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Polyphenol Glucosylating Activity in Cell Suspensions of Grape (*Vitis vinifera*)

MARK N. KRASNOW* AND TERENCE M. MURPHY

Section of Plant Biology, Division of Biological Sciences, University of California, One Shields Ave., Davis, CA, 95616

Stilbenes are phenolic molecules that have antifungal effects in the plant and antioxidant and anticancer effects when consumed in the human diet. Glycosylation of stilbenes increases their solubility and may make them more easily absorbed by the intestine. We have found an activity in extracts of cultured cells of *Vitis vinifera* (cv. Gamay Freaux) that glucosylates the stilbene resveratrol to form piceid. The Km for UDP-Glucose was 1.2 mM, and the Km for resveratrol was 0.06 mM, values similar to those of other phenolic glucosyltransferases. We investigated the resveratrol glucosylating activity of the enzyme extracted from cells grown under different light treatments (dark, visible light, light + ultraviolet (UVC) radiation) and found the activity to be unaffected or slightly reduced. In contrast, UVC light strongly stimulated extractable quercetin glucosyltransferase activity. These results, combined with analysis of phenolic compounds extracted from the differently treated cells, suggest that the resveratrol glucosyltransferase is distinct from the glucosyltransferase(s) active on other phenolics.

KEYWORDS: ***Vitis vinifera*; stilbene; resveratrol; glucosyltransferase**

INTRODUCTION

Stilbenoids are polyphenolic secondary metabolites produced in several plant families, including Vitaceae, Arachaceae, and Pinaceae. They are synthesized by the enzyme stilbene synthase (STS), which combines one molecule of hydroxycinnamoyl-CoA and three molecules of malonyl-CoA, yielding resveratrol as a product.

Stilbenoids are thought to play a phytoalexin role in the plants that produce them. Their accumulation can be induced by pathogen attack (*1*), UV light (*2*), and methyl jasmonate treatment (*3*, *4*). Stilbenes inhibit the germination and growth of fungal mycelia of *Botrytis cinerea*, *Plasmopara viticola*, and *Cladosporium cucumerinum in vitro* (*5*−*8*). Wheat and barley (non stilbenoid producing plants) transformed with a stilbene synthase gene showed increased resistance to fungal pathogens (*9*).

Stilbenoids have antioxidant properties in vitro (*10*) and have been shown to inhibit events in cancer initiation, promotion, and progression (*11*). Grapes and grape products constitute the greatest source of stilbenoids in the human diet (*12*). Stilbenoids, along with other phenolic compounds, may be responsible for the inverse relationship between moderate wine consumption and the occurrence of coronary heart disease and cancer (*13*). To act as antioxidants, these chemicals must be absorbed into the bloodstream; glycosylation may make phenolics more easily absorbed by the intestine. Glycosylated quercetin (a flavonol) was absorbed more than twice as well as the aglycone in human ileostomists (*14*); this may also be true of other phenolics, including stilbenoids. Glycosylation also increases the solubility of lipophilic molecules and allows for their storage within the plant.

Cell suspension cultures of *Vitis vinifera* (cv. Gamay Freaux) isolated from berry pulp fragments produce several stilbenoids. The primary stilbenoid accumulated by these cells is piceid (resveratrol-*O*-β-glucoside), though resveratrol is also accumulated to a lesser extent. Other studies using these cells have also identified the presence of pterostilbene, picetannol, the viniferins, and astringin (*15*−*18*). Only astringin is a glucoside; the others differ from resveratrol either by added hydroxyl groups (picetannol), methylation of hydroxyls (pterostilbene), or condensation (viniferins).

The enzyme UDP-glucose/flavonoid glucosyltransferase (UFGT) is capable of glycosylating anthocyanidins, producing the stable anthocyanins. We here report the extraction and characterization of an enzyme activity, analogous to that of UFGT, that glycosylates resveratrol to produce piceid (**Figure 1**).

MATERIALS AND METHODS

Plant Material. Cell cultures were established from the flesh of a *Vitis vinifera* (cv. Gamay Freaux) berry in 1978 by Dr. J. C. Pech (ENSA, Toulouse, France) and have been subcultured continuously since then. The growth medium consists of 20 g/L sucrose, 250 mg/L casein hydrolysate, macronutrients (*19*), micronutrients (*20*), vitamins (*21*), 0.2 mg/L Kinetin (0.93 μM), and 0.1 mg/L α-naphthalene acetic acid (0.54 μM). Cells were grown in 25 mL of media in 125 mL Erlenmeyer flasks. Cells were subcultured weekly at a 1:10 (v:v) ratio into new media and were incubated at 25 °C under continuous rotary shaking at 100 rpm (the standard growth conditions). Visible light treatment was constant irradiation from two F20 cool white fluorescent bulbs, at 30-cm distance (30 μmol photons/m^2). Dark treatments

* To whom correspondence should be addressed. E-mail: mnkrasnow@ucdavis.edu. Mailing Address: Plant Biology Section, University of California, One Shields Avenue, Davis, CA, 95616. Fax: (530) 752−5410.

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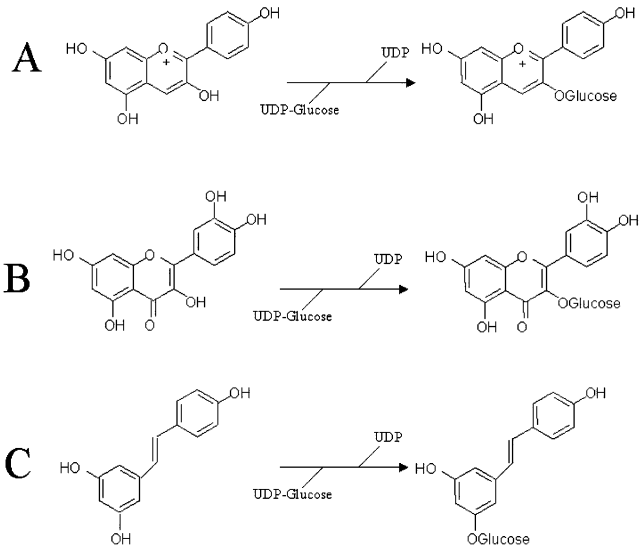

Figure 1. Reactions catalyzed by glucosyltransferases in grape cells. **A** is the UFGT reaction using cyanidin, producing cyanidin-3-glucoside. **B** is the UFGT reaction using quercetin, producing quercetin-3-glucoside. **C** is the resveratrol glucosylation reaction, producing piceid.

involved covering culture flasks with foil to exclude light. The ultraviolet (UV) treatment was a 10 min pulse of UVC (unfiltered mercury vapor lamp producing primarily 254 nm radiation) in a sterile hood applied to continuously shaken light-grown cultures on day six of growth. All experimental samples were harvested on day seven of culture.

Enzyme Extraction. All steps were carried out at 4 °C. Enzymes were extracted from vacuum filtered cells (3 g) by freezing them in liquid nitrogen and grinding them in the presence of glass beads (1.5 g) and PVPP (3 g). A 10-mL aliquot of the extraction buffer (0.2 M sodium phosphate (pH 6.8), 5 mM EDTA, and 1mM DTT) was added to the ground cells, and the resulting slurry was centrifuged at 12 000*g* for 20 min to remove insoluble material. The supernatant was collected and subjected to ammonium sulfate precipitation. The 30−80% ammonium sulfate pellet was resuspended in 500 μL of extraction buffer and dialyzed overnight against the same buffer. Samples were then assayed immediately or frozen at −20° C. Protein concentrations were estimated by the method of Bradford (*22*) with bovine serum albumin as a standard.

Phenolic Extraction. Seven-day old cells were harvested by vacuum filtration and freeze-dried. Phenolics were extracted from lyophilized cells using 50 μL of a 97% methanol/3% formic acid solution per milligram of cells. Cells were shaken and allowed to extract at 4 °C overnight. Insoluble cell material was removed by centrifugation at 12 000*g* for 10 min. The supernatant was filtered through a 0.45 μM pore filter prior to HPLC analysis.

HPLC Analysis. The method used was adapted from Donovan et al. (*23*) for separation of phenolics from fruits and juices. This method was able to differentiate piceid (retention time = 50 min) from resveratrol (retention time = 63 min).

Resveratrol Glucosylation Enzyme Assay. The method used was a modification of the UFGT enzyme assay of Lister et al. (*24*). The standard assay mixture consisted of 1.2 mM resveratrol (Sigma Chemical Co., St. Louis, MO), 50 mM tricine buffer (pH 8.5), 5 mM UDP-glucose, 6 mM dithiothrietol, and 50 μL of extract in a total volume of 250 μL. Samples were incubated at 30 °C for 3 h; then the reaction was stopped by the addition of 100 μL of 35% trichloroacetic acid. Products were extracted with ethyl acetate. The ethyl acetate was evaporated under vacuum, and the products were redissolved in 100 μL of methanol prior to HPLC analysis.

***β*-Glucosidase Digestion.** A 50-μL aliquot of cell extract or 60 μL of resveratrol glucosylation reaction product were incubated with 1 unit of β-glucosidase (Worthington Enzymes) in 0.1 M citrate buffer at pH 5 (total volume 500 μL). Digestion was carried out overnight at room temperature. Products were extracted with ethyl acetate and analyzed as described above.

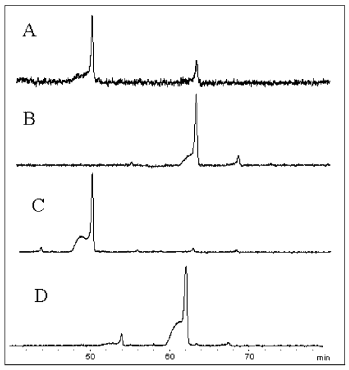

Figure 2. β-Glucosidase digestion of cell extract and resveratrol glucosylation product. **A** is the resveratrol glucosyltransferase reaction product. **B** is the reaction product treated with glucosidase. **C** is phenolic cell extract. **D** is cell extract treated with glucosidase Samples were incubated overnight at room temperature before HPLC analysis. The retention time (*X*-axis) for piceid is 51 min, and for resveratrol is 63 min. The peak at 55 min in the cell extract is due to an anthocyanin acylated with a hydroxycinnamic acid. *Y*-axis is absorbance at 316 nm.

Photoisomerization of Piceid and Resveratrol. A 750-μL aliquot of cell extract and 500 μL of 125 mM resveratrol were irradiated overnight at 12 °C using a tungsten halogen lamp (1300 μmol photons/m^2-s). Duplicate samples were kept in darkness. Phenolics were analyzed by HPLC as previously described.

RESULTS AND DISCUSSION

Resveratrol Glucosyltransferase Characterization. The cell enzyme extract was capable of glycosylating resveratrol, producing piceid. The identity of the reaction product was verified by its co-chromatography with the major stilbenoid extracted from the cells, which has been shown by other studies to be piceid (*3*, *16*). In confirmation, when cell phenolic extracts and resveratrol glucosylation products were subjected to β-glucosidase digestion, resveratrol was produced (**Figure 2**). The resveratrol glucosylation reaction was linear with time up to 5 h of incubation at 35 °C and was linear with enzyme concentration up to 250 and 500 μg protein (data not shown). Reaction mixtures with enzyme heated to 95 °C for 10 min showed no glucosyltransferase activity (data not shown).

Glucosyltransferase activity toward resveratrol was strongly pH dependent, with the maximal activity at pH 8.5−9 (**Figure 3**). The K_ms of the enzyme for its two substrates, resveratrol and UDP-glucose, were 0.06 mM and 1.2 mM, respectively, at pH 8.5 (**Figure 4**).

Glucosyltransferase Activities and Phenolic Accumulation Under Different Environmental Conditions. The concentration of piceid was highest in the dark grown cells. Visible light treatment reduced piceid accumulation. Light + UVC further decreased piceid concentration but strongly stimulated accumulation of resveratrol and another suspected stilbenoid in these cells (**Figure 5**). Correspondingly, resveratrol glucosyltransferase activity was highest in extracts of the dark grown cells and lower in extracts of cells grown under light and light + UV exposure (**Table 1**).

HPLC analysis of phenolic extracts of cells grown under different light treatments showed a clear increase in anthocyanin accumulation by visible light, with a lesser additional increase upon UVC exposure (**Figure 5**). UFGT activity in extracts was assayed using quercetin (a flavonol) as a substrate. Ford et al. (*25*) showed that purified UFGT accepts quercetin as a substrate; quercetin is preferred, because anthocyanidins are unstable at

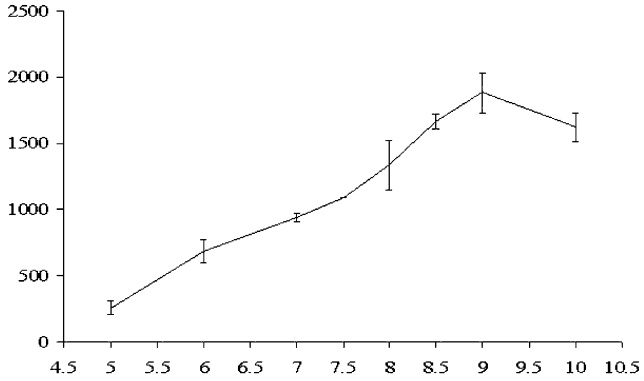

Figure 3. Resveratrol glucosylation pH optimum. Sodium phosphate (0.05 M) was used for pH 5, 6, and 7. Tricine (0.05 M) was used for pH 7.5, 8, 8.5, 9, and 10. All assays contained 1.2 mM resveratrol and 5 mM UDP-glucose. The same enzyme preparation was used for all pH analyses. No activity was observed at any pH without enzyme addition. All assays were done in duplicate, except pH 8.5 and 9, which were done in quadruplicate. *Y*-axis is mAU at 316 nm.

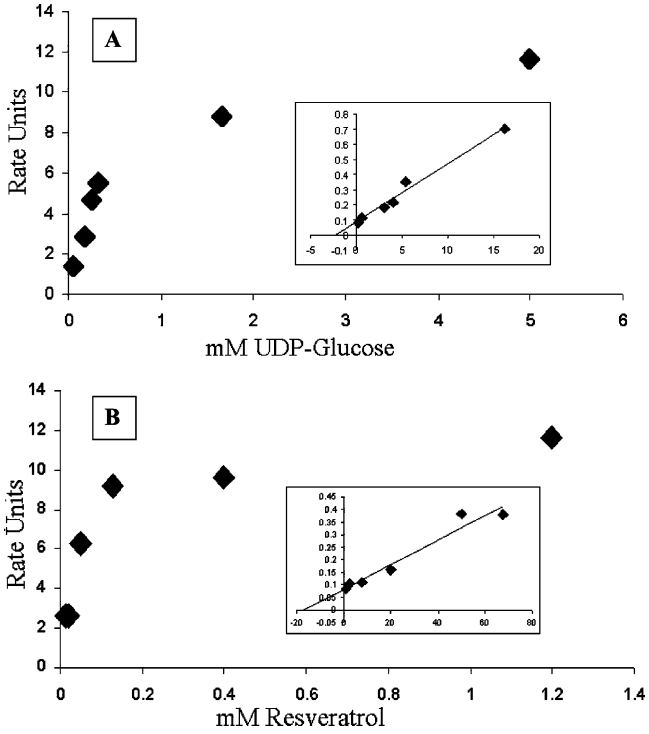

Figure 4. **A** Dependence of the reaction on UDP-Glucose concentration. The resveratrol concentration was kept constant at 1.2 mM. **B** Dependence of the reaction on resveratrol concentration. The UDP-glucose concentration was kept constant at 5 mM. All assays were done in triplicate. Insets are Lineweaver–Burk plots of data (*Y*-axis = 1/rate, *X*-axis = 1/mM substrate).

the pH at which the glucosyltransferase assay was performed. The glucosylating activity toward quercetin was similar from the light and dark grown cells and was increased dramatically by UVC treatment. This different responsiveness to light with respect to quercetin and resveratrol glucosylation activity suggests that there are at least two glucosyltransferase enzymes active in the cultures. The rates of reactions for the different substrates under different light conditions were also different. Quercetin glycosylation occurred much more quickly, with rates ranging between 82 and 172 nkatal/mg protein. Rates for resveratrol glycosylation were much slower, between 15.2 and 19.3 nkatal/mg protein (**Table 1**).

To investigate whether the observed changes in the concentrations of resveratrol and piceid were caused by direct photochemical reactions, cell extracts (containing primarily trans-piceid) and resveratrol (trans-form) were irradiated with visible light. Trans-resveratrol isomerizes in visible light to the cis form (*12*). Irradiated resveratrol showed a decrease in the peak at 63 min (trans form), with a concomitant increase in the peak at 68 min (cis form), which was absent when the sample was incubated in the dark (**Figure 6**, parts **A** and **B**). Because visible light isomerizes trans-resveratrol to the cis form, the observed increase of trans-resveratrol in light and light + UV treated cells could not have come from direct photochemical isomerization. Piceid showed no such effect when irradiated (**Figure 6**, parts **C** and **D**). The addition of the glucose moiety prevents the light induced isomerization. In light of this finding, the observed decrease of piceid also cannot be accounted for by photochemical isomerization of trans-piceid.

Is There More Than One Phenolic Glucosyltransferase? It is possible that the observed activity of this enzyme is due to the UFGT enzyme using resveratrol as a substrate. The observed pH maxima of the enzyme activity is in agreement with those of other glucosyltransferase enzymes (*26*−*28*). The *Vitis vinfera* UFGT enzyme has maximal activity at pH 8 (*25*). The Kms for substrates (both UDPG and flavonoid/stilbenoid) are in agreement with the calculated value for *Vitis vinifera* UFGT enzyme (*25*,*28*). There was glucosyltransferase activity toward quercetin in these cells, though no glycosylated flavonols are accumulated. It is possible that, in planta, which phenolic glucosides accumulate is determined by which substrates are provided to this multifunctional glucosyltransferase enzyme, and the provision of substrates is controlled either by altering activity of earlier biosynthetic enzymes, or by compartmentalization of enzymes, substrates, or enzyme products. Specifically, the accumulation of piceid in these cultures might occur because a very active stilbene synthase enzyme provides resveratrol substrate to a multifunctional glucosyltransferase enzyme capable of utilizing both anthocyanidins and stilbenoids, both planar molecules, as substrate.

Alternatively, in grape cells, there may be several glucosyltransferase enzymes, each of which is more specific toward one class of phenolic substrates. In grapes, UFGT transcript accumulates just as anthocyanins begin to accumulate at verasion (*29*). Enzymatic studies by Ford et al. (*25*) with this purified enzyme show that it accepted several flavonoid substrates, but anthocyanidins were acted upon more than 40-fold faster than any other. They also found no activity using resveratrol as a substrate.

Evidence from other studies points to the existence of several differentially regulated glucosyltransferase activities in *Vitis*. Flavonol glucosides accumulate in response to sunlight after anthocyanin accumulation had already begun (*30*). Piceid accumulation can be strongly induced by methyl jasmonate treatment in leaves and grapes both pre- and post-verasion (*4*). Enzymatic analysis of crude grape cell extracts showed six separable quercetin glycosylating activities, all showing lower activity toward anthocyanidins as substrates (*31*). Grapes that do not accumulate anthocyanins still accumulate other phenolic glucosides.

Our results support the hypothesis that there is more than one glucosyltransferase. The observation that a reduction in resveratrol glucosylating activity correlated with reduced piceid accumulation suggests that resveratrol glucosyltransferase activity directly determined the amount of piceid produced, but resveratrol glucosylating activity had no correlation with the anthocyanins accumulated. Dark-grown cells showed an increase

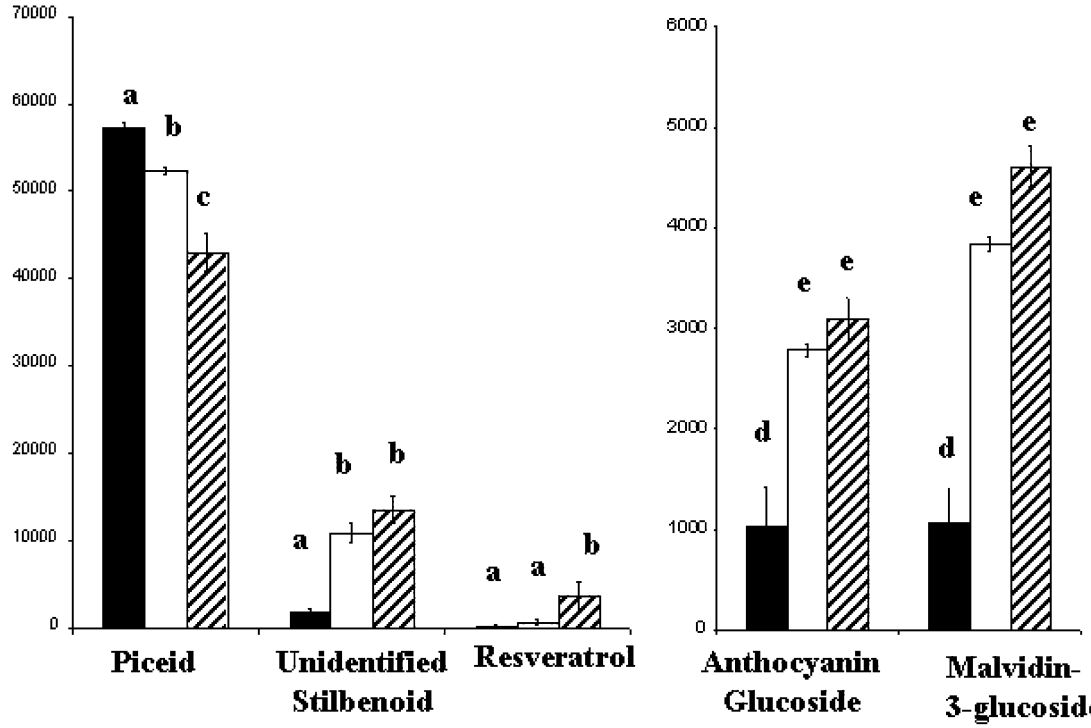

Figure 5. The effect of different light treatments of cultured cells on three stilbenoids and two anthocyanins extracted from the cells. Bars are the mean of triplicate samples ± SE. Statistical analysis was a *t*-test at $p = 0.01$ level. *Y*-axis is mAU at 316 nm for stilbenes on left graph and is mAU at 520 nm for anthocyanins on right graph. Black bars are dark grown cells, white bars are light grown cells, and hatched bars are light + UVC treated cells.

Table 1. Rates of Glucosylation of Resveratrol and Quercetin Under Different Light Treatments by Cell Enzyme Extracts

treatment	glucosylation of resveratrol	glucosylation of quercetin
dark	19.37 ± 3.31[b]	82.40 ± 4.73[c]
light	15.23 ± 3.04[b]	90.54 ± 3.33[c]
light + UVC	15.66 ± 1.37[b]	172.06 ± 10.51[d]

[a] All units are nkatal/mg total protein. Results are averages of triplicate samples ± standard error. [b] –*d*Values in a column followed by the same letter did not differ by *t*-test at the $p = 0.01$ level of confidence.

in resveratrol glucosylating activity and piceid accumulation, relative to light-grown cells, but a strong decrease in anthocyanin glucosides that were accumulated, suggesting a decrease in UFGT activity using anthocyanidins as substrates. Activity toward quercetin as a substrate had a different response to the light treatments, with UV strongly stimulating glucosyltransferase activity toward this flavonol. The relative rates of glucosylation with respect to stilbenes versus quercetin were also markedly different.

On the basis of their responsiveness to environmental stimuli, there is evidence of three distinct glucosyltransferase activities in our cell extract. The activity of resveratrol glucosylation decreased upon visible light irradiation of the cultures. This correlated with the observed decrease in accumulation of piceid in these cells. A quercetin glucosylating enzyme was stimulated only when irradiated with UV light, with similar activity in dark grown or light grown cells. The observed increase in anthocyanin accumulation in light irradiated cultures suggested an increase in the activity of an anthocyanidin utilizing glucosyltransferase upon light or light + UV treatment.

Several glucosyltransferase genes, all producing enzymes capable of glycosylating phenolic substrates would be expected to have homology to one another. In Southern blot analyses of *Vitis vinifera* genomic DNA, UFGT was one of the few enzymes

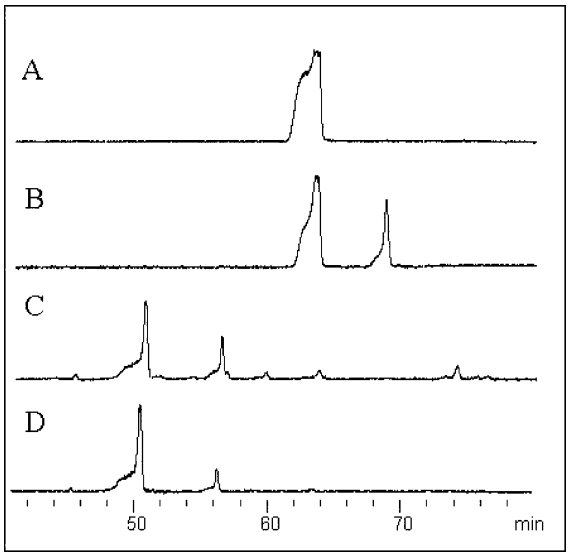

Figure 6. The effect of visible light irradiation of trans-resveratrol and cell extract containing trans-piceid. All irradiations were carried out at 12 °C using a tungsten halogen lamp (1300 μmol photons/m^2-s). All chropmatographs were monitored for absorbance at 316 nm. **A** is trans resveratrol incubated in the dark **B** is trans resveratrol incubated overnight under constant irradiation. Retention time for trans-resveratrol is 63 min, for cis-resveratrol is 68 min. **C** is cell extract incubated in the dark. **D** is cell extract irradiated overnight. Peaks at 55 and 59 min in cell extracts are due to absorbance by anthocyanins acylated with hydroxycinnamic acids.

in the phenolic pathway found only as a single band (*32*). It is possible that the heterologous probe used by this group failed to recognize other glucosyltransferase genes. Alternatively, there might be a single gene that can be differentially spliced producing proteins with different substrate specificities.

Plants must deal with an ever-changing environment and with their own developmental changes. Being immobile, many of

these alterations take the form of the synthesis of specific chemical compounds. Grapes are capable of synthesizing many distinct polyphenolic glucosides, including piceid, flavonol glucosides, and anthocyanins. Each of these compounds presumably has a function in the plant. Anthocyanins attract animals to distribute the seeds of mature red fruit (*33*). It is important that biosynthesis of these pigments be controlled developmentally, so that pigment accumulation does not begin until the seeds are mature and ready to be dispersed. Flavonol glucosides have been shown to be synthesized in the dermal tissues of many plant genera in response to UV light; the flavonols absorb strongly in the UV wavelengths, protecting underlying tissues from UV-induced damage (*34*, *35*). It is unclear why grape cells accumulate piceid, although it is more water soluble than resveratrol and the glucosylation prevents photochemical isomerization of resveratrol moiety. Possibly a better reason to accumulate the glucoside is that the glucosylation protects resveratrol from oxidation by fungal tyrosinase (*36*). Assuming that these glucosides have specific roles for the plant at specific developmental stages or environmental conditions, it is important for the plant to stringently regulate the glucosylation of polyphenolic compounds. Having several specific glucosyltransferases that are responsive to different environmental cues allows for the fine-tuning of the chemical compounds produced by the plant to best suit its growing conditions.

We have shown, for the first time, that there is a glucosyltransferase activity toward resveratrol in grape cells. We have characterized this activity and found it to be similar to other phenolic glucosyltransferases, but we suggest that it is a distinct enzyme.

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