT activities, which amounted to 27.90 and 27.09 nkat · mg protein^{-1}, respectively, in the control assay (= 100%).

Table III. Effects of inorganic ions, EDTA, 2-ME and SH group inhibitors.

Reagent[a]	Concentration [mM]	Relative activity[b] [%] F3GT	F7GT
Control	–	100	100
$MgCl_2$	1	99	79
	10	60	36
$MnCl_2$	1	11	18
	10	3	3
$CaCl_2$	1	91	67
	10	60	59
$CoCl_2$	1	27	5
$ZnCl_2$	1	2	2
$CuCl_2$	1	3	3
KCl	1	95	85
	10	84	70
2-ME	14	134	144
$MgCl_2$ + 2-ME	10 + 14	108	112
EDTA	1	91	83
	10	33	42
PCMB	1	7	9
PCMB + 2-ME	1 + 14	93	91
NEM	1	3	6
NEM + 2-ME	1 + 14	85	91
PMA	1	34	60
DTE	10	53	50
Iodoacetamide	1	87	74
Iodoacetate	1	85	79

3-O-glucosylation reaction took place with the substrates such as the 7-O-glucosides of kaempferol and quercetin. On the other hand, isorhamnetin, quercetin, kaempferol 3,4′-dimethyl ether and myricetin could function as substrate for F7GT, and moreover, flavonol 3-O-glycosides, such as the 3-O-glucosides and 3-O-rhamnosylglucoside of kaempferol and quercetin, were accepted for 7-O-glucosylation though less efficiently. Hydroquinone, aescretin and caffeic acid could function limitedly as substrate for F7GT, but not at all for F3GT.

The effects of cations and SH-group inhibitors on both F3GT and F7GT activities were studied. As shown in Table III, the results show that there was no requirement for Mg^{2+}, Ca^{2+}, and K^+ with

[a] PCMB = *p*-chloromercuribenzoate, NEM = N-ethylmaleimide, PMA = phenylmercuriacetate, DTE = dithioerythritol.
[b] The enzymes F3GT and F7GT used were the fractions after ion-exchange chromatography on a Mono Q™ column. Kaempferol was used as substrate for both assays of F3GT and F7GT activities, which amounted to 4.87 and 5.38 nkat · mg protein^{-1}, respectively, in the control assay (= 100%).

kaempferol as the substrate and their cations showed an inhibitory effect on both enzyme activities at higher concentration (10 mM). Furthermore, no significant difference between F3GT and F7GT was observed for the effect of other divalent cations and SH-group inhibitors on both activities. Other cations, such as Zn^{2+}, Cu^{2+}, Mn^{2+} and Co^{2+}, strongly inhibited both of the 3-O- and 7-O-glucosylations at 1 mM concentration. F3GT and F7GT activities were strongly inhibited by SH-group reagents, such as 1 mM N-ethylmaleimide and 1 mM *p*-chloromercuribenzoate, and the addition of 14 mM 2-ME resulted in almost completely recovery from their inhibitions in the presence of the reagents. 14 mM 2-ME itself stimulated the activities of both enzymes.

Discussion

A preparation of enzymes extracted from the young leaves of *Paederia scandens* var. *mairei* catalyzed the 3-O- and 7-O-glucosylations of 4′,5,7-trihydroxyflavonol (kaempferol), using UDP-D-glucose as the glucosyl donor. It has, in fact, been reported that both of the 3-O-glucoside and the 7-O-glucoside of kaempferol or quercetin are present in the leaves of *P. scamdens* var. *mairei* (Ishikura *et al.* 1990). Results presented in the present study prove that the O-glucosyl transferases, F3GT and F7GT, are two distinct enzymes. F3GT and F7GT are separable according to their different charges on a Mono Q™ column, but both enzymes were not separated on a column of Sephadex G-100. This indicates that F3GT and F7GT have the same M_r, estimated to be approximately 43 kDa. M_r of both enzymes ranges within the reported values of UDP-D-glucose: flavonoid glucosyl transferases from various plant sources between 40 and 59 kDa [1, 2, 6–8]. M_r of F3GT and F7GT is the same as that of flavonol O-glucosyl transferase from the *Euonymus* leaves [6], but somewhat different from that (55 kDa) of parsley, from which F7GT has, so far, solely been reported [4]. The pH optimum of F3GT and F7GT was studied in several buffers between pH 6.0 and 9.0. Optimum activity was found to be at 8.0 in imidazole-HCl buffer for both enzymes. This pH optimum is in close agreement with that of other sources which ranges within the reported values of 7.5–8.5 [1]. The effects of divalent cations on the two enzymes showed that there is no requirement for cations with kaempferol, and also with quercetin and other phenols (data not shown). Both F3GT and F7GT activities are very susceptible to inhibition by SH group reagents, indicating the presence of SH group at the active site of the enzymes. Similarly to the previous study [6], 2-ME was very effective on both activities of F3GT and F7GT, and storage of those enzymes without 2-ME caused complete inactivation during only three days. By chromatofocusing on a Mono P™ column F3GT and F7GT gave the different p*I*s of 5.12 and 4.50, respectively, which are not significantly different from the pH range of 4.3–5.6 for the other flavonoid O-glucosyl transferases [6, 8–12].

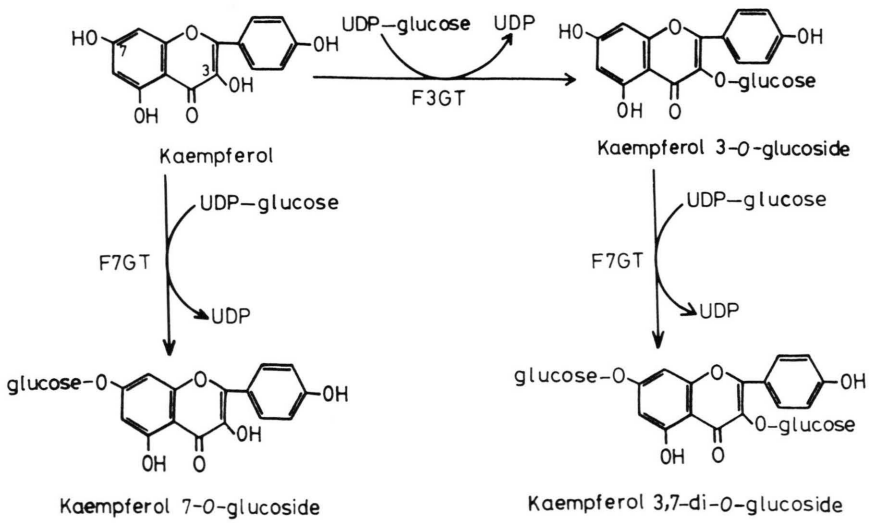

Fig. 5. A proposed pathway for the enzymatic synthesis of kaempferol O-glucosides in *P. scandens* var. *mairei*.

F3GT and F7GT had very low K_m values of 1.14 μM and 7.69 μM, respectively, for kaempferol, indicating a high affinity for those substrates. However, the K_m values of F3GT and F7GT for the co-substrate UDP-D-glucose were 13.33 μM and 15.39 μM, respectively. Both enzymes could hardly function with ADP-D-glucose and GDP-D-glucose. Among the acceptors tested, kaempferol was the best substrate for both F3GT and F7GT activities. Although some other flavonols could function as substrate for F3GT and F7GT, no 3-O-glucosylation reaction took place with the 7-O-glucosides of kaempferol and quercetin. On the other hand, flavonol 3-O-glycosides, such as the 3-O-glucosides of kaempferol and quercetin, were accepted for the 7-O-glucosylation though much less efficiently. Therefore, it seems likely that the 7-O-glucosylation of kaempferol or quercetin occurs subsequent to the 3-O-glucosylation. Fig. 5 shows a proposed pathway for their enzymatic synthesis of kaempferol O-glucosides in *P. scandens* var. *mairei*. This is inconsistent with the sequential glycosylation steps proposed by Sutter and Grisebach [4], using the cell suspension cultures of parsley, that is the sequential steps of 7-O- and then 3-O-glucosylations to give the 3,7-di-O-glucoside of quercetin or kaempferol.

[1] J. Ebel and K. Hahlbrock, in: The Flavonoids: Advances in Research (J. B. Harborne and T. J. Mabry, eds.), p. 641, Chapman and Hall, London 1982.
[2] W. Heller and G. Forkmann, in: The Flavonoids, Advances in Research Since 1980 (J. B. Harborne, ed.), p. 399, Chapman and Hall, London 1988.
[3] K. Hahlbrock and H. Grisebach, in: The Flavonoids (J. B. Harborne, T. J. Mabry, and H. Mabry, eds.), p. 866, Chapman and Hall, London 1975.
[4] A. Sutter and H. Grisebach, Biochim. Biophys. Acta **309,** 289 (1973).
[5] N. Ishikura, Z.-Q. Yang, K. Yoshitama, and K. Kurosawa, Z. Naturforsch. **45c,** 1081 (1990).
[6] N. Ishikura and Z.-Q. Yang, Z. Naturforsch. **46c,** 1003 (1991).
[7] N. Ishikura and K. Yamamoto, Plant Cell Physiol. **31,** 1109 (1990).
[8] Y. Sun and G. Hrazdina, Plant Physiol. **95,** 570 (1991).
[9] J. Köster and W. Barz, Arch. Biochem. Biophys. **212,** 98 (1981).
[10] K. L. Bajaj, V. de Luca, H. Khouri, and R. K. Ibrahim, Plant Physiol. **72,** 891 (1983).
[11] G. Hrazdina, Biochim. Biophys. Acta **955,** 301 (1988).
[12] C. A. McIntosh, L. Latchinian, and R. L. Mansell, Arch. Biochem. Biophys. **282,** 50 (1990).