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Determination of the Substrate Specificity of *Citrus paradisi* Flavonol Specific 3-O-Glucosyltransferase Mutant D344P

By

Nathan Richard Spaulding

An Undergraduate Thesis Submitted in Partial Fulfillment
of the Requirements for the
University Honors Scholars Program
Honors College
East Tennessee State University

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Acknowledgments

I would like to thank Dr. Cecilia McIntosh, my thesis mentor, for her guidance, patience, and help; Dr. Ranjan Chakraborty and Dr. Dhirendra Kumar, my thesis readers, for their support; and my lab mates for all their help with experiments. Additionally this research was funded in part by an NSF MCB grant 1120268 and an ETSU RDC grant awarded to C.A.M. and a student-faculty collaborative grant awarded to N.R.S and C.A.M. through the ETSU Honors College.

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Abstract

Plants produce a vast array of secondary metabolites. A group of phenolic compounds, the flavonoids, are metabolites ubiquitous among plants and are known to aid in processes such as plant reproduction, UV defense, pigmentation and development. In relation to human health, flavonoids have been found to possess anti-inflammatory, anti-cancer, and antioxidant properties. Flavonoid's ability to participate in so many interactions is due in part to their subclass variation and further chemical modification. One such modification is glucosylation, where a glucose molecule is added to the flavonoid substrate. The enzymes that catalyze these reactions are known as glucosyltransferases (GT). *Citrus paradisi* contains a glucosyltransferase that is specific for adding glucose to the 3-O position of flavonols (Cp-F3-*O*-GT). To further understand the reactions it catalyzes, Cp-F3-*O*-GT structure was modeled against an anthocyanidin/flavonol 3-*O*-GT found in *Vitis vinifera* to identify candidate amino acids for mutations. Mutants were then generated using site-directed mutagenesis, and one mutant, D344P, was constructed by an aspartate being replaced with a proline. Biochemical characterization of the mutant D344P protein was performed in order to determine whether the mutation has an effect on the substrate specificity of Cp3-O-GT. An initial quick-screening assay using radioactive UDP-glucose as a sugar donor suggested there may have been an expansion of substrate acceptance. The time course assays did not support observation. Additionally, results show that D344P protein has decreased activity with flavonols as compared to the wild-type Cp3-O-GT. with no expansion of substrate specificity. Homology models supported experimental results.

Introduction

Plants produce a myriad of chemical molecules known as secondary metabolites. The major groups of these molecules are phenolics, alkaloids, and terpenoids. The phenolics contain an important subgroup of molecules known as flavonoids. Composed of 15 carbons, flavonoids are synthesized through a series of chemical reactions starting with phenylalanine (Figure 1). The biosynthesis pathway can lead to nine subclasses of flavonoids (Owens and McIntosh, 2011). Flavonols, flavanones, anthocyanidins, flavones, and the rest of the nine subclasses can be distinguished by varying oxidation levels in the heterocyclic ring structure (Figure 2). The over 10,000 known natural flavonoids exist due to these subclasses as well as varying substituents such as hydroxyl, methyl, and sugar groups on the rings (Owens and McIntosh, 2011). Not all plants make all subclasses and many plants have specific flavonoid composition (Owens and McIntosh, 2011).

Flavonoids have a wide array of functions in plants. They have been found to play a role in UV defense (Owens and McIntosh and reference therein, 2011). Their role in UV defense was confirmed when *Arabidopsis thaliana* mutants that produced lower amounts of flavonoids became over sensitive to UV light (Li et al, 1993). In addition to UV defense, flavonoids play a role in fruit pigmentation. Anthocyanins are common red and blue pigmenting agents in plants. They are responsible for the red coloration in grapes and blood orange (Owens and McIntosh, 2011). Flavonoids also affect many different interactions such as plant-microbe interactions, plant-insect interactions and allelopathy (Simmonds, 2003; Steinkellner et al, 2007; Treutter 2005; Weir et al, 2004).

One notable example of these interactions is the involvement of flavonoids in the attraction of nitrogen-fixing bacteria to legume root nodules (Peters et al, 1986).

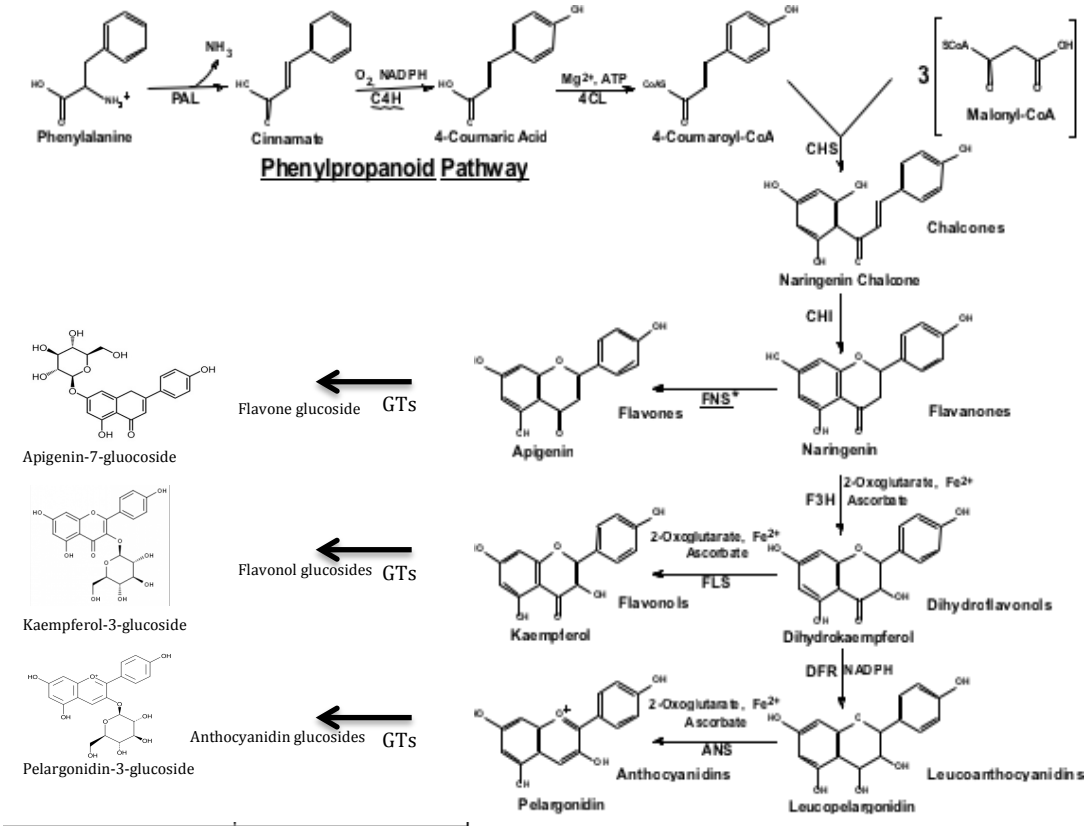


Figure 1: Biosynthesis pathway of flavonoids in citrus (adapted from Owens and McIntosh, 2011). PAL: phenylalanine ammonia-lyase; C4H: cinnamate-4-hydroxylase; 4CL: 4-coumarate: CoA ligase; CHS: Chalcone synthase; CHI: Chalcone isomerase; FNS: Flavone synthase; F3H: Flavanone 3-hydroxylase; FLS: Flavonol synthase; DFR: Dihydroflavonol 4-reductase; ANS: Anthocyanidin synthase; GTs: Glucosyltransferase.

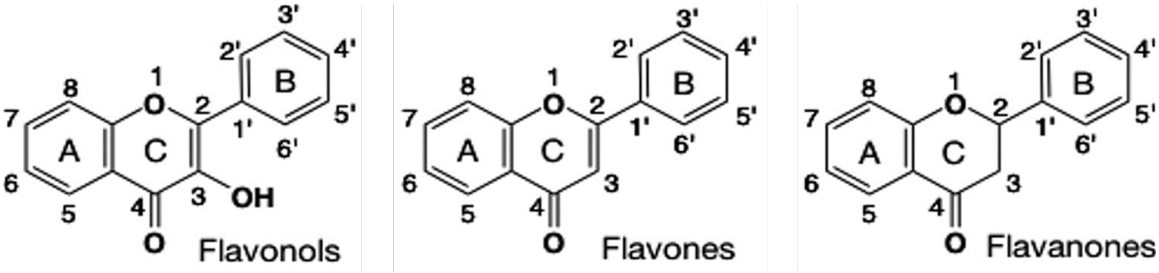


Figure 2: Some basic flavonoid subclass structures.

Furthermore, flavonoids can have many beneficial effects when consumed by humans. These molecules have been found to have anti-cancer, anti-inflammatory, anti-

bacterial, and anti-oxidant properties (Middleton et al, 2000; Jarial et al, 2016; Cushnie and Lamb, 2011). Additionally, HIV inhibition using flavonoids is being investigated (Cole et al, 2016).

Chemical modification is common among flavonoid compounds. These modifications include processes such as methylation, hydroxylation, and many others (Dixon and Pasinetti, 2010, Owens and McIntosh, 2011). One common flavonoid modification that occurs in nature is glucosylation. Glucosylation is the biochemical process in which a glucose molecule is added to an acceptor molecule (Figure 3). Enzymes known as glucosyltransferases (GTs, EC 2.4.1. x) catalyze these glucosylation reactions. Among these GTs, the largest group, GT1, uses UDP-sugars as donor substrates (Vogt and Jones, 2000; Yonekura-Sakakibara and Hanada, 2011). These enzymes are characterized by a 44 amino acid motif referred to as the plant secondary product glycosyltransferase (PSPG) box that constitutes the UDP-sugar-binding domain on the N-terminal portion of the enzyme (Hughes and Hughes, 1994; Vogt and Jones, 2000; Devaiah et al, 2016). The presence of a PSPG box can be used to identify putative GT genes in plants.

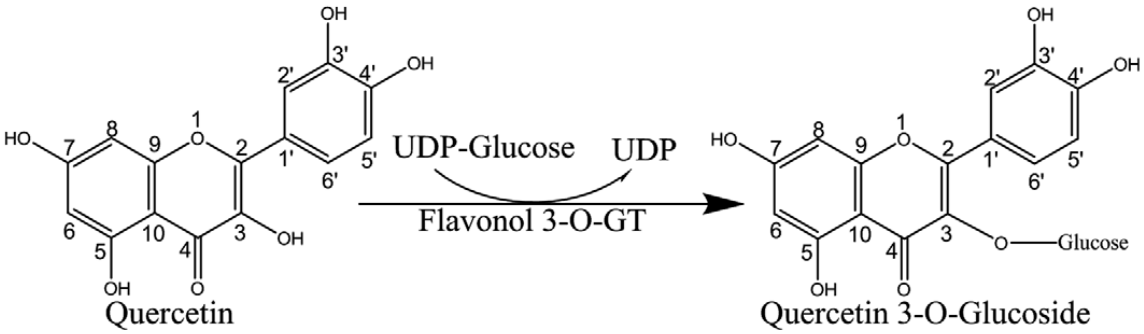


Figure 3: *Citrus paradisi* flavonol-specific 3-O-glucosyltransferase reaction (Owens and McIntosh 2009).

Though these GT enzymes share motifs and some similarities, they can have very different substrate specificities. Some GTs have very strict substrate specificities and can only glucosylate one subclass of flavonoid or even one particular molecule. For example, the flavonol-specific glucosyltransferase from *Citrus paradisi,* Cp-F3-*O*-GT, will only glucosylate flavonols and only at the hydroxyl group at the third carbon (Owens and McIntosh, 2009). In contrast to Cp-F3-*O*-GT, the well-studied, crystallized GT from *Vitis vinifera* (VvGT1) is able to glucosylate both flavonol and anthocyanidin substrates at the 3-OH position and thus has a broader specificity (Offen et al, 2006).

While these two enzymes, Cp-F3-*O*-GT and VvGT1, are relatively similar for GTs, 56% sequence identity and 87% homology, they vary in substrate specificity. The high percentage of similarity suggests that the difference is not solely caused by the primary amino acid sequence but is affected significantly by tertiary structure. When looking at the structures of these enzymes, several differences can be seen even though they have relatively the same shape (Figure 4). Understanding the effects of enzyme structure and function can provide valuable insight into the workings of enzyme substrate specificity. The effects of structure on enzyme function can be analyzed through point mutation analysis (Devaiah et al, 2017).

In the case of this research, the amino acid sequences and tertiary structures of Cp-F3-*O*-GT and VvGT1 were compared to identify potential residues for point mutation. At position 344, Cp-F3-*O*-GT contains an aspartate while VvGT1 contains a proline at the equivalent position (Figure 5). Because aspartate is a negatively charged amino acid and proline contains a cyclic structure which does not allow for conformational freedom in the protein backbone, a mutation at this position could cause a

structural change in the grapefruit enzyme. Using site directed-mutagenesis, a Cp-F3-*O*-GT mutant was generated with the aspartate in Cp-F3-*O*-GT changed to the proline as in the VvGT1 and given the name D344P. The hypothesis tested in this research is that the mutation D344P will change the substrate specificity of the Cp-F3-*O*-GT. The change of aspartate to a proline could affect the enzyme's ability to glucosylate flavonoid substrates through a change in structure. The results will provide key information to the field by showing how a change in structure can modify this enzyme's ability to glucosylate and its specificity.

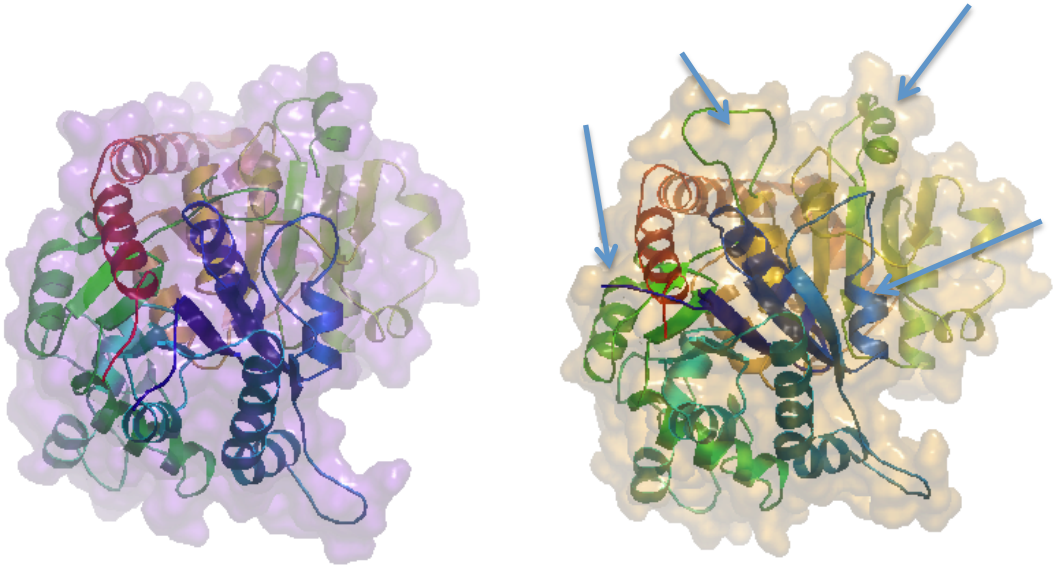
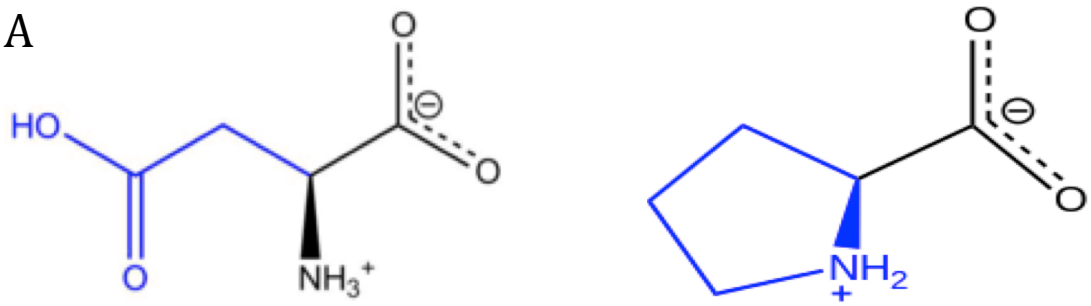


Figure 4: The crystal structure of VvGT1 (left) (Offen et al, 2006) and homology model of Cp-F3-*O*-GT (right). Arrows point to differences found in the Cp-F3-*O*-GT structure,

A



B

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V.vinifera      --MSQTTTNPHVAVLAFPFSTHAAPLLAVVRRLAAAAPHAVFSFFSTSQSNASIFHDSM-
C.paradisi      MAQTQSQPRPHIAVLNFPFSTHASSVLSIIKRLAVSAPTALFTFFSTPQSNKALFSTGQQ
D344P           MAQTQSQPRPHIAVLNFPFSTHASSVLSIIKRLAVSAPTALFTFFSTPQSNKALFSTGQQ
                   :*:  .**:*** *******: :*::::***.:** *:*:**** *** ::*  .

V.vinifera      HTMQCNIKSYDISDGVPEGYVFAGRPQEDIELFTRAAPESFRQGMVMAVAETGRPVSCLV
C.paradisi      RHLPSNVKPYDVSDGVPEGHVFSGKRQEDIELFMNAADANFRKAVEAAVAETGRPLTCLV
D344P           RHLPSNVKPYDVSDGVPEGHVFSGKRQEDIELFMNAADANFRKAVEAAVAETGRPLTCLV
                : : .*:* **:*******:**:*: ******* .**  .**:.:  ********::***

V.vinifera      ADAFIWFAADMAAEM-GVAWLPFWTAGPNSLSTHVYIDEIREKIGVSGIQGREDELLNFI
C.paradisi      TDAFIWFAAEMAREWNNVPWVPCWPAGPNSLSAHLYTDIIRDKIGTQS-QNQDQQLIHFI
D344P           TDAFIWFAAEMAREWNNVPWVPCWPAGPNSLSAHLYTDIIRDKIGTQS-QNQDQQLIHFI
                :********:** *   * *:* * *******:*:* * **:***... * ::::*:.**

V.vinifera      PGMSKVRFRDLQEGIVFGNLNSLFSRMLHRMGQVLPKATAVFINSFEELDDSLTNDLKSK
C.paradisi      PGMNKIRVADLPEGVVSGDLDSVFSVMLHQMGRQLPKAAAVFINSFEELDPELTNHLKTK
D344P           PGMNKIRVADLPEGVVSGDLDSVFSVMLHQMGRQLPKAAAVFINSFEELDPELTNHLKTK
                ***.*:*. ** **:* *:*:*:** ***:**: ****:************ .***.**:*

V.vinifera      LK-TYLNIGPFNLITPPPV--------VPNTTGCLQWLKE--RKPTSVVYISFGTVTTPPP
C.paradisi      FNNKFLSVGPFKLLLASDQQPSSATDLDDEYGCLAWLDKQKKKPASVAYVSFGTVATPSP
D344P           FNNKFLSVGPFKLLLASDQQPSSATDLDDEYGCLAWLDKQKKKPSVAYVSFGTVATPSP
                :: .:*.:***:*:             : :  *** **.:  :** **.*:*****:** *

V.vinifera      AEVVALSEALEASRVPFIWSLRDKARVHLPEGFLEKTRGYGMVVPWAPQAEVLAHEAVGA
C.paradisi      NEIVAIAEALEANKVPFIWSLRHRSQANLPNGFLERTRSDGIVVDWAPQVNVLAHEAVGV
D344P           NEIVAIAEALEANKVPFIWSLRHRSQANLPNGFLERTRSDGIVVPWAPQVNVLAHEAVGV
                *:**::*****.:********.::::..**:****:**. *:** ****.:********.

V.vinifera      FVTHCGWNSLWESVAGGVPLICRPFFGDQRLNGRMVEDVLEIGVGIEG-GVFTKSGLMSC
C.paradisi      FVTHCGWGSILESIAAGVPMIGRPFFGDQRINGRMMEQVWGVGVGVDGGGICTKEGLLSS
D344P           FVTHCGWGSILESIAAGVPMIGRPFFGDQRINGRMMEQVWGVGVAVDGGGICTKEGLLSS
                ******* *: **:*.***:* ********:****:*:*  :** ::* *: **.**:*.

V.vinifera      FDQILSQEKGKKLRENLRALRETADRAVGPKGSSTENFITLVDLVSKPKDV
C.paradisi      LDLILCQEKGIKIREKVTKLKQLCQNAIGPGGSSMQNLDALVDMISRSY--
D344P           LDLILCQEKGIKIREKVTKLKQLCQNAIGPGGSSMQNLDALVDMISRSY--
                :* **.**** *:***::  *:: .:.*:** *** :*: :*****:*:
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Figure 5: A) Amino acid structures of aspartate (left) and proline (right). B) Sequence alignment between Cp-F3-*O*-GT (GQ141630), VvGT1 (AAB81683), and the mutant D344P. Arrows indicate location 344 where the mutation was located.

Materials and Methods

Materials:

Materials purchased from Sigma (St. Louis, Mo, USA) included quercetin, quercetin 3-*O*-glucoside, kaempferol 3-*O*-glucoside, naringenin and dihydroquercetin, acid washed glass beads (pore size-0.5 mm), and ethidium bromide (EtBr). Kaempferol, naringenin-7-*O*-glucoside, hesperetin, eriodictyol, isosakuranetin, apigenin, luteolin, diosmetin, scutallerein, fisetin, myricetin, gossypetin, cyanidin chloride, and 4'-acetoxy-7-hydroxy-6-methoxyflavonol were purchased form Indofine (Hillsborough, NJ, USA). Phenylmethylsulfonyl fluoride (PMSF) was purchased from MP Biomedicals (Solon, OH). Materials purchased through Perkin Elmer included UDP-[U-14C] glucose (specific activity 293mCi/mol). UDP-glucose was purchased from Calbiochem (Gibbstown, NJ, USA).

Scale Up Expression of Recombinant Cp-F3-O-GT (WT) and Mutant D344P:

All media were prepared with recipes found in Kandel, 2016. A 3ml volume of yeast extract peptone dextrose medium (YPD) (Kandel, 2016) containing 100 µg/mL zeocin was prepared and a glycerol stock stored at -80°C of either WT or D344P mutant containing yeast, *Pichia pastoris,* was added to media. The inoculated media was allowed to incubate overnight in a shaker for 16-18 hours at 30°C and 250 rpm. After incubation, a 500-mL sample was placed in a 1 L flask containing 200 mL of buffered glycerol-complex medium (BMGY) (Kandel, 2016). The culture bearing flask was then incubated at 30°C and 250 rpm until the $O.D._{600}$ was 2-6, typically 18-24 hours. Once an $O.D._{600}$ of 2-6 was achieved, the culture was transferred into different sterile 50 mL tubes and the cell pellets were collected by centrifuging for 10 min at 2800 xg. Cell pellets

were resuspended in buffered methanol-complex medium (BMMY) (Kandel, 2016) to wash and then centrifuged for 10 min at 2800 xg to collect cell pellets. These cell pellets were resuspended in 50 mL BMMY and were then placed in a 1 L baffled flask, sealed with cheesecloth, and allowed to incubate at 30°C in a shaker at 250 rpm until a given time for optimal protein content as determined by time course SDS PAGE and western blot analysis, 12 hr and 18 hr for D344P and WT, respectively. The cultures were harvested by centrifuging for 10 min at 2800 xg and cells were stored at -80°C until use.

Enzyme Purification:

The cell pellets collected from the scale up procedure were resuspended in 5 mL of breaking buffer (50mM sodium phosphate buffer at pH 7.5, 1mM EDTA, 5% glycerol, 5 mM βME, and 1 mM PMSF). A French press was used to lyse the cells at 1120 psi pressure. Before use, French press was washed with water. Cells were lysed for a minimum of 4 times and were kept on ice the entire time. Lysed cultures were centrifuged at 13,000 xg for 20 min at 4°C, and the supernatant was collected and kept on ice. All purification chromatography was performed at 4°C. A 2.5 ml volume of crude protein extract was added to a 8.3 ml bed volume PD-10 column, previously equilibrated with 25 mL of equilibration buffer (50 mM sodium phosphate buffer at pH 7.5, 300mM sodium chloride and 5 mM βME), and the flow-through was discarded. The protein was then eluted and collected using 3.5 mL of equilibration buffer. This process was repeated again to obtain 7.0 mL of eluted protein. Next, a 2 mL bed volume TALON IMAC cobalt metal affinity resin column was precisely equilibrated with 25 mL of equilibration buffer, and the 7.0 mL of eluted protein was run through the column with the flow through being discarded. The column was then washed with equilibration buffer until

O.D. $_{280}$ of the eluate was less than 0.09 mg/ml. Once a low enough O.D. was obtained, the bound protein was eluted in 2 mL fractions using elution buffer (50mM sodium phosphate buffer pH 7.5, 300mM sodium chloride, 5mM βME, and 150 mM imidazole). The protein concentrations of the fractions were determined through use of a Nanodrop spectrophotometer at 280nm. Fractions with higher concentrations were pooled and then were concentrated and desalted using Amicon Centricon 30 MWCO centrifugal filters (Millipore, Billerica, MA, USA) and centrifuging at 2800 xg at 4°C for 10 minutes or until volume reached 500 µL. After the desired volume was reached, 2 mL of assay buffer (50 mM sodium phosphate buffer pH 7.5, 14 mM βME) was added in the Amicon and was centrifuged again at 2800 xg at 4°C for 10 minutes. The final concentrated protein, 250 µL, was collected and the concentration was determined. Enzyme was stored on ice throughout and samples were collected throughout the process for SDS-PAGE and western blot analysis.

Glucosyltransferase Activity:

Activity of glucosyltransferase was assayed by determining incorporation of ^{14}C-glucose by the enzyme as previously described (McIntosh et al, 1990). For initial substrate screening, 0.025 µCi of UDP-[U-^{14}C] glucose diluted to 20,000 cpm/10µL in water was used. The reaction mixture consisted of 5 µL of flavonoid aglycone (50nmol/5µL) in ethylene glycol monomethyl ether, 10 µL of UDP-^{14}C glucose, 50 µL of 50 mM phosphate buffer containing 14 mM βME, and 10 µL of enzyme (3 µg/10 µL) for a total reaction volume of 75 µL. Reactions were placed in a 37 °C water bath for 5 minutes. After 5 minutes, reactions were terminated by adding 15 µL of 6N HCl to the reaction and vortexing. The reaction product was extracted by adding 250 µL EtOAc to

the reaction, thoroughly mixing, centrifuging for a few seconds and placing a 150 µL aliquot of the top layer into 2 mL of CytoScint scintillation cocktail (Thermo Fisher). Results were measured by a Beckman LS 6500 scintillation counter. Incorporation was corrected for by setting up a ratio of 150/250 = cpm measured/ x, where x is the incorporation occurring.

Time course assays were conducted with 5 µL of flavonoid aglycone (50nmol/5µL) in ethylene glycol monomethyl ether, 10 µL of UDP-glucose (100nmol/10 µL) having 50,000cpm/10 µL, 50 µL of 50 mM phosphate buffer containing 14 mM βME, and 10 µL of enzyme at concentrations of 1.0 µg/reaction and 0.5 µg/reaction. Reaction mixtures were placed in a 37 °C water bath and were run for time points of 0, 5, 10, 15, 30, and 60 minutes. Incorporation was determined through the same methods as the screening assay. Pico-moles of product was calculated by (cpm incorporated/500)*1000.

SDS-PAGE and Western Blot Analysis:

For visualization of protein samples, 10% SDS-PAGE gels were run as described in McIntosh et al, 1990. Western blot analysis was run using antibody against C-Myc tags as described in Devaiah et al, 2016.

Homology Modeling and Docking:

The crystal structure of the *Vitis vinifera* flavonoid-3-*O*-glucosyltransferase (Offen et al. 2006) was used as a template for homology modeling. Three-dimensional structures were generated using UCSF Chimera and EasyModeller 4.0 was used to generate models with different molpdf (molecular probability distribution), DOPE (discrete optimized protein energy), and Ga341 scores. Docking analysis was performed

on the model with the lowest DOPE score. Swiss PDB viewer was used to refine for energy minimization for molecular docking. PyRx was used to dock protein with desired ligands and models were analyzed using Autodock 1.5.6 MGL tools software.

Results and Discussion

To test the hypothesis that the mutation D344P would broaden the substrate specificity of Cp-F3-*O*-GT, several experiments were performed. First, a screening assay was performed to identify the mutant protein's potential activity with different substrates. Next, the substrates that showed potential activity in the screening were tested with a time course assay to confirm activity. Finally homology models were obtained to analyze the predicted structures of the enzyme-binding sites.

Acceptor Substrate Activity Screening:

To test the substrate specificity broadening hypothesis, Cp-F3-*O*-GT (wild-type, WT) and D344P (mutant) proteins were reacted with radioactively labeled C^{14} UDP-Glucose and 14 flavonoid substrates from 6 subclasses (Table 1). This assay provides a quick method to view an enzyme's general activity patterns. The initial screening suggested that the mutant enzyme may have decreased activity when compared to the wild-type and the same patterns are seen in the verification (Table 1). In terms of the substrate specificity, the screening suggested a potential activity with flavone diosmetin and flavanones isosakuranetin and hesperetin. These activities need confirmation due to the sensitivity of the screening assay (Owens and McIntosh, 2009; McIntosh et al, 1990).

Table 1: Acceptor Substrate Screening of wild-type Cp-F3-*O*-GT and Mutant D344P. Activity expressed as percent relative activity to wild-type quercetin for the screening group and expressed as percent relative activity to the mutant with quercetin for the verification group. Substrates marked with a star were selected to have the activity confirmed.

Flavonoid Subclass	Flavonoid Substrate	Screening		Verification
		D344P	WT	D344P
Flavonol	Quercetin*	41	100	100.0
	Kaempferol*	18	51	61.2
	Fisetin*	19	40	55.4
	Gossypetin*	9	22	18.3
Flavanone	Naringenin	2	3	3.0
	Hesperetin*	4	3	13.5
	Eriodictyol	1	2	1.8
	Isosakuranetin*	3	2	14.0
Flavone	Apigenin	2	2	1.4
	Luteolin	0	2	2.1
	Diosmetin*	4	2	92.6
	Scutellarin	2	5	
Dihydroflavonol	Dihydroquercetin	3	2	3.4
Isoflavone	4'acetoxy-7-hydroxy-6-methoxy isoflavone	5	2	1.4
Anthocyanidin	Cyanidin chloride	1	4	3.6

Time Course Confirmational Assay:

Due to the sensitivity of the screening assay, some substrates which appear to show activity may be false positives. The time course assay is able to confirm whether the screening assay results are accurate. The use of unlimiting donor substrate UDP-glucose allows for this confirmation. In addition, the assay is able to provide information on rate of product formation.

The assay using the WT Cp-F3-*O*-GT and quercetin (Figure 6) agreed with the original characterization, exhibiting a preference toward quercetin as a substrate (Owens and McIntosh, 2009; Devaiah et al, 2016). The assay performed with D344P protein and quercetin supported the information shown in the screening assay. The assay showed that

incorporation occurred with the mutant enzyme, but at levels below that of the wild-type (Figure 6).

The reaction with WT remained linear for 5 min. The WT had apparent specific activities of 2508.32 pmol/min/µg and 1598.16 pmol/min/µg for 0.5 and 1.0 micrograms of enzyme respectively during this time. The higher activity for 0.5 micrograms might be caused by the enzyme competing against itself for donor substrate. For future kinetic assay 0.5 micrograms should be used. Mutant D344P remained linear for 30 min and had apparent specific activities of 361.33 pmol/min/µg and 224.11 pmol/min/µg for 0.5 and 1.0 micrograms of enzyme respectively. The specific activity of the mutant decreased substantially compared to the wild-type. Thus, results confirmed the activity for D344P with quercetin was reduced compared to the WT enzyme. The reduction in activity suggests that the mutation changed the tertiary structure of Cp-F3-*O*-GT in such a way that quercetin could still bind to the active site but in a position that prevents optimum usage. It can be noted that the apparent specific activities for both WT and D344P were less at higher concentrations of enzyme. This decrease could be caused by the enzyme competing against itself for substrate molecules. For future kinetic analysis, 0.5 micrograms of enzyme should be used.

Time course assay results for the enzymes with kaempferol showed a similar trend (Figure 7). Wild-type enzyme again produced results similar to those previously recorded (Owens and McIntosh, 2009). For the D344P mutant enzyme results, incorporation can be observed, but at amounts much lower than the wild-type. The WT reaction was linear for 5 min. WT had specific activities of 831.84 pmol/min/µg for reaction with 1.0 micrograms of enzyme and 1336.88 pmol/min/µg for reaction with 0.5

micrograms of enzyme. The D344P reaction was linear for 30 minutes and had apparent specific activites of 86.5 pmol/min/µg and 96.6 pmol/min/µg for 1.0 and 0.5 micrograms of enzyme, respectivly. There is at least a 10x decrease in the apparent specific activity between the two enzymes. These results support the decrease of activity for kaempferol in the mutant observed in the initial screening and support the idea that the mutation affected the structure in such a way that the mutant protein's ability to bind to and transfer glucose to kaempferol was affected.

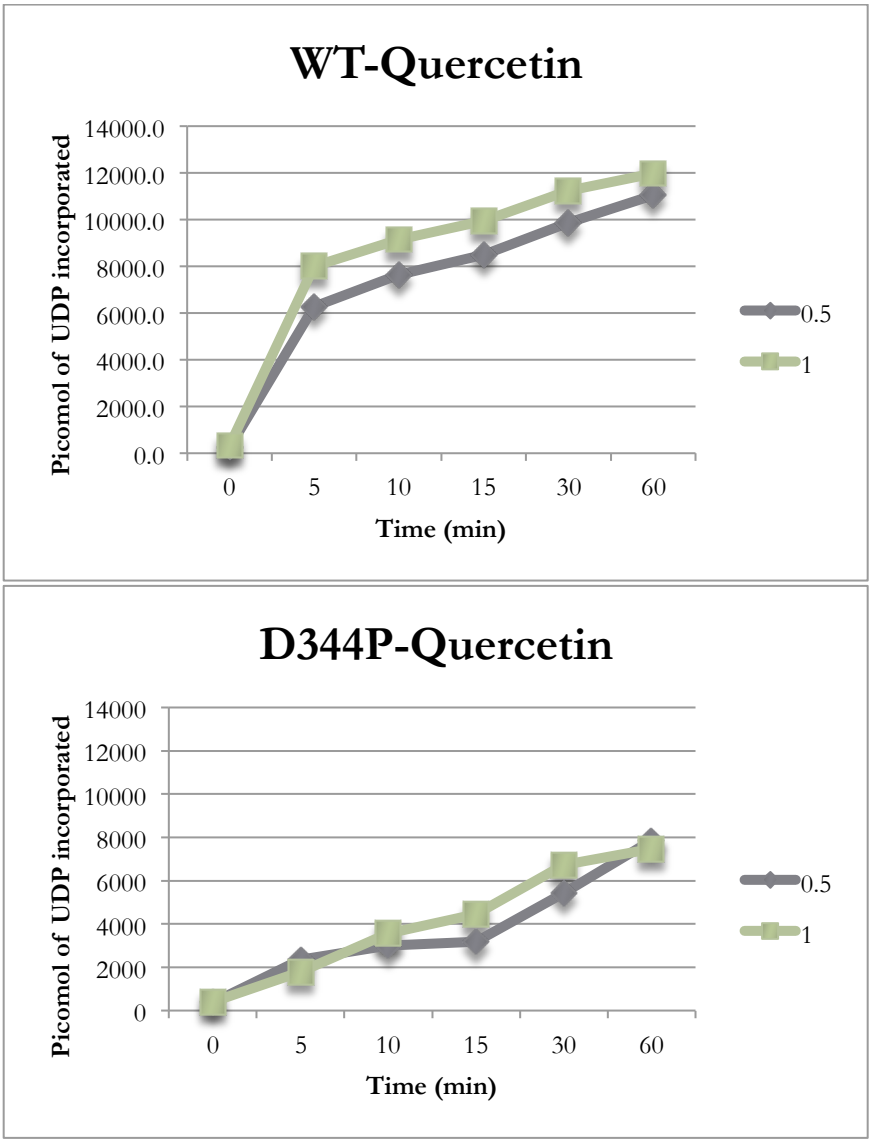

Figure 6: Time course assay for quercetin. The grey line represents 0.5 microgram of enzyme per reaction and the green line represents 1.0 microgram of enzyme per reaction.

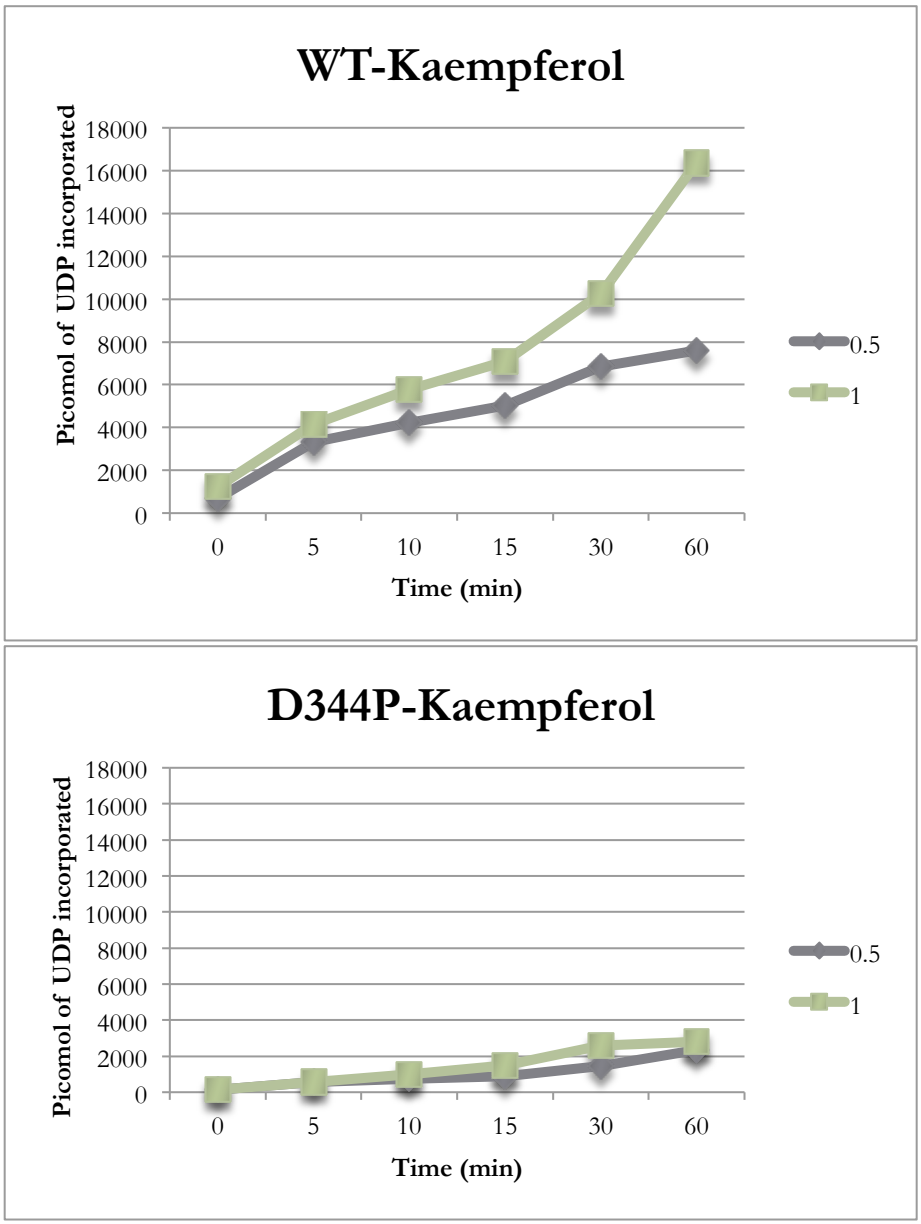

Figure 7: Time course assay with Kaempferol. The grey line represents 0.5 microgram of enzyme per reaction and the green line represents 1.0 microgram of enzyme per reaction.

Fisetin continued to show the same trends as seen with quercetin and kaempferol (Figure 8). The WT enzyme showed the activity with fisetin similar to those described previously (Owens and McIntosh, 2009). The WT reactoin remained linear for 5 minutes. The apparent specific activity of the 1.0 micrograms reacton was 485.84

pmol/min/μg. For 0.5 micrograms of WT enzyme, the apparent specific activity was 655.68 pmol/min/μg. The D344P enzyme had incorporation occur at a lower level than the wild-type. The reaction of the mutant was linear for 5 min. The mutant had a specific activity of 99.32 pmol/min/μg for 1.0 micrograms of enzyme and 125.32 pmol/min/μg for 0.5 micrograms of enzyme. The activity of the mutant can be confirmed to be reduced for this substrate, the mutant having around 5x less activity than the WT.

Gossypetin, the last flavonol tested in the screening, showed similar results to the other flavonols. The WT was not tested for this mutant due to sufficent characterization in previous research (Owens and McIntosh, 2009; Devaiah et al, 2017; Kandel, 2016). For the mutant, the reaction was linear for 5 min, and the specific activity of 0.5 micrograms of mutant enzyme is 532.77 pmol/min/μg and for 1.0 micrograms of enzyme specific activity is 225.99 pmol/min/μg. The mutant transferred glucose from UDP at low levels, supporting the decrease of activity for the enzyme, meaning the change in structure affected the enzyme's ability to function.

The screening assay suggested that the mutant could act on diosmetin. Upon examination of the time course assay, it became apparent that the activity of the mutant with diosmetin was a false positive. No incorporation with diosmetin occurred with the wild-type or the mutant enzyme assay (Figure 10). The lack of incorporation shows a lack of a broadened substrate specificity in the flavone subclass. The mutation hence did not cause a structural change that broadened substrate specificity to include flavones.

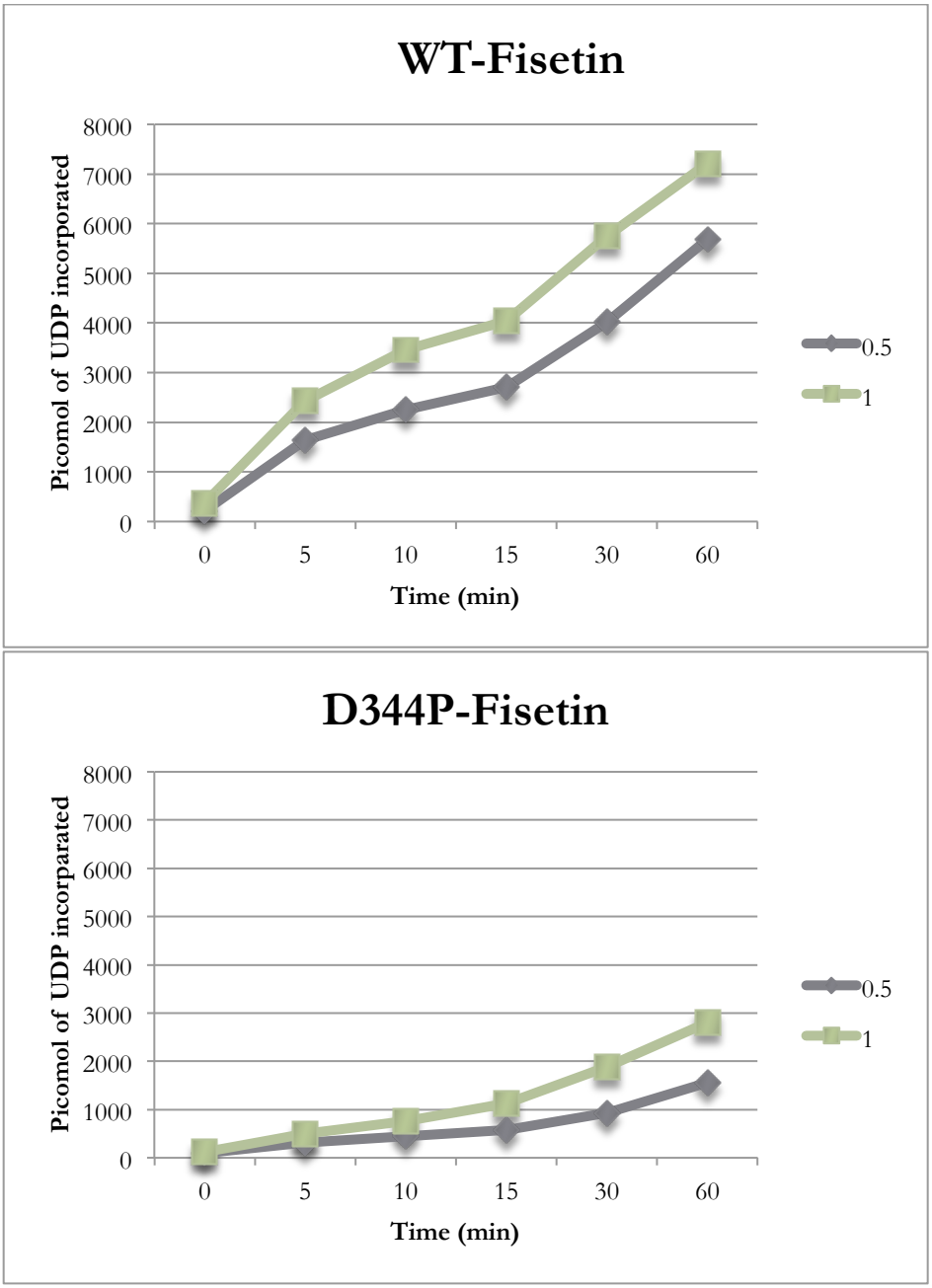

Figure 8: Time course assay with Fisetin. The grey line represents 0.5 microgram of enzyme per reaction and the green line represents 1.0 microgram of enzyme per reaction.

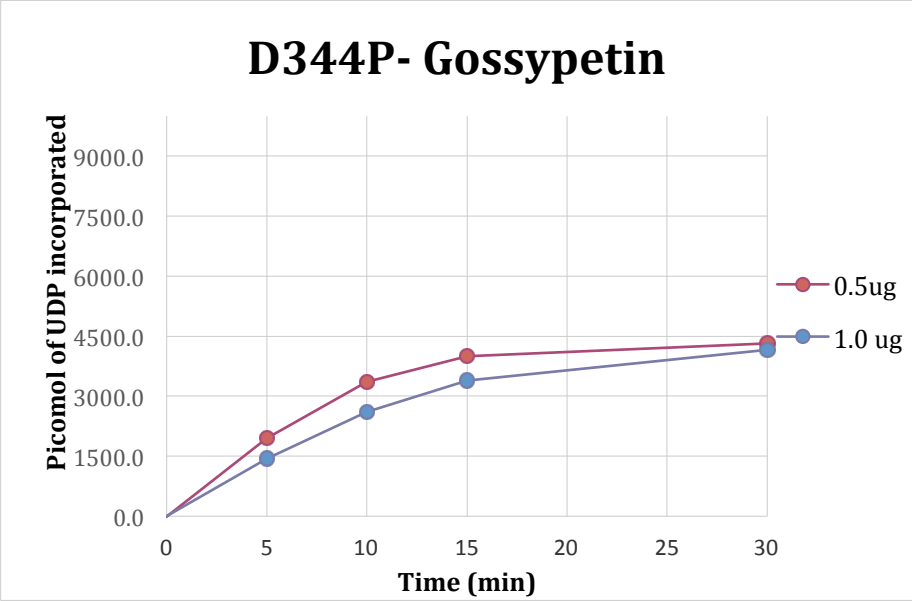

Figure 9: Time course assay with gossypetin. The red line represents 0.5 microgram of enzyme in the reaction and the blue line represents 1.0 microgram per reaciton. The reaction only goes to thirty minutes do to a loss of reaction mixture.

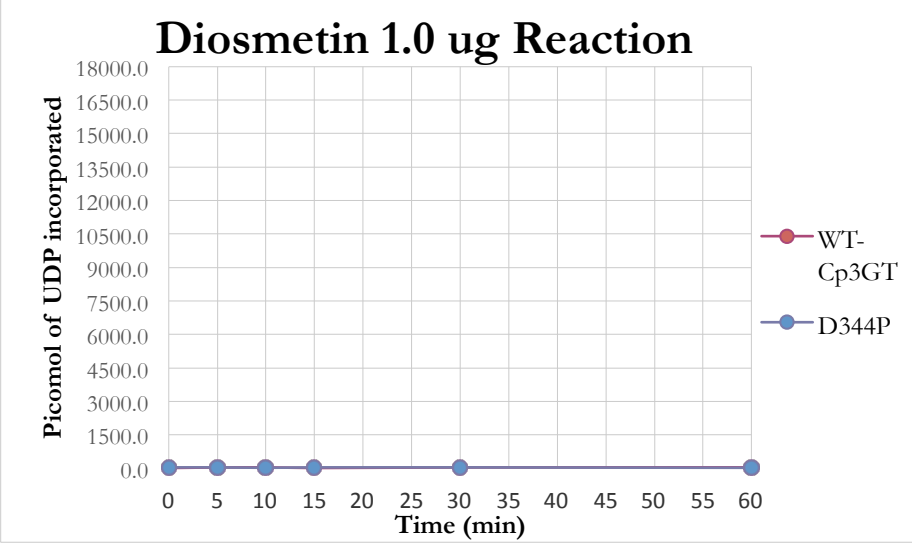

Figure 10: Time course assay with diosmetin. Note the lack of activity.

Other flavonoids tested in time course assays were the flavanone substrates isosakuranetin and hesperetin. Isosakuranetin (Figure 11) did not show any incorporation. Hesperetin (Figure 12) produced the same lack of incorporation. The mutant was unable to transfer glucose to either of the flavanone substrates supporting a lack of broadened specificity with this subclass.

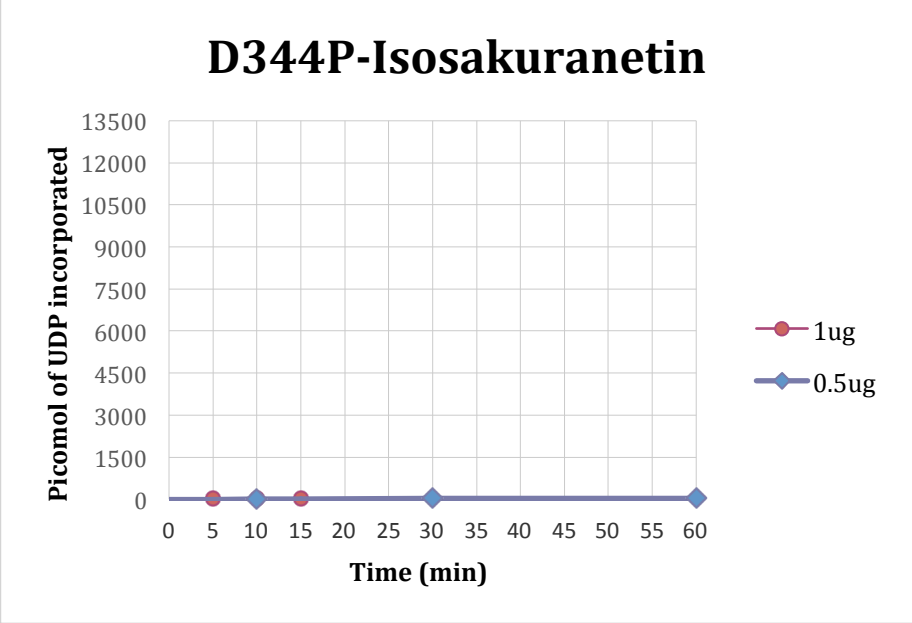

Figure 11: Time course assay with isosakuranetin. No activity was detected

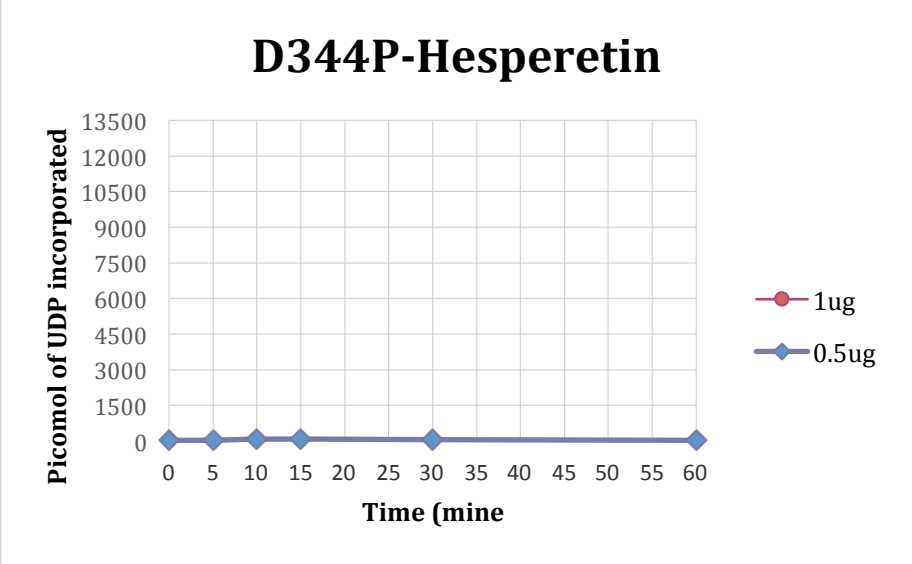

Figure 12: Time course assay with hesperetin. No activity was detected.

Overall, the time course assays were able to determine that D344P had decreased activity as compared to the wild-type and had no broadening of substrate specificity. The mutant retained the flavonol specificity of the wild-type and had a decreased activity with these substrates as compared to the wild-type.

In Silico Analysis of Enzyme Structure

To understand what is happening with substrate binding at the catalytic site, in silico docking analysis was used. Using the crystallized VvGT1 structure as a base, homology models of the substrate binding site of the wild-type Cp-F3-*O*-GT and mutant D344P were created. For each substrate for each enzyme, 9 models were created and were ranked based off of energy minimization. The catalytic residues important to substrate binding in VvGT1 were found to be His 20, Ser 18, and Asp 119 (Offen et al, 2006). These residues can be translated into the Cp-F3-*O*-GT and D344P models as His 22, Ser 20, and Asp 122. His 22 is thought to be the key for allowing the glucosylation to occur at the hydroxyl group on the third carbon, Ser 20 allows for stability in the binding of the substrate to the active site, and Asp 122 helps the reaction with the substrate and His 22 to occur. Additionally, a distance of under 5 Angstroms is preferred for catalytic activity to occur in VvGT1, so it was used as a credential for activity (Offen et al, 2006).

The first docking substrate used on the wild-type and mutant enzyme models was quercetin (Figure 13). The wild-type is known to react with quercetin. This reaction preference is supported by the distances under 5 A° between the catalytic residues and the quercetin substrate in the model.

The first mutant model generated did not have distance under 5 A° for any of the three catalytic residues (Figure 14). The distance of Ser 20 to the substrate was 6.966 A°, the distance of Asp 122 to His 22 was 5.354 A°, and the distance between the 3 hydroxyl group of quercetin and His 22 was 6.966 A°. Based off of these distances, it would appear that the model would predict no activity would occur for the mutant enzyme, but clear incorporation was observed by D344P with quercetin in the time course assay. The

incorporation seen in the time course assay would mean that the most energy minimalized model does not correctly portray the reaction occurring or that factors such as the binding of the sugar donor or distance of the sugar donor form the acceptor substrate are able are such that a reaction could still occur.

In comparison, the ninth model generated by the modeling program for D344P and quercetin shows at that the distance between His 22 and quercetin is 2.610 A° (Figure 15). Because the His 22 distance falls under 5 A° and the Ser 20 and Asp 122 distances are close to 5 A°, this model could potentially be a more accurate description of the binding occurring at the catalytic site. Because the Cp-F3-*O*-GT protein is a globular, fluid molecule, its shape can move allowing for residue distance to fall under the 5 A° distance needed for reaction preference to occur. Additionally, the distances of the acceptor substrate in the binding pocket could affect the reactive capabilities of the model. The ability of the mutant enzyme to transfer glucose from UDP-glucose to quercetin at a 6-fold lower specific activity can be explained by its potentially longer binding distances between the catalytic residues and the substrate. Along with conditions such as donor substrate binding distance, the fluidity of the molecule could allow the reaction to occur even though two of the three the acceptor substrate binding distances are slightly over 5 A°.

The enzymes docked with kaempferol revealed a similar trend (Figure 16). WT contains distances under 5 A° supporting its ability to glucosylate kaempferol. Though the distances are all under 5 A° for both the WT with quercetin and kaempferol, factors not explored in these models such as distance from UDP-glucose could explain the WT ability to glucosylate quercetin at a higher rate than kaempferol.

The first model of the mutant with kaempferol had distances over 5 A° with all substrates (Figure 17). His 22 had a distance of 6.077 A°, Ser 20 6.804 A°, and Asp 122 5.354 A°. Enzyme fluidity could allow for the distances to fall below 5 A°, but since the distances between the substrate and His 22 and Ser 20 are large, catalytic activity from this model would most likely come from unexamined factors such as donor distance.

The fifth model of D344P and kaempferol generated a model containing more favorable distances (Figure 18). His 22 had a distance of 3.600 A° from kaempferol. Asp 122 and Ser 20 had distance of 5.354 A° and 5.200 A°, respectively. Enzyme fluidity could allow the Asp 122 and Ser 20 distances to fall under 5 A° supporting its ability to glucosylate kaempferol but at a lower efficiency, a 10-fold lower specific activity, than the WT. These distances make the fifth model more favorable for depicting D344P's binding with kaempferol.

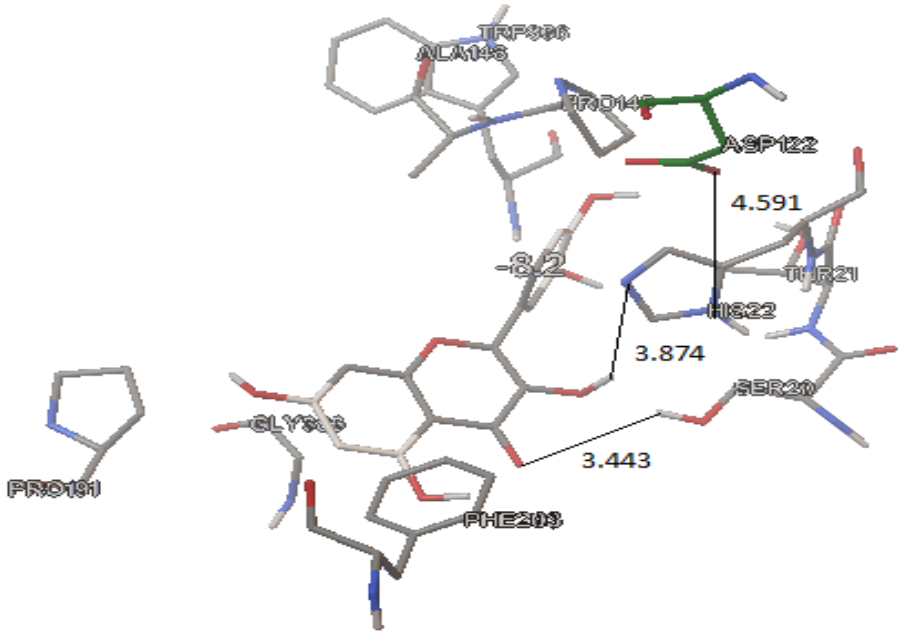

Figure 13: Homology model of Cp-F3-*O*-GT WT enzyme catalytic region docked with quercetin.

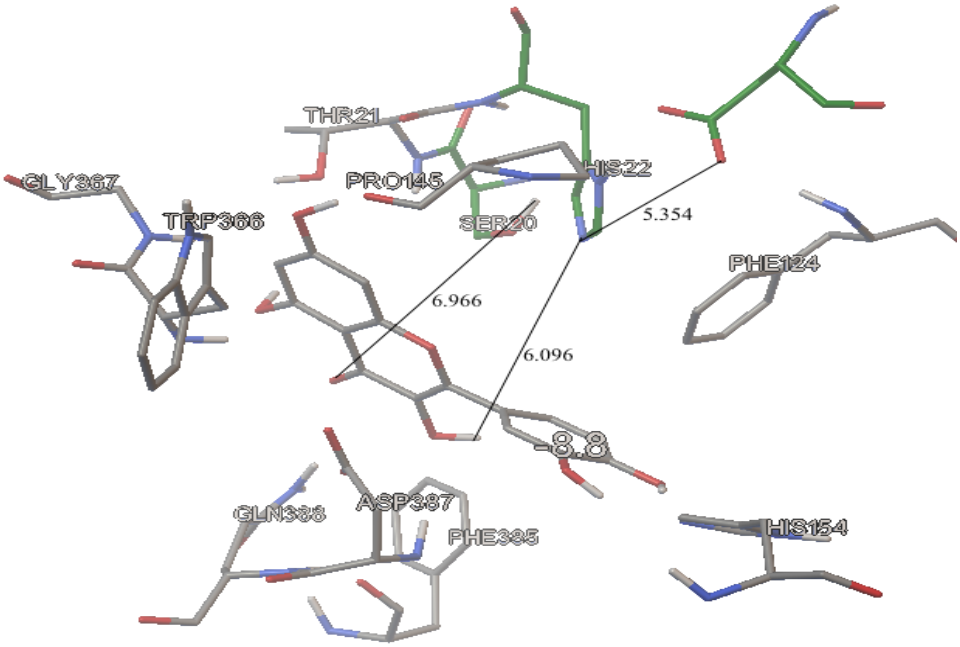

Figure 14: First model of the mutant D344P docked with quercetin. Though this is the most energy minimized model, the distances of the substrate from the residues suggests a lack of a reaction.

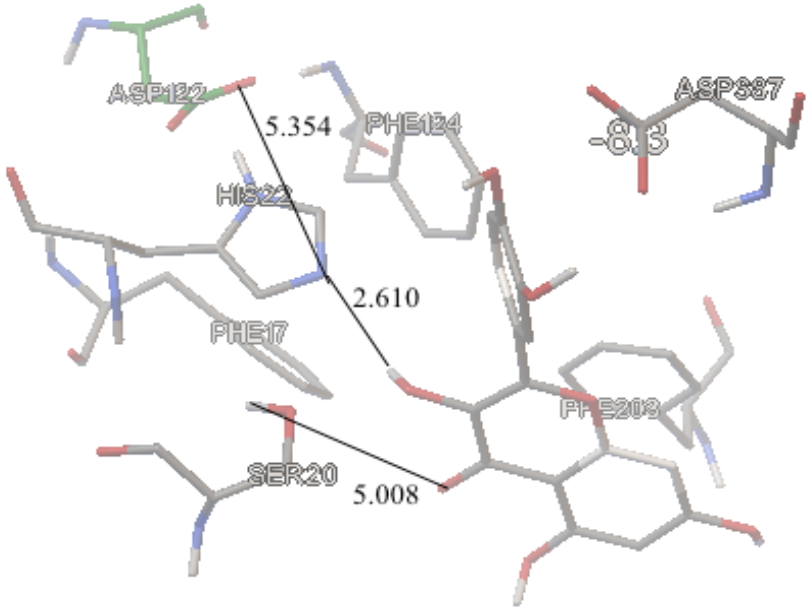

Figure 15: Modeling of the catalytic region of mutant D344P docked with quercetin. This model produced the most favorable distances for the reactivity in D344P.

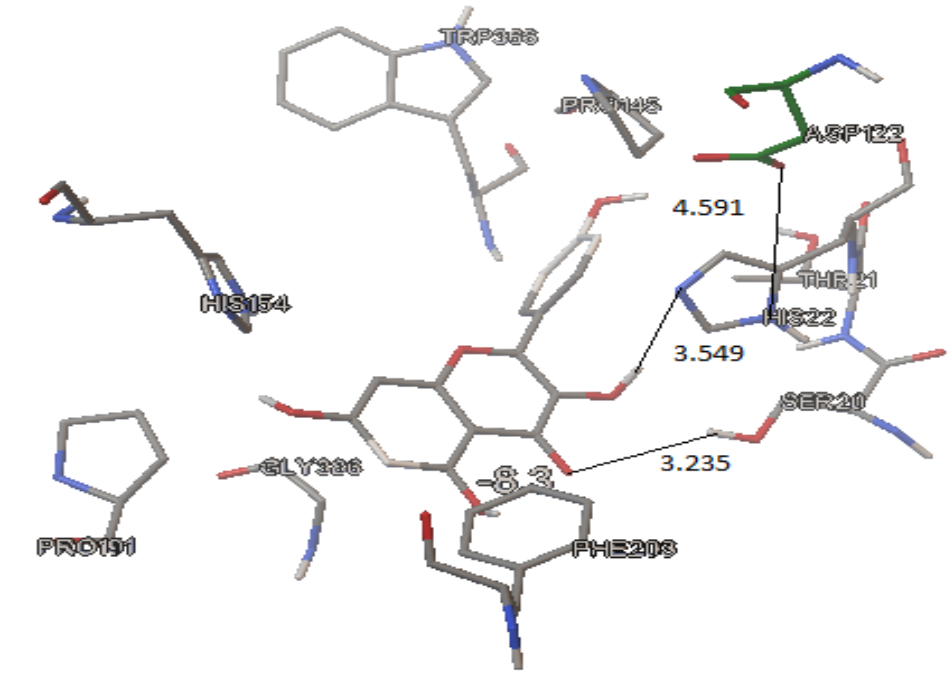

Figure 16: Homology model of the WT catalytic site docked with kaempferol.

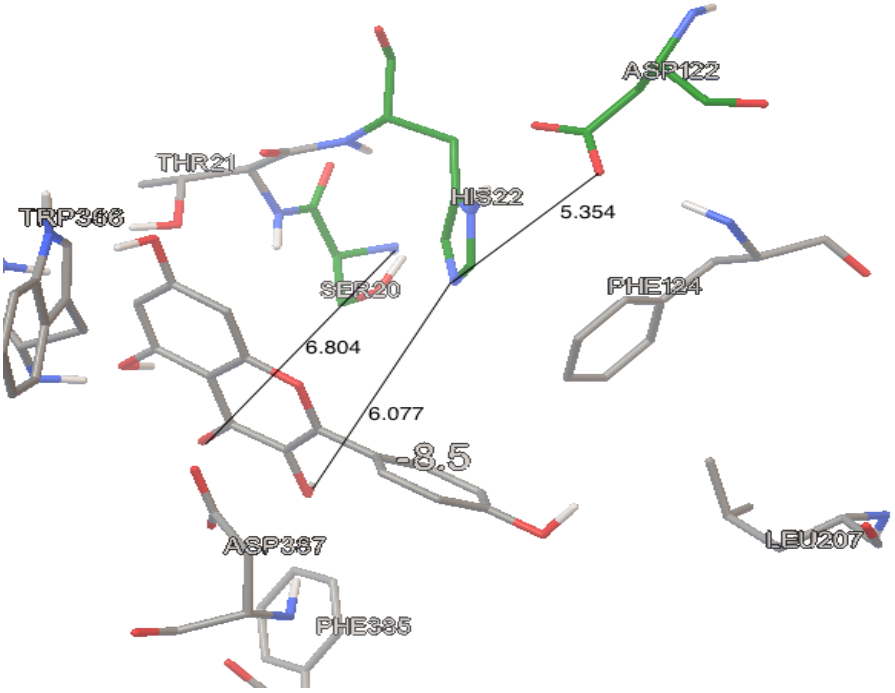

Figure 17: First modeling of the catalytic region of mutant D344P docked with kaempferol. Though it was presented as the first possible model, distances are over the 5 A° distance indicating a reaction does not occur.

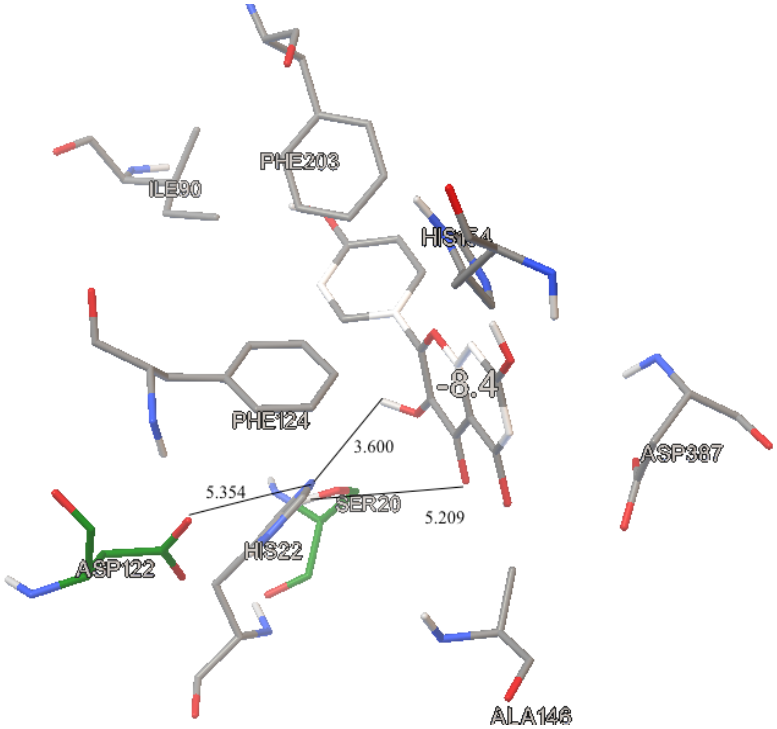

Figure 18: Homology model number 5 of mutant D344P docked with kaempferol. The distance between His 22 and the 3 hydroxyl group of kaempferol is under A°, making it a more favorable model of the reaction.

The results of these models reveal that the mutation likely changed the tertiary structure of Cp-F3-*O*-GT. This change is evident in the different predicted distances of His 22, Ser 20 and Asp122 between the WT and mutant models. The change in structure did not allow for substrate specificity broadening and decreased the mutant's activity with flavonols.

Summary and Directions for Future Research

To understand the relationship between structure and function in GT substrate specificity, mutant D344P of Cp-F3-*O*-GT was created by comparing the amino acid sequences and structures of Cp-F3-*O*-GT and VvGT1. It was hypothesized that mutant D344P would have an expanded substrate specificity due to a structural change caused by replacing an aspartate with a proline. The initial screening assays suggested that the mutant D344P had potential expanded activity with diosmetin (flavone) and isosakuranetin and hesperetin (flavanones). Time course confirmational assays revealed that these potential substrates identified by initial screening were false positives and that the mutant D344P had reduced activity with the flavonol substrates. Homology models of the mutant D344P and wild-type Cp-F3-*O*-GT support a change in the structure that affected acceptor substrate binding occurred. These results do not support the hypothesis originally proposed.

Further stud of the mutant D344P can continue to reveal the information related to the structure/function relationship of the mutation to the enzyme Cp-F3-*O*-GT. Understanding the structure/function relationship can lead the way for the development of custom enzymes for novel product creation. The effects of the mutation D344P can be further analyzed through several methods. First, performing high-performance liquid chromatography on reaction products could provide insight into the exact compounds being made. Product analysis would allow for aspects of regiospecificity to be explored.

Additionally, determination of optimum pH, optimum reaction temperature, V_{max}, and K_m can elaborate more kinetic difference of the mutant compared to the wild-type. Understanding kinetic properties reveal more about how the reactions occur. For the

purposes of creating custom enzymes, kinetic information could determine if a product could be made efficiently.

Finally, crystallization of Cp-F3-*O*-GT and D344P would give a definite structure of the Cp-F3-*O*-GT and mutant enzymes. Co-crystallizing the enzymes with acceptor and donor substrates would allow the distances of catalytic residues and desired substrate for a given conformation. It would allow for a deeper analysis unobtainable with homology modeling.

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