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Uptake and metabolism of flavonols during in-vitro germination of *Petunia hybrida* (L.) pollen

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Abstract. Flavonol-deficient petunia pollen [conditionally male fertile (CMF) pollen] is unable to germinate but application of nanomolar concentrations of flavonol aglycones completely restores function (Mo et al. 1992). In this study a chemically synthesized radioactive flavonol, [4′-*O*-^{14}C]kaempferide, was used as a model compound to study the metabolism of flavonols during the first few hours of pollen germination. [4′-*O*-^{14}C] Kaempferide was as efficient at inducing CMF pollen germination as kaempferol and quercetin, the aglycone form of the endogenous flavonols in petunia pollen. Analysis by high-performance liquid chromatography (HPLC) of extracts from both in-vitro-germinated pollen and the germination medium showed that more than 95% of the applied radioactivity was recovered as three kaempferide 3-*O*-glycosides *and* unmetabolized kaempferide; no flavonol catabolites were detected. Only HPLC fractions that contained the aglycone, or produced it upon acid hydrolysis, could induce CMF pollen germination in vitro. Structurally diverse flavonols could be classified according to how efficiently the aglycone was internalized and glycosylated during pollen germination. The ability of an individual flavonol to restore germination correlated with the total uptake of flavonols but not with the amount of glycoside formed in the pollen. Thus this study reinforces the conclusion that flavonol aglycones are the active compound for inducing pollen germination.

Key words: *Petunia* (pollen) – Pollen germination – Flavonol radiolabeled

Introduction

Flavonols are aromatic compounds that have an essential role in plant fertility and fecundity in maize and petunia (Taylor and Jorgensen 1992; Vogt et al. 1994; Ylstra et al. 1994). Flavonol-deficient plants are conditionally male-fertile (CMF); they are self-sterile due to a lack of pollen germination and tube growth but the defect can be reversed by providing the pollen with flavonol aglycones (Taylor and Jorgensen 1992). Biochemical complementation has been achieved by adding kaempferol, a flavonol aglycone, to an in-vitro suspension of CMF pollen or to the CMF stigma at the time of self-pollination (Mo et al. 1992; Vogt et al. 1994; Vogt et al. 1995). We developed a bioassay based on the stimulation of pollen germination by compounds added to an in-vitro suspension of CMF pollen and used the assay to show that only flavonol aglycones could restore pollen function (Mo et al. 1992; Vogt et al. 1995). Structure-activity analysis of methylated and glycosylated flavonols determined that hydroxyl groups at the 3-, 5- and 7- positions of the flavonol molecules were essential for rapid pollen germination. In addition, the number of hydroxyl groups in the B-ring (Fig. 1A) correlated with the concentration of flavonol required for full germination (Mo et al. 1992). The more-hydrophilic molecules were less efficient in stimulating CMF pollen germination, while substitution of one or more of the hydroxyl groups with the less-polar methoxyl group markedly enhanced pollen germination (Vogt et al. 1995).

An essential objective towards understanding how flavonols stimulate pollen germination is to identify the inducing molecule. However, this determination has been confounded by the finding that only flavonol aglycones can complement CMF pollen but wild-type

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Abbreviations: CMF = conditionally male fertile; FAB-MS = fast atom bombardment mass spectroscopy; GM = germination medium; [4′-*O*-^{14}C]kaempferide = ^{14}C-labeled kaempferide; kaempferol 3-*O*-gal-glc = kaempferol 3-*O*-(2″-*O*-glucopyranosyl)-β-D-galactopyranoside; quercetin-3-*O*-gal-glc = quercetin 3-*O*-(2″-*O*-glucopyranosyl)-β-D-galactopyranoside; RT = retention time

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pollen contains only flavonol diglycosides, which in our system are incapable of stimulating germination (Pollak et al. 1993; Vogt et al. 1995; Taylor et al. 1997). During pollen development, flavonol aglycones are produced in the tapetal layer of the anther wall, released into the locule, taken up and glycosylated by enzymes associated with the pollen grain. The major flavonols in petunia pollen were determined to be kaempferol and quercetin 3-*O*-(2″-*O*-glucopyranosyl)-β-D-galactopyranoside [kaempferol and quercetin 3-*O*-gal-glc (Vogt and Taylor 1995; Zerback et al. 1989)]. The flavonol glycosides in pollen are structurally distinct from those in the corolla; the uniqueness is conferred, not at the aglycone level, but by different sugars and the type of interglycosidic linkage (Wiering and de Vlaming 1984; Brugliera et al. 1994; Kroon et al. 1994). Vogt and Taylor (1995) demonstrated that, coincident with the biochemical induction of CMF pollen germination, the added kaempferol or quercetin are metabolized to the same flavonol diglycosides that are present in wild-type pollen. Two pollen-specific activities were identified: UDP-galactose: flavonol 3-*O*-galactosyltransferase (F3GalTase) added galactose at position 3 of the flavonol C-ring. Then a UDP-glucose: glucosyltransferase (F3GlcTase) catalyzed the formation of a 1→2 linkage between glucose and galactose. Glycosylation renders the flavonol molecule more polar and water-soluble and is thought to aid in detoxification and/or transport to storage organs. In somatic cells, flavonol glycosides accumulate in the vacuoles, but in pollen their cellular location is unknown.

Pollen tube growth is rapid, requiring the synthesis of large amounts of cell wall material. The possibility that flavonols may function as components of the extending pollen tube wall could explain the germination and tube growth requirement for these molecules. Cell-wall-associated flavonol glycosides have been detected in the needles of conifers (Strack et al. 1989). In pulse-chase experiments ^{14}C-labeled CO_2 was incorporated into soluble kaempferol 3-*O*-glucoside which was detected 3 weeks later in an insoluble extracellular fraction (Heilmann and Strack, 1990). In other tracer studies using suspension-cultured plant cells, flavonols with free 3- and 4′-hydroxyl groups were degraded under specific conditions to 2,3-dihydroxy flavones (Hösel et al. 1972; Hösel and Barz 1972; Muhle et al. 1976) which could be further decomposed to form benzoic acid derivatives (Hösel et al. 1975; Miller and Schreier 1985). This type of molecule has been shown to undergo cross-linking and incorporation into the cell wall (Muhle et al. 1976 Fry 1986). However, a requirement of flavonols (or their metabolites) for cell wall formation has not been established.

The metabolic fate of flavonol aglycones during the initial stages of pollen germination and tube growth may suggest how these small hydrophobic molecules act. Since CMF plants do not contain flavonols, unlabeled compounds can be used in metabolism studies. However, a radioactive flavonol is desirable, especially if the flavonol C15 skeleton is catabolized during germination. Therefore, we chemically synthesized [4′-*O*-^{14}C]kaempferide, and used this tracer and a series of unlabeled flavonol aglycones, to determine the structure and abundance of the flavonol metabolites that are formed during pollen germination. This study also excluded the pollen wall as a site of flavonol accumulation.

Materials and methods

Plant material. The *Petunia hybrida* L. plants used in this study were described by Taylor and Jorgensen (1992).

Synthesis of [4′-O-^{14}C]kaempferide. [4′-*O*-^{14}C]Kaempferide was prepared by [^{14}C]diazomethane-mediated methylation of robinin (kaempferol-3-*O*-rhamnosyl galactoside 7-*O*-rhamnoside) according to Markham (1982). Four milligrams of [^{14}C]diazald (N-methyl-N-nitroso-*p*-toluenesulfonamide, 1480 $MB_2 \cdot mmol^{-1}$; Sigma Chemical Co., St. Louis, Mo., USA) and 350 mg unlabeled diazald (Aldrich Chemical Co., Milwaukee Wis., USA) were dissolved in 500 μL of 10 N KOH in a sealed glass vial (Black 1983). The released diazomethane was transferred by a nitrogen stream and collected in five 2-mL fractions of diethyl ether. Robinin at 15 mg/500 μL in methanol:dimethyl sulfoxide (DMSO) (10:1, v/v) was added to the combined diethyl ether-diazomethane solution. Methoxylation was complete within 2 min, and excess solvent and unreacted diazomethane were evaporated under nitrogen. To hydrolyze the glycosyl moieties, the residue was dissolved in 4 mL methanol:4 N HCl (1:1), incubated at 60 °C for 1 h, diluted with 10 mL water, and after 2 h of incubation on ice, the yellow precipitate was recovered by centrifugation at 3000 · *g* for 20 min. The methanol-solubilized material was purified by HPLC on a Nova-Pak C_{18} Radial-Pak cartridge (20 cm long, 8 mm i.d.; Waters, Milford Mas., USA) using a linear gradient elution of 5%–90% (v/v) acetonitrile (CH_3CN) in 5% acetic acid in 39 min. The radioactive compound with the same retention time (RT) as kaempferide (17.8–19.5 min) was purified by analytical HPLC using a reverse-phase column (Nova-Pak C18; 150 mm long, 3.9 mm i.d.; particle size 4 μm). The specific activity of HPLC-purified [^{14}C]kaempferide was determined from liquid scintillation counting, 365-nm-peak area, and spectrophotometry as 15.9 $MB_2 \cdot mmol^{-1}$.

Treatment of pollen with [4′-O-^{14}C]kaempferide and unlabeled flavonols. Pollen from 16 anthers harvested from stage 10 (Pollak et al. 1993) CMF flowers was suspended in 8 mL germination medium (GM; pH 5.0) (Mo et al. 1992). The pollen suspension was incubated with 2.5 μM [4′-*O*-^{14}C]kaempferide in DMSO (ca. 21000 dpm) at room temperature with shaking (100–130 rpm). Aliquots were harvested at 1, 15, 60 or 240 min after treatment by centrifugation, the pellet washed with 0.5 mL of GM, recentrifuged, and extracted twice with 0.5 mL methanol and once with 0.5 mL 50% methanol. The GM and the pollen GM wash (0.5 mL) were combined and extracted twice with 4 mL ethyl acetate followed by 2 × 4 mL *n*-butanol. The organic fractions were dried, the residue dissolved in 100 μL methanol and 50-μL aliquots were analyzed by HPLC. The eluant from the entire HPLC gradient was collected in 0.5-mL fractions and the radioactivity in each fraction was determined by liquid scintillation counting.

For experiments with nonradioactive flavonols, pollen from 20 anthers was suspended in 10 mL of GM and the appropriate flavonol in DMSO added to the desired final concentration. After incubation, aglycones and glycosides in the pellet and the GM were extracted as previously described and analyzed by HPLC.

Flavonols sources were: myricetin, Sigma Chemical Co. robinin, quercetin, galangin and isorhamnetin, Spectrum Chemical (Gardena, Calif., USA); kaempferol and kaempferide, Apin Chemical (Abingdon, Oxon, UK); morin, Aldrich; rhamnetin,

Indofine Chemical Co. (Belle Mead, N.J., USA). All flavonols were purified by HPLC prior to use.

Analysis by HPLC. The HPLC system was as described by Pollak et al. (1993) and Vogt et al. (1994) with the following modifications: solvent A, 2% (v/v) acetic acid in H_2O; solvent B, 2% (v/v) acetic acid in CH_3CN. Flavonols were separated with a gradient of 0–2 min 5% B in A; 2–22 min, 5–90% B in A; 22–23 min, 90% B in A. Flavonol aglycones were detected at a UV absorbance of 365 nm (375 nm for myricetin) and 350 nm for flavonol glycosides. Flavonol aglycones were quantified by comparison with calibrated peak areas of purified aglycone standards; glycosides were quantified from peak areas of the corresponding aglycones, applying a correction factor for peak shift due to glycosylation. Quantification was linear between 0.1 and 5 nmol.

Isolation and purification of kaempferide glycosides from germinating pollen. Kaempferide glycosides were isolated on a preparative scale for biochemical and structural characterization. Pollen from 500 CMF anthers was incubated for 4 h with gentle shaking (90 rpm) in 125 mL GM supplemented with 10 μM kaempferide. The pollen was collected by centrifugation and the decanted GM was collected and processed separately. The pellet was extracted thrice with 5 mL methanol followed by a single wash with 5 mL of 70% (v/v) methanol; the combined extracts were dried and dissolved in methanol. Kaempferide glycosides in the extract were partially purified by two rounds of preparative TLC using a cellulose matrix (Eastman Kodak, Rochester, N.Y., USA) and 15% (v/v) acetic acid as solvent followed by analytical TLC using an MN-Polygram polyamide-6 matrix (Machery-Nagel & Co.-Düren, Brinkmann Instruments, N.Y., USA) and methanol:acetic acid:H_2O (90:5:5), by vol. as the solvent. The kaempferide glycosides were localized by UV fluorescence, excised from the TLC plate and extracted with methanol.

The decanted GM was extracted twice with 20 mL ethyl acetate followed by two extractions with 20 mL *n*-butanol; the combined extracts were dried, the residue dissolved in 5 mL methanol, diluted to 100 mL with water and purified by solid-phase extraction (C_{18} Sep-Pak; Waters).

The kaempferide glycosides in both the pollen and GM extracts were purified by preparative HPLC. The nonradioactive kaempferide glycosides were identified by co-chromatography with the corresponding radiolabeled kaempferide glycosides isolated from GM and pollen.

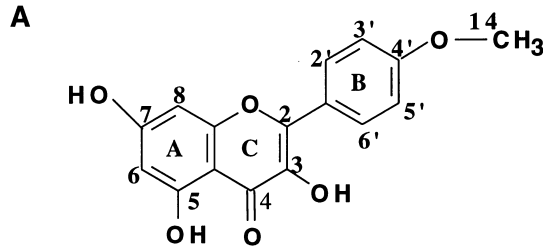
Identification of flavonol aglycones and glycosides in pollen and GM extracts. Flavonols were identified by comparing the UV absorption spectrum (235–435 nm) of the HPLC-resolved compounds with a flavonol spectral library (994 photodiode array detector; Waters). Three identical sets of HPLC fractions (50 × 0.5 mL each) were collected from each experiment. The amount of radioactivity was determined in one set. The second set was used for the *unhydrolyzed* pollen rescue by evaporating each fraction to dryness and dissolving in sufficient DMSO to yield a final concentration of 2–10 μM when 1 μL was added to 100 μL of a CMF pollen suspension. The third set of fractions (*hydrolyzed* pollen rescue) was evaporated to dryness, dissolved in 50 μL methanol adjusted to 2 N HCl and incubated at 95 °C for 30 min. The hydrolysate was dried, the residue extracted with ethyl acetate, dried again, dissolved in DMSO and added to a CMF pollen suspension as described above. Germination of CMF pollen was scored after 4 h incubation.

The identity of the sugar moiety of the kaempferide glycoside was determined by cellulose TLC using a solvent of pyridine:butanol:H_2O:benzene (5:5:3:1, by vol.) and glucose and galactose standards as described by Lewis and Smith (1969). The sugars released from 100 nmol of hydrolyzed peak 3 (Fig. 2) were visualized with a solution of *p*-anisidine hydrochloride (1%, w/v) and sodium hydrosulfite (0.1, w/v) in *n*-butanol: methanol (9:1, v/v). Glycosides of kaempferol and quercetin were identified by comparison of HPLC RTs and UV-spectra with the corresponding glycosides purified from V26 pollen. The position of sugar attachment to the kaempferide molecule was determined spectrophotometrically as described in Mabry et al (1970).

Sixty micrograms of purified kaempferide glycoside (peak 3, Fig. 2) isolated from the GM was analyzed by fast atom bombardment mass spectroscopy (FAB-MS) to determine the molecular mass using the conditions and instrumentation described in Vogt and Taylor (1995).

Results

Synthesis of [4′-O-^{14}C]kaempferide. Radiolabeled flavonols are not commercially available; therefore a prerequisite of this study was to synthesize this tool. Three design considerations were that: (i) the molecule should rescue CMF pollen at concentrations similar to kaempferol and quercetin, the endogenous petunia pollen flavonol aglycones (Vogt et al. 1995; Zerback et al. 1989); (ii) synthesis of the compound must be feasible with readily available starting chemicals and laboratory equipment; and (iii) the radioactive moiety should not be vulnerable to enzymatic or non-enzymatic cleavage. Figure 1A shows the structure of kaempferide (kaempferol 4′-*O*-methyl ether), a flavonol aglycone that fulfills all three requirements.



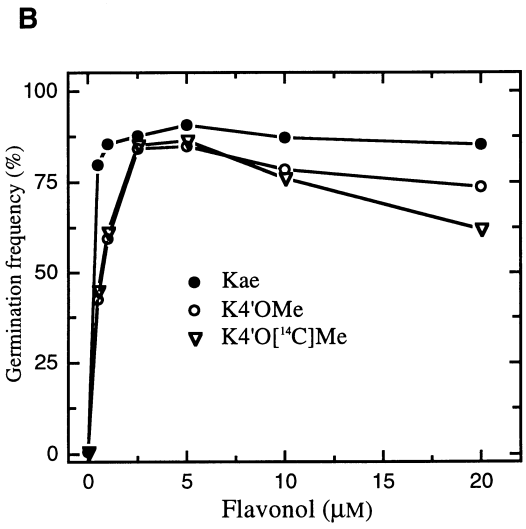


Fig. 1A,B. Structure and rescue activity of [4′-*O*-^{14}C]kaempferide. **A** [4′-*O*-^{14}C]kaempferide. **B** In-vitro CMF-pollen rescue activities of kaempferol (*Kae*), kaempferide (*K4′OMe*) and radioactive kaempferide (*K4′O*[^{14}C]*Me*). Data are means of two experiments. *Me*, methyl group

[4′-*O*-^{14}C]Kaempferide was synthesized by introducing a [^{14}C]methyl group to the 4′-OH position of kaempferol using the highly reactive methyl donor, diazomethane, generated from [^{14}C]diazald (Markham 1982; Black 1983). Diazomethane attacks oxygen of hydroxyl (OH) groups with acidic protons; therefore, the OH groups at the 3, 7, and 4′ positions will be modified under conditions which will not methylate the 5-OH which is less reactive because of hydrogen bonding with the C4 carbonyl oxygen. By protecting critical functional OH groups (C3 and C7) with sugar residues which are subsequently removed by acid hydrolysis, and varying the reaction time, the methyl group can be introduced specifically at the C4′-OH group, which is non-essential for bioactivity. Robinin which has an acidic 4′-OH group and protected 3-*O* and 7-*O* positions, was used as the starting material. The purified [4′-*O*-^{14}C]Kaempferide showed HPLC RT and spectral properties identical to those of authentic kaempferide.

Flavonol-induced pollen germination is concentration dependent and saturable (Mo et al. 1992). The endogenous flavonols of petunia pollen show a ten fold difference in their CMF pollen-rescue ability; kaempferol, the most active, induces a maximum response at 0.5 to 1 μM and quercetin is required at 5–10 μM. Kaempferide in both the labeled and unlabeled form, induced maximum germination at 2.5 μM (Fig. 1B). Therefore, for metabolism studies, we used flavonol concentrations of 2.5 and 5 μM, the lowest level which elicits a full response and allows reliable detection of flavonol metabolites.

Metabolic fate of [4′-O-^{14}C]kaempferide in germinating CMF pollen. The metabolites of [4′-*O*-^{14}C]kaempferide formed during CMF germination were analyzed by HPLC, TLC, UV-spectral analysis, and liquid scintillation counting of extracts from the pollen and the GM at various times after addition of the tagged molecule. Figure 2A,B shows the UV absorbance and radioactive profile of the HPLC-separated extracts from pollen and GM after 4 h incubation with 2.5 μM [4′-*O*-^{14}C]kaempferide. The radioactivity was distributed among four peaks in extracts from both pollen and the GM in which the pollen was incubated; no radioactivity was found in the void volume, or in washes of the HPLC column. The major radiolabeled compound (peak 4) present in both the pollen and GM had an RT (18 min) and UV-spectral properties identical to kaempferide. When the HPLC fraction corresponding to peak 4 was added to a CMF pollen suspension, it induced germination (Fig. 2C,D) which was not increased by acid hydrolysis of the fraction (compare Fig. 2C and E). Thus we conclude that peak 4 represents the unaltered [4′-*O*-^{14}C]kaempferide.

Three additional radioactive compounds were detected: peaks 1 and 2 in the pollen extract and peaks 1 and 3 in the GM extract (Fig. 2A,B). Ultraviolet-spectral analysis (Mabry et al. 1970) of the purified compounds indicated that all three were flavonols which were glycosylated at the C3 position (Fig. 1). Analysis by HPLC of acid hydrolysates of the glycosides confirmed that the flavonol backbone of these compounds was kaempferide. These results are consistent with the ability of the specific HPLC fractions to stimulate germination: only kaempferide (peak 4) could induce CMF pollen germination in the unhydrolyzed samples (Fig. 2C,D), but when the glycoside fractions were hydrolyzed to release aglycone, they also induced germination (Fig. 2E,F). Analysis of peak 1 in both pollen and GM using both shift reagents (Mabry et al. 1970) and HPLC clearly demonstrated that these two peaks represent the same compound. Peak 2 in the pollen extract and peak 3 in the GM extract had RTs of 12.4 and 13.2 min, respectively; therefore, they are assumed to be different kaempferide 3-*O*-glycosides.

Thin-layer chromatography and FAB-MS analysis were used to determine that the predominant glycoside represented by peak 3, was kaempferide 3-*O*-monogalactoside. A single sugar which co-migrated with a galactose standard was detected by TLC of an acid hydrolysate and the FAB-mass spectrum of the unhydrolyzed fraction had a protonated molecular ion at m/z 463 and a fragment ion at m/z 301 corresponding to the loss of a single glycosyl moiety (there was no signal at m/z beyond 600). Since only small amounts of kaempferide glycosides 1 and 2 could be purified, the glycosyl moieties of these compounds were not determined. However, based on their RTs they are assumed to be 3-*O*-di- and/or 3-*O*-triglycosides of kaempferide.

To test whether kaempferide is catabolized to molecules which do not absorb at 365 nm, are nonradioactive, but can rescue CMF pollen, all HPLC fractions from [4′-*O*-^{14}C]kaempferide-rescued pollen and GM extracts were tested for their ability to stimulate CMF pollen germination. The data presented in Fig. 2, panels C–F confirm that only the radioactive kaempferide aglycone or the glycoside fractions, after hydrolysis, are bioactive.

Time course of flavonol glycoside accumulation. Germination of flavonol-rescued CMF pollen is rapid (Mo et al. 1992) and shows virtually the same kinetics as wild-type pollen: both require 30 min hydration followed by swelling at the germination pore and tube outgrowth 3–5 min later. The uptake of [4′-*O*-^{14}C]kaempferide into the pollen grain and distribution of radioactive derivatives between the pollen and GM during the initial stages of pollen germination is shown in Table 1. Within 1 min of addition of [4′-*O*-^{14}C]kaempferide to hydrated CMF pollen, $>15\%$ of the radiolabel is associated with the pollen. This precedes visual evidence of pollen tube outgrowth. Maximum accumulation (≈25%) of the aglycone in pollen occurs after 1 h when the length of the pollen tube is equivalent to about five pollen grain diameters.

During the 4 h incubation, the progressive disappearance of radiolabel from the GM is accompanied by an increase in kaempferide aglycone and kaempferide glycosides 1 and 2 in the pollen. This confirms our earlier observation that exogenously added flavonol aglycones are rapidly taken up by the pollen and glycosylated by pollen-specific enzymes (Vogt and

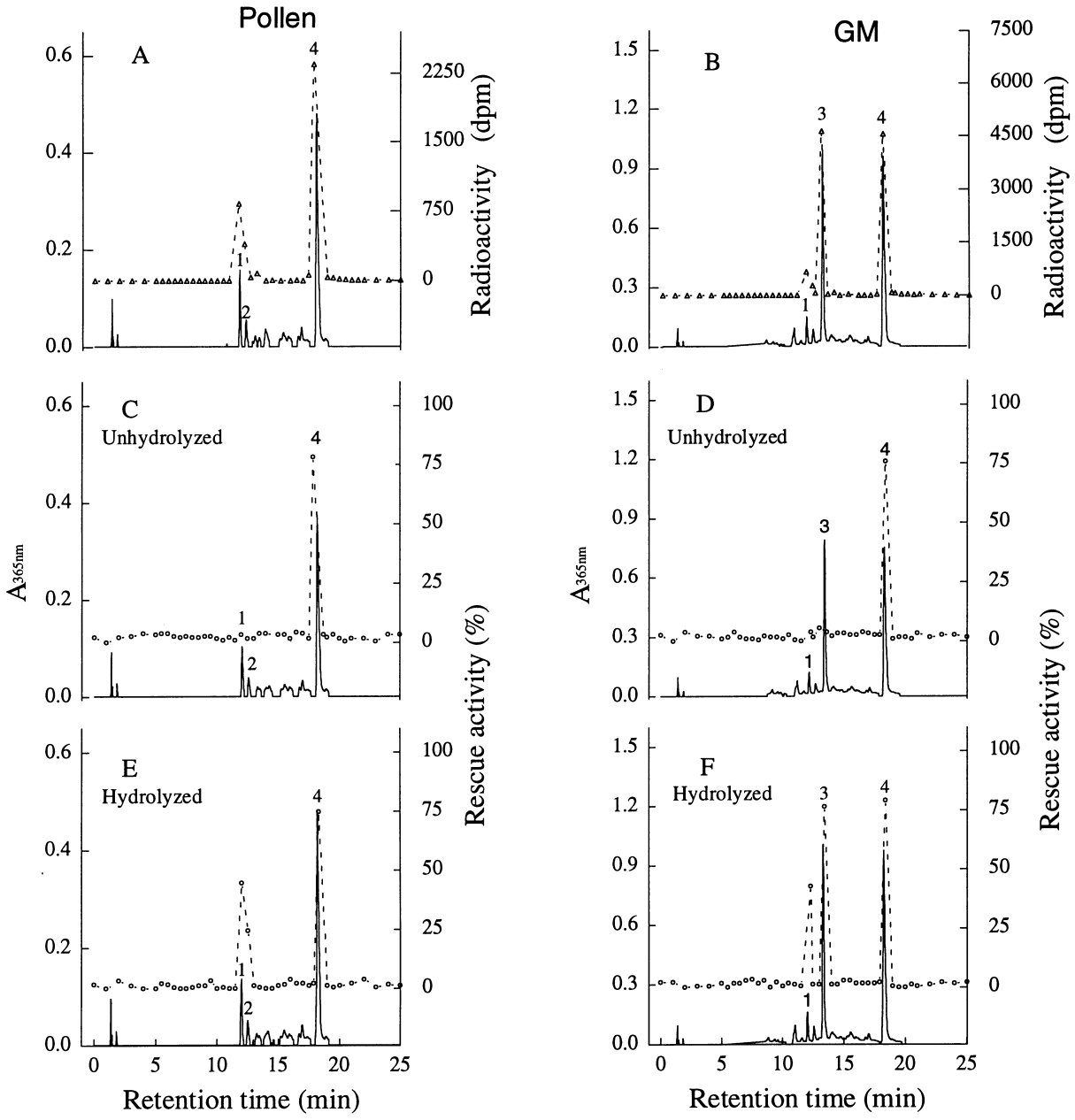


Fig. 2A–F. Radioactivity and rescue activity in HPLC fractions after 4 h incubation of CMF pollen with 2.5 μM 4′-*O*-^{14}C]kaempferide. The HPLC profile (——) and the total radioactivity (△·····△) and percent pollen germination (○·····○) were determined in each of fifty 0.5-mL fractions. **A** Radioactivity in HPLC fractions of pollen. **B** Radioactivity in HPLC fractions of GM. **C** Rescue activity in unhydrolyzed HPLC fractions of pollen. **D** Rescue activity in unhydrolyzed HPLC fractions of GM. **E** Rescue activity in acid-hydrolyzed HPLC fractions of pollen. **F** Rescue activity in acid-hydrolyzed HPLC fractions of GM

Taylor 1995). Unexpectedly, kaempferide glycosides also accumulated in the GM. Substantial amounts of [4′-*O*-^{14}C]kaempferide 3-*O*-galactoside were detected within 15 min of aglycone addition and accounted for about 33% of the added [4′-*O*-^{14}C]kaempferide after 4 h incubation. Accumulation of this glycoside pool depends on the presence of an intact pollen grain; it is not formed when the kaempferide is incubated in conditioned GM from which the pollen has been removed.

The hypothesis that flavonols are incorporated into the growing pollen wall is refuted by the small amount of residual radioactivity recovered in the methanol-insoluble pollen fraction. It accounted for only 0.05–0.4% of the total radioactivity; based on the concentration of kaempferide required to elicit CMF pollen germination (Fig. 1B) and the specific activity of the added [4′-*O*-^{14}C]kaempferide, this amount of radioactivity would not be sufficient to induce CMF pollen germination. It is noteworthy that the amount of cell-wall-associated radioactivity did not markedly increase with pollen tube growth and the concomitant formation of new cell wall.

The experimental results illustrated in Fig. 2 and Table 1 show that throughout the entire 4-h incubation, more than 95% of the added radioactivity was recovered

Table 1. Time course of [4′-*O*-^{14}C] kaempferide and glycoside accumulation in pollen and GM. Pollen suspension (8 mL) in GM was incubated with 20 nmol (ca. 21 000 dpm) of [4′-*O*-^{14}C]kaempferide, aliquots removed at indicated times, extracted and analyzed by HPLC and liquid scintillation counting. Data are means ± SE from four experiments

Incubation Time (min)	1[a]	15	60	240
Radioactivity (dpm) in:				
Pollen				
[4′-*O*-^{14}C] kaempferide				
– aglycone	3150 ± 100 (15.0[b])	4140 ± 110 (19.7)	4635 ± 235 (22.1)	3000 ± 300 (14.3)
– glycoside 1	50 ± 25 (0.2)	125 ± 15 (0.6)	140 ± 30 (0.7)	610 ± 40 (2.9)
– glycoside 2	35 ± 10 (0.2)	70 ± 15 (0.3)	190 ± 10 (0.9)	270 ± 25 (1.3)
residue	10 ± 3 (0.05)	25 ± 4 (0.1)	35 ± 2 (0.2)	75 ± 7 (0.4)
GM				
[4′-*O*^{14}C]kaempferide				
– aglycone	16640 ± 310 (79.2)	15170 ± 105 (72.2)	12990 ± 465 (61.9)	6860 ± 495 (32.7)
– glycoside 1	30 ± 10 (0.1)	85 ± 35 (0.4)	160 ± 30 (0.8)	1115 ± 595 (5.3)
– glycoside 3	140 ± 55 (0.7)	510 ± 60 (2.4)	1315 ± 110 (6.3)	6975 ± 525 (33.2)
residue	150 ± 10 (0.7)	190 ± 30 (0.9)	230 ± 20 (1.1)	725 ± 220 (3.5)
Total Recovery	20180 ± 350 (96.1)	20360 ± 310 (97.0)	19770 ± 200 (94.1)	20355 ± 790 (96.9)

[a]The 1-min samples were harvested 1 min after radioactive kaempferide addition but the time involved in aliquoting, washing and centrifugation extended the exposure to about 7 min
[b]Percentage of total radioactivity (21 000 dpm) added

as kaempferide aglycone and glycosides. Thus the only significant metabolic products formed during germination are the glycosides, suggesting that flavonol catabolites are not responsible for pollen germination.

Pollen uptake and O-glycosylation of structurally-distinct flavonols. The apportionment of the bulk of the kaempferide 3-*O*-galactoside to the GM instead of the germinating pollen was unexpected. In wild-type pollen, diglycosides of kaempferol and quercetin accumulate to high levels but the aglycone is undetectable (Pollak et al. 1993). To determine if kaempferide is uniquely metabolized, formation of the conjugate and the subsequent partitioning of glycoside and aglycone were determined for a group of structurally distinct flavonols. All but one of the compounds induce CMF pollen germination, albeit to vastly different extents (Table 2). Although the amount and distribution of the aglycone and glycosides vary, the flavonols can be classified into distinct overlapping groups. Group 1 includes galangin, kaempferol and isorhamnetin and is characterized by the accumulation of large amounts of both the aglycone and glycosides in pollen. Quercetin, morin and myricetin constitute a second group in which flavonols, especially the glycosides, accumulate to significantly lower levels in pollen. Kaempferide shows a unique pattern that combines characteristics of both groups: large amounts of aglycone (group 1) but very little glycoside (group 2), accumulate in the pollen. When the structural features of the two groups are compared, the common feature is that all group 2 compounds are more polar than group 1 compounds. Uptake is likely hindered by the increased polarity and this in turn results in low internal glycoside levels. However the distribution of kaempferide and kaempferide metabolites belies the hypothesis that glycoside formation is solely dependent on pollen aglycone levels. A more likely explanation is that the distribution and relative amounts of the aglycone and glycoside are the result of differences both in uptake and in substrate usage by the F3GalTase enzyme. Alternatively, the chemical properties of the individual flavonols may target them to different pollen compartments, some of which may be inaccessible to the conjugating enzyme. Interestingly, rhamnetin (7-methyl quercetin), which does not stimulate pollen germination because the 7-OH group required for activity is blocked, is taken up and glycosylated efficiently. Taken together, this experiment shows that there is a correlation between the ability to induce germination and aglycone uptake, but not to glycosylation.

Time and concentration dependence of glycoside formation in CMF pollen. Kaempferide induces the same biological response as the endogenous pollen flavonols, kaempferol and quercetin, but it appears to be processed differently. The accumulation of glycosylated and non-glycosylated kaempferide, kaempferol, and quercetin were compared in both the GM and pollen at various

Table 2. Distribution of flavonol accumulation in pollen after 1 h incubation. The CMF pollen suspensions (5 mL) were incubated with 5 μM flavonol aglycone for 1 h, extracted and the extracts analyzed by HPLC. Data are means ± SE from three experiments. Results are expressed as pmol of flavonol in pollen from one CMF anther (approx. 35 000 pollen grains · anther^{-1})

Flavonol aglycone structure[a]	Germination frequency[b] (%)	Flavonol aglycone and glycosides in pollen (pmol · anther^{-1})		
		Aglycone	Glycoside(s)	Total
Galangin				
3,5,7-OH	88.7 ± 0.5	354 ± 12	171 ± 22	525 (21%)[c]
Kaempferol				
3,5,7,4′-OH	87.8 ± 0.9	358 ± 24	358 ± 29	716 (29%)
Isorhamnetin				
3,5,7,4′-OH, 3′OMe	88.8 ± 1.3	192 ± 14	680 ± 49	872 (35%)
Kaempferide				
3,5,7-OH, 4′OMe	85.2 ± 2.7	727 ± 29	27 ± 2	754 (30%)
Quercetin				
3,5,7,3′,4′-OH	83.5 ± 2.3	268 ± 11	36 ± 3	304 (12%)
Morin				
3,5,7,2′,4′-OH	58.4 ± 3.1	173 ± 17	22 ± 2	195 (8%)
Myricetin				
3,5,7,3′,4′,5′-OH	< 5	135 ± 3	ND[d]	135 (5%)
Rhamnetin				
3,5,3′,4′-OH, 7OMe	< 5	183 ± 33	822 ± 31	1005 (40%)

[a]Me = methyl group
[b]Germination frequency after 4 h incubation.
[c]Percent of added flavonol aglycone · anther^{-1}
[d]ND = not detectable

times during germination (Fig. 3A) and at different concentrations (Fig. 3B). The time course of flavonol accumulation (Fig. 3A) shows that 1 min after adding the flavonols to pollen suspensions, the aglycone forms of all three compounds were detected in the pollen. Twice as much kaempferide accumulated (410 pmol) as kaempferol (220 pmol) or quercetin (160 pmol). Unlike kaempferide 3-*O*-galactoside, which accumulated in the GM, the major glycosides of kaempferol and quercetin were associated with the pollen. Analysis by HPLC confirmed that they co-migrate with kaempferol- and quercetin 3-*O*-gal-glc from wild-type pollen. The low

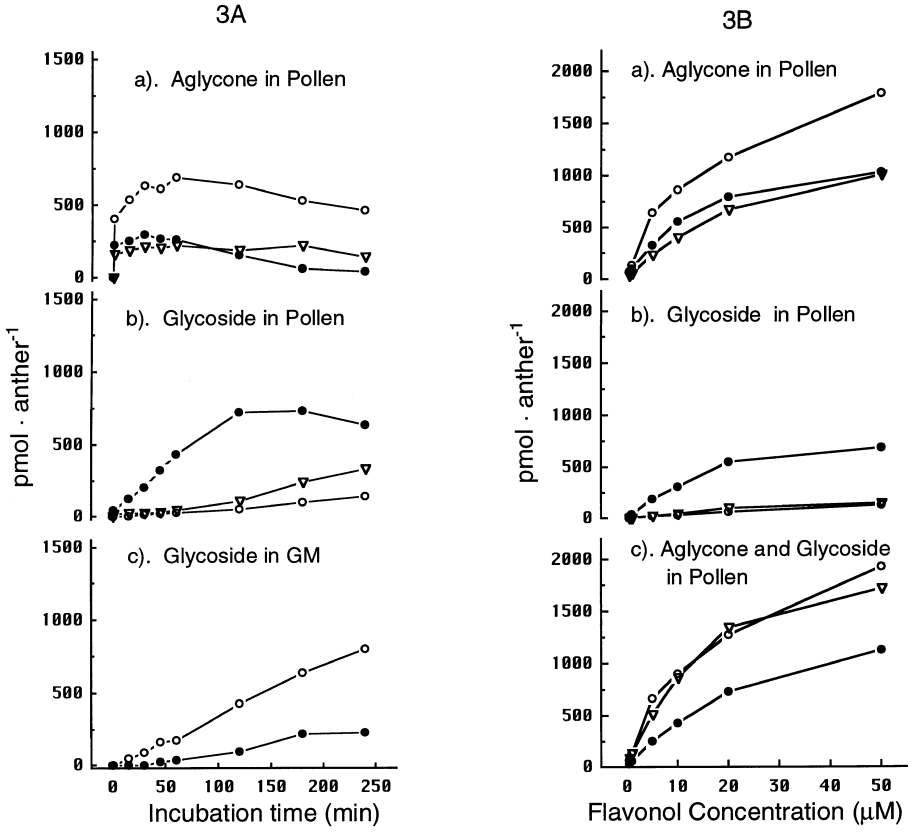


Fig. 3A,B. Time and concentration dependence of glycoside formation in CMF pollen. (○—○), Kaempferide and its glycosides; (●—●)kaempferol and its glycosides; (▽—▽), quercetin and its glycoside. **A** Time courses of aglycone and glycoside accumulation of kaempferide, kaempferol and quercetin in pollen and GM. The extracts were analyzed by HPLC as described in *Materials and methods.* Results are expressed as pmol of flavonol in pollen from one anther (approx. 35 000 pollen grains · anther^{-1}) or in 0.5 mL GM. Glycosides represent the sum of all glycosylated species. **B** Effect of increasing flavonol concentration on uptake and glycosylation by CMF pollen. Incubation time was 30 min

level of kaempferol 3-*O*-gal-glc that was detected in GM after 1.5 h incubation (Fig. 3A, panel c) was probably released into GM from burst pollen grains.

The uptake of kaempferide, kaempferol and quercetin aglycones by CMF pollen was concentration dependent and unsaturable at concentrations ranging from 0.5 μM to 50 μM (Fig. 3B). On the other hand glycoside formation saturated at 20 mM for kaempferol. Increasing concentrations of kaempferide or quercetin failed to enhance the low level of glycoside formation in the pollen. Thus formation of flavonol glycosides in rescued CMF pollen is not limited by uptake, a finding that may have bearing on the accumulation of flavonols in wild-type pollen where the aglycone and the intermediate monoside are never detected, only the diglycoside.

Discussion

Previous studies of flavonoid metabolism used labeled substrates which were isolated from cell suspensions grown in the presence of a radioactive precursor. This is an inefficient approach for producing specific molecules especially if the precursor is incorporated into several classes of secondary metabolites. Our studies on the fate of the flavonol molecule during pollen germination necessitated the production of a labeled molecule with the precise structural features required for biological activity. Therefore, we opted to synthesize a germination-inducing flavonol by incorporation of a ^{14}C-labeled methyl group into the molecule via a diazomethylation reaction. The compound chosen for these experiments, kaempferide, does not occur naturally in petunia pollen; however, its use as a model flavonol is justified because it stimulates germination with virtually the same efficiency as kaempferol and it can be synthesized by chemical modification of existing flavonols, whereas kaempferol cannot.

We found that more than 95% of the added [4′-*O*-^{14}C]kaempferide rapidly accumulated in the germinating pollen or was conjugated to form three glycosides. No breakdown products were detected and negligible amounts of radioactivity were associated with the methanol-insoluble pollen residue. After 4 h of pollen tube growth, the surface area of the tube greatly exceeds that of the pollen grain (≈100:1). If flavonols (or an oxidative derivative) were incorporated into the growing pollen tube wall, it would cause a large increase in the amount of radioactivity associated with the methanol-insoluble fraction. Thus the low absolute amount and the modest increase (eight fold) of radioactivity measured during the 4-h incubation excludes a structural role for flavonols in pollen cell wall formation during germination.

These studies confirmed that formation of flavonol 3-*O*-glycosides is the dominant metabolic pathway in germinating CMF pollen (Table 1). The major metabolite, the 3-*O*-galactoside, is identical to the intermediate monoside that is predicted from the structure of the wild-type pollen diglycoside, confirming that initial flavonol metabolism in CMF pollen is the same as that which occurs during normal pollen development. However, flavonol metabolism in rescued CMF pollen differs from that in the wild type in an important detail: the substantial levels of the aglycone and galactoside that accumulate in CMF pollen are never detected in developing or germinating wild-type pollen (Pollak et al. 1993). Our inability to detect these intermediate products in wild-type pollen may be due to their presence at levels below our detection limit, a short half-life, or simply because we have not sampled pollen at the appropriate developmental moment.

Mo et al. (1992) showed that flavonol aglycones could be grouped into three classes according to the minimum concentration required to induce maximum levels of pollen germination: the most effective flavonols show full rescue activity at 1 μM, e.g., galangin, kaempferol, and isorhamnetin; quercetin and morin are required at 5–10 μM concentration to elicit a full response and some compounds, e.g. myricetin, are required at 100 μM. Previous structure-activity analyses showed that less-polar functional groups in the B-ring strongly influenced the ability of individual flavonols to stimulate germination (Mo et al. 1992; Vogt et al. 1995). In the present study (Table 2) we have demonstrated a three-way correlation between the concentration of the aglycone in the pollen grain, the polarity of the molecule (as reflected in B-ring hydroxylation) and the effectiveness of each flavonol to rescue pollen germination. This strongly implicates the aglycone as the germination-inducing species.

On the other hand, no correlation was observed between glycosylated flavonol levels in pollen and the extent of germination (Table 2). Our choice of radiolabeled substrate was fortuitous in this regard. Kaempferide stimulated pollen germination at concentrations comparable to kaempferol and was efficiently internalized, yet did not accumulate as the glycoside in pollen. However, when the GM was analyzed for flavonol metabolites it was apparent that kaempferide was an efficient substrate for F3GalTase. It is not known why the 3-*O*-galactoside of kaempferide is excluded from the pollen while kaempferol 3-*O*-galactoside is readily internalized. Minor amounts of kaempferide glycosides of unknown structure do accumulate in pollen. Differences in pH optima and solubilization requirements (Vogt and Taylor 1995) of F3GlcTase, which converts the 3-*O*-galactoside to the diglycoside, suggest that it is located in a separate pollen compartment from F3GalTase. Flavonol 3-*O*-galactosides, formed by the action of a membrane-associated F3GalTase with a catalytic domain on the extracellular side of the plasma membrane, must be internalized and presented to the F3GlcTase protein. Perhaps the proximity of the 4′ methyl group of kaempferide to the galactose residue at the 3 position may sterically interfere with some aspect of internalization; alternatively kaempferide 3-*O*-galactoside may be a poor substrate for F3GlcTase. Either alternative would result in the accumulation of the kaempferide derivative in the external medium. These possibilities can be tested by determining the location and substrate specificity of the purified F3GlcTase protein.

Although we excluded the pollen tube wall as an accumulation site, our efforts to pinpoint the site of flavonol action were not successful. Previous localization experiments with a fluorescent reagent showed bright yellow patches of fluorescing material in the cytoplasm of germinating wild-type and flavonol-rescued CMF pollen; an association with a particular cellular structure was not discernible (Vogt and Taylor 1995; Ylstra et al. 1994). In addition, this type of analysis cannot distinguish between flavonol aglycones and glycosides. Due to the diffusive nature of flavonols, the ideal molecule for these studies would be a photoreactive flavonol which could be immobilized to a specific cellular compartment(s) by cross-linking to proteins.

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