Purification, Cloning, and Heterologous Expression of a Catalytically Efficient Flavonol 3-O-Galactosyltransferase Expressed in the Male Gametophyte of Petunia hybrida*

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Flavonoids are plant-specific molecules that are required for pollen germination in maize and petunia. They exist in planta as both the aglycone and glycosyl conjugates. We identified a flavonol 3-O-galactosyltransferase (F3GalTase) that is expressed exclusively in the male gametophyte and controls the formation of a pollen-specific class of glycosylated flavonols. Thus an essential step to understanding flavonol-induced germination is the characterization of F3GalTase. Amino acid sequences of three peptide fragments of F3GalTase purified from petunia pollen were used to isolate a full-length cDNA clone. RNA gel blot analysis and enzyme assays confirmed that F3GalTase expression is restricted to pollen. Heterologous expression of the F3GalTase cDNA in Escherichia coli yielded active recombinant enzyme (rF3GalTase) which had the identical substrate specificity as the native enzyme. Unlike the relatively nonspecific substrate usage of flavonoid glycosyltransferases from sporophytic tissues, F3GalTase uses only UDP-galactose and flavonoids to catalyze the formation of flavonol 3-O-galactosides. Kinetic analysis showed that the $k_{cat}/K_m$ values of rF3GalTase, using kaempferol and quercetin as substrates, approaches that of a catalytically perfect enzyme. rF3GalTase catalyzes the reverse reaction, generation of flavonoids from UDP and flavonol 3-O-galactosides, almost as efficiently as the forward reaction. The biochemical characteristics of F3GalTase are discussed in the context of a role in flavonol-induced pollen germination.

Flavonoids are a large group of 15-carbon molecules that are formed by the condensation of 4-coumaryl-CoA, derived from phenylalanine of the shikimic acid pathway, with 3 molecules of malonyl-CoA in a reaction catalyzed by chalcone synthase. Subsequent reactions give rise to the various classes of flavonoids which differ in the oxidation state of the central pyran ring and by modifications such as glycosylation, acylation, and methylation to the three rings (1) (Fig. 1). Flavonoids perform a variety of functions including protection from ultraviolet light (2), signaling between leguminous roots and nitrogen-fixing soil bacteria (3), and the visible anthocyanin pigments in flowers attract pollinators (4). More recently, an essential role in plant reproduction was established for the flavonol class (Fig. 1) (5). In flowering plants when pollen germinates on a receptive stigma it extrudes a tube which rapidly grows by apical extension to transport the sperm cells to the embryo sac for fertilization. Chalcone synthase mutants in maize and petunia produced viable pollen which lacked flavonoids and was unable to germinate or produce a functional pollen tube. However, the mutant pollen was conditionally male fertile (CMF)

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Glycosyltransferase activities. F3GalTase forms the flavonol 3-O-galactoside (II) from the flavonol (I) and UDP-galactose. F2*GT forms the flavonol 3-O-glucosylgalactoside (III) from II and UDP-glucose. The flavonol (I) shown is kaempferol.

The two enzyme activities responsible for formation of the pollen-unique class of flavonol glycosides were identified as a F3GalTase, which used UDP-galactose as a substrate to convert flavonols to the flavonol 3-O-galactosides, and a flavonol 3-O-galactoside-2′-O-glucosyltransferase (F2*GT) which transfers glucose from UDP-glucose to the 2′ position of galactose to form the flavonol glucosylgalactoside (Fig. 1). Both glycosyltransferase activities were simultaneously extracted from pollen and catalyzed the formation of the flavonol diglycosides in vitro (7). This study also demonstrated that kaempferol, quercetin, and UDP-galactose were the only endogenous substrates of F3GalTase, and that the flavonol 3-O-galactoside is the only endogenous substrate that the F2*GT activity glucosylates (e.g., it does not glucosylate the flavonol). Importantly, formation of the flavonol 3-O-galactoside was a prerequisite to formation of the flavonol diglycoside (7).

Glycosylation is one of the final steps in flavonoid biosynthesis (1, 13) and converts a chemically reactive molecule into a less reactive form. Presumably, the enhanced solubility of flavonoid glycosides aids their intracellular transport and sequestration (14, 15). Although the cellular location of flavonoid glycosides is unknown, another class of flavonoids, the anthocyanidin glycosides are targeted to the vacuole following conjugation with glutathione (16). Glycosyl conjugation might also function to provide a reservoir of active molecules. The activity of plant growth regulators and other signal molecules is proposed to be regulated by interconversion between the active aglycone and the inactive glycosyl conjugate (15). The alternative roles of glycosylation are not mutually exclusive and in the case of flavonols there is evidence for both. High levels of flavonols are less reactive (8) and flavonols induce germination but the glycosylated flavonol glycosides accumulate in pollen (5, 8, 17, 18).

We want to establish the mechanism by which flavonols induce pollen to germinate and the role of glycosyl conjugate formation in this process. F3GalTase activity was deemed to be a critical component of flavanol-mediated pollen germination because: 1) F3GalTase is the sole activity in petunia pollen that metabolizes flavonols; 2) formation of flavonol glucosylgalactosides in petunia pollen is strictly dependent on F3GalTase activity; and 3) F3GalTase activity, through its forward and reverse reactions, has the potential to modulate the abundance of the active molecule. We used a directed approach, exploiting amino acid sequences in the native protein, to isolate a F3GalTase cDNA clone. Heterologous expression of the F3GalTase cDNA in Escherichia coli resulted in obtaining soluble, active F3GalTase which was used for biochemical characterization. The resulting information was used to construct a model of how F3GalTase activity might function during flavonol-induced pollen germination.

**EXPERIMENTAL PROCEDURES**

**Plant Material**—Pollen from anthers of stage 2–9 flowers (17) of the flavonoid-deficient line of Petunia hybrida, termed CMF, previously characterized by Taylor and Jorgensen (6) was used as a source of both native F3GalTase and total RNA for PCR and Northern blot analyses.

**FIG. 1. Flavonol glycoside biosynthesis in petunia pollen.** The biosynthesis of flavonol glycosides in petunia pollen is catalyzed by two glycosyltransferase activities. F3GalTase forms the flavonol 3-O-galactoside (II) from the flavonol (I) and UDP-galactose. F2*GT forms the flavonol 3-O-glucosylgalactoside (III) from II and UDP-glucose. The flavonol (I) shown is kaempferol.
All manipulations following excision of the protein from the gel were performed at the Protein and Nucleic Acid Facility at Stanford University Medical Center.

cDNA Library Construction—cDNA was synthesized using the SuperScript Lambda System (Life Technologies, Inc.) from 2 μg of poly(A)-enriched RNA isolated from stage 6 petunia pollen. Ten micrograms (1:100 dilution) of the cDNA (20 ng) was ligated into 0.5 μg of NotI/Sall-digested ZipLox DNA (Life Technologies, Inc.) and the resulting constructs were packaged in vitro using the Gigapack II System (Stratagene). The library was amplified by plating on E. coli host strain Y1090 and contained 2.3 × 10^9 phage particles.

Isolation and Cloning of F3GalTase RACE PCR Products—To obtain a nucleotide probe for screening the petunia pollen cDNA library, a RACE PCR protocol (21) was performed using total RNA isolated from stage 6 pollen and the following primers in the 5’ to 3’ direction: primer 1, CTGAGAGAAGACTGCTAGCAGCTTGAAGAACC(T)Tφ; 2, CTTAGAGAAGACTCCTGAGCAGCTATGTGAGC; 3, TCTGAGGCCTTGAAACAGGC; 4, AAYTTYITAYAGTNNGGCC; 5, GTNGCNACNCCNCCNCC; 6, GGICCIACCTTTGATTTTACATTTA; 7, CAAATCAACGGGATCCACG; 8, AACTTGCTACTGTTGTCGCC; 9, GCTAGCTGGCAATAGTGCATATTACATTACGGTATG.

First strand cDNA synthesis was performed in 1× reaction buffer (50 mM Tris-HCl, pH 8.3, 7.5 mM KCl, 3 mM MgCl2, and 10 mM dNTP) containing 5 μg of total RNA, 10 μL of RNasin, 25 μM of primer 1, 0.1 μg of oligo dT, and 200 units of SuperScript II. RNAase H reverse transcriptase (Life Technologies, Inc.) in 20 μL final volume. The reaction was incubated for 50 min at 45 °C and terminated by heating at 70 °C for 15 min. After cooling on ice, 2 μL of E. coli RNase H was added and the mixture was incubated for an additional 20 min at 37 °C.

The RNAse H-treated cDNA was diluted into 0.5 μL of TE buffer and stored at −80 °C. A 3’-RACE PCR product was generated using 3 μL of the cDNA as template, 25 pmol of primers 2 and 4, and 2.5 units of Taq DNA polymerase (Life Technologies, Inc.) in 50 μL of reaction volume. The addition of the other components were as described by the manufacturer.

The cycling parameters were: 2 min hot start at 94 °C, 5 min at 45 °C, 20 min at 72 °C, one cycle; 60 s at 94 °C, 60 s at 45 °C, 1.25 min at 72 °C, 35 cycles; final extension for 10 min at 72 °C. One μL of a 1:1000 dilution (in TE) of the reaction was used as template in a nested PCR experiment using 25 pmol of primers 3 and 5 and other reaction components as described previously. Cycling conditions were: 2 min hot start at 94 °C; 60 s at 94 °C; 30 s at 45 °C, 1 min at 72 °C; 30 cycles; final extension for 10 min at 72 °C. The most abundant amplicon at about 600 bp was ligated into pCR2.1 (Invitrogen) and transformed into E. coli TOP10F competent cells according to the manufacturer’s instructions.

For expression, the resulting plasmid (pETF3GalTase) in E. coli BL21(DE3) cells was grown at 37 °C in LB medium + 30 μg ml−1 kanamycin. The resulting clone was selected on LB agar plates containing kanamycin and ampicillin. The cells were harvested by centrifugation, resuspended in 0.1 M sodium phosphate buffer, pH 8.0, 0.5 M KCl, 1% (v/v) Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 0.01 M imidazole, 2.0 mg ml−1 lysozyme, and frozen in liquid nitrogen. The cells were thawed and after 5 min at room temperature, the lysate was adjusted to 5 mM MgCl2 and DNase I was added. A final concentration of 0.1 μg ml−1. The lysate was incubated for an additional 10 min and the cell debris was pelleted by centrifugation at 12,000 × g for 5 min (4 °C). The supernatant was decanted and Ni-NTA-agarose resin (Qiagen) was added (0.01 ml of the 50% slurry, as supplied by the manufacturer, per μL of lysate) and mixed with the lysate for 1 h at room temperature. The resin was pelleted by brief centrifugation, washed twice, and eluted with 0.2 M imidazole according to the manufacturer’s instructions. One μL Triton X-100 was included in the wash and elution buffers. The N-terminal 6× His-tag was then removed using thrombin at 10 units mg−1 rF3GalTase, in elution buffer, at 4 °C for 16 h. To terminate the reaction, phenylmethylsulfonyl fluoride was added to 1 mM. rF3GalTase was diluted 10-fold in wash buffer, excluding the imidazole, and incubated with Ni-NTA-agarose resin as described previously for 1 h at room temperature. The resin was pelleted by centrifugation and rF3GalTase in the supernatant was quantified using the BCA protein assay (Pierce).

The F3GalTase Enzyme Assay—rF3GalTase activity in the forward direction was measured in 50 mM HEPES-OH, pH 7.5, 75 mM KCl, 50 μM MnCl2, 1 mM DTT, 5% glycerol, 5 mM UDP-galactose, and 2.5 μg of purified rF3GalTase in a 50-μL final volume. The mixture was incubated at 43 °C for 3 min and the reaction was initiated by adding various concentrations of kaempferol or quercetin in dimethyl sulfoxide (final dimethyl sulfoxide concentration, 1%), and vortexed briefly to mix. After 3 min, the reaction was terminated with 2 volumes of 100% methanol and the assay mixture centrifuged at 12,000 × g for 5 min. The amount of flavonol 3-O-galactoside in 50 μL of the supernatant was determined by HPLC. Quantification was performed by integrating the peak area and using the first order regression equation derived from calibration curves of kaempferol 3-O-galactoside and quercetin 3-O-galactoside standards. One unit of rF3GalTase activity is defined as that amount of enzyme which catalyzes the formation of 1 pmol of 3-O-galactoside in 1 min using the described conditions. rF3GalTase activity in the reverse direction was measured as described previously except that 50 mM MES-Tris buffer, pH 6.0, and 5 mM UDP were used. The reaction was initiated by adding various concentrations of flavonol 3-O-galactoside (in dimethyl sulfoxide). The calibration curves for quantification of flavonols were based on kaempferol and quercetin.

2 V. Guyon, J. Astwood, and L. P. Taylor, submitted for publication.
FIG. 2. SDS-PAGE of proteins in fractions from the native F3GalTase purification protocol. Lane 1, crude homogenate; lane 2, High-Q preparative anion exchange chromatography eluate; lane 3, Mono-Q HR 5/5 anion exchange chromatography eluate; lane 4, Mono-Q HR 5/5 anion exchange chromatography eluate; lane 5, UDP-diphospho-hexanolamine affinity chromatography eluate. Proteins were size fractionated through 12% (w/v) acrylamide and visualized by silver staining. The migration of standard proteins of known mass are shown on the right-hand side of the gel.

FIG. 3. Nucleotide and deduced amino acid sequence of the petunia pollen F3GalTase cDNA. The nucleotide sequence of the NotI/SalI insert of the pF3GalTase1 plasmid is shown as well as the deduced amino acid sequence of the putative open reading frame. Sequenced peptides 1, 2, and 3 obtained from endopeptidase-treated native F3GalTase are underlined and the initiation codon and polyadenylation signal are in bold.

amino-agarose resulted in a near homogenous F3GalTase preparation which was sufficient for determining that the size of the F3GalTase peptide was about 45 kDa. However, the yield of active protein from the column was unacceptable for further biochemical characterization, so preparative SDS-PAGE was used to obtain sufficient material for protein sequencing. The 45-kDa protein in the Mono HR 5/5 eluate was gel purified and subjected to proteolytic digestion with Lys-C endopeptidase followed by N-terminal sequencing of the HPLC-purified peptide fragments. The sequence of three peptides, P1, P2, and P3 are shown in Fig. 3.

Synthesis and Cloning of F3GalTase RACE PCR Products and Isolation of the Full-length F3GalTase cDNA—The sequence of a 3′-RACE product, generated using degenerate oligonucleotide primers to the native F3GalTase protein sequence, showed a significant level of positional identity with glucosyltransferases from several plant species and mammalian tissues. The sequence of these partial F3GalTase sequences is a highly conserved region in the C terminus of glucosyltransferases from numerous eukaryotic and prokaryotic sources (22, 26, 30). To increase the likelihood of recovering a F3GalTase clone, we synthesized a 5′-RACE product with the expectation it would more likely contain gene-specific sequences. The deduced amino acid sequence of the resulting 725-bp PCR product

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(pF3GalTase5’ RACE) contained the sequence of peptide 1 and residues 1–9 of peptide 2 (Fig. 3). When pF3GalTase5’ RACE was used as a hybridization probe to screen the pollen cDNA library, a 1.431-kbp cDNA clone (pF3GalTase1, Fig. 3) containing a single open reading frame of 1.353 kbp was isolated. The translated peptide of the pF3GalTase1 cDNA clone is 451 residues with a predicted molecular mass of 48.9 kDa and contains all of the sequences of peptides 1–3 (Fig. 3). Residues 1–30 potentially comprise a cleavable signal sequence (PSORT program) reducing the mass of the peptide to 45.7 kDa, which corresponds to the observed mass of native F3GalTase (Fig. 2).

The deduced peptide sequence of pF3GalTase1 showed significant identity with several plant glycosyltransferases and an alignment with representatives of two dicot and two monocot flavonoid glycosyltransferases as well as a mammalian ceramide galactosyltransferase are shown in Fig. 4. The highest identity was with a grape flavonoid glucosyltransferase (53%) (26) and a mung bean flavonoid galactosyltransferase (49%) (29). Lower but significant identity was observed with the flavonoid glucosyltransferases from maize (37%) (24, 25) and barley (36%) (31). Although F3GalTase has significant identity with other flavonol glycosyltransferases, it contains putative regulatory motifs that are not present in any of the four plant sequences in Fig. 4. The predicted motifs were determined using the MOTIF program and include a single amidation site, two cAMP/cGMP-dependent protein kinase phosphorylation sites, six casein kinase II phosphorylation sites, four protein kinase C phosphorylation sites, four protein kinase C phosphorylation sites, eight N-myristoylation sites, and six N-glycosylation sites. It is unknown if native F3GalTase is modified at any of these sites.

The high level of sequence identity in the region spanning residues 328 to 370 in the petunia F3GalTase sequence has been noted in a number of glycosyltransferases from bacteria, plants, and animals. Based solely on the similar function of these proteins, part or all of the region has been proposed as a UDP-binding site (30) or a plant secondary product glucosyltransferase gene signature sequence (22, 26). The conservation of these sequences across kingdoms is highlighted by the fact that even though the overall positional identity with a rat brain ceramide galactosyltransferase is 19%, within this 43-amino acid sequence it rises to 44% (32, 33). The predicted amino acid sequences of 11 glycosyltransferases, all of which are predicted to conjugate small organic molecules such as flavonoids, auxins, and zeaxanthin, were aligned using GCG PILEUP with a Blosum 62 matrix (data not shown). From residues 328 to 370 the overall identity for the 10 plant and one bacterial protein was greater than 50% and included contiguous residues ((WA/CPQ) and (HC/AGWS)) with 90% positional identity. These residues are obvious targets for site-directed mutagenesis to determine their potential role in enzyme activity.

Tissue-specific and Developmental Expression of F3GalTase mRNA—

RNA gel blot analysis was used to determine the tissue-specific and developmental expression of F3GalTase mRNA. Fig. 5 shows that F3GalTase mRNA accumulation was restricted to pollen; transcripts were not detected, even after prolonged exposure of the autoradiograph, in any of the sporophytic tissues tested (seedling, leaf, stigma/style, and corolla). The lack of F3GalTase mRNA in sporophytic tissues was corroborated by an inability to detect F3GalTase enzyme activity in total soluble protein extracts from these tissues (data not shown). Thus, F3GalTase is expressed specifically in the male gametophyte where it modifies a molecule required for functional pollen.

A developmental profile of F3GalTase mRNA accumulation showed that transcripts were present throughout pollen development with the highest steady state levels in stage 4 of V26.
and, in stage 6 of CMF, pollen. In wild-type (V26) pollen during the proergic phase of development, i.e., after the pollen is released from the anther and during germination, F3GalTase mRNA levels decreased to about 6% of the levels in stage 4 pollen. This pattern of F3GalTase expression parallels the accumulation of flavonol diglycosides which peak at stage 9 and decrease with maturity in V26 anthers (stage 10–12) (17).

F3GalTase cDNA Expression in E. coli and Substrate Usage of the Recombinant Enzyme—The pF3GalTase1 plasmid was used as a template to construct, via PCR, an insert for expression in E. coli. rF3GalTase was expressed in a soluble active form by growth for 16 h at 22 °C in the presence of 5.0 μM IPTG (Fig. 6). At temperatures above 22 °C or at IPTG concentrations above 5.0 μM, rF3GalTase expression resulted in the formation of inclusion bodies and no detectable rF3GalTase activity in the soluble fraction. In the absence of the pETF3GalTase plasmid, rF3GalTase activity in crude lysates from BL21(DE3) cells was not detectable.

The recombinant enzyme retained the same substrate specificity as native F3GalTase (7, 18), accepting only UDP-galactose and flavonols as substrates. In addition to the endogenous flavonols kaempferol and quercetin, rF3GalTase also conjugated galangin, iso-rhamnetin, kaempferide, rhamnetin, morin, myricetin, and fisetin (Table I). Importantly, all of these flavonols induce pollen germination. Other flavonoids including the flavone apigenin, the flavanone naringenin, the dihydrolavonol dihydroquercetin, and the anthocyanidins pelargonidin, delphinidin, and cyanidin were tested and none were used as substrates by rF3GalTase.

Substrate usage was determined for the reverse reaction catalyzed by F3GalTase and was shown to be as specific as the forward reaction. rF3GalTase assayed in the reverse direction accepted only kaempferol 3-O- and quercetin 3-O-galactosides as substrates and not the respective flavonol 3-O-glycosides, similar to that reported for native F3GalTase (18).

Temperature/pH Optimum and Native Molecular Mass of F3GalTase—rF3GalTase activity was determined at temperatures from 35 to 60 °C and maximum activity was observed at 50 °C (Fig. 7). However, incubation of the enzyme at 50 °C for 15 min prior to initiating the reaction resulted in a 26% reduction of rF3GalTase activity compared with rF3GalTase assayed without preincubation (data not shown). Preincubation at 43 °C for the same amount of time resulted in only a 5% reduction of activity so to avoid potential artifacts caused by thermal inactivation of the enzyme, rF3GalTase assays were conducted at 43 °C.

The activity of rF3GalTase in both the forward and reverse
Steady-state Kinetic Analyses of rF3GalTase—Purified rF3GalTase was used to determine the $K_m$, $V_{max}$, $k_{cat}$, and $k_{cat}/K_m$ values of kaempferol, quercetin, and UDP-galactose in the forward direction at pH 7.5 and kaempferol-3-O-galactoside, quercetin-3-O-galactoside, and UDP in the reverse direction at pH 6.0 (Table II). Using a saturating concentration of UDP-galactose, the $K_m$ for kaempferol was calculated as 1.1 $\mu$M with a $V_{max}$ of 75 units mg$^{-1}$, whereas for quercetin the $K_m$ value was 0.96 $\mu$M and the $V_{max}$ was 73.4 units mg$^{-1}$. Using a saturating concentration of flavonol, the $K_m$ of UDP-galactose was determined to be 426 $\mu$M with a $V_{max}$ of 75.1 units mg$^{-1}$ when kaempferol was the co-substrate and a $K_m$ value of 387 $\mu$M with a $V_{max}$ of 70.5 units mg$^{-1}$ when quercetin was the co-substrate. The $K_m$ values of kaempferol-3-O-galactoside, quercetin-3-O-galactoside, UDP with kaempferol-3-O-galactoside as the co-substrate, and UDP with quercetin-3-O-galactoside are similar to the values reported for a flavonol GalTase from mung bean leaf tissue (28).

The calculation of $k_{cat}$ and $k_{cat}/K_m$ values determined the turnover number and the catalytic efficiency, respectively, of rF3GalTase with each of the substrates tested. The values reported in Table II indicate that rF3GalTase is highly efficient: with the flavonol substrates, the catalytic efficiency is only 1.6–1.8-fold less than the lower end of a diffusion-controlled limit ($10^8$–$10^{12}$ M$^{-1}$ s$^{-1}$) of a chemical reaction rate in solution. The $k_{cat}/K_m$ values of rF3GalTase using kaempferol and quercetin in the forward direction, and quercetin-3-O-galactoside in the reverse reaction, is comparable to carbonic anhydrase when carbon dioxide is the substrate ($8.3 \times 10^7$ M$^{-1}$ s$^{-1}$) (37), or fumarase when malate is the substrate ($3.6 \times 10^7$ M$^{-1}$ s$^{-1}$) (38). Carbonic anhydrase and fumarase are enzymes considered to have attained virtual catalytic perfection. Because rF3GalTase is a reversible enzyme, its activity is reduced by substrate concentration.
by the presence of the reaction products in either direction. So that maximum catalytic rates can be measured throughout the linear portion of the assay, the reaction must be assayed, in the forward or reverse directions, with an excess of substrates. If the rF3GalTase reaction could be coupled with another enzyme which uses, for example, either UDP or kaempferol 3-O-galactoside as substrates at rates equal to or greater than the rF3GalTase catalyzed production of these compounds, then the $k_{cat}/K_m$ of the rF3GalTase catalyzed reaction might be increased to the level where it achieves catalytic perfection.

**DISCUSSION**

The flavonols that induce pollen germination are rapidly metabolized by F3GalTase, a gametophytically expressed enzyme which catalyzes the reversible addition of kaempferol and queretin to UDP-galactose to form the respective flavonol 3-O-galactosides. We exploited amino acid sequences in the native protein to isolate a full-length F3GalTase cDNA from a petunia pollen cDNA library. RNA gel blot analysis and enzyme assays of various tissues showed that F3GalTase is expressed exclusively in pollen. Conclusive proof that the cDNA encoded F3GalTase was provided by heterologous expression in *E. coli* and demonstration that the recombinant enzyme retained the substrate specificity of the native enzyme in both the forward and reverse reactions.

The availability of active rF3GalTase in a pure form allowed us to determine substrate usage and steady-state kinetic parameters not possible with a partially purified enzyme. The ability to unambiguously determine these characteristics helped to clarify the role of F3GalTase during pollen germination. We found that F3GalTase uses only UDP-galactose and flavonols to catalyze the formation of flavonol 3-O-galactosides in pollen. The only other flavonol galactosyltransferase described to date is active in sporophytic tissue; the highest abundance of the mRNA, as well as enzyme activity, was detected in the leaves of mung bean (28, 29). In addition this enzyme is relatively nonspecific because it accepts four classes of flavonoids and two nucleotide sugars as substrates.

The total exclusion of other flavonoids as substrates, including the closely related anthocyanidins, makes F3GalTase unique among flavonoid glycosyltransferases, all of which show some activity toward more than one class of flavonoid (26, 28, 29, 39–41). The paradigm of a single activity with broad substrate usage has its historic roots in the UDP glucose:flavonoid glucosyltransferase activity encoded by the maize *Bronze-1* gene. Genetic and biochemical analysis of various maize tissues, including pollen, demonstrated that the UDP glucose: flavonoid glucosyltransferase encoded by the *Bz1* gene was able to conjugate both flavonol and anthocyanidin substrates (39–41). *Bz-1* was the first flavonoid glucosyltransferase cloned and served as a hybridization probe to isolate virtually every flavonoid glucosyltransferase gene described to date. However, very few of these enzymes have been purified or tested for substrate usage so their identity rests solely on similarity with the *Bz1* gene product. The view that flavonoid glycosylation results from the ubiquitous expression of a single activity with broad substrate acceptance is most certainly simplistic. The characteristics of F3GalTase indicate that conjugate formation is a more complex process, with highly specific activities expressed in a tissue-specific and/or developmentally controlled manner.

Understanding the role of flavonol glycoside formation in pollen germination requires that we reconcile the finding that flavonols, which is the only form that induces pollen germination *in vitro*, are not detected in developing wild-type pollen which accumulates only the diglycoside. The inability of flavonol glycosides to be internalized precludes a direct test of their germination-inducing properties *in vitro*. The finding that F3GalTase is relatively efficient in catalyzing both the forward and reverse transglycosylation reactions, added to our present understanding, allowed us to construct the following model of flavonol metabolism during pollen germination. As pollen develops within the anther, expression of F3GalTase located in the pollen wall facilitates the rapid uptake and conjugation of flavonols from the locular fluid. The inability to detect the galactoside in developing pollen requires that the F2"GT activity is highly efficient or a mechanism exists to channel the intermediate.

If the high levels of flavonol glucosylgalactosides that accumulate in wild-type pollen function as a latent pool of the active molecule, then a mechanism is required to re-generate the flavonol when the pollen is ready to germinate on the stigma. Although we have shown that F3GalTase can form flavonols from flavonol 3-O-galactosides, numerous attempts to detect a reverse F2"GT activity in pollen extracts have been unsuccessful. This, coupled with the fact that catabolism of metabolites rarely proceeds via a reversal of the biosynthetic pathway through which they formed, makes it unlikely that formation of the flavonol proceeds via a simple reversal of the pathway in Fig. 1.

The two most common mechanisms to remove a glycosyl moiety are hydrolysis by glycosidases or transglycosylation with UDP as the acceptor substrate. We propose that both activities are used in pollen and have preliminary evidence that intact pollen can catalyze the reverse F3GalTase reaction *in vitro*. When CMF pollen was incubated with kaempferol 3-O-galactoside or queretin 3-O-galactoside and increasing concentrations of UDP, kaempferol or queretin was detected in the surrounding germination medium in amounts that increased as UDP levels increased (18). Coincident with the increased production of kaempferol or queretin in the pollen suspension, the percentage of germinated grains increased in a UDP concentration-dependent manner. When the same experiment was repeated with the corresponding flavonol 3-O-gluconides and UDP, flavonols were not detected and the pollen did not germinate (18).

Pollen is released from the anther in a metabolically quiescent state induced by the loss of water that occurs just before dehiscence. Upon contact with the stigma exudate, the rapid rehydration of the pollen is accompanied by a dramatic increase in metabolic activity (42). It has been previously demonstrated that numerous plant tissues, including pollen, contain glucosidases (43–48) which remove glucose molecules from various glucosides, including flavonoids. Glucosidase activity in hydrated pollen could catabolize the flavonol glucosylgalactosides to the respective flavonol galactosides. F3GalTase activity in the reverse direction could then generate flavonols from the flavonol 3-O-galactosides, thus producing the molecule required for pollen to germinate. In further support of this model, we have recently detected queretin in mature V26 pollen hydrated in germination medium for 30 min. Alternatively, these data are not inconsistent with a model that involves the activation of a flavonol-mediated signaling pathway early in pollen development when flavonols accumulate in the anther. In this scenario, a second, non-flavonol signal would be stored until activated by hydration of the pollen on the stigma.

REFERENCES

1. Heller, W., and Forkmann, G. (1994) in *The Flavonoids: Advances in Research Since 1986* (Harborne, J. B., ed) pp. 499–535, Chapman and Hall Ltd., London
2. Li, J., Ou-Lee, T.-M., Raba, R., Amundson, R. G., and Last, R. L. (1993) *Plant...

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3. K. D. Miller and L. P. Taylor, manuscript in preparation.
4. L. P. Taylor, unpublished observations.
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34019

3. Burn, J., Rossen, L., and Johnston, A. W. B. (1987) *Genes Dev.* 1, 456–464
4. Harborne, J. B. (1980) in *Secondary Plant Products* (Bell, E. A., and Charlwood, B. V., eds) pp. 329–395, Springer-Verlag, Berlin
5. Mo, Y., Nagel, C., and Taylor, L. P. (1992) *Proc. Natl. Acad. Sci. U. S. A.* 89, 7213–7217
6. Taylor, L. P., and Jorgensen, R. (1992) *J. Hered.* 83, 11–17
7. Vogt, T., and Taylor, L. P. (1995) *Plant Physiol.* 108, 903–911
8. Vogt, T., Wellenweber, E., and Taylor, L. P. (1995) *Phytochemistry* 38, 589–592
9. Zerback, R., Bokel, M., Geiger, H., and Hess, D. (1988) *Phytochemistry* 26, 897–899
10. Wiering, H., and de Vlaming, P. (1984) in *Secondary Plant Products* (Sink, K. C., ed) pp. 39–65, Kluwer Academic Publishers Group, Dordrecht, Netherlands
11. Sembdner, G., Atzorn, R., and Schneider, G. (1994) *Plant Mol. Biol.* 26, 1459–1481
12. Mars, K. A., Allenito, M. R., Lloyd, A. M., and Walbot, V. (1995) *Nature* 375, 397–400
13. Pollak, P. E., Vogt, T., Mo, Y., and Taylor, L. P. (1993) *Plant Physiol.* 102, 925–932
14. Taylor, L. P., Strenge, D., and Miller, K. D. (1998) in *Flavonoids in the Living Petunia* (Sink, K. C., ed) pp. 49–68, Springer-Verlag, Berlin
15. Xu, P., Vogt, T., and Taylor, L. P. (1997) *Planta* 202, 257–265
16. Harborne, J. B., and Williams, C. A. (1988) in *The Flavonoids, Advances in Research Since 1980* (Harborne, J. B., ed) pp. 303–328, Chapman and Hall, London
17. Bandurski, R. S., Cohen, J. D., Slovin, J., and Reinecke, D. M. (1995) in *Plant Hormones: Physiology, Biochemistry, and Molecular Biology* (Davies, P. J., ed) pp. 39–65, Kluwer Academic Publishers Group, Dordrecht, Netherlands
18. Harborne, J. B. (1980) in *J. Hered.* 83, 1–17
19. Zerback, R., Bokel, M., Geiger, H., and Hess, D. (1988) *Phytochemistry* 26, 897–899
20. Wiering, H., and de Vlaming, P. (1984) in *Secondary Plant Products* (Sink, K. C., ed) pp. 39–65, Kluwer Academic Publishers Group, Dordrecht, Netherlands
21. Sembdner, G., Atzorn, R., and Schneider, G. (1994) *Plant Mol. Biol.* 26, 1459–1481
22. Mars, K. A., Allenito, M. R., Lloyd, A. M., and Walbot, V. (1995) *Nature* 375, 397–400
23. Pollak, P. E., Vogt, T., Mo, Y., and Taylor, L. P. (1993) *Plant Physiol.* 102, 925–932
24. Taylor, L. P., Strenge, D., and Miller, K. D. (1998) in *Flavonoids in the Living Petunia* (Sink, K. C., ed) pp. 49–68, Springer-Verlag, Berlin
25. Xu, P., Vogt, T., and Taylor, L. P. (1997) *Planta* 202, 257–265
26. Harborne, J. B., and Williams, C. A. (1988) in *The Flavonoids, Advances in Research Since 1980* (Harborne, J. B., ed) pp. 303–328, Chapman and Hall, London
27. Bandurski, R. S., Cohen, J. D., Slovin, J., and Reinecke, D. M. (1995) in *Plant Hormones: Physiology, Biochemistry, and Molecular Biology* (Davies, P. J., ed) pp. 39–65, Kluwer Academic Publishers Group, Dordrecht, Netherlands
28. Harborne, J. B. (1980) in *J. Hered.* 83, 1–17
29. Zerback, R., Bokel, M., Geiger, H., and Hess, D. (1988) *Phytochemistry* 26, 897–899
30. Wiering, H., and de Vlaming, P. (1984) in *Secondary Plant Products* (Sink, K. C., ed) pp. 39–65, Kluwer Academic Publishers Group, Dordrecht, Netherlands
31. Sembdner, G., Atzorn, R., and Schneider, G. (1994) *Plant Mol. Biol.* 26, 1459–1481
32. Mars, K. A., Allenito, M. R., Lloyd, A. M., and Walbot, V. (1995) *Nature* 375, 397–400
33. Pollak, P. E., Vogt, T., Mo, Y., and Taylor, L. P. (1993) *Plant Physiol.* 102, 925–932
34. Taylor, L. P., Strenge, D., and Miller, K. D. (1998) in *Flavonoids in the Living Petunia* (Sink, K. C., ed) pp. 49–68, Springer-Verlag, Berlin
35. Xu, P., Vogt, T., and Taylor, L. P. (1997) *Planta* 202, 257–265
36. Harborne, J. B. (1980) in *J. Hered.* 83, 1–17
37. Zerback, R., Bokel, M., Geiger, H., and Hess, D. (1988) *Phytochemistry* 26, 897–899
38. Wiering, H., and de Vlaming, P. (1984) in *Secondary Plant Products* (Sink, K. C., ed) pp. 39–65, Kluwer Academic Publishers Group, Dordrecht, Netherlands
39. Sembdner, G., Atzorn, R., and Schneider, G. (1994) *Plant Mol. Biol.* 26, 1459–1481
40. Mars, K. A., Allenito, M. R., Lloyd, A. M., and Walbot, V. (1995) *Nature* 375, 397–400
41. Pollak, P. E., Vogt, T., Mo, Y., and Taylor, L. P. (1993) *Plant Physiol.* 102, 925–932
42. Taylor, L. P., and Jorgensen, R. (1992) *J. Hered.* 83, 11–17
43. Zerback, R., Bokel, M., Geiger, H., and Hess, D. (1988) *Phytochemistry* 26, 897–899
44. Wiering, H., and de Vlaming, P. (1984) in *Secondary Plant Products* (Sink, K. C., ed) pp. 39–65, Kluwer Academic Publishers Group, Dordrecht, Netherlands
45. Sembdner, G., Atzorn, R., and Schneider, G. (1994) *Plant Mol. Biol.* 26, 1459–1481
46. Mars, K. A., Allenito, M. R., Lloyd, A. M., and Walbot, V. (1995) *Nature* 375, 397–400