Flavonol 3-O-Glycosyltransferases Associated with Petunia Pollen Produce Gametophyte-Specific Flavonol Diglycosides

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Wild-type petunia pollen accumulates high levels of flavonol 3-O-glycosides. Pollen from conditionally male-fertile petunia has no flavonols and is unable to germinate. Pollen function is restored both in vivo and in vitro by providing flavonol aglycones, but not flavonol glycosides, to the pollen. In the present study, incubation of an in vitro suspension of conditionally male-fertile pollen with kaempferol or quercetin resulted in the accumulation of kaempferol and quercetin 3-O-glycosides in the pollen. We identified two glycosyltransferase activities associated with the intact pollen grain that catalyze the formation of a gametophyte-specific class of flavonol glycosides. Feeding studies showed that product formation was highly specific for flavonols with an unsubstituted 3-hydroxyl group and quercetin 3-O-glycosides in the pollen. We identified two glycosyltransferase activities in a cell-free extract, it was necessary to add Triton X-100, 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine, and ATP to the pollen at pollination. Flavonol-deficient pollen can be biochemically complemented in vitro with nanomolar concentrations of flavonol aglycones (Mo et al., 1992) or in vivo by the kaempferol that accumulates in wild-type stigma exudates upon pollination or wounding (Vogt et al., 1994). The ability of the flavonol-deficient pollen to utilize an exogenous source of flavonols to restore full function led us to coin the term CMF (Taylor and Jorgensen, 1992). CMF pollen provides us with a model system to understand the uptake and metabolism of flavonols during pollen development.

A bioassay based on addition of plant extracts or purified compounds to an in vitro suspension of CMF pollen established that flavonol aglycones were the only component necessary for in vitro pollen function. This study showed that all of the flavonols in wild-type petunia anthers were conjugated to sugars; no flavonol aglycones were detected at any stage (Pollak et al., 1993).

Glycosylation is one of the final steps in flavonol biosynthesis (Heller and Forkmann, 1994). The addition of sugars to the relatively nonpolar flavonol aglycones produces a bulkier, water-soluble molecule. The enzymes responsible for adding sugars to flavonol aglycones are UDP monosaccharide:flavonoid glycosyltransferases. These enzymes show exquisite substrate and site specificity (Kleinehollenhorst et al., 1982; Bar-Peled et al., 1991; Ishikura and Mato, 1993; Ishikura et al., 1993). The most common site on the flavonol molecule for glycosyl addition is carbon 3 of the C-ring, although other sites, especially the hydroxyl at carbon 7, are often substituted (Ishikura et al., 1993). Zerbach et al. (1989) found that the predominant flavonols in the pollen of a cyanidin (+) variety of petunia were kaempferol and quercetin 3-O-(2'-O-β-D-glucopyranosyl)-β-D-galactopyranoside. On the other hand, flavonol (and anthocyanin) glycosides isolated from petunia corollas are structurally different, having been identified as flavonoid 3-O-rutinosides (rhamnosyl 1→6 glucoside) (Wiering and de Vlaming, 1984; Brugliera et al., 1994; Kroon et al., 1994).

Petunia pollen lacking flavonols does not function because it is unable to germinate and/or sustain tube growth (Taylor and Jorgensen, 1992). However, the defect was overcome by applying kaempferol, a flavonol aglycone, to the pollen at pollination. Flavonol-deficient pollen can be biochemically complemented in vitro with nanomolar concentrations of flavonol aglycones (Mo et al., 1992) or in vivo by the kaempferol that accumulates in wild-type stigma exudates upon pollination or wounding (Vogt et al., 1994). The ability of the flavonol-deficient pollen to utilize an exogenous source of flavonols to restore full function led us to coin the term CMF (Taylor and Jorgensen, 1992).

Flavonols are present in the pollen of many, if not all, species and they can account for a large percentage (2–5%) of the dry weight (Wiermann and Gubatz, 1992). The vast majority of pollen flavonols are present as the sugar-conjugated form, flavonol 3-O-glycosides (Stanley and Linzens, 1974; Ceska and Styles, 1984; Pollak et al., 1993). A developmental profile of flavonol accumulation in petunia anthers showed that flavonol 3-O-glycosides began to accumulate at the uninucleate-microspore stage and reached peak levels when the binucleate pollen was accumulating starch. Almost 4000 pmol of flavonol 3-O-glycosides accumulated in each anther, a level that persisted until dehis-
pounds able to induce germination (Mo et al., 1992). A structure-activity analysis showed a correlation between functional groups at specific sites on the flavonol molecule and the ability to restore pollen germination in vitro. Flavonols with substitutions at the 3 position in the C-ring were not effective in stimulating germination (Mo et al., 1992; Vogt et al., 1995). The specificity of flavonol aglycones for stimulating pollen tube growth in vitro was also shown using cultured tobacco pollen (Ylstra et al., 1992).

To elucidate the role of flavonols in pollen germination, the biosynthesis and metabolic fate of flavonols in anthers must be understood. Toward this goal we used CMF pollen to identify and characterize two unique glycosyltransferase activities that convert added flavonol to flavonol 3-O-diglycosides. The involvement of the glycosyltransferase activities in normal flavonol metabolism was confirmed by showing that structurally identical flavonol 3-O-diglycosides were formed by both CMF and wild-type pollen. The flavonol 3-O-diglycosides that accumulate in pollen are structurally and chemically distinct from the flavonol 3-O-glycosides that are formed in somatic tissues of the same plant. The implications of gametophyte-localized glycosyltransferases for the formation of pollen-unique flavonols are discussed in terms of the flavonol requirement for pollen germination.

**MATERIALS AND METHODS**

**Plant Material**

The CMF and wild-type (V26) petunia used in this study were described by Taylor and Jorgensen (1992). Developmentally staged flower buds from which anthers and pollen were harvested were described by Pollak et al. (1993).

**Glycosyltransferase Assay**

**Intact Pollen**

CMF pollen at the appropriate developmental stage (stage 6 for most analyses) was collected in GM (Mo et al., 1992) or 50 mM Mes, pH 6.0, by gently squeezing the anthers with a spatula. The pollen suspension (8 mL) from 250 anthers was transferred to a fresh tube, and the crushed anthers were washed with 1 to 2 mL of GM or Mes to recover the residual pollen. Pollen was recovered from the combined suspensions by centrifugation at 1500g for 10 min, washed with 5 mL of GM or Mes to remove tapetal contamination, reconstituted, and suspended in 10 mL of GM or Mes. Glycosyltransferase activity in intact pollen grains or pollen washes was assayed by incubating 200 μL of the pollen suspension or wash solution with the appropriate flavonol substrate at 100 to 250 μM. Neither UDP-Glc nor UDP-Gal was added to the standard intact pollen assay. After 30 min of incubation at 25°C with gentle rotation (150 rpm), the reaction was terminated with 300 μL of MeOH, vortexed, and centrifuged at 10,000g for 10 min. Aliquots were immediately assayed by HPLC, and the individual flavonol peaks were monitored at 365 nm. MeOH was added to control reactions before the addition of any substrate.

**Disrupted Pollen**

Pollen from 50 anthers was released into 1 mL of Mes (50 mM, pH 5.5) as described above, and the crushed anthers were washed with an additional 200 μL of Mes buffer. The combined pollen suspension was centrifuged at low speed to collect the pollen, which was washed with 1 mL of Mes buffer and reconstituted. The pollen was suspended in 1 mL of Mes buffer, brought to 1% Triton X-100, and disrupted for 100 s in a minibead beater (Biospec Products, Bartlesville, OK). After 5 min on ice, the disrupted pollen extract was centrifuged at full speed in a microfuge for 5 min. The resulting cell-free extract was assayed for glycosyltransferase activity in a 100-μL volume containing 0.5 mM UDP-Glc and UDP-Gal, 100 to 250 μM kaempferol or other substrates (5 mM stock in DMSO), and 85 μL of the pollen extract. Where indicated, the cell-free extract was passed over gel-filtration columns of Sephadex G10 or Bio-Gel P6 (Bio-Rad) to remove low molecular weight compounds including the endogenous flavonols in V26 pollen extracts. When the standard assay was modified to test additives or deletions, the reaction volume was made up to 100 μL with either GM or Mes buffer. After incubation at 25°C for 20 min, the reaction was terminated by adding 200 μL of MeOH, vortexing, and centrifuging. A 50-μL aliquot was analyzed by analytical HPLC. The same protocol was used to prepare cell-free extracts and to determine glycosyltransferase activity in corolla, root, stigma, and leaves.

**Preparative HPLC Isolation of Kaempferol and Quercetin 3-O-(2'-O-β-D-Glucopyranosyl)-β-D-Galactopyranoside**

To obtain sufficient material (approximately 3 mg) for FAB-MS and 1H- and 13C-NMR analyses, pollen from 2,500 CMF anthers was used to isolate kaempferol and quercetin 3-O-(2'-O-β-D-glucopyranosyl)-β-D-galactopyranoside. The compounds were prepared enzymatically by incubating for 3 h at a 10-mL pollen suspension (300 anthers worth) supplemented with the corresponding flavonol aglycone (300 μM). The reaction was terminated by centrifugation at 1,500g for 10 min, and the pollen pellet was resuspended in 3 mL of 80% MeOH, extracted for 1 h at room temperature, vortexed, and centrifuged at 10,000g for 20 min. The supernatant was concentrated in a Speedvac (Savant, Farmingdale, NY) reconstituted in 1 mL of MeOH, and 200-μL aliquots were subjected to HPLC purification using an RP Novapak C18 semipreparative (7.8 × 200 mm) column (Waters RCM 8 × 10 with a 10-cm extension tube) at a flow rate of 3 mL/min. The solvent system consisted of solvent A, H2O (5% acetic acid), and solvent B, acetonitrile (5% acetic acid), with a gradient elution of 5% B in A to 90% B in A in 39 min. The compounds that eluted with retention times of 10.5 min (peak 2) and 11 min (peak 1) were collected, evaporated to dryness in a Speedvac, and subjected to FAB-MS, 1H-NMR, and 13C-NMR analyses. Glycoside hydrolysis and aglycone identification were performed as described by Pollak et al. (1993). Sugar identification was accomplished by TLC analysis according to the method of Hansen (1975).
Analytical HPLC

HPLC analysis of MeOH extracts of pollen was performed as described by Vogt et al. (1994).

FAB-MS

Positive mode FAB mass spectra were generated with a VG7070EHF mass spectrometer (Fisons Instruments, Danvers, NJ) using a glycerol matrix.

NMR Spectrometry

$^1$H-NMR spectra were obtained at ambient temperature with a Bruker AMX-300 NMR spectrometer (Billerica, MA) operating at a resonance frequency of 300.13 MHz. Samples were dissolved in DMSO-$d_6$. $^1$H spectra were referenced to the residual DMSO signal at 2.49 ppm.

$^{13}$C-NMR spectra were obtained at ambient temperature with a Varian VXR500S NMR spectrometer operating for carbon at a resonance frequency of 125.7 MHz and ambient temperatures. The perdeuterated DMSO solvent (DMSO-$d_6$) signal at 39.5 ppm was used as a reference signal. The complete set of $^{13}$C- and $^1$H-NMR coordinates can be obtained from L.P. Taylor.

Microscopy

CMF pollen was incubated with kaempferol (1 µM final) for 1.5 h, and then quercetin (5 µM final) was added for an additional 0.5 h of incubation. At the end of the 2 h of incubation, the pollen suspension was mixed with diphenylboric acid 2-aminoethyl ester (0.5% in 50% MeOH) (Sigma) and photographed immediately and after an additional 5 h of incubation at room temperature as described by Vogt et al. (1994). Kaempferol at 1 µM and quercetin at 5 µM give equivalent levels of pollen germination (Mo et al., 1992), but the fluorescence of the quercetin-diphenylboric acid 2-aminoethyl ester complex is more intense and thus easier to visualize than the corresponding kaempferol adduct (Neu, 1957).

RESULTS

Formation of Flavonol Glycosides by Flavonol-Supplemented CMF Pollen

CMF pollen is devoid of flavonols and unable to germinate (Taylor and Jorgensen, 1992), but adding nanomolar concentrations of kaempferol to an in vitro suspension leads to rapid pollen germination and tube growth (Mo et al., 1992). When analyzed by HPLC, a MeOH extract of the kaempferol-supplemented CMF pollen contained, in addition to the kaempferol peak, a more polar compound (Fig. 1B, peak 1) with $A_{350}$ and $A_{325}$ maxima. Formation of peak 1 was absolutely dependent on adding kaempferol to the CMF pollen suspension. It was, however, not dependent on pollen germination; omitting boric acid from the GM prevented pollen tube growth but not formation of peak 1. Adding sodium azide (0.05%), a general metabolic inhibitor, to the CMF pollen suspension completely abolished formation of the novel peak. Incubation of CMF pollen with quercetin, another flavonol aglycone, produced a second new peak with an elution and spectral profile typical of a flavonol 3-O-glycoside (Fig. 1C, peak 2). The retention time and spectral characteristics of the compounds formed by the CMF pollen were identical with the two major flavonol glycosides (Fig. 1A, peaks V1 and V2) from wild-type (V26) petunia pollen (Pollak et al., 1993). Acid hydrolysis of peak 1 and peak 2 yielded kaempferol and quercetin, respectively. These results suggested that incubation of CMF pollen with the appropriate aglycone led to the formation of kaempferol and quercetin glycosides in the pollen grain.

Biochemical and Structural Analysis of Pollen Flavonol Glycosides

To determine the chemical structure of the kaempferol and quercetin conjugates formed by CMF pollen, peaks 1 and 2 were isolated on a preparative scale by HPLC. The
two major flavonol glycosides from extracts of V26 pollen (peaks V1 and V2) were isolated for use as reference standards. Acid hydrolysis, TLC, FAB-MS, and "H-NMR and "C-NMR spectroscopy identified peak 1 as kaempferol 3-O-glucosyl-galactoside. Similarly, peak 2 was identified as quercetin 3-O-glucosyl-galactoside (Fig. 2).

FAB-MS was used to determine the molecular mass of the kaempferol and quercetin glycosides from V26 pollen and peak 1 and peak 2 from the CMF pollen extracts. The FAB mass spectrum of the compounds represented by peaks 1 and V1 showed a molecular ion of m/z = 610 [M]+, corresponding to the unfragmented molecular ion. High-mass fragment ions were also observed at m/z = 448 and 286, suggestive of a diglycoside composed of hexoses of molecular mass 180. FAB-MS of peaks 2 and V2 yielded unit mass values of m/z = 626 [M]+ and fragment ions at m/z = 464 and 302, indicating that the quercetin derivative was a similar diglycoside. Hydrolyzed extracts of the compounds represented by peaks 1, 2, V1, and V2 were analyzed by TLC to identify the sugar residues. Each extract produced two spots of equal intensity that co-migrated with authentic Gal and Glc standards. Acid hydrolysis, TLC, FAB-MS, and "H-NMR and "C-NMR spectroscopy identified peak 1 as kaempferol 3-O-glucosyl-galactoside and peak 1 and peak 2 from the CMF pollen extracts. The FAB mass spectrum of the compounds represented by peaks V1 and V2 yielded unit mass values of m/z = 626 [M]+ and fragment ions at m/z = 464 and 302, indicating that the quercetin derivative was a similar diglycoside. Hydrolyzed extracts of the compounds represented by peaks 1, 2, V1, and V2 were analyzed by TLC to identify the sugar residues. Each extract produced two spots of equal intensity that co-migrated with authentic Gal and Glc standards, indicating that the flavonol 3-O-glycosides were diglycosides of Gal and Glc. "H-NMR spectrometry confirmed that both Gal and Glc were in the β-configuration.

The ease of generating the aglycone by acid hydrolysis and the UV spectrum of peaks 1 and 2 suggested that the sugars were O-linked at position 3 of the flavonol skeleton and that all other possible sites were unsubstituted (Markham, 1982). "C-NMR spectrometry was used to determine the site of glycosylation, the sugar order, and the interglycosidic linkage. The chemical shifts (δ) confirmed that substitution was at position 3 and that the sugars were joined in a 1→2 interglycosidic linkage between the C2" of Gal and C1" of Glc (Fig. 2). The use of Gal to form the pyranoside and the 1→2 interglycosidic linkage is rather uncommon in flavonol 3-O-glycosides (Harborne and Williams, 1988). The chemical and spectral shifts from the "C- and "H-NMR analyses of kaempferol and quercetin 3-O-(2"-O-β-D-glucopyranosyl)-β-D-galactopyranoside formed by CMF pollen were virtually identical with the values obtained for the flavonol glycosides isolated from V26 pollen and Corylus avellana pollen and with literature values for reference compounds (Strack et al., 1984; Zerback et al., 1989).

These analyses unambiguously showed that the flavonol glycosides formed by CMF pollen are kaempferol and quercetin 3-O-(2"-O-β-D-glucopyranosyl)-β-D-galactopyranoside and that they are identical with the flavonol glycosides that accumulate in wild-type V26 petunia pollen. The occurrence of these unusual flavonol glycosides in petunia pollen was first recorded by Zerback et al. (1989) in a cyanidin (+) variety of petunia. Flavonol 3-O-diglycosides with the 1→2 linkage are not confined to the Solanaceae; they have also been identified in pollen from several other plant families (Prattviel-Sosa and Percheron, 1972; Strack et al., 1984; Meurer et al., 1988; Ferreres et al., 1989).

**Flavonol 3-O-Glycosyltransferases Catalyze Glycoside Formation during Pollen Development**

The formation of diglycosides of kaempferol and quercetin by the intact CMF pollen indicates that one of the final modifications to the flavonol molecule is catalyzed by the gametophyte. Flavonol 3-O-diglycosides are formed by the sequential addition of sugar residues (Bar-Peled et al., 1991). The structure of the pollen flavonol 3-O-glycoside indicates that two activities must be expressed by CMF pollen: a F3GalT adds Gal to the flavonol aglycone at position 3 of the C-ring, and a second activity, a glucosyltransferase (F3GT), adds Glc to the flavonol 3-O-glycoside by a 1→2 linkage. We define this dual activity as F3GalT-F3GT. Flavonoid glycosyltransferases use UDP-activated sugars as the glycosyl donors (Heller and Forkmann, 1994). Since neither UDP-Glc nor UDP-Gal was added in the intact pollen assay, we assume that the activity associated with CMF pollen uses the endogenous pool of nucleotide sugars.

Each mature V26 anther contains more than 2000 pmol of kaempferol 3-O-glycosides and 2000 pmol of quercetin 3-O-glycosides (Pollak et al., 1993). If F3GalT-F3GT activity is involved in producing these compounds, then kaempferol and quercetin 3-O-(2"-O-β-D-glucopyranosyl)-β-D-galactopyranoside formation by CMF pollen and flavonol 3-O-glycoside accumulation in V26 anthers should show similar developmental profiles. Anther and pollen development in CMF plants proceeds at the same rate as V26; therefore, the same stages are comparable. High levels of F3GalT-F3GT activity were detected in CMF pollen at the binucleate microspore stage (stage 3) onward through maturity (Fig. 3). Peak product formation was measured at stage 6, when the binucleate pollen has a prominent exine and the tapetal layer of the anther wall has largely disintegrated. The activity remained elevated throughout pollen development and even increased slightly during in vitro germination of CMF pollen. These data correlated well with flavonol 3-O-glycoside accumulation in V26 anthers, in which product was first detected at stage 3, and by stage 6, each V26 anther accumulated about 1000 pmol of

![Figure 2. Flavonol 3-O-(2"-O-β-D-glucopyranosyl)-β-D-galactopyranoside.]
kaempferol 3-O-glucosyl-galactoside and 1000 pmol of quercetin 3-O-glucosyl-galactoside (Pollak et al., 1993).

Enzyme Activity in a Cell-Free Extract of CMF Pollen

The flavonoid glycosyltransferases described to date are soluble enzymes (Heller and Forkmann, 1994). However, initial attempts to recover activity from ruptured pollen grains were unsuccessful; F3GT activity was completely abolished and F3GalT activity was substantially reduced. This loss occurred whether or not nucleotide sugars were added to the extract. However, when the CMF pollen was disrupted in a buffer containing 1% Triton X-100, F3GalT-F3GT activity was retained. At concentrations of less than 1% (0.05, 0.1, 0.2, and 0.5%), the detergent was ineffective and addition of Triton X-100 without disruption of the pollen did not release activity from the pollen grain (data not shown). Stabilizing agents such as glycerol, PEG, and MeOH (5%) were unsuccessful in retaining F3GalT-F3GT activity when added to a cell-free extract prepared without Triton X-100.

In contrast to intact pollen, F3GalT-F3GT activity in the cell-free extract was absolutely dependent on addition of UDP-Glc and UDP-Gal. Other sugar donars were not tested. The pH dependency of F3GalT-F3GT activity from disrupted pollen was determined over a range of pHs from 4.0 to 8.5. Maximum activity was measured at pH 5.5 in Mes buffer (0.5 mm), with a second peak of activity at pH 8.0 (0.5 mm Tris) (data not shown). The presence of two peaks may represent different pH optima for the two activities present in the extract. The effect of different cations on F3GalT-F3GT activity was tested; 1 mm Cu$^{2+}$ and Zn$^{2+}$ completely inhibited the F3GalT-F3GT activity (data not shown). Cu$^{2+}$- and Zn$^{2+}$-mediated inhibition of flavonoid glycosyltransferases was previously reported in Vigna seedlings and Paederia leaves (Ishikura and Mato, 1993; Ishikura et al., 1993). Other bivalent cations (Mn$^{2+}$, Ca$^{2+}$, and Mg$^{2+}$) neither enhanced nor reduced activity.

Substrate Specificity

The substrate specificity of the transferase activity associated with intact and disrupted CMF pollen was determined (Fig. 4). Although substrate use was set relative to kaempferol in both types of analyses, the absolute activity level in intact pollen and the cell-free extract are not directly comparable because substrate usage by intact pollen must account for uptake and access to the enzyme(s). This is evident in the usage pattern of kaempferol, quercetin, and myricetin, which were glycosylated by intact CMF pollen with decreasing efficiency, but the difference was abolished with the disrupted pollen extract. These compounds constitute a series of increasing hydrophilicity with one, two, and three hydroxyl groups, respectively, in the B-ring. Presumably, the more hydrophilic myricetin is a poor substrate for intact pollen because it cannot penetrate the pollen grain as easily as the less polar compounds. Substrate accessibility is also reflected in the acceptance pattern of the highly polar, glycosylated substrates (Fig. 4). Intact pollen was unable to glycosylate either 3-O-mono-

Figure 3. Developmental time course of kaempferol 3-O-(2''-O-β-D-glucopyranosyl)-β-D-galactopyranoside formation in CMF pollen. Stage 1 to 2, 0- to 15-mm bud and uninucleate, vacuolate microspores; stage 3 to 4, 16- to 25-mm bud, binucleate pollen, and tapetum disintegrating; stage 5 to 6, 26- to 35-mm bud; stage 7 to 8, 36- to 50-mm bud and prominent exine on microspores; stage 9 to 10, 51- to 60-mm bud, prominent pores on pollen, and stigma receptive; G, in vitro germinated CMF pollen supplemented with kaempferol. Values are means ± SE of three experiments.

Figure 4. Substrate usage by intact CMF pollen and a cell-free (disrupted) extract prepared from CMF pollen. Assay conditions are described in "Materials and Methods." Substrate acceptance is set relative to kaempferol (100%). nt, Not tested; Que, quercetin; Kae, kaempferol.
glycosides or a 7-O-diglycoside, but disrupted pollen efficiently converted the monoglycoside to a diglycoside and produced a 3-O-glycosylated derivative of the 7-O-rhamnosylglycosides. Evidently, the polar and bulky sugar derivatives cannot traverse the pollen wall to gain access to the F3GalT-F3GT proteins.

O-Methylation at position 7 or 3' of the flavonol skeleton did not interfere with glycosylation at position 3 in either intact or disrupted pollen. Di-O-methylated substrates were actually glycosylated more efficiently than monomethyl ethers by intact pollen (compare quercetin 7,3'-di-O-Me with either quercetin 3'- or 7-O-Me). Because methyl-substituted flavonols are less polar than nonmethylated compounds, this enhanced usage may result from an accumulation of the hydrophobic, polymethylated molecules on the lipophilic coating of the pollen wall. As expected, 3-O-methylation prevented 3-O-glycoside formation by either intact or disrupted pollen; neither kaempferol 3-O-Me nor quercetin 3-O-Me was accepted as a substrate. This corroborates the specificity of glycosylation for position 3 of the flavonoid molecule.

Flavonols lacking at least one hydroxyl group in the B-ring (galangin, quercetin 3',4'-dimethyl ether, and kaempferol 4'-monomethyl ether) were poor substrates (less than 5% activity) for the cell-free F3GalT-F3GT activity (Fig. 4). This suggests that glucosyl- or galactosyltransferase activity, or both, prefers an electrophilic group in the B-ring of the substrate. Ibrahim et al. (1987) also noted a relationship between B-ring substitutions and the ability of a flavonol to act as a substrate for an O-methyl transferase.

We found no product formation with representatives of other flavonoid classes, including apigenin (flavone), naringenin (flavanone), and dihydroquercetin (dihydroflavonol), as well as p-hydroxy benzoic and p-coumaric acid.

The sugar specificity of the F3GalT and F3GT activities in the cell-free extract was determined by measuring product formation in the presence of a single hexose donor and either the aglycone or monoglycoside as acceptor. F3GalT was absolutely dependent on UDP-Gal for activity; no product was formed when only UDP-Glc was supplied. Although F3GT showed absolute specificity for forming a 1→2 interglycosidic linkage, it did not show a marked preference for UDP-Glc or UDP-Gal as the second sugar donor. However, as shown in Figure 4 (rightmost two substrates), a galactopyranoside was the preferred acceptor. Cell-free extracts of CMF pollen incubated with kaempferol 3-O-galactoside or kaempferol 3-O-glucoside as substrates formed more than twice as much kaempferol 3-O-glucosyl-galactoside as kaempferol 3-O-glucosyl-glucoside (276 versus 122%).

A time course of the accumulation of the monoglycoside and diglycoside products by the disrupted pollen extract showed that formation of the latter was dependent on the formation of the galactoside (Fig. 5). The diglycoside and the monoglycoside are easily distinguished by their characteristic retention times on the HPLC chromatogram. After a 1-min incubation, measurable amounts of kaempferol 3-O-galactoside were present, but no diglycoside had formed. Following an initial lag phase, the glucosyl-galactoside was detected (2 pmol min⁻¹ anther⁻¹) after a 4-min incubation when about 8 pmol of monoside had accumulated. Thereafter, diglycoside formation was linear up to 30 min and increased at the expense of monoglycoside accumulation.

Petunia corollas accumulate flavonol 3-O-glycosides, which have been structurally identified as rhamnosyl 1→6 glucosides (Wiering and de Vlaming, 1984; Brugliera et al., 1994; Kroon et al., 1994). To provide support for the hypothesis that F3GalT-F3GT activity is pollen specific, we analyzed the flavonol glycosides formed by an extract of CMF corollas. As expected, the major product was kaempferol 3-O-glucoside with a minor amount of an unidentified diglycoside (data not shown). Extracts of roots, leaves, or stigmas showed no evidence of kaempferol or quercetin 3-O-(2'→4')-diglycoside; kae 3-O-gal-glu) and kaempferol 3-O-galactoside (monoglycoside; kae 3-O-gal) formation with disrupted pollen extracts. Quantitation by HPLC was described by Vogt et al. (1994).

Figure 5. Time course of kaempferol 3-O-glucosyl-galactoside (diglycoside; kae 3-O-gal-glu) and kaempferol 3-O-galactoside (monoglycoside; kae 3-O-gal) formation with disrupted pollen extracts. Quantitation by HPLC was described by Vogt et al. (1994).

Localization of Added Flavonols

Fluorescent microscopy of quercetin-supplemented CMF pollen was used to confirm that the added flavonols were
internalized and not restricted to the outer pollen wall. Diphenylboric acid 2-aminoethyl ester reacts with hydroxyl groups in the B-ring of quercetin and kaempferol to produce a vivid orange and green fluorescence, respectively (Neu, 1957). Examination of the germinated CMF pollen 30 min after adding quercetin showed that the aglycone was closely associated with the hydrophobic pollenkitt that coats the pollen exine. Several hours later, when much of the added quercetin had been converted to the diglucoside, the fluorescence was restricted to the cytoplasm of the pollen grain (Fig. 6). This latter fluorescence pattern is identical with V26 pollen, which contains only kaempferol 3-O-glycosides and quercetin 3-O-glycosides (Pollak et al., 1993), and confirms that glycosylation at position 3 does not interfere with formation of a complex with the diphenylboric acid 2-aminoethyl ester reagent.

**DISCUSSION**

Adding flavonol aglycones to flavonol-deficient pollen leads to the formation of unique flavonol 3-O-glycosides. Structural analysis of the products formed upon feeding kaempferol and quercetin to CMF pollen confirmed that they were identical with the flavonol 3-O-glycosides that accumulate in wild-type petunia pollen. Thus, we have identified two glycosyltransferase activities that are responsible for the formation of flavonol glycosides in pollen. F3GalT catalyzes the transfer of Gal to the 3-hydroxyl position of a flavonol aglycone, and F3GT forms a 1→2 interglycosidic bond between Glc and the galactoside. As a result, high levels of a gametophyte-specific class of flavonol glycosides accumulate in pollen; there is no evidence that pollen forms the more prevalent 1→4- and 1→6-linked diglycosides or the monoglycosides that predominate in somatic tissues (Wiering and de Vlaming 1984; Brugliera et al., 1994; Kroon et al., 1994; T. Vogt and L.P. Taylor, unpublished data).

**A Model for Flavonol Biosynthesis in Anthers and Pollen**

Pollen development is characterized by interactions between the developing gametophyte and the surrounding sporophytic tissue. We propose a model of flavonol biosynthesis that separates flavonol aglycone formation in the sporophyte (tapetum) and flavonol glycoside accumulation in the gametophyte (pollen). There is substantial genetic, molecular, and biochemical evidence that the biosynthetic enzymes for flavonol aglycones are active in the tapetum but are not expressed in pollen (Herdt et al., 1978; Kleinenhollenhorst et al., 1982; Kehrel and Wiermann, 1985; Beerhues et al., 1989, 1993; Taylor and Jorgensen, 1992). The findings reported here suggest that conversion of the non-polar flavonol aglycones to the highly polar flavonol 3-O-glycosides is mediated by glycosyltransferase activities localized in the intact pollen grain. Several observations support this conclusion: (a) F3GalT and F3GT activities are associated with the intact pollen grain; (b) wild-type pollen accumulates structurally unique flavonol 3-O-glycosides and CMF pollen forms the same flavonol 3-O-glycosides when incubated with the appropriate flavonol aglycone; (c) highly polar, glycosylated flavonols are unable to rescue CMF pollen, presumably because they cannot enter the pollen grain; and (d) fluorescence labeling shows that flavonol 3-O-glycosides accumulate within the pollen cytoplasm in both wild-type and flavonol-supplemented CMF pollen. Our model proposes that during development kaempferol and quercetin are produced by enzymes active in the tapetum and released into the locule by diffusion or cell rupture. The flavonol aglycones are then internalized in the pollen grain by an unknown mechanism. Uptake may be mediated by the pollen glycosyltransferases or may occur by simple or facilitated diffusion, with subsequent glycosylation reactions taking place in the pollen cytoplasm. In either event the result is the accumulation of a gametophyte-specific class of flavonols. Thus, compartmentalization of enzymes in the biosynthetic pathway to different tissues ensures gametophyte-specific product accumulation.

The pollen glycosyltransferases tightly control formation of the pollen-specific flavonol 3-O-glycosides at multiple levels: the absolute requirement of the F3GalT for UDP-Gal, the preference of F3GT for UDP-Glc, and the specificity of F3GT for the 2′ position of the acceptor flavonol 3-O-galactoside. Although the F3GT can utilize UDP-Gal...
and glucopyranosides in vitro, only the glucosyl-galactoside is formed in vivo. This may be due to compartmentalization and/or high concentrations of a readily accessible pool of UDP-Glc (3.5 mM) as described for Nicotiana alata pollen (Schlüpmann et al., 1994).

The two enzyme activities were not separated in the cell-free extract, but the data presented in Figure 5 and the fact that flavonol 3-O-galactoside is not detected in vivo suggest that product formation involves sequential channeling (Hrazdina and Jensen, 1992). In this scenario F3GalT is readily accessible to flavonol aglycons from the anther locule (or the CM) as well as UDP-Gal from an unknown pollen compartment. In this regard it is worth noting that some F3GalT activity is removed from the intact CMF pollen in the wash solution (T. Vogt and L.P. Taylor, unpublished data). We postulate that F3GalT and F3GT are in close proximity and that F3GT is membrane associated. This would explain why the 1→2-linking enzyme cannot be removed from the pollen grain without the use of detergent. However, efforts to solubilize the F3GT with 0.5 M salt, which can remove peripheral membrane proteins, was ineffective (T. Vogt and L.P. Taylor, unpublished data).

A plasma membrane or cell surface-associated flavonol glucosyltransferase (or possible transferase complex) is highly unusual; all flavonoid transferases described to date are soluble proteins (Heller and Forkmann, 1994). A putative membrane or surface-localized F3GalT-F3GT that produces a pollen-specific flavonol might function in surface-mediated recognition of flavonols during pollen germination. In animal systems, membrane-bound galactosyltransferases mediate cell surface interactions, including tissue organization, neuronal outgrowth, and fertilization (Cooke and Shur, 1994). A similar function in plants merits further investigation.

Is there a direct link between glycosylation of flavonols and their ability to stimulate pollen germination? The glycosylation of kaempferol, quercetin, and myricetin in intact pollen mirrors the efficiency of these compounds to promote CMF pollen rescue (Mo et al., 1992). However, the activity of the cell-free extract indicates that the extent of pollen rescue does not correlate with the ability of these three flavonols to act as F3GalT-F3GT substrates. Moreover, rescue activity requires a free hydroxyl group at position 7 of the aglycone (Vogt et al., 1995), but 7-O-methylated quercetin derivatives are excellent substrates for glycosylation. In addition, galangin is a poor substrate for the cell-free activity but induces complete pollen rescue (Mo et al., 1992). Although an absolute correlation does not exist between glycosylation and germination, we cannot rule out that the reaction is not required for pollen germination. This would require analyzing pollen development in mutants devoid of F3GalT-F3GT activity, and to our knowledge no such mutants exist.

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