5-2012

Cloning, Expression, and Biochemical Characterization of Recombinant Putative Glucosyltransferases Clone 3 and 8 from Grapefruit (Citrus paradisi)

Deborah Hayford
East Tennessee State University

Cloning, Expression, and Biochemical Characterization of Recombinant Putative Glucosyltransferases Clone 3 and 8 from Grapefruit (*Citrus paradisi*)

A thesis

presented to

The faculty of the Department of Biological Sciences

East Tennessee State University

in partial fulfillment

of the requirement for the degree

Master of Science in Biology

---

by

Deborah Hayford

May 2012

Cecilia A. McIntosh, PhD, Chair

Dhirendra Kumar, PhD

Daniel K. Owens, PhD

Ranjan Chakraborty, PhD

Key words: Flavonoids, *Citrus paradisi*, Glucosyltransferase, Inclusion Bodies, Phenolics

ABSTRACT

Cloning, Expression, and Biochemical Characterization of Recombinant Putative Glucosyltransferases Clone 3 and 8 from Grapefruit (*Citrus paradisi*)

by

Deborah Hayford

The grapefruit plant, *Citrus paradisi*, tends to accumulate high levels of flavonoid glycosides such as flavanones and flavones. Flavonoids have a vast array of important functions in plants and also in humans. Glucosyltransferases (GTs) are enzymes responsible for glucosylation reactions. In our pursuit to study the structure and function of flavonoid GTs, we have used molecular approaches to identify, clone, express, and functionally characterize the enzymes. This research was designed to test the hypothesis that PGT3 is a flavonoid glucosyltransferase and is subject to biochemical regulation. PGT3 has been tested for GT activity with compounds representing subclasses of flavonoids as well as some simple phenolics. Results indicate GT activity with 6 substrates, p-hydroxybenzoic acid, vanillin, vanillic acid, p-hydroxyphenylpyruvate, gentisic acid, and catechol. A second project designed to clone putative PGT8 into the *Pichia* expression system has been completed.

ACKNOWLEDGEMENTS

I first and foremost want to thank God for the gift of life. I would want to extend my sincere thanks to my graduate committee members Dr. Cecilia McIntosh, Dr. Ranjan Chakraborty, Dr. Dhirendra Kumar, and Dr. Daniel Owens for their profound guidance and advice throughout my research.

I would also want to express my appreciation to Dr. Cecilia McIntosh for her mentoring, guidance, and support throughout the entire research. Her continuous encouragement, advice, and instruction helped me to complete this thesis research. I would also want to thank Dr. Daniel Owens for teaching me most laboratory techniques and also for his advice and suggestions throughout this research. I would also want to thank the faculty of biology department and all graduate students both past and present for support and encouragement during the past 2 years. My warmest thanks go to my family for the support, advice and love they have given me. Last but not the least, I would want to thank Daniel Ahyia Oduro for his care and love that kept me strong throughout this masters program.

CONTENTS

Page

ABSTRACT 2

ACKNOWLEGMENTS 3

LIST OF TABLES 12

LIST OF FIGURES 14

Chapter

1. INTRODUCTION 17

Plant Secondary Metabolites 17

The Flavonoids 18

Roles of Flavonoids in Plants 21

Flavonoids and Human Health 26

Flavonoid Biosynthesis 27

B ring Synthesis 28

A ring Synthesis 29

Modification of Flavonoids 32

Glycosylation of Flavonoids 32

Importance of Glycosylation 33

Glucosyltransferases 35

Citrus paradisi Glucosyltransferase Research 39

Chapter Page

Hypotheses ……41

2. MATERIALS AND METHODS……..42

Materials……42

Chemicals and Reagents…….....42

Cells and Vectors .....……43

Kits.. .....……43

Buffers……..43

Culture Media……..44

Flavonoid and Other Phenolic Substrates for Enzyme Assays …….....44

Methods ……45

Heterologous Expression of Recombinant PGT3 in *E.coli*……...45

Optimizing the Expression of Recombinant PGT3 in pCD1 Vector in *E.coli* …….45

Preparation of Total and Soluble Protein Fractions ……..46

Analysis by SDS-PAGE……...47

Western Blotting…… 48

Expression with Varying Concentrations of IPTG……..49

Chapter Page

Culture Scale-Up……50

Purification of Recombinant PGT3 Protein by IMAC ……50

Silver Staining……52

Cloning of PGT3 into *Pichia* pastoris Vectors……52

Primer Design……52

Amplification of PGT3……..55

Cloning of Modified PGT3 into TOPO VECTOR……57

Analyzing Transformants……...57

Selected Colonies for Constructs 1, 2, and 3……58

Isolation of Plasmid DNA by Minipreps……...58

Restriction Digests of Plasmid Constructs and Vectors……59

Gel Purification of PGT3 Constructs and Vectors……60

Cloning PGT3 into Expression Vectors, pPICZA and pPICZAα……60

Ligation of pPICZ Vectors and PGT3 Inserts …….....61

Selection of Positively Transformed Colonies……..63

Midiprep of Vectors and Designated Clones for Constructs 1, 2, and 3……...65

Chapter Page

Phenol: Chloroform Extraction……66

Transformation into *Pichia pastoris*……..67

Transformation by Electroporation……68

Direct PCR Screens of *Pichia* Transformants……...69

Expression of Recombinant PGT3 Protein in *Pichia pastoris*……...69

Test Inductions for Expression of PGT3 Protein……...70

Preparation of Samples for Analysis ……71

Analysis by SDS-PAGE and Western Blot ……..71

Scale-Up of Expression ……72

Test Purification using Talon Resin……...73

Screening rPGT3 Protein for GT Activity ……...74

Concentrating using Centricon-30……74

Preparation of Substrates and UDP -^{14}C glucose……...75

Screening of rPGT3 Protein using Flavonoids as Substrates……76

Expression of PGT8 in *Pichia pastoris*……79

Chapter Page

Cloning of PGT8 into *Pichia* pastoris Vectors……79

Primer Design……..79

Amplification of PGT8……80

Cloning of Modified PGT8 into TOPO Vector……80

Analyzing Transformants……...81

Isolation of Plasmid DNA by Minipreps……...81

Restriction Digests of PGT8 Plasmid and pPICZA Vector……...82

Gel Purification of PGT8 Construct and pPICZA Vector……82

Cloning PGT8 into Expression Vector, pPICZA……..83

Ligation of pPICZA Vector and PGT8 ……83

Selection of Positively Transformed Colonies……..84

3. RESULTS……85

Optimizing the Expression of Recombinant PGT3 in pCD1 Vector in *E.coli* ……85

Expression With and Without Betaine and Sorbitol……85

Expression with Varying Concentrations of IPTG…… 86

Enrichment/Purification of Soluble Recombinant PGT3 Protein using IMAC……88

Chapter Page

Cloning PGT3 into pPICZ Vectors for Expression in *Pichia pastoris*……. 89

Isolation of PGT3 and pPICZ vectors…….. 89

Modification of the Ends of PGT3 from pCR®4-TOPO Vector…….. 90

Cloning Modified PGT3 into TOPO……. 91

Restriction Digests of Plasmid Constructs ……92

Gel Purification of PGT3 Constructs and Vectors…… 97

Ligation of pPICZ Vectors and PGT3 Inserts for Constructs 1, 2, and 3……..98

Midiprep of Vectors and Designated Clones for Constructs 1, 2, and 3…….104

Transformation into *Pichia pastoris*……106

Expression of Recombinant PGT3 Protein in Yeast……107

Purification of rPGT3 using Immobilized Metal Affinity

Chromatography (IMAC)……109

Screening rPGT3 Protein for GT Activity……. .111

Screening rPGT3 Protein Using Flavonoid and Simple Phenolic

Substrates……112

Cloning PGT8 into pPICZA Vector……125

Chapter Page

Isolation of PGT8 and pPICZA Vector……..125

Modification of the Ends of PGT8 from pCR®4-TOPO Vector……126

Cloning Modified PGT8 into TOPO……...126

Gel Purification of PGT8 Construct and pPICZA Vector…….130

Ligation of pPICZA with PGT8 Construct……...131

4. DISCUSSION……133

Expression and Characterization of PGT3……..135

Cloning PGT8 into pPICZA Vector……140

Directions for Future Research……140

REFERENCES……143

APPENDICES…….157

APPENDIX A: Abbreviations……157

APPENDIX B: Stock Solutions and Media Recipes……...159

APPENDIX C: Staining Solution Recipes……..165

APPENDIX D: Gel Recipes……166

APPENDIX E: Buffer Recipes……167

APPENDIX F: Sequences……………………………………………………………172

VITA……………………………………………………………………………………173

LIST OF TABLES

Table Page

1. The 6 Most Commonly Found Anthocyanins…….....22

2. PGT3 Clone Primer Sequences Designed For PCR Amplification……..54

3. PCR Conditions Used for Amplification of PGT3 sequences……...55

4. Reaction Mixtures for Ligation of pPICZA Vector and PGT3-A9 Insert…….61

5. Reaction mixtures for Ligation of pPICZAα1 Vector and PGT3-B15 Insert……62

6. Reaction mixtures for Ligation of pPICZAα2 Vector and PGT3-C15 Insert……62

7. Amount of Plasmid DNA Quantified from Midipreps……..66

8. Flavonoid and Simple Phenolic Substrates Screened with rPGT3 for Activity……76

9. RP-HPLC Conditions used for rPGT3 Activity Assay with Phenolic Compounds……..78

10. PGT8 Clone Primer Sequences Designed For PCR Amplification…….79

11. PCR Conditions Used for Amplification of PGT8 sequence……...80

12. Reaction Mixtures for Ligation of pPICZA Vector and PGT8 insert……..83

13. Protein Microassay of Expressed rPGT3 at 0-96 hr Postinduction……...109

14. Screening Activity of rPGT3 using Flavonoid and Simple Phenolic Substrates……..114

Table Page

15. Retention Time of Standards, Experimental groups and Controls for

Simple Phenolic Compounds Tested……117

LIST OF FIGURES

Figure Page

1. The Carbon Skeleton Structure of Flavonoids Without Modification ……. 18

2. The Chemical Structures of the Different Classes of Flavonoids. ….....19

3. The Anthocyanidin Skeleton Structure ……. ……. 22

4. The Synthesis of 4-coumaroyl CoA from Phenylalanine …… 28

5. Formation of Naringenin Chalcone …….. …….. 29

6. Flavonoid Biosynthetic Pathway …….. ……...30

7. Synthesis of Flavanone Neohesperidosides and Rutinosides in Citrus…….35

8. Formation of Quercetin 3-O-Glucoside by a Flavonol-Specific-3-O-GT…….36

9. Alignment of PSPG Box Sequences from Grapefruit Putative Glucosyltransferase Clone 3 and other Plant Glucosyltranferases…….38

10. Schematic Diagram Showing Cloning of Construct 1 ……53

11. Schematic Diagram Showing Cloning of Construct 2 ……54

12. Schematic Diagram Showing Cloning of Construct 3 ……55

13. Expression Levels of PGT3 in LB Media Containing Betaine and Sorbitol. …….86

14. Expression Levels of rPGT3 in LB Media without Betaine and Sorbitol……...86

15. Expression Levels of Recombinant PGT3 under Varying Concentrations of IPTG……...87

16. Enrichment of rPGT3 using 1 mL Metal Affinity Chromatography Column (IMAC……88

17. Enrichment of rPGT3 using 5 mL Imobilized Metal Affinity Column (IMAC).....89

18. Miniprep of PGT3 Plasmid, pPICZA and pPICZAα Vectors from pCR4-TOPO/TOP10…..90

19. Modified and Amplified PGT3……91

20. Confirmation of Modifed PGT3-TOPO Transformed Colonies by PCR Screens……..92

Figure Page

21. Restriction Digests of PGT3-TOPO Plasmid DNA……93

22. Alignment of PGT3 Sequence with PGT3-TOPO (construct1) Sequence using Bioedit……94

23. Alignment of PGT3 Sequence with PGT3-TOPO (construct2) Sequence using Bioedit……95

24. Alignment of PGT3 Sequence with PGT3-TOPO (construct1) Sequence using Bioedit……96

25. Restriction Digests of PGT3-TOPO Constructs and pPICZ Vectors for DNA Plasmid Purification……97

26. Gel Purified PGT3 Inserts and pPICZ Vectors Prior to Ligation…….....98

27. DNA Agarose Gel Electrophoresis for Verification of Transformed pPICZ Vectors Containing PGT3 Inserts using PCR Screening…….....99

28. Verification of Presence of PGT3 Insert in pPICZ Vectors using Restriction Digestions……100

29. Alignment of PGT3 Sequence with PGT3-pPIPCZA (construct1) Sequence using Bioedit……...101

30. Alignment of PGT3 Sequence with PGT3-pPICZAα (construct2) Sequence using Bioedit……...102

31. Alignment of PGT3 Sequence with PGT3-pPIPCZAα (construct2) Sequence using Bioedit……...103

32. Completely Linearized Empty Vectors and Vectors containing rPGT3……...105

Figure Page

33. DNA Agarose Gel Electrophoresis Showing Purification of Plasmid DNA by Phenol: Chloroform Extraction……...106

34. DNA Gel Electrophoresis of Transformed *Pichia pastoris* Strain X33 Containing pPICZ/PGT3 Constructs……..107

35. Expression of rPGT3 (using Construct1) in *Pichia pastoris*……108

36. Silver Stained SDS-PAGE Analysis Showing Purification of rPGT3 Expressed in *Pichia pastoris*……110

37. Western Blot Analysis Showing Detection of rPGT3 Expressed in *Pichia pastoris*……...110

38. Purification of rPGT3 Expressed in *Pichia pastoris* and Fractions……111

39. Representative Western Blot of Enriched rPGT3 Expressed in *Pichia pastoris*……112

40. HPLC Chromatograms for rPGT3 Enzyme Assays with 12 Phenolic Compounds……124

41. Miniprep of PGT8 from pCR4-TOPO/TOP10 and pCD1/TOP10……..125

42. Modified and Amplified PGT8……126

43. Confirmation of Modifed PGT8-TOPO Transformed Colonies by PCR Screens…….127

44. Verification of Presence of mPGT8 Insert in TOPO Vector……...128

45. Alignment of PGT8 sequence with PGT8-TOPO Sequence using Bioedit…….129

46. Restriction Digests of PGT8-TOPO Construct and pPICZA Vector for DNA Purification……130

47. Representative DNA Agarose Gel Showing Purified PGT8 Insert for Ligation with pPICZA Vector……131

48. Alignment of PGT8 sequence with PGT8-pPICZA sequence using Bioedit……...132

CHAPTER 1

INTRODUCTION

Plant Secondary Metabolites

There are 2 major classes of metabolites existing in plants. These are primary metabolites and secondary metabolites (Korkina 2007). Primary metabolites include carbohydrates, lipids, amino acids, and proteins that are necessary for basic cellular function and reproduction (Kutchan 2001 and ref. therein). Plants produce a variety of other organic compounds called secondary metabolites that have crucial roles in plant development as well as in the interaction of a plant with its biotic and abiotic environment (Kutchan 2001 and ref. therein).

Plant secondary metabolites have been described as being antibiotic, antifungal, antiviral, able to protect plants from pathogens (phytoalexins), and toxic to some plants (allelopathy) (Bourgard et al. 2001). They are also known to have important UV absorbing properties therefore preventing plants from damaging light (Li et al. 1993).

There are many types of compounds that are categorized as secondary metabolites. They are often classified under several major large molecular families such as the phenolics, alkaloids, and terpenoids (Bourgaud et al. 2001). There are about 30,000 terpenoids, 12,000 alkaloids, and over 10,000 phenolics of which flavonoids are a part (Martens et al. 2010 and ref. therein). Flavonoids represent a large family of low molecular weight secondary polyphenolic compounds that are widely distributed throughout the plant kingdom ranging from mosses to angiosperms (Koes et al. and ref. therein 1994). This important group of secondary metabolites has attracted the interest of many scientists over the years for several reasons such as the vast occurrence,

complex diversity, and numerous functions of the compounds (Forkman and Martens 2001; Martens et al. 2010 and ref. therein).

The Flavonoids

The flavonoids are a group of naturally occurring plant phenolic compounds with a basic C_6-C_3-C_6 carbon structure (McIntosh 1990 and ref. therein). This carbon skeleton contains 2 benzene rings (A and B) joined by a heterocyclic ring (C) (Fig. 1).

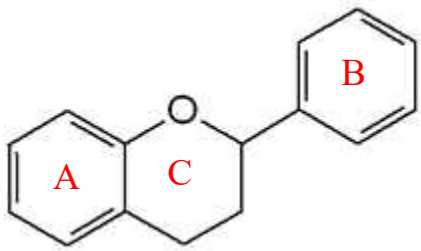


Figure 1. The Carbon Skeleton Structure of Flavonoids Without Modification. Rings A, B, and C are labeled.

The oxidation state of the heterocyclic ring and the position of ring B are important in the classification of flavonoids (Fig. 2). These classes include chalcones, aurones, flavanones, flavones, isoflavones, flavonols, dihydroflavonols, leucocyanidins, and anthocyanidins (Heller and Forkman 1993).

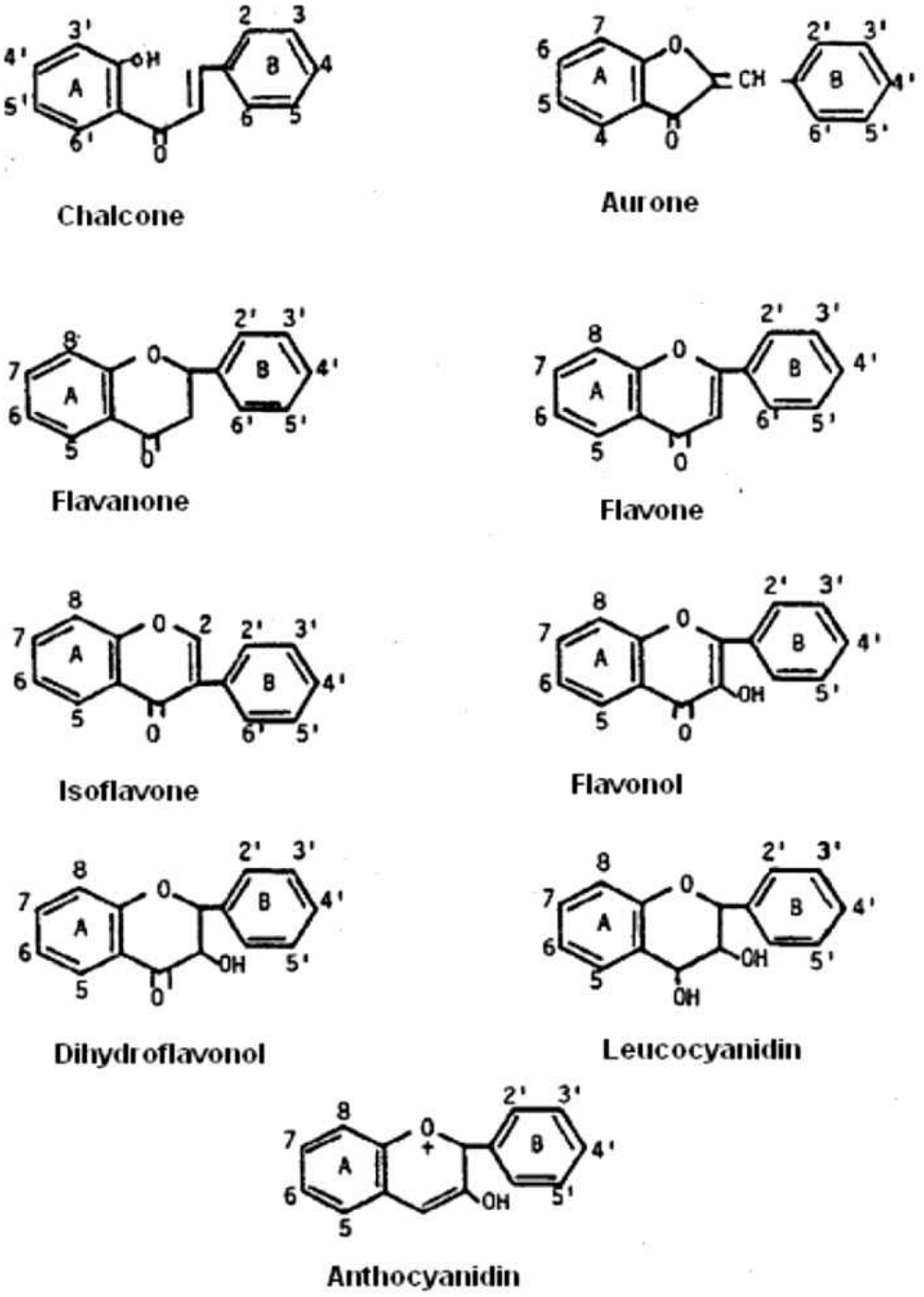


Figure 2. The chemical structures of the different classes of flavonoids. (McIntosh 1990 used with permission)

Among all the classes, the chalcones have been shown to be the first true flavonoid in the pathway (Heller and Forkman 1993 and ref. therein; Owens and McIntosh 2011 and ref. therein). Chalcone is the key step to flavoniod biosynthesis because chalcones are the precursor to the rest of the flavonoids. Chalcones can be isomerized into flavanones, and from these intermediates, different classes of flavonoids can be synthesized based on the oxidative state of the heterocyclic ring (Marais et al. 2007; Owens and McIntosh 2011 and ref. therein).

Another subclass of flavonoids, flavanones, is widely accumulated in citrus species (Jourdan et al. 1985 and ref. therein). Naringenin is one of the most common flavanones in citrus species and it exists in glycosidic forms (Kesterson and Hendrickson 1957). Naringin is accumulated mostly in grapefruit and pummel and is a major contributor to the bitterness in grapefruit and processed grapefruit products (Mansell et al. 1983 and ref. therein). Naringin is also known to account for 40 to 70% of the dry weight of young green grapefruit and leaves (Kesterson and Hendrickson 1957: Jourdan et al. 1985). Isoflavones are the largest group of naturally occurring isoflavonoids (Dewick 1993). Two of the naturally occurring plant isoflavones, genestein and daidzen, are found in soybeans and other leguminous plants where they function as phytoalexins (Jung et al. 2000). The isoflavonoids are a distinct subclass of flavonoids with a limited distribution in the plant kingdom (Harborne 1993; Livingstone et al. 2011 and ref. therein). Isoflavonoids are predominantly found in legumes (Liu et al. 2003).

Dihydroflavonols have a wide distribution in the plant kingdom and have been described in ferns, gymnosperms, and angiosperms (Bohm 1993 and ref. therein; Ashihara et al. 2010 and ref. therein). Dihydrokaempferol and dihydroquercetin are the most common members of the group (Harborne 1993). Quercetin, a flavonol, is known to have over 350 different conjugate forms in plants (Jones et al. 2003 and ref. therein). Aurones constitute the smallest subclass of

flavonoids (Harborne 1988). The distribution of flavones in plants is diverse, they can be found in various parts of plants, above and below ground, seeds, fruits, leaves, barks, stem, roots, and several other parts (Martens and Mithofer 2005). Apart from flavones being present in angiosperms, they have also been described within the gymnosperms (Harborne and Baxter 1999 and ref. therein).

Anthocyanins are plant colorants and are perhaps one of the most important groups of plant pigments that are soluble in water (Harborne 1993 and ref. therein). They are responsible for most of the pigmentation found in flowers and leaves and these pigmentation serve various functions (Gould et al. 2005 and ref. therein). They are predominantly found in solution within the vacuole (Pecket and Small 1980).

Roles of Flavonoids in Plants

Flavonoids can exist in both colored and colorless forms and the visual effect of color on humans and animals may be contributed by flavonoids (Brouillard and Dangles 1993). Chalcones and aurones give yellow and orange colors to tissues in which they are found (Ribereau-Gayon 1975). Furthermore, aurones have been shown to give more color to flowers than flavones (Brouillard and Cheminat 1988).

Flavonoids are involved in many critical processes in plants. Anthocyanins, a class of flavonoids, are glycosides of anthocyanidins (Kong et al. 2003 and ref. therein). There are 6 substitution patterns that can occur on the B ring (Fig. 3) (Kong et al. 2003). These substitution patterns influences the color of anthocyanins (Table 1). There are over 17 naturally occurring anthocyanidins (aglycones). Six of them are commonly found in higher plants (Table 1) and they are pelargonidin, peonidin, cyanidin, malvidin, petunidin, and delphinidin (Kong et al. 2003 and

ref therein). The glycosides of cyanidin, delphinidin, and pelargonidin are the most predominant in nature occurring in 80% of pigmented leaves, 69% of fruits, and 50% of flowers (Kong et al. 2003 and ref. therein).

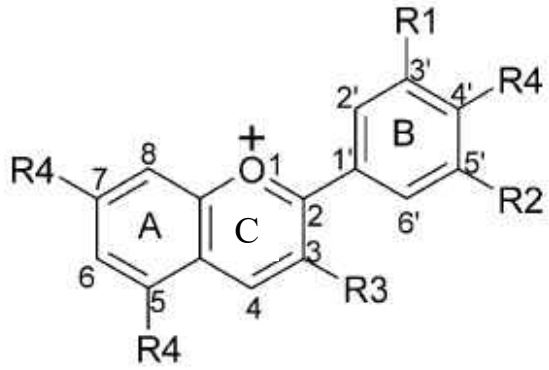


Figure 3. The Anthocyanidin Skeleton Structure. R1 and R2 are OH, H or OMe, R3 is H or a Glycosyl and R4 is Glycosyl or OH (Kong et al. 2003 and ref. therein).

Table 1. The 6 Most Commonly Found Anthocyanins (Adapted from Kong et al. 2003)

Name	Abbreviation	3	5	6	7	3^1	4^1	5^1	Color
Perlagonidin	Pg	OH	OH	H	OH	H	OH	H	O.red
Peonidin	Pn	OH	OH	H	OH	OMe	OH	H	O.red
Cyanidin	Cy	OH	OH	H	OH	OH	OH	H	O.red
Malvidin	Mv	OH	OH	H	OH	OMe	OH	OMe	B.red
Petunidin	Pt	OH	OH	H	OH	OMe	OH	OH	B.red
Delphinidin	Dp	OH	OH	H	OH	OH	OH	H	B.red

Note: B.red indicates bluish red and O.red indicates orange red.

A commonly known physiological function of anthocyanin pigments and flavonol copigments is providing beautiful pigmentation in flowers, fruits, and leaves (Winkel-Shirley 2001 and ref. therein; Gould et al. 2005 and ref. therein). This coloration aids in the attraction of pollinators and seed dispersers (Winkel-Shirley 2001 and ref. therein; Gould et al. 2005 and ref.

therein). An important function of these pigments is to attract insects and other animals that may help in cross pollination (Brouillard 1993). The unique structure and combination of various flavonoids in different plant species produce visible and UV reflection spectra that can be detected by insects and larger animals, facilitating successful pollination and fertilization (Yu et al. 2006). Flavones can also be used as copigments of delphinidin derivatives in blue flowered plants and this contributes to the attraction of pollinators (Harborne and Williams 2000 and ref. therein).

Anthocyanins have also helped in the understanding of the phenomenon of cosuppression, particularly in *Petunia hybrida* (Winkel–Shirley 2001 and ref. therein). Cosuppression can be defined as the silencing of a gene by the presence of a related gene (Melche 1997). An example is shown in *Petunia hybrida* where transformation of Petunia with a chalcone synthase gene led to the formation of petunia flowers with less pigmentation (Van der Krol et al. 1990 and ref. therein). Historically, flavonoids have been an attractive research subject. The eye catching anthocyanin pigments have been very useful for genetic experiments including Gregor Mendel's study of the inheritance of genes where the traits seed coat color and flower color were significant to the study of genetics (Schijlen et al. 2004). Anthocyanins can also act as antioxidants, phytoalexins, and as antibacterial agents (Kong et al. 2003).

Anthocyanins may also play important roles with other flavonoids such as in the resistance of plants to insect attack (Harborne 1988; Simmonds, 2003 and ref.therein). An example was shown in cotton leaves where cyanidin 3-glucoside, the most widespread anthocyanin imparted protection against tobacco budworm (Hedin 1983 and ref. therein). Another example reviewed in Simmonds, 2003, anthocyanin was associated with the expression of red spots in sorghum upon attack by aphids serving as a response mechanism to offer

resistance against further attack. Anthocyanins have been shown to be electron donors for reactive oxygen species example hydrogen peroxide (Casati and Walbot 2003 and ref. therein). This shows that anthocyanins have the potential for in vitro activity. Studies have been conducted to investigate the range of sensitivity of various bacterial strains to flavonoids by Ulanowska et al. 2006. Flavonoids from various subgroups were used in this study such as isoflavones (genisten and daidzein), flavones (apigenin), flavanone (naringenin), and flavonol (kaempferol). Effects of various flavonoids on growth of cultures of 3 model bacterial species (*Escherichia coli*, *Vibrio harveyi*, and *Bacillus subtilis*) were used and strong inhibition of growth of cultures of a gram negative bacterium, *V. harveyi*, was observed (Ulanowska et al. 2006). The most severe effects were caused by genisten, apigenin, and kaempferol (Ulanowska et al. 2006).

The ability of flavonols to absorb UV light is another important physiological function of these compounds that has attracted interest in recent years owing to the deterioration of the earth's ozone layer (Jordan 1996 and ref. therein). Plants, in their capture of energy from sunlight for photosynthesis, are exposed to harmful UV radiation (Jordan 1996: Reuber et al. 1996). The photons in these wavelengths can cause damage to DNA, RNA, and proteins that may result in mutagenesis, cell damage, and death (Bieza and Lois 2001). It has been noted that flavonoids act as sunscreens for plants that may be vulnerable to UV radiation exposure (Jordan 1996: Reuber et al. 1996). Flavonoids are found in the epidermal layer of leaf tissue and in pollen and are known to be rapidly synthesized when pollen and leaf tissue is exposed to UV-B radiation (Ryan et al. 2002).

Flavonoids are also known to be involved in a variety of other interactions between plants and microorganisms, functioning as defense factors (phytoalexins) and as signaling molecules

(Korkina 2007). Flavones and flavanones, which are present in many major land plant lineages, play a role as signaling molecules between legumes and nitrogen fixing rhizobia (Martens and Mithofer 2005). Flavonoids such as apigenin, luteolin, eriodictyol, and naringenin were identified as inducers of rhizobium nodulation genes (Peters and Long 1988).

Flavonoids can act as feeding deterrents (Harbone 1993 and ref. therein) to phytophagous insects at relatively low concentrations (Harbone 1993 and ref. therein). For example, flavonoids inhibit the larvae of some insects from feeding and they can repress the development of corn earworm moth, *Heliothis zea* (Martens and Mithofer 2005 and ref. therein). There is a rather thin dividing line between attraction and repellence. In some instances, a flavonoid may act as an attractant to one insect and a deterrent to another (Harbone 1993 and ref.therein).

A variety of antifungal flavonoids, including flavanones and flavonols, have been identified in the sapwood of tree species (Kemp and Burden 1986). These antifungal flavonoids are found usually after wounding or fungal attack (Kemp and Burden 1986 and ref. therein). These compounds pinosylvin and its monomethyl ether have been observed to be induced in pinus species and are considered to be phytoalexins (Kemp and Burden 1986 and ref. therein). Isoflavanoids predominantly found in legumes such as beans, peas, alfafa, and clover are also attributed with antifungal properties (Winkel 1996). The isoflavone genistein is known to have antifungal activity and is shown to be induced during disease as a disease response (Yu et al. 2000 and ref. therein).

Complex isoflavonoid derivatives such as rotenoids, rotenone, deguelin, and amorphigenin from Amorpha, Lonchocarpus, Derris, and Tephrosia species are characterized with insecticidal and parasiticidal properties (Lambert et al. 1993). These complex isoflavonoid

derivatives are also accumulated in the leaves of *Tephrosia vogelii* and the leaves of this plant have 4% of dry mass as rotenoids (Lambert et al. 1993).

Flavonoids and Human Health

Flavonoids have attracted the interest of many due to the promise of being powerful antioxidants that may protect the human body from free radicals because flavonoids have hydrogen radical donating abilities (reviewed in Aghel and Beiranvand 2008). Hesperidin, a flavanone diglycoside, is found in sweet orange and lemon (Hendrickson and Kesterson 1964). Hesperidin may be associated with potential benefits in the prevention of diseases such as decreasing capillary permeability as well as anti-inflammatory, antimicrobial, and anticarcinogenic effects (Aghel and Beiranvand 2008). Hesperidin been also been associated with control of oedema and excess swelling of legs due to accumulation of fluids (Aghel et al. 2008 and ref. therein).

Epidemiological studies strongly suggest that the intake of flavonoids from the diet is helpful in the prevention of atherosclerosis and its related events including coronary heart disease (Kris-Etherton et al. 2002; Terao et al. 2008). Isoflavonoids have also been linked to the anticancer benefits of soy-based foods (Liu 2004 and ref. therein). Isoflavonoids such as genisten and diadzen are reported to have health benefits such as the reduction of osteoporosis, relief of menopausal symptoms, lowering the risk of certain cancers, and lowering the risk of coronary heart disease (Jung et al. 2000 and ref. therein). Isoflavonoids have also been shown to increase the levels of good choloestrol (HDL) and lower the levels of bad cholesterol (LDL) thus its use aids in the prevention of artherosclerosis (This et al. 2011 and ref. therein).The chemical structure of genisten and diadzen is similar to estradiol (Yum et al. 2011 and ref. therein). The

structural similarity gives these compounds estrogenic effects (Yum et al. 2011 and ref. therein). The stilbenes and anthocyanins in red wine have been shown to exhibit an inverse effect on coronary heart diseases (Kris-Etherton et al. 2002 and ref. therein).

Naringin, a flavanone diglycoside produced in large quantities in the leaves and fruit tissues of grapefruit, is responsible for much of the bitter taste in grapefruit and grapefruit products (Owens and McIntosh 2011 and ref. therein). The bitterness that this compound imparts to grapefruit and its processed products makes it an interesting area of research and investigation (Kesterson and Hendrickson 1957; Berhow et al. 1998; Owens and McIntosh 2011 and ref. therein). Naringin has been reported to have antioxidative and metal chelating properties (Yilmaz et al. 2011 and ref. therein). It has been reported that naringin may have the ability to reduce genomic damage made by exposure to cadmium (Yilmaz et al. 2011 and ref. therein).

Flavonoid Biosynthesis

Various enzymes are involved in the synthesis of the different classes of flavonoids. There are 2 main pathways that synthesize key precursors for flavonoid production. The shikimate/arogenate pathway leads to the production of phenylalanine and the hydroxycinnamate acid pathway leads to the formation of one of the critical precursor molecules for flavonoid biosynthesis, 4 –Coumaroyl-CoA (Fig. 4) from phenylalanine (Heller and Forkmann 1993 and 1988 and ref. therein; Owens and McIntosh 2011 and ref. therein).

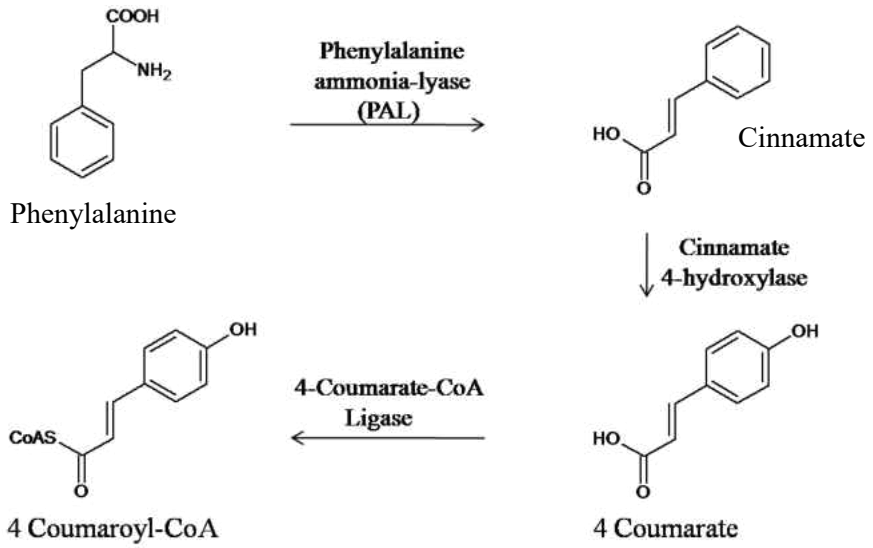


Figure 4. The Synthesis of 4-coumaroyl CoA from Phenylalanine. PAL= Phenylalanine ammonia-lyase (Adapted from Koukol and Conn 1961); Cinnamate 4-hydroxylase (Russell and Conn 1967); 4-Coumarate-CoA ligase (Hahlbrock and Grisebach 1970).

B Ring Synthesis

Phenylalanine is a product of the Shikimate pathway and is the origin of the B ring in the flavonoid skeletal structure (Koukol and Conn 1961). It has been shown that phenylalanine undergoes a series of reactions involving transamination, reduction, and dehydration (Koukol and Conn 1961). Phenylalanine ammonia lyase (PAL) deaminates phenylalanine to trans-cinnamic acid (Koukol and Conn 1961). Cinnamate 4-hydroxylase (C4H) catalyzes the synthesis of p-hydroxycinnamate from trans-cinnamate to produce 4-coumarate (Potts et al. 1973 and ref. therein). C4H is a member of the cytochrome P-450 monooxygenase superfamily (Potts et al. 1973 and ref. therein). The conversion of cinnamate to p-coumaric acid (4-coumarate) consumes molecular oxygen and NADPH (Kyun Ro et al. 2001). The next enzyme in the biosynthetic pathway is coumaroyl CoA ligase (4CL), which converts p-coumarate to its coenzyme-A ester,

thus forming 4 coumaroyl CoA (Knobloch and Hahlbrock 1977). This reaction consumes ATP and uses Mg^{2+} as a cofactor (Ragg et al. 1981).

A Ring Synthesis

In the biosynthesis of the A ring of the flavonoid structure, a molecule of 4- coumaroyl CoA is condensed with 3 molecules of malonyl-CoA resulting in naringenin chalcone (Fig.5). This is catalyzed by chalcone synthase (Kreuzaler and Hahlbrock 1972; Heller and Forkman; 1988 and ref.therein).

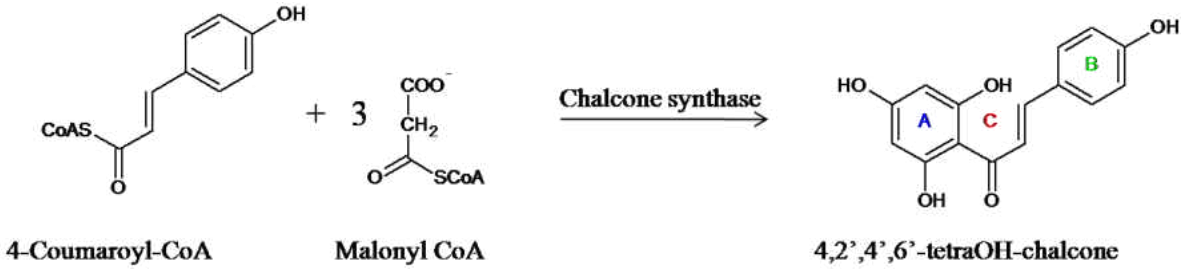


Figure 5. Formation of the Naringenin Chalcone (Kreuzaler and Hahlbrock, 1972).

Chalcone is the first true flavonoid compound synthesized in the pathway and is the precursor to all other flavonoids (Heller and Forkman 1993 and ref. therein). After the synthesis of chalcones, the enzyme chalcone isomerase (CHI) isomerizes chalcones to flavanones (Moustafa and Wong 1967). Flavonones are the first branch metabolite in the pathway. The isomerization of chalcones to flavanones can occur in solution giving a racemic mixture or be catalyzed by CHI producing a specific epimer (Moustafa and Wong 1967).

Flavanones can be converted into flavones by the enzyme flavone synthase (FSI), (Fig. 6). This conversion is known to be catalyzed by 2 different isomeric enzyme systems (Kochs and Griesebach 1986). In one reaction, flavanone was converted to flavone by the enzyme obtained from cell suspension cultures of parsley; this enzyme was classified as a 2-

oxoglutarate dependent dioxygenase because it required 2-oxoglutarate, Fe^{2+}, and ascorbate as cofactors (Britsch et al. 1981 and ref. therein). In another study, the enzyme isolated from flowers of *Anthirrhinum majus* catalyzed the conversion of flavanone to flavones was localized in the microsomal fraction and required NADPH as cofactor (Stotz and Forkmann 1981). The NADPH- dependent flavone synthase, a P450 enzyme, was also isolated from flower extracts of *Verbena hybrida* and *Taraxacum officinale* (Stotz and Forkmann 1981and ref. therein).

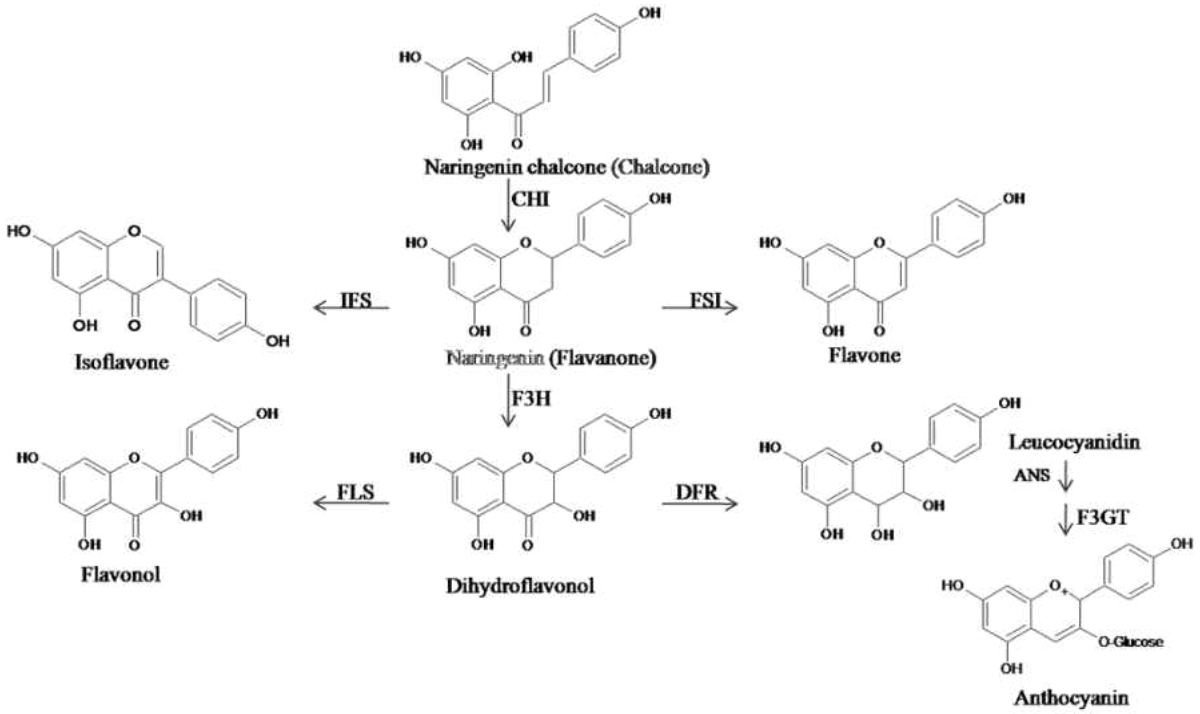


Figure 6. Flavonoid Biosynthetic Pathway. CHI= chalcone isomerase, IFS= Isoflavone synthase, FSI= flavone synthase, F3H= flavanone 3- hydroxylase, DFR= dihydroflavonol 4-reductase, FLS= flavonol synthase, ANS= anthocyanidin synthase and F3GT= flavonoid 3-O-glucosyltransferase (Figure adapted from Heller and Forkmann 1999).

Isoflavones originate from a flavanone precursor by a 2, 3 -migration of the B ring (Fig. 6) (Griesebach and Doerr 1960; Grisebach and Brandner 1961). The conversion of flavanones to isoflavones has been shown to occur in 2 steps (Kochs and Griesebach 1986). In

the first step, (2 S) - naringnenin (flavanone) is converted to an intermediate compound by the enzyme isoflavone synthase (IFS), which is localized in the microsomal fractions of elicitor induced soybean cell suspension cultures (Kochs and Griesebach 1986). This reaction requires NADPH and molecular oxygen as cofactors. In the second step, the intermediate compound is converted to the isoflavone genisten (Kochs and Griesebach 1986).

Flavanone 3-hydroxylase isolated from cell cultures of *Haplopappus gracilis* and *Petunia hybrida* has been shown to catalyze the conversion of *(2S)*-naringenin (flavanone) to (2R, 3R)-dihydrokaempferol (dihydroflavonol) (Fritsch and Griesebach 1975: Britsch and Griesebach 1986). Hydroxylation of (*2S*)-flavanone to (2R, 3R)-dihydroflavonol is a prominent step in the synthesis of flavonols and anthocyanins (Britsch and Griesebach 1986 and ref. therein). The enzyme preparation of flavanone 3-hydroxylase (F3H) from flowers of *Mattiola incana* was shown to require 2-oxoglutarate, Fe^{2+}, and ascorbate as cofactors (Heller and Griesebach 1980). Crude extract from cell cultures of parsley was also shown to contain flavanone 3-hydroxylase and flavonol synthase (FLS) and it catalyzed the conversion of flavanones to flavones and dihydroflavonol to flavonols using the same cofactors (Britsch et al. 1981). Dihydroflavonols are the second branch metabolite in the pathway (Fig. 6).

Flavonol synthase, (FLS) is classified as a 2-oxoglutarate-dependent dioxygenase that converts dihydroflavonols to flavonols (Fig. 6) (Britsch et al. 1981 and ref. therein). The enzymic conversion of dihydroflavanols to leucocyanidin is catalyzed by the enzyme, dihydroflavonol 4-reductase, (DFR) (Stafford and Lester 1982). Crude soluble protein extracts from *Douglas fir* have been shown to catalyze the conversion of (+)-dihydroquercetin to its 3, 4-diol (leucocyanidin) (Stafford and Lester 1982). DFR was shown to be an NADPH-dependent enzyme (Stafford and Lester 1982).

The final steps in the formation of anthocyanins have not been fully elucidated. However, it has been postulated that the precursor for anthocyanin formation is leucoanthocyanidins (Harborne 1986; Saito et al. 1999). Leucoanthocyanidins are converted to the anthocyanidin flavylium cation by hydroxylation subsequently followed by 2 dehydration reactions (Harborne 1986 and ref. therein). The enzymic conversions leading to the formation of anthocyanins from leucocyanidins has however not yet been fully shown *in vitro* (Forkmann 1991; Saito et al. 1999). However in a recent study, cDNA sequence from a perennial herb, *Perilla frutescens* that encodes anthocyanin synthase (ANS) was shown to share similarities in the amino acid region with family 2-oxoglutarate dependent oxygenases. This putative ANS was recombinantly expressed and the rANS catalyzed conversion of leucocyanidin to anthocyanin (Saito et al. 1999).

Modification of Flavonoids

At every branch point of flavonoid biosynthesis, flavonoids can be converted into other classes of flavonoids through the enzymes of the core biosynthetic pathway (Fig. 6) or derivatized to give the distinct compounds found in plants (McIntosh et al. 1990). Modification of flavonoids such as glycosylation, methylation, acetylation, and hydroxylation occurs within each flavonoid class as well as sulfation, prenylation, and C- glycosylation (Heller and Forkman 1993 and ref. therein; Schijlen et al. 2004 and ref. therein; Owens and McIntosh, 2011 and ref. therein). These modifications are essential to the functions of flavonoids in plants.

Glycosylation of Flavonoids

Most naturally occurring flavonoids exist in glycosylated forms; this suggests that glycosylation is an important plant biochemical process for flavonoid production (McIntosh and

Mansell 1990; Kramer et al. 2003; Owens and McIntosh 2011 and ref. therein). Glycosylation is a prominent modification reaction and is usually the last step in the biosynthesis of natural compounds (McIntosh and Mansell 1990: Vogt and Jones 2000 and ref. therein). Glycosylation in coordination with hydroxylation, acylation, and methylation reactions contributes to the variety and complexity of plant secondary metabolites (Vogt and Jones 2000 and ref. therein). The enzymes leading to glycoside formation, the glycosyltransferases, transfer nucleotide-diphosphate –activated sugars to low molecular weight substrates and this can result in changes in the activity of the acceptor molecule and affect the subcellular localization of the acceptor molecule (Campbell et al. 1997; Lim et al. 2002; Kramer et al 2003 and ref. therein). The activated sugars are usually UDP-glucose but UDP-galactose and UDP-rhamnose are also found (Vogt 2000 and ref. therein). The attachment of sugar to the flavonoid aglycone can be directly to the C15 carbon structure resulting in a C-C bond or can attach to a hydroxyl group in this case forming a C-O bond (Harborne and Williams 1988 and ref. therein). The formation of glycosides has been shown to occur on almost every position on the C-15 backbone, however, the 3, 5, and/or 7 hydroxyl positions have been predominant (Harborne 1988; Harborne 1993 and ref. therein; Berhow 1998; Vogt and Jones 2000 and ref. therein; Owens and McIntosh 2011 and ref.therein).

Importance of Glycosylation

Glycosylation plays an essential role in plant metabolism by controlling the bioactivity, storage, and transport of plant natural products such as secondary metabolites, hormones, etc. (Jones et al. 2000 and ref. therein; Shao et al. 2005 and ref. therein). The addition of sugars to aglycones may lead to an enhancement of water solubility and stability of the compounds

(Paquette et al. 2003 and ref. therein; Wang et al. 2009 and ref. therein; Owens and McIntosh, 2011 and ref. therein).

Another major importance of glycosylation is the organoleptic properties it can endow particularly in *Citrus* species, where the accumulation of certain flavonoid glycosides results in bitter, tasteless, sweet, or bittersweet products (Horowitz and Gentili 1969). Grapefruit accumulates mainly the bitter flavanone 7-O-neohesperidosides (example, naringin) and orange and lemons mainly accumulate the tasteless flavanone 7-O-rutinosides (example, hesperidin) (Barthe et al. 1988; McIntosh et al. 1990; Owens and McIntosh 2011 and ref. therein). During the formation of naringin, naringenin is glucosylated by a 7-O- glucosyltransferse (Fig. 7). A 1-2 rhamnosyltransferase, rhamnosylates naringenin 7-O- glucoside to form the bitter flavonoid diglycoside compound (Fig. 7). This accounts for the bitter taste in grapefruit (Horowitz and Gentili 1963; Horowitz et al. 1986 and ref. therein). Interestingly if rhamnose is attached to the 6-OH position of the glucose attached to prunin, it results in a tasteless product (Fig. 7) (Horowitz and Gentili 1963; Horowitz et al. 1986 and ref. therein; McIntosh and Mansell 1990; Owens and McIntosh, 2011). Naringin has been shown to be the most abundant flavonoid in grapefruit and can account to about 40-70% of the dry weight of young green grapefruit and leaves (Jourdan et al. 1985 and ref. therein; Owens and McIntosh 2011 and ref. therein).

The glycosylation pattern of flavonoids tends to affect bioavailability in food (Kramer et al. 2003 and ref. therein). An example is shown with quercetin, a flavonol present in many foods where it exists as the diglycoside rutin (quercetin-3-*O*-rutinoside) making it relatively unavailable for absorption through the intestine (Hollerman et al. 1997). It is suggested that the monoglucosides of these compounds are readily absorbed in the intestine through an active transport system whereas the diglycosides are absorbed only after the sugar moieties have been

cleaved off by microflora in the colon (Scalbert and Williamson 2000). Thus, the conversion of quercetin diglycosides into monoglucosides could be a gateway to enhance the bioavailability of quercetin in foods (Olthoff et al. 2000; Kramer et al. 2003 and ref. therein).

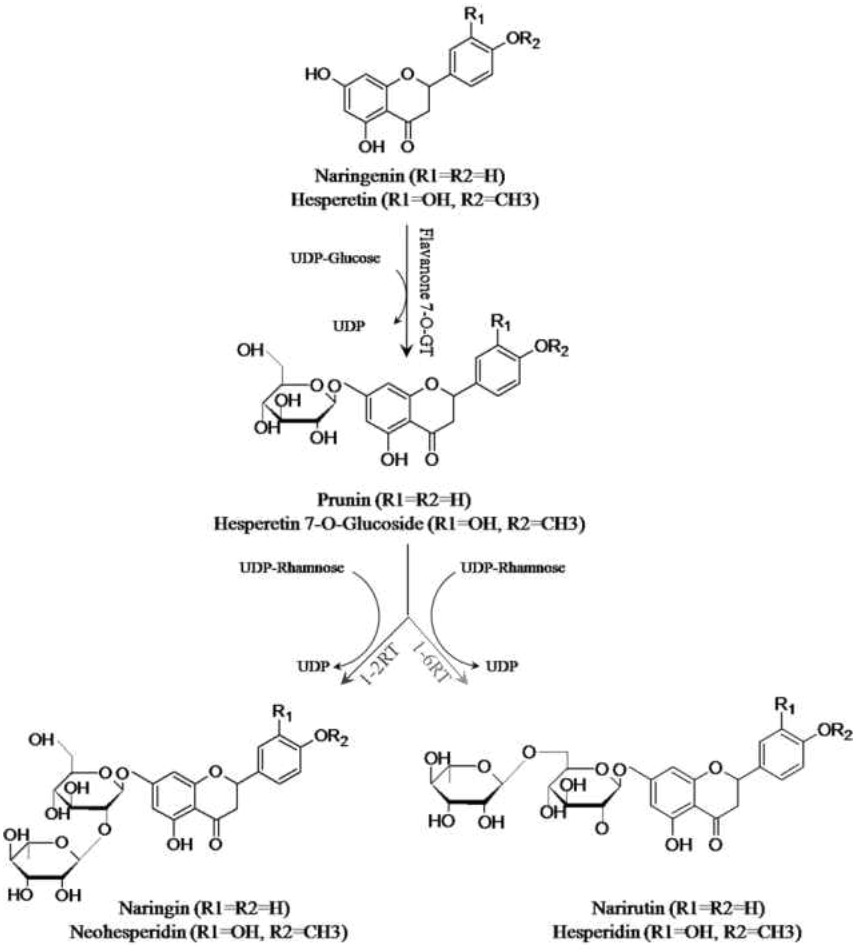


Figure 7. Synthesis of Flavanone Neohesperidosides and Rutinosides in Citrus (Figure adapted from Owens and McIntosh 2011 and ref. therein).

Glucosyltransferases

One of the types of glycosylation is glucosylation. Glucosylation is a modification reaction in plants that leads to the formation of glucosides. The enzymes responsible for this formation are called glucosyltransferases (GTs). They accomplish this by transferring UDP

activated glucose to a corresponding acceptor molecule such as a flavonoid (Fig. 8) (McIntosh, 1990 and ref. therein; Owens and McIntosh 2009; Owens and McIntosh 2011 and ref. therein). Glucosylation is important because it increases solubility and hence transport (Asen and Jurd 1967 and ref. therein; Shao et al. 2005 and ref. therein; Owens and McIntosh 2011 and ref. therein), regulates the bioavailability of the compounds for other metabolic processes, and also stabilizes structures (Asen and Jurd 1967; McIntosh 1990 and ref. therein; Li et al. 2008 and ref. therein; Owens and McIntosh 2011 and ref. therein).

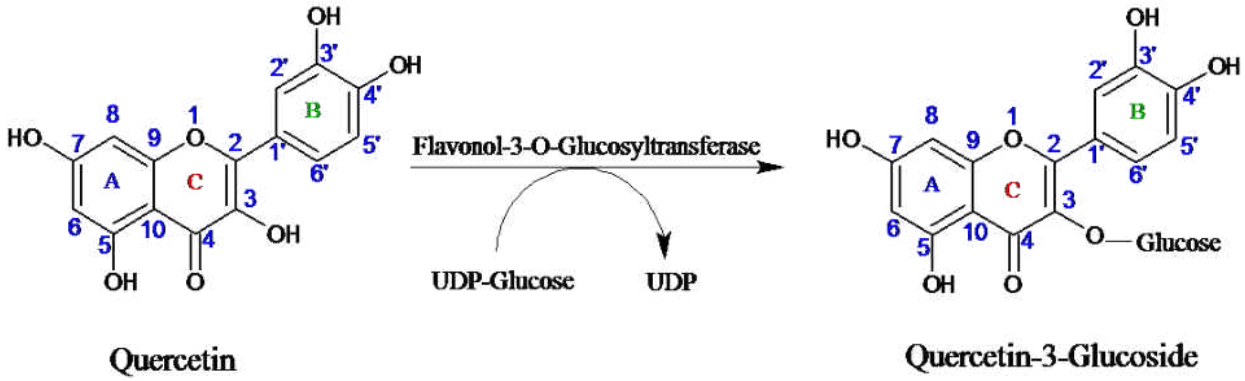


Figure 8. Formation of Quercetin 3-O-Glucoside by a Flavonol-specific-3-O-GT. Glucosylation reaction showing the formation of a Quercetin 3-O- Glucoside by the action of a flavonol specific 3-O-GT enzyme from grapefruit (Figure adapted from Owens and McIntosh 2009).

Because GTs comprise a large family of enzymes, they have been classified based on the degree of primary sequence identities (Osmani et al. 2009 and ref. therein). Family 1 GTs use UDP activated sugar as a donor in the glycosylation reactions and are referred to as UGTs (UDP-dependent glucosyltransferases) (Mackenzie et al. 1997; Lim and Bowles 2004; Osmani et al. 2005). Due to the ability of plants to synthesize bioactive low molecular weight compounds, many of which are glucosylated at specific postions, different glucosides are present in plants and have been identified (Osmani et al. 2005). The grape plant alone has been shown to contain over 200 glucosides (Sefton et al. 1994). Likewise in Arabidopsis, over 120 UGT putative

encoding genes have been identified (Paquette et al. 2003). Although these UGTs have been identified though primary sequence analysis, only a few of them have been biochemically characterized (Knisley et al. 2009 and ref. therein).

Glucosyltransferases, which are involved in secondary plant metabolism, are usually soluble enzymes with a molecular weight between 45- 60 kDa with an optimum pH range for activity between 6.5-8.5 (McIntosh 1990; Vogt and Jones 2000; Owens and McIntosh 2011 and ref. therein). For example, a flavanone-specific 7-*O*- glucosyltransferase isolated and characterized from grapefruit (*Citrus paradisi*) seedlings was shown to have an optimum pH of 7.5-8.0 and an apparent molecular weight of 54.9 kDa (McIntosh et al. 1990). In another instance, a flavonol-specific 3-O- glucosyltransferase (Fig. 8) from *Citrus paradisi*, heterologously expressed in *E.coli*, had a pH optimum of 7.5 and a molecular weight of 51.2 kDa (Owens and McIntosh 2009).

Glucosyltransferases (GTs) involved in secondary metabolism share a UDP sugar binding motif called a PSPG box (Fig. 9) (Hughes and Hughes 1994; Mackenzie et al. 1997). The PSPG box consists of a short stretch of approximately 44 amino acids. Although there is some degree of sequence identity within the PSPG box of plant GTs involved in natural product formation (Fig. 9), the overall sequence similarity among flavonoid GTs is low (Vogt and Jones 2000 and ref. therein; Owens and McIntosh 2009; Owens and McIntosh 2011). Located at the N-terminal region of the PSPG box are the highly conserved amino acids W X P Q. Located at the C-terminus of the PSPG box is the amino acid Q. GTs involved in secondary metabolism have been postulated to undergo a transfer reaction where there is an inversion of an anomeric sugar from an α linkage in UDP-glucose to a β configuration in the resulting glycoside (Kapitinov and Yu 1999). Two highly conserved amino acids, histidine and glutamic acid are postulated to be

involved in the sugar inversion (Vogt and Jones 2000 and ref. therein). This highly conserved histidine residue is present in the active site of all UGT structures (example, His22 in UGT71G1) (Wang 2009 and ref. therein). The highly conserved residue is observed to be close to the glucose moiety of the sugar donor and acceptor (Wang 2005 and ref. therein).

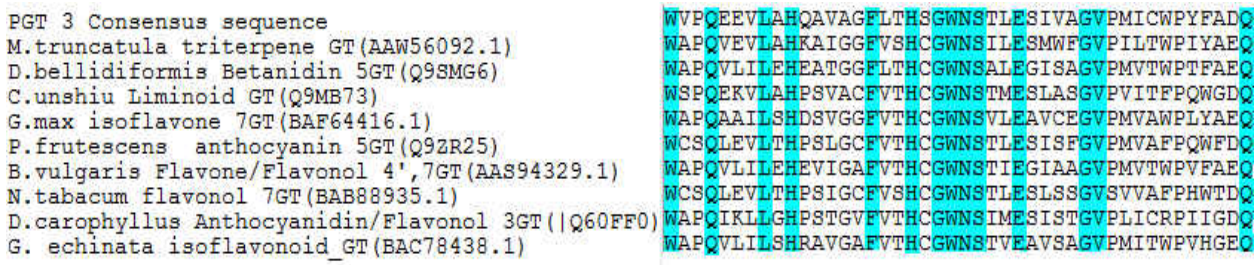


Fig. 9 Alignment of PSPG Box Sequences from Grapefruit Putative Glucosyltransferase Clone 3 and other Plant Glucosyltranferases. Regions shaded blue indicate conserved amino acid. These glucosyltransferases include putative PGT3 and 9 GTs that have been biochemically characterized.

Several putative plant GT sequences can be identified from plant genomic databases by use of sequence similarity with the PSPG box (Knisley et al. 2009; Vogt and Jones 2000 and ref. therein). Even though there is some degree of homology within the PSPG box, comparison of overall nucleotide or amino acid sequence of these enzymes tends to be low (Vogt 2000; Sarker et al. 2007; Owens and McIntosh 2009 and ref. therein). Only a few of the putative GTs produced by these genes have been biochemically characterized, a majority of which are 3-O-GTs (Kinsley et al. 2009 and ref. therein). A comparison of amino acid sequences of flavonoid 3-O-GT from *Perilla frutescens*, *Vitis vinifera*, and *Ipomoea purpurea* showed an overall amino acid homology of 25-31% and 65-75% homology within the PSPG Box (Sarker et al. 2007). This is evidence that, while similarity searches may be useful in the identification of putative flavonoid glucosyltransferase clones, heterologous expression and biochemical characterization of the resulting proteins remains the only way to conclusively establish biochemical function (Owens and McIntosh, 2009 ; Osmani et al. 2009; Owens and McIntosh 2011).

Citrus paradisi Glucosyltransferase Research

Grapefruit, *Citrus paradisi,* is a model plant of choice in this research because it tends to accumulate high levels of glycosylated flavonoids, particularly flavanones and flavones, in fruits and other vegetative tissues (Jourdan et al. 1985, Owens and McIntosh 2011 and ref. therein). Citrus diglycosides are known to be produced in high levels such as the flavanone naringin and flavone glycosides (McIntosh et al. 1990; Berhow et al. 1998 and ref. therein; Sarker 2007 and ref. therein; Owens and McIntosh 2011 and ref. therein). The preponderance and variety of flavonoid glycosides found in Citrus has resulted in an interest in the biosynthesis of these derivatives (Lewinsohn et al. 1986; McIntosh 1990; McIntosh and Mansell 1990; Berhow et al. 1998; Owens and McIntosh 2009; Owens and McIntosh 2011 and ref. therein). The global interest in flavonoids from grapefruit can be attributed in part to the organoleptic properties of grapefruit and its processed products (Mansell et al. 1983). Grapefruit is also known to produce flavonol 3-*O* glucosides, chalcone glycosides, flavonol 7-*O*- glycosides, and C glycosylated flavonoids (Nogata et al. 1994; Berhow, 1998).

A flavanone specific 7-*O*-glucosyltransferase has been isolated and characterized from *Citrus paradisi* seedlings (McIntosh and Mansell 1990). This enzyme was noted to catalyze the glucosylation of the 7-OH group of naringenin to form prunin and was purified and characterized (McIntosh et al. 1990; McIntosh and Mansell 1990). Subsequent purification of this enzyme revealed the presence of other flavonoid GTs that showed activity with flavonoid compounds (flavonols, flavones, chalcone, flavonols) representing all the classes with the exception of anthocyanins (McIntosh et al. 1990). In a similar vein, a flavonol specific 3-O-glucosyltransferase from *Citrus paradisi* was cloned and biochemically characterized (Owens and McIntosh 2009).

The study of enzymes through structural and functional analysis provides a basis for understanding enzyme substrate interaction and also helps in the identification of fundamental amino acids that may be involved in the binding of the enzyme to the substrate (Li et al. 2007 and ref. therein ; Osmani et al 2009; Owens and McIntosh 2009). In Shao et al. (2005) the elucidation of the crystal structures of UDP flavonoid/triterpene GT UGT71G1 from *Medicago truncatula* bound to UDP –glucose revealed certain key residues that are involved in the recognition of substrates. Further studies done with mutagenesis confirmed the involvement of those key residues and the roles they played in substrate binding and enzyme activity (Shao et al. 2005). Also, in Offen et al. (2006), the crystal structure of a UDP-glucose: flavonoid 3-O-glucosyltransferase (VvGT1) from grape revealed key domains that may be involved in substrate binding, donor recognition, and the catalytic activity of the enzyme. The crystal structures of these GTs revealed the presence of conserved key amino acids in or near the PSPG box which may be responsible for the recognition of an activated sugar donor (Shao et al. 2005 and ref. therein; Offen et al. 2006 and ref. therein; Li et al. 2007 and ref. therein).

Purification and characterization of flavonoid GTs directly from grapefruit tissues has yielded active enzymes (Mcintosh et al. 1990; McIntosh and Mansell 1990). However, due to the labile nature of the enzyme and the minute levels of enzyme present in the tissues, accruing enough for purification and direct protein sequencing is quite cumbersome and nearly impossible (McIntosh et al. 1990; McIntosh and Mansell 1990; Tanner 2000). To overcome this, the use of molecular approaches to identify, clone, heterologously express, and characterize resulting proteins has been used (Sarker et al. 2007; Owens and McIntosh 2009; Lin 2011). In Owens and McIntosh (2009) a recombinant flavonol 3-O-GT was characterized and shown to glucosylate the flavonol aglycones kaempferol, myricetin, and quercetin. The enzyme was regioselective,

produced a single product that corresponded to glycosylation at the 3 hydroxyl position and was confirmed by product identification using HPLC and TLC to be a flavonol 3-O-GT (Owens and McIntosh 2009). A host of other grapefruit glucosyltransferase clones have been studied using molecular approaches (Sarker 2004; Strong 2005; Mallampalli 2009; Lin 2011).

A directionally cloned EST library was created from young grapefruit leaves and mined for putative GT clones, (Sarker 2004). Out of this, PGT3, a full length clone was obtained (Sarker 2004 and Kiser 2005). The focus of our research is to identify putative flavonoid glucosyltransferase clones, to establish the structure/function relationships of these enzymes, to study the glucosylation patterns of flavonoid and other phenolic GTs, and the results obtained would add to the knowledge of glucosyltransferases.

Hypotheses

This thesis research was designed to test the hypothesis that putative glucosyltransferase clone 3 (PGT3) is a flavonoid glucosyltransferase and may be subject to biochemical regulation. A presentation of results obtained from optimization of the expression of PGT3 in *E.coli* and purification of resulting protein is reported in this thesis. Also in this thesis, results obtained from cloning and expression in *Pichia pastoris,* purification, and biochemical characterization of PGT3 protein are reported.

Another project was designed to test that the expression of putative flavonoid glucosyltransferase clone 8 (PGT8) protein in yeast, *Pichia pastoris* will result in more soluble protein than in *E.coli*. This clone had previously been expressed in *E.coli*; however, the majority of the protein expressed was packed in insoluble inclusion bodies. Results obtained from cloning are reported.

CHAPTER 2

MATERIALS AND METHODS

Materials

Chemicals and Reagents

Zeocin™ , Luria-Bertani broth powder, agar, ampicillin sodium salt, chloramphenicol, isopropyl-ß-D-thiogalactopyranoside (IPTG), tris base, β-mercaptoethanol (βME), tetramethylethylenediamine (TEMED, electophoresis grade), ammonium persulfate (APS), coomassie brilliant blue, ponceau s, ethidium bromide (EtBr, 10 mg/mL) for DNA gel electrophoresis, nitro-blue tetrazolium chloride (NBT), nitrocellulose membrane (0.45µm pore size), ethyleneglycol monomethylether, dimethylformamide, Whatman chromatography paper (3MM CHR), 5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt (BCIP), sodium phosphate (NaH2PO4 · H2O), phenol, chloroform, isoamyl alcohol, 100% ethanol, acrylamide 40% solution (acrylamide: bis-acrylamide, 19:1), glacial acetic acid, peptone powder, yeast extract, and yeast nitrogen base powder, were purchased from Fisher Scientific (Pittsburgh, PA). Amido black stain 10B was purchased from Corning (Palo Alto, CA). Thioredoxin-tag (trx-tag) monoclonal antibody, and goat anti-mouse IgG alkaline phosphatase (AP) conjugates were purchased from Novagen (Madison, WI). GoTaq DNA polymerase, 5 X green / colorless buffer for polymerase chain reactions (PCR), deoxyribonucleotide triphosphate (dNTP), T4 DNA ligase enzyme, ligation buffer, and restriction enzymes *Xba1, Xho1, Kpn1, Apa1*, *and Bstx1* were purchased from Promega (Madison, WI). The Mini Trans Blot system for Western blotting, the Mini-PROTEIN Tetra gel electrophoresis system, and micro pulser cuvettes were from Bio-rad (Hercules, CA). TALON Metal Affinity Resin was purchased from Clonetech (Mountain Veiw,

CA). Amicon Centricon 30 centrifugal filters were purchased from Millipore (Billerica, MA). All primers were synthesized by Integrated DNA Technology and ordered through the Molecular Biology Core Facility (ETSU, TN). Lyticase enzyme (from *Arthobacter luteus*) lyophilized powder and acid washed glass beads (pore size 0.5mm) were purchased from Sigma Aldrich (St. Louis, MO).

Cells and Vectors

The pCD1 vector and *E.coli* BL21 (DE3) RIL cells were obtained from Brenda Winkel, at Virginia Polytechnic Institute and State University (Blacksburg, VA). One shot Top 10 competent *E.coli* cells were purchased from Invitrogen (Carlsbad, CA). The pPICZA and pPICZAα vectors and X33 parent strain of *Pichia pastoris* were a kind gift from Sanja Roje at Washington State University (Pullman, WA). Competent yeast cells were prepared as described in the easy select *pichia* invitrogen manual.

Kits

The Silver Stain Plus Kit was purchased from Bio-Rad (Hercules, CA). The Wizard SV Gel and PCR clean-up system kit was from Promega (Madison, WI). TOPO TA Cloning® Kits were purchased from Invitrogen (Carlsbad, CA). QIAprep spin Miniprep kits were from Qiagen (Valencia, CA). Quantumprep plasmid mididrep kit was purchased from Qiagen (Valencia, CA).

Buffers

IMAC elution buffer contained 150 mM imidazole, 0.3 M NaCl, and 5 mM BME in 50mM phosphate buffer, pH 7.5), IMAC equilibration/ wash buffer contained 50mM sodium phosphate, 0.3 M NaCl and 5mM BME, pH 7.5) and IMAC MES (2-(N-morpholino)

ethanesulfonic acid) buffer (pH 5.0) were prepared as described in appendix E. TANK (Tris-glycine-SDS) Buffer (pH8.3), Western blot transfer buffer (pH 8.3), TBS-T buffer, AP buffer (pH 9.5), TAE (Tris-Acetate EDTA) buffer, 50mM Tris-HCl buffer (pH 7.5) was prepared. A phosphate buffer (pH 7.5) that contained 50mM βME was prepared. The breaking buffer contained 50 mM sodium phosphate (pH 7.4), 1mM PMSF (phenylmethylsulfonyl fluoride), 1mM EDTA (Ethylenediaminetetraacetic acid), and 5% glycerol. Also, PBS-T buffer was prepared (see appendix E).

Culture Media

LB (Luria-Bertani), low salt LB medium, YPDS (yeast extract peptone dextrose sorbitol), YPD (yeast extract peptone dextrose) liquid media, and plates were prepared as described in Appendix B. Buffered glycerol-complex media (BMGY), and buffered methanol-complex media (BMMY) were prepared as described in the appendix. Selective LB media contained 100mg of ampicillin antibiotic per liter. Selective YPDS media contained 25 mg of zeocin antibiotic per liter.

Flavonoid and Other Phenolic Substrates for Enzyme Assays

Chalcone (4, 2', 4', 6'-tetra-OH-chalcone) was synthesized from naringenin according to Moustapha and Wong (1966) by C. McIntosh. Naringenin, prunin, hesperetin, apigenin, apigenin-7-O-glucoside, luteolin, kaempferol, quercetin, 4'-methoxyflavonol, and 4'-acetoxy, 7-hydroxy-6-methoxyisoflavone were taken from stock supplies of Dr. Cecilia McIntosh at East Tennessee State University (Johnson City, TN). Eriodictyol, isosakuranetin, fisetin, gossypetin, diosmetin, scutellarein, umbelliferone, scopotelin, esculetin, sinapic acid, catechol, phloroacetophenone, and 2, 4-dihydroxybenzaldehyde were purchased from Indofine

(Hillsborough, NJ). Vanillin, 2-hydroxycinnamic acid, vanillic acid, 4-hydroxybenzoic acid, and gentisic acid were purchased from Sigma, (St.Louis, MO). Uridine-5'-diphospho-[U-^{14}C]-glucose (UDP-G; 261 mCi/ mMol) was obtained from ICN (Irvine, CA).

Methods

Heterologous Expression of Recombinant PGT3 in *E.coli*

PGT3 clone was obtained through EST mining of a directionally cloned young grapefruit cDNA library (RoySarkar, 2004; Kiser, 2005) and had previously been cloned into pCD1 vector for expression (Mallampalli, 2009). Preliminary work done on the expression of PGT3 in *E.coli* (Mallampalli, 2009) resulted in the successful expression of the protein; however, the majority of the protein remained insoluble in inclusion bodies. Potential optimization strategies to overcome this challenge tested by varying expression conditions such as postinduction culture media (LB, M9) and temperature (25°C, 15°C, 11°C, or 4^{0}C) did not increase production of soluble PGT3 protein (Mallampalli, 2009). Therefore another optimization strategy, variation of media composition, was tested in this research.

Optimizing the Expression of Recombinant PGT3 in pCD1 Vector in *E.coli*

In order to overcome the challenge of inclusion bodies, variation of media composition such as the use of osmotic reagents, (betaine and sorbitol) and variation of the concentration of the inducer IPTG were tested. Frozen BL21 (DE3) RIL cells containing PGT3 expression construct in pCD1 were cultured overnight in 2 vials, both containing 10 mL LB medium 100 µg/mL ampicillin, 34 µg/mL chloramphenicol with 1 of the vials containing betaine and sorbitol in final concentrations of 2.5 mM and 660 mM, respectively (Arakawa and Timasheff, 1985; Blackwell and Horgan, 1991; Picaud et al., 2007). The cultures were incubated at 37 ^{o}C and 250

rpm. The next day, 1 mL each of the overnight culture was diluted (100 fold) with fresh 100 mL medium. The cultures were incubated at 37 ^{o}C at 250 rpm and the OD_{600} of each culture was measured every half hour using a spectrophotometer until the OD_{600} reached the range of 0.5 - 0.7. A 500 µL aliquot was taken from each vial to represent the preinduction samples and these were centrifuged at 10,000 × g for 10 minutes at 4 ^{o}C. The pellets were saved for future analysis. A 100 µl aliquot of IPTG (inducer) was added to each of the remaining cultures to a final concentration of 1.0 mM, and the cultures were incubated at 25 ^{o}C for 24 hours at 250 rpm.

Every 6 hours, a 1 mL and a 15 mL aliquot of postinduction sample were taken from each culture. The 1 mL postinduction samples were used for preparation of total protein and were microfuged at room temperature 10000 x g. The cell pellets were stored at -20 ^{o}C for subsequent analysis by SDS-PAGE. The 15 mL sample was used for preparation of soluble protein and was centrifuged at 4 ^{o}C in a Sorvall RC-5B refrigerated super speed centrifuge (Fisher Scientific) at 13000 x g for 10 minutes to collect the cells. The supernatants were discarded and pellets were stored at –80 ^{o}C for further analysis.

Preparation of Total and Soluble Protein Fractions

The 500 µL preinduction cell pellets and 1mL postinduction cell pellets for cultures with and without betaine and sorbitol each were resuspended in 250 µL of 2X SDS-PAGE sample buffer (described in appendix E). Microfuge tubes containing the resuspended pellets were vortexed vigorously, boiled for 15 minutes and microfuged for 10 minutes at 13000 x g at room temperature. A 200 µL aliquot was put into a fresh microfuge tube, microfuged for 10 minutes at 13000 x g, and a 100 µL aliquot was carefully pipetted and stored at -20 ^{o}C for subsequent SDS-PAGE and Western blot analysis.

The isolation of soluble protein was done as follows. The postinduction cell pellets were removed from -80°C freezer, thawed, and resuspended in 850 µL ice-cold 50 mM phosphate buffer (pH 7.5) containing 14 mM βME (β-mercaptoethanol). The resuspended cells were lysed by sonicating on ice with a dismembrator model 500 with microtip (Fisher Scientific), pulsing 10 times for 5s each with a 60s recovery. The lysed samples were centrifuged at 4°C at 13000 x g for 10 minutes. The pellets were discarded and the supernatants were put in sterile 1.5 mL tubes and analyzed for expression of soluble recombinant PGT3 protein by SDS-PAGE and Western Blot.

Analysis by SDS-PAGE

The preinduction and postinduction samples, soluble and insoluble fractions from cultures induced with and without betaine and sorbitol were mixed with sample buffer in this manner. A 15 µL aliquot of each soluble fraction was mixed with 5 µL of 4 X SDS-PAGE sample buffer. A 6 µL aliquot of each insoluble fraction was mixed with 6 µL of 2X sample buffer. Also, a sample of broad range molecular weight protein marker diluted 20 fold in 2X sample dye was prepared. All samples, including the broad range molecular weight protein marker, were boiled for 5 minutes and microfuged for a minute at 13000 X g at room temperature. The samples were loaded onto 10% SDS-PAGE gels and electrophoresed in 1X tank buffer for 45 minutes at 200 Volts. After 45 minutes, the gel was removed from the electrophoresis tank. Prior to staining, the stacking gel was cut off and discarded. The separating gel was then stained with coomassie brilliant blue R250 and destained with gel destaining solution. Preparation of staining and destaining solutions used is described in Appendix C.

Western Blotting

Before Western blotting, nitrocellulose membranes (0.45 μm pore size) were soaked in transfer buffer containing 20% methanol (appendix E) for at least 30 minutes to equilibrate the membrane. Immediately before assembling the cassette, sponges placed on both sides of the blotting cassette and soaked in transfer buffer carefully removing air bubbles. The SDS-PAGE gel was sandwiched between 2 sponges, Whatman paper (3 MM CHR), and the nitrocellulose membrane, all cut with the same dimension. The sandwich was clamped together and placed in the gel transfer box and electro blotted with cooling pack placed in a Mini Trans Blot system containing 1X transfer buffer (with 20% methanol) for 90 minutes at 100 Volts. After 90 minutes, the nitrocellulose membrane was stained with ponceau s stain (see appendix C) for one minute to observe bands and the protein marker lane was cut off and saved. The protein marker was washed with several changes of water to remove the ponceau s stain, restained with amido black for a couple of minutes and the membrane was stored in the dark. The rest of the membrane was washed with several changes of water to remove the ponceau s stain. The destained nitrocellulose membrane was blocked with 30 mL of blocking solution (containing 1.5gms fat free milk and 3 mL of TBS-T) for 30 minutes at room temperature with gentle shaking. Blocking prevents nonspecific binding of proteins to the nitrocellulose membrane. After that, the nitrocellulose membrane was washed 3 times, 5 minutes each with 20 mL of 1X-TBST. The membrane was then incubated in 10 mL Trx-tag monoclonal antibody that recognizes the protein of interest based on the presence of Trx-tag (2μL of Trx-tag monoclonal antibody in 1X TBS-T) at room temperature for 30 minutes with gentle shaking. The membrane was subsequently washed 3 times with 20 mL of 1X TBS-T for 5 minute each. The primary antibody was detected by incubating in a secondary antibody solution (1μL of goat antimouse IgG HRP

conjugate in 10 mL of 1X TBS-T) for 30 minutes at room temperature with gentle shaking. The membrane was again washed 5 times each with 20 mL of 1X TBS-T for 5 minutes to remove loosely bound secondary antibody after which 15 mL of 1X alkaline phosphatase buffer (pH 9.5) substrate containing 60µL of NBT and 60µL of BCIP was added to detect the presence of PGT3 recombinant protein. The development reaction was terminated with deionized water after signals are seen (within a minute or two unless otherwise noted) and an image of the membrane was taken and recorded.

Expression with Varying Concentrations of IPTG

In order to test the effect of the concentration of IPTG on induction of soluble rPGT3 protein, rPGT3 was expressed under varying concentrations of IPTG. The usual concentration of IPTG (1 mM) was used and concentration of 5 mM and 0.2 mM were tested. All culture inductions were done on cells growing in LB$amp_{100mg/L}$$chloramphenicol_{34mg/L}$. Frozen BL21 (DE3) RIL cells containing PGT3 expression construct in pCD1 were cultured overnight in 10 mL LB medium 100 µg/mL ampicillin, 34 µg/ mL chloramphenicol. The next day, 3 mL of the overnight culture was diluted with 300 mL of fresh LB$amp_{100mg/L}$$chloramphenicol_{34mg/L}$. The cultures were incubated at 37 ^{o}C at 250 rpm and the absorbance of the culture at OD_{600} was measured every half hour using a spectrophotometer until the OD_{600} reached the range of 0.5-0.7. A 500 µL aliquot was taken from the culture prior to induction to represent preinduction sample and microfuged at 10,000 × g for 10 minutes at 4^{o}C and the pellets saved for future analysis. The remainder of the culture, approximately 300 mL, was divided into 3 erlenmeyer flasks, labeled, and each culture induced with a final concentration of 0.2 mM, 1.0 mM, or 5 mM IPTG, respectively. The cultures were incubated at 25 ^{o}C, 250 rpm, at 24 hours, a 15 ul aliquot of

postinduction sample was taken from each culture. The cell pellets were lysed and analyzed by SDS-PAGE and Western blot as previously described.

Culture Scale-Up

To scale up recombinant PGT3 production, induction of rPGT3 protein expression was done as previously described with the following changes: 1000 mL of culture was induced with a final concentration of 1 mM IPTG and incubated at 25 ^{o}C, 250 rpm for 24 hours. The cell pellets were harvested by centrifugation and stored as previously described. The cell pellet was resuspended in 35 mL of ice cold lysis buffer (50 mM phosphate buffer, 14 mM βME). The resuspended cells were then lysed with a French press at 4^{o}C, 3 times at 2000 psi. The crude lysate was then centrifuged at 4^{o}C for 20 minutes in a sorvall RC-5B refrigerated superspeed centrifuge at 13000 x g. The supernatant from this step was carefully transferred into a clean 50 mL conical tube without disturbing the pellet and kept on ice for protein purification.

Purification of Recombinant PGT3 Protein by IMAC

Soluble recombinant PGT3 (crude) was purified by immobilized metal affinity chromatography (IMAC) using the TALON purification system (TALON Metal Affinity Users Manual) for purification by eluting polyhistidine tagged protein (PGT3) with increasing concentrations of imidazole in the elution buffer. Purification was done initially using a column with 1mL bed volume. The manufacturer's instructions were followed with a few changes. All buffers used contained 5 mM βME. The column was washed with 10 bed volumes of 1X equilibration buffer (50mM sodium phosphate, 300mM sodium chloride and 5mM BME, pH. 7.0). Soluble rPGT3 (crude) sample, (10 mL) was loaded onto the column and 2 mL flow through fractions of the sample was collected. After the sample had passed through the column,

the column was washed with 20 mL of equilibration/ wash buffer and 2 mL fractions were collected. After this, 10 mL of elution buffer containing 10 mM imidazole (pH 7) was used to remove weakly bound proteins and 2 mL fractions were collected. Subsequently, 10 mL of elution buffer containing 150 mM imidazole (pH 7) was used to elute tightly bound polyhistidine tagged proteins from the column and 2 mL fractions were collected. The column was then washed with 5 mL of MES buffer (pH 5) to regenerate the column by removing any remainder of imidazole that may be bound to the column. To prepare the column for another use, it was washed with 10 bed volumes of deionized distilled water allowing 2 mL to remain in the column and the column was capped. Fractions obtained from purification were analyzed by SDS-PAGE and Western Blot as previously described.

Following purification of soluble recombinant rPGT3 by IMAC with a column of 1 mL bed volume, a column of 5 mL bed volume was used as a means to attempt further enrichment of rPGT3 protein. The previously described protocol was used with the following changes. Before loading the sample on the column, the column was equilibrated with 50 mL of equilibration wash buffer (as described in appendix E). The sample was passed through the column and 2 mL fractions of flow through were collected. Subsequently, 35 mL of wash buffer with the same composition as previously described was used and 5 mL fractions were collected. Weakly bound proteins were removed with elution buffer containing 10 mM imidazole and 5 mL fractions were collected. Tightly bound polyhistidine tagged proteins were eluted with elution buffer containing 150 mM imidazole and 5 mL fractions were collected. The column was rinsed with 20 mL of MES buffer (pH 5) and subsequently washed with 50 mL of deionized water. Fractions were analyzed by SDS-PAGE and Western Blot to determine which fractions contained the most enriched rPGT3 protein.

Silver Staining

Fractions obtained from the purification step were analyzed by SDS-PAGE and stained with Bio-Rad silver stain plus kit following manufacturer's instructions. This was done to detect fractions that contained the most expressed PGT3 and also to assess purification.

Cloning of PGT3 into *Pichia pastoris* Vectors

Primer Design

PGT3 cloned into TOPO vector and held in competent TOP10 cells (Kiser, 2005) was isolated by miniprep with the Qiagen miniprep kit by following the manufacturer's instructions. To be able to clone PGT3 into pPICZ and pPICZα vectors, PGT3 had to be modified with primers to contain appropriate restriction sites. Cloning into the 2 different vectors, PGT3 was modified using 3 strategies termed construct 1, 2, and 3.

In Construct 1 (Fig 10), PGT3 sequence was modified with the forward primer (CSP 134F: 5'CATGGGTACCATGGAAGAAAAGCCTAAATCTC3'). The primer included a linker, colored in red, and Kpn1 restriction site, colored in blue. The underlined sequence represents the start codon of PGT3 sequence. The reverse primer (CSP 135R: 5' CGCGGGCCCTGCTTGGG AGCTCATCATC 3') also included the Apa1 restriction site (colored blue). These primers introduced the Kpn1 and Apa1 restriction sites on the 5' and 3'ends of PGT3 sequence (Table 3). After PGT3 was modified with the primers using PCR amplification, it was digested with the Kpn1 and Apa1 restriction enzymes to create sticky ends (Fig. 10). The vector pPICZA, into which construct 1 was to be cloned, was also digested with Kpn1 and Apa1 restriction enzymes to create compatible sticky ends with which the modified PGT3 sequence was to be ligated. The

pPICZA vector results in intracellular expression of rPGT3 protein in the presence of an inducer (methanol) (Easy select *Pichia* manual, Invitrogen).

Fig. 10. Schematic Diagram Showing Cloning of Construct 1 (strategy used to clone PGT3 into pPICZA vector for intracellular expression of recombinant PGT3 protein).

The Construct 2 strategy (Fig 11), was developed by modifying the 5' and 3' ends of PGT3 sequence with forward/sense primer, CSP 134F (5'CATGGGTACCATGGAAGAAA AGCCTAAATCTC 3'). This included a linker, colored in red, and the Kpn1 restriction site, colored in blue. The start codon of PGT3 sequence is underlined (Table 3). The reverse primer CSP 137R; 5'CGCTCTAGACGTGCTTG GGAGCTCATCATC 3') included the Xba1 restriction site, colored in blue (Table 2). The modified PGT3 sequence was cloned into pPICZα vector by digesting pPICZα vector with Kpn1 and Xba1 restriction enzymes to create sticky ends that would be compatible with the sticky ends of PGT3. The vector, also equipped with an alpha factor signal sequence, leads the expression through a secretory pathway resulting in protein being secreted into the culture media. This strategy referred to construct 2 was meant to drive expression of PGT3 protein through the secretory pathway in the presence of an inducer methanol ((Easy select *Pichia* manual, Invitrogen). However, during the expression of rPGT3 through the secretory pathway, 14 extra amino acids are expressed along with PGT3 protein. A cleavage site (Kex 2) is cleaved upon expression of the recombinant protein.

Fig 11. Schematic Diagram Showing Cloning of Construct 2 (strategy used to clone PGT3 into pPICZα vector for extracellular expression of recombinant PGT3 protein).

Table 2. PGT3 Clone Primer Sequences Designed For PCR Amplification. The underlined areas correspond to PGT3 5' and 3'end sequences. Areas in red represent linker regions, areas in blue represent restriction sites. C represents construct. Annealing temperatures are in parenthesis.

C	Sense Primer/Forward Primer	Antisense Primer/Reverse Primer
1	CSP 134F (60°C) 5'CATGGGTACCATGGAAGAAAAGCCTAAATCTC3'	CSP 135R (58°C) 5' CGCGGGCCCTGCTTGGGAGCTCATCATC 3'
2	CSP 134F (60 °C) 5'CATGGGTACCATGGAAGAAAAGCCTAAATCTC 3'	CSP 137R (58°C) 5'CGCTCTAGACGTGCTTG GGAGCTCATCATC 3'
3	CSP136F (60 °C) 5'CATGCTCGAGAAAAGAATGGAAGAAAAGC CTAAATCTC 3'	CSP 137R (58°C) 5'CGCTCTAGACGTGCTTG GGAGCTCATCATC 3'

In the strategy used for construct 3 (Fig. 12), the ends of PGT3 sequence were modified with the forward primer (CSP 136F:5'CATGCTCGAGAAAAGAATGGAAGAAAAGC CTAAATCTC 3'). This included a linker, (colored in red) Xho1 restriction site (colored in blue), and the start codon of PGT3 sequence (underlined) as shown in Table 2. The reverse primer (CSP 137R: CGCTCTAGACGTGCTTG GGAGCTCATCATC 3') included an Xba1 restriction site. These primers introduced the Xba1 and Xho1 restriction sites to the 5' and 3'ends of the sequence. The modified PGT3 sequence was cloned into pPICZα vector by digesting pPICZα vector with Xba1 and Xho1 restriction enzymes to create sticky ends that would be compatible with the sticky ends of PGT3. This strategy, referred to construct 3, was also meant to drive expression of PGT3 protein through the secretory pathway in the presence of an inducer

methanol but this time, expression of PGT3 would be without extra amino acids (Fig. 12). To do this, clone-specific primers were designed to have the yeast consensus sequence for proper initiation of translation of expressed recombinant protein. The primers were each diluted to a stock concentration of 200 mM by mixing in an appropriate volume of sterilized deionized water. From the stock, a 20 µM working solution was prepared by diluting 5 µL of the stock with 45 µL of sterilized deionized water to make a final volume of 50 µL and stored in -20^{o} C for later use.

Fig 12. Schematic Diagram Showing Cloning of Construct 3 (strategy to get rPGT3 into pPICZα vector for expression through the secretory pathway without extra amino acids).

Amplification of PGT3

PGT3 was modified by PCR amplification using an Eppendorf heated-lid gradient thermal cycler and a 1000 fold dilution of miniprepped PGT3 as template (0.001µg/µL). The PCR reaction consisted of the following; 2.5 µL of PGT3 template, 10 µL of 5X colorless Go-taq buffer, 2.5 µL of 20 µM sense primer, 2.5 µL of 20 µM , antisense primer, 1 µL of Go-Taq enzyme and 31 µL of deionized still water to make a total of 50 µL reaction. The primers had similar annealing temperatures so they were all subjected to the same PCR conditions. The PCR cycles were as shown in Table 3.

Table 3. PCR Conditions Used for Amplification of PGT3 sequences.

Steps	Temperature	Time	
1	94 °C	1 min	
2	94 °C	30 sec	X30
3	58 °C	30 sec	
4	72 °C	2 min	
5	72 °C	3 min	

The PCR reactions were done with an initial denaturation step, 1 for 1 minute at 94°C followed by 30 cycles (94°C for 30 seconds, 56 °C for 30 seconds, 72 °C for 2 minutes), and a final extension step, 5, at 72°C for 3 minutes. After the final extension step, the samples were automatically kept at 4 °C by the Eppendorf heated-lid gradient thermal cycler. The amplified PCR products were analyzed using DNA agarose gel (0.8% agarose gel which contained ethidium bromide) electrophoresis. To do the analysis, a 5 µl aliquot of each of the PCR products was mixed with 1 µl of 6X loading dye. Also, to be able to determine the size of the PCR products, 5 µl of 1Kb DNA ladder (Promega;containing loading dye) was mixed with 1 µl of deionized distilled water and electrophoresed on the gel for 60 minutes at 100 volts. An image of the modified PGT3 was viewed using a Gel Documentation Imaging System from UVP (Upland, CA) and the images were recorded. The PCR products of modified PGT3 were gel purified using the Wizard SV Gel and PCR clean-up kit.

The pPICZA and pPICZAα vectors were isolated from frozen TOP10 cells, miniprepped, and digested with appropriate restriction enzymes to have compatible sticky ends with the modified PGT3 sequences. The digested vectors were analyzed by DNA agarose gel

electrophoresis and the image viewed using a gel documentation imaging system and the images recorded.

Cloning of Modified PGT3 into TOPO VECTOR

Modified PGT3 (mPGT3) PCR products for all 3 constructs were cloned into pCR®4-TOPO vector using the TOPO TA Cloning Kit (Invitrogen) following manufacturer's instructions. A 4 µL aliquot of modified PGT3 PCR product, 1 µL of TOPO® Vector and 1 µL of TOPO® salt solution were incubated for 5 minutes at room temperature. A 2 µL aliquot of each TOPO cloning reaction was transformed into 50 µL of one shot TOP 10 competent *E.coli* cells (Invitrogen) according to manufacturer's instructions. For positive control of the transformation step, a 1 µL aliquot of PUC19 was incubated with 50 µL of one shot TOP 10 competent *E.coli* cells. After transformation, 250 µL of SOC medium (appendix B) was added to each transformed mix and shaken at 37°C, 225 rpm for an hour for the cells to recover. A 100 µL aliquot of each transformation mix was spread on a prewarmed $LB_{amp\ (100mg/L)}$ plate. For the positive control, a 1:10 dilution of the transformed mix was done in $LB_{amp\ (100mg/L)}$ liquid and a 100 µL aliquot was spread on $LB_{amp\ (100mg/L)}$. The plates were incubated overnight at 37°C.

Analyzing Transformants

Colonies obtained were analyzed by selecting 17 colonies from the PGT3-pCR4 TOPO transformed plates. Colonies were selected using sterile toothpicks and streaked onto $LB_{amp\ (100mg/L)}$ plates to make duplicates and for further T streaking to obtain single colonies. The toothpicks were each submerged in corresponding labeled 1.5 mL eppendorf tubes containing 100 µL of sterile distilled water to be used as templates for amplification by PCR for screening. The samples for each construct were boiled for 5 minutes to lyse the cells. A PCR master mix

containing 4 µL of 10 mM dNTPs, 20 µL of 20 mM sense primer T3, 20 µL of 20 mM antisense primer T7, 40 µL of 5 X Go-Taq buffer (green), 94 µL of sterile dH_20, and 2 µL of Taq polymerase was made. A 9 µL aliquot of PCR master mix added to 1 µL of the lysed cells. A negative PCR control was done by replacing cell lysate with sterile distilled water. The Tm for PCR reaction was 58 ^oC, all other conditions used for PCR reaction program were the same as previously described. Analyses of the PCR products were performed using DNA agarose gel electrophoresis as previously described.

Selected Colonies for Constructs 1, 2, and 3

Positively transformed colonies for constructs 1, 2, and 3 were selected as previously described. For each construct, four colonies were selected for isolation of plasmid DNA, purification, and digestion. The colonies selected for construct 1 were labeled A2, A6, A9, and A18. Those colonies selected for construct 2 were labeled B1, B3, and B15, and those selected for construct 3 were labeled C6, C13, C15, and C17.

Isolation of Plasmid DNA by Minipreps

Plasmid DNA of the selected colonies for the 3 constructs was miniprepped from overnight bacterial cell cultures. Single PCR screened colonies were inoculated into 10 mL LB $_{amp\ (100mg/L)}$ liquid media and incubated at 37^oC at 250 rpm overnight. A 500 µL aliquot of the overnight culture was added to 500 µL glycerol to make a final concentration of 50 % glycerol and was stored at -80 ^oC. The remainder of the culture was subjected to plasmid DNA purification using a QIA-prep Spin Miniprep Kit according to the manufacturer's instructions. The following modifications were made. Upon final elution, the QIA-prep spin columns (Qiagen) were placed in clean 1.5 mL eppendorf tubes and 50 µL of sterile deionized water was

added to the center of each spin column, incubated for 1 minute at room temperature, and then centrifuged at 13000 x g for 60 seconds in a microfuge to elute the DNA. Plasmid DNA from pPICZA and pPICZAα were eluted just as described above. A 30 µL aliquot of miniprepped PGT3-pCR4 TOPO samples for all 3 constructs were sent for sequencing at the University of Tennessee sequencing facility (Knoxville) for verification of the presence of modified PGT3 constructs in TOPO vector.

Restriction Digests of Plasmid Constructs and Vectors

After isolation of plasmid DNA for all 3 constructs and plasmids had been digested to confirm the presence of PGT3 inserts, larger digestion reactions were carried out to obtain enough DNA for gel purification. Construct 1 (colony A2) restriction digestion reaction consisted of the following components; 14 µL of miniprep plasmid DNA, 2 µL of Kpn1 restriction enzyme, 2 µL of Apa1 restriction enzyme, and 2 µL of 10X buffer A. Construct 2 (colony B15) restriction digestion reaction consisted of the following; 14 µL of miniprep plasmid DNA, 2 µL of Kpn1, 2 µL of Xba1, and 2 µL of 10 X multicore buffer. Construct 3 restriction digestion reaction was made up of 14 µL of miniprep plasmid DNA, 2 µL of Xho1, 2 µL of Xba1, and 2 µL of 10 X buffer D for a final volume of 20 µL.

The corresponding vectors, pPICZA and pPICZAα prepared for ligation with constructs 2 and 3 were also digested as follows: restriction digest reaction for pPICZA was composed of 23 µL of miniprep pPICZA vector, 2 µL of Kpn1, 2 µL of Apa1 and 2 µL of 10X buffer A. Restriction digest reaction for pPICZAα (construct 2) was composed of 23 µL of miniprep plasmid DNA, 2 µL of Kpn1, 2 µL of Xba1 and 2 µL of 10X multicore buffer. Restriction digestion reaction for pPICZAα (construct 3) was composed of 23 µL of miniprep plasmid DNA,

2 µL of Xho1, 2 µL of Xba1, and 2 µL of 10X buffer D for a final volume of 30 µL. All reactions were incubated at 37 ^{o}C for 4 hours and the digestion reactions terminated at 65 ^{o}C for 5 minutes. To each of the digested constructs, 4 µL of 6X sample dye was each added and to the digested vectors, 6 µL of 6X sample dye was added. The samples were electrophoresed on 0.8% agarose gel containing EtBr as previously described.

Gel Purification of PGT3 Constructs and Vectors

Digested constructs and corresponding vectors were gel purified using the Wizard SV Gel and PCR clean-up system (Promega) following the manufacturer's instructions. The gel purified PGT3 constructs and accompanying vectors were analyzed by DNA gel electrophoresis using 0.8% agarose gel containing EtBr as previously described to confirm complete purification of the inserts and vectors and also to confirm that the sizes of the inserts and vectors were correct.

Cloning PGT3 into Expression Vectors, pPICZA and pPICZAα

Two different volumes, 5 µL and 2 µL, of the gel purified inserts and corresponding vectors were loaded on agarose gel, electrophoresed as previously described. The bands were visualized using a Gel Documentation Imaging System (UVP Bioimaging Systems, Upland, CA) and an image was recorded, printed, and used for quantification of the concentration of DNA. To quantify the concentration of the bands of inserts and vectors, a comparison of the intensities of the bands with the intensity of the bands in the Exact Gene Low Range plus DNA ladder (quantitative DNA standard) was done.

Ligation of pPICZ Vectors and PGT3 Inserts

The concentrations of digested pPICZA vector (15.5 ng/µL) and the PGT3-A9 insert (15.5 ng/µL), pPICZAα1 vector (28 ng/µL), PGT3-B15 insert (41 ng/µL), pPICZAα2 vector (31 ng/µL), PGT3-C15 insert (41 ng/µL) were determined as previously described. Tables 4, 5, and 6 shows the ligation reaction ratios. The reactions were incubated at 15 ^{o}C for 4 hours. Different ligation ratios were calculated by using the formula provided in (Promega catalog, Part# 9PIM180), as shown below.

$$\frac{\text{ng of vector X kb (size of insert)}}{\text{kb (size of vector)}} \text{ X molar ratio of } \frac{\text{insert}}{\text{vector}} = \text{ng of insert to be used in the ligation reaction}$$

Table 4. Reaction Mixtures for Ligation of pPICZA Vector and PGT3-A9 Insert (Construct 1).

Molar ratio of insert to vector	volume of insert used (µL)	volume of vector used (µL)	volume of T4 DNA ligase (µL)	volume of ligase buffer (µL)	volume of sterile water (µL)	total volume (µL)
1:1	2.9	6.5	1	1.5	5.8	17.7
2:1	5.8	6.5	1	1.5	2.9	17.7
3:1	8.70	6.5	1	1.5	-	17.7

Table 5. Reaction mixtures for Ligation of pPICZAα1 Vector and PGT3-B15 Insert (Construct 2).

Molar ratio of insert to vector	volume of insert used (µL)	volume of vector used (µL)	volume of T4 DNA ligase (µL)	volume of ligase buffer (µL)	volume of sterile water (µL)	total volume (µL)
1:1	2.5	7.2	1	1.5	3.6	15.8
2:1	4.1	7.2	1	1.5	2	15.8
3:1	6.1	7.2	1	1.5	-	15.8

Table 6. Reaction mixtures for Ligation of pPICZAα2 Vector and PGT3-C15 Insert (Construct 3). Molar ratio of insert to vector.

Molar ratio of insert to vector	volume of insert used (µL)	volume of vector used (µL)	volume of T4 DNA ligase (µL)	volume of ligase buffer (µL)	volume of sterile water (µL)	total volume (µL)
1:1	2.7	6.5	1	1.7	5.4	17.3
2:1	5.4	6.5	1	1.7	2.7	17.3
3:1	8.1	6.5	1	1.7	-	17.3

In order to maintain the concentration of the insert and vector, the ligation reactions were done in a minimum total volume as possible. Therefore the total volumes for the ligation reactions were not the same for all constructs but consistent within each. After the ligation reaction, 2 µL of each reaction mixture was transformed into 16.67 µL of Top 10 competent cells

(holding cell line) using the heat shock method per the manufacturer's instructions and incubated on ice for 30 minutes. After 30 minutes, the reactions were heat shocked for 30 seconds at 42 ^{o} C and immediately incubated on ice for 2 minutes. An 83.3 µL aliquot of prewarmed plain low salt LB medium was added to each reaction mixture, capped tightly, and incubated with shaking at 225 rpm, 37 ^{o}C in a vertical position for 1 hour to allow the cells to recover. For transformation control, 1 µL of PUC 19 was added to 16.67 µL of Top 10 competent yeast cells and heat shocked as previously described. After an hour of incubation of the transformed mixtures, a 100 µL aliquot of each reaction was spread evenly on prewarmed low salt $LB_{zeocin\ (50mg/L)}$ selective plates and incubated overnight at 37^{o} C. The transformed PUC 19 mixture was diluted 10 fold with prewarmed plain low salt LB medium and a 100 µL aliquot was spread evenly on prewarmed plates, incubated as previously described.

Selection of Positively Transformed Colonies

Zeocin containing LB plates incubated overnight at 37^{o} C and colonies that had grown were analyzed by PCR screening. The negative control PUC 19 served as a means to compare the effectiveness of the transformation procedure. For each ligation ratio used for transformation, 34 colonies were randomly selected with sterile toothpicks and submerged into labeled microfuge tubes containing 100 µL of sterile water. The toothpicks were subsequently streaked on fresh selective low salt $LB_{zeocin(50mg/L)}$ plates to make replicates. The samples were boiled for 5 minutes to lyse the cells and a 1 µL aliquot used as a template for PCR amplification. The primers used for PCR amplification were clone specific primers designed to have the yeast consensus sequence for proper initiation of translation of the expressed recombinant protein. A PCR master mix containing 4 µL of 10 mM dNTPs, 20 µL of 20 mM sense primer, 20 µL of 20 mM antisense primer, 40 µL of 5 X Go-Taq buffer (green), 94 µL of sterile dH_20, and 2 µL of

Taq polymerase was made. A 9 µL aliquot of PCR master mix added to 1 µL of the lysed cells. A negative PCR control was done by replacing cell lysate with sterile distilled water. The Tm for PCR reaction was 58 ^{o}C, all other conditions used for PCR reaction program were the same as previously described (Table 3). To be able to save and continue research with the clones obtained from PCR screens, 50% glycerol stocks of 5 positively transformed colonies for each construct were made and stored at -80 ^{o}C.

In order to confirm that these clones contained PGT3 inserts of expected size, recombinant plasmids for construct 1, 2, and 3 were miniprepped from an overnight culture of each clone in LB. With construct 1, a 2 µL aliquot of the miniprep was digested with 1µL of Kpn1 (10µg/µL), 1 µL of Apa1 (10µg/ µL), 1 µL of 10X promega buffer A and 5µL of sterile water at 37^{o}C for 4 hours. With construct 2, a 2 µL aliquot of miniprep was digested in 1 µL of Kpn1 (10µg/µL), 1 µL of Xba1 (10µg/µL) 1µL of 10X promega multicore buffer and 5µL of sterile water at 37^{o}C for 4 hours. A 2 µL aliquot of construct 3 miniprep was digested with 1 µL of Xba1 (10µg/µL) and 1 µL of Xho1 (10µg/µL) in 1 µL of 10X promega buffer D and 5µL of sterile water at 37^{o}C for 4 hours. At 2 hours into the digestion process, a 1 µL aliquot each respective restriction enzyme was added to the reaction to aid in complete digestion of the plasmids. The digestion reactions were terminated after 4 hours in a 65 ^{o}C water bath for 5 minutes. Samples of digested plasmids were analyzed with DNA agarose gel electrophoresis (0.8%) containing EtBr as previously described to confirm the presence of PGT3 inserts.

A 30 µL aliquot of each purified recombinant PGT3-pPICA, pPICZAα1 and pPICZAα2 was sent for sequencing at University of Tennessee sequencing facility (Knoxville) for verification of the presence of PGT3 insert in frame with appropriate vectors and all fusion tags. Results from sequencing were analyzed with Bioedit Sequence Alignment Program to confirm

ligation of PGT3 in pPICZ vectors; all restriction sites were identified. Start codons of insert and all fusion tags were confirmed to be in reading frame. The following clones were selected out of the many positively transformed clones that were verified by sequencing to contain PGT3 insert for subsequent experiments: Construct 1, clone labeled, NL3, construct 2, clone labeled B23, construct 3, clone labeled, C54.

Midiprep of Vectors and Designated Clones for Constructs 1, 2, and 3

To transform *Pichia pastoris*, 5-10 µg of plasmid DNA is required (Easy select *Pichia* manual, Invitrogen). In order to elute sufficient plasmid DNA for transformation of PGT3-pPICZ into *Pichia pastoris* X33 strain for expression of recombinant PGT3, a plasmid midiprep was done using Quantum Prep Plasmid Midiprep Kit according to manufacturer's instructions. However, elution of plasmid DNA was done with some modifications. The quantum spin column was put in a sterile 2 mL eppendorf tube and a 500 µL aliquot of sterile DNase free water was added to the column prior to elution. To determine the concentrations of the eluted plasmid DNA after midiprep, a 2 µL aliquot of each of the purified plasmid DNA electrophoresed on 0.8% agarose gel and the intensity of the bands were compared. To quantify the concentration of the bands of digested inserts, a comparison of the intensities of the bands with the intensity of the bands in the Exact Gene plus DNA ladder (quantitative DNA standard) was done (Table 7). .

Table 7. Amount of Plasmid DNA Quantified from Midipreps. The concentration of plasmid DNA was quantified.

Construct	Vector (µg/10 µL)	Insert (µg/10 µL)
1	17	11.50
2	11	17
3	10.5	12.5

A 300 µL aliquot of purified plasmid DNA of each of the constructs were linearized by restriction digestion prior to transformation into yeast. To obtain an enzyme that would cut one time in the 5' *AOX1* region to linearize the vectors within the 5' *AOX1* region, a restriction map of PGT3 was made and the only enzyme that did not cut within the PGT3 gene sequence was *Bst*X1(Promega). The digestion reaction was incubated at 37^{o} C for 4 hours with 3 µL of *Bst*X1 enzyme and 30 µL of Buffer D. The digestion reaction was terminated by incubation at 65 ^{o}C for 5 minutes and a 2 µL aliquot was analyzed along with a 5µL aliquot of 1Kb DNA ladder (Promega; containing loading dye) for marker control by DNA gel electrophoresis to confirm complete linearization. The gel was visualized as previously described.

Phenol: Chloroform Extraction

Prior to transformation into *Pichia,* completely linearized plasmid DNAs (rPGT3 in pPICZA) were subjected to phenol: chloroform extraction and ethanol precipitation to remove proteins from nucleic acids and concentrate the sample. The phenol: chloroform solvent was prepared as described in appendix E. The procedure for extraction was as follows; first, an equal volume of phenol: chloroform (500µL phenol, 500µL chloroform) was added to 300 µL of plasmid DNA sample in 1.5 mL eppendorf microfuge tube; second, the contents of the tube were

mixed until an emulsion was formed; third, the mixture was centrifuged at 12000 X g for 1 minute at room temperature; fourth, the aqueous phase was transferred to another clean microfuge tube without disturbing the organic phase, an equal volume of chloroform added, and steps 2 -4 were repeated. To recover the DNA by precipitation with ethanol, a quarter volume of 7.5 M sodium acetate was added to the aqueous DNA sample and 2 volumes of ice cold 100% ethanol were added. The sample was mixed vigorously by shaking and placed in a -80 ^{o}C freezer for 15 minutes. Next, the sample was centrifuged for 15 minutes at 14000 x g, 4 ^{o}C. The supernatant was discarded and 250 µL of cold 80% ethanol was gently added to the pellet. This was microfuged for 10 minutes at 14000 X g at 4 ^{o}C. The supernatant was carefully drawn off and the pellet dried in a speedvac. The dried pellet was resuspended in 10 µL of sterile deionized distilled water. For the purpose of determining the concentration of the DNA, a 0.5 µL aliquot of the eluted DNA and a 100 fold dilution of it were loaded on a 0.8% agarose gel and electrophoresed against a 10 µL aliquot of 1Kb Exact gene DNA ladder for verification of insert and vector sizes and for quantification.

Transformation into *Pichia pastoris*

Transformation into *Pichia* required completely linearized plasmid DNA that had also been purified with phenol: chloroform extraction. Prior to transformation, competent yeast cells were prepared as follows; an overnight culture *Pichia pastoris* strain X33 was grown at 30 ^{0}C , 250 rpm in YPD (yeast peptone dextrose) medium. A flask with 500 mL freshly prepared YPD medium (appendix B) was inoculated with 0.5 mL of the overnight culture. The 500 mL cell culture was grown overnight at 30 ^{o}C, 250 rpm to reach an OD_{600} of 1.3-1.5. As soon as the required optimal density was reached, the cell culture was centrifuged at 4 ^{o}C for 5 minutes in a Sorvall RC-5B refrigerated super speed centrifuge (Fisher Scientific) at 1500 X g. The cell pellet

was resuspended in 500 mL aliquot of ice cold sterile water and centrifuged to wash the cells. The cells were again resuspended in 250 mL aliquot of ice cold sterile water and centrifuged. After this, the pellet was resuspended in 20 mL of ice cold 1M sorbitol, centrifuged as previously described, and the pellet was resuspended in 0.5 µL aliquot of ice-cold 1M sorbitol to obtain a final volume of 1.5 mL. The competent yeast cells were stored on ice and used same day (Easy select *Pichia* Manual, Invitrogen).

Transformation by Electroporation

Prior to electroporation, 0.2 cm micro pulser cuvettes (Bio-rad) were incubated on ice and the protocol provided by the manufacturer was duly followed (Easy select^{TM} Pichia manual, Invitrogen). An 8µg sample of linearized DNA for constructs 1, 2, and 3 were each mixed with 80 µL of freshly prepared competent yeast cells. Each mixture was transferred to a previously incubated ice-cold 0.2 cm micro pulser cuvette on ice for 5 minutes and the cells were pulsed at 1.5 kV (Delorme, 1989). The same procedure was done with the vectors pPICZA and pPICZAα and these were transformed to serve as a negative control for expression of recombinant PGT3. A 1 mL aliquot of ice-cold sorbitol was quickly added to each cuvette and each sample transferred into a sterile 15 mL tube. The transformed samples in the 15 mL tubes were then incubated at 30 ^{o}C without shaking, for 2 hours. A 25 µL, 50 µL, 100 µL, and 200 µL aliquot of the transformed mix each were spread evenly on prewarmed labeled YPDS plates (as described in appendix B) containing zeocin $_{100\mu g\ /ml.}$ For all transformations, a negative control reaction was done by replacing linearized plasmid DNA with sterile deionized water. All plates were incubated for 4 days at 28 ^{o}C until colonies were observed. The plates with colonies were stored at 4 ^{o}C .

Direct PCR Screens of *Pichia* Transformants

A PCR test of the *Pichia* clones for insertion of PGT3 gene was done by randomly selecting 17 colonies for PGT3-pPICZA/NL3 (construct 1), 17 colonies for PGT3-pPICZAα1/ B23 (construct 2), and 17colonies for PGT3-pPICZA α2/C54 (construct 3). The PCR screens were done as previously described with a few modifications. A sterile toothpick was used to select single colonies and the toothpick was resuspended in a 1.5 mL microcentrifuge tube containing 5 µL of sterile distilled water. In order to lyse the cells, a 1.5 µL aliquot of 6U / µL solution of lyticase was added to the cell culture and incubated at 30 ^{o}C for 10 minutes. The samples were then frozen in -80 C for 10 minutes. A PCR master mix was prepared as previously described with clone specific primers (Table 4), and a 9 µL aliquot of the PCR master mix was added to a 1 µL aliquot of the cell lysate. The PCR conditions were the same as previously mentioned (Table 3) with an annealing temperature of 58 ^{o}C. To select positively transformed *Pichia* clones, a 10 µL aliquot of each of the PCR products were analyzed by 0.8 % agarose gel electrophoresis. Stocks of positively transformed *Pichia* clones containing PGT3 insert were saved in 50% glycerol and stored at -80 ^{o}C for future use.

Expression of Recombinant PGT3 Protein in *Pichia pastoris*

Prior to the expression of Recombinant PGT3 protein, 5 positively transformed *Pichia* clones were cultured overnight in YPDS liquid media (appendix B). After visualization of cloudiness of the cell culture, a 500 µL aliquot of the culture was mixed in 500 µL of glycerol and stored in a -80 ^{o}C freezer. To test the expression of recombinant PGT3 protein, test inductions were performed.

Test Inductions for Expression of PGT3 Protein

Recombinant PGT3 *Pichia* strains that had been confirmed to contain PGT3 insert were tested for expression of soluble protein at different post induction times. Using a single transformed colony, a 25 mL aliquot freshly prepared BMGY media was inoculated in a 250 mL baffled flask. The culture was incubated to generate biomass at 30 ^{o}C in a shaking incubator at 250 rpm until the culture reached an OD_{600} of 2-6 (approximately 22 hours). (All media used were prepared as described in appendix B). After that, the cell culture was centrifuged in a Sorvall RC-5B refrigerated superspeed at 3000 X g for 5 minutes at room temperature to pellet the cells. The pelleted cells were resuspended in 150 mL of prewarmed BMMY to reach an OD_{600} of 1. The cell culture (150mL) was put in a 1000 mL baffled flask. Prior to induction, a 1 mL aliquot of the cell culture was placed in a 1.5 eppendorf tube to represent preinduction sample. This was microcentrifuged at 13000 X g for 3 minutes at room temperature, the supernatant discarded, and the pellets were stored at -80 ^{o}C. To drive the expression of recombinant PGT3, the remaining 149 mL of cells were induced by adding 100 % methanol to a final concentration of 0.5 % and the flask was covered with 2 layers of cheesecloth for maximum aeration. The cell culture was then incubated at 30 ^{o}C, 250 rpm for 4 days. To maintain induction conditions, 100% methanol was added to the cell culture to make a final concentration of 0.5 % every 24 hours. In order to ascertain which timepoint of induction was best for the expression of recombinant PGT3, a 1 mL aliquot of cell culture was taken every 6 hours for the first day (6, 12, 18, 24 hours), then every 12 hours for the second to the fourth day (36, 48, 60, 72, 84, 96 hours). Each sample was microcentrifuged to pellet the cells and the supernatant discarded. The pellets were frozen at 80 ^{o}C after harvesting. Samples from vectors pPICZA and pPICZAα that

were transformed into yeast to serve as a negative control for expression of recombinant PGT3 were collected in the same manner.

Preparation of Samples for Analysis

The yeast cell samples transformed with rPGT3 in pPICZA collected at the various postinduction times indicated above were prepared for analysis of protein expression through the intracellular pathway by SDS-PAGE and Western Blot analysis. The yeast cells had to be lysed to get access to the protein. The cells were thawed on ice, a 1mL aliquot of breaking buffer (50 mM sodium Phosphate, 1 mM PMSF, 1 mM EDTA, 5% glycerol, pH 7.4) was added to each sample, and the cells resuspended. In order to break the cells, an equal volume of acid washed glass beads (size 0.5mm) was added and the samples were vortexed for 30 seconds, incubated on ice for 30 seconds, repeated for 8 cycles. The samples were then centrifuged at 4 ^{o}C for 10 minutes at 14000 x g and the supernatant was transferred into a clean 1.5 mL eppendorf tube on ice. Samples to be run on SDS-PAGE gels and Western Blot were prepared in this manner:50 µL of supernatant was mixed with 50 µL of 2X sample buffer. The sample mixture was then boiled for 10 minutes and centrifuged for a minute at 13000 x g at room temperature. Preinduction samples were prepared the same way as postinduction samples.

Analysis by SDS-PAGE and Western Blot

Samples were run on SDS-PAGE as previously described with the following modifications: 20 µL aliquot of the prepared samples, 20 µL of pre-induced samples and 5ul broadrange molecular weight standards were loaded on gels. To test for negative control of expression, samples prepared from yeast cells transformed with pPICZA empty vector (induced using the same conditions for recombinant PGT3 constructs) were loaded on gels to show the

background of native *Pichia* proteins that may be present intracellularly and to differentiate recombinant PGT3 protein from background yeast proteins.

To detect recombinant PGT3 expression by Western blot analysis, the procedure was carried out as previously described but with the following changes. A 20 µL aliquot of each sample prepared as mentioned above was run on SDS-PAGE and antimyc monoclonal antibody was used as a primary antibody and goat anti-mouse IgG-AP conjugate (in 10 mL of 1X PBS-T) as secondary antibody. The preparation of blocking buffer was prepared as previously described using 1X PBS and blocking was done for 1 hour. Incubation with primary and secondary antibodies was done in PBS-T. Incubation with the primary antibody was for 2 hours at room temperature and 1 hour with secondary antibody. All washes were done in 1X PBS-T, 3 times for 5 minutes. Development of the blot was done with 15 mL of alkaline phosphatase buffer containing 60 µL of Nitro-BT and 60 µL of NBT solution. The blot was developed for about 2 minutes (unless noted otherwise) and the reaction stopped by immersion of the blot in sterile water. Analysis of SDS-PAGE and Western Blots was done as previously described.

Scale-Up of Expression

Results from methanol test inductions indicated the best postinduction time for expression of recombinant PGT3 to be 6 hours. With that in mind, a scale up of the expression culture was done by growing the same volume of culture for 6 hours and harvesting the entire 150 mL culture. Prior to harvesting the cells, the cell culture was divided into 2 and placed in 500 mL centrifuge tubes and centrifuged at 3000 x g for 5 minutes at room temperature. The cell pellet obtained was stored in a -80 ^{o}C freezer.

Test Purification using Talon Resin

A 6-hour postinduction pellet of recombinant PGT3 was resuspended in 5 mL breaking buffer (50mM sodium phosphate, 1mM PMSF, 1mM EDTA, 5 % glycerol, 5mM BME, pH 7.4). The resuspended sample was then lysed with a French press at 2000 psi 3 times. The lysis was done with a French press because of the volume of cells used and also because the cells needed to be at 4 ^{o}C to prevent degradation or denaturation of proteins. The cell lysate was then collected into a 50 mL centrifuge tube and centrifuged in a sorvall RC-5B superspeed centrifuge for 20 minutes, 4^{0}C at 13000 x g. The pellet was discarded and the supernatant was saved on ice and desalted prior to purification to remove excess salts that may interfere with the IMAC column. To desalt the supernatant containing recombinant PGT3 protein, a prepacked PD-10 column was washed with 25 mL of equilibration / wash buffer ((50mM sodium phosphate, 300mM sodium chloride, 5mM BME, pH. 7.5). During the process, the column was never allowed to run dry. Afterwards, a 2.5 mL aliquot of protein sample was applied to the column and the first 2.5 mL flow-through sample collected and discarded. The protein was then eluted with 3.5 mL of equilibration/wash buffer (50mM sodium phosphate, 300mM sodium chloride, 5mM BME, pH 7.5) and 3.5 mL of eluate sample collected and saved. The PD-10 column was then rinsed with 25 mL of equilibration/wash buffer (50mM sodium phosphate, 300mM sodium chloride, 5mM BME, pH. 7.5), leaving 1 mL of liquid on top. This was done to prepare the column for future use.

The 3.5 mL of desalted eluate sample obtained from the PD-10 column was purified further using immobilized metal affinity chromatography (Talon resin). A 4 mL IMAC column was washed with 40mL of equilibration wash buffer (50mM sodium phosphate, 300mM sodium chloride and 5mM BME, pH 7.5). The eluate sample from the desalting step was then run

through IMAC column adjusting the flow rate so that it took 15 minutes (minimum) for the sample to pass through the column. The rationale behind this was to allow the protein to bind well to the column. Then, 1 mL fractions of the flow- through sample (4 fractions) were collected. The column was washed with equilibration wash buffer (same composition as above) and 2 mL fractions collected. The absorbance of each wash fraction was checked using a spectrophotometer at OD_{280}. An absorbance of almost zero indicated that sufficient washes had been done. The column was then eluted with 10 mM imidazole (10 mM imidazole, 0.3 M NaCl, and 5 mM BME in 50mM phosphate buffer, pH 7.5) to remove loosely bound proteins and 2mL fractions were collected, while monitoring the absorbance at OD_{280} as above. Afterwards, elution with 150 mM imidazole (150 mM imidazole, 0.3 M NaCl, and 5 mM BME in 50mM phosphate buffer, pH 7.5 was done and 2mL fractions were collected (10 fractions). All fractions obtained from the purification steps were analyzed by SDS-PAGE and Western Blot to determine fractions containing the most enriched rPGT3 protein.

Screening rPGT3 Protein for GT Activity

The optimal expression condition for PGT3 was used to express protein to be tested for activity with flavonoid substrates and phenolics. Prior to screening for GT activity, the expressed protein was purified by IMAC as described. Aliquots of samples obtained were analyzed by SDS-PAGE after enzyme assays had been done.

Concentrating using Centricon-30

The fractions obtained from purification of rPGT3 were pooled, desalted, and concentrated using a centricon -30 in a Sorvall RC-5B refrigerated superspeed centrifuge at 4000 x g at 4^{o}C until the sample was highly concentrated. To the concentrated sample, 1500 µL

of 50 mM Phosphate buffer containing 14 mM BME (pH 7.5) was added and the samples centrifuged again. Another 1500 µL aliquot of 50 mM phosphate buffer containing 14 mM BME (pH 7.5) was added and the sample centrifuged further to concentrate it (final volume approximately 1.5 mL). The sample was then transferred into a 2 mL microfuge tube on ice ready to be used for GT activity screening. A portion of the desalted and concentrated fractions and all fractions obtained from each purification step were kept for analysis by SDS-PAGE and Western blot. About 800 µL of the enzyme was saved in 40% glycerol at -20 ^{o}C to test the stability of the enzyme.

Preparation of Substrates and UDP -^{14}C glucose

The substrates to be tested (Table 8) were diluted to a concentration of 50 nmoles / 5 ul in 100 % ethylene glycol monomethylether. Substrate samples were stored at -20 ^{O}C, capped tightly and wrapped in paraffin in a non-self-defrosting freezer. A working solution of ^{14}C-UDP-glucose was prepared by mixing 0.45 µL of ^{14}C stock and 9.55 µL of phosphate buffer (50 mM) per reaction to reach 20000 cpm/ 10 µL.

Table 8. Flavonoid and Simple Phenolic Substrates Screened with rPGT3 for Activity.

Flavonoid sub-class	Substrate
Chalcone	4,2',4'6'- tetra-OH-chalcone
Flavanones	Naringenin Hesperitin Eriodictyol Isosakuranetin
Glucoside	Prunin
Flavones	Apigenin Luteolin Diosmetin Scutallarein
Glucoside	Luteolin-7-O glucoside
Flavonols	Kaempferol Quercetin Fisetin Gossypetin 4' methoxy flavonol
Isoflavone	4'-acetoxy-7-hydroxy-6-methoxy isoflavone
Phenolics	Esculetin Umbelliferone Scopoletin
Simple phenolics	2'4 -dihydrobenzaldehyde Catechol

Screening of rPGT3 Protein for GT Activity using Flavonoids as Substrates

A 10ul aliquot of the enriched enzyme and 50ul of phosphate buffer (50 mM phosphate, (pH 7.5) containing 14 mM βME) was incubated with 10µL of ^{14}C-UDP-glucose and 5µL (50nmol) substrate dissolved in 100 % ethylene glycol monomethylether. The reaction was incubated at 37 ^{o}C for 15 minutes (n=2). After 15 minutes, the reaction was stopped by addition of 15µL of 6M HCL and vortexed for 10 seconds. To separate any radiolabelled flavonoid glycosides from the unincorporated UDP-^{14}C glucose, 250 µL of ethyl acetate was added to the sample, vortexed for 10 seconds, and partitioned into organic and liquid phases. The organic

phase would contain the flavonoids and the aqueous phase the unincorporated UDP-^{14}C- glucose. A 100-150 µL aliquot of the organic phase was then pipetted into a 2 mL CytoScint scintillation fluid. The amount of incorporated radiolabelled glucose was counted in a Beckman LS 6500 scintillation counter and counted to determine cpm in 250 µL. For negative control assays, 60 ul of phosphate buffer was used in place of the enzyme. This was incubated with 10ul of UDP-^{14} C glucose and 5ul of aglycone dissolved in 100 % ethylene glycol monomethyether. Positive control reactions consisted of 60 µL grapefruit leaf extract, 5 µL substrate and 10 µL of UDP-^{14} C-glucose. Another control reaction was done where grapefruit leaf extract and rPGT3 was incubated to see if any inhibitors were present in the PGT3 sample. The reaction was incubated as above.

A number of phenolic aldehydes and phenolic acids have been shown to be present in grapefruit (Fieldman and Hanks 1965; Ahmad and Hopkins 1993). Most of these compounds, according to the Merck Index, are highly water soluble. Thus, their glucosides will also be water soluble. Knowing this, activity screens using ethyl acetate to separate the glucosides from the unincorporated UDP-glucose will not be feasible. To test the activity of rPGT3 with simple phenolic substrates, reverse phase high performance liquid chromatography (HPLC) was used to separate and identify glucosides from aglycones. The reaction mixture was made up of 35 µL of enriched PGT3 enzyme, 5 µL of substrate (50 nmol) and10 µL (100 nmol) UDP-glucose in 50 mM phosphate buffer (pH 7.5) containing 14 mM BME (pH 7.5). The reaction was then incubated at 37^{o}C for 30 minutes. The reaction was stopped after 15 minutes by the addition of 200 µL methanol to precipitate the proteins. The precipitated protein was removed by centrifuging at 10000 x g, for 15 minutes at 4 ^{o}C. The supernatant was dried by use of a speedvac concentrator from Thermo Electron Corporation (Waltham, MA). The dried extracts were

resusupended in milliQ water and run through a C18 RP-HPLC with isocratic elution using Waters 1525 Binary HPLC Pump ,Waters 2487 Dual λ Absorbance Detector, and Waters Symmentry® C18 column (5µm, 4.6 × 150 mm) from Waters (Milford, MA). The Solvents used are shown in Table. 9. The experiment was duplicated for each substrate tested and also negative control reactions were performed. The enzyme for the negative control reaction was denatured by boiling prior to the addition of substrate and UDP-glucose. Table 9 shows the conditions used for RP-HPLC.

Table 9. RP-HPLC Conditions used for Testing rPGT3 Activity with Phenolic Compounds (Adpted from Ahmad and Hopkins 1993)

	Mobile phase	Detection wavelength (nm)
Gensitic Acid	10% ACN	330
Catechol	15% ACN	280
p-Hydroxybenzoic acid	15% MeOH	255
Caffeic acid	30%MeOH	322
Scopoletin	30%MeOH	340
p-Hydroxyphenylacetic acid	15% MeOH	215
p-Hydroxyphenylpyruvic acid	15% MeOH	215
o-Coumaric acid	30% MeOH	325
p-Coumaric acid	30% MeOH	308
Ferulic acid	30% MeOH	324
Vanillic acid	10% ACN	260
Vanillin	10% ACN	273

Note : ACN: Acetonitrile; MeOH: Methanol. The wavelength of detection is λ_{max} of the substrates. Mobile phase was diluted with 0.1M phosphate buffer (pH 3).

Expression of PGT8 in *Pichia pastoris*

Cloning of PGT8 into *Pichia* pastoris Vectors

Primer Design

PGT8 (see appendix F) was previously cloned into TOPO vector and held in competent TOP10 cells by D. Owens and was isolated by miniprep with the Qiagen miniprep kit by following the manufacturer's instructions. To be able to clone PGT8 into pPICZA vector, PGT8 had to be modified with primers to contain appropriate restriction sites on the 5' and 3' ends.

PGT8 sequence was modified with the forward primer (CSPF: 142 5' CATGGGTACC ATGGGAACTGAATCTCTTGT 3'), the primer included a linker, colored in red, and Kpn1 restriction site, colored in blue. The underlined sequence represents the start codon of PGT3 sequence. The reverse primer (CSP 143R: 5' CGCGGGCCCATACTGTACACGTGTCCGTC 3'). These primers introduced the Kpn1 and Apa1 restriction sites on the 5' and 3'ends of PGT8 sequence (Table 10). After PGT8 was modified with the primers using PCR amplification, it was digested with the Kpn1 and Apa1 restriction enzymes to create sticky ends. The vector, pPICZA was also digested with Kpn1 and Apa1 restriction enzymes to create compatible sticky ends with which the modified PGT8 sequence was to be ligated. Use of the pPICZA vector results in intracellular expression of rPGT8 protein in the presence of an inducer (methanol).

Table 10. PGT8 Clone Primer Sequences Designed For PCR Amplification. The underlined areas correspond to PGT8 5' and 3'end sequences. Areas in red represent linker regions, areas in blue represent restriction sites. Annealing temperatures are in parenthesis.

Sense Primer/Forward Primer	Antisense Primer/Reverse Primer
CSP 142 F (64^{O}C)	CSP 142 R (56^{O}C)
5'CATGGGTACCATGGGAACTGAATCTCTTGT 3'	5'CGCGGGCCCATACTGTACACGTGTCCGTC 3'

Amplification of PGT8

PGT8 was modified by PCR amplification using an Eppendorf heated-lid gradient thermal cycler and a 1000 fold dilution of miniprepped PGT8 as template (0.001µg/µL). The PCR reaction consisted of the following; 2.5 µL of PGT8 template (PGT8 in TOPO vector), 10 µL of 5X colorless Go-taq buffer, 2.5 µL of 20 µM sense primer, 2.5 µL of 20 µM , antisense primer, 1 µL of Go-Taq enzyme and 31 µL of deionized still water to make a total of 50 µL reaction. The primers had similar annealing temperatures so they were all subjected to the same PCR conditions. The PCR cycles were as shown in Table 11.

Table 11. PCR Conditions Used for Amplification of PGT8 sequence.

Steps	Temperature	Time	
1	94 °C	1 min	
2	94 °C	30 sec	X30
3	56 °C	30 sec	
4	72 °C	2 min	
5	72 °C	3 min	

Cloning of Modified PGT8 into TOPO Vector

Modified PGT8 (mPGT8) PCR product was cloned into pCR®4-TOPO vector as a holding vector for amplification using the TOPO TA Cloning Kit (Invitrogen) following manufacturer's instructions. A 4 µL aliquot of modified PGT8 PCR product, 1 µL of TOPO® Vector and 1 µL of TOPO® salt solution were incubated for 5 minutes at room temperature. A 2 µL aliquot of each TOPO cloning reaction was transformed into 50 µL of One Shot TOP

10 competent *E.coli* cells (Invitrogen) according to manufacturer's instructions. Transformation reaction was done as previously described for PGT3. A 100 µL aliquot of the transformation mix was spread on a prewarmed $LB_{amp\ (100mg/L)}$ plate. For the positive control, a 1:10 dilution of the transformed mix was done in $LB_{amp\ (100mg/L)}$ liquid and a 100 µL aliquot was spread on $LB_{amp\ (100mg/L)}$. The plates were incubated overnight at 37°C.

Analyzing Transformants

Colonies obtained were analyzed by selecting 17 colonies from the PGT8-pCR4 TOPO transformed plates. Colonies were selected using sterile toothpicks and streaked onto $LB_{amp\ (100mg/L)}$ plates to make duplicates and for further T streaking to obtain single colonies as previously described. The samples for each construct were boiled for 5 minutes to lyse the cells. A PCR master mix containing 4 µL of 10 mM dNTPs, 20 µL of 20 mM sense primer T3, 20 µL of 20 mM antisense primer T7, 40 µL of 5 X Go-Taq buffer (green), 94 µL of sterile dH_20, and 2 µL of Taq polymerase was made. A 9 µL aliquot of PCR master mix added to 1 µL of the lysed cells. A negative PCR control was done by replacing cell lysate with sterile distilled water. The Tm for PCR reaction was 56 °C, all other conditions used for PCR reaction program were the same as previously described (Table 11). Analyses of the PCR products were performed using DNA agarose gel electrophoresis as previously described.

Isolation of Plasmid DNA by Minipreps

Plasmid DNA of the 2 positve colonies was miniprepped from overnight bacterial cell cultures. Single colonies were inoculated into 10 mL $LB_{amp\ (100mg/L)}$ liquid media and incubated at 37°C at 250 rpm overnight. A 500 µL aliquot of the overnight culture was added to 500 µL glycerol to make a final concentration of 50 % glycerol and was stored at -80 °C. The remainder

of the culture was subjected to plasmid DNA purification using a QIA prep Spin Miniprep Kit as previously described. The pPICZA plasmid DNA was isolated as previously described. A 30 µL aliquot of miniprepped PGT8-pCR4 TOPO samples was sent for sequencing at the University of Tennessee sequencing facility (Knoxville) for verification of the presence of modified PGT8 constructs in TOPO vector.

Restriction Digests of PGT8 Plasmid and pPICZA Vector

Several samples of PGT8-TOPO were sent for sequencing to confirm presence of PGT8 in TOPO vector. One sample confirmed to have PGT8 in frame with TOPO vector was minipreped and digested with Kpn1 and Apa1 restriction enzymes to confirm the presence of PGT8 insert. Larger digestion reactions were carried out to obtain enough DNA for gel purification. Restriction digestion reaction consisted of the following components; 14 µL of miniprep plasmid DNA, 2 µL of Kpn1 restriction enzyme, 2 µL of Apa1 restriction enzyme, and 2 µL of 10X buffer A.

Gel Purification of PGT8 Construct and pPICZA Vector

Digested PGT8 construct and pPICZA vector were gel purified using the Wizard SV Gel and PCR clean-up system (Promega) following the manufacturer's instructions. The gel purified PGT8 construct and pPICZA vector were analyzed by DNA gel electrophoresis using 0.8% agarose gel containing EtBr as previously described to confirm complete purification of the insert and vector and also to confirm that the size of the insert and vector was correct.

Cloning PGT8 into Expression Vector, pPICZA

Ligation of pPICZA Vector and PGT8

The concentration of digested pPICZA vector and PGT8 insert was determined as previously described. Table 12 shows the ligation reaction ratios. The reactions were incubated at 15^{o}C for 4 hours. The ligation ratios were calculated using the formula provided in Promega catalog (part #9PIM180), as previously described.

Table 12. Reaction Mixtures for Ligation of pPICZA Vector and PGT8 insert.

Molar ratio of insert to vector	volume of insert used (µL)	volume of vector used (µL)	volume of T4 DNA ligase (µL)	volume of ligase buffer (µL	total volume (µL)
1:1	21	18	4	2	49
2:1	42	18	3	6.5	69.5

The total volume of the ligation mixture was unusually large because, the yield obtained after purification of the pPICZA vector and PGT8 insert was very low, thus the concentration was low. A large volume was needed to get the required concentration after quantification of the band intensity. After the ligation reaction, 2µL of the reaction mixture was transformed into 50 µL of top 10 competent cells (holding cell line) using the heat shock method per the manufacturer's instructions as previously described. After transformation, a 100 µL aliquot of the transformed mixture was spread evenly on prewarmed $LB_{zeocin\ (100mg/l)}$ plates and incubated at 37 ^{o}C overnight.

Selection of Positively Transformed Colonies

Single colonies from zeocin resistance LB plates that were incubated overnight were selected and cultured overnight in LB_{zeocin} liquid media incubated at 37 ^{o}C, 250 rpm. Plasmid DNA was isolated from the cultures by miniprep as previously described. A 2µL aliquot of the miniprep was digested with 1 µL of Kpn1 (10 µg/µL), 1 µL of Apa1 (10 µg/µL), 1 µL of 10X promega buffer A and 5 µL of sterile water at 37^{o}C for 4 hours as previously described. Samples of digested plasmids were analyzed with DNA agarose gel electrophoresis as previously described to confirm the presence of PGT8 insert. A 30 µL aliquot of purified rPGT8 was sent for sequencing at University of Tennessee sequencing facility (Knoxville) for verification of the presence of PGT8 insert in frame with pPICZA vector and fusion tags. Results from sequencing were analyzed with Bioedit Sequence Alignment Program to confirm ligation of PGT8 in pPICZA vector as previously described.

CHAPTER 3

RESULTS

Optimizing the Expression of Recombinant PGT3 in pCD1 Vector in *E.coli*

Expression With and Without Betaine and Sorbitol

In order to overcome the challenge of inclusion bodies, several strategies were employed to optimize the expression of soluble recombinant PGT3. One of strategies was variation of media composition. With regards to variation of media composition, recombinant PGT3 was expressed in the presence and absence of betaine and sorbitol. Betaine and sorbitol are osmotic reagents that have been shown to increase the expression of some soluble proteins by increasing osmotic stress (Blackwell and Horgan 1991). This tends to prevent the packing of soluble proteins into insoluble inclusion bodies. The postinduction cell pellets containing recombinant PGT3 protein was resusupended in lysis buffer, lysed by sonicating and analyzed by SDS-PAGE and Western blotting. Figures 13 and 14 shows the expression levels of rPGT3 in LB media with and without betaine and sorbitol. The optimal expression of rPGT3 at 25^{o}C in LB media containing betaine and sorbitol was 6 and 18 hours postinduction. Several attempts were made to reproduce the results however this was not consistent with subsequent experiments. Expression of rPGT3 in LB media without betaine and sorbitol resulted in maximum expression of rPGT3 at 25^{o}C, 24 hours postinduction. A comparison of the expression of rPGT3 in the media with and without betaine and sorbitol showed that the optimum media for expression of rPGT3 was without betaine and sorbitol. All subsequent inductions were done for 24 hours without betaine and sorbitol in the culture media.

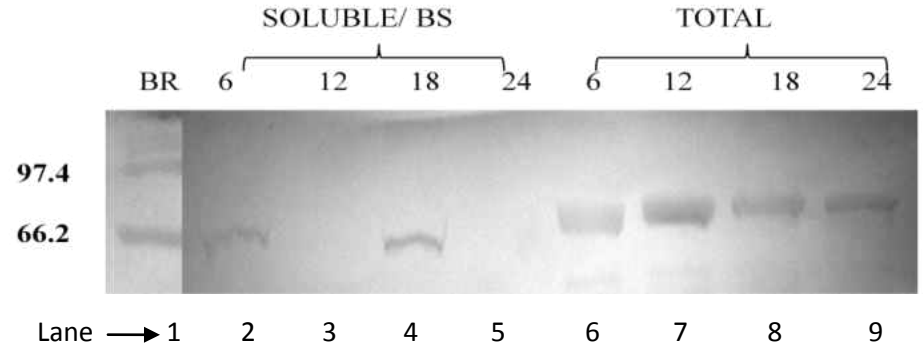


Fig 13. Expression Levels of PGT3 in LB Media Containing Betaine and Sorbitol. Western blots showing levels of soluble and total rPGT3 protein using LB media containing betaine and sorbitol. PGT3 protein was detected using antibodies directed against the thioredoxin fusion partner. Lane 1 is protein molecular weight marker, lanes 2 – 5 are soluble protein sample, and lanes 6-9 show total proteins expressed. Hours postinductions are noted at the top of each lane.

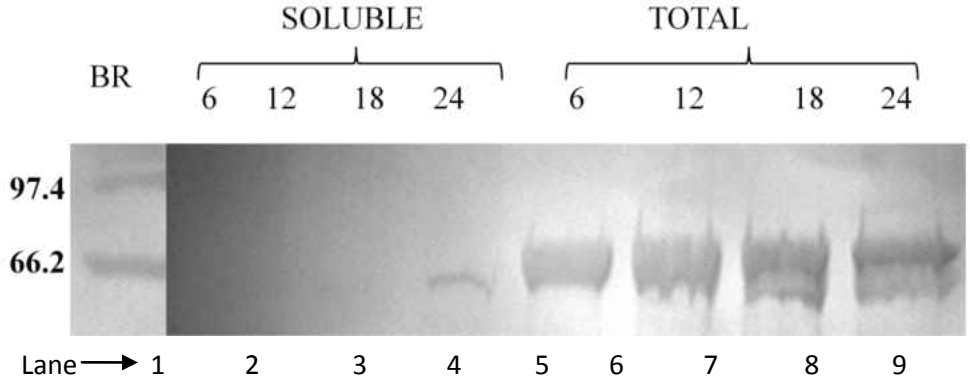


Fig 14. Expression Levels of rPGT3 in LB Media without Betaine and Sorbitol. Western blots showing levels of soluble and total rPGT3 protein using LB media without the addition of betaine and sorbitol. PGT3 protein was detected using antibodies directed against the thioredoxin fusion partner. Lane 1 shows protein molecular weight marker; lanes 2 – 5 are soluble protein samples; and lanes 6-9 shows total rPGT3 proteins expressed.

Expression with Varying Concentrations of IPTG

In this strategy, different concentrations of the inducer IPTG were used for expression of recombinant PGT3. The inducer IPTG has been used in varying concentrations (0.005-5) mM per liter for expression of foreign genes in *E.coli* (Donovan et al. 1996 and ref. therein). The use of higher concentrations is sometimes done in an attempt to fully induce the lac promoter (Donovan et al. and ref. therein). To test the effect of varying concentrations of IPTG in this

experiment, a lower concentration of 0.2 mM, the usual 1mM concentration, and a higher concentration, 5 mM, were tested. Postinduction cell pellets were resuspended in lysis buffer and lysed using a dismembrator model 500 with microtip (Fisher Scientific). Preinduction, total protein, and soluble protein fractions were analyzed by SDS-PAGE and Western blotting. Figure 15 shows the levels of expression of recombinant PGT3 under the varying concentrations of IPTG. Results show that varying the concentration of IPTG did not increase production of soluble rPGT3.

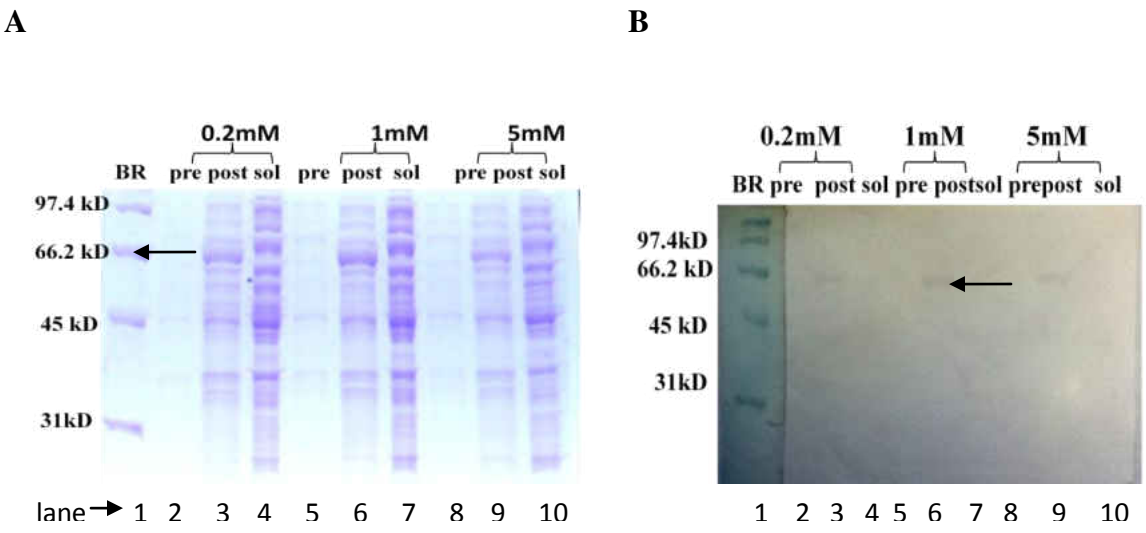


Fig 15. Expression Levels of Recombinant PGT3 under Varying Concentrations of IPTG. A. Coomasie stained SDS-PAGE showing the expression levels of PGT3 protein. Lane 1, molecular weight marker; lane 2, preinduced sample; lane 3, total recombinant PGT3 protein; and lane 4, soluble recombinant PGT3 proteins expressed with a final IPTG concentration of 0.2 mM; lanes 5-7, pre induction sample, total and soluble recombinant PGT3 respectively from cultures induced with 1mM IPTG. Lanes 8-10, preinduction, total and soluble recombinant PGT3 expressed with a final IPTG concentration of 5 mM. B. Western blot detecting the expression of recombinant PGT3 protein. PGT3 protein was detected using antibodies directed against the thioredoxin fusion partner. Lane 1 shows protein molecular weight marker; lanes 2-4 indicates preinduction, total and soluble recombinant protein expressed under 0.2 mM final concentration of IPTG; lanes 5-7 indicates preinduction, total and soluble recombinant PGT3 expressed under 1 mM final concentration of IPTG; lanes 8-10 indicates preinduction, total and soluble recombinant PGT3 expressed under 5 mM final concentration of IPTG.

Enrichment/Purification of Soluble Recombinant PGT3 Protein using IMAC

In an attempt to enrich the trace soluble recombinant PGT3 protein, 24 hour postinduction cell pellets were resuspended in lysis buffer and lysed. The cell lysate was centrifuged and 10 mL of the supernatant were run through a 1 mL IMAC column. Fractions were analyzed by SDS-PAGE and Western blot to test for enrichment of rPGT3 and to identify the fractions that contained the most purified rPGT3 protein. Figure 16 shows fractions obtained via IMAC. Results show that most of the rPGT3 was found in the flow-through (data not shown) and the 10 mM imidazole fractions (Fig 16 A, lanes 3-7) and was not binding to the column hence the majority of rPGT3 was lost. This may have been due to an overload of rPGT3 on the 1 mL IMAC column. To test this, a 5 mL IMAC column was used to enrich rPGT3 (Fig. 17). Results confirm enrichment of rPGT3. However yields of enriched rPGT3 were insufficient to permit screening for GT activity.

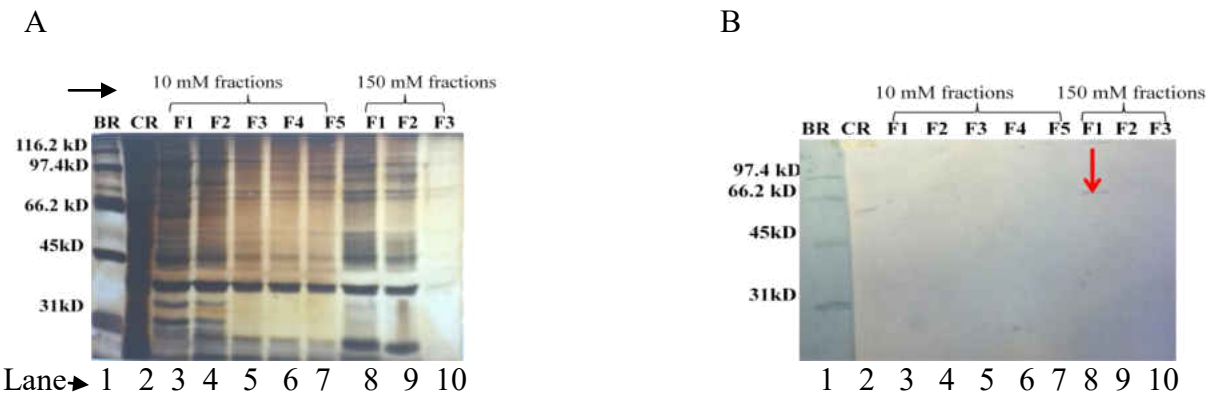


Fig 16. Enrichment of rPGT3 using 1 mL Metal Affinity Chromatography Column (IMAC). A. Representative Silver stained SDS PAGE of proteins eluted with 10 and 150 mM imidazole after metal affinity chromatography. Lane 1, Broad range molecular weight marker; lane 2, crude PGT3 lysate; lanes 3-7, washes with 10mM Imidazole; lanes 8-10, proteins eluted with 150 mM imidazole buffer. B. Representative Western blot. Lane 1, broad range marker; lane 2, crude PGT3 lysate; lanes 3-7, washes with 10 mM imidazole; lanes 8-10, rPGT3 proteins eluted with 150 mM imidazole buffer. Lane 8, marked with red arrow indicates the fraction with the most enriched PGT3.

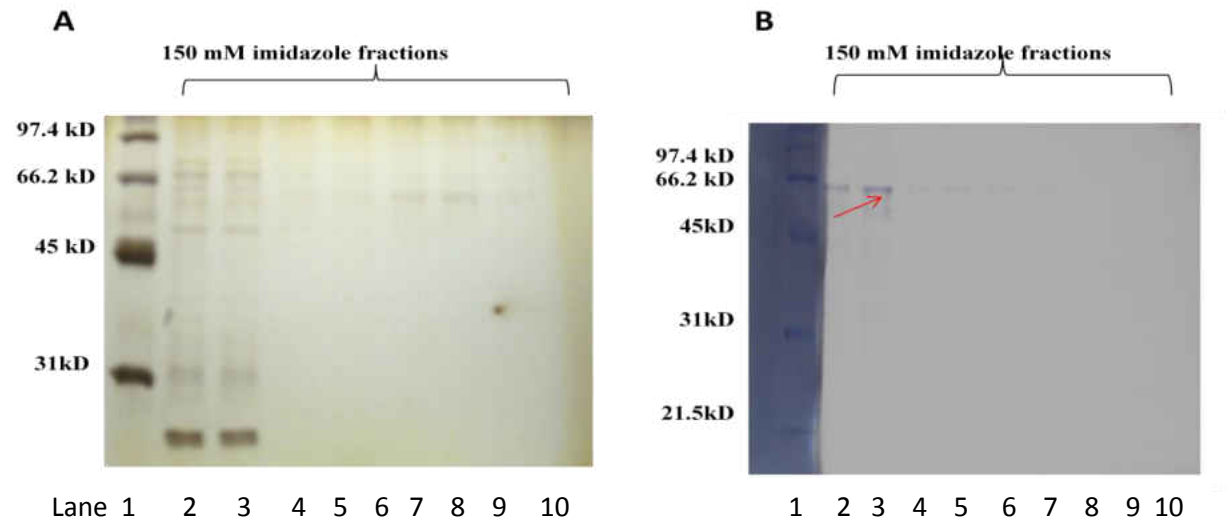


Fig 17. Enrichment of rPGT3 using 5 mL Imobilized Metal Affinity Column (IMAC)**.** A. Representative silver stained SDS PAGE of proteins eluted with 150 mM imidazole after metal affinity chromatography. Lane 1, Broad range marker, lane 2-10, proteins eluted with a buffer containing 150mM Imidazole. B. Representative Western blot of PGT3 proteins eluted with 150 mM imidazole after metal affinity chromatography.

After several attempts to optimize the expression of rPGT3 in *E.coli* results obtained for yield of soluble protein were not reproducible. Thus, expression in yeast was tested.

Cloning PGT3 into pPICZ Vectors for Expression in *Pichia pastoris*

Isolation of PGT3 and pPICZ vectors

In order to subclone PGT3 into pPICZA vectors, the PGT3 sequence and the multiple cloning site of pPICZA and pPICZAα vectors were studied to identify appropriate cloning sites. Cultures of PGT3 in pCR4-TOPO/TOP10, pPICZA vector, and pPICZAα vector in *E.coli* were miniprepped to isolate the plasmids. Results confirm plasmid isolation was successful (Fig.18).

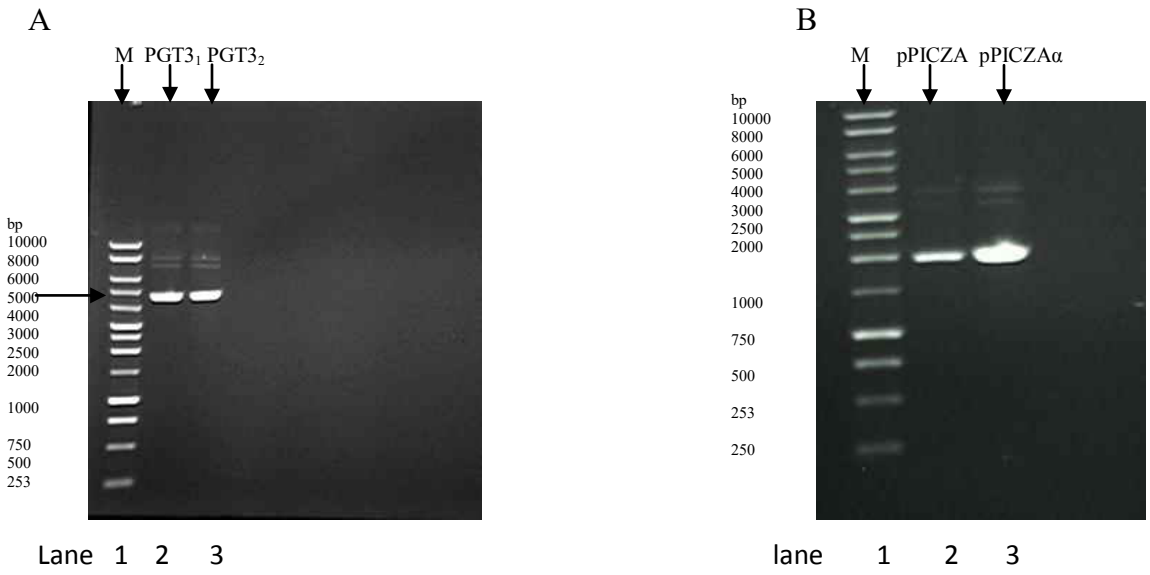


Fig. 18. Miniprep of PGT3 Plasmid, pPICZA and pPICZAα Vectors from pCR4-TOPO/TOP10. A. DNA agarose gel (0.8%) electrophoresis showing the mimiprep yields of PGT3/TOPO. Lane 1, kb DNA ladder; lane 2 and 3, PGT3/pCR4-TOPO/TOP10 clones. B. Miniprep of pPICZA and pPICZAα vectors. Lane 1, 1 kb DNA ladder; lane 2 and 3 pPICZA and pPICZAα vectors, respectively.

Modification of the Ends of PGT3 from pCR®4-TOPO Vector

The 5' and 3' ends of PGT3 sequence were then modified with primers (Table 2; chapter 2) and the PCR products were analyzed with DNA agarose gel electrophoresis (Fig 19). The sizes of the bands in lanes 2, 3, and 4 were calculated as 1493bp, 1490bp, and 1500 bp, respectively. This was compared with the estimated size of PGT3 (1495 bp) to confirm that the PCR product was the size of full length PGT3. See appendix F nucleotide sequence of PGT3.

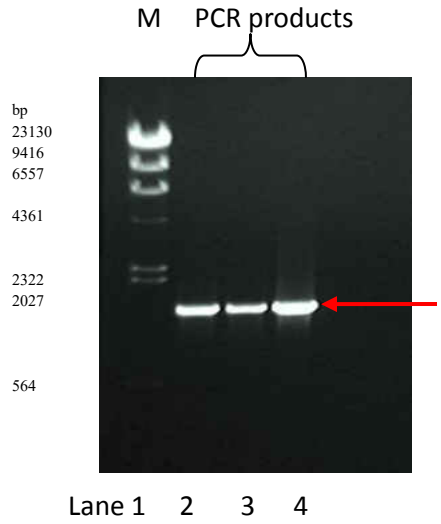


Fig. 19. Modified and Amplified PGT3. DNA agarose gel (0.8%) showing the PCR products obtained after modifying PGT3 with appropriate primers (Table 2) to get PGT3 cloned into *Pichia pastoris* using 3 cloning strategies. Lane 1: 5 ul of lambda Hind III marker; lane 2, 2 ul of construct 1 PCR product; lane 3, 2ul of construct 2 PCR product; and lane 4, 2ul of construct 3 PCR product. Arrow indicates the PCR products.

Cloning Modified PGT3 into TOPO

The PCR products obtained for constructs 1, 2, and 3 were subcloned into pCR®4-TOPO vector and transformed into TOP 10 competent cells as described in the material and methods section. Single colonies were selected from LB $_{amp\ (100mg/L)}$ plates and screened using clone specific primers to confirm the transformation of TOPO vector containing PGT3 into TOP 10 cells (Fig. 20). Results showed a 1500 bp (Fig. 20) amplification product that corresponded to the estimated size of PGT3 in some colonies. These colonies were 2, 6, and 9 for construct 1; colonies 1, 2, 3, 4, 5, 6, 11, 14, 15, and 16 for construct 2; and, colonies 6, 10, 13, 15, and 17 for construct 3.

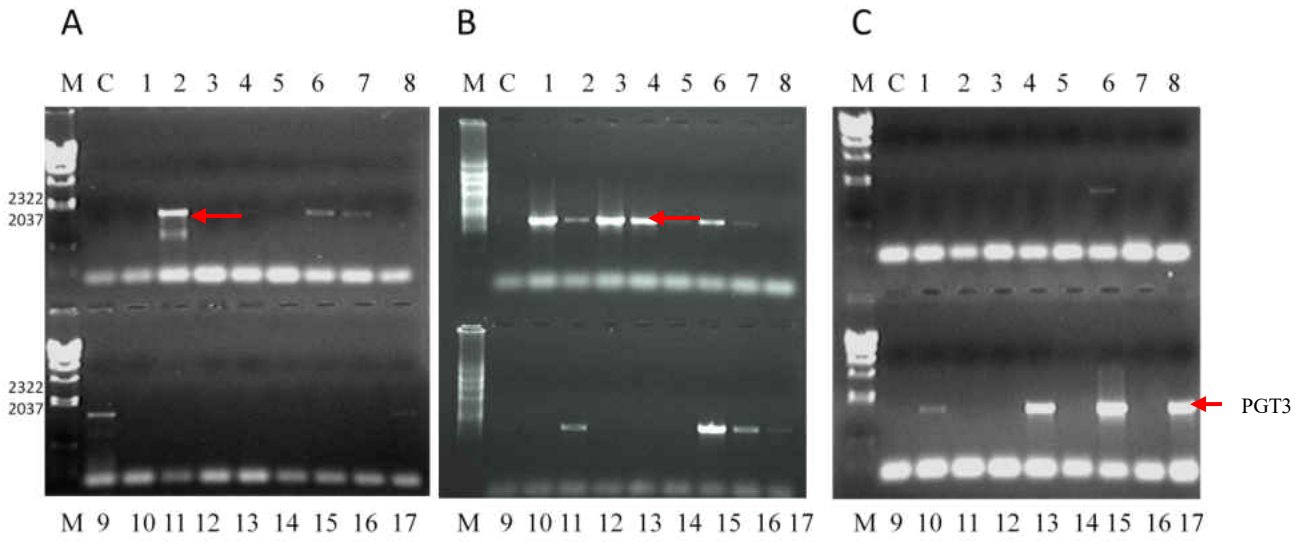


Fig. 20. Confirmation of Modifed PGT3-TOPO Transformed Colonies by PCR Screens. A, B, and C are all representative DNA agarose gels (0.8%) showing PCR products for colonies obtained for constructs 1, 2, and 3, respectively corresponding to the the size of PGT3 (1495 bp). Lane marked M indicates Lambda Hind III marker; lane marked C indicates a negative control (PCR reaction run without a template).

Representative colonies were selected for plasmid isolation and digestion. With regards to construct 1, clonies 2, 6, and 9 were selected. Colonies 1, 3, and 15 were selected for construct 2. Colonies 13, 15, and 17 were selected for construct 3. The plasmid DNA from each selected colony was isolated by miniprep as described in the materials and methods section.

Restriction Digests of Plasmid Constructs

After isolation of the plasmid DNA for all 3 constructs, each isolate was digested with appropriate restriction enzymes to confirm the presence of PGT3 insert in TOPO vector. Digested and undigested samples were run on 0.8% agarose gel for analysis (Fig. 21). Upon analysis, the digested colonies had a band that corresponded to the size of TOPO vector (3596 bp) and another band that corresponded to the size of PGT3 (1495 bp) (Fig. 21). Aliquots of the plasmid DNA for all 3 constructs were sent for sequencing. Results confirmed the presence of PGT3 insert in TOPO vector (Fig. 22, 23, and 24).

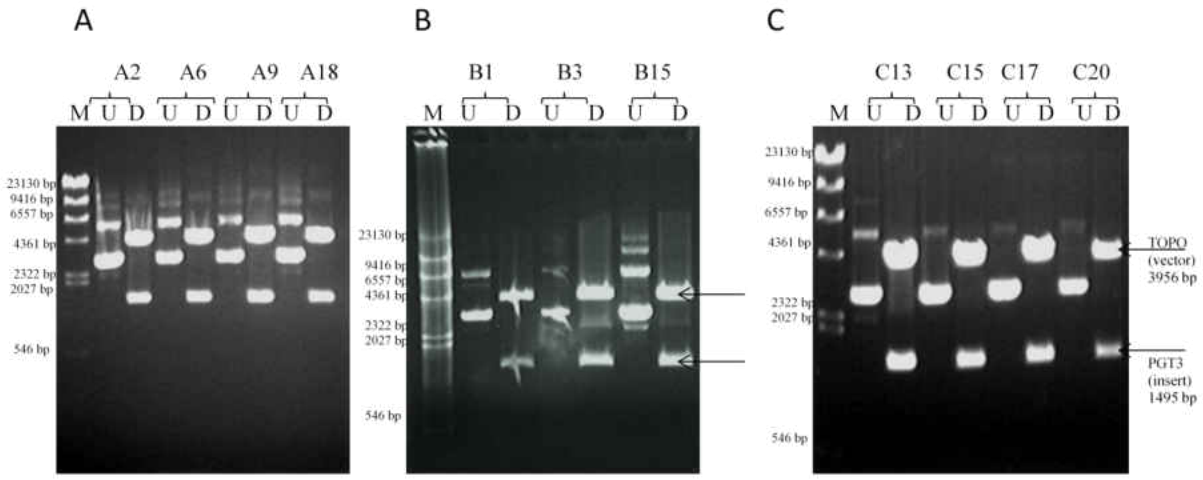


Fig. 21. Restriction Digests of PGT3-TOPO Plasmid DNA. Plasmid DNA was isolated from positive colonies that contained modified PGT3-TOPO and plasmids were digested with restriction enzymes to verify presence of insert in vector A. Representative DNA agarose gel electrophoresis showing plasmid DNA isolation and restriction digestion of PGT3 construct 1 clone 2, 6, 9, and 18 using Kpn1 and Apa1 restriction enzymes B. Representative DNA agarose gel electrophoresis showing plasmid DNA isolation and restriction digestion of PGT3 construct 2 clones, 1, 3, and 15 using Kpn1 and Xba1 C. Shows isolation and digests of PGT3 construct 3 clones, 13, 15, 17, and 20 using Xba1 and Xho1. Note; UD= undigested plasmid D= digested plasmid.

A.

```
PGT3TOPO5'    GTATTAGCGG CGCGATTCGC CCTTCATGGG TACCATGGAA GAAAAGCCTA
PGT3          ---------- ---------- ---------- ----ATGGAA GAAAAGCCTA

                   ....|....| ....|....| ....|....| ....|....| ....|....|
                            60         70         80         90        100
PGT3 TOPO     AATCTCCTCA TATCCTGATC TTTCCTCTCC CATGCCAAAG CCATGTGAAT
PGT3          AATCTCCTCA TATCCTGATC TTTCCTCTCC CATGCCAAAG CCATGTGAAT

                   ....|....| ....|....| ....|....| ....|....| ....|....|
                           110        120        130        140        150
PGT3 TOPO     TCCATGCTCA AGCTTGCCGA GATTTTTGGC TTAGCTGGCC TAAAAGTGAC
PGT3          TCCATGCTCA AGCTTGCCGA GATTTTTGGC TTAGCTGGCC TAAAAGTGAC
```

B.

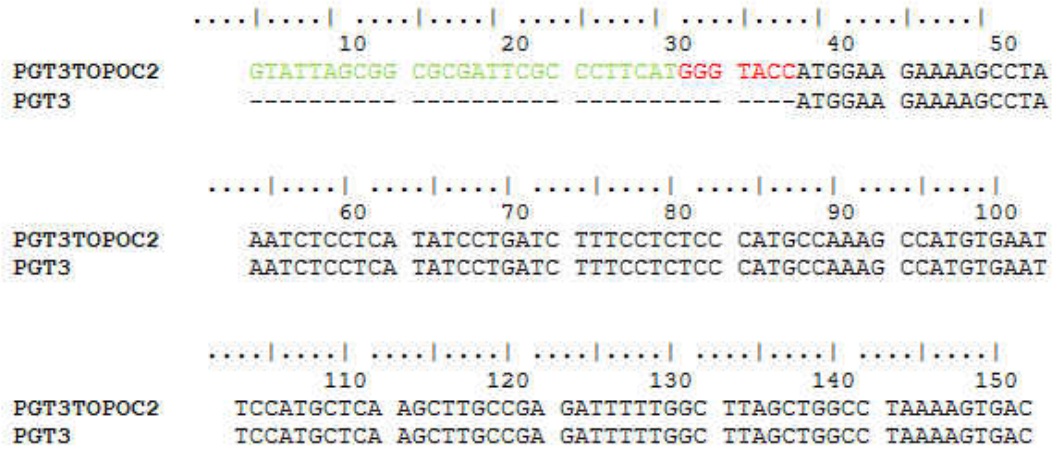
```
                         1310       1320       1330       1340       1350
C1 3'         AAGGAGGAGT TTATGAGAGC AGCTGATCGG ATGGCTACAA TGGCTAGAAC
PGT3          AAGGAGGAGT TTATGAGAGC AGCTGATCGG ATGGCTACAA TGGCTAGAAC

                   ....|....| ....|....| ....|....| ....|....| ....|....|
                         1360       1370       1380       1390       1400
C1 3'         CACTGCTAAT GAAGGTGGAC CATCTTATTG TAATTTGGAC CGTCTGATTG
PGT3          CACTGCTAAT GAAGGTGGAC CATCTTATTG TAATTTGGAC CGTCTGATTG

                   ....|....| ....|....| ....|....| ....|....| ....|....|
                         1410       1420       1430       1440       1450
C1 3'         ATGATATTAA GATGATGAGC TCCCAAGCAT GGGCCCGCGA AGGGCGAATT
PGT3          ATGATATTAA GATGATGAGC TCCCAAGCAT GA
```

Fig. 22. Alignment of PGT3 Sequence with PGT3-TOPO (construct1) Sequence using Bioedit. A. 5' Alignment; region in red represent restriction enzyme site. Region in green represents TOPO vector sequence. B. 3'Alignment of PGT3 Sequence with PGT3-TOPO (construct1) Sequence using Bioedit. The regions in red represent restriction enzyme site.

A.



B.

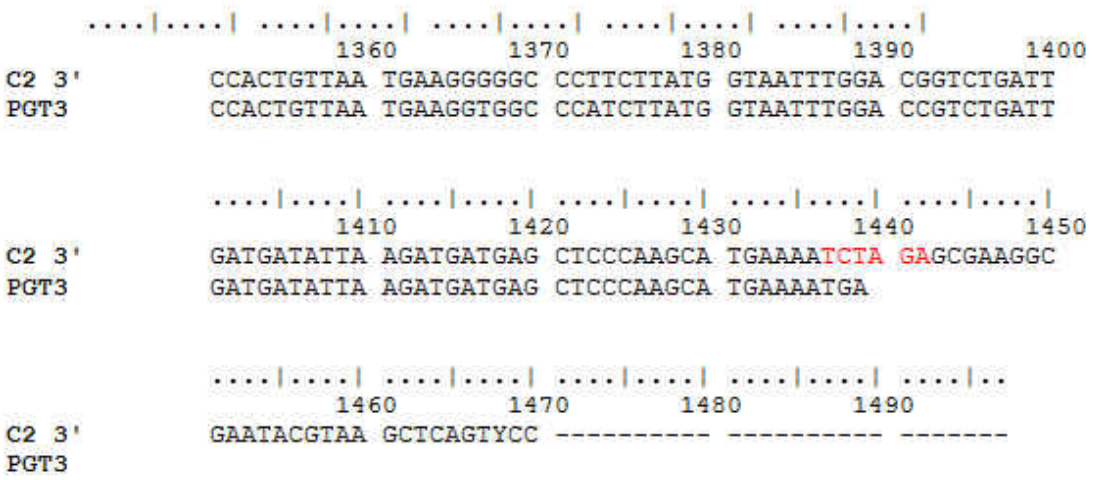
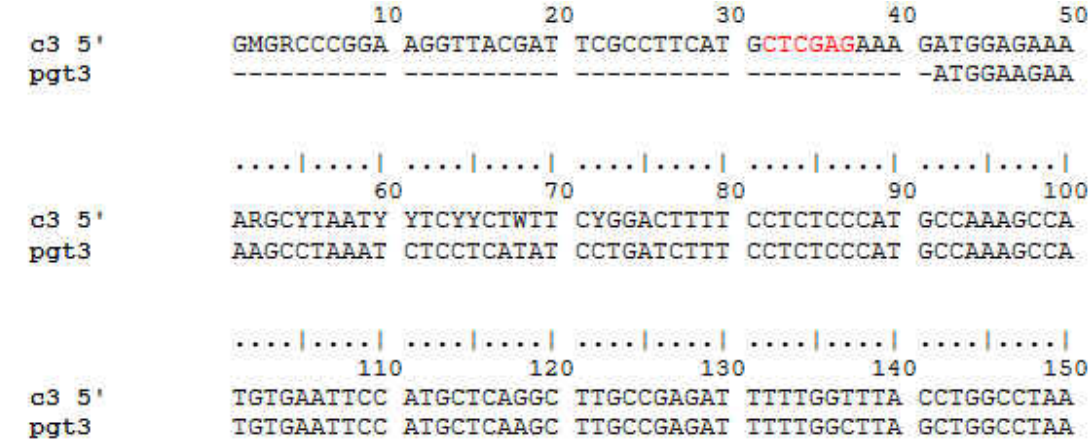


Fig. 23. Alignment of PGT3 Sequence with PGT3-TOPO (construct2) Sequence using Bioedit. A. 5' Alignment; region in red represent restriction enzyme site. Region in green represents TOPO vector sequence. B.3' Alignment of PGT3 Sequence with PGT3-TOPO (construct2) Sequence using Bioedit. The regions in red represent restriction enzyme site.

A.



B.

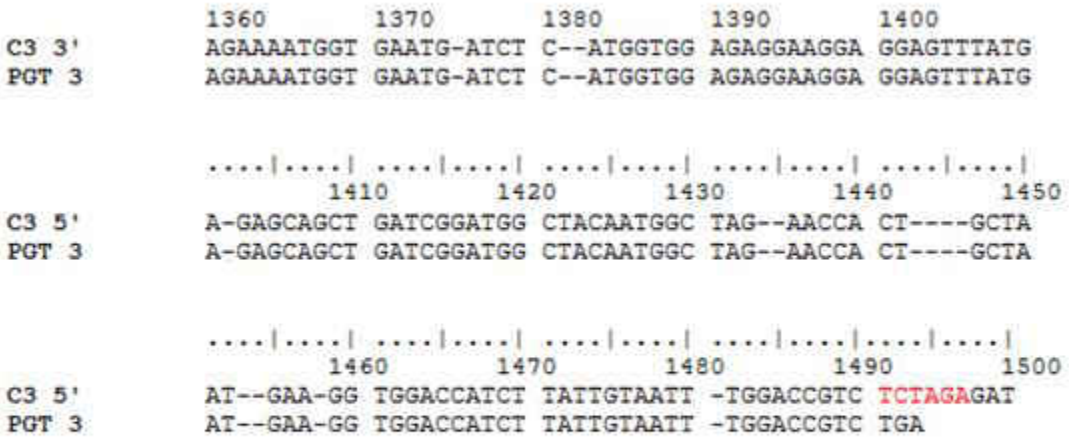


Fig. 24. Alignment of PGT3 Sequence with PGT3-TOPO (construct3) Sequence using Bioedit. A. 5' Region: The area in red represent restriction enzyme site. Region in green represents TOPO vector sequence. B. 3' Region: The regions in red represent restriction enzyme site.

Gel Purification of PGT3 Constructs and Vectors

After confirmation of the presence of PGT3 insert in TOPO. Larger volumes (14 µL) of restriction digests of clones 2, 15, and 13 (representing constructs 1, 2, and 3) were performed and the bands were gel purified (Fig. 25). The corresponding vectors, pPICZA, pPICZAα 1, and pPICZAα 2 with which construct 1, 2 and 3 were to be ligated with respectively were also digested. Prior to ligation of the purified PGT3 and pPICZ vectors, the gel purified PGT3 constructs and accompanying vectors were each analyzed by DNA gel electrophoresis using 0.8% agarose gel containing EtBr as previously described, to confirm complete purification of the inserts and vectors and also to confirm that the sizes of the inserts and vectors were correct (Fig. 26). Results (Fig. 26 A) confirmed that plasmid DNA from respective colonies selected for constructs 1, 2, and 3 contained an insert of expected size for PGT3. Figure 26 B confirmed purification of pPICZ vectors.

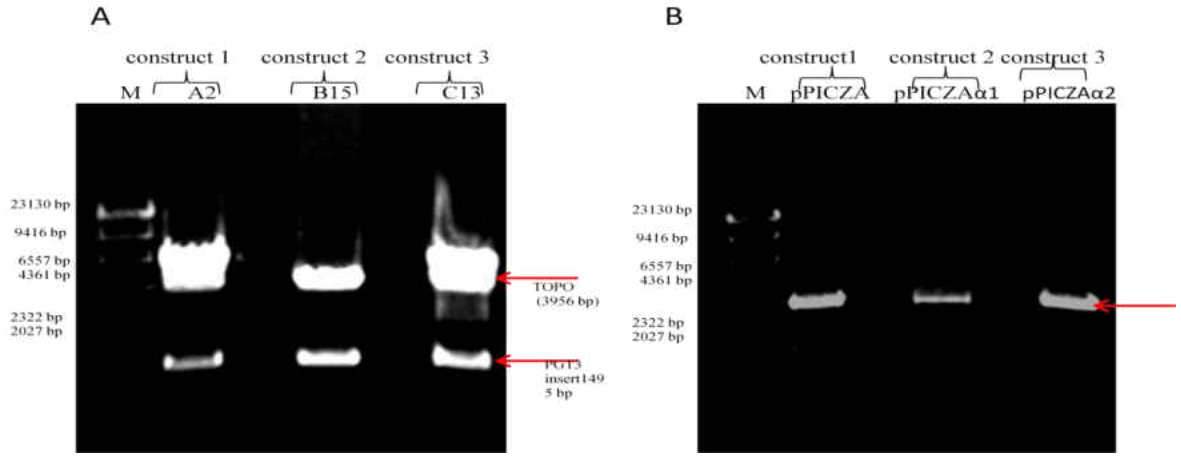


Fig. 25. Restriction Digests of PGT3-TOPO Constructs and pPICZ Vectors for DNA Plasmid Purification. A. Representative DNA agarose gel electrophoresis showing restriction digestion of clones representing construct 1, 2, and 3 PGT3 inserts. Digestion with restriction enzymes Kpn1 and Apa1 for construct 1; Kpn1 and Xba1 for construct 2, Xba1 and Xho1 for construct 3. Note separation of bands PGT3 (1495 bp), from TOPO vector (3596 bp). B. Representative DNA agarose gel electrophoresis showing restriction digestion of pPICZA, pPICZAα1, and pPICZAα2 vectors. The vectors were digested with restriction enzymes to make them compatible for ligation with their corresponding PGT3 inserts.

Ligation of pPICZ Vectors and PGT3 Inserts for Constructs 1, 2, and 3

Prior to ligation, aliquots of digested PGT3 inserts and corresponding pPICZ vectors were run on DNA agarose gel to aid in the quantification of the concentration of DNA. A 2 ul and 5 ul aliquot of representative purified plasmids of PGT3 inserts and pPICZ vectors were run together with Lambda Hind III marker and 1 kb DNA Plus marker (Fig. 26). The concentration of DNA of the inserts and vectors were estimated by comparing the intensity of each band with the intensity of the Lambda Hind III markers (Fig. 26, M2). The concentration of purified pPICZA vector was 15.5 ng/µL and the PGT3-A9 insert concentration 15.5 ng/µL. Also, pPICZAα1 vector had a concentration of 28 ng/µL and PGT3-B15 insert was 41 ng/µL. With regard to pPICZAα2 vector, its concentration was 31ng/µL and PGT3-C15 insert was 41 ng/µL.

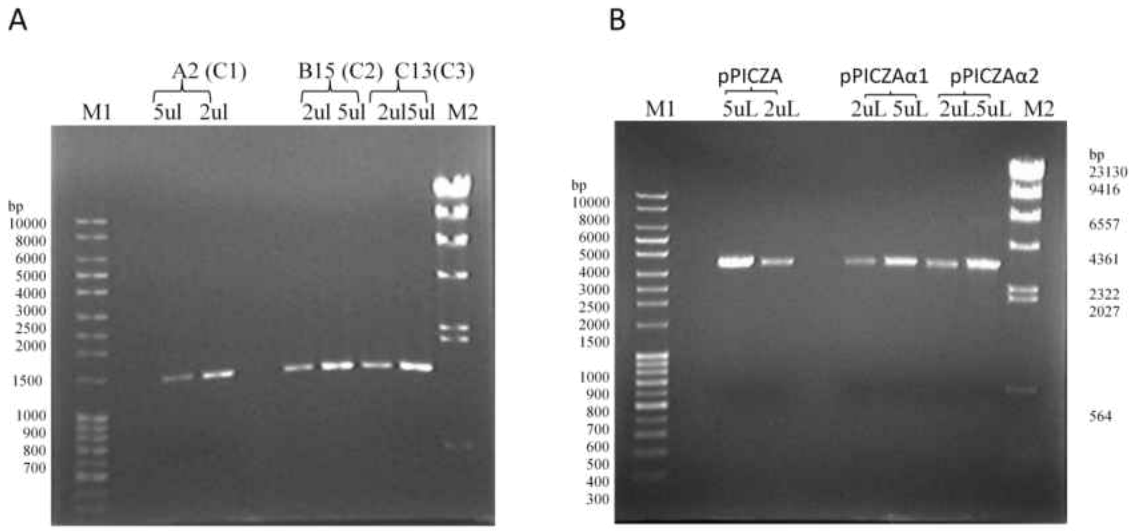


Fig. 26. Gel Purified PGT3 Inserts and pPICZ Vectors Prior to Ligation. A. Representative DNA agarose gel electrophoresis showing purified PGT3 inserts ready for ligation with pPICZ vectors. Constructs annotated as C1, C2, and C3, respectively with different volumes run on agarose for quantification of DNA concentration. B. Representative DNA agarose gel electrophoresis of purified pPICZ vectors. M1 represents 1 kb DNA ladder, M2 represents lambda Hind III marker.

After ligation of PGT3 inserts with corresponding pPICZ vectors, the ligation reactions were transformed into TOP 10 competent cells and resulting colonies were screened by PCR with clone specific primers to confirm transformation. Results of the PCR screens are shown in figure 27. Note that the positive colonies showed a band corresponding to the estimated size of PGT3 (1495 bp). Gel A, B, and C are representations of construct 1, 2, and 3, respectively (Fig. 27).

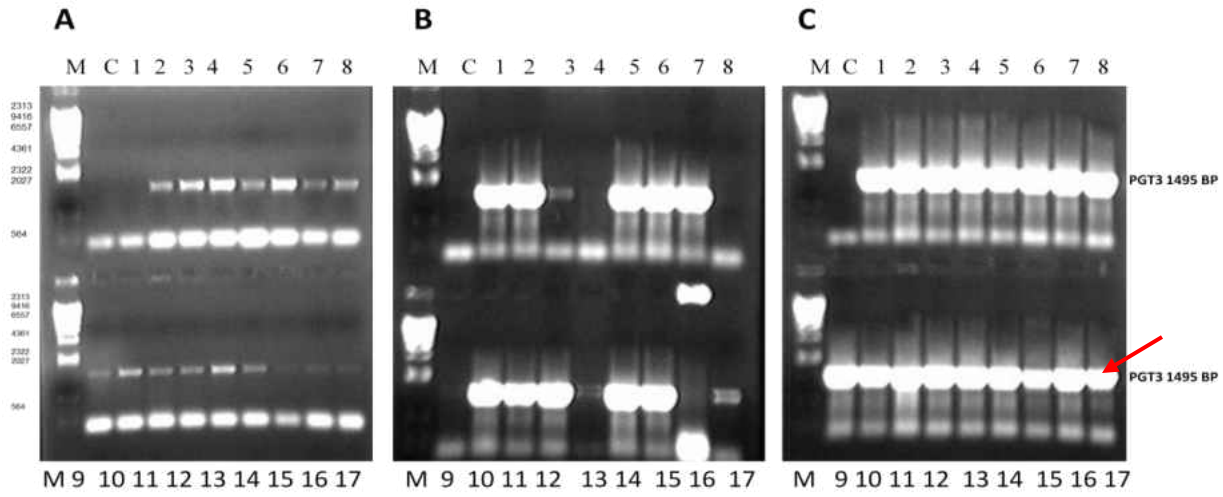


Fig. 27. DNA Agarose Gel Electrophoresis for Verification of Transformed pPICZ Vectors Containing PGT3 Inserts using PCR Screening. A, B, and C are representatives of construct 1, 2 and 3 respectively. M represents Lambda Hind III marker, C, represents negative control (no template) for PCR reaction. Lane numbers correspond to different colonies for each construct.

Plasmid DNA of positive clones were isolated and digested for further verification of correct sized insert (Fig. 28). Results of sequencing analysis confirmed the ligation of PGT3 and pPICZ in frame with the C terminal fusion tags (Figs. 29-31).

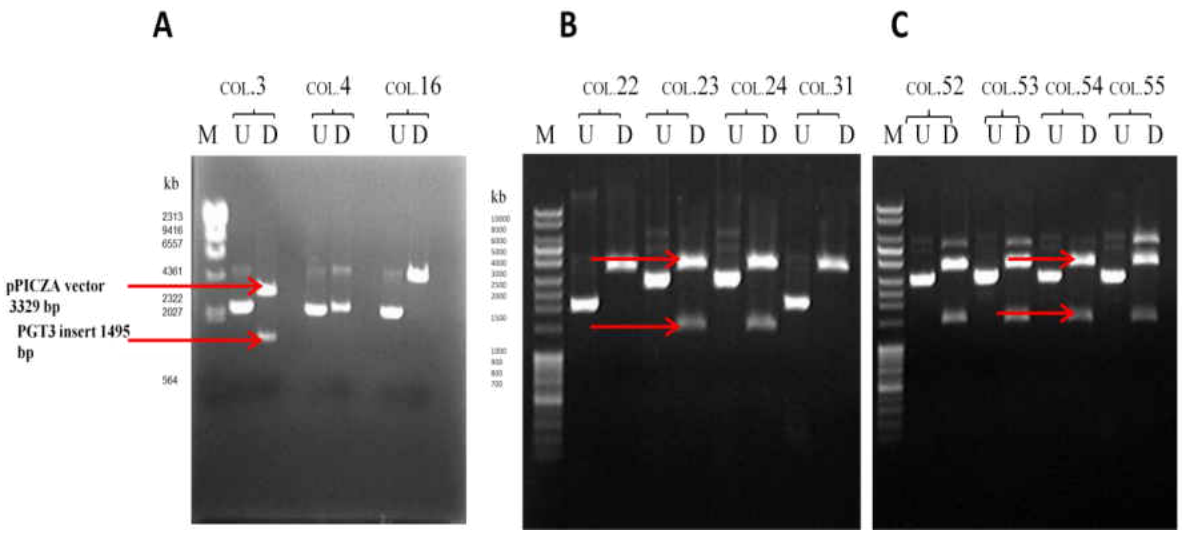
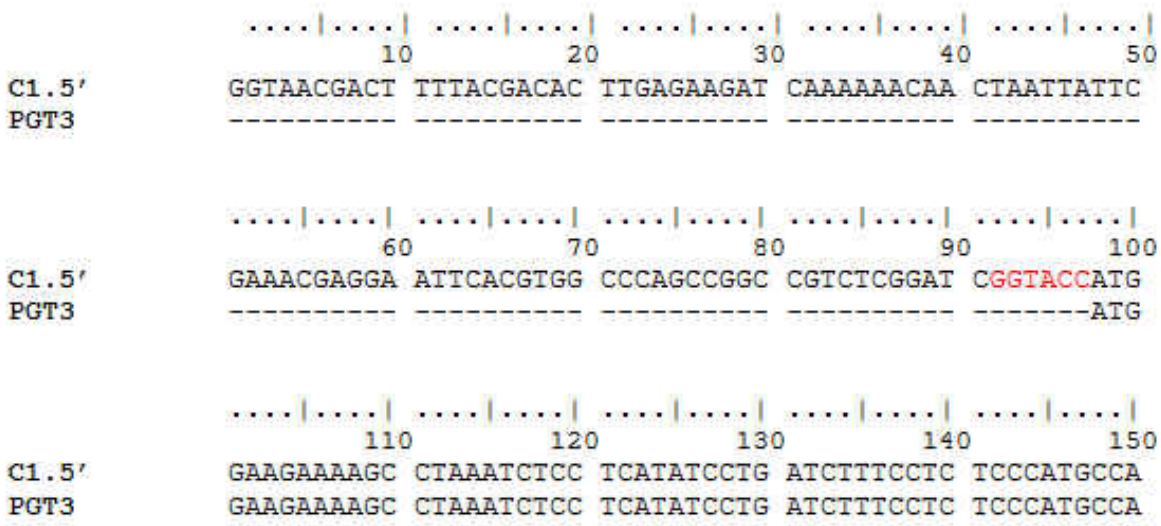


Fig. 28. Verification of Presence of PGT3 Insert in pPICZ Vectors using Restriction Digestions. Representative DNA agarose gel electrophoresis showing plasmids selected for restriction digestion. Plasmids that contained the insert showed a separation of insert (1495 bp) and vector (3329 bp). A, B, and C are representatives of construct 1, 2 and 3 respectively. Digestion with restriction enzymes Kpn1 and Apa1 for construct 1; Kpn1 and Xba1 for construct 2, Xba1 and Xho1 for construct 3. M indicates Lambda Hind III marker. U indicates undigested plasmid, UD indicated digested plasmid, col represents colony.

A.



B.

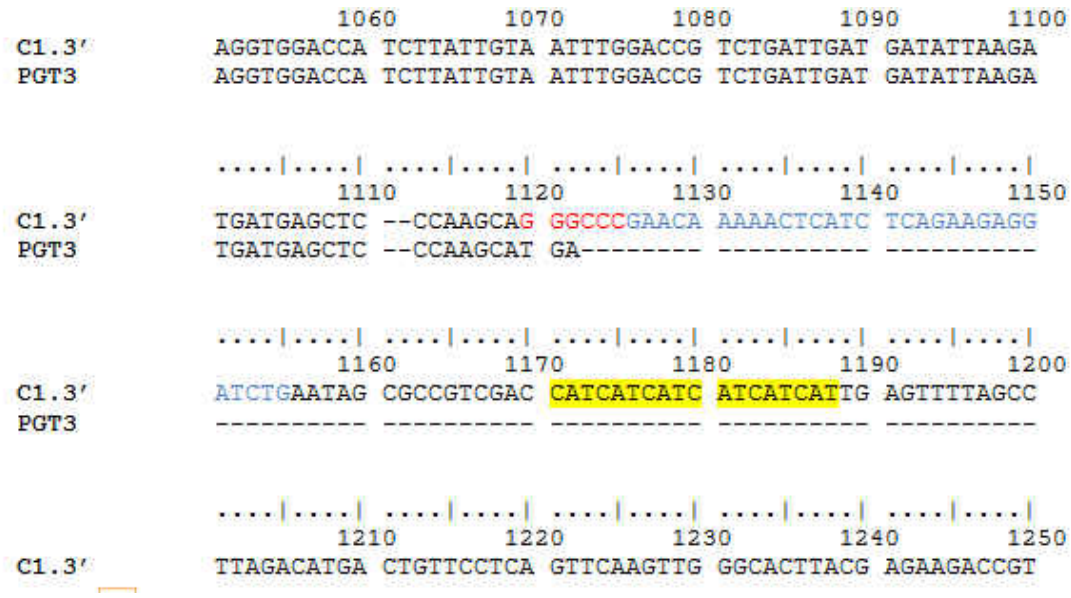
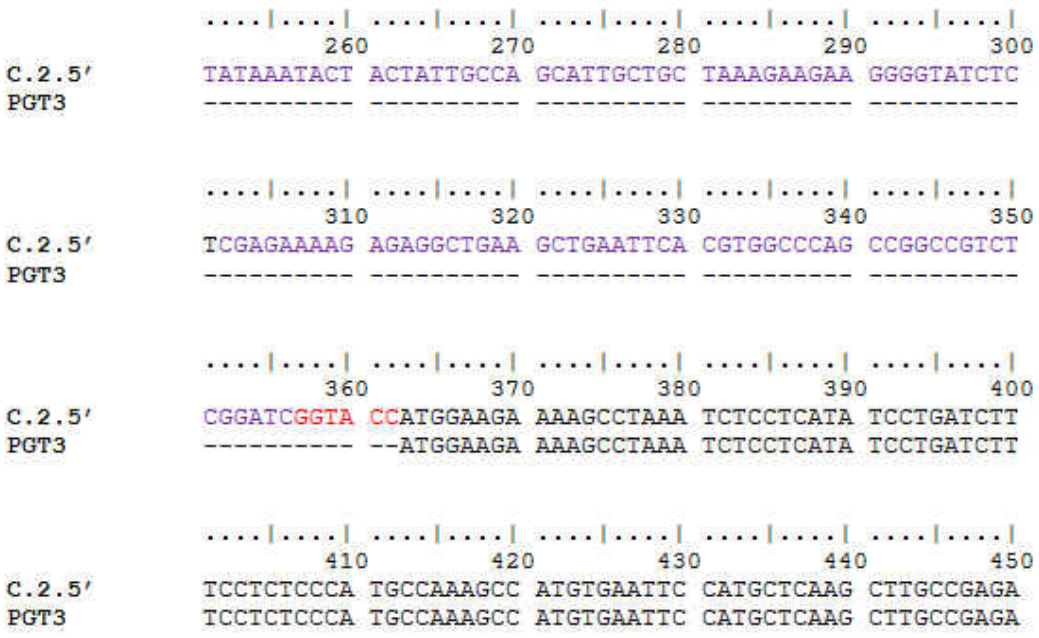


Fig 29. Alignment of PGT3 Sequence with PGT3-pPIPCZA (construct1) Sequence using Bioedit. A. 5' alignment; region in red represent restriction enzyme site. B. 3' Alignment of PGT3 sequence with PGT3-pPICZA (construct1) sequence using Bioedit. The regions in red represent restriction enzyme site. Region in blue represents the c-myc epitope tag, and yellow represents polyhistidine tag sequences in the pPICZA vector.

A.



B.

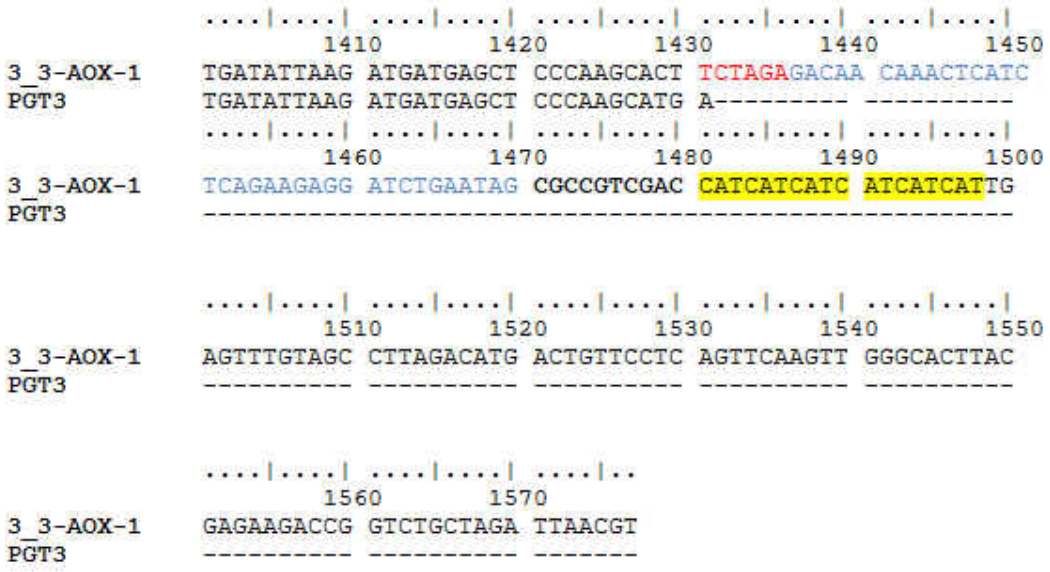


Fig 30. Alignment of PGT3 Sequence with PGT3-pPICZAα (construct2) Sequence using Bioedit. A. 5’ Alignment; regions in red represent restriction enzyme site; region in purple represents the alpha factor signal sequence. B. 3’ Alignment of PGT3 sequence with PGT3-pPICZAα (construct2) sequence using Bioedit. The regions in red represent restriction enzyme site; region in blue represents the c-myc epitope tag.

A.

B.

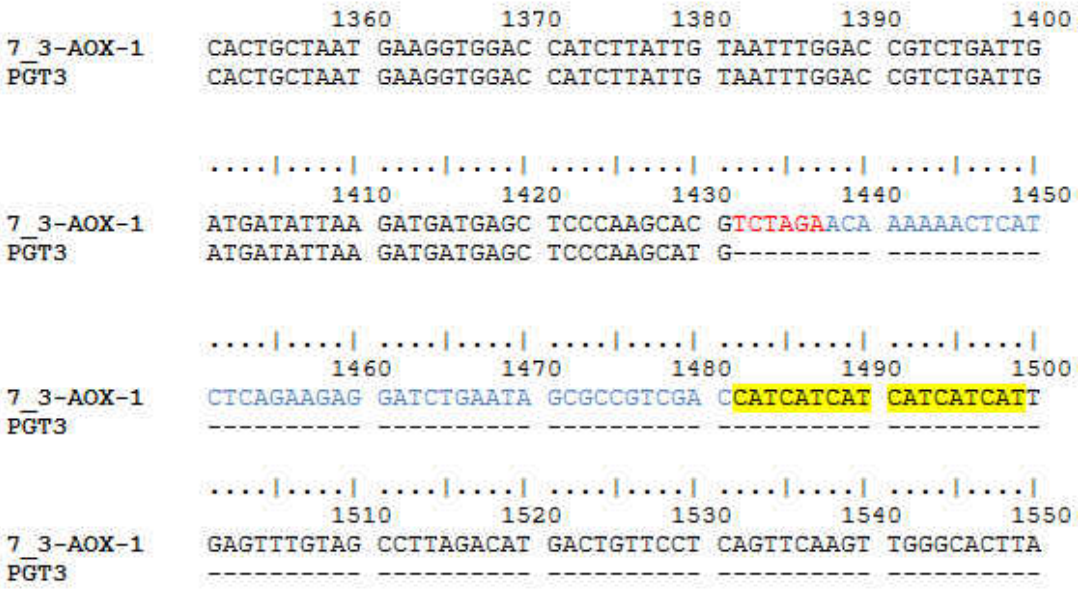


Fig 31. Alignment of PGT3 Sequence with PGT3-pPIPCZAα (construct 3) Sequence using Bioedit. A. 5' Alignment: regions in red represent restriction enzyme site; region in purple represents the alpha factor signal sequence. B. 3' Alignment of PGT3 sequence with PGT3-pPIPCZAα (construct3) sequence using Bioedit. The regions in red represent restriction enzyme priming site; region in blue represents the c-myc epitope tag.

Midiprep of Vectors and Designated Clones for Constructs 1, 2, and 3

Sequencing analysis confirmed successful ligation of PGT3 inserts with corresponding pPICZ vectors (Fig.29-31). The recombinant plasmids PGT3-pPICZA, PGT3-pPICZAα1, and PGT3-pPICZAα2 were labeled NL3, B23, and C54. These represented construct 1, 2, and 3 and were used for further experiments. In order to transform *Pichia pastoris*, 5-10 µg of linearized DNA is required (Invitrogen Pichia manual, 25-0172). To obtain sufficient amount of plasmid DNA for this process, a plasmid midiprep was performed as described in the materials and methods section. To determine the concentrations of the eluted plasmid DNA after midiprep, an aliquot of each of the purified plasmid DNA of rPGT3 and vectors were run on DNA agarose gel (0.8%). The concentration of plasmid DNA for construct 1 insert was estimated to be 11.25 µg and 16.89 µg for pPICZA vector. The concentration of plasmid DNA for construct 2 insert was estimated to be 16.8µg and 10.45 µg for pPICZAα1 vector. The concentration of plasmid DNA for construct 3 insert was 12.49 µg and 10.45 µg for pPICZAα2 vector.

A 300 µL aliquot of each of the purified plasmid midiprep DNA for the constructs and corresponding vectors was subjected to restriction digestion with BstX1 enzyme to linearize the plasmids. A DNA gel (0.8%) electrophoresis was run to confirm complete linearization (Fig. 32). Results show completely linearized rPGT3 inserts and vectors. Sizes of bands correspond to pPICZA (3.3 kb), pPIZAα (3.6 kb), and rPGT3 (≈ 4.8 kb). Note C1, C2, and C3 represent construct 1, 2, and 3, respectively.

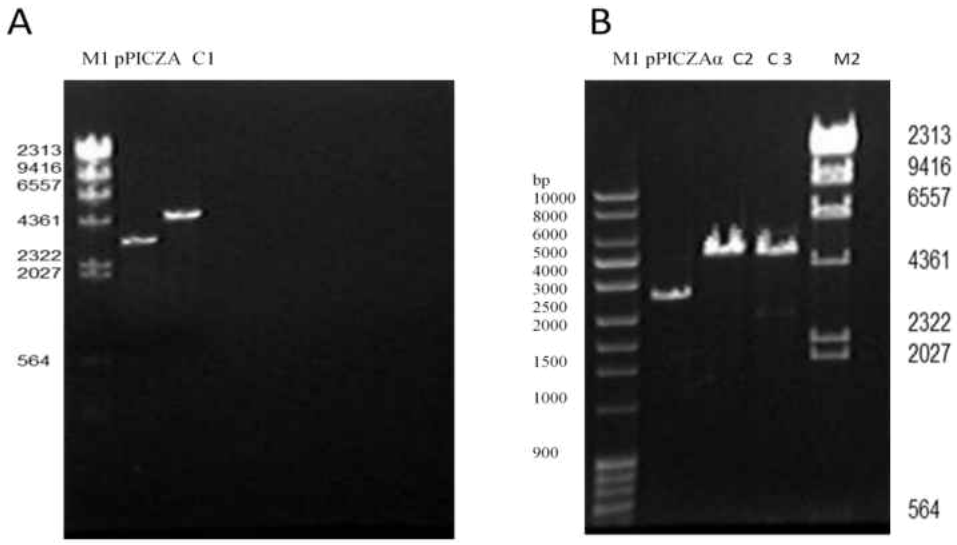


Fig. 32 Completely Linearized Empty Vectors and Vectors containing rPGT3. A. Represents DNA agarose gel electrophoresis of construct 1 containing rPGT3 and empty pPICZA vector. B. Represents DNA agarose gel electrophoresis of construct 2 and 3 containing rPGT3. Size of linearized bands corresponds to size of PGT3 plus empty vector. M1 indicates Lambda Hind III marker, M2 indicates 1 kb DNA ladder. C1 represents constructs 1, C2 represents construct 2, and C3 represents construct 3.

Before transformation into the *Pichia pastoris* genome, the linearized plasmids were subjected to phenol: chloroform extraction and ethanol precipitation procedure to remove proteins from nucleic acid and to concentrate the sample. To quantify the concentration of plasmid DNA, a 1 ul aliquot of the plasmid DNA extracted by phenol chloroform was run on a DNA agarose gel and image recorded (Fig. 33). The results from purification by phenol: chloroform showed a highly concentrated DNA bands.

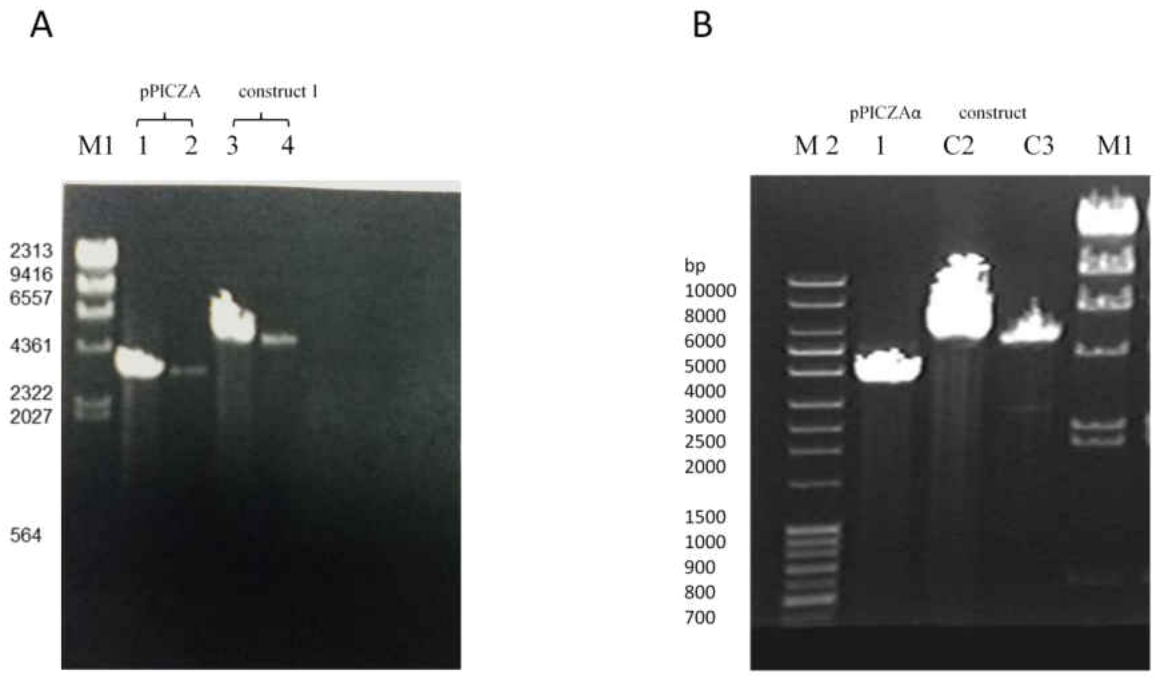


Fig. 33. DNA Agarose Gel Electrophoresis Showing Purification of Plasmid DNA by Phenol: Chloroform Extraction. A. Lane 1 and 2 are 0.5 ul and 100 fold dilution of plasmid DNA of pPICZA vector, respectively. Lane 3 and 4 are plasmid DNA of construct 1 (containing rPGT3). Lane marked 3 indicates a 0.5 ul of plasmid DNA, lane 4 is a 100 fold dilution of plasmid DNA. B. Represents Plasmid DNA of pPICZAα plasmid and plasmid DNA of construct 2 and 3, respectively. M1 indicates Lambda DNA Hind III marker, M2 indicates 1 kb DNA ladder, C2 and C3 represent constructs 2 and 3, respectively.

Transformation into *Pichia pastoris*

Linearized recombinant plasmids were transformed into competent yeast cells using electroporation. Empty vectors of pPICZA and pPICZAα vectors were also transformed into freshly made competent yeast cells to serve as a negative control for the expression of rPGT3. Selection of transformed *Pichia* clones was done using PCR screens. Colonies selected for PCR screens were used as templates to amplify the region of PGT3 sequence present (Fig.34). Results show *Pichia* cells that were transformed showed a 1495 bp band corresponding with estimated size of PGT3. Note bands in gel A, B, and C is a representative of constructs 1, 2, and 3 respectively. For further study, colonies A2, B3, and C12 were used.

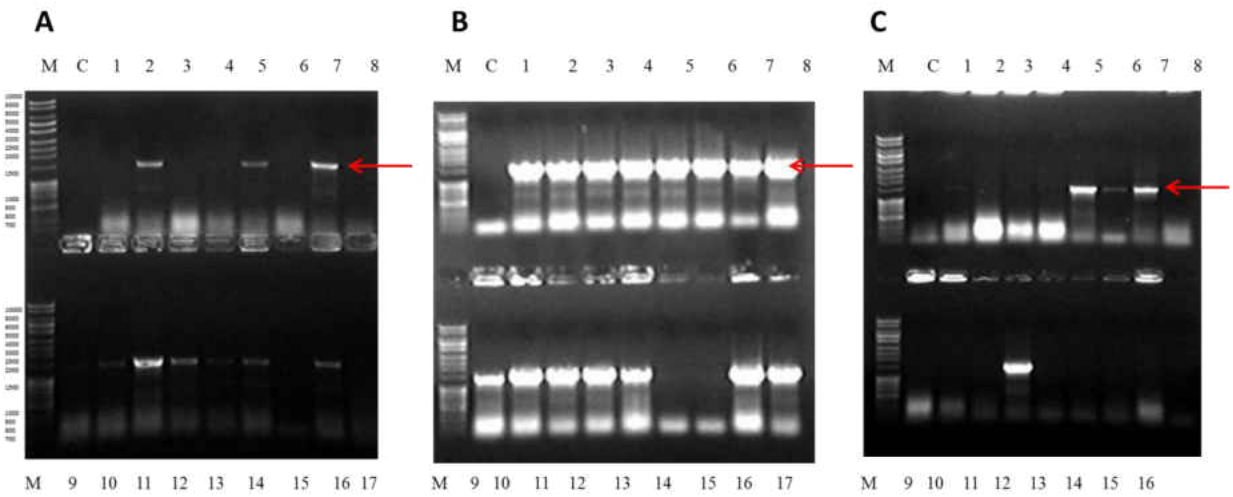


Fig. 34. DNA Gel Electrophoresis of Transformed *Pichia pastoris* Strain X33 Containing pPICZ/PGT3 Constructs. PCR screens were done using clone specific primers. A, B, and C represent colonies selected for screening of constructs 1, 2, and 3, respectively. Lane M represents 1 kb DNA ladder; C represents negative control for PCR reaction. Note: arrow points to bands corresponding to PGT3 size 1495 bp.

Expression of Recombinant PGT3 Protein in Yeast

In order to test the conditions for expression of recombinant PGT3 protein, test inductions were carried out. Recombinant PGT3 *Pichia* strains that had been confirmed to contain PGT3 insert were tested for expression of soluble protein at different postinduction times (0-96 hours) under control of the AOX1 gene promoter. In the presence of methanol, the AOX1 promoter is activated and this drives the expression of rPGT3 (Invitrogen Pichia manual). For a negative control, yeast transformed with empty pPICZA vector was treated with methanol to show the background expression of native *Pichia* proteins. Expression of rPGT3 was confirmed by Western blot analysis of samples taken at the various postinduction times for construct 1(Fig. 35). The results show expression of rPGT3 in *Pichia* at 6-60 hours postinduction. The expressed protein was detected with c-myc antibody. The band sizes correspond with rPGT3 including tags (58.5 kD).

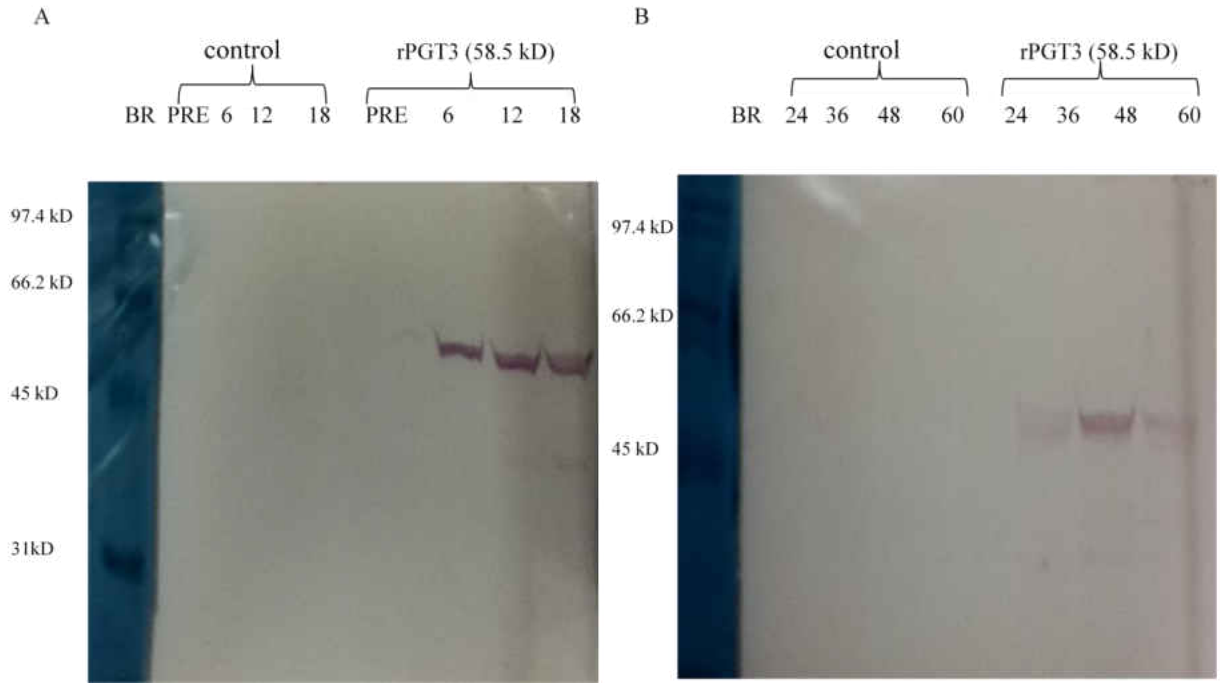


Fig. 35. Expression of rPGT3 (using Construct1) in *Pichia pastoris.* Test inductions of PGT3 in *Pichia pastoris* at 30°C. Samples were collected at different post induction times. A. Representative Western Blot showing detection of expressed rPGT3 from 6-18 hours post induction using C-myc antibody against the C-myc epitope tag. B**.** Western blot showing detection of expressed rPGT3 at 24-60 hours post induction. Lane marked PRE indicates preinduction sample; lane marked control represents expression of yeast transformed with an empty vector. A total volume of 5ul of protein was loaded in each well. The blot was developed for about 2 minutes.

After test inductions were done, microassay of the amount of protein in samples collected at the various postinduction time points was determined (Table 13). The concentration of protein was shown to increase steadily from the 6th hour up to the 24th hour after which the protein concentration started to decline up to the 96th hour. The intensity of the bands on the Western blot (Fig. 35) showed the 6th hour to have the most rPGT3 when compared with the other bands indicating that rPGT3 yield was most enriched at that time. Subsequent inductions were done for 6 hours at 30^{o}C.

Table 13. Protein Microassay of Expressed rPGT3 at 0-96 hr PostInduction

Post induction time/hr	Protein Concentration µg/ µL	Amount of Protein Loaded on Gel µg
6	2.6	13
12	2.8	14
18	4.6	23
24	5.2	26
36	3.3	16.5
48	2.6	13
60	2.2	11
72	1.6	8
84	1.4	7
96	1.2	6

Purification of rPGT3 using Immobilized Metal Affinity Chromatography (IMAC)

Due to the fact that rPGT3 was equipped with C-terminal 6 His tags, purification could be done using an ion metal affinity chromatography technique. To enrich rPGT3, a 6 hour postinduction yeast cell pellet was resusupended in breaking buffer containing BME to prevent the formation of disulfide bridges. The breaking buffer also contained PMSF to prevent the action of proteases if present in the protein sample. The buffer also had a pH of 7.5 which has been shown to be an optimum pH for activity of glucosyltransferases. After resuspension of the cell pellet, it was lysed by French press at 4^{o}C. The crude lysate was centrifuged, and a 2.5 mL aliquot desalted to remove excess salts that may interfere with the binding of the protein to the IMAC column. The desalted eluate was immediately used for purification via IMAC. Fractions

were analyzed by SDS-PAGE (Fig. 36) and Western blot (Fig.37). Results show an enrichment of rPGT3 from crude fraction (CR, Fig 36A.) Most contaminating bands observed in crude rPGT3 and flow-through samples (Fig.36 A, F1-F4) were sufficiently removed by several washes as seen in fractions W1-W9 (Fig. 36.A and B). Enriched rPGT3 was achieved in fractions F2-F4 on SDS-PAGE (Fig.36B) and detected by Western blot (Fig. 37B).

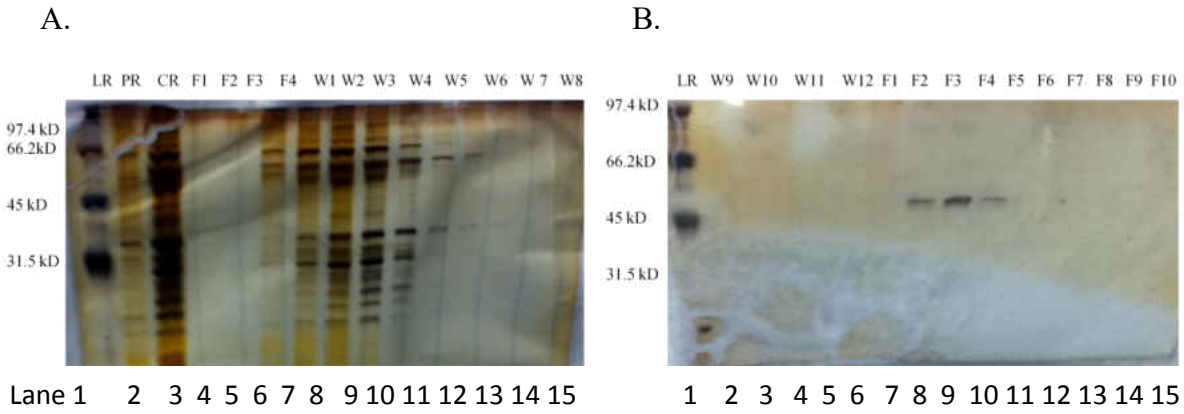


Fig. 36. Silver Stained SDS-PAGE Analysis Showing Purification of rPGT3 Expressed in *Pichia pastoris*. A. Representative fractions obtained. Lane 1, broad range protein molecular weight marker, lane 2, preinduced sample, lane 3, crude rPGT3, lane 4-8 rPGT3 flow throughs , lane 9-15, washes with equilibration wash buffer to remove contaminating proteins B. Representative of fractions obtained. Lane 1, protein low range molecular weight marker, lane 2-5, washes. Lane 6-15, fractions eluted with 150 mM imidazole. Gels were silver stained.

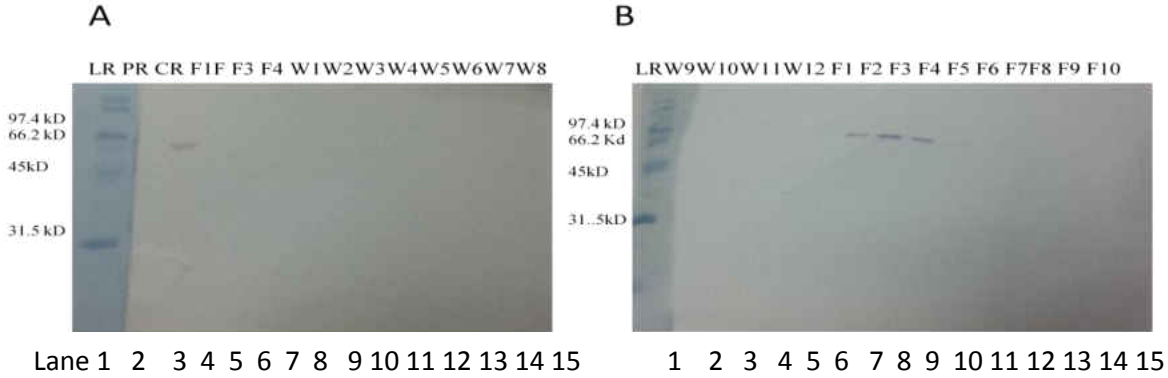


Fig. 37.Western Blot Analysis Showing Detection of rPGT3 Expressed in *Pichia pastoris*. A. Representative fractions obtained. Lane 1, low range protein molecular weight marker, lane 2, preinduced sample, lane 3, crude rPGT3, lane 4-8 rPGT3 flow throughs , lane 9-15, washes with equilibration wash buffer to remove contaminating proteins. B. Representative of fractions obtained. Lane 1, protein low range molecular weight marker, lane 2-5, washes with equilibration buffer. Lane 6-15, fractions eluted with 150 mM imidazole.

Screening rPGT3 Protein for GT Activity

In order to test activity of rPGT3 protein, rPGT3 protein purified by IMAC was used; however, the preparation was scaled up to increase yield. Bearing in mind the trend observed with the test purification, 12 washes of 2ml fractions were used to wash the column prior to eluting rPGT3 with 150 mM imidazole. Aliquots of samples obtained were saved and analyzed by SDS-PAGE and Western Blot after enzyme screening assays had been done. The fractions observed from previous purification of PGT3 under the same conditions of expression to contain the most enriched PGT3 were pooled, desalted and concentrated.

A portion of the desalted and concentrated fractions and all fractions obtained from the purification step was kept for analysis by SDS-PAGE (Fig. 38) and Western blot (Fig. 39). Results show that rPGT3 was enriched and fractions F1-F4 were pooled, desalted, concentrated, and used for activity screens. The enriched rPGT3 fractions were detected using antibodies directed againt the c-myc epitope tag (Fig. 39).

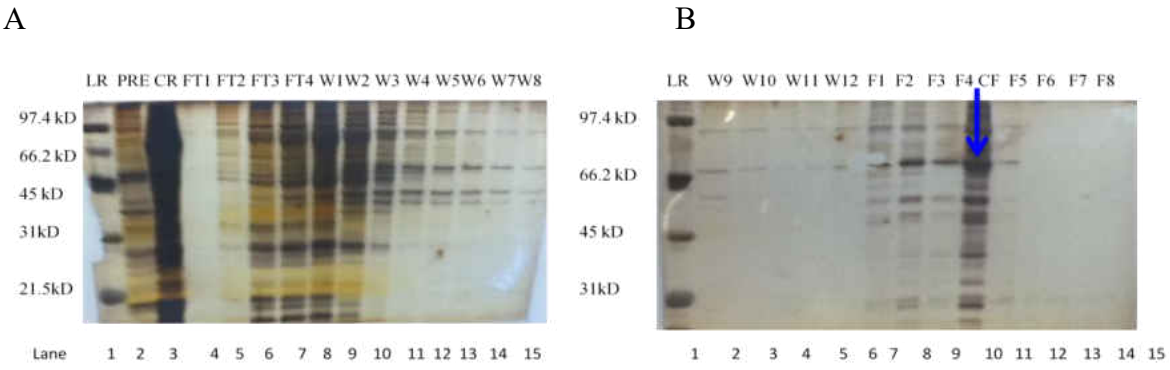


FIG. 38. Purification of rPGT3 Expressed in *Pichia pastoris* and Fractions. A. Lane 1, low range protein molecular weight marker, lane 2, preinduced sample, lane 3, crude rPGT3, lane 4-8 rPGT3 flow throughs , lane 9-15, washes with equilibration wash buffer to remove contaminating proteins. B. Lane 1, protein low molecular weight marker, lane 2-5, washes with equilibration buffer to remove contaminating and unbound proteins. Lane 6-9, rPGT3 protein eluted with 150 mM imidazole. Lane 10 (CF), pooled, desalted, and concentrated fractions (6-9) for enzyme assay of rPGT3. Lane 11- 15 subsequent fractions eluted with 150 mM imidazole**.** Gels were silver-stained.

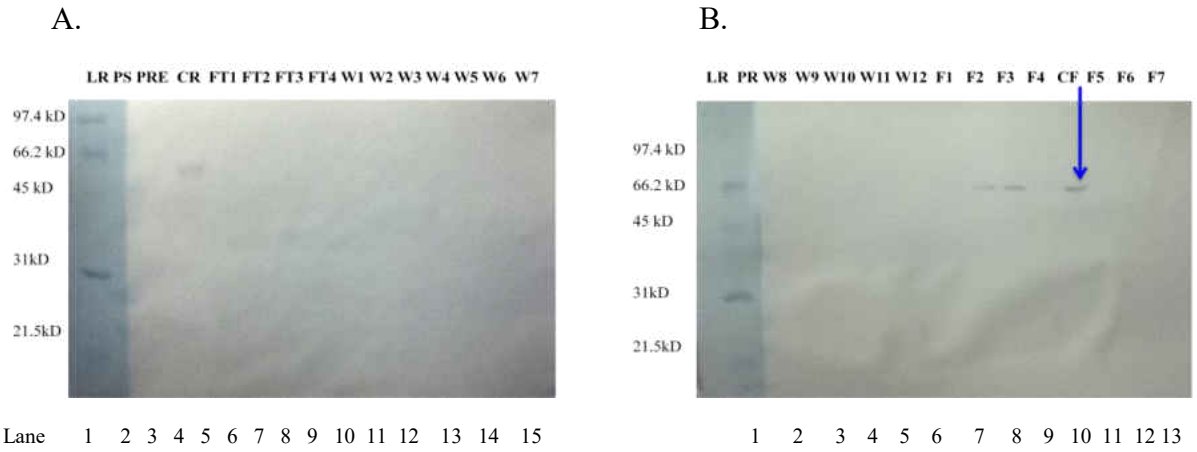


FIG. 39. Representative Western Blot of Enriched rPGT3 Expressed in *Pichia pastoris*. A. Lane 1, low range protein molecular weight marker, lane 2, preinduced sample, lane 3, crude rPGT3, lane 4-8 rPGT3 flow throughs , lane 9-15, washes with equilibration wash buffer to remove contaminating proteins. B. Lane 1, protein low molecular weight marker, lane 2-5, washes with equilibration buffer to remove contaminating and unbound proteins. Lane 6-9, rPGT3 protein eluted with 150 mM imidazole. Lane 10 (CF), pooled, desalted, and concentrated fractions (6-9) for enzyme assay of rPGT3. Lane 11- 13 subsequent fractions eluted with 150 mM imidazole.

Screening rPGT3 Protein Using Flavonoid and Simple Phenolic Substrates

A total of 18 compounds representing flavonoid substrates and 3 compounds representing phenolics were used as initial substrates for screening GT activity of rPGT3. The reactions were run along with 2 sets of negative controls. In one negative control reaction, no rPGT3 was added to the reaction, and in the other, rPGT3 was added after addition of HCL to denature and inactivate the enzyme. Two sets of positive control reactions were tested. In one, grapefruit leaf extract was used as the source of GT enzyme. And in the other, grapefruit leaf extract and rPGT3 were both used. The second positive control was done to see if there would be a decrease in the glucose incorporation when compared with that obtained in the reaction with only grapefruit leaf extract. This would test for an inhibiting factor in the rPGT3 sample. The incorporation of radiolabelled UDP- C^{14} glucose onto flavonoid subtrates was analyzed using a scintillation counter. Results show average cpm incorporation of the substrates tested against rPGT3, the

incorporation measured in the negative control reactions (Table 14). Results with positive controls showed an incorporation of radiolabelled glucose unto kaempferol by glucosyltransferases present in the crude grapefruit leaf extract. The positive control reaction that contained rPGT3 did not show much difference in the amount of radiolabelled glucose incorporated. Results from testing flavonoid substrates with rPGT3 gave insignificant counts of activity (Table 14).

Table 14. Screening Activity of rPGT3 using Flavonoid and Simple Phenolic Substrates. (Note: 20000 cpm of C^{14} labeled UDP-glucose was used per reaction)

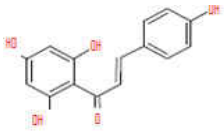
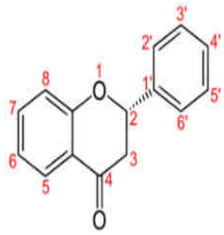
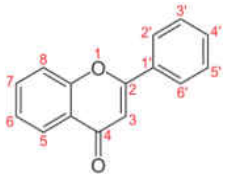
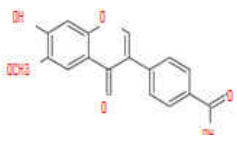
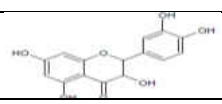
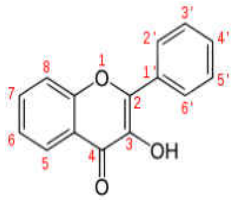
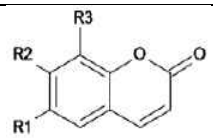
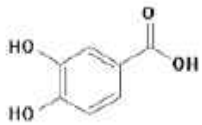
Group	Subclass	Structure	Compound	Substitutions	Average cpm (n=2) Incorporation 250µL ethyl acetate).
Flavonoid	Chalcone		2'4,4',6' Tetrahydroxy-chalcone		22
	Flavanone		Naringenin	4'=5=7=OH	20
			Hesperetin	3'=5=7=OH, 4'= OCH3	25
			Eriodictyol	3'=4'=5=7=OH	45
			Isosakuranetin	5=7=OH,4'= OCH3	20
			Prunin	4'=5=OH, 7= -O-glucose	40
	Flavone		Apigenin	4'=5=7=OH	9
			Luteolin	3'=4'=5=7=OH	35
			Diosmetin	3'=5=7= OH, 4'=OCH3	12
			Scutallarein	4'=5=6=7=OH	30
			Luteolin-7-O-glucoside	3'=4'=OH, 7=-O-glucose	26
	Isoflavone		4'-acetoxy-7-hydroxy-6-methoxy-isoflavone		23
	Dihydrofla-vonol		Dihydroquercetin		12

Table 14 Continued. Screening Activity of rPGT3 using Flavonoid and Simple Phenolic Substrates. (Note: 20000 cpm of C^{14} labeled UDP-glucose was used per reaction).

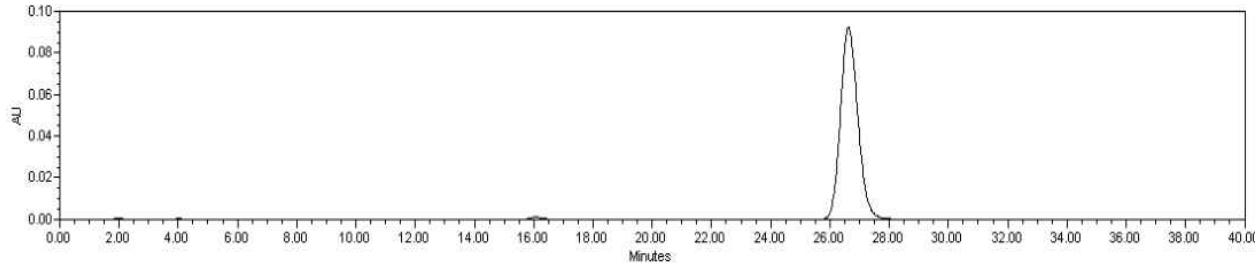
Group	Subclass	Structure	Compound	Substitutions	Average cpm (n=2) Incorporation (250µL ethyl acetate).
Flavonoid	Flavonol		Kaempferol	5=7=4'=OH	30
			Quercetin	3'=4'=5=7=OH	28
			Fisetin	4'=5'=7=OH	14
			Gossypetin	3'=4'=5=7=8=OH	35
			4'methoxy-flavonol	4'=OCH3	20
Phenolics	Coumarin		Umbelliferone	R1=R3=H, R2=OH	66
			Esculetin	R1=R2=OH, R3=H	53
Simple phenolics			2'4' dihydro-benzaldehyde		34
Controls	Positive control	Grapefruit leaf extract+ rPGT3+kaempferol			1058
		Grapefruit leaf extract+kaempferol			1188
	Negative control	Denatured rPGT3+kaempferol			52
		No rPGT3+kaempferol			42

After initial screens of activity or rPGT3 using flavonoid substrates, the preferred flavonoid substrate of rPGT3 was not found. Subsequent screens using 12 compounds representing simple phenolic substrates were tested. Substrate standard samples were each run and experimental groups were run twice through the HPLC column. Two sets of positive control

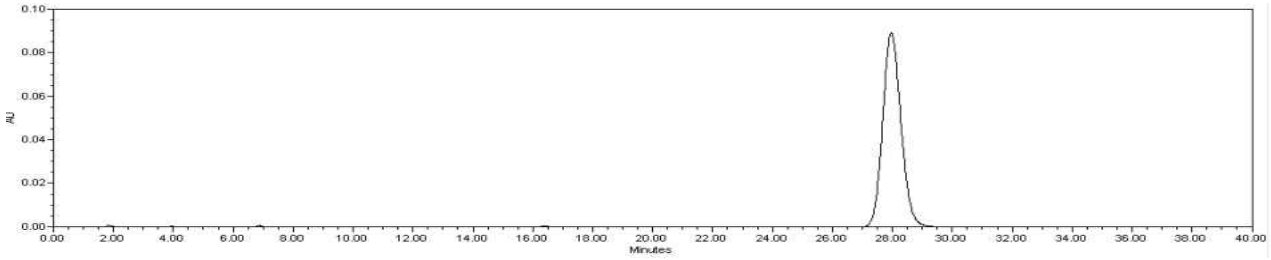
reactions were tested. In one, grapefruit leaf extract was used as the source of GT enzyme. And in another grapefruit leaf extract and rPGT3 were both used. The second positive control was done to see if there would be an inhibiting factor in the rPGT3 sample. One negative control reaction was run through the column along with the control, experimental, and standard groups. The elution time between each standard and each duplicate of the experimental group was noted and compared. Results show a shift in substrate retention time thus a conversion of some of the substrate to form glucosides in experimental groups of p-hydroxybenzoic, catchol, gentisic acid, vanillin, vanillic acid, and p-phenylpyruvic acid. The difference between the retention times of the aglycones and glucosides is shown in table 15 (Ahmad and Hopkins 1993). In order to confirm that rPGT3 is using these substrates as preferred substrates, a repeat of these experimental groups was run alongside negative control reactions.

Table15. Summary of Difference between Retention Times of Aglycones and Glucosides (Adapted from Ahmad and Hopkins, 1993).

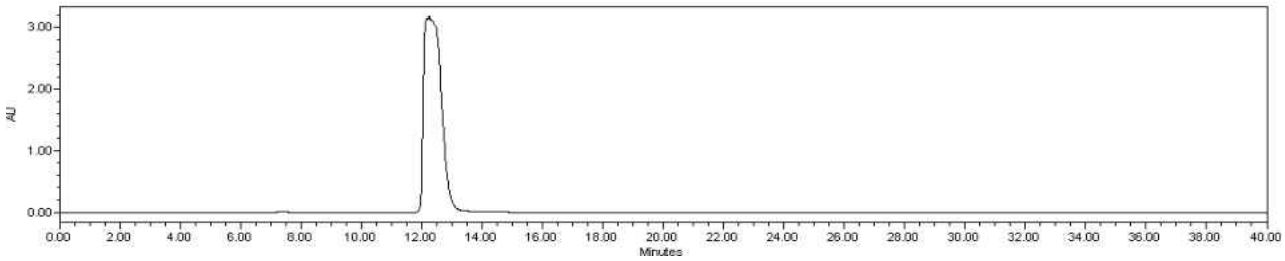
	Retention time (min)		
Compound	Aglycone	Glucoside	Difference between Aglycone and glucoside
p-Coumaric acid	9.3	4.6	1.3
Caffeic acid	6.2	4.9	4.7
Ferulic acid	11.2	5	6.2
p-Hydroxybenzoic-acid	10.1	5.5	4.6
Vanillic acid	7.9	4.3	3.6
Vanillin	9.1	4.5	4.6
Catechol	8.8	6.0	2.8
Scopoletin	13.1	5.5	8.0
Quercetin	18.6	6.0	12.6



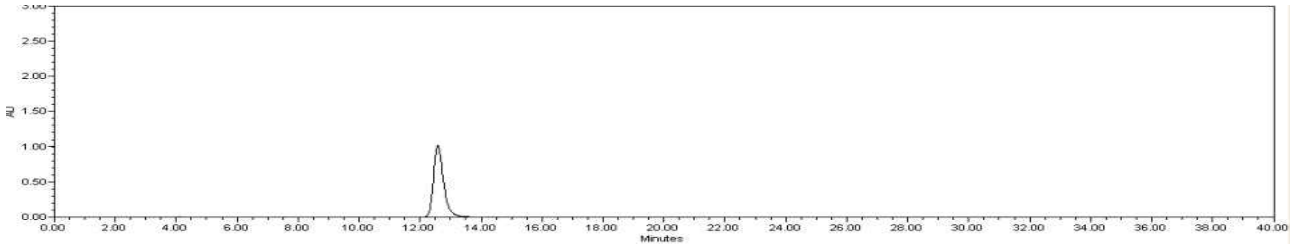
I. o-Coumaric standard. Retention time is 27.5 minutes. λ = 325



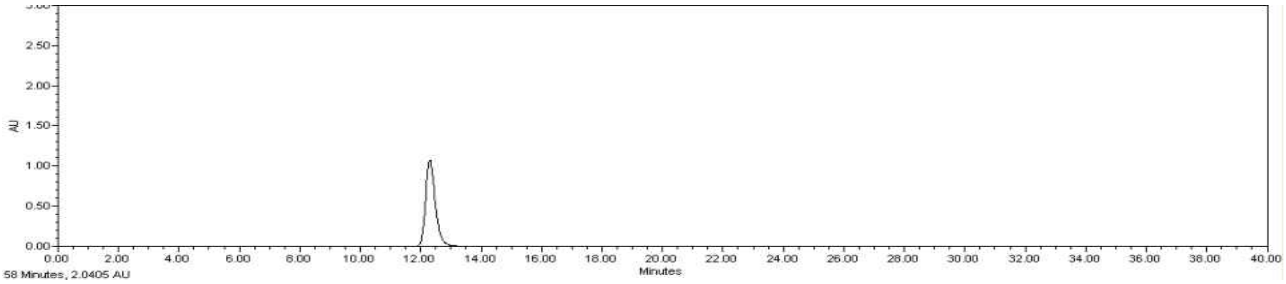
II. o-Coumaric acid experimental. Retention time of major peak is 27.6 minutes. λ = 325



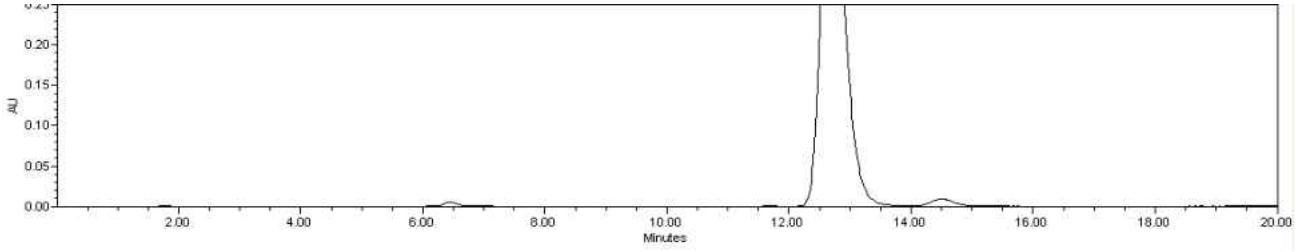
III. p-Coumaric acid standard. Retention time of major peak is 12.216. λ = 308



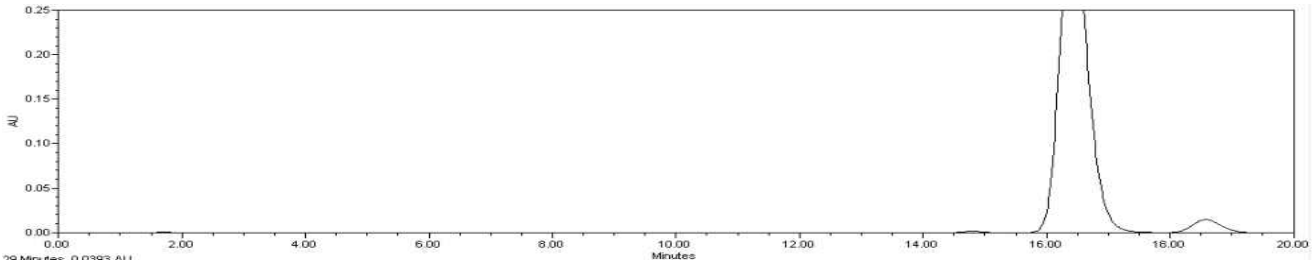
IV. p-Coumaric acid negative control. Retention time of major peak is 12.241. λ = 308



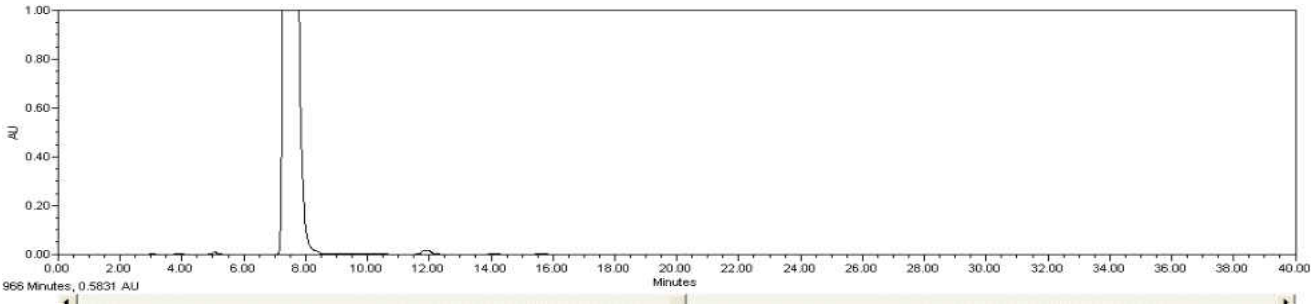
V. p-Coumaric acid experimental. Retention time of major peak is 12.302. λ = 308



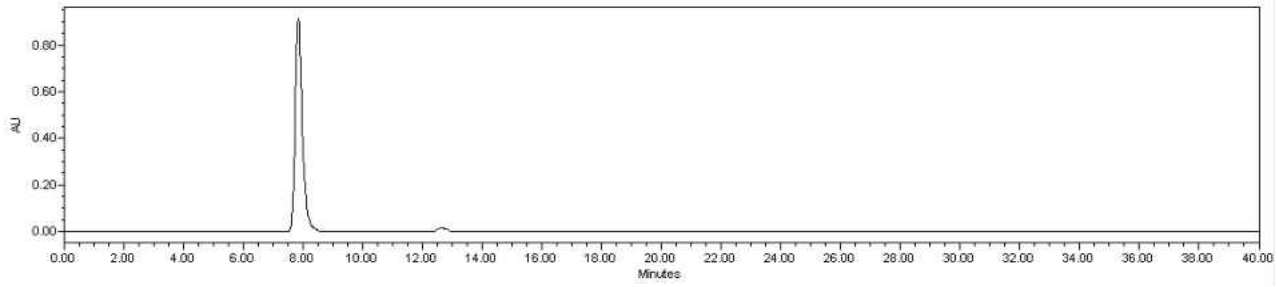
VI. Ferulic acid standard. Retention time is 13.466. λ = 324



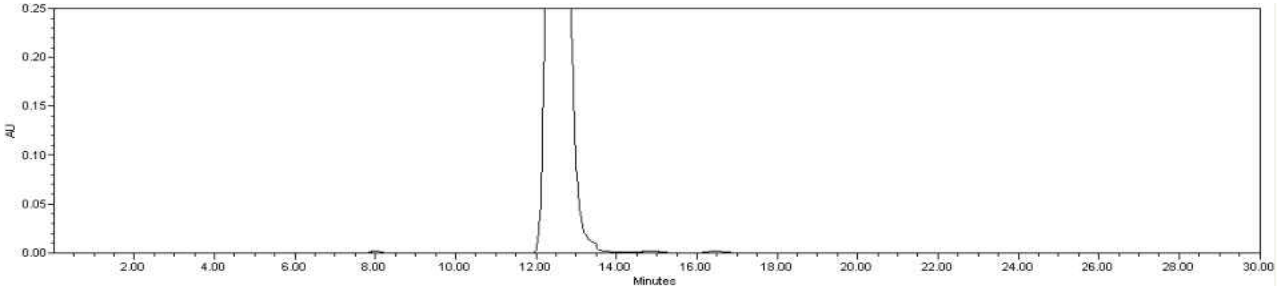
VII. Ferulic acid experimental. Retention time is 16.390. λ = 324



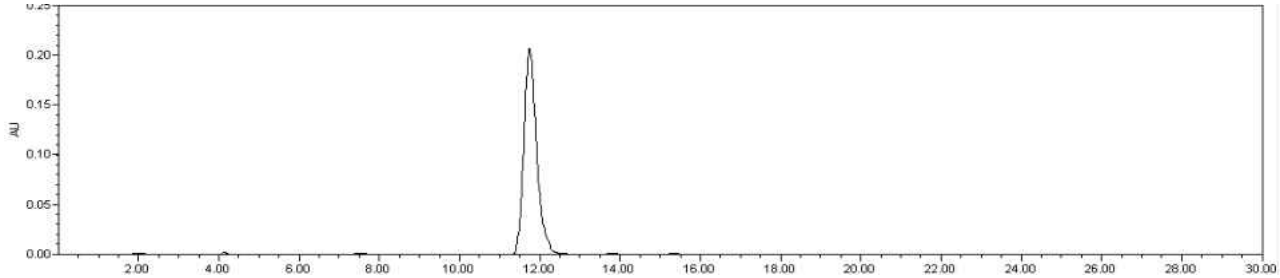
VIII. Caffeic acid standard. Retention time is 7.375. λ = 322



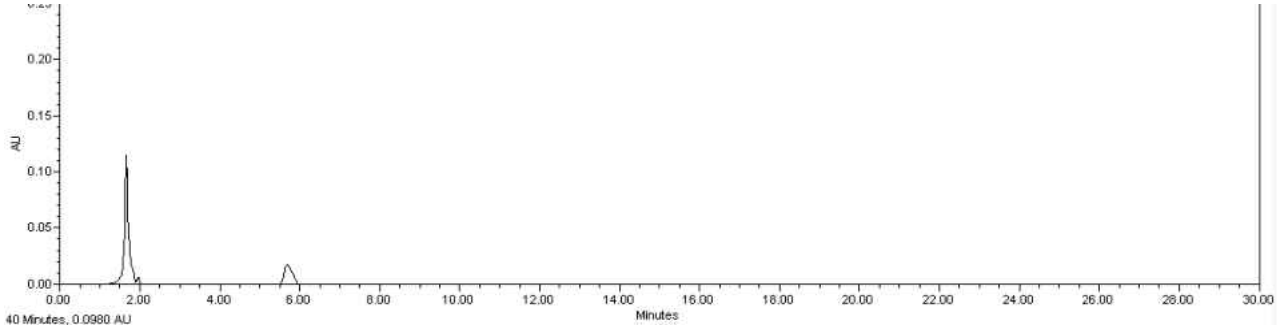
IX. Caffeic acid experimental. Retention time is 7.641. λ = 322



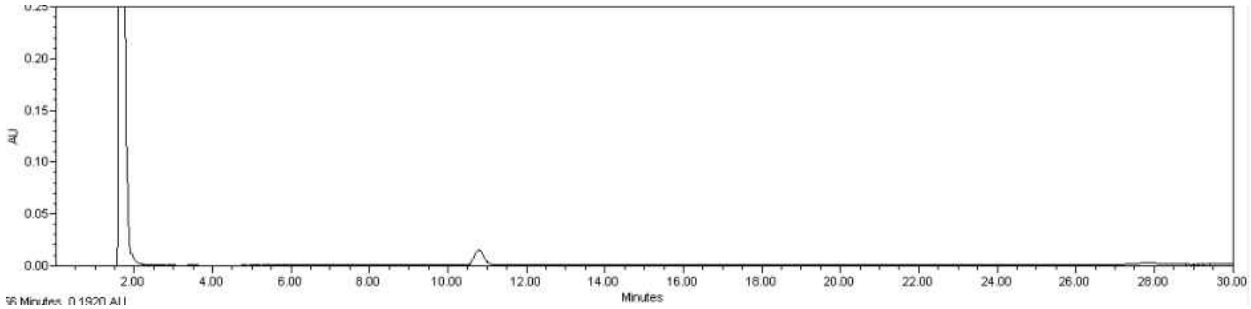
X. Scopoletin standard. Retention time is 12.477. λ = 340



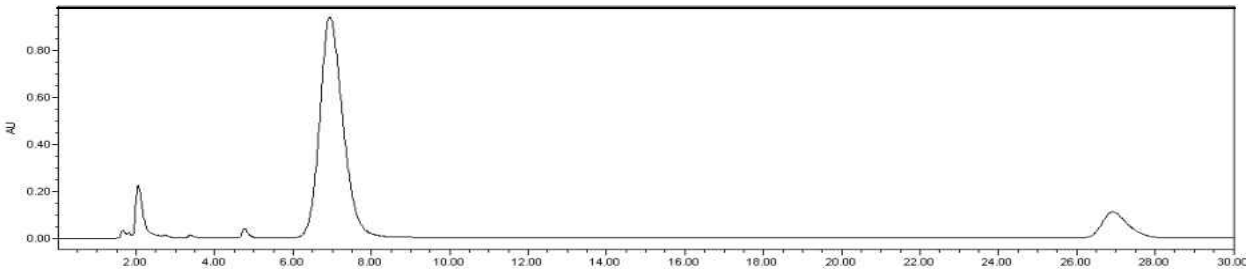
XI. Scopoletin expermental. Retention time is 12.040. λ = 340



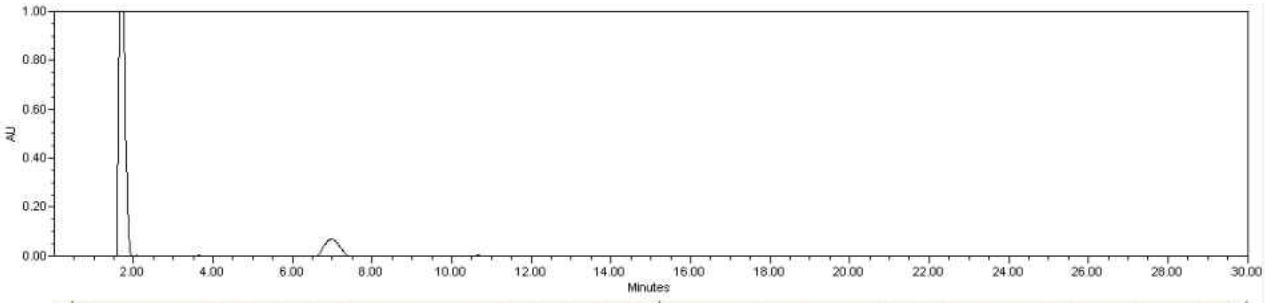
XII. p-Hydroxyphenylacetic standard. Retention time is 1.9. λ = 215



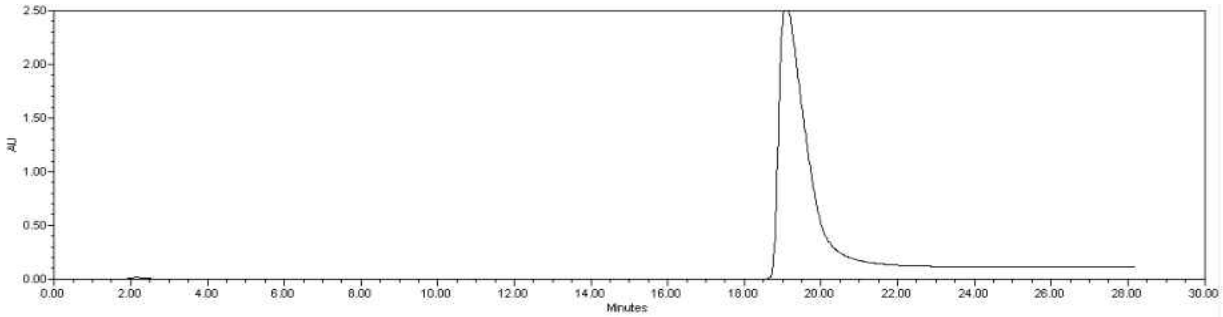
XIII . p-Hydroxyphenylacetic acid experimental. λ = 215



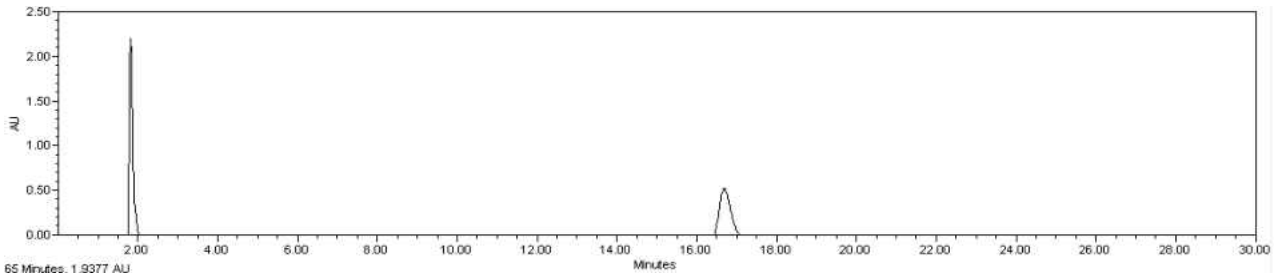
XIV. p-hydroxyphenylpyruvic acid standard. Retention time of major peak is 6.940. λ = 215



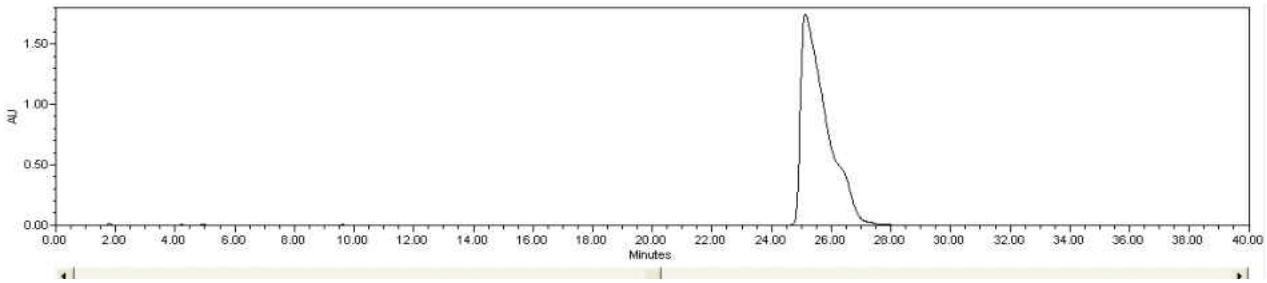
XV. p-hydroxyphenylpyruvic acid experimental. Retention time of major peak is 1.688. λ = 215



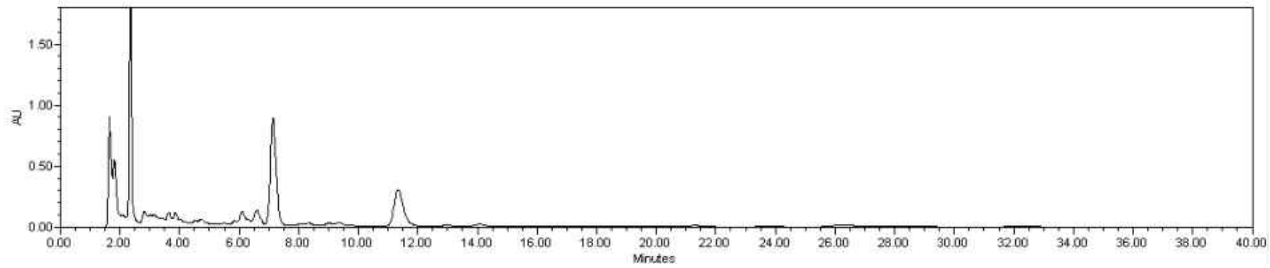
XVI. p-hydroxybenzoic acid standard. Retention time is 19.094. λ = 255



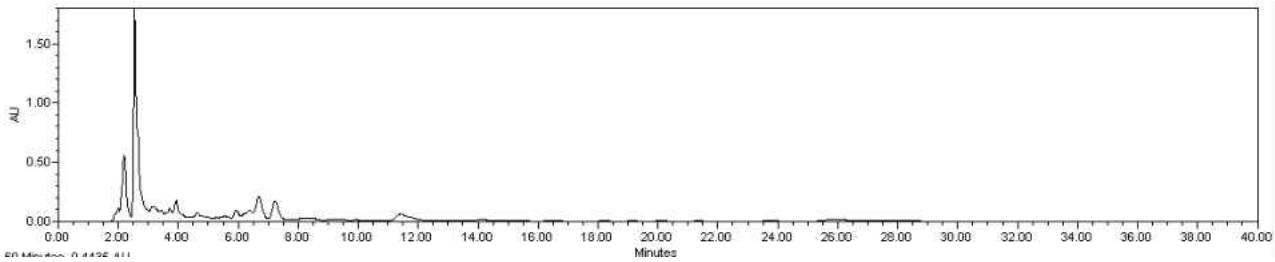
XVII. p-hydroxybenzoic acid experimental. Retention time of major peak is 1.799 and retention time of second peak is 16.229. $\lambda = 255$



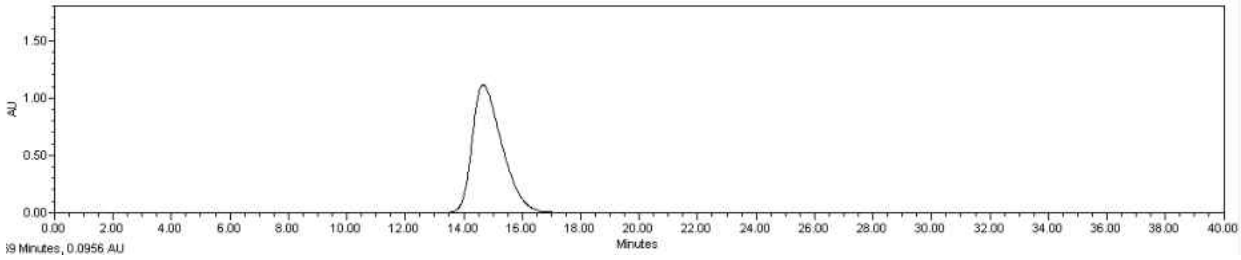
XVIII. Retention time of quercetin standard is 25.139.



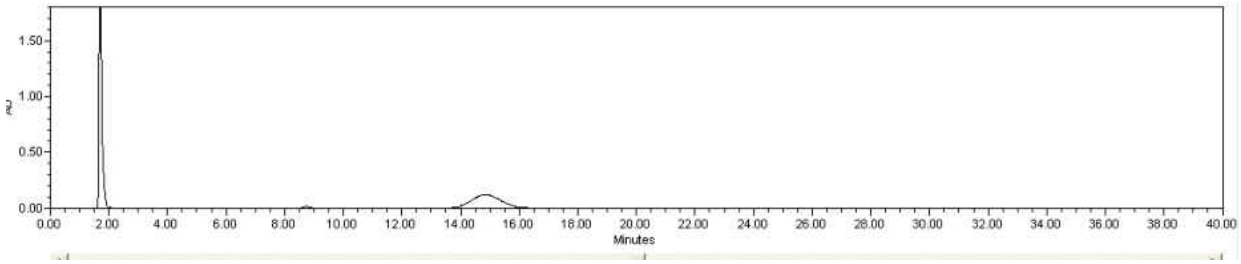
XIX. Positive control reaction using quercetin and crude grapefruit leaf extract as the source of enzyme. Note disappearance of quercetin peak.



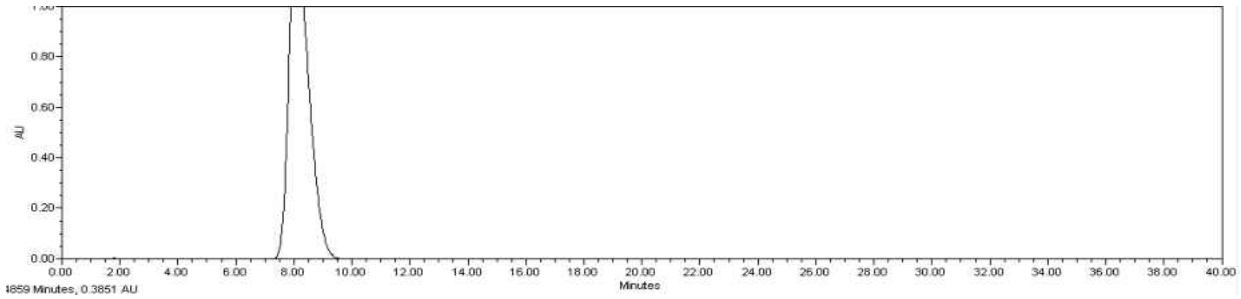
XX. Positive control reaction 2 (quercetin, crude grapefruit leaf extract, and rPGT3). Note disappearance of quercetin peak



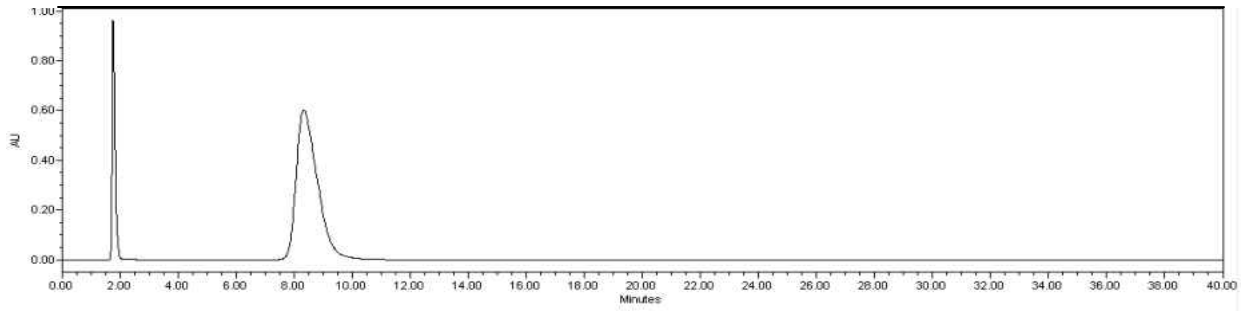
XXI. Vanillin standard. Retention time is 14.664. λ = 273



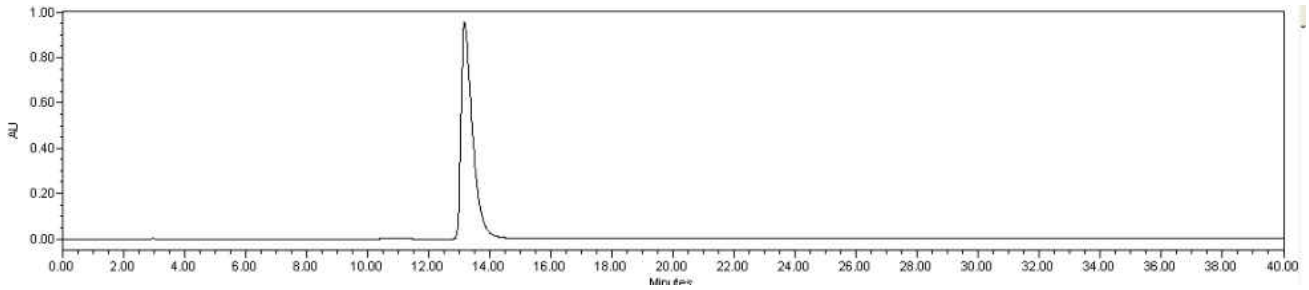
XXII. Vanillin experimental. Retention time of major peak is 1.740 and the peak height of substrate has reduced. λ = 273



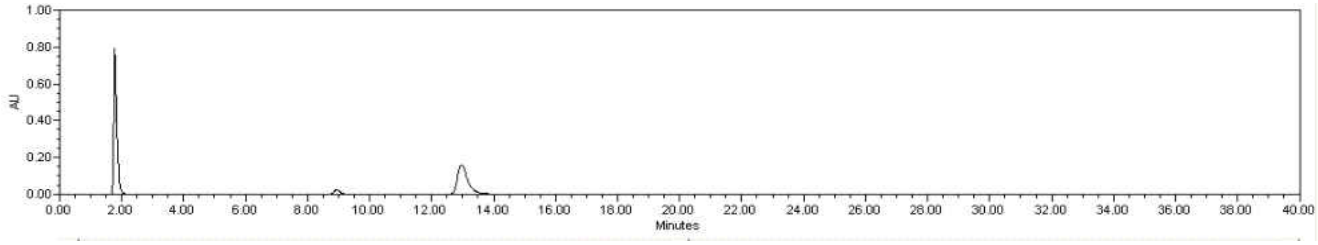
XXIII. Vanillic acid standard. Retention time is 8.079. λ = 260



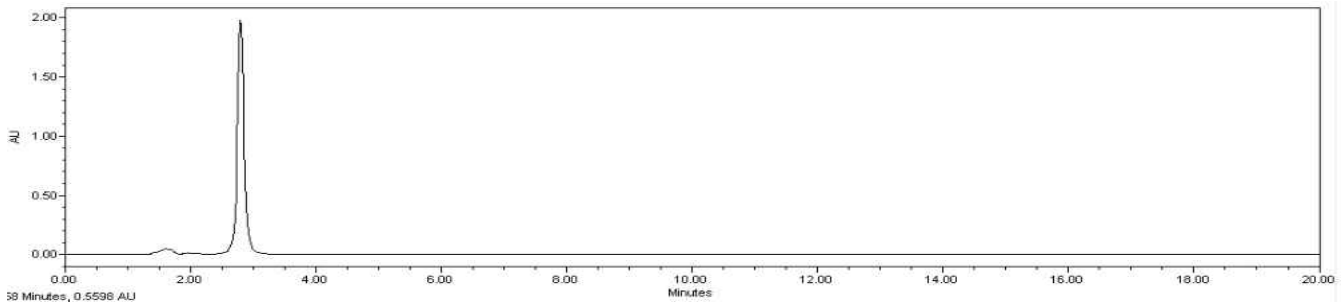
XXIV. Vanillic acid experimental. Retention time of major peak is 1.9 and retention time of second peak is 8.331. λ = 260



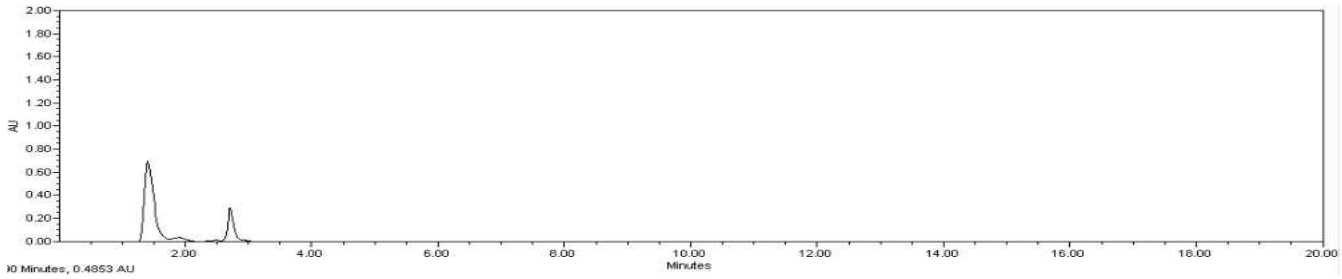
XXV. Gentisic acid standard. Retention time is 13.172. λ = 330



XXVI. Gentisic acid experimental. Retention time of major peak is 2.0 and peak height of substrate has reduced. λ = 330



XXVII. Catechol standard. Retention time is 2.799. λ = 280



XXVIII. Catechol experimental. Retention time of major peak is 1.409 and peak height of standard has reduced. λ = 280

Fig. 40. HPLC Chromatograms for rPGT3 Enzyme Assays with 12 Phenolic Compounds. Note the production of glucosylated phenolics were identified through the comparison with phenolic standards.

Cloning PGT8 into pPICZA Vector

Isolation of PGT8 and pPICZA Vector

Prior to sub-cloning PGT8 into pPICZA vector, PGT8 in pCR4-TOPO/TOP10 and pPICZA vector were miniprepped to isolate the plasmids (Fig.41). Results show miniprep of pPICZ vector from TOPO vector and PGT3 insert from TOPO (3000 bp bands in lane 2 and 3) and pCD1 vector (4000 bp bands in lane 4 and 5) respectively.

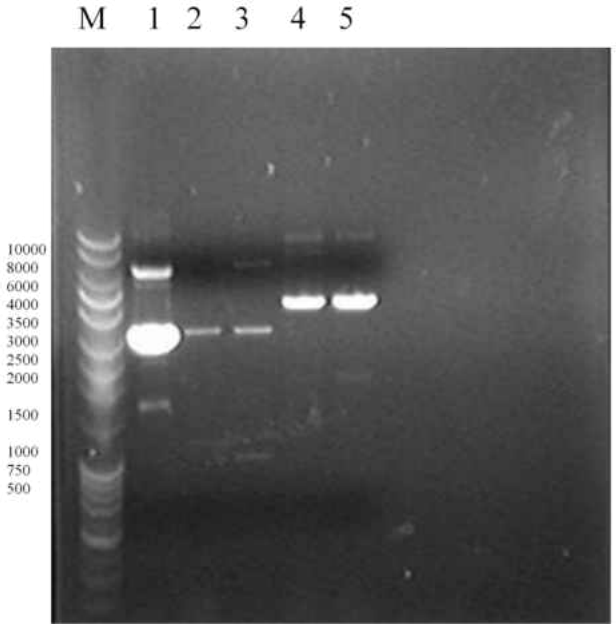


Fig. 41. Miniprep of PGT8 from pCR4-TOPO/TOP10 and pCD1/TOP10. DNA agarose gel (0.8%) electrophoresis showing the miniprep of PGT8. Lane M represents 1 kb DNA ladder, used as a marker. Lane 1; positive control for the miniprep reaction (PGT11). Lane 2 and 3 are PGT8/pCR4-TOPO/TOP10 clones. Lane 4 and 5 are PGT8/pCD1-TOP10 clones, respectively.

Modification of the Ends of PGT8 from pCR®4-TOPO Vector

The 5' and 3'ends of PGT8 sequence were then modified with primers (Table 10) and the PCR products were analyzed with DNA agarose gel electrophoresis (Fig 42). The sizes of the bands in lanes 1, 2, 3, and 4 were calculated as 1530, 1535, 1535, and 1540 bp, respectively. This was compared with the estimated size of PGT8 (1536bp) to confirm that the PCR product was full length.

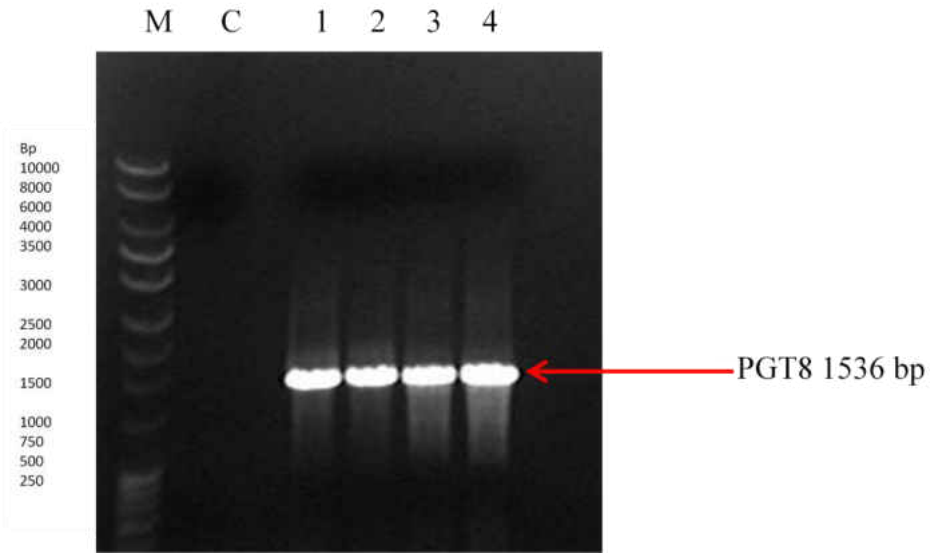


Fig. 42. Modified and Amplified PGT8. DNA agarose gel (0.8%) showing the PCR products obtained after modifying PGT8 with appropriate primers to get PGT8 cloned into *Pichia pastoris*. Lane M: 5 ul 1 kb DNA plus marker, lane 1-4 are PCR products of PGT8 samples. Arrow indicates the calculated band size of one of the PCR products.

Cloning Modified PGT8 into TOPO

The mPGT8 PCR products were subcloned into pCR®4-TOPO vector and transformed into TOP 10 competent cells as described in the material and methods section. Single colonies were selected from LB $_{amp\ (100mg/L)}$ plates and screened using clone specific primers to confirm the

transformation of mPGT8-TOPO vector into TOP 10 cells. The colonies shown by PCR screens to contain PGT8 showed an approximately 1500 bp (Fig.43) amplification product that corresponded to the expected size of PGT8. Results show all colonies contained PGT8 with the exception of colonies 4 and 5 (Fig.43).

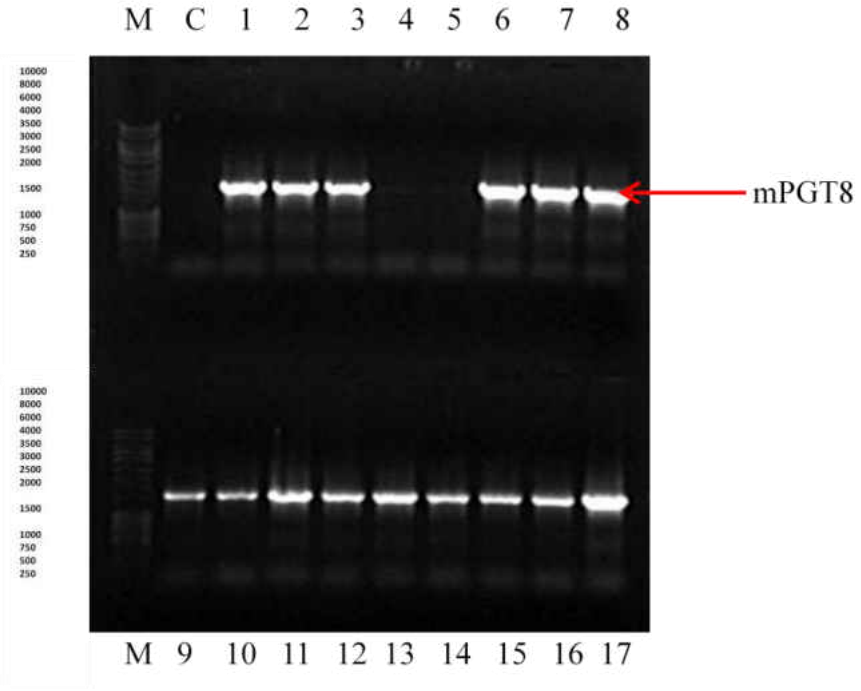


Fig. 43. Confirmation of Modifed PGT8-TOPO Transformed Colonies by PCR Screens. Lane M marked M is 1kb molecular weight marker; lane C is a negative control (PCR reaction run without a template). Lanes 1-17 are colonies selected for screening.

The plasmid DNA from colonies 7, 9, and 13 were isolated by miniprep as described in the materials and methods section. Also a miniprep of pPICZA vector was performed. Restriction digestion of pPICZA and PGT8 were carried out to confirm the presence of PGT8 in TOPO vector. Results verified the presence of PGT8 vector in TOPO vector (Fig. 44 and also the digested PGT8-TOPO plasmid contained PGT8 insert of expected size. The digested sample (labelled D in Fig. 44) showed a separation of 2 bands, one corresponding to the size of TOPO

and the other the size of PGT8. Plasmid DNA of PGT8-TOPO was isolated and sent for sequencing. Results of sequencing confirmed presence of PGT8 in the TOPO vector (Fig.45).

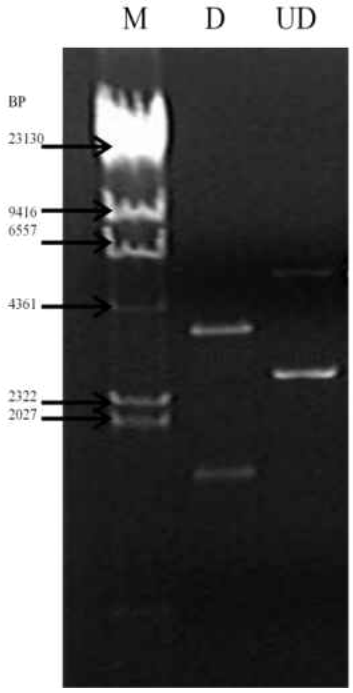
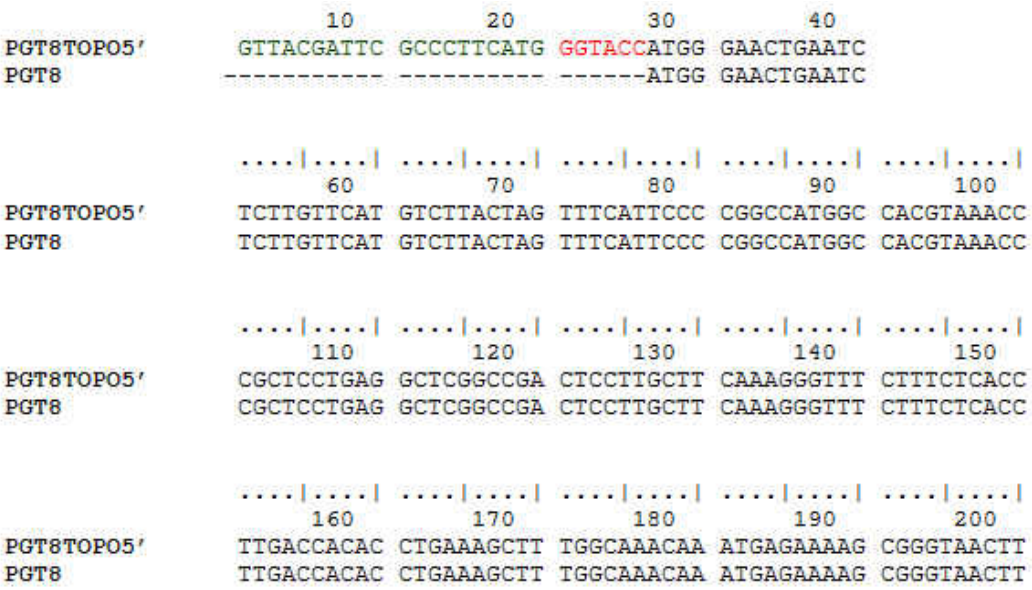


Fig. 44. Verification of Presence of mPGT8 Insert in TOPO Vector. Representative DNA agarose gel electrophoresis showing restriction digestion of PGT8 in TOPO. Plasmid DNA was eluted and digested for primary verification of correct sized insert and vector. Lane marked D, is digested PGT8-TOPO. Note the separation of insert (1536 bp) and vector (3956 bp). Lane M indicates Lambda Hind III marker. U indicates undigested plasmid.

A.



B.

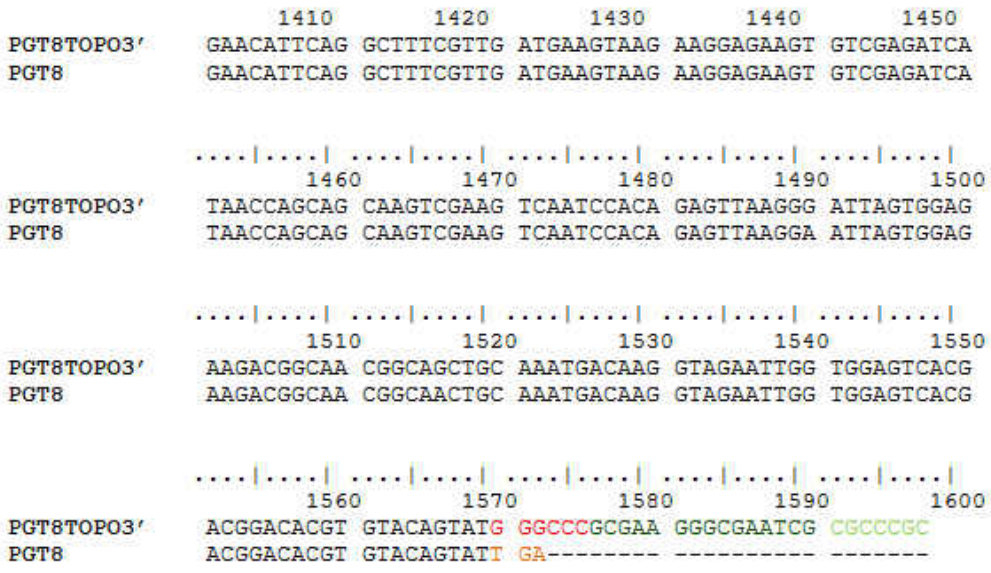


Fig. 45. Alignment of PGT8 sequence with PGT8-TOPO Sequence using Bioedit. A. 5' region. The regions in red represent restriction enzyme priming site, green represent TOPO vector 3' sequence and orange represents stop codon. B. 3' region. The area in red represent restriction enzyme site and green represents the 5' region of TOPO vector.

Gel Purification of PGT8 Construct and pPICZA Vector

After confirmation of the presence of PGT8 insert in TOPO (Fig. 45). Larger volumes (14 µL) of restriction digests of PGT8-TOPO and pPICZA vector were performed as described in the materials and methods in order to isolate the mPGT8 band.The mPGT8 band and the linearized pPICZA plasmid were gel purified. Prior to ligation of the purified PGT8 and pPICZA vector, the gel purified PGT8 construct and vector were each analyzed by DNA gel electrophoresis using a 0.8% agarose gel containing EtBr as previously described (Fig.46). The digested PGT8 (1536bp) in lanes 2 and 3, separated from TOPO (3956 bp). Also, digested pPICZA plasmid approximately 3600 bp separated from the undigested pPICZA. This was cut and purified.

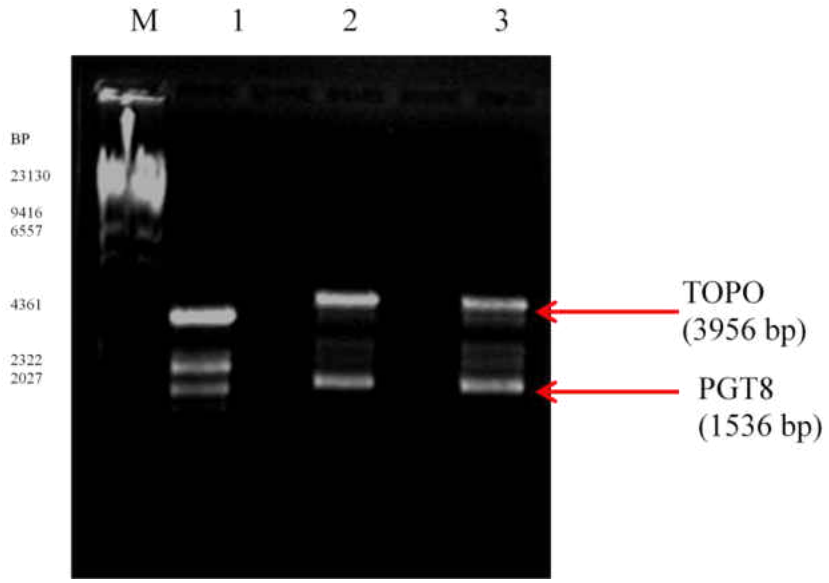


Fig.46. Restriction Digests of PGT8-TOPO Construct and pPICZA Vector for DNA Purification. Lane M is lambda hind III marker; lane 1 is digested pPICZA vector; lane 2 and 3 are digested PGT8-TOPO plasmids. Digestions were done with restriction enzymes Kpn1 and Apa1. The vector was digested with restriction enzymes to make it compatible for ligation with PGT8 insert.

Ligation of pPICZA with PGT8 Construct

Prior to ligation, aliquots of digested PGT8 inserts and pPICZA vector were run on DNA agarose gel to aid in the quantification of the concentration of DNA. A 2 ul and 5 µL aliquot of representative purified plasmids of PGT3 inserts and pPICZ vectors were run together with Lambda Hind III marker and 1 kb DNA plus marker on a DNA agarose gel (Fig. 47). After ligation of PGT8 insert with pPICZA vector, the ligation reactions were transformed into TOP 10 competent cells. The transformed cells were plated on $LB_{zeocin\ (100mg/l).}$ Single colony cells were miniprepped and plasmid DNA isolated. The plasmid DNA was sent for sequencing. Results of sequencing (Fig.48) showed the presence of PGT8 in-frame in the pPICZA vector.

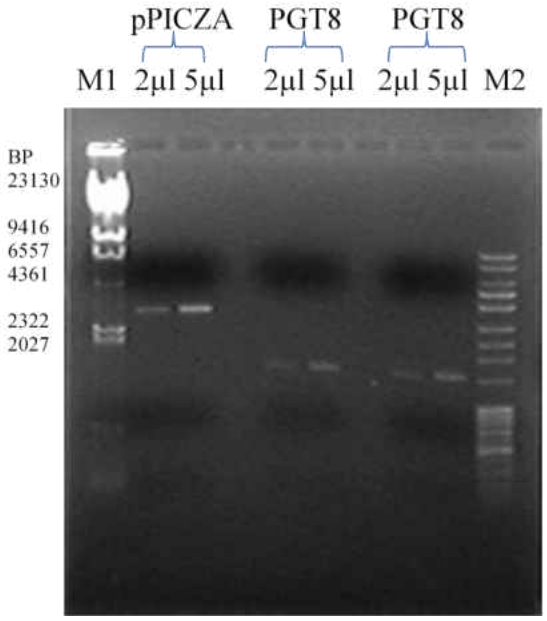
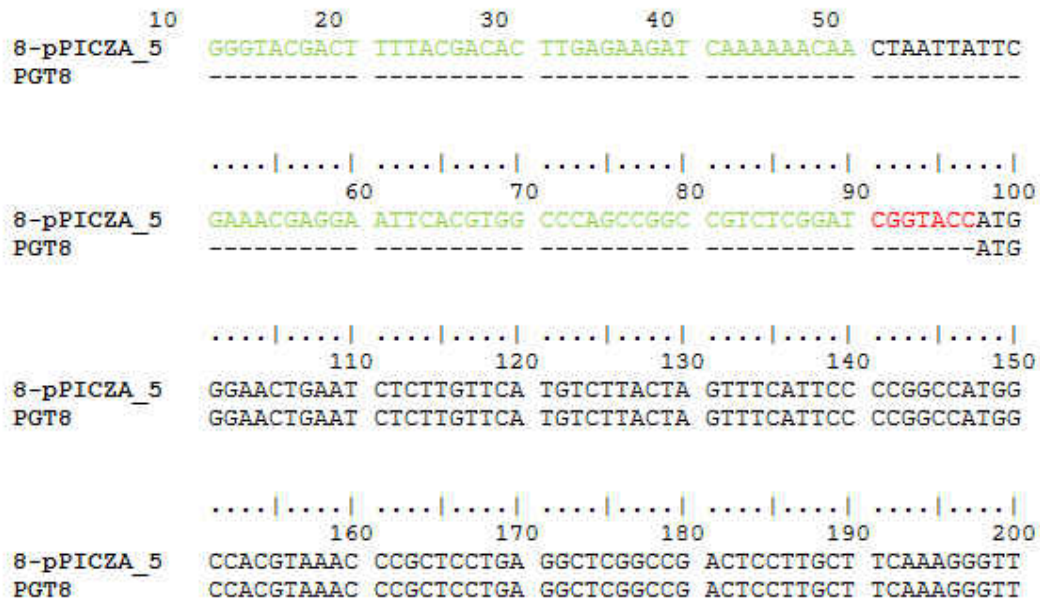


Fig. 47. Representative DNA Agarose Gel Showing Purified PGT8 Insert for Ligation with pPICZA Vector. Different volumes run on agarose for quantification of DNA concentration are noted. M1 represents represents lambda Hind III marker and M2 represents 1 kb DNA plus molecular weight ladder.

A.



B.

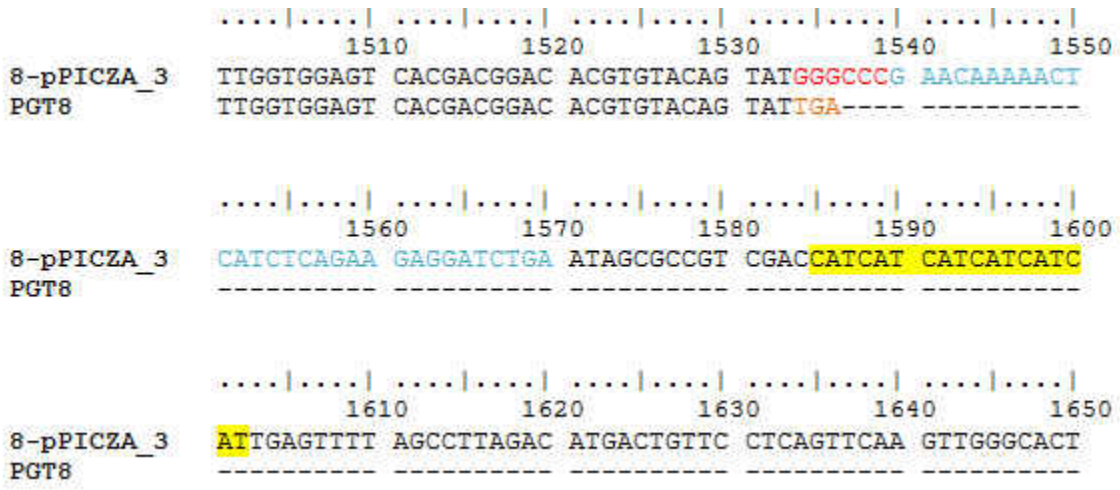


Fig. 48 Alignment of PGT8 sequence with PGT8-pPICZA sequence using Bioedit. A. 5' region. The area in red represent restriction enzyme priming site, green represent pPICZA vector 3' sequence.B. 3' Alignment of PGT8 sequence with PGT8-pPICZA sequence using Bioedit. The regions in red represent restriction enzyme priming site, blue represents c-myc epitope tag, area marked yellow represents plyshistidine tag, and orange represents the stop codon of PGT8.

CHAPTER 4

DISCUSSION

Plant secondary metabolites play crucial roles in the development and survival of plants (Kutchan 2001 and ref. therein; Fall et al. 2011 and ref. therein). Plant secondary metabolites are classified under several major large families such as the phenolics, alkaloids, and terpenoids (Bougard et al. 2001; Martens 2010 and ref. therein). Flavonoids are phenolic secondary metabolites with a wide diversity of compounds distributed throughout the plant kingdom (Koes et al. and ref. therein; Schijlen et al. 2004 and ref. therein).

Most naturally occurring flavonoids exist in glycosylated forms; this suggests that glycosylation is a key plant biochemical process (McIntosh and Mansell 1990; Kramer et al. 2003; Owens and McIntosh, 2011 and ref. therein). Glucosylation is a modification reaction in plants that leads to the formation of glucosides. Glucosyltransferases (GTs) are the enzymes that add sugars to aglycones to form glucosides (McIntosh 1990; Owens and McIntosh 2009; Owens and McIntosh 2011 and ref. therein). Glucosylation increases solubility and hence transport of compounds to parts of plants for biochemical processes (Asen and Jurd 1967; Shao et al. 2005 and ref. therein; Owens and McIntosh 2011 and ref. therein).

Grapefruit tends to accumulate high levels of glycosylated flavonoids (McIntosh 1990; McIntosh et al. 1990; Berhow et al 1998; Owens and McIntosh 2009; Owens and McIntosh 2011 and ref. therein). A flavanone-specific 7-O-GT noted to catalyse the glucosylation of the 7-OH group of naringenin to form prunin was isolated and characterized from *Citrus paradisi* seedlings (McIntosh and Mansell 1990). From this work several GTs from *Citrus paradisi* were shown to have the ability of attaching sugars to flavones, flavonols, flavanones, and chalcones (McIntosh

et al 1990). Also a flavonol specific 3-O-glucosyltransferase from *Citrus paradisi* was cloned and biochemically characterized (Owens and McIntosh 2009). This makes grapefruit *Citrus paradisi* a model plant to study flavonoid GTs and their characterization.

Purification and characterization of flavonoid GTs directly from tissues of grapefruit has resulted in the elucidation of active enzymes (McIntosh and Mansell 1990; McIntosh et al. 1990). However due to the labile nature of the enzyme and the minute levels of the enzyme present in the tissues, enough enzyme could not be accrued for purification and subsequent direct amino acid sequencing of the enzyme (McIntosh et al. 1990; McIntosh and Mansell 1990; Tanner 2000 and ref. therein). In order to overcome this, several plant putative GT sequences can be identified from plant genomic databases using the PSPG box as a motif for similarity (Knisley et al. 2009; Vogt and Jones 2000 and ref. therein). Although there is some degree of sequence identity within the PSPG box of plant GTs involved in natural product formation, the overall sequence similarity among flavonoid GTs is low (Owens and McIntosh 2009 and ref. therein; Sarker et al. 2007). The use of amino acid sequences alone cannot be used to predict specific functions and biochemical assays remain the only way to conclusively establish function. A combination of bioinformatics and biochemical assays remains the only way to conclusively establish function to these enzymes (Owens and McIntosh 2009 and ref. therein).

This research was designed to test the hypotheses that grapefruit plant glucosyltransferase clone 3 is a flavonoid glucosyltransferase and is subject to biochemical regulation (if activity is found, biochemical characterization such as kinetics, pH, cofactors, inhibitors, and temperature will be studied). It was also designed to test that the expression of grapefruit plant glucosyltransferase clone 8 in yeast, *Pichia pastoris* will be more soluble than in *E.coli.*

Expression and Characterization of PGT3

A directionally cloned EST library created from young grapefruit leaves and mined for putative clones resulted in the isolation of PGT3, a full-length clone (Sarker 2004). The presence of a PSPG box made PGT3 a possible candidate for plant secondary metabolite glucosyltransferase.

The use of prokaryotic expression systems such as the *E.coli* expression system may perhaps be the most common of all for protein expression due to its many advantages (Waugh 2005 and ref. therein). Some of advantages are the fact that the genetics of *E.coli* is well known, it is relatively cheap and simple to work with, it has the ability to produce high densities and also allows for purification strategies due to the production of tagged recombinant fusion proteins that can be purified using metal affinity columns (Sorensen and Mortensen 2004 and ref. therein, Sahdev et al. 2008). Another added advantage of expressing protein in *E.coli* using the pCD1 vector is the thireodoxin fusion tag could be cleaved off the expressed protein before testing for activity. It is possible that the size and structure of the tag could affect the activity of the protein (Owens and McIntosh 2009). These advantages make *E.coli* expression system a reasonable first choice for expression of target proteins.

With this in mind, PGT3 was cloned into pCD1 vector and expressed as a recombinant fusion protein with thireodoxin and 6xHis tags. The expressed PGT3 protein was confirmed with SDS-PAGE and Western blot; however, the majority of the recombinant protein expressed was packed in insoluble inclusion bodies (Fig. 14). Although the *E.coli* expression system is endowed with many advantages, it is not a perfect system. One notable disadvantage of expressing target proteins in *E.coli* is that, overproduction of target proteins induces a stress response in *E.coli*

which tends to pack majority of the recombinant target protein insoluble inclusion aggregates (Waugh D 2005, Sorensen and Mortensen 2004 and ref. therein). Many eukaryotic proteins do not express in a useful form (inclusion bodies) when expressed in *E.coli* perhaps due to the absence of a proper folding mechanism (Oganesyan et al. 2007). The production of target proteins as insoluble inclusion bodies poses a major setback in protein expression. To enhance the solubility of recombinant proteins, various optimization strategies such as variation of media composition, variation of the concentration of inducing agents, lowering of temperature at which the target protein is expressed, and variation of media composition (Waugh 2005 and ref. therein).

In this research, attempts were made to vary media composition by expressing PGT3 in the presence and absence of betaine and sorbitol (Figs.13 and 14) and variation of inducer IPTG (Fig.15). The rationale behind this was to increase the expression of soluble PGT3 protein to test for flavonoid GT activity. Betaine and sorbitol are osmotic reagents that have been shown to increase the expression of some soluble proteins by increasing osmotic stress (Blackwell and Horgan 1991). This tends to prevent the packing of soluble proteins into insoluble inclusion bodies. The amount of soluble PGT3 protein expressed in the absence of betaine and sorbitol was more than when expressed in the presence of betaine and sorbitol. Variation of inducer IPTG affected the expression of total protein but had no significant effect on the expression of soluble recombinant PGT3. Overall, the optimization strategies employed to enhance the solubility of rPGT3 in *E.coli* did not yield enough protein to be tested for GT activity.

To be able continue with this research, it was imperative to find a way of producing enough soluble rPGT3 protein to test for flavonoid GT activity. Therefore, PGT3 was expressed in *Pichia* a eukaryotic expression system. The *Pichia* expression system was used because, as a

eukaryotic expression system, it had a better protein folding and processing mechanism thus do not produce insoluble inclusion bodies, it is relatively simple to use and inexpensive as *E.coli* (it uses methanol for induction which is inexpensive), and it had an advantage of 10-100 fold higher heterologous protein expression levels (Cereghino and Cregg 1999). Although GTs are not known to undergo post-translational modification, if proteins that need to undergo post-translational modifications are expressed in *Pichia*, this is taken care of by using the pPICZα. The above features made *Pichia* expression system the next choice for expressing rPGT3 protein.

Expression of recombinant proteins in *Pichia* is under the control of the AOX1 gene promoter and the expression of the AOX1 gene is induced by methanol (Invitrogen Pichia manual, 25-0172). In the presence of methanol, the AOX1 promoter is activated and this drives the expression of rPGT3. Recombinant proteins expressed using *Pichia* are secreted into the media or intracellularly expressed, depending on different vectors and cloning strategies. Two vectors, pPICZ and pPICZα, expresses recombinant protein in the intracellular and secretory pathway, respectively (Invitrogen Pichia manual, 25-0172). In this research, PGT3 was cloned into both pPICZA and pPICZAα. The expression of PGT3 using both vectors was tested. Test inductions were done where samples were taken from the 6^{th} hr to 96^{th} hour. The rationale behind this was to determine the time post induction when the optimal soluble rPGT3 was expressed. Confirmation of expression was done by analyzing samples from the various time points (Fig. 35). Based on the Western blot analysis the optimum time for expression of rPGT3 was the 6^{th} hour postinduction in construct 1 (PGT3 cloned in pPICZA). Expression levels of constructs 2 and 3 (PGT3 in pPICZAα) was minimal and insufficient to be detected by Western blot analysis. Due to this, expression of rPGT3 using pPICZA was chosen for further experiments.

Soluble rPGT3 protein was enriched by immobilized metal affinity chromatography. The rationale for enrichment of soluble proteins was to remove contaminating proteins which might affect enzyme activity. Enrichment of PGT3 was possible as a result of the selective binding of the poly-his tagged PGT3 with the cobalt metal ion in the IMAC column. Loosely bound proteins were removed with equilibration wash buffers, signifying removal of all contaminants. Tightly bound poly histidine tagged PGT3 protein was eluted with high concentration of imidazole (Figs. 36 and 37). Prior to enrichment by immobilized metal affinity chromatpgraphy, cell lysate was desalted to get rid of the salts from the breaking buffer which could potentially affect the binding of the poly histidine tagged proteins to the IMAC coluum. Also, fractions obtained from the enrichment process were analyzed by SDS-PAGE and Western blot analysis. The goal was to identify which fractions contained the most enriched rPGT3 (Figs. 36 and 37). The most enriched fractions were pooled, desalted (to get rid of the salts such as imidazole and sodium chloride which is known to affect enzyme activity), and concentrated prior to enzyme activity assays.

The hypothesis of this research was to test that clone 3 is a putative flavonoid glucosyltransferase. Thus, the enriched pooled, desalted, and concentrated fraction of rPGT3 was screened for flavonoid GT activity. The rPGT3 was first tested with compounds representing various subclasses of flavonoids (Table 14). Because flavanone glycosides tend to be accumulated in grapefruit, naringenin and hesperetin representing flavonones were selected for screens. The glycosides of naringenin and hesperetin had been shown to accumulate in grapefruit (McIntosh and Mansell 1990 and ref. therein; Owens and McIntosh 2011 and ref. therein). Luteolin and apigenin, 2 compounds from the flavone group, were tested knowing that glycosides of these 2 compounds have been shown to accumulate in grapefruit (McIntosh and

Mansell 1990). The rationale behind this was that, if activity was found with any of one of the compounds within a flavonoid subclass, the possiblility of the preferred substrate of the enzyme being in that flavovonid group was greater. With this in mind, several substrates from each flavonoid subclass were tested. A total of 18 flavonoid substrates representing all the flavonoid subclasses were tested. However, no significant activity was detected with any of the compounds. Results with positive controls showed an incorporation of radiolabelled glucose onto kaempferol by glucosyltransferases present in the crude grapefruit leaf extract. This was observed with an average incorporation of radiolabelled glucose (1058 cpm in 250 µL of ethy acetate) in a reaction that contained 20000 cpm of radiolabelled glucose. Comparing this to the average cpm incorporation of the flavonoid substrates (Table 14), there was a significant activity in the positive controls but no significant activity with rPGT3. This suggests that rPGT3 does not use any of the flavonoid compounds tested as a preferred substrate.

Although this research was designed to test the hypothesis that rPGT3 was a flavonoid GT, it is likely that PGT3 might be able to glucosylate other secondary metabolites such as phenolic compounds and phenolic glucosides that have been shown to be present in citrus species (Fieldman and Hanks 1965). In order to test this, rPGT3 protein was screened for GT activity with a few phenolic compounds (coumarins and simple phenolics) however no significant activity was found (Table 14). At this stage the compounds that had been tested ruled out the possibility of them being substrates for PGT3. Thus, the way forward was to screen even more phenolic compounds that have been found to be present in grapefruit.

Additional candidate substrates were found in the literature reporting other glucosides found in grapefruit (Fieldman and Hanks 1965; Ahmad and Hopkins 1993; Gardana et al. 2008). These compounds (12 substrates) were tested with rPGT3 for activity. Due to the high water

solubility of these compounds, glucosides formed would be equally water soluble so separation of glucosides from aglycones could not be done with ethyl acetate extraction.

A reverse phase high performance liquid chromatography (HPLC) using a C-18 column and Isocratic elution was used for the separation of glucosides and aglycones (Ahmad and Hopkins 1993) (See Table 16, and Fig. 40). Out of the 12 substrates tested, there were indications of activity with 6 (p-hydroxybenzoic acid, vanillin, vanillic acid, p-hydroxyphenylpyruvate, catechol, and gentisic acid). The chromatograms of the reactions with these substrates showed a reduction in the peak height of the standard and/ or formation of new peaks. A newer peak with a shorter retention time is indicative of a more polar compound that may be a glucoside. These compounds and other related compounds have been shown to influence growth, dormancy, and/ or shown to be increased in response to pathogens (Fieldman and Hanks 1965; Fraissinet-Tachet et al. 1998 and ref. therein). Thus the presence of these glucosides implies they may perform these physiological functions in *Citrus paradisi.*

Cloning PGT8 into pPICZA Vector

Putative flavonoid glucosyltransferase clone 8, previously expressed in *E.coli*, was subcloned into pPICZA vector for expression in *Pichia*. The expression of PGT8 in *E.coli* resulted in majority of the expressed protein as insoluble inclusion bodies; however, some trace activity with liminoids and quercetin was found (Owens and McIntosh personal communication). Results confirm that PGT8 has been successfully cloned into pPICZA vector.

Directions for Future Research

If confirmation of GT activity is found with any of the 6 phenolic substrates, identification of products will be done and characterization of the enzyme will be conducted.

Biochemical characterization including enzyme kinetics, optimal reaction conditions (such as pH, buffer, and temperature) and testing for cofactors and inhibitors will be done.

On the other hand, if the result is not reproducible, the following suggestions can be done. First and foremost, more work on screening additional candidate substrates for PGT3 should be done. Some simple phenolic substrates that could be tested include m-coumaric acid, salicylic acid, and sinapic acid. The rationale behind testing these substrates is that glucosides of these compounds were shown to be present in roots and leaves of *Citrus sp* (Fieldman and Hanks 1965). More candidate substrates from each of the flavonoid subclasses could also be tested because rPGT3 was not tested with all flavonoids. Hence, testing a couple of substrates within a particular subclass may not reveal the best substrate for PGT3. The preferred substrate of PGT3 may only be shown after testing a lot more substrates from each class of flavonoid group. For example, phloretin (related to chalcone) has been shown to be present in leaves and roots of citrus species and could also be tested.

Again, if no activity is found with any of the 6 simple phenolic substrates, removal of fusion tags prior to screening for activity can be considered. The characterization of rPGT3 was done with fusion tags (c-myc and polyhistidine) attached to the protein at the C-terminus. The total size of the myc epitope and polyhistidine tag is only about 2.5 kDa (Invitrogen Pichia manual, 25-0172). The presence of tags, independent of size, could interfere with the biological activity or influence the behavior of a protein (Waugh 2005 and ref. therein; Owens and McIntosh 2009). The removal of tags prior to screening for activity would serve as a basis for testing the hypothesis that the presence of a tag could interfere with the biological activity of a protein. This can only be done if the vector being used has advantage of being modified to remove encoded tags, for example the pCD1 vector (Owens et al. 2008). Perhaps *Pichia*

expression vectors could be modified to include a thrombin cleavage site in order to remove fusion tags.

The third option to try if there is no activity found with any of the 6 simple phenolic substrates would be to consider the type of UDP sugar. The sugar donor used in this research was UDP-glucose. If the preferred sugar for PGT3 is UDP-galactose, activity with any of the substrates would not be found until we use the preferred sugar. Thus, other sugar donors such as UDP-galactose, UDP-mannose, UDP-xylose, and UDP-rhamnose could be tested (Offen et al. 2006; Osmani et al. 2009). Of these potential sugars mentioned, UDP-rhamnose may be first choice because its glycosides containing rhamnosyl residues were found in grapefruit tissues (Owen and McIntosh 2011 and ref. therein). However, the lack of commercial availability of UDP-rhamnose means that in order to test this substrate, the sugar would have to be synthesized in the laboratory if possible.

With regards to PGT8, the next step would be to transform PGT8-pPICZA into *Pichia* genome for expression of rPGT8 protein. After transformation, test inductions for expression could be done to determine the optimum conditions for production of rPGT8 protein. Successful expression of PGT8 will then lead to the next step which will be to enrich rPGT8 by immobilized metal affinity chromatography making use of the polyhistidine tag as a means of purification. Once enrichment is achieved, rPGT8 should be tested first against limonin monolactone subtrates as well as with flavonoid substrates. The rationale for testing limonin monolactones are because limonoate glucosides have been shown to be widely distributed in citrus (Hasegawa et al. 1989; Hasegawa et al. 1991 and ref. therein). Also some activity with quercetin was found with rPGT8 expressed in *E.coli* (McIntosh and Owens personal communication).

REFERENCES

Aghel N, Ramezani Z, Beiranvand S. 2008. Hesperidin from *Citrus sinensis* Cultivated in Dezful, Iran. *Pakistan Journal of Biological Sciences*. *20*: 2451-2453.

Ahmad SA, Hopkins TL. 1993. B-Glucosylation of Plant Phenolics by Phenol B-Glucosyltransferase in Larval Tissue of the Tobacco Hornworm, *Manduca sexta* (L.). I*nsect Biochemistry and Molecular Biology*. *23*: 581-589

Asen S, Jurd L. 1967. The Constitution of a Crystalline, Blue Cornflower Pigment *Phytochemistry*. *6*: 577-584

Ashihara H, Deng WM, Crozier WA. 2010. Distribution and Biosynthesis of Flavan 3-ols in *Camellia sinensis* Seedlings and Expression of Genes Encoding Biosynthetic Enzymes. *Phytochemistry*. *71*: 559–566.

Barthe A, G Jourdan SP, McIntosh CA, Mansell RL, 1988. Radioimmunoassay for the Quantitative Determination of Hesperidin and Analysis of its Distribution in *Citrus sinensis*. *Phytochemistry*, *27*: 249-254.

Berhow M, Tisserat B, Kanes K, Vandercook C, 1998. Survey of Phenolic Compounds Produced in Citrus. USDA ARS *Technical Bull* 158.

Bioedit software: http://www.mbio.ncsu.edu/bioedit/bioedit.html, accessed Febuary, 2012

Bieza K, Lois R. 2001. An Arabidopsis Mutant Tolerant to Lethal Ultraviolet-B Levels shows Constitutively Elevated Accumulation of Flavonoids and other Phenolics. *Plant Physiology*. *126*: 1105-1115.

Bohm AB, 1993. The Minor Flavonoids, Chapter 9 in *Advances in Flavonoid Research since 1986*, Harborne (Editor), Chapman & Hall. 388-433.

Bourgaud F, Gravot A, Milesi S, Gontier E. 2001. Production of Plant Secondary Metabolites: A Historical Perspective. *Plant Sci. 161*: 839-851.

Britsch L, Heller W, Grisebach H. 1981. Conversion of Flavanone to Flavones, Dihydroflavanol and Flavanol with an Enzyme System from Cell Cultures of Parsley, *Z. Naturfosch*. *36*: 742-750

Brouillard R, Cheminat A. 1988. Flavonoids and Plant Color. In Plant Flavonoids in Biology and Medicine II: Biochemical, cellular and medicinal properties (eds V. Cody, Middleton E, J.B.Harborne and Beretz), Alan R.Liss, New York, p.93

Brouillard R, Dangles O**,** 1993. Flavonoids and Flower Colour. Chapter 3 In: Harborne JB (Ed) *The Flavonoids: Advances in Research* since 1986, pp 565-588

Campbell JA, Davies GJ, Bulone V, Henrisatt B. 1997. A Classification of Nucleotide-Diphospho-sugar Glycosyltransferases based on Amino Acid Sequence Similarities. *Biochem J*. *326:* 929-939.

Casati P, Walbot V. 2003. Gene Expression Profiling in Response to Ultraviolet Radiation in Maize Genotypes with Varying Flavonoid Content. *Plant Physiology*. *132:* 1739-1754

Cereghino JL, Cregg JM. 1999. Heterologous Protein Expression in the Methylotrophic Yeast *Pichia pastoris. FEMS Microbiology. 24*: 45-66

Dewick P. 1993. Isoflavonoids, Chapter 5 in *Advances in Flavonoid Research since 1986* Harborne (Editor), Chapman & Hall, pp. 118-232

Delorme E. 1989. Transformation of *Saccharomyces cerevisiae* by Electroporation. *Applied and Environmental Microbiology*. *55*: 2242-2246.

Donovan RS, Robinson CW, Glick BR. 1996. Optimizing Inducer and Culture Conditions for Exression of Foreign Proteins under the Control of the Lac Promoter. *Journal of Industrial Microbiology*. *16* :145-154.

Durren R.L, McIntosh C.A. 1999. Flavanone -7-0-glucosyltransferase Activity from *Petunia hybrida. Phytochemistry*. *52* :793-798.

Easy Select Pichia Expression Kit for Expression of Recombinant Proteins using pPICZA and pPICZα. in *Pichia pastoris*, 2009 User manual, version 1, 25-0172.

Fall LAD, Solomon PS. 2011. Role of Cereal Secondary Metabolites Involved in Mediating the Outcome of Plant-Pathogen Interactions. *Metabolites*.*1*: 64-78.

Fieldman AW, Hanks RW. 1965. Phenolic Compounds in Roots and Leaves of Four Citrus Cultivars. *Nature*. *207*: 985-986

Forkman G, Martens S. 2001. Metabolic Engineering and Applications of Flavonoids. *Current Opinion in Biotechnology*. *12*: 155-160

Forkmann G. 1991. The Anthocyanins Chapter 1 in *Advances in Flavonoid Research since 1986* Harborne (Editor), Chapman & Hall, pp 1-19.

Forkmann G, Heller W, Grisebach H. 1980. Anthocyanin Biosynthesis in Flowers of *Matthiola incana* Flavanone 3- Flavonoid 3$^{'}$- hydroxylases. *Z. Natureforsch 35*: 691-695

Fraissinet-Tachet L, Baltz R, Chong J, Kauffmann S, Fritig B, Saindrenan P. 1998. Two Tobacco Genes Induced by Infection, Elicitor and Salicylic Acid Encode Glucosyltransferases Acting on Phenylpropanoids and Benzoic Acid Derivatives, including Salicylic Acid *FEBS Letters*. *437*: 319-323.

Fritsch H, Grisebach H. 1975. Biosynthesis of Cyanidin in Cell Cultures of *Happlopapus gracilis*. *Phytochemistry*.*14*: 2437-2442

Gardana C, Nalin F, Simonetti P. 2008. Evaluation of Flavonoids and Furanocoumarins From *Citrus bergamia* (Bergamot) Juice and Identification of New Compounds. *Molecules*. *13*: 2220-2228

Gould KS, Lister C. 2005. Flavonoid Functions in Plants. Chapter 8 in *Flavonoids:* Chemistry Biochemistry and Applications. Andersen, OM, and Markham, K. R. (Editors), Taylor & Francis, Inc, Philadelphia, pp 397-425

Hahlbrock K, Grisebach H. 1970. Formation of Coenzyme A Esters of Cinnamic Acids with an Enzyme Preparation from Cell Suspension Cultures of Parsley. *FEBS Lett*. *1*: 62-64.

Harborne JB, Baxter H. 1999. *Handbook of Natural Flavonoids* vol, 2 , Wiley, Chichester.

Harborne JB, Williams CA. 1988. Distribution and Evolution of Flavonoids in the Monocotyledons. Chapter 8 in *The Flavonoids: Advances in Research Since 1980.* Harborne, J. B. (Editor), Chapman and Hall, New York, 304-328

Hasegawa S, Bennet RD, Herman Z, Forg CH, Ou P. 1989. Limonoid Glucosides in Citrus. *Phytochemistry*. *28*: 1717-1720

Hasegawa S, Ou P, Forg CH, Herman Z, Coggins CW Jnr, Atkin DR. 1991. Changes in the Lomonoate A Ring Lactose and Limonin 17-β-D-Glucopyranoside Content of Naval Oranges during Fruit Growth and Maturation. *J. Agric Food Chem*. *39*: 262-265

Hedin PA, Jenkins JN, Collum DH, White WH, Parrot WL. 1983. In P. A. Hedin (Ed.), Plant Resistance to Insects. *American Chemical Society*, Washington, DC, pp 347-365

Heller W, Forkmann G. 1988. Biosynthesis. Chapter 11 in *The Flavonoids: Advances in Research Since 1980.* Harborne, J. B. (Editor), Chapman and Hall, New York, 399-425

Heller W, Hahlbrock K. 1980. Highly Purified "Flavanone Synthase" from Parsley Catalyzes the Formation of Naringenin Chalcone. *Arch Biochem Biophys*. *600*: 617-619

Heller W, Forkmann G. 1993. Biosynthesis of Flavonoids. In *The Flavonoids: Advances in Research Since 1986***,** Harborne, J.B., (ed.), Chapman and Hall, London, pp. 499-536.

Hollerman PCH, Bijsman MCNP, Van-Gameran Y, Cnossen EPJ, de- Vries JHM, Katan MB. 1999. The Sugar Moeity is a Major Determinant of the Absorbption of Dietary Flavonoid Glycosides in Man. *Free Radic. Res. 31*: 569-573

Horowitz RM, Gentili B. 1969. Taste and Structure in Phenolic Glycosides *J Agric Food Chem*. *17*: 696-700.

Horowitz RM. 1986. Taste Effects of Flavonoids. In "*Plant Flavonoids in Biology and Medicine"*, Cody, V., Middleton Jr, E., and Harborne, J.B, (eds.), *213* of "Progress in Clinincal and Biological Research", Alan R, Liss Inc., NY pp. 163-176.

Hughes J, Hughes MA.1994. Multiple Secondary Plant Product UDP-glucose Glucosyltransferase Genes Expressed in Cassava (*Manihot esculenta Crantz)* Cotyledons. *DNA Seq*. *5*: 41–49.

Jones P, Messner B, Nakajima JI, Schaffner RA, Saito K. 2003. UGT73C6 and UGT78D1, Glycosyltransferases Involved in Flavonol Glycoside Biosymthesis in *Arabidopsis thaliana*. *The Journal of Biological Chemistry*.*278*: 43910-43918

Jordan, B.R., 1996. The Effects of Ultraviolet-B Radiation on Plants: A Molecular Perspective *Adv. Bot. Res*. *22*: 98–162.

Jourdan PS, McIntosh CA, Mansell RL.1985. Naringin Levels in Citrus Tissues II. Quantitative Distribution of Naringin in *Citrus paradisi* MacFad. *Plant Physiol 77*: 903-908.

Jung W, Yu O, Lau SMC, Keefe OP, Odell J, Fader G, McGonigle B. 2000. Identification and Expression of Isoflavone Synthase, the Key Enzyme for Biosynthesis of Isoflavones in Legumes. *Nat Biotechnol*. *18*: 208-212.

Kemp MS, Burden RS. 1986. Phytoalexins and Stress Metabolites in the Sapwood of Trees.

Kesterson JW, Hendrickson R. 1957. Naringin a Bitter Principle of Grapefruit. *Fla Agric Exp* Stn Tech Bull *511*.

Kiser SJ. 2005. Cloning, Expression, and Functional Analysis of a Putative Glucosyltransferase EST Sequence from Grapefruit Leaves. East Tennessee State University. Honor‘s Thesis.

Knisley D, Seier E, Lamb D, Owens DK, McIntosh CA. 2009. A Graph-theoretic Model based on Primary and Predicted Secondary Structure Reveals Functional Specificity in a Set of Plant Secondary Product UDP-glucosyltransferases. In Proceedings of the 2009 International Conference on Bioinformatics, Computational Biology, Genomics and Chemoinformatics, (BCBGC-09), Loging, W., Doble, M., Sun, Z., and Malone, J., (eds.), pp. 65-72, ISRST

Knobloch KH, Hahlbrock K. 1977.4-Coumarate: CoA ligase from Cell Suspension Cultures of Petroselinum hortense Hoffm. Partial Purification, Substrate Specificity, and Further Properties. *Arch Biochem Biophys. 184:*237-248.

Kochs G, Grisebach H. 1986. Enzymic synthesis of isoflavones, *Eur J. Biochem 155*: 311- 318

Kochs G, Grisebach H. 1986. Induction and Characterization of a NADPH-dependent Flavone Synthase from Cell Cultures of Soybean. *Z, Naturforsch*. *42*: 343-348

Koes RE, Quattrocchio F, Mol JNM. 1994. The Flavonoid Biosynthetic Pathway in Plants; Function and Evolution. *Phytochemistry.16*: 123-132.

Kong, J. M., Chia, L.S., Ngoh-Khang, Goh Chia T.F, Brouillard, R, 2003 Analysis and Biological Activities of Anthocyanins. *Phytochemistry*. *64*: 923-933

Korkina L.G., 2007. Phenylpropanoids as Naturally Occurring Antioxidants: from Plant Defense to Human Health. *Cellular and Molecular Biology*.*53*: 15-25.

Koukol J, Conn EE. 1961. The Metabolism of Aromatic Compounds in Higher Plants. *The Journal of Biologcial Chemistry, 236:* 2692-2698

Kramer MC, Prata TNR, Willets GM, Luca DV, Stephens CJ, Graser G. 2003. Cloning and Regiospecificity Studies of Two Flavonoid Glucosyltransferases from *Allium cepa*, *Phytochemistry. 64*: 1069-1076.

Kris-Etherton PM, Hecker KD, Bonanome A, Coval SM, Binkoski AE, Hilpert KF, Griel AE, Etherton TD. 2002. Bioactive Compounds in Foods: their Role in the Prevention of Cardiovascular Disease and Cancer. *Am. J. Med*. *113*: 71–88.

Kreuzaler F, Hahlbrock, K. 1972. Enzymatic Synthesis of Aromatic Compounds in Higher Plants: Formation of Naringenin (5, 7, 4'-trihydroxyflavanone) from p-Coumaroyl Coenzyme A and Malonyl Coenzyme A. *FEBS Lett*. *1*: 69-72.

Kutchan TM**.** 2001. Ecological Arsenal and Developmental Dispatcher: the Paradigm of Secondary Metabolism. *Plant Physiol 125***:** 58–60

Lambert N, Trouslot MF, Nef-Campa C, Chrestin H. 1993. Production of Rotenoids by Heterotrophic and Photomixotrophic Cell Cultures of *Tephrosia vogelii*. *Phytochemistry*. *34*:1515-1520.

Lewinsohn EB, Mazur Y, Gressel J. 1986. Glucosylation of Exogenous Flavanones by Grapefruit (*Citrus paradisi*) Cell Cultures. *Phytochemistry*. *25*: 2531-2535.

Li J, Ou-lee T, Raba R, Amundson RG, Last R. 1993. Arabidopsis Flavonoid Mutants are Hypersensitive to UV-B Irradiation. *Plant Cell*. *5*: 171-179.

Li L, Modolo LV, Escamilla-Trevino LL, Achnine L, Dixon RA, Wang X. 2007. Crystal Structure of *Medicago truncatula* UGT85H2 – Insights into the Structural Basis of a Multifunctional (Iso)flavonoid Glycosyltransferase. *J Mol Biol*. *370*: 951–963

Li Z, Ngojeh G, DeWitt P, Zheng Z, Chen M, Lainhart B, Li V, Felpo P. 2008. Synthesis of a Library of Glycosylated Flavonols. *Tetrahedron Letters*.*49*: 7243-7245.

Lim E.K, Bowles DJ. 2004. A Class of Plant Glycosyltransferases involved in Cellular Homeostasis. *The EMBO Journal 23*: 2915–2922

Lim EK, Doucet CJ, Li Y, Elias L, Worral D, Spencer SP, Ross J, Bowles DJ. 2002. The Activity of Arabidopsis Glycosyltransferases toward Salicylic acid, 4- Hydroxybenzoic acid and other Benzoates. *Journal of Biological Chemistry. 277*: 586-592

Lin Z. 2011. Expression and Characterization of Putative Plant Secondary Product Glucosyltransferase Clone 10 from *Citrus paradisi* and Resolution of Multiple Bands in PGT5/6. East Tennessee State University. MS Thesis.

Liu CJ, Huhman D, Sumner WL, Dixon RA. 2003. Regiospecific Hydroxylation of Isoflavones by Cytochrome P450 81E Enzymes from *Medicago truncatula*. *The Plant Journal*. *36*: 471-484.

Livingstone MJ, Zolotarov Y, Stromvik VM. 2011. Transcripts of Soybean Isoflavone 7-O-Glucosyltransferase and Hydroxyisoflavanone Dehydratase Gene Homologues are at Least as Abundant as Transcripts of their Well Known Counterparts. *Plant Physiology and Biochemistry. 49*: 1071-1075.

Marais JPJ. Deavours B, Dixon RA, Ferreira D. 2007. The Stereochemistry of Flavonoids. Chapter 1 in *The Science of Flavonoids.* E. Grotewold (Editor), Springer, New York, pp 1-4.

Mackenzie PI, Owens IS, Burchell B, Bock KW, Bairoch A, Belanger A, Fournel-Gigleux S, Green M, Hum DW, Iyanagi T et al. 1997. The UDP Glycosyltransferase Gene Superfamily: Recommended Nomenclature Update based on Evolutionary Divergence. *Pharmacogenetics*. *7*: 255–269

Mallampali VS. 2009. Expression and Biochemical Function of Putative Flavonoid GT Clones from Grapefruit and Identification of New Clones Using the harvEST Database. East Tennessee State University. MS Thesis.

Mansell RL, McIntosh CA, Vest SE.1983. An Analysis of the Limonin and Naringin Content of Grapefruit Juice Samples Collected from Florida State Test Houses, *J. Agric Food Chemistry*. *31*:156-162

Martens S, PreuB A, Marten U. 2010. Multifunctional Flavonoid Dioxygenases: Flavonol and Anthocyanin Biosynthesis in *Arabidopsis thaliana* L.*Phytochemistry 71*: 1040-1049.

Martens S, Mithofer A. 2005. Flavones and Flavone Synthases. *Phytochemistry*. *66* :2399-2407.

McIntosh CA, Mansell RL.1983. Distribution of Limonin during the Growth and Development of Leaves and Branches of *Citrus paradisi*. *J. Agric Food Chem. 31*:319-325

McIntosh CA, Mansell RL. 1990. Biosynthesis of Naringin in *Citrus paradisi*: UDP-Glucosyltransferase Activity in Grapefruit Seedlings. *Phytochemistry*. *29*: 1533-1538

McIntosh, C.A. 1990. Biosynthesis of Naringin in *Citrus paradisi*: UDP Glucosyltranferase Activity in Grapefruit Seedlings. University of South Florida, Ph.D. Dissertation.

McIntosh CA, Latchininan L, Mansell RL. 1990. Flavanone Specific 7-O-glycosyltransferase Activity in *Citrus Paradisi* Seedlings: Purification and Characterization. *Archives of Biochemistry and Biophysics*. *282*: 50-57

Melche U. 1997. Genetic Code: Overview. In R. McClenaphan (Ed), Encyclopedia of Genetics Pasadena CA: Salem Press, pp 406-412

Merck Index. Merch & Co., Inc. Ninth Edition, 1976

Moustapha E, Wong E. 1966. Purification and Properties of Chalcone-Flavanone Isomerase from Soya Bean Seed. *Phytochemistry*. *6*: 625-632

Nogata Y, Ohta H, Yoza KI, Berhow M, Hasegawa S. 1994. High Performance Liquid Chromatographic Determination of Naturally Occurring Flavonoids in Citrus with a Photodiode-array Detector. *J Chromatogr A*. *667*: 59-66.

Offen W, Martinez-Fleites C, Yang M, Kiat-Lim E, Davis BG, Tarling CA, Ford CM, Bowles DJ, Davies GJ. 2006. Structure of a Flavonoid Glucosyltransferase Reveals the Basis for Plant Natural Product Modification. *EMBO J*. *25*: 1396–1405

Oganesyan N, Ankoudinova I, Kim SH, Kim R. 2007. Effect of Osmotic Stress and Heat Shock in Recombinant Protein Overexpression and Crystallization. *Protein Expression and Purification 52*: 280-258

Osmani SA, Bak S, Moller BL. 2009. Substrate Specificity of Plant UDP-Dependent Glycosyltransferases Predicted from Crystal Structures and Homology Modeling. *Phytochemistry.70*: 325-247

Othloff MR, Hollman PCH, Vree TB, Katan MB. 2000. Bioavailability of Quercetin-3-O-Glucoside and Quercetin-4'-Glucoside do not Differ in Humans. *J.Nutr. 130*: 1200-1203

Owens DK, McIntosh CA. 2011. Biosynthesis and Function of Citrus Glycosylated Flavonoids In "*Recent Advances in Phytochemistry*"*41*: 67-95, Springer publishing

Owens DK, McIntosh CA. 2009. Biosynthesis and Function of Citrus Identification Recombinant Expression and Biochemical Characterization of a Flavonol 3-0-Glucosyltransferase Clone from *Citrus paradisi*. *Phytochemistry*.*70*: 1382-1391

Owens DK, Crosby KC, Runac J, Howard BA, Winkel BS. 2008. Biochemical and Genetic Characterization of Arabidopsis Flavanone 3beta-hydoxylase. *Plant Physiol. Biochem* . *46*: 833-843

Paquette S, Moller BL, Bak S. 2003. On the Origin of Family 1 Plant Glycosyltransferases *Phytochemistry*. *62*: 399-413.

Pecket RC, Small CJ. 1980. Occurrence, Location and Development of Anthocyanoplasts. *Phytochemistry*. *19*: 2571-2576.

Peters KN, Long RS. 1988. The Role of Plant Compounds in the Regulation of Rhizobium Nodulation Genes. Chapter 5 in *Recent Advances in Phytochemistry* vol 22. Conn (Editor) Plenum press pp 83-97.

Potts MRJ, Weklych R, Conn E. 1974. The 4-Hydroxylation of Cinnamic Acid by Sorghum Microsomes and the Requirement for Cytochrome P-450. *The Journal of Biological Chemistry*. *249*: 5019-5026

Ragg HK, David N, Hahlbrock K. 1981. Coordinated Regulation of 4-coumarate: CoA ligase and Phenylalanine Ammonia-lyase mRNAs in Cultured Plant Cells. *The Journal of Biological Chemistry. 256*: 10061-10065

Reuber S, Bornman JF, Weissenbo CKJ. 1996. A Flavonoid Mutant of Barley (Hordeumvulgare L) Exhibits Increased Sensitivity to UV-B Radiation in the Primary Leaf. *Plant Cell Environ. 19*: 593–601.

Ribereau-Gayon P. 1972. In *Plant phenolics*. V. H. Heywood (Editor). Hafner, New York

Sarkar RT, Strong CL, Sibhatu MB, Pike LM, McIntosh CA. 2007. Cloning, Expression, and Characterization of a Putative Flavonoid Glucosyltransferase from Grapefruit (*Citrus paradisi)* Leaves. Chapter 18 In *"Concepts in Plant Metabolomics"*. B.J. Nikolau, and Wurtele, E.S. (eds.), Springer, pp. 247-257.

Sarker RT. 2004. cDNA Cloning, Expression and Characterization of a Putative Glucosyltransferase (GT) from Grapefruit (*Citrus paradisi)* Seedlings. East Tennessee State University. Master's Thesis.

Ryan GK, Swinny Ewald EM, Kenneth R, Winefied C. 2002. Flavonoid Gene Expression and UV Photoprotection in Transgenic and Mutant Petunia Leaves *Phytochemistry*. *59*: 23–32

Russell DW. 1971. The Metabolism of Aromatic Compounds in Higher Plants. X. Properties of the Cinnamic Acid 4-hydoxylase of Pea Seedlings and Some aspects of its Metabolic and Developmental Control. *The Journal of Biological Chemistry*. *246*: 3870–78.

Saito1 K, Kobayashi M, Gong Z, Tanaka Y, Yamazaki M. 1999. Direct Evidence for Anthocyanidin Synthase as a 2-oxoglutarate-dependent Oxygenase: Molecular Cloning and Functional Expression of cDNA from a red formas of *Perilla frutescens. The Plant Journal. 17:* 181-189

Sahdev S, Khattar SK, Saini KS. 2008. Production of Active Eukaryotic Proteins through Bacterial Expression Systems: A Review of the Existing Biotechnology Strategies. *Mol Cell Biochem*. *307*: 249-64.

Scalbert A, Williamson G. 2000. Dietary Intake and Bioavailabilty of Polyphenols. *J.Nutr*. *130*: 2073-2085.

Schijlen EGWM, Vos CH, Tunen RD, Van JA, Bovy GA. 2004. Modification of Flavonoid Biosynthesis in Crop Plants. *Phytochemistry*. *65*: 2631-2648.

Sefton MA, Francis IL, Williams PJ. 1994. Free and Bound Volatile Secondary Metabolites of *Vitis vinifera* Grape cv. Sauvignon Blanc. *J. Food Sci*. *59*: 142-147.

Shao H, He X, Achnine L, Blount WJ, Dixon AR, Wang X. 2005. Crystal Structures of a Multifunctional Triterpene/Flavonoid Glycosyltransferase from *Medicago truncatula*. *Plant Cell*. *7*: 3141-3154.

Simmonds MSJ. 2003. Flavonoid–insect Interactions: Recent Advances in Our Knowledge *Phytochemistry. 64*: 21–30.

Stafford AH, Lester HH. 1982. Enzymic and Non-enzymic Reduction of (+)-Dihydroquercetin to its 3, 4-diol. *Plant Physiology*. *70*: 695-698

Terao J, Kawai Y, Murota K. 2008. Vegetable Flavonoids and Cardiovascular Disease. *Asia Pac Journal of Clin Nutr*. *17*: 291-293

This PC, Leclercq PDG, Jacquot Y. 2011. A Critical View of the Effects of Phytoestrogens on Hot Flashes and Breast Cancer Risk. *Maturitas*. *70*: 222-226.

Ulanowska K, Tkaczyk A, Konopa G, Wegrzyn G. 2006. Differential Antibacterial Activity of Genisten Arising from Global Inhibition of DNA, RNA and Protein Synthesis in Some Bacterial strains. *Arch Microbiol*. *184*: 271-278.

Van der Krol AR, Mur LA, Beld M. 1990. Flavonoid Genes in *Petunia*: Addition of a Limited Number of Gene Copies may lead to a Suppression of Gene Expression. *Plant Cell*. *4*: 291-299.

Vogt T, Jones P. 2000. Glycosyltransferases in Plant Natural Product Synthesis: Characterization of a Supergene Family. Reviews, *Trends in Plant Science*. *5*: 380-38

Wang X. 2009. Structure, Mechanism and Engineering of Plant Natural Product Glycosyltransferases, *FEBS Letters 583*: 3303–3309

Waugh DS. 2005. Making the Most of Affinity Tags, *Trends in Biotechnology*. *23*: 316-320

Winkel –Shirley B. 2001. Flavonoid Biosynthesis. A Colorful Model for Genetics, Biochemistry Cell Biology and Biotechnology. *Plant Physiology*. *126*: 485-493.

Winkel SB. 1996. Flavonoid Biosynthesis: New Functions for an Old Pathway. *Trends in Plant Science*. *11*: 377-378

Yılmaz D, Aydemir NC, Vatan O, Ece T, Rahmi B. 2012. Influence of Naringin on Cadmium-induced Genomic Damage in Human Lymphocytes *In Vitro*. *Toxicology and Industrial Health*. 28: 114–121.

Yu Oliver, Matsuno M, Subramanian S. 2006. Flavonoid Compounds in Flowers Genetics and Biochemistry, Floriculture, in *Ornamental and Plant Biotechnology*: Advances and Topical Issues, Ed 1. Global Science Books, London, pp 283–293

Yu O, Woosuk J, June S, Robert AC, Gary MF, McGonigle B**,** Odell JT. 2000. Production of the Isoflavones Genistein and Daidzein in Non-Legume Dicot and Monocot Tissues. *Plant Physiol. 124*: 781-793

Yum, MK, Mi YJ, Daeho C, Tae SK. 2011. Suppression of Dendritic Cells Maturation and Functions by Daidzein, a Phytoestrogen, *Toxicology and Applied Pharmacology*. *257:* 174–181

APPENDICES

APPENDIX A: Abreviations

BME - β-mercaptoethanol

bp - base pairs

BMGY - buffered glycerol complex-medium

BMMY - buffered methanol-complex medium

cDNA - complementary deoxyribonucleotide acid

CHI - chalcone isomerase

CHS - chalcone synthase

cpm - counts per minute

CSP - clone specific primer

DFR - dihydroflavonol reductase

DNA - deoxyribonucleic acid

dNTPs - deoxyribnucleoside triphosphate

EDTA - ethylenediaminetetraacetic acid

EtBR - ethidium bromide

EST - expressed sequence tag

F3GT - flavonoid 3-O-glucosyltransferase

F3H - flavanone 3β-hydroxylase

FLS - flavonol synthase

FSI - flavone synthase I

GT - glucosyltransferase

IFS - isoflavone synthase

LB - luria-bertani

PAL - phenylalanine ammonia lyase

PBS - phosphate buffered saline

PCR - polymerase chain reaction

rpm - revolutions per minute

SDS-PAGE - sodium dodecyl sulphate-polyacrylamide gel electrophoresis

YPD - yeast extract peptone dextrose

YPDS - yeast extract peptone dextrose

APPENDIX B: Stock Solutions and Media Recipes

10X YNB:

134 grams of yeast nitrogen base (YNB)

Add 1000 mL of distilled water

Heat solution to dissolve completely

Filter sterilize the solution and store at 4°C

500X B (0.02% Biotin):

20 milligrams biotin

Add 100 mL ddH_2O

Filter sterilize the solution and store at 4 °C

10X D (20% Dextrose):

200 grams D-glucose

Add 1000 mL ddH_2O

Filter sterilize and store at room temperature

10X M (5 % Methanol):

5 mL methanol

Add 95 mL ddH_2O

Filter sterilize and store at 4°C

10X GY (10 % Glycerol):

100 mL glycerol

Add 900 mL ddH_2O

Filter sterilize and store at room temperature

Plain liquid LB medium:

25 grams LB broth powder

Add sterile ddH_2O to 1000 mL

Adjust pH to 7.5

Autaclave and store at 4^oC

Liquid LB medium with antibiotics

25 grams LB broth powder

Add sterile ddH_2O to 1000 mL

Adjust pH to 7.5

Autaclave and add 0.1 gram of Amplicillin and/ or 0.034 Chloramphenicol

Store at 4^oC

LB-Agar plate with antibiotics:

12.5 grams of LB broth powder

7.5grams of agar

Add sterile ddH2O to 1000 mL

Adjust pH to 7.5

Autoclave and add 0.1 gram Ampicillin and/ or 0.034 gram Chloramphenicol

Store at 4 ^OC

Plain liquid low salt LB medium:

10 grams tryptone

5 grams yeast extract

5 grams NaCl

Adjust pH to 7.5

Add sterile ddH2O to 1000 mL

Autoclave for 20 minutes

Store at 4^{o}C

Liquid low salt LB medium with antibiotics:

10 grams tryptone

5 grams yeast extract

5 grams NaCl

Adjust pH to 7.5

Add sterile ddH2O to 1000 mL

Autoclave for 20 minutes

Allow to cool to about 55 ^{o}C

For low salt LB with Zeocin, add 25 μg/ ml final concentration

Store at 4 ^{o}C. If media contains Zeocin store in the dark

Low Salt LB Agar Plates:

To plain low salt liquid LB media, add 15 grams agar per liter of medium

Add sterile ddH2O to 900 mL

Autoclave for 20 minutes

Allow medium to cool to about 55 ^{o}C

Pour into plates, allow medium to harden

Invert plates and store 4 ^{o}C.

If Zeocin is added, store in the dark.

Plain liquid YPD medium:

10 grams yeast extract

20 grams peptone

Add sterile ddH2O to 900 mL

Autoclave for 20 minutes

Add 100 mL 10X D

Liquid YPD medium with Zeocin

10 grams yeast extract

20 grams peptone

Add sterile ddH2O to 900 mL

Autoclave for 20 minutes

Allow medium to cool to about 60 ^{o}C

Add 100 mL 10X D

Add 25 mg of Zeocin

Store medium at 4^{o}C in the dark

YPD-Agar plate with Zeocin

To a liter of plain YPD liquid medium

Add 15 grams of agar

Autoclave for 20 minutes

Allow medium to cool to about 55^{o}C

Add 25 mg of Zeocin

Pour medium in plates and allow to harden

Store plates containing Zeocin in the dark at 4^{o}C

Liquid YPDS medium with Zeocin:

10 grams yeast extract

182.2 grams sorbitol

20 grams peptone

Add sterile ddH2O to 900 mL

Autoclave for 20 minutes

Add 100 mL 10X D

Cool solution to ≈ 60^{o}C

Add 100 mg of Zeocin and store medium at 4^{o}C in the dark

YPDS-Agar plates with Zeocin:

10 grams yeast extract

182.2 grams sorbitol

20 grams peptone

15 grams of agar

Add sterile ddH2O to 900 mL

Autoclave for 20 minutes

Add 100 mL 10X D

Cool solution to ≈ 60^{o}C

Add 100 mg of Zeocin and pour into plates.

Store plates in the dark at 4^{o}C

BMGY medium:

10 grams yeast extract

20 grams peptone

Dissolve above in 700 mL distilled water

Autoclave 20 minutes

Cool to room temperature and add the following:

100 mL sterile 1M potassium phosphate buffer, pH 6

100 mL sterile 10X YNB

2 mL sterile 500X B

100 mL sterile 10X GY

Store media at 4^{o}C

BMMY medium:

10 grams yeast extract

20 grams peptone

Dissolve above in 700 mL distilled water

Autoclave 20 minutes

Cool to room temperature and add the following:

100 mL sterile 1M potassium phosphate buffer, pH 6

100 mLsterile 10X YNB

2 mL sterile 500X B

100 mL sterile 10X M

Store media at 4^{o}C

APPENDIX C: Staining Solution Recipes

Ponceau S staining solution:

0.5 grams Ponseau S

10 mL acetic acid

Add ddH_2O to 100 mL

Amido Black stain B:

0.1 gram Amido Black 10B

10 mL acetic acid

Add ddH_2O to 100 mL

Coomassie Blue staining solution:

0.006 grams Coomassie Brilliant Blue G250

10 mL acetic acid

40 mL methanol

Add ddH_2O to 100 mL

Destaining solution

300 mL methanol (30%)

100 mL acetic acid (10%)

Add 600 mL ddH_2O to make a liter

APPENDIX D: Gel Recipes

SDS-PAGE Gel (10 %)

Running/ Separating Gel Composition:

1.25 mL 40% Acrylamide (acrylamide: bis-acrylamide, 19:1)

1.25 mL 4X running buffer (pH 8.8)

2.33 mL distilled water

50 µL 10% SDS

50µL APS

10µL TEMED (add last)

Stacking Gel composition:

200 µL 40% Acrylamide (acrylamide: bis-acrylamide, 19:1)

500 µL 4X stacking gel buffer (pH 6.8)

1.84 mL distilled water

20 µL 10% SDS

20 µL APS

5 µL TEMED (add last)

0.8/% Agarose Gel

0.24 grams Agarose powder

Add 30 mL of 1X TAE buffer

Heat until agarose melts

APPENDIX E: Buffer Recipes

4X SDS-PAGE Running Buffer:

36.5 grams (1.5 M) Tris-base

Adjust pH to 8.8

Add ddH_2O to 200 mL

4X SDS-PAGE Stacking Gel Buffer:

3 grams (1.5 M) Tris-base

Adjust pH to 6.8

Add ddH_2O to 200 mL

SDS-PAGE Running/ Tank Buffer:

30 gramsTris Base

144 grams Glycine

10 grams SDS

Add ddH_2O to 1000 mL

Adjust pH to 8.3

10% SDS Solution:

10 grams SDS

Add ddH_2O to 100 mL

2X SDS-PAGE Sample Buffer:

25 mL 1M Tris-base Buffer (pH 6.9)

4 grams SDS

20 mL glycerol

Add ddH_2O to 100 mL

To every 1mL of above mixture, add 20 µl βME before use

4X SDS-PAGE Sample Buffer:

2 mL 1M Tris-base Buffer (pH 6.9)

2 mM 20% SDS Solution

4 mL 50% glycerol

0.2 mg Bromophenol Blue

To every 1 mL of above mixture, add 50 µl βME before use

10X TBS-T Buffer:

8.76 grams sodium chloride

10 mL of 1M Tris-base solution (pH 8)

500 µl of tween 20

Add ddH_2O to 1000 mL

10X PBS Buffer:

76 grams NaCl (1.3M)

10 grams Na_2HPO_4 (70 mM)

4.1 grams NaH_2PO_4 (30 mM)

Add ddH_2O to 1000 mL and adjust pH to 7.2

1X PBS-T Buffer:

100 mL 10X PBS

Add ddH_2O to 1000 mL

Add 0.05 % of tween 20 and stir to mix completely

10X Western blot Transfer Buffer:

30 grams Tris base

144 grams glycine

Add ddH_2O to 1000 mL

Alkaline Phosphatase Buffer:

5.8 grams of NaCl

1.02 grams of $MgCl_2$

100 mL 1M Tris base solution

Adjust pH to 9.5

Add ddH_2O to 1000 mL

50 mM Phosphate Buffer (pH 7.0):

0.2918 grams of Monosodium Phosphate

0.7733 grams of Disodium Phosphate

Add ddH_2O to 1000 mL

IMAC Equilibration/ Wash Buffer (pH 7.5):

0.2918 grams of Monosodium Phosphate

0.7733 grams of Disodium Phosphate

17.532 grams of NaCl

Adjust pH to 7.5

Add ddH_2O to 1000 mL

IMAC Elution Buffer (pH 7.5):

0.2918 grams Monosodium Phosphate

0.7733 grams Disodium Phosphate

17.532 grams NaCl

10.21g Imidazole

Adjust pH to 7.5

Add ddH_2O to 1000 mL

IMAC MES Buffer (pH 5.0):

3.9046 grams 2-(N-morpholine)-methanesulfonic acid (MES)

Adjust pH to 5.0

Add ddH2O to 1000 mL

50X TAE Buffer:

50 mL EDTA (pH 8.0)

28.6 mL Glacial Acetic Acid

121 grams Tris Base

Add ddH2O to 1000 mL

Breaking Buffer:

6 grams sodium phosphate (monobasic)

372 mg EDTA

50 mL glycerol

Adjust pH to 7.4

Add ddH2O to 1000 mL

Store at 4 ^{o}C

To each 57 mL of the above mixture, add 10 mg of PMSF right before use.

1 M Potassium Phosphate Buffer, pH 6:

132 mL 1 M K_2HPO_4

868 mL 1M $KHPO_4$

Adjust pH to 6.0

Autoclave for 20 minutes and store at room temperature

Phenol: Chloroform:

Combine equal parts of phenol and 0.5M Tris-HCL pH 8.0

Add 0.1 % 8-hydroxyquinoline 2- carboxylic acid

Stir above mixture for 15 minutes

Remove the upper-most layer of the mixture

Add an equal volume of 0.1M Tris-HCl to the mixture

Stir for 15 minutes, repeat above steps until pH of phenol: chloroform is above 7.8

APPENDIX F: Sequences

PGT3 Nucleotide Sequence (1495 bp) :

atggaagaaaagcctaaatctcctcatatcctgatctttcctctcccatgccaaagccatgtgaattccatgctcaagcttgccgagatttttggc
ttagctggcctaaaagtgaccttcctaaactccaagcacaaccacgaacgcctcatccggtacacggatatccatgaccgttttctgcagtatt
ctgaattccaatttaagaccatctcagatggtcttccagcggatcacccacgagcaggtgaccgattgatggagatattcgattctttgagtttg
aataccaggccacttctcaaacagatgctgattgacactagcccgccagtgagctgcatcattggagatgcatgtatggaatttgtggtagac
gttgctactgaacttgaaatccctgtaatccattttcgtgctataagtgcttgtagcttctgggcatatttctctatccctgaaatgatccaagcagg
cgagcttcctatgaaagcctacgatgaagacatggatcgtctgataacaaaagttccaggcatggaaacttttctgagattccgagatctccc
cagcttttgccgagtgagtgacgtaaccgaccgagatttgcaagttcttaagaacgtgacccaacagtcccctcgaacccatgctttgatact
caacacgtttgaagacctagaagaaccaattctgtcacatatacgcaccaaatgccccaaagtgtacacaataggacctcttcacttgcagct
caagacaagactcgcctcaaacgtgatctcatcgtcatcccaaaagtctctaaacagtctctgggaagtggacagaagttgcattgaatggtt
tggtaaacagcctgtgcggtctgtactgtacgtaagctttggtagcattacactgttgaaaagggaacagctgattgagttttggcacggtcttg
tagacagtaagcagcggttcttgtgggtcattaggccagattctgtgattggcgaaggtgacgccccggcagagcttgtggagggcacgaa
ggagagaggtcatttggttagttgggtcccacaggaggaggtactggctcaccaggctgtggctggattcttgacacacagcggctggaac
tcaacattggagagtatagtagccggggtgccaatgatttgctggccttactttgctgatcaacagatcaatagtaggtttgtgagtgaagtgtg
gaatcttggattggatatgaaggatgtgtgcgatagaaatgttgttgagaaaatggtgaatgatctcatggtggagaggaaggaggagtttat
gagagcagctgatcggatggctacaatggctagaaccactgctaatgaaggtggaccatcttattgtaatttggaccgtctgattgatgatatt
aagatgatgagctcccaagcatgaaaacatatagctaaggttagtgcaagatgttactgctagattaaataaattttaaagtctcgag

PGT8 Nucleotide Sequence (1536 bp):

atgggaactgaatctcttgttcatgtcttactagtttcattccccggccatggccacgtaaacccgctcctgaggctcggccgactccttgcttc
aaagggtttctttctcaccttgaccacacctgaaagctttggcaaacaaatgagaaaagcgggtaacttcacctacgagcctactccagttgg
cgacggcttcattcgcttcgaattcttcgaggatggatgggacgaagacgatccaagacgcggagatcttgaccaatacatggctcaacttg
agcttattggcaaacaagtgattccaaaaataatcaagaaaagcgctgaagaatatcgccccgtttcctgcctgatcaataacccatttatccct
tgggtctctgatgttgctgaatccctagggcttccgtctgctatgctttgggttcaatcttgtgcttgttttgctgcttattaccattactttcacggttt
ggttccatttcctagtgaaaaagaacccgaaattgatgttcagttgccgtgcatgccactactgaagcatgatgaagtgcctagcttcttgcatc
cgtcaactccttatcctttcttgagaagagctattttggggcagtacgagaatcttggcaagccgttttgcatattgttggacactttctatgagctt
gagaaagagattatcgattacatggcaaaaatttgccctattaaacccgtcggccctctgttcaaaaaccctaaagctccaaccttaaccgtcc
gcgatgactgcatgaaacccgatgaatgcatagactggctcgacaaaaagccaccatcatccgttgtgtacatctctttcggcacggttgtct
acttgaagcaagaacaagttgaagaaattggctatgcattgttgaactcggggatttcgttcttgtgggtgatgaagccgccgcctgaagact
ctggcgttaaaattgttgacctgccagatgggttcttggagaaagttggagataagggcaaagttgtgcaatggagtccacaagaaaaggtg
ttggctcaccctagtgttgcttgctttgtgactcactgcggctggaactcaaccatggagtcgttggcatcgggggtgccggtgatcaccttcc
cgcaatggggtgatcaagtaactgatgccatgtatttgtgtgatgtgttcaagaccggtttaagattgtgccgtggagaggcagagaacagg
ataatttcaagggatgaagtggagaagtgcttgctcgaggccacggccggacctaaggcggcggagctgaaggagaacgcgctgaagt
ggaagaaggaggcggaggaagctgtggccgatggtggctcgtcggataggaacattcaggctttcgttgatgaagtaagaaggagaagt
gtcgagatcataaccagcagcaagtcgaagtcaatccacagagttaaggaattagtggagaagacggcaacggcaactgcaaatgacaa
ggtagaattggtggagtcacgacggacacgtgtacagtattga

VITA

DEBORAH HAYFORD

Personal Data:	Date of Birth: December 12th 1981
	Place of Birth: Accra, Ghana, West-Africa
	Marital Status: Single
Education	Master of Science in Biology (May 2012)
	East Tennessee State University, Johnson City,
	Bachelor of Science in Biology (June, 2005)
	University of Cape-Coast, Ghana, West-Africa
Professional Experience	Research Assistant (2010-2012), East Tennessee State University
	The McIntosh Lab
	Teaching Assistant (2011)
	Department of Biological Sciences, ETSU, Johnson City, TN
	Biology Lab Instructor

Presentations:

Deborah Hayford, Daniel K. Owens, Cecilia McIntosh. "Heterologous Expression and Characterization of Recombinant Putative Glucosyltransferase Clone 3 from Grapefruit, *Citrus paradisi*." Presented at the 50th Anniversary of the Phytochemical Society of North America (PSNA), Kona, Hawaii (December, 2011).

Deborah Hayford, Daniel K.Owens, Cecilia McIntosh. "Heterologous Expression and Characterization of Recombinant Putative PGT3 from Grapefruit, *Citrus paradisi."* Appalachian Student Forum, East Tennessee State University, Johnson City, Tennessee (April 2011)

Deborah Hayford. "Heterologous Expression and Biochemical Characterization of Recombinant Putative Glucosyltransferase Clone 3 from Grapefruit, *Citrus paradisi."* Presented to the Department of Biological Sciences, East Tennessee State University, Johnson City, Tennessee (October, 2010)

Deborah Hayford, Daniel K.Owens, Cecilia McIntosh. "Heterologous Expression of Recombinant PGT3 from Grapefruit, *Citrus paradisi."* Presented at the 49th Annual meeting of Phytochemical Society of North America (PSNA), St Petersburg, FL (July, 2010).

Honors and Awards:

Active member of Phytochemical Society of North America (PSNA)

Active member of ETSU Graduate Professional Students Association (GPSA)

Phytochemical Society of North America /National Science Foundation Travel award to attend 50th Aniversary of PSNA (December 10-15, 2011)

ETSU Graduate Professional Students Association Travel award (December. 2011)

Phytochemical Society of North America Travel award (July, 2010)

ETSU Graduate Professional Students Association Travel award (July, 2010)