Cloning and Characterization of Vitis vinifera UDP-Glucose:Flavonoid 3-O-Glucosyltransferase, a Homologue of the Enzyme Encoded by the Maize Bronze-1 Locus That May Primarily Serve to Glucosylate Anthocyanidins in Vivo*

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We report here the cloning and optimized expression at 16 °C and the characterization of a Vitis vinifera UDP-glucose:flavonoid 3-O-glucosyltransferase, an enzyme responsible for a late step in grapevine anthocyanin biosynthesis. The properties of this and other UDP-glucose:flavonoid 3-O-glucosyltransferases, homologues of the product encoded by the maize Bronze-1 locus, are a matter of conjecture. The availability of a purified recombinant enzyme allowed for the unambiguous determination of the characteristics of a flavonoid 3-O-glucosyltransferase. Kinetic analyses showed that $k_{cat}$ for glucosylation of cyanidin, an anthocyanidin substrate, is 48 times higher than for glucosylation of the flavonol quercetin, whereas $K_m$ values are similar for both substrates. Activity toward other classes of substrates is absent. Cu$^{2+}$ ions strongly inhibit the action of this and other glucosyltransferases; however, we suggest that this phenomenon in large part is due to Cu$^{2+}$-mediated substrate degradation rather than inhibition of the enzyme. Additional lines of complementary biochemical data also indicated that in the case of V. vinifera, the principal, if not only, role of UDP-glucose:flavonoid 3-O-glucosyltransferases is to glucosylate anthocyanidins in red fruit during ripening. Other glucosyltransferases with a much higher relative activity toward quercetin are suggested to glucosylate flavonols in a distinct spatial and temporal pattern. It should be considered whether gene products homologous to Bronze-1 in some cases more accurately should be referred to as UDP-glucose:anthocyanidin 3-O-glucosyltransferases.

Despite the widespread occurrence of glycosylated secondary metabolites, including flavonols (3), anthocyanins (4), monoterpnes (5), plant hormones (2), and metabolites of systemic fungicides (6), isolation and characterization of purified enzymes responsible for their metabolism have only been reported in a couple of select instances (7, 8). The most widely studied groups of plant glucosyltransferases are those associated with the biosynthesis of flavonoid glucosides, including flavonol glucosides, flavanone glucosides, and anthocyanins (3, 4, 9, 10). Earliest reports included the detection of an anthocyanidin and flavonol glucosylating activity in endosperm extracts of maize (Zea mays), (11–13). This work formed the basis for identification by transposon tagging of the gene product of the maize Bronze-1 locus (14). cDNAs encoding flavonoid glucosyltransferases have been isolated from a number of plant species utilizing homology to the maize Bronze-1 cDNA. In the grapevine, Sparvoli and co-workers (15) cloned a partial cDNA from the variety Lambrusco based upon homology to a putative flavonoid glucosyltransferase of Antirrhinum majus, itself identified by homology to the maize cDNA (16). Recently, Boss et al. (17–19) used the “Sparvoli” cDNA to detect the expression of anthocyanidin glucosyltransferase mRNA during the development of berries of Vitis vinifera variety Shiraz and to show that red and white grapes, and color mutants (sports) of several varieties, differed in whether or not they express the UFGT1 gene. Although several reports on the characteristics of expression levels and transgenesis of the UFGT gene have appeared, a clear picture of the properties of the corresponding gene product is not available. Thus, despite the suspected pivotal role played by UFGT in pigment accumulation, this enzyme has never been subjected to a thorough characterization in a purified and guaranteed homogeneous state. Indeed, a confusing picture of the nature and extent of flavonoid glucosyltransferases has arisen in the very extensive literature on these activities. For example, Jonsson et al. (20), Teusch et al. (21), and Hrazdina (22) conclude a common identity of UDP-glucose:anthocyanidin 3-O-glucosyltransferase and UDP-glucose:flavonol 3-O-glucosyltransferase with the enzyme significantly more active toward the flavonols. In contrast, preliminary analysis of

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1 The abbreviations used are: UFGT, UDP-glucose:flavonoid 3-O-glucosyltransferase; rUFGT, recombinant UDP-glucose:flavonoid 3-O-glucosyltransferase; IMAC, immobilized metal affinity chromatography; UDP-glucose, uridine-5'-diphosphoglucose; HPLC, high pressure liquid chromatography; PIPES, 1,4-piperazinediethanesulfonic acid; MES, 4-morpholineethanesulfonic acid; CAPS, 3-(cyclohexylamino)propanesulfonic acid; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)]-ethylglycine.
a Gentiana triflora recombinant UFGT in crude bacterial lysates indicated a preference for anthocyanins over flavonols (23). Additionally Do et al. (24), recently concluded that a partially purified UFGT from grapes exhibits activity with anthocyanins but not at all with flavonols.

The confusing picture concerning the multiplicity and properties of anthocyanidin/flavonol 3-O-glucosyltransferases undoubtedly stems from the repeated inability of investigators to purify any such enzyme to homogeneity. Additionally, the highly labile nature of anthocyanidins but not of flavonol substrates at the basic pH optimum of most glucosyltransferases has inevitably contributed further to the confusion regarding the relative activities and observed specificities of these enzymes.

We report here the cloning of a cDNA encoding a full-length UFGT from grapes of V. vinifera, the world’s largest fruit crop with annual production of 60 million tons (25). Following development of a protocol which employs very slow growth of *Escherichia coli* at 16 °C, expression of the cDNA and subsequent purification of a histidine-tagged rUFGT in an active form has permitted, for the first time, the unambiguous determination of the properties of a member of this ubiquitous enzyme family and allowed us to highlight some of the pitfalls that could contribute to erroneous conclusions regarding anthocyanidin glucosyltransferases. It has been demonstrated that UFGT exhibits much higher catalytic efficiency against anthocyanidins than against flavonols such as quercetin and kaempferol. Additional data also strongly indicate that in the case of grapes, the principal role of UFGT is to glucosylate anthocyanidins in red fruit, whereas products of other genes serve to glucosylate flavonoids in a distinct spatial and temporal pattern.

**EXPERIMENTAL PROCEDURES**

**Biochemicals and Reagents—**All biochemicals were of analytical grade or higher. Flavonoid substrates and authentic glucosides were obtained from Fluka, Castle Hill, New South Wales, Australia; Apin Chemicals, Oxford, UK; Carl Roth GmbH, Karlsruhe Germany; Extra-grade or higher. Flavonoid substrates and authentic glucosides were employed here. mRNA Cloning of Full-length *V. vinifera* UFGT cDNA—Employing mRNA isolated from whole *V. vinifera* cv. Shiraz grape berries 10 weeks after flowering (26), a Superscript Choice cDNA synthesis kit (Life Technologies, Inc.), ZAP II arms pre-digested with EcoRI (Stratagene) and EcoRI adaptors, a cDNA library was prepared according to the manufacturer’s instructions by C. Davies (CSIRO Plant Industry, Adelaide, Australia). An aliquot of the amplified library, consisting of approximately 150,000 plaques, was screened using a 532-base pair partial cDNA clone for UFGT from grape (15) as a probe. Hybridization was carried out overnight at 65 °C in Church-Gilbert buffer (27); washings, also at 65 °C, were in 2× SSC. This would allow the detection of nucleic acid species with up to approximately 30% mismatched sequences (28). Two hybridizing clones were isolated, and their nucleotide sequences determined using standard protocols for chain termination sequencing (29) of both encoding and non-encoding strands.

**Synthesis and Purification of Recombinant UFGT—**PCR amplification reactions were carried out using 1× Vent DNA polymerase buffer, 0.2 mM dNTPs, 1 μM each forward (UFGTF1 5′-GGCGATTCCGATGTCCAAACCAAGCAACACCGCTGCGTGCTG-3′) and reverse (UFGTR1 5′-GTCCTACAACAAAAAGGATGTTACCGGCTACCAAGCTT-GAATTCCGG-3′) DNA primer, 2 mM MgSO₄, and 0.8 units of Vent DNA polymerase (New England Biolabs) in a 20-μl final volume with approximately 1 ng of plasmid pGUT3 DNA template. Thermal cycling parameters were 94 °C, 5 min, 30 × (94 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min) and a final 5 min at 72 °C. After digestion of the 1.4-kb PCR product, transformation, production of colonies *Novi* and *BamHII*, the DNA was ligated into pET14b (Novagen, Madison, WI) and used to transform competent cells of *E. coli* strain JM109. Plasmid was prepared from recombinant clones and used to transform the *E. coli* BL21 pLysS strain (30) to yield the clone pET14-VVUFGT used in the subsequent production of recombinant UFGT.

Soluble, active UFGT was expressed from pET14-VVUFGT following growth and induction at 16 °C. Briefly, L broth (100 ml) held at 16 °C and containing ampicillin and chloramphenicol was inoculated with 100 μl of overnight culture (grown at 37 °C) and grown until an *A₅₀₀* between 0.4 and 0.6 was reached (typically 24–40 h). Induction of recombinant protein expression was initiated by the addition of isopropyl-β-thio-β-D-galactopyranoside to 0.4 mM, and cultures were grown for a further 24 h. Cells were harvested by centrifugation at 14,000 × g for 15 min, and the drained pellets stored at −20 °C. Bulk growth (1-liter scale) was carried out in 5-liter Ehrenmeyer flasks using 5 ml of an overnight starter culture.

Frozen cells (about 0.8–1.0 g per 50 ml of culture) were thawed and resuspended in lysis buffer (20 mM Tris-Cl, pH 8.0, 100 mM NaCl, 10 mM imidazole, 3 mM peroxidase, 3 ml per cell weight) in 10-ml tubes followed by three freeze/thaw cycles using liquid nitrogen. Phenylmethylsulfonyl fluoride was added to 1.5 mM, DNase I to 690 units ml⁻¹, and the modified mixture thoroughly by passage through an 18-gauge syringe needle 10 times before incubation on a mixing wheel for 20 min at room temperature. The suspension was transferred to 2-ml tubes and centrifuged for 20 min at full speed in a bench top microcentrifuge. The resulting supernatant and pellet contained, respectively, soluble and insoluble protein fractions.

Recombinant UFGT was purified from the soluble fraction by IMAC using the Talon system (CLONTECH, Palo Alto, CA), utilizing the 6-histidine tag placed at the N terminus of the recombinant protein. Protein in the soluble fraction was bound to the affinity resin and eluted using a gradient containing 10 mM imidazole as described in manufacturer’s protocol. Additional Do et al. (29) of both encoding and non-encoding strands.

**Properties of UFGT**

**Enzyme Assays—**Glucosylation was routinely assayed in a modification of the buffer system reported by Do et al. (24). In a final assay volume of 200 μl, the reaction conditions were 100 mM buffer (Tris-Cl, pH 8.0, or HEPES-OH, pH 8.0), 10 mM poly(ethylene glycol) 3400, 14 mM 2-mercaptoethanol, 2 mM dithiothreitol, 9 mM UDP-glucose, 100 μM flavonol or anthocyanidin acceptor substrate and enzyme. The amount of enzyme used was varied according to the acceptor substrate under study. Except in the case of anthocyanidin substrates, assays were routinely started with the addition of the enzyme and were incubated at 30 °C for up to 6 min (anthocyanidin substrates) and 10 min (flavonol substrates). Samples were taken at two time points to ensure linearity of all data points and hence to measure initial rates of activity. Flavonol and anthocyanidin glucosides were prepared fresh as required from stock solutions stored at −20 °C. Unless stated otherwise, the solvent used for substrate dilution was 2-methoxyethanol (ethylene glycolmono- methyl ether (2-methoxyethanol)).

Reactions were stopped by acidification. For flavonol acceptor substrates, 50 μl of glacial acetic acid was used, and for anthocyanidins, 150 μl of 5% HCl was added to the reaction mixture.

Routine activity results were analyzed by reversed-phase HPLC using a Beckman System Gold apparatus (128 diode array detector module, 126 pumps, 507E autosampler) and Gold Nouveau software. A 250 × 4.5 mm Vydac 218TP C18 column was used and maintained at 30 °C for up to 6 min (anthocyanidin substrates) and 10 min (flavonol substrates). Samples were taken at two time points to ensure linearity of all data points and hence to measure initial rates of activity. Flavonol and anthocyanidin glucosides were prepared fresh as required from stock solutions stored at −20 °C. Unless stated otherwise, the solvent used for substrate dilution was 2-methoxyethanol (ethylene glycolmono- methyl ether (2-methoxyethanol)).

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The radioactive donor substrate UDP-[U¹⁴C]glucose was used for the determination of the rate and stoichiometry of glucosylation. Assay incorporation data were not available. UDP-[U¹⁴C]glucose (10.6 GBq mmol⁻¹, DuPont) was added to a final concentration of 2.5 μM and supplemented with “cold” UDP-glucose to 100 μM. For analysis by liquid scintillation counting or thin layer chromatography, flavonol glucosides were extracted into 500 μl of ethyl acetate to remove unincorporated donor substrate. Liquid scintillation counting of ethyl acetate extracts (200 μl, duplicates) was performed using Ultima Gold XR mixture
(Cannock Packard) in a LS6000TD counter (Beckman). TLC analysis was performed upon ethyl acetate extracts following concentration at 70 °C in a Centrivap Concentrator (Labconco) under reduced pressure. Residues were dissolved into 20 μl of ethyl acetate. TLCs were run on Silica Gel 60 F254 plates (Merek) in a solvent comprising 55 parts ethyl acetate, 2 parts formic acid, 2 parts water (32). Developing time was 20 min. A dried at 80 °C for 1 h and exposed to storage phosphor imaging plates (Molecular Dynamics). Glucosidase formation from anthocyanidin, phenol, and monoterpen acceptor substrates was assessed after separation using SepPak disposable reverse phase cartridges (Millipore) to remove the unincorporated UDP-[U-14C]glucose prior to liquid scintillation counting or TLC assay. Briefly, acidified assay mixtures were loaded onto pre-charged SepPak cartridges, washed with 5 ml of MilliQ water, and eluted in 2–3 ml of ethanol (33). After lyophilization under reduced pressure, residues were dissolved into 20 μl of ethyl acetate. For assays with anthocyanidin substrates, ethanol used for elution was acidified to 0.1% v/v HCl.

Kinetic Measurements—$K_m$ values of the recombinant glucosyltransferase were determined for the donor substrate UDP-glucose and the acceptors quercetin, cyanidin, malvidin, and delphinidin. Additionally, $V_{max}$ was determined for quercetin and cyanidin. For the measurement of the $K_m$ for UDP-glucose, 200 μM quercetin was used as the acceptor substrate, and the concentration of UDP-glucose was varied from 0.25 to 200 μM. $K_m$ values were determined with 9.5 μM UDP-glucose as the donor substrate, and the concentration of the acceptor varied from 1 to 200 μM as applicable. Assays were as described above. Additionally, for the determination of the $K_m$ for cyanidin, reducing agents were omitted from the reaction mixture, and cyanidin, rather than enzyme, was added to start the reaction. Experimental data from HPLC analyses were integrated using Gold Nouveau software and for cyanidin and quercetin glucosylation, converted to specific activities by reference to standard curves obtained using the authentic glucosides. Data were transformed and plotted as Lineweaver-Burk graphs to allow calculation of $K_m$ and $V_{max}$ values.

Substrate Specificity—The activity of the recombinant glucosyltransferase with alternative donor and acceptor substrates was tested. Nucleotide sugars ADP, CDP, GDP, and TDP-glucose, UDP-glucuronic acid, UDP-galactose, UDP-, and UDP-xylene were tested for their ability to support glycosylation of quercetin. Alternative acceptor substrates tested, with the donor substrate UDP-glucose, were kaempferol, fisetin, morin, myricetin, isorhamnetin (flavanons), quercetin-3-O-glucoside (flavanol glucoside), pelargonidin, delphinidin, peonidin, and malvidin (anthocyanidins), cyanidin-3-O-glucoside (anthocy-

Metal Inhibition Studies—The effect of divalent metal ions upon the glucosylation of quercetin and cyanidin was studied using radiolabeled UDP-[U-14C]glucose and liquid scintillation counting. Standard 200-μl assays performed in the absence of added reducing agents were supplemented with CuCl₂, MgCl₂, CaCl₂, MnCl₂, and ZnCl₂, to final concentrations of 0.01, 0.1, and 10 mM as specified.

Product Inhibition Assays—The inhibition of glucosyltransferase activity was tested for quercetin and cyanidin glucosylation. In both cases the cognate and alternative glucosides were added individually to glucosylation assays at concentrations between 1 and 100 μM. Product formation was determined by HPLC analysis.

Production of Antibodies, Western Transfer, and Immunoblotting—Rabbit antibodies were produced by the School of Biochemistry, La Trobe University, Melbourne. rUFGT (approximately 500 μg) was mixed with an equal volume of Freund’s complete adjuvant and injected intramuscularly. The rabbit was given two boosts at 4-week intervals before blood samples were obtained. Serum was separated from red cells by centrifugation at 3000 × g for 15 min at 4 °C and stored in 0.02% sodium azide at 4 °C. Total soluble protein extracts of leaves and berries of grapevine varieties as specified were prepared by a modification of the method of Hawker (34, 35). Full details of this protocol will appear elsewhere. Following preparation, extracts were desalted on a Bio-Gel P6 column and concentrated in a stirred cell unit (Amicon) using a YM30 membrane. Proteins were resolved by SDS in 12%, 2.63% C (w/v) Tris-Gly-gels (36) and blotted onto nitrocellulose membranes (MSE Laboratories, Westbro, MA) using a semi-dry transfer unit (Biorad, Hercules, CA). A 1:1 dilution of antibody was used for immunoblotting experiments. Immoblottlabs were probed with anti-rUFGT antibodies and horseradish peroxidase-coupled goat anti-rabbit antibodies (Promega) before detection using ECL™ reagents (Amershrem Pharmae Biotech) and horseradish peroxidase-coupled goat anti-rabbit antibodies (Promega) before detection using ECL™ reagents (Amershrem Pharmae Biotech).

RESULTS

Cloning and Identification of Full-length cDNAs Encoding UFGT from V. vinifera cv. Shiraz, Overexpression and Purification of Soluble Active Recombinant UFGT—By using the partial 532-base pair grape cDNA previously isolated by Sparvoli et al. (15), two clones homologous to putative plant UFGT sequences were isolated from a screen of approximately 150,000 plaque forming units of a V. vinifera cv. Shiraz post- 

The recombinant UFGT (rUFGT) was therefore expressed as a soluble, active protein in E. coli using the pET14b 6-His fusion vector system. Proteins expressed from sequences cloned into pET14b contain an additional 20 NH₂-terminal amino acid residues arising from the vector, including the 6-His “tag” used for metal-chelate affinity purification and a recognition site for the protease thrombin. Preliminary growth and induction trials at 30 and 25 °C yielded protein that was expressed mainly as insoluble inclusion bodies (data not shown). Further reduction of the growth and induction temperature to 16 °C resulted in the expression of significant amounts of a protein, the size of which corresponded to that of rUFGT from V. vinifera UFGT versus Bronze-1 encoded UFGT) to 25% (V. vinifera UFGT versus solanidine glucosyltransferase). Positional identity is not surprisingly greater in the C-terminal third of the protein where the suspected UDP-glucose binding domain, common to all glucosyltransferases, is located (46% V. vinifera UFGT versus Bronze-1 encoded UFGT, 35% V. vinifera UFGT versus solanidine glucosyltransferase) (38). Quite clearly, sequence comparisons of the N-terminal parts of these various glucosyltransferases do not allow solid conclusions to be drawn regarding the enzymic properties of the encoded enzyme, particularly because there exists very little correlation between protein/gene sequences and biochemical characterization of the encoded products.

When soluble protein extract prepared from cells containing putative rUFGT was assayed for enzymic activity, significant quercetin glucosylation was observed, suggesting that an ac-
tive enzyme had been successfully expressed. Uninduced cell lysate and lysate prepared from \textit{E. coli} BL21 pLysS cells that were not transformed with pET14-\textit{V. vinifera} UFGT did not contain any glucosyltransferase activity against quercetin (data not shown).

Purification of rUFGT by IMAC from 5 g of cell paste proved straightforward, yielding large quantities of the recombinant enzyme. The inclusion of 15 mM imidazole in the wash buffer resulted in recovery of an essentially homogeneous preparation of rUFGT (Fig. 2, lane 5). Purified recombinant UFGT prepared in this way was stored at \(-80^\circ\text{C}\), where it was stable for many months. At least three freeze-thaw cycles could be tolerated without apparent loss of activity. Additionally, the recombinant enzyme was stable when stored for at least 120 h both at \(20^\circ\text{C}\) and \(4^\circ\text{C}\) and showed only a 30% decrease in quercetin glucosylation after the same period of storage at room temperature (data not shown).

\textbf{Pitfalls in the Assay of Glucosyltransferase Activities—}Assaying for enzyme activities with flavonol and anthocyanidin substrates in particular has inherent pitfalls that appear to be not always fully recognized, and this can in some cases lead to erroneous conclusions concerning the properties of the enzymes.
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under consideration. For example, in our initial assays of the recombinant enzyme, it proved very difficult to achieve linearity between time points when assaysing for glucosylation of cyanidin at pH values where activity could be observed (data not shown). The possibility that product inhibition was responsible for this phenomenon was effectively discounted by supplementing assays with the product. No effect upon the extent of cyanidin nor quercetin glucosylation was observed by the prior addition to the assay of either quercetin glucoside or cyanidin glucoside to a final concentration of 100 μM. Likewise the use of very low enzyme concentrations failed to achieve linearity between assays typically performed for 5, 10, and 20 min (data not shown). Given the relatively stable nature of rUFGT described above, these observations indicate that loss of linearity is due either to substrate or product instability during the assay procedure.

In detailed chemical studies Brouillard (39) demonstrated that both very fast and slower interconversions of tautomeric anthocyanidin forms occur in aqueous solutions, particularly at pH values above 4. The red flavilyum form AH+ occurs as the predominant species at pH values below approximately 2.5. If the pH is raised above 4, the blue quinoidal anhydrobase A, a kinetic product, forms instantaneously. This unstable intermediate rapidly decolorizes in aqueous solution (seconds) through the formation of a carbinol pseudobase B arising from nucleophilic attack by water at the 2-position of the C15 flavonoid backbone. Re-acidification at any time during this process to a pH below 2 will fully restore the red flavilyum form of the anthocyanidin. However, if the anthocyanidin solution is left for extended times (minutes) at pH 4 and above, it undergoes conversion to an open chain cis-chalcone and then the final thermodynamic product the trans-chalcone. At this point, re-acidification of the flavilyum form cannot be achieved by simple re-acidification (39). The observation that very little of the red flavilyum form of the anthocyanidin substrate cyanidin was re-acidification (39). The observation that very little of the red flavilyum form of both substrate and product is the predominant equilibrium product and is stable for extended periods (39). Duplicate HPLC determinations of individual assays analyzed at the beginning and end of batch HPLC runs (up to 48 h apart) gave essentially equal data sets, suggesting that no decomposition of anthocyanidin glucosides was occurring at this low pH (data not shown).

Determination of pH Optima and Substrate Specificity of the Recombinant Enzyme—Incubation of rUFGT with either cyanidin or quercetin aglycones in the presence of 9 mM UDP-glucose donor substrate resulted in the formation of the respective 3-O-glucosides as determined by co-chromatography and diode array spectroscopy using authentic glucosides assayed by both TLC and HPLC analyses. Assays in the absence of the UDP-glucose donor substrate yielded no product (data not shown).

The pH optimum of the recombinant UFGT was analyzed for quercetin and cyanidin acceptor substrates and in both cases was observed to fall around pH 8.0 (data not shown). Quercetin glucosylation exhibited a marked buffer effect, with values in Tris buffer approximately 85% of those at the same pH in HEPES buffer. This effect was not seen with cyanidin glycosylation, but the instability of anthocyanidin substrates over much of the pH range studied inevitably impacted upon the final activity obtained.

The activity of the recombinant UFGT was tested against a wide range of donor and acceptor substrates. Of the donor substrates, only UDP-glucose was able to support the glycosylation of quercetin. No activity was observed with UDP-galactose, UDP-xyllose, UDP-glucuronic acid, UDP-mannose, ADP-glucose, CDP-glucose, GDP-glucose, or TDP-glucose (data not shown). This agrees with data obtained for non-homogeneous preparations of other flavonoid 3-O-glucosyltransferases showing exclusive acceptor-substrate specificity for anthocyanidins and flavonols (reviewed by Heller and Forkmann (40)).

The enzyme was specific for the glycosylation of flavonol and anthocyanidin substrates, with no activity observed for any other substrate tested. The range of substrates for which glucosides could be formed by enzymes in a total soluble protein extract prepared from V. vinifera variety Shiraz berries of equivalent developmental status to those used for the isolation of UFGT cDNAs was much greater than that catalyzed by rUFGT alone. Glucosylation of flavonols, a flavone, a flavanone, an isoflavone, and monoterpene was detected when total berry proteins were used in assays for glucosyltransferase ac-
No glucosylation was seen with dihydroflavonol or flavan-3-ol substrates (data not shown).

**Kinetic Parameters of the Recombinant Glucosyltransferase**—The kinetics of the recombinant glucosyltransferase were studied both for donor and acceptor substrates (Fig. 3). By using quercetin (200 μM) as the acceptor substrate, the dependence of activity upon the concentration of UDP-glucose was analyzed between 0.25 and 8 mM. A hyperbolic saturation curve was obtained, from which Lineweaver-Burk transformation gave a $K_m$ for UDP-glucose of approximately 1.9 mM and $V_{max}$ for quercetin glucoside formation of 18.9 nanokatals mg$^{-1}$. When the concentration of UDP-glucose was maintained constant at 9 mM and the concentration of quercetin varied between 1 and 40 μM, a $K_m$ for quercetin of approximately 15 μM was obtained. The $V_{max}$ estimated for quercetin glucoside formation under these conditions was also 18.9 nanokatals mg$^{-1}$.

When the concentration of cyanidin was varied between 1 and 80 μM with UDP-glucose maintained at 9 mM, a $K_m$ for cyanidin of 30 μM and a $V_{max}$ for cyanidin glucoside formation of 905 nanokatals mg$^{-1}$ (48 times that observed for quercetin glucosylation) were obtained. All data were determined and averaged from three independent assays. However, due to the instabilities described above associated with the use of anthocyanidin substrates, greater variability was observed in the apparent $K_m$ data for this substrate than for either quercetin or UDP-glucose. By using the same reaction conditions, the $K_m$ for delphinidin was determined to be approximately 16 μM and for malvidin approximately 35.7 μM. $V_{max}$ data were not obtained for these acceptors. Additionally, thrombin cleavage of the 6-His N-terminal tag, removing 17 non-UFGT amino acids, had no effect on the ratio of cyanidin to quercetin glucosylation (data not shown).

**Relative Activity Against Flavonol and Anthocyanidin Substrates**—The relative activity of rUFGT with a range of flavonol and anthocyanidin substrates was tested. As shown (Fig. 4), cyanidin and quercetin gave the highest rates of anthocyanidin and flavonol glucosylation, respectively. Anthocyanidin glucosylation rates were compared by HPLC analyses, with the total absorbance for each glucoside measured at the absorption maximum determined from the chromatogram of each product. These products were identified by comparison with authentic glucoside standards (cyanidin, malvidin, and peonidin) and by the appearance, when anthocyanidin substrate was incubated with rUFGT, of a peak exhibiting an approximately 10–20 nm lower absorbance maximum and a retention time shorter than the authentic anthocyanidin substrate (delphinidin and pelargonidin), as expected for a glucosylated product. Relative rates of flavonol glucoside formation were determined by liquid scintillation counting of ethyl acetate extracts from assays using radiolabeled UDP-[U$^{14}$C]glucose donor substrate. This allowed the accurate determination of total glucoside formation, otherwise not possible for those flavonol glucosides for which molar extinction coefficients or authentic samples were not available.

**Metal Inhibition Studies**—Many reports of the properties of partially purified glucosyltransferases include the effect of added divalent metal ions (10, 11, 20, 21, 24, 32, reviewed in Ref. 40). The addition of 1 mM Mg$^{2+}$ or Ca$^{2+}$ had only a slight effect upon the extent of quercetin glucosylation, whereas Cu$^{2+}$, Mn$^{2+}$, and Zn$^{2+}$ at the same concentration caused up to 96% reduction in the amount of quercetin glucoside formed. Additionally, Cu$^{2+}$ at both 0.1 and 0.01 mM was observed to
cause over 80% reduction in quercetin glucosylating activity. Although our preliminary experiments in the absence of added reducing agents suggested Cu$^{2+}$ inhibition of the recombinant glucosyltransferase itself, further investigation proved this to be incorrect. The addition of even 0.1 mM Cu$^{2+}$ to either quercetin or cyanidin glucosylation assays caused rapid and irreversible bleaching of the substrates, thus rendering the detection of product formation by HPLC and absorbance measurement impossible. Spectroscopic analyses of both quercetin and cyanidin before and after the addition of 1 mM Cu$^{2+}$ to the assay buffer showed the effect more clearly (Fig. 5). In each case, the colored component of the absorption spectrum was lost, leaving only the absorbance at around 280 nm arising from the phenolic ring. It seems likely that Cu$^{2+}$ is acting to catalytically convert the C$_15$ flavonoid into a compound not recognized by rUFGT. These data meant that it was not possible to attribute the observed lack of glucoside formation in the presence of Cu$^{2+}$ to specific inhibition of the glucosyltransferase itself.

**Comparison of Glucosyltransferase Activities of Cloned V. vinifera UFGT with Total Activities in Grapevine Leaves and Berries**—The specificity studies reported above clearly showed that the activity of rUFGT was likely to be restricted to glucosylation of flavonols and anthocyanidins, as originally suggested for the products of the Bronze-1 allele (41, 42). However, it does not follow that glucosylation of both these types of substrates in grapes is the consequence of rUFGT action in vivo, as has been assumed to be the case for certain lines of maize (43). Indeed, Dooner (44) and Styles and Ceska (45) reported accumulation of flavonol-3-glucosides in the endosperm, and coleoptiles, of bz-1/bz-1 lines of maize in which there was no detectable anthocyanin accumulation. In both cases the authors predicted the existence of a Bz-1-independent glucosyltransferase, which may be both tissue-specific (44) and with differing positional specificity to that encoded at the Bronze-1 locus (45). To investigate this in grapevine tissues, a comparison was made between the cyanidin-3-glucoside/quer-cetin-3-glucoside ratio made on the one hand by rUFGT and on the other hand by enzyme extracts from leaves and berries of the grapevine variety Muscat of Alexandria. The expression of the UFGT gene in berries of this white grape variety, in which anthocyanins are present only in trace amounts (46), cannot be detected by Northern blotting (19). The results of HPLC assays for glucoside formation from each acceptor substrate are given in Table I. This indicates very strongly that there must exist in leaves and berries additional enzyme activities capable of glucosylating quercetin at a greater rate than observed for rUFGT, since the ratio of cyanidin to quercetin glucosyltransferase activity is much smaller in both tissues (ratios of 1.1 and 2.4, respectively) than is seen for rUFGT (ratio of 48), an enzyme which may serve predominantly to glucosylate anthocyanidins at and after veraison in red berries. This became even clearer when extracts of berries from the red variety Shiraz (also the source of the rUFGT cDNA) and the white variety Muscat of Alexandria were compared for their ability to glucosylate anthocyanidins and flavonols (Table I). We found that while the total units and specific activity of quercetin glucosylating activity were practically identical in extracts made from these grapes, the extract from the red Shiraz berries had a specific activity for cyanidin glucosylation which was almost 25-fold higher than that seen in extracts from berries of Muscat of Alexandria. This strongly suggests a very minor role, if any, for UFGT in the production of flavonol glucosides in berries. That this indeed appears to be the case was further demonstrated by Western blotting of the extracts on which the enzymic assays had been performed (Fig. 6). Only red grapes are seen to contain significant quantities of immunoreactive UFGT, the abundance of which correlates with UDP-glucose:cytidine 3-O-glucosyltransferase activity and not at all with UDP-glucose:quercetin 3-O-glucosyltransferase activity (cf. lanes 1–4 in Fig. 6). The lack of immunoreactive material in the extracts from the white variety cannot be explained by changes in protein sequence of UFGT within varieties of the same species. Our previous work on other grape proteins indicates a very strong retention of immunological reactivity across V. vinifera varieties (47). Indeed, given the different electrophoretic mobilities of immunoreactive material from the two varieties, it is possible that UFGT is practically absent from the white fruit and that this constitutes the real reason anthocyanins do not accumulate in these white varieties since Northern analyses previously have shown most other genes in the anthocyanin biosynthesis pathway to be expressed (19).

**DISCUSSION**

Despite the great importance of glucosylation to many of the metabolic processes underlying plant growth, development, and response to environmental stress (2), the enzymes responsible for the formation of glucosides remain the subject of much speculation and uncertainty. Among the most important reasons for this have undoubtedly been the difficulties experienced in obtaining plant glucosyltransferases in a homogeneous state. Notwithstanding a few elegant examples (pummelo UDP-rhamnose:flavanone 7-O-glucosyltransferase (7) and corn indole-3-acetic acid glucoside synthase (8)), in the vast majority of reports on the characterization of specific plant
glucosyltransferases, the final state of purity achieved has been some way from homogeneity. This presents many problems for the assignment of specificities and is further complicated by the structural similarity of many distinct substrates, which may permit cross-reactivity under experimental conditions.

An alternative approach for the study of glucosyltransferases, capable both of yielding large amounts of protein and offering a simple way to achieve homogeneity, is to clone the cDNAs of the desired enzyme into a suitable expression system. At least four reports of the production of recombinant plant glucosyltransferases are known. However, in one of these (48), the enzyme was inactive, and in the other three no effort was made to purify the expressed enzyme from the background of host proteins (23, 49, 50).

We have now cloned cDNAs representing a *V. vinifera* glucosyltransferase. Initial attempts at obtaining an active recombinant protein from *E. coli* cells grown at 30 °C failed due to the exclusive accumulation of the rUFGT in insoluble aggregates. However, following expression at 16 °C, the encoded protein was shown to be soluble and after purification was shown to harbor UDP-glucose:flavonoid 3-O-glucosyltransferase activity when assayed in vitro. Importantly, this conclusion could not have been safely arrived at from amino acid sequence similarities alone as these never exceeded 50% positional identity. The availability of purified preparations of rUFGT permitted calculation of the protein concentration used in kinetic assays and thereby, for the first time, the determination of *k*<sub>cat</sub> values for flavonoid glucosylase formation. The *k*<sub>cat</sub> for cyanidin glucoside formation was calculated to be 47.63 s<sup>-1</sup>, while for quercetin

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**TABLE I**

| Variety           | Tissue       | Substrate | Activity | Ratio |
|-------------------|--------------|-----------|----------|-------|
| Muscat of Alexandria | Leaf         | Cyanidin  | 394.7    | 2.4:1 |
|                   |              | Quercetin | 167.3    |       |
|                   | Berry (22 °C | Cyanidin  | 76.1     |       |
|                   | Brix         | Quercetin | 69.7     | 1.1:1 |
| Shiraz            | Berry        | Cyanidin  | 1892.4   |       |
|                   | (24 °C Brix) | Quercetin | 79.6     | 24:1  |
| rUFGT             | Berry        | Cyanidin  | 905°     | 48    |
|                   |              | Quercetin | 19°      |       |

* Activity of purified recombinant UFGT expressed as nanokatals mg<sup>-1</sup>

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**FIG. 5.** The effect of Cu<sup>2+</sup> upon absorption spectra of flavonol and anthocyanidin substrates. Cyanidin (A and B) and quercetin (C and D) (20 μM) were prepared in 100 mM Tris-Cl, pH 8.0, 10 mM poly(ethylene glycol) 4000, 9 mM UDP-glucose, and absorption spectra were recorded from 240 to 640 nm (cyanidin, A) or 240 to 480 nm (quercetin, C). B and D, Cu<sup>2+</sup> (as CuCl<sub>2</sub>) was added to a final concentration of 1 mM and 5 min after mixing the spectra were re-recorded as described previously.

**FIG. 6.** The presence of UFGT in grapes correlates strongly with cyanidin glucosylating activity but not at all with quercetin glucosylating activity. Total soluble protein extracts from berries of the red grape variety Shiraz, and from berries and leaves of the white grape variety Muscat Of Alexandria, were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane followed by immunoblot analysis using rabbit antibodies raised against rUFGT. Shiraz berries: lane 1, 5.6 picokatals cyanidin glucosylating activity, 0.24 picokatals quercetin glucosylating activity; lane 2, 11.2 picokatals cyanidin glucosylating activity, 0.49 picokatals quercetin glucosylating activity; Muscat of Alexandria berries: lane 3, 0.43 picokatals cyanidin glucosylating activity, 0.40 picokatals quercetin glucosylating activity; lane 4, 0.86 picokatals cyanidin glucosylating activity, 0.80 picokatals quercetin glucosylating activity; Muscat of Alexandria leaves: lane 5, 1 picokatal cyanidin glucosylating activity, 0.41 picokatal quercetin glucosylating activity; and lane 6, 2 picokatals cyanidin glucosylating activity, 0.82 picokatals quercetin glucosylating activity; and lane 7, rUFGT, 10 ng.
trolling role in anthocyanin synthesis. In contrast to the situations exerted at earlier points in the biosynthetic pathway (reviewed where a white sport of the red grape variety Cabernet Sauvignon "sports" of white grape varieties showing accumulation of an-
deletable throughout berry development. Similarly, in transcripts of anthocyanidin biosynthetic genes in general were
correlated with the detectability of UFGT mRNA, whereas
obtained from a multitude of species across the plant kingdom.

We therefore suggest that the UFGT investigated here, from a physiological point of view, more appropriately be considered a UDP-glucose:anthocyanidin 3-O-glucosyltransferase. It appears that these considerations are applicable when assigning roles to homologues of UFGT in other systems where the role of
UFGT for production of flavonol glucosides might vary considerably depending on the genetic background, as seen for different
lines of maize bz-1/bz-1 mutant lines (44, 45).

The availability of protocols for production of active recombi-
binant glucosyltransferases at low growth temperature, com-
bined with good antibodies and carefully executed enzyme ass-
ays, should now facilitate a more rigorous assessment of the
properties and roles of "UFGT" homologues, a much studied
class of enzymes for which the genes and cDNAs have been obtained from a multitude of species across the plant kingdom.

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REFERENCES

1. 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 Ltd., London.
2. Bandurski, R. S., Cohen, J. D., Slomin, J., and Reinecke, D. M. (1995) in Plant Hormones: Physiology, Biochemistry, and Molecular Biology (Davies, P. J., ed) 2nd Ed., pp. 39–65, Kluwer Academic Publishers Group, Dordrecht, Netherlands.
3. Williams, C. A., and Harborne, J. B. (1993) in The Flavonoids: Advances in Research Since 1986 (Harborne, J. B., ed) pp. 357–395, Chapman & Hall Ltd., London.
4. Brouillard, R., and Dangles, O. (1993) in The Flavonoids: Advances in Research Since 1986 (Harborne, J. B., ed) pp. 565–588, Chapman & Hall Ltd., London.
5. Williams, P. J., Strauss, C. R., and Wilson, B. (1981) Am. J. Enol. Vitic. 32, 230–235.
6. Coleman, J. O. D., Blake-Kalff, M. M. A., and Davies, T. G. E. (1997) Trends Plant Sci. 2, 144–151.
7. Bar-Peled, M., Lewinsohn, E., Fluur, R., and Gressel, J. (1991) J. Biol. Chem. 266, 20983–20989.
8. Kowalczyk, S., and Bandurski, R. S. (1991) Biochem. J. 279, 509–514.
9. Lewinsohn, E., Britsch, L., Mazur, Y., and Gressel, J. (1989) Plant Physiol. (Bethesda) 91, 1323–1328.
10. McIntosh, C. A., and Mansell, R. L. (1990) Phytochemistry 29, 1533–1538.
11. Larson, R. L. (1971) Phytochemistry 10, 3073–3076.
12. Larson, R. L., and Lonergan, C. M. (1972) Plant Physiol. 50, 699–706.
13. Patron, S., and Lonergan, C. M. (1973) Cereal Res. Commun. 1, 13–22.
14. Fedoroff, N. V., Furtet, D. B., and Nelson, O. E. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 3825–3829.
15. Sparvoli, F., Martin, C., Scienza, A., Gavazzi, G., and Tonelli, C. (1994) Plant Mol. Biol. 24, 743–755.
16. Martin, C. R., Prescott, A., Mackay, S., Bartlett, J., and Vrijelandt, E. (1991) Plant J. 1, 37–49.
17. Boss, P. K., Davies, C., and Robinson, S. P. (1996) Plant Physiol. (Bethesda) 111, 1059–1066.
18. Boss, P. K., Davies, C., and Robinson, S. P. (1996) Aust. J. Grape Wine Res. 2, 163–170.
19. Boss, P. K., Davies, C., and Robinson, S. P. (1996) Plant Mol. Biol. 32, 565–569.
20. Jonsson, L. M. V., Arisman, M. E. G., Bastiaanet, J., Denker-Koopman, W. E., Gerats, A. G. M., and Schram, A. W. (1984) Z. Naturforsch. 39, 559–564.
21. Trusche, M., Forkmann, G., and Seyffert, W. (1986) Z. Naturforsch. 41, 699–706.
22. Hrazdina, G. (1988) Biochim. Biophys. Acta 955, 301–309.
23. Tanaka, Y., Yonekura, K., Fukushima-Minato, M., Fukui, Y., Fujihara, H., Ashikari, T., and Kusumi, T. (1996) Plant Cell Physiol. 37, 711–716.
24. Do, C. B., Cermier, F., and Nicholas, Y. (1995) Plant Sci. 112, 43–51.
25. Kanelis, A. K., and RoubaudlakisAngelakis, K. A. (1993) in Biochemistry of Fruit Ripening (Seymour, G., Taylor, S. J., and Tucker, G., eds) pp. 189–234, Chapman & Hall Ltd., London.
26. Davies, C., and Robinson, S. P. (1996) Plant Physiol. (Bethesda) 111, 275–283.
27. Church, G. M., and Gilbert, W. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 9232.
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1991–1995
28. Meinkoth, J., and Wahl, G. (1984) *Anal. Biochem.* **138**, 267–284
29. Sanger, F., Niesler, S., and Coulsen, A. R. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **74**, 5463–5467
30. Studier, F. W. (1991) *J. Mol. Biol.* **219**, 37–44
31. Schluin, K. E., Davies, K. M., Derales, S. C., Markham, K. R., Miller, R. M., Bradley, J. M., Mans, D. G., and Green, N. K. (1997) *Plant Sci.* **125**, 53–61
32. Parry, A. D., and Edwards, R. (1994) *Phytochemistry* **37**, 655–661
33. Williams, P. J., Cynkar, W., Francis, I. L., Gray, J. D., IJland, P. G., and Coombe, B. G. (1995) *J. Agric. Food Chem.* **43**, 37–44
34. Hawker, J. S. (1969) *Phytochemistry* **8**, 9–17
35. Hawker, J. S. (1969) *Phytochemistry* **8**, 337–344
36. Fling, S. P., and Gregerson, D. S. (1986) *Anal. Biochem.* **155**, 83–88
37. Harlow, E., and Lane, D. (1988) *Antibodies: A Laboratory Manual*, pp. 471–510, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
38. Hughes, J., and Hughes, M. A. (1984) *DNA Sequence*, pp. 41–49
39. Brouillard, R. (1982) in *Anthocyanins as Food Colors* (Markakis, P., ed), pp. 1–40, Academic Press, New York
40. Heller, W., and Forkmann, G. (1993) in *The Flavonoids: Advances in Research Since 1986* (Harborne, J. B., ed) pp. 499–535, Chapman & Hall Ltd., London
41. Dooner, H. K., and Nelson, O. E. (1977) *Biochem. Genet.* **15**, 509–519
42. Larson, R. L., and Coe, E. H., Jr. (1977) *Biochem. Genet.* **15**, 153–156
43. Dooner, H. K., and Nelson, O. E. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **74**, 5623–5627
44. Dooner, H. K. (1979) *Phytochemistry* **18**, 749–751
45. Styles, E. D., and Ceska, O. (1981) *Can. J. Genet. Cytol.* **23**, 691–704
46. Gholami, M., and Coombe, B. G. (1995) *Aust. J. Grape Wine Res.* **1**, 67–70
47. Tattersall, D. B., van Heeswijk, R., and Høj, P. B. (1997) *Plant Physiol.* (Bethesda) **114**, 759–769
48. Moens, C. P., Allen, P. V., Friedman, M., and Belknap, W. R. (1997) *Plant J.* **11**, 227–236
49. Szerszen, J. B., Szczynowski, K., and Bandurski, R. S. (1994) *Science* **265**, 1699–1701
50. Warnecke, D. C., Baltrus, M., Buck, F., Wolter, F. P., and Heinz, E. (1997) *Plant Mol. Biol.* **35**, 597–603
51. Holton, T. A., and Cornish, E. C. (1995) *Plant J.* **7**, 1071–1083
52. Price, S. F., Breen, P. J., Vallado, M., and Watson, B. T. (1995) *Am. J. Enol. Vitic.* **46**, 187–194
53. Harvath, D. M., and Chua, N. H. (1996) *Plant Mol. Biol.* **31**, 1061–1072
54. Purteke, D., Schiefelbein, J. W., Johnston, F., and Coe, E. H., Jr. (1988) *Plant Mol. Biol.* **11**, 473–481