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Cloning, Heterologous Expression in Yeast, and Biochemical Characterization of Recombinant Putative Glucosyltransferase Clones 9 and 11 from Grapefruit (Citrus paradisi)

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Cloning, Heterologous Expression in Yeast, and Biochemical Characterization of Recombinant

Putative Glucosyltransferase Clones 9 and 11 from Grapefruit (*Citrus paradisi)*

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A thesis

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Master of Science in Biology

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by

Anye Wamucho

May 2012

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ABSTRACT

Cloning, Heterologous Expression in Yeast, and Biochemical Characterization of Recombinant

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Flavonoids are plant secondary metabolites that play diverse roles in plants and human health. These compounds in most part exist in the glucosylated form. Grapefruit accumulates high levels of glucosylated flavonoids. Plant secondary product glucosyltransferases (GTs) catalyze the glucosylation reaction, but due to low homology at both the nucleotide and amino acid sequence level of different GTs, it is not possible to ascribe function based on sequence only. The hypotheses that PGT clones 9 and 11 are plant secondary product GTs and are biochemically regulated were tested. PGT 9 has been cloned into *Pichia pastoris* using the pPICZA and pPICZAα vectors, expressed, enriched, and screened for GT activity with a variety of phenolic substrates. Initial screens show catechol, gentisic acid, vanillin, and p-hydroxylphenylacetic acid as potential substrates for the PGT 9 protein. PGT 11 has been successfully cloned into pPICZA for transformation into yeast, expression, and subsequent characterization.

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I would like to dedicate this work most especially to my sister Zih Zila Wamucho who left us 2 years back. You were and will always be an inspiration to me.

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CHAPTER 1

INTRODUCTION

<u>Plant Secondary Metabolites</u>

Plants produce a myriad of organic chemical compounds that play essential roles such as pigmentation, pollination, seed dispersal, flavor (odor and taste), and interactions with other organisms such as insects, animals, and other plants (Bennet and Wallsgrove 1994 and ref. therein; Zhao et al. 2005 and ref. therein; Martens et al. 2010 and ref. therein; Kennedy and Wightman 2011). In the presence of stress, new compounds or modified versions of the pre-existing compounds are produced which help the plants fight off abiotic and biotic stresses such as high UV exposure and infections amongst others (Li et al. 1993; Cohen et al. 2001 and ref. therein; Cushnie et al. 2005).

Some of the organic chemical compounds produced in plants are classified as secondary metabolites. As opposed to primary metabolites being common to almost all plants, only a subset of secondary metabolite genes are found in some plants and as such can be used as taxonomic markers (Bennet and Wallgroves 1994 and ref. therein; Pichersky and Gang 2000 and ref. therein). For example, grapefruit and blonde orange do not accumulate anthocyanin but blood oranges do and it is a close relative to both (Owens and McIntosh 2009 and ref. therein). New secondary metabolites continue to be unraveled. This may be due to evolution of new genes that produce these new secondary metabolites (Pichersky and Gang 2000). Like all living organisms, plants are faced with several challenges that hinder their growth and survival. Over the years, plants have coped with such challenges by developing a myriad of defense mechanisms. Pests, infections, and extreme environmental conditions not suitable for plant growth and survival are amongst the challenges faced by plants. Herbivory is also one of the factors that drastically

affects plants as insects and animals tend to feed on them. This may explain the need for new

protective compounds in order for plants to survive these challenges.

There exist several different types of plant secondary metabolites with different functions

as earlier mentioned. Three main classes of secondary metabolites are: terpenoids, alkaloids, and

phenolic compounds (Zwenger and Basu 2008 and ref. therein). The largest group of plant

secondary metabolites are the terpenoids with ~30,000 compounds followed by the alkaloids

with ~ 12,000 compounds (Martens et al. 2010 and ref. therein). The third largest group

constitute phenolics of which there are well over 10,000 compounds (Martens et al. 2010 and ref.

therein).

Due to the high content of secondary metabolites in plants, research on the effect of these

molecules on human health has increasingly become popular as plants constitute a major part of

human and animal diet. Several findings suggest that these compounds have several beneficial

roles to humans. These compounds have been shown to be treatments for wounds, bacterial

infections, cancer risk, coronary heart disease, and strokes among others (Cook and Sammons

1996 and ref. therein; Hollman et al. 1999 and ref. therein; Sen et al. 2002; Brusselmans et al.

2005).

Flavonoids

Flavonoids constitute one of the largest groups of polyphenolic plant secondary

metabolites with 15 carbon atoms arranged in a 3-ring structure (Figure 1). The 3-ring structures

of flavonoids are labeled A, B, and C with a defined numbering of the carbon atoms. Rings A

and B are benzene rings joined by a C3 linkage (C6-C3-C6). The level of oxidation of the C3

ring divides flavonoids into different classes and this difference in oxidation is reflected in the

chemical properties of each class (Owens and McIntosh 2010 and ref. therein). The different flavonoid classes include chalcone, aurone, flavone, flavanone, isoflavone, flavonol, dihydroflavonol, and anthocyanidin (Figure 1).

Being polyphenolics, flavonoids have free hydroxyl groups on the rings through which different types of modifications occur resulting in a wide array of different types. Different substitutions may occur on different ring positions. Over 5000 different flavonoids were described by 2004 but ~5000 more have been described since then giving a total of ~10,000 flavonoids (Holden 2004 and ref. therein; Dixon and Pasinetti 2010 and ref. therein; Martens et al. 2010 and ref. therein). This diversity is as a result of the different types of modifications such as glycosylation, methylation, and acetylation on the free hydroxyl group of these compounds among others.

Roles of Flavonoids in Plants

Plants produce an array of secondary metabolites among which are the flavonoids. Flavonoids are ubiquitous in higher plants and are commonly found in fruits, vegetables, nuts, seeds, stems, flowers, roots, and leaves (Cushnie et al. 2005). These flavonoids have beneficial biological roles in plants.
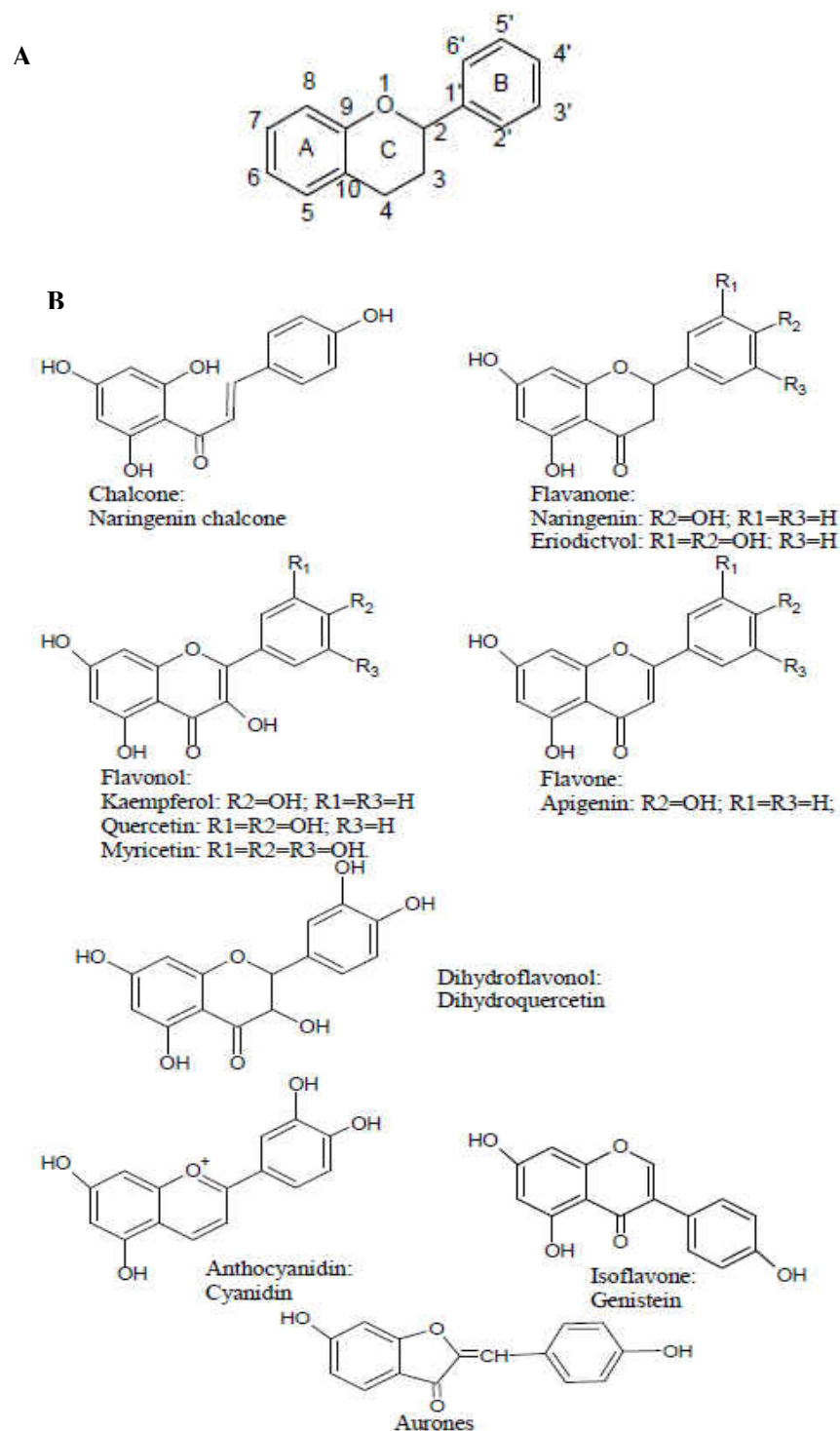
Figure 1. Chemical Structure of Flavonoids. A. Basic structure and numbering pattern of flavonoids. B. Flavonoid classes and examples based on different substitution patterns at different ring positions (Lin 2011 and ref. therein).

Plants are often exposed to harsh environmental conditions not favorable for growth. One of such is the increase in UV radiation in the biosphere that causes serious damage to plants. UV radiation is known to damage DNA, RNA, and proteins (Li et al. 1993 and ref. therein). It is also known that phenylpropanoid gene expression is induced in the presence of UV light (Li et al. 1993 and ref. therein). The phenylpropanoid pathway leads to the synthesis of flavonoids. Experiments have been carried out that show that plants are less tolerant to UV radiation when the synthesis of flavonoids is hindered. For example Arabidopsis flavonoid mutants that had mutations in the chalcone synthase gene (*tt4)* and chalcone isomerase gene (*tt5*) were shown to be hypersensitive to UV radiation when compared to wild type plants (Li et al. 1993). Flavonoids absorb this UV radiation and reduce the damage caused by it. High accumulation of quercetin-3-glycoside was observed in the skin of apples after being exposed to high UV, and it is worth noting that once these glycosides were synthesized in response to high UV exposure, they were retained for prolonged periods even when stored in darkness (Solovchenko and Schmitz-Eiberger 2003).

The idea that flavonol glycosides tend to be retained can be linked to recent investigations that show a decreasing level of expression of a flavonol glucosyltranferase gene in grapefruit leaves that is highly expressed in Stage 2 (young) cotyledons but not expressed in stage 3 (older) cotyledons (Daniels et al. 2011). This glucosyltransferase catalyzes the synthesis of flavonol 3-O –glucoside (Owens and McIntosh 2009). The flavonol glucosyltransferase gene is again expressed in stage 3 first (young) true leaves but not in stage 4 first true (older) leaves. The expression of this gene, however, increases again at stage 4 second true (young) leaves and in stage 5 young leaves (Daniels et al. 2011) probably due to increase in size of leaves and requirement for more quercetin glucoside to act as a sunscreen. Stage 2 refers to the appearance

of the first cotyledons, stage 3 to the appearance of the first true leaves, stage 4 to the first appearance of the second set of true leaves, and stage 5 refers to 1-4 year old plants (Daniels et al. 2011).

Plants also face problems such as bacterial and fungal infections. Flavonoids act as phytoalexins fighting off infections of plants by such microorganisms (Cohen et al. 2001 and ref. therein). Several classes of flavonoids have been shown to have antimicrobial activity (Bylka et al. 2004). The antimicrobial role of flavonoids was first determined from plants used by lay healers and physicians to treat human disease (Cowan 1999; Cushnie et al. 2005 and ref. therein). When the content of such plants were investigated, they turned out to have a high concentration of flavonoids. Leaves of alfafa contain a high concentration of isoflavonoid glycosides that upon infection with a fungal pathogen *Ascochyta imperfeti* are broken down by glucosidase activity to the corresponding aglycone that then act as phytoalexins preventing the spread of the pathogen (Cohen et al. 2001and ref. therein). Earlier studies had shown an increase in the mRNA expression of core enzymes involved in the phenylpropanoid pathway leading to the synthesis of flavonoids in response to fungal elicitor, wounding, and infection of beans (Mehdy and Lamb 1987 and ref. therein; Dixon et al. 2002 and ref. therein).

Propolis, a resinous material produced by cone-bearing plants, has a high flavonoid content especially galangin and pinocembrin (Cushnie et al. 2005). The high concentration of these flavonoids has been attributed to the antimicrobial properties of this plant material (Cushnie et al. 2005). These flavonoids have been shown to have different mechanisms by which they carry out their antimicrobial functions such as inhibition of nucleic acid synthesis (myricetin), inhibition of cytoplasmic membrane function (sophoraflavanone G), as well as inhibiton of energy metabolism (licochalcone A and C) (Cushnie et al. 2005). Some flavonoids

have also been shown to have antifungal activities acting as inhibitors of spore germination of plant pathogens (Cushnie et al. 2005 and referenced therein). Galangin, a flavonol found in propolis, has also been shown to have inhibitory activity against *Aspergillus flavus* (Cushnie et al. 2005 and ref. therein).

Acting as feeding deterrents, flavonoids tend to protect plants from herbivory and harmful insects that may cause considerable damage to the plant. To determine flavonoids with such properties, the feeding activity of insects are tested against these flavonoids. Several classes of flavonoids are known to act as feeding deterrents. For example, 9 isoflavonoids isolated from legume plants were shown to be feeding deterrents against 2 pasture scarabs, *Costelytria zealandica* and *Heteronychus arator* that cause severe economic damage to crops (Iwashina 2003 and ref. therein). Several other classes of flavonoids have also been shown to have antifeeding characteristics such as flavones, flavanones, flavonols, and dihydroflavonols (Iwashina 2003 and ref. therein). The bitterness of grapefruit in part is due to the flavanone naringin which is highly accumulated in young grapefruit tissues making up to 40-70% of the dry weight of young green fruits and leaves (Jourdan et al. 1985; McIntosh and Mansell 1990). The bitterness of grapefruit may serve as a feeding deterrent (Benavente-Garcia et al. 1997), although the compound is also found in juice (Mansell et al. 1983).

Pollination, in part, is carried out by insects, bats, and birds and this is very important in plant sexual reproduction. Seed dispersal is carried out by insect and animals (Cohen et al. 2001 and ref. therein). Most insects and animals are attracted to plants that portray beautiful colors and odors (Miller et al. 2011). Plant fruits and flowers have different colors depending on the kind of pigment present in these plants (Miller et al. 2011). For plants to be colored, the pigments must reflect light in the visible range. Anthocyanins act as visible pigments (orange, red, purple, and

blue) that attract insects, other pollinators, and seed dispersers (Iwashina 2003; Miller et al. 2011 and ref. therein). Some flavonoids act as pollinator attractants even though they are not visible pigments. For example, flavones and flavonols absorb light in the UV range but are still capable of acting as pollinator attractants due to the fact that some insects can discriminate UV light (Iwashina 2003).

Flavonoids have also been shown to play a role in pollen germination (Ylstra et al. 1992; Miller et al. 1999). Hence, they are not just only involved in attracting pollinators but play a central role in this process by being directly involved in the formation of these plant structures that help propagate the species in which they are involved. Flavonols are required for plant pollen germination in maize and petunia (Miller et al. 1999). The flavonol 3-O-galactosyltransferase gene is expressed exclusively in the male gametophyte of *Petunia hybrida* and controls the formation of a glycosylated flavonol that plays a role in pollen germination (Miller et al. 1999). It is worth noting that the same enzyme is not expressed in the female gametophyte (Miller et al. 1999). Flavonol-deficient plants have been shown to be self-sterile because the pollen fails to germinate or produce a functional tube but, when complemented with flavonol, the sterility was reversed indicating that flavonols play a key role in pollen germination (Taylor. 1997).

Flavonoids also play important roles in interaction of plants with other organisms such as microbes that are important for plant survival. For example, nitrogen-fixing symbiotic bacteria play an important role by providing fixed nitrogen to the plants. The plants then provide nutrients for the nitrogen-fixing bacteria (Cohen et al. 2001). Some N_2-defficient plants have been shown to produce flavonoids that attract nitrogen-fixing symbionts (Cohen et al. 2001 and ref. therein). For example, N_2-deprived alfafa increase the secretion of flavonoids from their roots and

Rhizobium meliloti a N_2-fixing bacteria has been shown to be attracted to alfafa-derived luteolin, 4',7-dihydroxyflavone, 4',7-dihydroxyflavanone, and 4,4'-dihydroxychalcone (Cohen et al. 2001 and ref. therein; Walker et al 2003 and ref. therein). Investigations have shown that these same compounds act as inducer for the *R. meliloti* nodulation genes (Cohen et al. 2001 and ref. therein). Earlier studies also identified flavonoids in the root exudates of legumes as one of the molecules that induced root nodulation in *R. meliloti* (Peters and Long 1988; Walker et al. 2003 and ref. therein).

Flavonoids are also implicated in plant-plant interaction acting as agents of allelopathy (Iwashina 2003 and ref. therein). Communication between plants is vital for their survival as they tend to compete for nutrients and other growth requirements such as water and light. Some plants secrete allelochemicals that prevent the germination of seeds of that same plant or related plant species thus reducing competition for growth nutrients. For example, leaf and stem extracts of *Trifolium pretense* with high isoflavonoid content have been shown to inhibit the germination of its own seeds and those of its close relatives *T. repense* and *T.hydrium* (Iwashina 2003 and ref. therein). Ten isoflavones and their glycosides were identified in the extract. Frond exudates of *Pityrogramma spp.* contain 2',6'- dihydroxy-4'-methoxychalcone, 2',6'-dihydroxy-4'-methoxydihydrochalcone, and izalpinin that inhibited spore germination and gametophyte development of *Pityrogramma calomeranos* (Iwashina 2003 and ref. therein). However, at some concentrations these compounds acted as stimulants for spore germination (Star 1980).

<u>Roles of Flavonoids in Human Health</u>

Flavonoids play important roles in human health. Most of the health benefits to humans are due to their ability to act as antioxidants or being able to interact and inhibit enzymes

involved in disease processes (Cook and Sammons 1996 and ref. therein; Brusselmans et al. 2005; Zhang et al. 2010). Most of the health benefits have been learned from epidemiological studies as well as *in vitro* experiments. For example, epidemiological studies have shown a protective effect of vegetables and fruits against cancer (Hollman et al. 1999 and ref. therein). Flavonoids are ubiquitously expressed in plants, and vegetables and fruits are dietary sources of these compounds.

Consumption of food products containing high amounts of flavonoids has been shown to lower the risk of various cancers and their mechanisms of action have been investigated. One of the mechanisms is by induction of cancer cell apoptosis (Brusselmans et al. 2005). Brusselmans and his colleagues discovered that this apoptosis was brought about by inhibition of the fatty acid synthase (FAS) activity required for the production of long chain fatty acids in cancer cells. Up-regulation of FAS occurs during early tumor development and RNA interference-mediated silencing of FAS had earlier been shown to inhibit lipogenesis leading to growth arrest and apotopsis of prostate cancer cells (De Schrivjer et al. 2005). Normal cells, on the other hand, have low levels of FAS because they obtain most of their saturated fatty acids from the diet. Some flavonoids that were shown to have anti-FAS activity were quercetin, luteolin, and kaempferol (Brusselmans et al. 2005).

Free radicals generated from cell processes pose a great problem to human health such as coronary heart disease and stroke (Cook and Sammons 1996 and ref. therein; Vaya et al. 2003 and ref. therein). Ness and Powles (1997), through epidemiological studies, showed that consumption of fruits and vegetable had a strong protective role against stroke and a weaker protective effect for coronary heart disease. For example, there is evidence that peroxidation of low density lipoproteins (LPO) is positively associated with atherogenesis (Cook and Samman

1996 and ref. therein). Peroxidation of LPO is brought about by free radicals and flavonoids have been shown, *in vitro,* studies to scavenge these radicals. This is an example of flavonoids acting as antioxidants (Cook and Sammons 1996 and ref. therein). Flavonoids also act as chelators of metals required for chemical reactions that produce reactive oxygen species and thus are capable of protecting and regenerating natural antioxidants such as tocopherols (Vaya et al. 2003 and ref. therein). Because LDL lipoperoxidation is the initiation step in atherogenesis, flavonoids are capable of preventing atherogenisis through their free radical scavenging activity or chelating properties.

The process of wound repair in animals requires the presence of oxygen and oxygen therapies are being used for wound healing (Sen et al. 2002). Molecular oxygen plays a central role in the pathogenesis and therapy of chronic wounds (Shetty et al. 2007). The production of reactive oxygen species as this molecular oxygen is used can lead to cytotoxicity and delay in wound healing (Senel et al. 1997). *Ocimum sanctum* is a plant that has been used for the traditional treatment of wounds (Shetty et al. 2007 and ref. therein). The chemical composition of this plant was investigated and the study of 12 different plant flavonoids (mainly orientin and vicenin) from this plant revealed that they had both *in vitro* and *in vivo* antioxidant properties (Shetty et al. 2007). The ability of these flavonoids to eliminate the reactive oxygen species was associated with the wound healing property of this plant (Shetty et al. 2007 and ref. therein).

Flavonoids have also been shown to have antiviral properties. Some flavonoids such as quercetin have been shown to be inhibitors of reverse transcriptase of RNA viruses. This is very important because current inhibitors of this enzyme, such as acyclovir, are very toxic to all cells and not only to virally infected cells (Havsteen 2002 and ref. therein). Flavonoids also have the potential of stimulating the production of interferons which fight against viruses (Havsteen 2002

and ref. therein). For example, *Trollius chinensis* Bunge is a flower used in China for the treatment of upper respiratory infections (Li et al. 2001). The antiviral activity of flavonoids isolated from this plant were tested and 2 flavonoids, orientin and vitexin, exhibited potent or moderate activity against Para 3 virus (Li et al. 2001).

<u>Flavonoid Biosynthesis</u>

The biosynthesis of flavonoids occurs through the phenylpropanoid pathway that gives rise to a variety of plant natural products. The first enzyme of this pathway is phenylalanine ammonia-lyase (PAL) that plays a crucial role at the interface between plant primary and secondary metabolism (Koukol and Conn 1961; Maier and Hasegawa 1969). Koukol and Conn first referred to the enzyme as phenylalanine deaminase because the enzyme catalyzed the deamination of phenylalanine to form trans-cinnamic acid (Koukol and Conn 1961). Cinnamate-4-hydroxylase (C4H), that requires NADPH and Cytochrome P-450 for its activity, catalyzes the 4-hydroxylation of cinnamic acid to produce *p*-coumaric acid (Potts et al. 1974). This is followed by the formation of 4-coumaryl CoA from *p*-coumaric catalyzed by 4-coumarate: CoA ligase (Ragg et al. 1961). All steps mentioned are summarized in Figure 2.
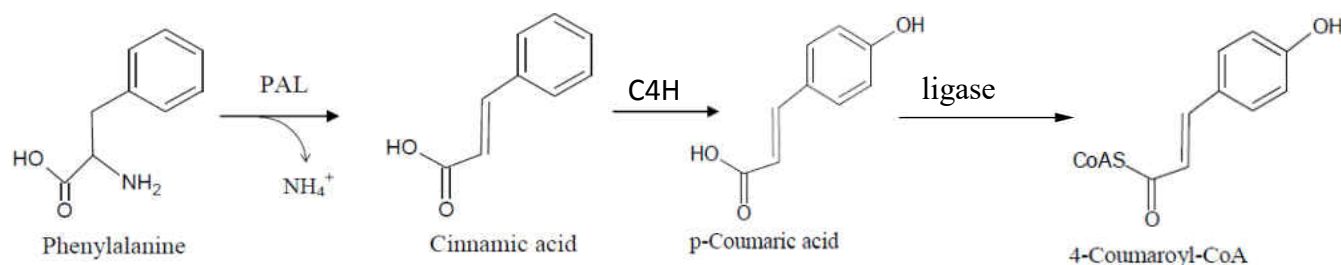
Figure 2. Formation of 4-Coumaryl CoA from Phenylalanine (Koukol and Conn 1961; Ragg et al. 1961; Maier and Hasegawa 1969; Potts et al. 1974).

Chalcone synthase (CHS) catalyzes the condensation of 3 molecules of malonyl CoA and one molecule of 4-coumaryl CoA (Figure 3) to form naringenin chalcone (Heller and Hahlbrock 1980). It is considered to be the key enzyme in flavonoid biosynthesis as it catalyzes the formation of the first flavonoid which serves as a backbone for the synthesis of other flavonoids. It was earlier referred to as flavanone synthase (Kreuzaler et al. 1979; Kreuzaler et al. 1983 and ref. therein) but was later changed to chalcone synthase after it was realized that the final reaction product is naringenin chalcone (tetrahydroxychalcone) and not naringenin flavanone (Heller and Hahlbrook 1980). This was probably because tetrahydroxychalcone undergoes spontaneous cyclization to form flavanone.
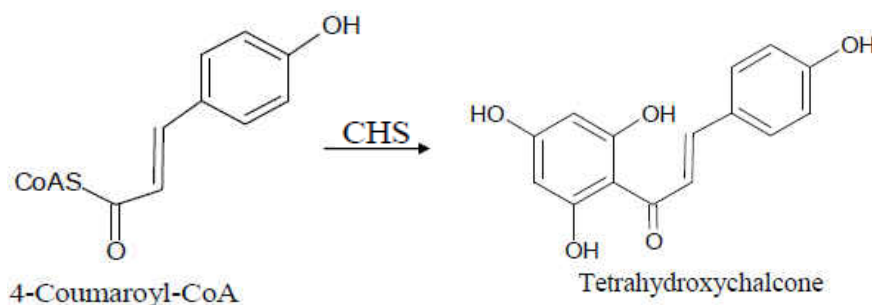
Figure 3. Formation of Naringenin Chalcone (Kreuzaler et al. 1979; Heller and Hahlbrock 1980).

Isomerization of chalcone to flavanone is catalyzed by chalcone isomerase (CHI) (Figure 4). This enzyme catalyzes the intramolecular cyclization of bicyclic chalcones into tricyclic flavanones (Moustafa and Wong 1966). This enzyme was referred to as chalcone cyclase by Raymond and Maier after purifying and characterizing the enzyme from *Citrus paradisi* (Raymond and Maier 1977; Jez and Noel 2002 and ref. therein; Mehdy and Lamb 1987 and ref. therein)
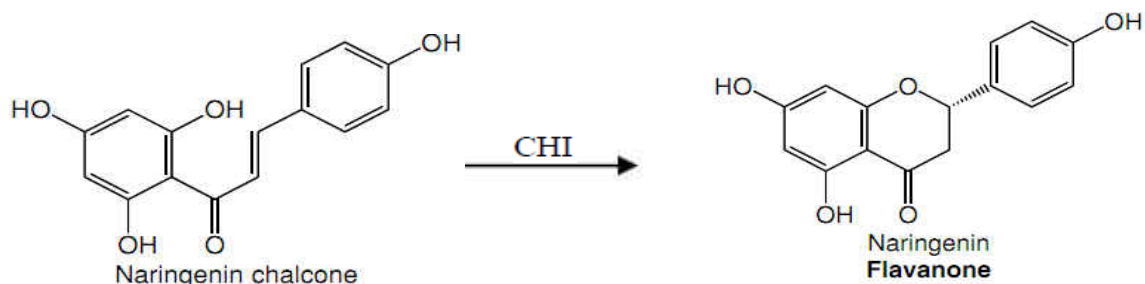
Figure 4. Cyclization of Chalcones to Flavanone (Moustafa and Wong 1966)

Flavanones then serve as a branch point for the formation of other flavonoids (Figure 5). Flavone synthase (FNS), a cytochrome P-450 dependent monooxygenase and NADPH-dependent enzyme, catalyzes the formation of flavones from flavanone (Grisebach and Kochs 1986). In the characterization of FNS from soybean cell cultures, it was realized that NADH could not substitute the requirement of NADPH by this enzyme (Grisebach and Kochs 1986). Two forms of FNS exist. One is FNS I which requires 2-oxoglutarate, O_2, and Fe^{2+} for its activity whereas FNS II requires NADPH and O_2 for its activity (Britsch et al. 1981; Grisebach and Kochs 1986; Martens and Mithofer 2006 and ref. therein). Isoflavone synthase (IFS) catalyzes the formation of isoflavone from naringenin and requires NADPH and O_2 for its activity. The activity of this enzyme was found to be in the microsomal fraction (Grisebach and Kochs 1986). Flavanone 3-β-hydroxylase (F3H) catalyzes the conversion of flavanone to dihydroflavanol (Britsch et al. 1981; Britsch and Grisebach 1986; Pelt et al. 2003). Conversion of flavanones to flavone, dihydroflavonol, and flavonol was observed in cell cultures of parsley in the presence of 2-oxoglutarate, Fe^{2+}, and ascorbate (Britsch et al. 1981).
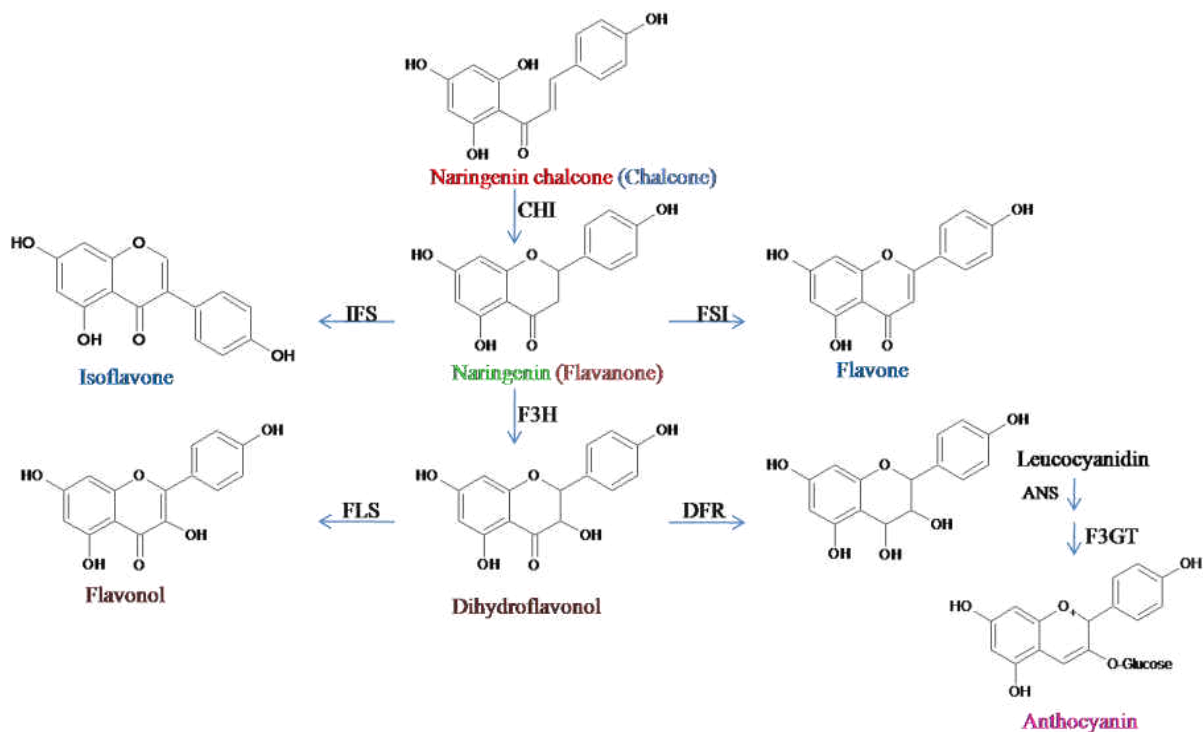
Figure 5. Flavonoid Biosynthetic Pathway (adapted from Heller and Forkmann 1988). CHI= chalcone isomerase (Moustafa and Wong 1966; Raymond and Maier 1977), IFS= Isoflavone synthase (Kochs and Grisebach 1986) FSI= flavone synthase (Britsch et al. 1981; Grisebach and Kochs 1986), F3H= flavanone 3β-hydroxylase (Britsch et al. 1981; Britsch and Grisebach 1986; Pelt et al. 2003), DFR= dihydroflavonol 4-reductase (Stafford and Lester 1982), FLS= flavonol synthase (Britsch et al. 1981; Lukacin et al. 2003), ANS=anthocyanidin synthase (Heller et al. 1985; Saito et al., 1999), and F3GT= flavonoid 3-O-glucosyltransferase. (Heller et al. 1985; Fukuchi-Mizutani et al. 2003; Owens and McIntosh 2010).
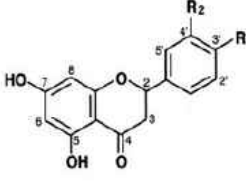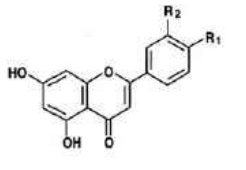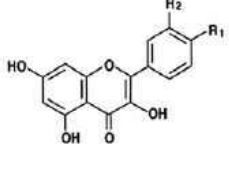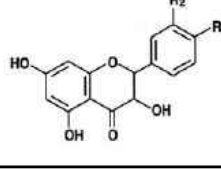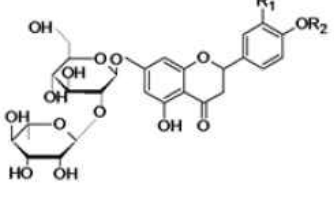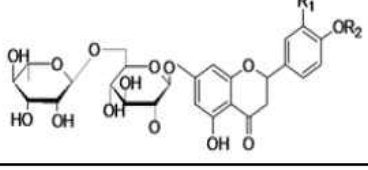
Synthesis of flavonols is catalyzed by the enzyme flavonol synthase (FLS) and it is a 2-oxogluatarate dependent dioxygenase acting on dihydroflavonols to yield flavonols (Britsch et al. 1981; Britsch and Grisebach 1986 and ref. therein; Lukacin et al. 2003). Dihyroflavonol reductase (DHR) converts dihydroflavonol to leucoanthocyanins from which anthocyanidins and anthocyanins are formed by anthocyanidin synthase (ANS) and UDP-3-O-glucosyltransferase (Heller et al. 1985: Saito et al. 1999, Fukuchi-Mizutani et al. 2003).

Citrus Flavonoids

Several classes of flavonoids have been found in *Citrus spp*. These classes of flavonoids include flavanones, flavones, flavonols, dihydroflavonols, and anthocyanidins, but the anthocyanidins are found only in blood oranges (Berhow 1998; Tripoli et al. 2007 and ref. therein). Among these different classes of flavonoids, the flavonols, flavones, and flavanones are the most abundant (McIntosh and Mansell 1990; Owens and McIntosh 2011) with the flavanones being the most common. Over 60 different individual flavonoids have been identified and a few examples are shown in Table 1(Benavente-Garcia et al. 1997; Berhow 1998).

Citrus species are known to produce and accumulate high levels of flavonoid glycosides especially flavanone and flavone glycosides (McIntosh and Mansell 1990; Owens and McIntosh 2011 and ref. therein). A good example of a prominent flavanone found in Citrus is naringin (flavanone diglycoside) which is a bitter compound (McIntosh and Mansell 1990 and ref. therein). Flavonoid composition is important in terms of the taste of fruits making them of interest because these fruits are of commercial importance. For example hesperidin forms sediments in juices resulting in undesired cloudiness, while naringin influences the bitterness of grapefruit juices (Kesterson and Hendrickson 1957, Hendrickson and Kesterson 1965; Mansell et al. 1983; Gattuso et al. 2007 and ref. therein).

Table 1**.** Some Principal Flavonoids Found in Citrus with Structure and Substitution Patterns (Benavente-Garcia et al. 1997, Berhow et al. 1998).

Class	Common Name	Substitution Pattern	
Flavanone aglycones	Naringenin	R1=OH	R2=H
	Eriodictyol	R1= R2=OH	
	Hesperitin	R1= O-Me	R2=OH
	Isosakuranetin	R1= O-Me	R2=H
Flavone aglycones	Apigenin	R1=OH	R2=H
	Luteolin	R1= R2=OH	
	Diosmetin	R1= O-Me	R2=OH
Flavonol aglycones	Quercetin	R1= R2=OH	
	Kaempferol	R1=OH	R2=H
	Isorhamnetin	R1= OH	R1=O-ME
Dihydroflavonol aglycone	Dihydrokaempferol	R1=OH	R2=H
Flavanone diglycoside (neohesperidoside)	Naringin	R1=R2=H	
Flavanone diglycoside (rutinoside)	Narirutin	R1=R2=H	

The distribution of these flavonoids within citrus tissues has been investigated and it has been confirmed that these flavonoids vary in concentration and type within the plant organs and tissues (Jourdan et al. 1985; McIntosh and Mansell 1997). For example, peels have been shown to have more flavonoids than seeds (Tripoli et al. 2007 and ref. therein). Flavanone glycosides have been found in the lemon seeds, and peels as well as tangerine seeds but are not present in the juices of these fruits (Tripoli et al. 2007 and ref. therein). The levels of flavanone glycosides naringin in *Citrus paradisi* and hesperidin in *Citrus sinensis* have been studied (Barthe et al. 1987; Jourdan et al. 1985). These studies showed that naringin and hesperidin were most concentrated in the young leaves of grapefruit and orange, respectively, and interestingly that hesperidin was found only in orange and not in grapefruit while naringin is found in grapefruit and not in orange (Barthe et al. 1987; Jourdan et al. 1985). Distribution of naringin in juices of different grapefruit cultivars has been studied in an attempt to determine what cultivar is more suitable to extract juice for consumption as this is one of the bitter compounds responsible for the bitterness of grapefruit (Mansell et al. 1983). It was realized that there was a statistically significant difference in the amount of naringin in the different juice samples.

Another importance of flavonoid composition of citrus is for human health benefits. These health benefits are mostly related to the antioxidant properties of flavonoids (Benavente-Garcia et al. 1997). The antioxidant property is achieved through their antiradical, anti-lipoperoxidation, and metal chelation activities (Tripoli et al. 2007 and ref. therein). Certain structural properties are important for the antioxidant properties of these flavonoids (Figure 6). The catechol structure of the B-ring confers greater stability to the aroxyl radicals and participates in electron dislocation, the 2, 3 double bond that is also involved in electron dislocation from the B ring, and the 3- and 5-hydroxyl groups (Tripoli et al. 2007 and ref.

therein). Other factors such as glycosylation, methylation, and free hydroxyl groups also influence the antioxidant properties (Benavente-Garcia et al. 1997). The health benefits of these compounds have drawn significant interest in the determination of citrus flavonoid composition and their properties (Benavente-Garcia et al. 1997; Tripoli et al. 2007 and ref. therein).
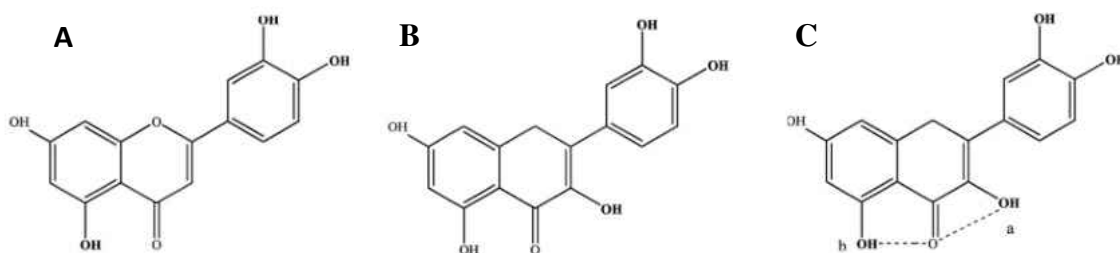
Figure 6. Important Antioxidant Structural Properties of Flavonoids. A. Catechol structure in B-ring. B. 2, 3 double bond. C. Presence of both 3 hydroxyl (a) and 5 hydroxyl (b) groups. (Benavente-Garcia et al. 1997; Tripoli et al. 2007).

Glycosylation

Glycosylation is the addition of sugars to compounds and this reaction is often catalysed by glycosyltransferases. Most glycosyltansferases are soluble proteins with a molecular weight between 45-65kDa under *in vitro* conditions (Vogt and Jones 2000 and ref. therein). Glycosyltransferases have been classified into subfamilies according to the degree of primary sequence identity and even though the primary structures of these enzymes are poorly conserved, the limited crystallized glycosyltransferases available show a highly conserved secondary and tertiary structure (Osmani et al. 2008 and ref. therein). Family one glycosyltransferases contain the plant UDP –dependent glycosyltransferases which glycosylate a wide variety of small plant molecules (flavonoids, alkaloids, terpenoids, and others) using UDP-activated sugars (Osmani et al. 2008 and ref therein). The activated sugar is usually UDP-glucose

but other activated sugars such as UDP-galactose, UDP-xylose, UDP-mannose, and UDP-glucuronic acid are also used by some enzymes (Vogt and Jones 2000; Osmani et al. 2008).

Glycosylation leads to the structural and functional modification of bioactive compounds. It enhances water solubility, physicochemical stability, and bioactivity as well as influencing the taste qualities of these compounds (Paquette et al. 2003 and ref. therein; Noguchi et al. 2008; Wang 2009 and ref. therein; Owens and McIntosh 2009; Owens and McIntosh 2011 and ref. therein). Another important aspect is the substrate specificity exhibited by the different glycosyltransferases as well as transfer of specific types of sugars (Osmani et al. 2008; Owens and McIntosh 2011). Depending on the type of sugar used as well as position of glycosylation, a particular compound may exhibit different biological activity or chemical property.

Glycosylation has several biological roles. With respect to flavor, this is a very important reaction in the *Citrus spp*. Naringin and narirutin are both formed by the rhamnosylation of naringenin 7-O glucoside (McIntosh and Mansell 1990; McIntosh et al. 1990; Bar-Peled et al. 1991, Frydman et al. 2004). For naringin, rhamnosylation occurs on the 2-OH group of the glucose molecule while rhamnsoylation occurs on the 6-OH group of the glucose unit for narirutin. This difference in position of rhamnosylation gives rise to a bitter naringin and a taseteless narirutin (Owens and McIntosh 2011 and ref. therein). The synthesis of both compounds is illustrated in Figure 7.
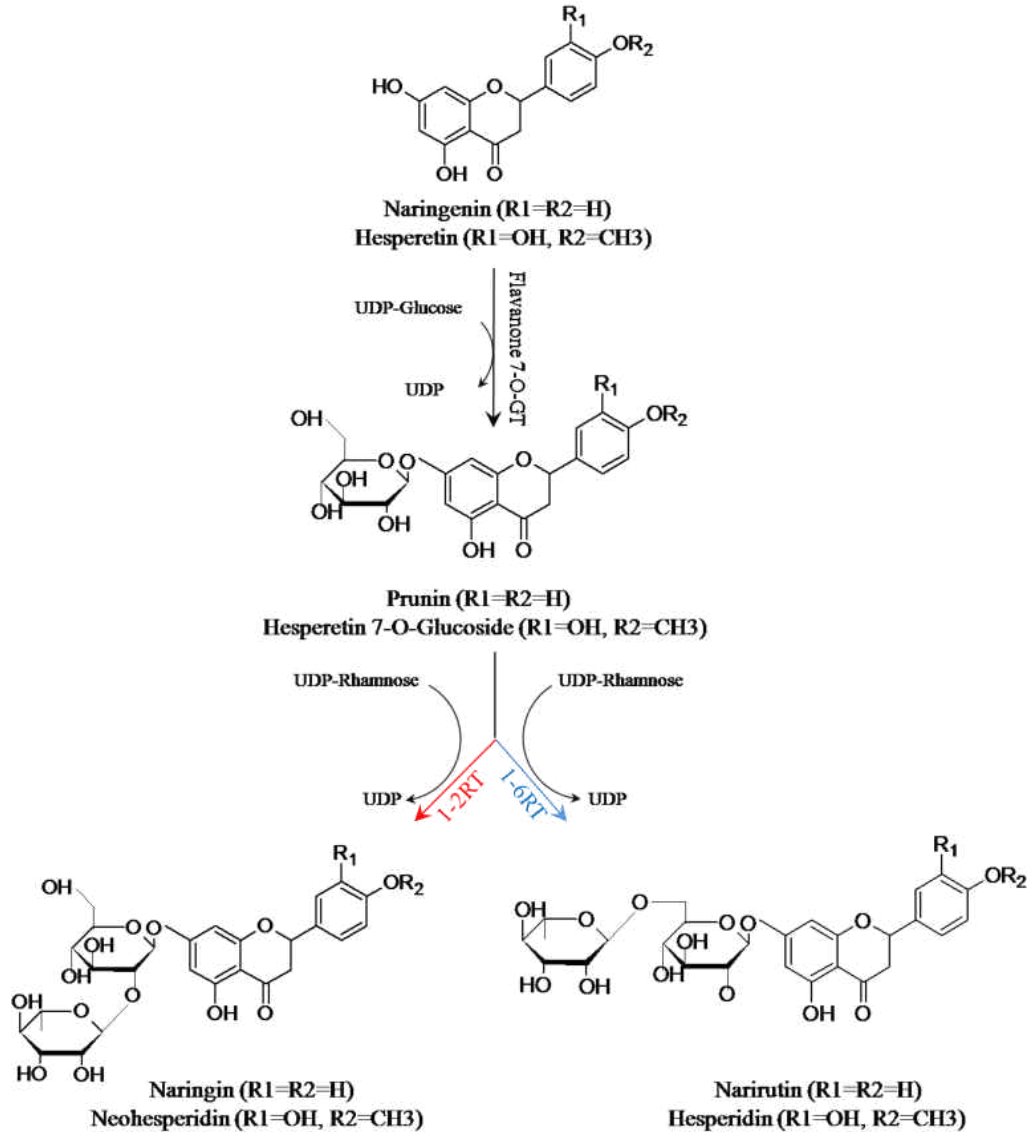
Figure 7. Glycosylation of Flavonoids Influences Taste Properties in Citrus (adapted from Owens and McIntosh 2011). Bitter naringin and tasteless narirutin differ only in position of the rhamnosyl on the glucose moiety. (McIntosh and Mansell 1990; McIntosh et al. 1990; Bar-Peled et al. 1991, Frydman et al. 2004; Owens and McIntosh 2011 and ref. therein).

Flavonoids are not always synthesized at the location where their activity is needed and as such must be transported to areas where they are needed (Zhao and Dixon 2009). Flavonoids are found in most plant cell compartments such as cytosol, vacuole, endoplasmic reticulum, chloroplast, nucleus, and small vesicles and may be transported from one compartment to another (Zhao and Dixon 2009). Anthocyanins are primarily found in the vacuole (Zhao and

Dixon 2009 and ref. therein). Glycosylation is one of the many chemical modifications of

flavonoids that enhance their transport (Zhao and Dixon 2009). Even though other requirements

such as acylation are essential for the transport of flavonoids, glycosylation alone is sufficient for

the uptake of most flavonoids into the vacuole through certain pumps (Zhao and Dixon 2009 and

ref. therein). Analysis of phloematic fluids revealed that naringin and neohespeiridin are

transported from one part of the tree to another (Castillo et al. 1992) but the effect of

glucosylation on the transport of these compounds from tissue to tissue is yet to be investigated.

Glycosylation has also been shown to play a vital role in anthocyanin pigmentation. A

good example is the glucosylation of delphinidin (an anthocyanin) that is a required for the

acylation of this compound (Fukuchi-Mizutani et al. 2003). This results in stabilization as well

as increasing the intensity of the blue color.

Glucosylation and Glucosyltransferases

Glucosylation is one of the most prominent glycosylations that occur in plant secondary

product metabolism. This involves the transfer of a glucose sugar from a UDP-activated glucose

molecules such as flavonoids (Owens and McIntosh 2009 and ref. therein; Owens and McIntosh

2011). This reaction is catalyzed by a group of enzymes known as glucosyltransferases (GTs).

An example of this type of reaction is illustrated in Figure 8 below.
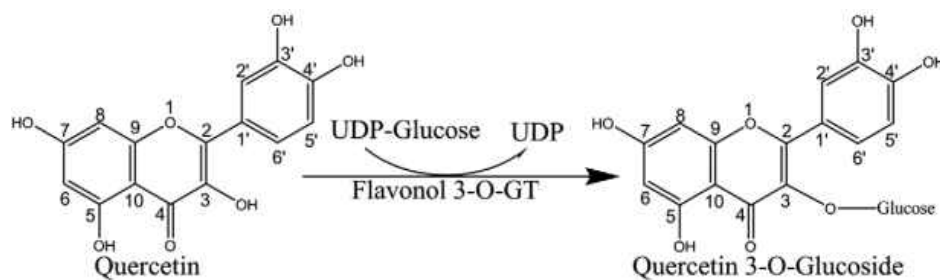
Figure 8. Synthesis of Quercetin 3-O-glucoside from Quercetin . Catalyzed by a Flavonol 3-O-Glucosyltransferase isolated from *Citrus paradisi* (Owens and McIntosh 2009).

Glucosyltransferases share a loosely conserved motif known as the Plant Secondary

Glucosyltransferase (PSPG) Box (Vogt and Jones 2000 and ref. therein). This motif recognizes

and binds to the glucose moiety of UDP-glucose and helps bring about glucosylation of the

acceptor molecule (Owens and McIntosh 2009). This motif spans 44 amino acids with some, not

all, the amino acids conserved within different glucosyltranferases as shown in Figure 9 (Vogt

and Jones 2000). The PSPG box is near the C-terminus of these enzymes. Despite similarities in

the PSPG domain, the overall sequence identities of glucosyltransferases is low (Owens and

McIntosh 2009 and ref. therein; Vogt and Jones 2000). For example, PGT 1 which is now

thought to be a shorter version of PGT 9 was compared with a limonoid GT from *Citrus unshiu,*

there was only a 33% identity for the entire sequence as opposed to 61% identity for the PSPG

box region only (RoySarkar 2004).

Nterm < WAPQVEVLAHPAVGCFVTHCGWNSTLESISAGVPMVAWPFFADQ >Cterm

Figure 9. Consensus PSPG Box (adapted from Vogt and Jones 2000). Highly conserved amino
acids in blue (identity>50%) or red (identity>80%)

Origin of Putative Glucosyltransferase Clones 9 and 11

Grapefruit has been shown to accumulate high levels of glucosylated flavonoids. It also

contains almost all the enzymes involved in the core flavonoid biosynthetic pathway and has also

been shown to have diverse glucosyltransferases (McIntosh and Mansell 1990; McIntosh et al.

1990). This makes grapefruit a potential source for diverse glucosyltransferases clones.

Several glucosyltransferase clones have been identified from *Citrus paradisi* using

different approaches in the McIntosh lab and named PGT 1- 11 (RoySarkar 2004; Strong 2005;

Mallampalli 2009; Lin 2011; Owens and McIntosh 2011). Putative GTs are identified,

expressed, and biochemically characterized (Owens and McIntosh 2009). The expression levels of different putative genes in *Citrus paradisi* have been looked into and the levels expressed differ from tissue to tissue as well as at different developmental stages (Daniels et al. 2011).

After identifying a putative glucosyltransferase, it is cloned into an expression vector such as pCD1 (Owens and McIntosh 20011 and ref. therein) and transformed into *E.coli* cells for expression of soluble proteins. The pCD1 vector is a modified version of the pET 32a altered such that the fusion tag can be cleaved off (Owens et al. 2008 and ref. therein). Following expression of these proteins, they are purified and their activities screened against flavonoids as well as other plant secondary metabolites. If activity is found with a substrate, then the kinetics of these different enzymes are also studied.

Glucosyltransferases have been shown to be substrate specific and determining the substrate for each enzyme is also of interest to the lab. Plant UDP-dependent glycosyltransferases (UGTs) with high amino acid similarity and from the same family have been shown to function differently. For example UGT73A5 and UGT71F2 from *Dorotheanthus bellidiformis* show only about 20% overall similarity but glucosylate the same set of acceptor molecules (Osmani et al. 2008 and ref. therein). On the contrary, 2 UGTs from *Allium cepa* (AcUGT73G1 and AcUGT73J1) fall in the same family of UGTs but the former exhibits very broad acceptor specificity, whereas the latter has a very narrow specificity. Therefore, it is clear that substrate specificity cannot be predicted solely based on degree of amino acid sequence identity (RoySarkar et al. 2007, Osmani et al. 2008 and ref therein; Owens and McIntosh 2009; Owens and McIntosh 2011). Flavonoid GTs differ in substrate specificity with a few exceptions and this class of enzymes also exhibit different chemical properties such as pH optima and pI's

(McIntosh 1990 and ref. therein). It is therefore necessary to express these proteins and screen for their activity against a wide array of substrates to assign biochemical function.

The substrate specificity of one of these putative GT enzymes (PGT 7) was successfully determined using this method. This was flavonol 3-O-glucosyltransferase (GQ141630) with its preferred substrate being quercetin (Owens and McIntosh 2009). It also had 57.9% and 40.7% relative activity with kaempferol and myricetin, respectively, both of which are flavonols. It showed relatively low or no activity for other flavonoid classes indicating that this enzyme is flavonol specific. PGT 10 has also been successfully expressed and a wide array of substrates screened to test for substrate activity and specificity (Lin 2011). No GT activity was detected.

One of the major problems faced with expression in *E.coli* has been the presence of inclusion bodies into which the protein of interest is packed in an insoluble form (Mallampalli 2009, Owens and McIntosh 2011 and ref. therein). This has been observed for other recombinant glucosyltransferases (Owens and McIntosh 2009 and ref. therein). Despite this problem, sometimes enough soluble protein is obtained that can be used to test for the activity of the enzyme (Owens and McIntosh 2009).

Several techniques have been used to identify new clones from *C. paradisi*. Techniques used include SMART RACE PCR to amplify genes from a cDNA library of young grapefruit leaves in which partial 5' and 3' sequences were obtained (Sibhatu 2003; Strong 2005; RoySarkar et al. 2007), full sequences obtained by PCR using clone specific primers and "walking out" to the gene ends, full length clones obtained by designing primers to the extreme ends and using PCR (Sibhatu 2003; RoySarkar 2004; Strong 2005; RoySarkar et al. 2007; Cantrell 2006; Epling 2007), directional cloning and screening of a grapefruit EST cDNA library (RoySarkar 2004), and mining the harvEST database which contains expressed sequence tags

(EST) from different Citrus species (Mallampalli 2009 and ref therein). Mallampalli (2009)

showed that it was possible to obtain new clones by bioinformatic analysis of the harvEST

database. Five new clones were identified that did not match or correspond to previously

identified clones. Among these 5 candidate unigenes was Cit. 1332.1 corresponding to PGT 9.

Clone specific primers were designed against these unigenes and 3 of them (PGT 9, PGT 10, and

PGT 11) were successfully amplified from young grapefruit leaf cDNA. It is possible that the

other 2 clones may not be expressed in grapefruit leaf as these enzymes have been shown to be

expressed in a tissue specific manner, or that the genes are not conserved at the 5' or 3' termini.

Because these genes are developmentally regulated as well, it is also possible that the stage of

leaves used may not have been expressing at that developmental stage. Also, considering that the

harvEST database consists of ESTs from different *Citrus spp*, the other 2 clones may be

expressed in different *Citrus spp* and not grapefruit. PGT clones 9 and 11 consensus sequences

contained the conserved PSPG box motif confirming it to be a putative secondary product

glucosyltransferase (Figure 10). Given that the harvEST database consist of ESTs from different

Citrus spp. and that the sequence obtained from young grapefruit cDNA library differed from the

consensus sequence only by one base suggests that PGT 9 may be evolutionary conserved within

the different Citrus species (Figure 11).

      The unmodified PGT clones 9 and 11 sequences (Figures 11 and 12) were cloned into

pCR4 TOPO vector and transformed into Top 10 competent *E. coli* cells (Mallampalli 2009).

This served as a holding cell line for these sequences.
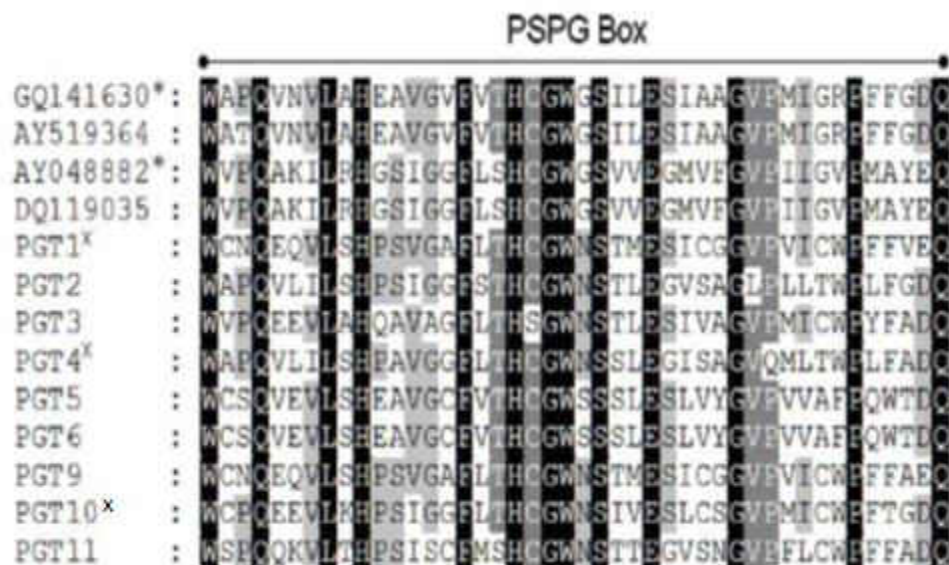
Figure 10. Alignment of PSPG box of Citrus Flavonoid Glucosyltransferases and Putative Plant Secondary Metabolite GT Clones. PGT 1-6, 9-11. *Citrus paradisi* cv. Duncan putative glucosyltransferase. GQ141630. *Citrus paradisi* cv. Duncan flavonol 3-O-glucosyltransferase. AY048882. *Citrus maxima* flavonoid 1-2 rhamnosyltransferase. AY519364. *Citrus sinensis* putative flavonoid 3-O-GT. (*). Biochemically characterized. (x). Not a flavonoid glucosyltransferases based on current evidence. The dark shaded regions show highly conserved amino acids and the shaded regions show less conserved amino acids (adapted from Owens and McIntosh 2009).

PGT 9 was modified using primers designed against the 5' and 3' ends of the sequence, cloned into pCD1 vector, and transformed into *E.coli* for expression and characterization. Successful expression was achieved but most of it was packed in insoluble fraction as inclusion bodies (Mallampalli 2009). This has been one of the major challenges faced in the biochemical characterization of these enzymes. The amount of soluble protein obtained was not sufficient to test for activity. Different temperatures, culture media, and time postinduction of cultures were tested to optimize the production of soluble proteins but no significant change in the amount of soluble proteins was observed. Attempts were made to enrich the soluble protein fraction using immobilized metal affinity chromatography but not enough was obtained to be able to screen for GT activity (Mallampalli 2009).

Figure 11. Alignment of PGT 9 Consensus Sequence with that Amplified from Young Grapefruit Leaf cDNA. There was only one base difference at position 1260 shown in a rectangle (Mallampalli 2009).

```
PGT 11      ATGAGCAGTC CCCATGTTGT GGTAATACCG AATCCAGAAC AAGGGCATGT

            AATTCCTTTA CTAGAGTTGT CACAAAATTT GGCCAAGCAT GGCCTGAGAA

            TCACATTTGT GAACTCGGAG TATAATCACA ATCGGGTATT GGAATCATTA

            GAAGGAAAGA ATTACATTGG GGAGCAAATC CATCTGGTGT CGATCCCTGA

            TGGAATCGAA CCATGGGATG ACAGGAGTGA TATGAGAAAG TTGTTAGAAA

            AACGTTTACA GGTTATGCCT GGAAAGCTGG AGGGGCTCAT AGAAGAGATC

            CATGGAAGAG GAGGTGAAAA AATAGCTTGT CTCATTGCAG ATGGGGCAGC

            GGGGTGGGCT ATTGAAGTCG CTGAGAAGAT GAAACTCAGG CGGGCGGTTG

            TAGTGATAAC CTCAGCAGCA ACAGTGGCCT TGACATTCAG TATCCCTAAG

            CTGATAGAGG ATGGGGTCAT TAACAGTAAC GGGACTCCAA TCAAGGAACA

            AATGATTCAA TTGGCACCAA ATATGCCTGC AATAAGCACT GGAGAGCTAT

            TTTGGACTCG CTTTGGTGAC TTGACCATGC AAAAAATCTT CTTTGATTTT

            ATGGTGCAAA ACATGAGAGC GACGAGAGCG GTAGATTTCC AGTTATGCAA

            CTCAACATAC GAGCTGGAGG GTGGAGCATT CAGTATGATT CCGGAGCTTC

            TTCCAATAGG ACCACTTTTG GCGAGCAACC GGTTAGGAAA TTCAGCAGGA

            TACTTTTTGC CAGAAGACTC AAAATGTGTG GAATGGCTAG ACCAACAGCA

            AGCAAACTCA GTCATTTATG TTGCTCTGGG GAGCCACACG GTACTCGAGC

            AAAATCAGTT CCAAGAATTA GCATTAGGAC TTGAAATCTG CAACAGAGCG

            TTCTTATGGG TGGTGAGGCC AGATATTACC AATGATGCAA ATGATGCATA

            CCCTGAAGGA TTTCGAGAGA GAGTAGCCGC TCGAGGACAG ATGATCAGTT

            GGTCTCCTCA GCAGAAGTTT CTCACCCACC CTTCCATTTC CTGTTTCATG

            AGCCACTGCG GTTGGAATTC CACCACGGAA GGTGTGAGCA ACGGAGTCCC

            TTTCCTGTGT TGGCCATTCT TTGCTGACCA ATTCATGAAC ACCACCTACA

            TTTGTGATGT TTGGAAGGTG GGATTGAGGC TTGAAAGAAA CCAAAGTGGT

            ATTATCGGAA GAGAAGAAAT TAAGAACAAG GTGGATCAAG TGCTGGGTGA

            TCAAAATTTT AAGGCAAGGG CCTTAAAGCT TAAGGAAAAA GCCCTCAGTA

            GTGTCAGAGA AGGTGGTTCA TCCAACAAGG CAATTCAGAA TTTCGTTCAA

            TCCATCAAGC AATGGCCCGC ATGA
```

Figure 12. Full Length Sequence of PGT 11 Amplified from Young Grapefruit Leaf cDNA (Mallampalli 2009).

<u>Hypotheses</u>

This research is designed to test the hypothesis that PGT 9 is a plant secondary product glucosyltransferase specifically a flavonoid glucosyltransferase. This entails expression of the soluble recombinant protein in order to screen for its activity against a variety of citrus secondary metabolites especially flavonoids as these are highly accumulated in the glycosylated form. This research is also designed to test the hypothesis that PGT 9 is under biochemical regulation as previous studies have shown that these GTs are inhibited by UDP and activated by sulfhydryl-blockers (McIntosh and Mansell 1990; McIntosh et al. 1990 and ref. therein; Vogt 2000). Results of cloning of PGT 11 into yeast for expression and biochemical characterization are reported in this work.

CHAPTER 2

MATERIALS AND METHODS

Materials

Chemicals and Reagents

Zeocin^{TM} , Luria-Bertani broth powder, agar powder, 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
#3, 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 and 5 X green / colorless buffer for
polymerase chain reactions (PCR), deoxyribonucleotide triphosphate (dNTP), T4 DNA ligase
enzyme and ligation buffer, and restriction enzymes Xba1, Xho1, Kpn1, Apa1, and Bstx1 were
purchased from Promega (Madison, WI). Mini Trans Blot system for Western blotting, Mini-
PROTEAN Tetra gel electrophoresis system, and micro pulser cuvettes were from Bio-rad
(Hercules, CA). TALON Metal Affinity Resin was purchased from Clonetech (Mountain View,
CA). Amicon Centricon 30 centrifugal filters were purchased from Millipore (Billerica, MA).

All primers were synthesized by Integrated DNA Technologies. Lyticase enzyme from *Arthobacter luteus* (lyophilized powder) and acid washed glass beads (pore size 0.5mm) were from Sigma Aldrich.

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 appendix.

Kits

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

Buffers

IMAC elution buffer containing 10 or 150 mM imidazole, 0.3 M NaCl, and 5 mM BME in 50mM phosphate buffer (pH 7.5), IMAC equilibration/ wash buffer (containing 50mM sodium phosphate, 0.3M sodium chloride, 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 (pH 8.3), 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), and 50mM phosphate buffer (pH 7.5, containing 50mM βME) were prepared as described in the appendix. Breaking buffer (containing 50mM Sodium phosphate, pH 7.4, 1mM PMSF (phenylmethylsulfonyl fluoride), 1mM EDTA (Ethylenediaminetetraacetic acid) and 5% glycerol), and PBS-T buffer were also prepared as described in the appendix.

Culture Media

LB (Luria-Bertani), low salt LB, YPDS (yeast extract peptone dextrose sorbitol), and 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 Appendix B.

Substrates for Enzyme Screening Assays

Chalcone (4, 2', 4', 6'-tetra-OH-chalcone), naringenin, hesperetin, apigenin, luteolin, kaempferol, quercetin, myricetin, 4'-methoxyflavonol, cyanidin, 4'-acetoxy-7-hydroxy-6-methoxyisoflavone were from stock supplies of Dr. Cecilia A. 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-14C]-glucose (UDP-G; 261 mCi/ mMol) was obtained from ICN (Irvine, CA).

<div align="center">

Methods

Optimization of Soluble Recombinant PGT 9 Expression in *E. Coli*

</div>

Frozen cells of BL21 (DE3) RIL containing PGT 9 expression construct in pCD1 vector were inoculated using a sterile toothpick into 10ml of LB $_{amp(100mg/L), chlr (34 mg/L)}$ (Luria-Broth media containing ampicillin at 100 mg/l and chloramphenicol at 34 mg/l final concentration) liquid medium containing 2.5mM betaine and 660mM sorbitol and incubated at 37^oC, 250 rpm overnight. The next morning, 100ml of fresh LB $_{amp(100mg/L), chlr (34 mg/L)}$ medium containing the same concentration of betaine and sorbitol was inoculated with 1ml of the overnight culture and grown at 37^oC with shaking at 250 rpm to an OD_{600} between 0.5-0.7. A 1ml aliquot was removed to represent the preinduction culture sample. The remaining culture was treated with IPTG to a final concentration of 1mM, and the cultures were incubated at 25^oC (250 rpm). Postinduction samples (500µl for total protein determination and 15mL for soluble protein isolation) were collected after 6, 8, 12, and 24 hours. The 500µl samples for determination of total protein were centrifuged at room temperature in an eppendorf centrifuge (Fisher Scientific) at 10,000 X g for 10 minutes and pellets for SDS-PAGE stored at -20^oC overnight. The 15ml samples for detection of soluble protein were centrifuged at 4°C in a Sorvall RC-5B refrigerated super speed centrifuge (Fisher Scientific) at 10,000 X g for 10 minutes to collect the cells. The supernatants were discarded and pellets were stored at -80^oC. Culture conditions such as temperature and time of sample collection were also altered in the experiment to determine the conditions with the highest soluble protein yield. Temperatures of 20^oC, 25^oC, and 30^oC were used in combination with betaine and sorbitol.

Isolation of Total and Soluble Recombinant Proteins

Total cellular protein was prepared for analysis as follows: the postinduction pellets were resuspended in 250µl of 2X SDS-PAGE sample buffer. Tubes containing resuspended pellets were vortexed to achieve complete resuspension, boiled for 15 minutes, and centrifuged at 10,000 X g for 15 minutes at 4°C. A 200µl aliquot was transferred to a microcentrifuge tube and stored at -20°C for SDS-PAGE analysis.

Isolation of soluble protein was performed as follows. The postinduction cell pellets were removed from -80°C freezer, thawed, and resuspended in 1ml cold 50mM phosphate buffer (pH 7.5) containing 14mM βME (β-mercaptoethanol). Cells were lysed by sonicating the resuspended pellets by pulsing on ice 10 times for 5s each with a 60s recovery using a sonic dismembrator model 500 with microtip (Fisher Scientific). The sonicated samples were centrifuged at 10,000 X g for 20 minutes at 4°C. The pellets were discarded and each supernatant was placed in a clean 1.5ml tubes and analyzed for expression of soluble rPGT 9 protein using SDS-PAGE and Western blotting.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed as follows: For total protein sample, 6µl of sample was mixed with 6µl of 2X SDS-PAGE, boiled for 5 minutes and centrifuged 1 minute at 10,000 X g. For the soluble protein sample, 15µl samples was mixed with 5µl of 4X SDS-PAGE sample buffer, boiled for 5 minutes, centrifuged for 1 minute at 10,000 X g. Broad range marker was diluted 20 folds by mixing 1µl of the stock with 19µl of 2X dye. For the prestained marker, 10µl was used. All samples and markers were analyzed on an acrylamide gel (5% stacking gel, 10% separating gel). The samples were electrophoresed for 45 minutes at 200volts.

<u>Western Blot</u>

Prior to Western blotting, the nitrocellulose membrane was soaked in transfer buffer (containing 20% methanol) for 20 minutes as recommended by the manufacturer. The SDS-PAGE gel was placed onto the nitrocellulose membrane. The gel and membrane were sandwiched between paper and sponge and clamped tightly together after ensuring no air bubbles had been formed between the gel and membrane. The sandwich was placed in the transfer tank (containing an ice pack) and 1X transfer buffer (containing 20% methanol) was added and the gel was electroblotted for 1 hour at 100V. After 1 hour, the nitrocellulose membrane was stained with ponceau S stain for 1-2 minutes to visualize the protein markers. The area containing the protein markers was separated from the rest of the membrane using a clean razor blade. The ponceau S was removed by inserting the membrane in distilled water and shaking gently until all the stain was removed.

The remaining membrane containing the samples of interest was blocked with 30ml of blocking solution (containing 1.5g of fat free milk powder and 3ml of 1X TBS-T) for 30 minutes at room temperature with gentle shaking. Next, the nitrocellulose membrane was washed with 20ml of 1X TBS-T, 3 times for 5 minutes each, and then incubated in primary antibody at room temperature (2μl of Trx-tag monoclonal antibody in 10ml of 1X TBS-T) for 30 minutes with gentle shaking. After incubation, the membrane was washed with 20ml of 1X TBS-T, 3 times for 5 minutes each, and then the membrane was incubated in secondary antibody (1μl of Goat Anti-Mouse AP conjugate in 10ml of 1X TBS-T) for 30 minutes at room temperature with gentle shaking. The membrane was then washed with 20ml of 1X TBS-T, 5 times for 5 minutes each to remove unbound secondary antibody. Substrate solution was prepared as follows: 0.083g of NBT was mixed with 700μl of dimethylformamide (70%) and 300μl of distilled water. 0.042g of

BCIP was mixed with 1ml of dimethylformamide (100%). The developing solution was prepared by adding 60μl of the prepared BCIP and 60μl of the NBT to 15ml of AP buffer (7.5) to make up the developing solution. The developing solution was prepared 1 minute prior to usage. The developing solution was then poured over the washed membrane and watched closely for bands at room temperature while shaking gently. It took 6 minutes for bands to develop. The reaction was stopped by rinsing with distilled water.

Enrichment of Soluble Recombinant PGT 9

Several attempts were made to enrich the soluble fraction of recombinant PGT 9 (rPGT 9) using IMAC. Collection of samples at different times postinduction and as well as different culture temperatures (15ºC, 20ºC and 25ºC) were carried out.

Cells were cultured at 25ºC and samples collected at 6 hours postinduction using the procedure described above. The –80ºC frozen cell pellet was thawed and resuspended in 10ml of lysis buffer (50mM phosphate buffer; 5mM βME). The resuspended sample was lysed using a French press 3 X at 2000 psi (Fisher Scientific) while keeping the sample on ice. The lysed sample was collected in a 50ml conical centrifugal tube and centrifuged for 20 minutes, 4ºC at 13000 X g in a Sorvall RC-5B refrigerated superspeed centrifuge. The supernatant containing soluble rPGT 9 protein was transferred carefully into a fresh 50ml tube without disturbing the pellet and labeled "Crude".

Soluble rPGT 9 protein (crude) was enriched by immobilized metal affinity chromatography (IMAC) using the TALON system (TALON Metal Affinity User's Manual, Version PR651843, Clontech). The enrichment was performed using 5ml column bed volume. The column was equilibrated with 10ml of equilibration wash buffer. A total of 10ml sample was

applied to the column and 2ml fractions were collected. After applying sample to column, the

column was washed with 30ml of equilibration wash buffer and 5ml fractions were collected.

Weakly bound proteins were eluted using 30ml of 10mM imidazole and 5ml factions collected.

The tightly bound polyhistidine tagged proteins were eluted using 150mM imidazole buffer. The

fractions were analyzed by SDS-PAGE and the gels silver stained using silver stain plus (Bio-

Rad) according to the manufacturer's instructions.

Expression of PGT 9 in Methylotropic Yeast (*Pichia Pastoris*)

Cloning of PGT 9 into pPICZA and pPICZAα Vectors

Primer Design and Cloning Strategies. Primers were designed to modify the ends of PGT

9 to introduce restriction sites found in the mulitiple cloning site (MCS) of pPICZA and

pPICZAα vectors. Three different types of modifications were carried out requiring four primers,

each primer introducing a different restriction site for digestion to obtain sticky ends. The primer

combinations for the 3 different modifications are shown in Table 2.

Clone 1 was modified for ligation with pPICZA vector for intracellular expression of

PGT 9. There is no start codon upstream of the PGT 9 sequence when inserted into this vector.

Transcription begins at the PGT 9 start codon hence a native N-terminal structure (Figure 13).

Restriction enzymes used for digestion of modified PGT 9 and vector were Kpn I and Apa I

(Table 2). The rPGT 9 would have a c-myc epitope and a polyHis tag (Figure 13).

Clone 2 was modified for ligation with pPICZAα for secretion of expressed PGT 9. With

this vector, rPGT 9 would be expressed with the secretory signal peptide attached to the N-

terminus but this signal is cleaved at the Kex2 cleavage site from the yeast cells. This design was

such that 14 additional amino acid residues would be attached to the final rPGT 9 after cleavage

of the secretory signal hence the N-terminal would not be native (Figure 14). The set of

restriction enzymes used for this clone were Kpn I and Xba I (Table 2). These enzymes do not

cut within the PGT 9 sequence. rPGT 9 would be expressed with the c-terminal tags as well

(Figure 14).

Clone 3 was also designed for ligation with pPICZAα for secretion of expressed rPGT 9

(Table 2). The difference between clone 2 and clone 3 is that the Kex2 cleavage site is

introduced immediately adjacent to the rPGT 9 start codon such that cleavage of the secretory

signal would leave a native N-terminal of rPGT 9 with no additional amino acids (Figure 15). C-

terminal tags would also be present.

Table 2. Primer Combinations and Restriction Sites Introduced. Green=linkers,
purple=restriction sites, red=PGT 9 start codon, Blue=Kex2 cleavage site (site at which secretory
signal is cleaved after secretion of heterologous protein into the media).

Strategy	Sense primer	Antisense primer
1 pPICZA	(CSP120F) (64^{0}C)(Kpn I restriction site) 5'CATGGGTACCATGGATCCAGCCGGAGCAAG 3'	(CSP121R)(64^{0}C)(Apa I restriction site) 5'CGCGGGCCCGTGAAGGGTTTCGGTTCCGG 3'
2 pPICZAα	(CSP120F) (64^{0}C)(Kpn I restriction site) 5'CATGGGTACCATGGATCCAGCCGGAGCAAG 3'	(CSP123R) (64^{0}C)(Xba I restriction site) 5'CGCTCTAGACGGTGAAGGGTTTCGGTTCCGG 3'
3 pPICZAα	(CSP122F) (64^{0}C)(Xho I restriction site and Kex2 cleavage site) 5'CATGCTCGAGAAAAGAATGGATCCAGCCGGAGCAAG 3'	(CSP123R) (64^{0}C) (Xba I restriction site) 5'CGCTCTAGACGGTGAAGGGTTTCGGTTCCGG 3'
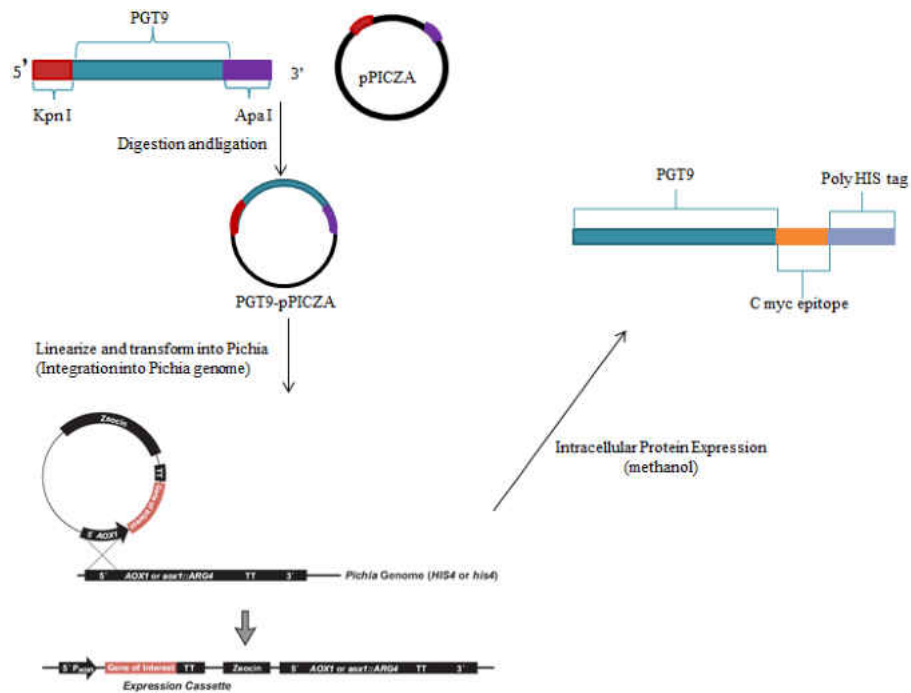
Figure 13: Cloning Strategy 1 for PGT 9. Recombinant PGT 9 expressed with a native N-terminal but not secreted out of the yeast cells (modified from Invitrogen Manual).
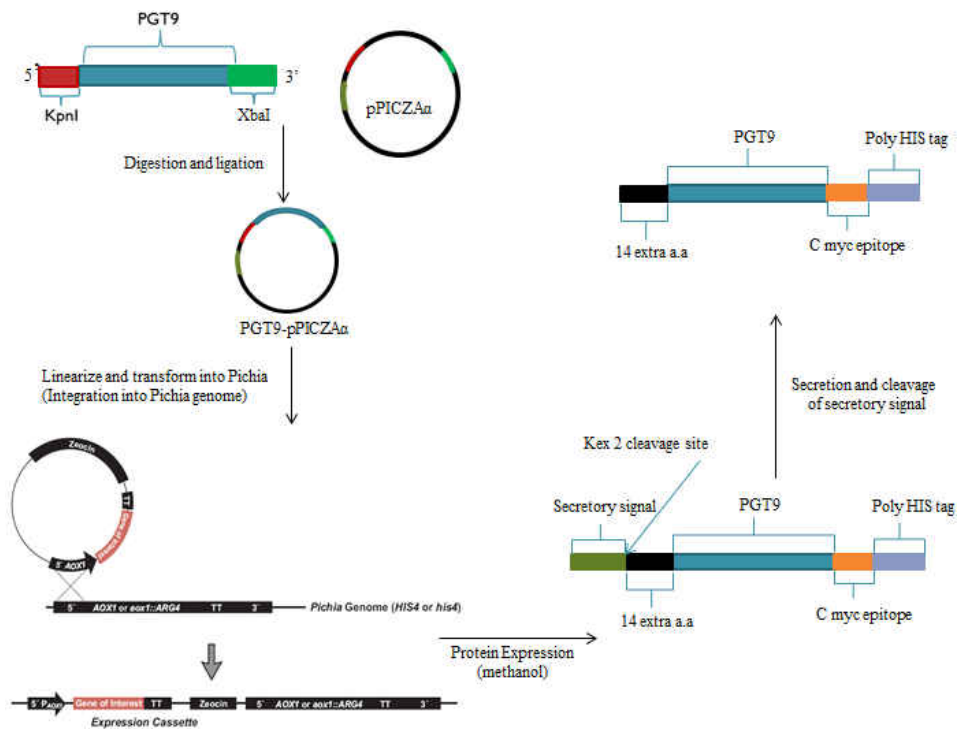
Figure 14. Cloning Strategy 2 for PGT 9. Recombinant PGT 9 expressed and secreted out of the yeast cells with 14 extra amino acids at the N-terminus (modified from Invitrogen Manual).Not drawn to scale.
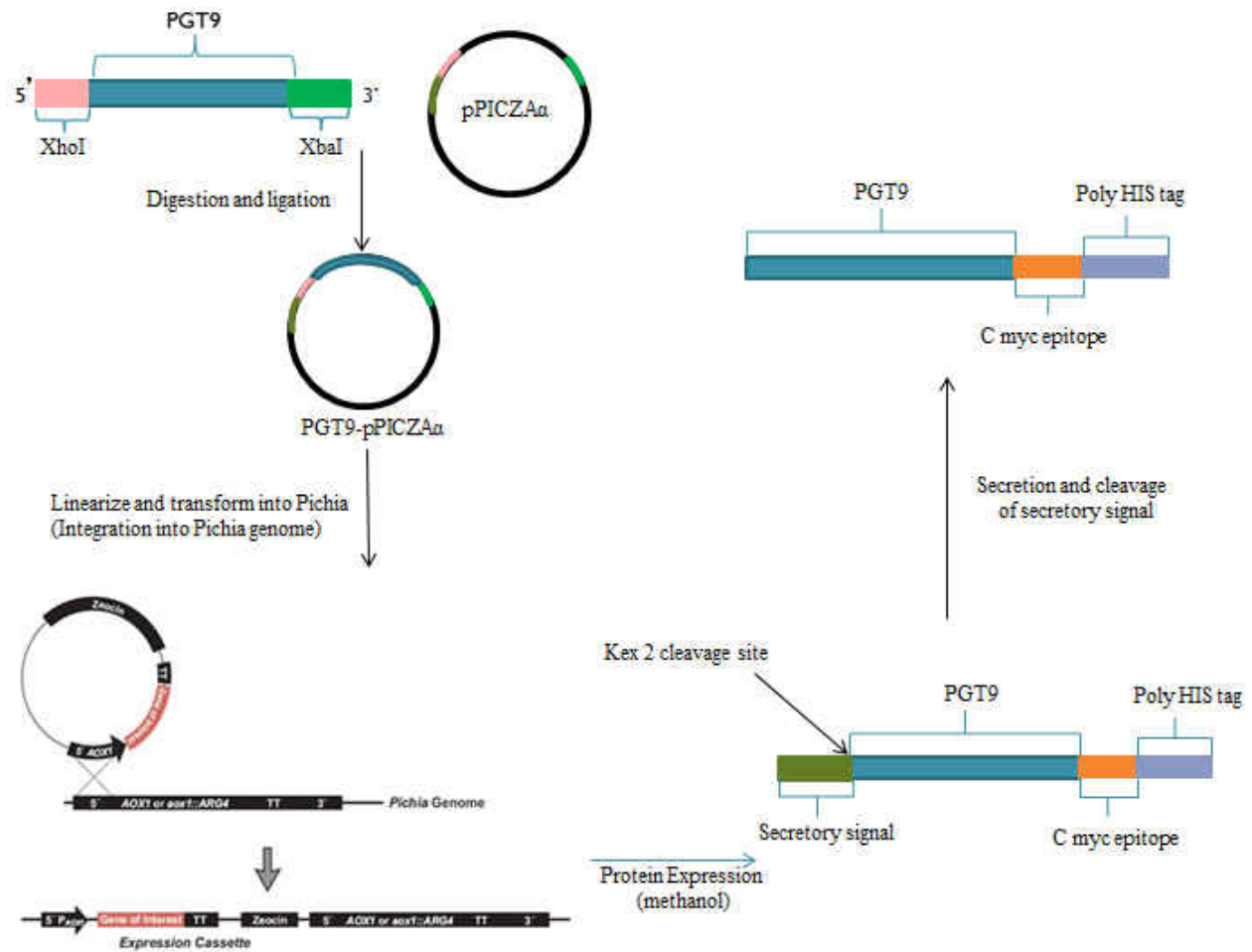
Figure 15. Cloning Strategy 3 for PGT 9. Recombinant PGT 9 expressed and secreted out of the yeast cells with a native N-terminus (modified from Invitrogen Manual). Not drawn to scale.

Plasmid (PGT 9-Topo) DNA Isolation from *E.coli*

In order to modify ends of PGT 9, Top 10 *E. coli* cell containing unmodified PGT 9 in pCR4 TOPO were cultured in 10ml of LB $_{amp(100mg/L)}$ [Luria-Broth media containing ampicillin at 100 milligrams/liter] overnight at 37°C, 250rpm. The next morning, a plasmid miniprep was carried out following the QIAprep Spin Miniprep kit protocol. Low salt LB media was also prepared (2.5g NaCl, 2.5g yeast extract, 5g tryptone, 12.5mg zeocin, pH 7.5) for culture of TOP 10 cells containing pPICZA and pPICZAα vectors. A 10ml aliquot of this media was inoculated with the TOP 10 *E.coli* cells and cultured overnight at 37°C, 250 rpm and miniprep was carried

out the following morning. A 2µl aliquot of each miniprep sample was mixed with 3µl of water and 1µl of 6X dye. The sample was run on a 0.8% agarose gel at 100V for 30 minutes.

PCR Modification of 5' and 3' Ends of PGT 9

A stock concentration of 200µM for each primer was prepared by first mixing and then centrifuging the tubes containing the primers in an eppendorf centrifuge for 1minute at 10,000 X g and then adding the appropriate amount of sterile distilled water. A 20µM working solution (final volume of 50µl) was prepared by adding 5µl of stock solution to 45µl of sterile distilled water. The 200µM stock and 20µM working solutions of primers were stored at -20˚C.

Plasmid DNA (PGT 9-TOPO) isolated from *E. coli* cells was used as template for a PCR amplification reaction using clone-specific primers as shown in Table 2. PCR tubes contained 2.5ul of 20µM solution of each appropriate clone-specific primer (CSP), 2.5µl of a 1:1000 diluted plasmid, 1µl of Taq polymerase, 10µl of 5X colorless buffer, and 31µl of sterilized deionized water. PCR steps, temperatures, and cycles are shown in Table 3. PCR products were analyzed using a 0.8% DNA agarose gel stained with ethidium bromide.

TOPO Cloning of PCR Modified PGT 9

PGT 9 PCR product was cloned into pCR®4-TOPO vector using the TOPO TA Cloning Kit (Invitrogen) according to manufacturer's instructions. The TOPO cloning reaction consisted of 4µl aliquot of gel purified PGT 9 PCR products, 1µl of salt solution, and 1µl of TOPO vector and the reaction was incubated for 30 minutes at room temperature. The TOPO cloning reaction was subsequently transformed into One-Shot Top 10 competent *E. coli* cells as follows: a 2µl aliquot of the TOPO cloning reaction was transferred into tube containing 50µl competent cells.

The procedures for transformation were carried out following the manufacturer's instructions (Invitrogen). For the positive ligation control, 1µl of pUC19 was added to 50µl of One-Shot competent cells.

Table 3. PCR Steps Used in the Modification of 5' and 3' Ends of PGT 9.

Step	Temperature (^{o}C)	Time (seconds)	# of Cycles
Initiation	94	60	1
Denaturation	94	30	
Annealing	64	30	30
Elongation	72	120	
Final Elongation	72	300	1

After transformation, 250µl of S.O.C medium was added to each transformation reaction. The reactions were then incubated at 37ºC at 225rpm for 1hour and 100 µl of culture containing transformation reaction was spread onto each LB$_{amp(100mg/L)}$ plate. For the pUC19 control, the transformation was diluted 1:10 into LB$_{amp(100mg/L)}$ liquid media and 100µl was spread on each LB$_{amp(100mg/L)}$ plate. Plates inoculated with the transformation samples were incubated overnight at 37°C.

Individual colonies from the PGT 9-pCR4 TOPO transformed plates were carefully picked using a sterile toothpick and streaked onto plates containing LB$_{amp(100mg/L)}$ media to make replicates. The plate with cells containing PGT 9 modified with Kpn I/Apa I was labeled X. The

plate with cells containing PGT 9 modified with Kpn I/Xba I was labeled Y and that containing cells modified with Xho I/Xba I was labeled Z. As opposed to several colonies growing on plates X and Z, only 7 colonies grew on plate Y. Each toothpick was then immediately placed into a sterile 0.5ml eppendorf tube containing 100 µl of sterile distilled water. The samples were boiled for 5 minutes to lyse the cells and 1µl of cell lysate was added to 9µl of PCR master mix. The PCR master mix contained the following: 4µl of 10mM dNTPs, 10µl of 20µM T3 sense primer, 10µl of 20µM T7 antisense primer, 40µl of 5 X green Go-Taq buffer, 94µl of sterile dH20, and 2µl of Taq polymerase. The conditions for the PCR reaction were as previously described (Table 3). Sterile dH_2O was used in place of cell lysate as a negative control. The PCR products were analyzed using a 0.8% DNA agarose gel stained with ethidium bromide.

Three positive colonies from each plate were T-streaked on selective LB $_{amp(100mg/L)}$ agar plates to obtain single colonies. Single colonies were selected from the streaks and inoculated in 10ml of LB $_{amp(100mg/L)}$, and grown overnight at 37^oC at 250 rpm. From the overnight culture, 500µl was collected and added to 500µl of sterile glycerol (50% glycerol final concentration) and stocks were stored at -80°C. From the remaining culture, 5ml was used to carry out minipreps using QIA prep Spin Miniprep Kit according to the manufacturer's instructions to isolate plasmid DNA containing modified PGT 9. Plasmid DNA was eluted with 50µl of distilled water. A 2µl aliquot was mixed with 3µl of distilled water and 1µl of 6 X dye and analyzed on a 0.8% agarose gel stained with ethidium bromide.

Restriction Digest of Inserts and Vectors

In order to confirm the presence of PGT 9 in the TOPO vector, digestion was also carried out using the appropriate pairs of restriction enzymes. A 2μl aliquot of miniprep plasmid DNA from colonies having the different modified PGT 9 sequences were digested. The digestion mix was made up of 2μl of plasmid, 1μl of appropriate buffer, 1μl of each restriction enzyme, and 5μl of distilled water. This was incubated at 37°C for 2 hours. Each digest was then analyzed on a 0.8% agarose gel stained with ethidium bromide. After 2 hours, 1μl of each enzyme was added to the digestion mix and incubated again for 2hours. Once plasmids were confirmed to have the modified PGT 9 sequences by agarose gel electrophoresis using a 0.8% agarose gel, one colony for each modification was selected for further use. Colony X6 (Kpn I/Apa I restriction site), Y2 (Kpn I/ Xba I restriction sites), and Z13 (Xho I/ Xba I restriction sites) were selected.

In order to carry out ligation reactions, larger volume restriction digests of the rPGT 9 plasmids with corresponding vectors were carried out (Table 4). From the minipreps, 16μl of each insert (PGT 9-TOPO) and each vector were digested with restriction enzymes to obtain sticky ends for ligation. After incubating the digestion mix at 37°C for 2hours, 1μl of each enzyme was added to the reaction and incubated for another 2hours at 37°C. This was to ensure complete digestion of the inserts and vectors. X6 and pPICZA were digested with Kpn I/Apa I, Y2 and pPICZAα were digested with Kpn I/Xba I and Z13 and pPICZAα were digested with Xho I/Xba I (Table 4). The digestion reactions were stopped by incubating the tubes at 65^{0}C for 5 minutes. The entire sample from each digestion reaction was loaded onto a 0.8% agarose gel.

Table 4. Digestion Reaction Set-up for Different Cloning Strategies for PGT 9.

Ligation Pair	Insert/Vector (16µl of each)	Restriction Enzymes (1µl of each)	Buffer (2µl of each)
A	X6	Kpn I/Apa I	A
A	pPICZA	Kpn I/Apa I	A
B	Y2	Kpn I/ Xba I	A
B	pPICZAα	Kpn I/ Xba I	A
C	Z13	Xho I/ Xba I	D
C	pPICZAα	Xho I/ Xba I	D

PGT 9 and vector bands were cut out of the agarose gel and gel purified following the Promega Wizard SV Gel and PCR clean-up system. From the gel-purified bands, 2ul and 5ul of each insert and vector were loaded on a 0.8% agarose gel as previously described alongside quantitative Lambda/Hind III and 1kb plus exACTGene DNA markers to determine the concentration of each and also to confirm the size of the insert and vectors. The concentrations of each gel purified digest were determined and recorded in Table 5.

Ligation of Digested PGT 9 with Vectors and Transformation into Top 10 Competent *E.coli*

After determining the concentration of the vectors and insert, ligations were carried out using different molar ratios (insert:vector) of 1:1, 3:1, and 6:1. The ligation mix was incubated at 15°C for 4hours. The concentrations of each digested rPGT 9, pPICZA and pPICZAα were determined and are recorded in Table 5. The different volumes used for the different ratios and different cloning strategies are shown in Tables 6, 7, and 8.

Table 5. Concentration of Vectors and Inserts Used During Ligation for Each Cloning Strategy as Determined Using Quantitative DNA Marker.

Strategy	Vector/ Insert	Concentration (ng/µl)
1	X6	18.0
	pPICZA	16.5
2	Y2	15.5
	pPICZAα	13.0
3	Z13	15.5
	pPICZAα	18.0

The different volumes used in the ligation procedure were calculated using a formula provided in the Promega catalog (Part# 9PIM180) as shown below.

$$\frac{\text{ng of Vector X kb (size of insert)}}{\text{kb (size of vector)}} \; X \; \frac{\text{molar ratio of insert}}{\text{vector}} = \text{ng of insert to use in the reaction}$$

A total of 100ng of vector was used as recommended in the user manual (Promega catalog, Part# 9PIM180catalog). The volume corresponding to this was also calculated by simply dividing the required amount (100ng) by the concentration of the vector. Least possible volumes were used for each ligation procedure.

Table 6. Reaction Mixtures for Ligation of pPICZA Vector and PGT 9 Insert (Strategy 1)

Ratio of Insert to Vector(µl)	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	1.23	3.2	1	1	3.57	10
3:1	3.7	3.2	1	1	1.1	10
6:1	7.4	3.2	1	1.5	1.9	15

Table 7. Reaction Mixtures for Ligation of pPICZAα Vector and PGT 9 Insert (Strategy 2)

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	7.7	1	1.5	2.1	15
3:1	7.9	7.7	2	2.1	1.3	21
36:1	15.8	7.7	2	3.0	1.5	30

Table 8. Reaction Mixtures for Ligation of pPICZAα Vector and PGT 9 Insert (Strategy 3).

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	5.6	1	1.2	1.5	12
3:1	7.9	5.6	2	1.9	1.6	19
6:1	15.8	5.6	2	2.6	-	26

After the ligation reactions were incubated, 2μl of the products were transformed into separate aliquots of Top10 *E. coli* cells to serve as holding cell lines. The procedure for transformation was as described before, but only 16.6μl of Top10 cells were used for each transformation using the heat shock method. After adding 2μl of the cloning product to the 16.6μl of Top10 cells, it was incubated on ice for 30 minutes. The cells were then heat shocked at 45°C for 30 seconds and incubated on ice again for 2 minutes. Plain low salt LB was added to the transformation sample instead of SOC media due to high salt concentration in the SOC media which could inhibit the antibiotic zeocin leading to false positives. Because the volume of Top10 cells used was only 16.6μl, 83.3μl of the low salt LB was used to resuspend the cells. They were incubated at 37°C in a vertical position for 1 hour while shaking at 225rpm for the cells to recover. For the negative control, 1μl of pUC 19 was transformed into 16.6μl of Top10 cells and the heat shock process followed as described. After, 1 hour of recovery, 100μl of the cells were evenly plated on pre-warmed LB$_{zeocin\ (25mg/L)}$ agar plates and incubated overnight at 37°C. A 1:10 dilution for the cells transformed with pUC 19 was carried out and a 100μl aliquot was evenly plated on low salt LB$_{zeocin\ (25mg/L)}$ to serve as the negative control.

PCR Screening for Positive Colonies

PCR screens were used to screen colonies that had grown overnight at 37°C on the selective low salt LB$_{zeocin\ (25\ mg/L)}$. To confirm transformation, colonies were selected at random from the different plates containing cells transformed with different ligation ratios. Sterile toothpicks were used to select these colonies and each colony was resuspended in 100μl of sterile dH$_2$O and then streaked on a selective LB$_{zeocin\ (25mg/L)}$ to make replicates. The samples were boiled for 5 minutes to lyse the cells and release the vector which should contain the insert

if the ligation procedure was successful. This was used as the template for the PCR reaction. Clone-specific primers were used in the PCR reaction to amplify the inserts if they were present in the vector. A master mix was prepared as previously described in "TOPO CLONING OF PCR MODIFIED PGT 9" using the clone-specific primers corresponding to each cloning strategy; all primers had an annealing temperature of 64°C. Same PCR cycles in Table 3 were used and the final PCR product analyzed on a 0.8% agarose gel. Positive colonies were selected from the plates that contained the replicates and T-streaked on selective low salt LB$_{zeocin\ (25\ mg/L)}$ plates to get single colonies. Single colonies obtained were subsequently cultured overnight at 37°C while shaking at 250 rpm in selective low salt LB$_{zeocin\ (25\ mg/L)}$ media.

Aliquots of 500μl of overnight cultures for each cloning strategy were saved in 50% glycerol (final concentration) at -80°C. For cloning strategy 1, designed such that PGT 9 would not be secreted into the media, four colonies labeled PX 18 S. C. 6 (X6) and 7 (X7) (Positive X Colony 18 single colonies 6 and 7), PX 23 S. C. 15 (X15), and PX 18 S. C. 9 (X9) were saved. For strategy 2, designed such that PGT 9 would be secreted into the media with 14 extra amino acids at the N-terminal, PY A, B, C, and D (Positive Colony Y single colonies A, B, and D) were saved. For Strategy 3, designed such that PGT 9 is secreted into the media with a native N-terminal, PZ 1, 4, 9, and 17 (Positive Colony Z single colonies 1, 4, 9, and 17) were saved.

In order to confirm presence of PGT 9 in pPICZA and pPICZAα vectors, 5ml of each overnight culture was used to carry out minipreps to isolate the vectors containing the PGT 9 insert. The recombinant vectors were digested with corresponding restriction enzymes to confirm the presence of the modified PGT 9 inserts in the pPCIZA and pPICZAα vectors. For digestion of 5μl of the PX S. C. miniprep samples (strategy 1), 1μl of Kpn I (10μg/μl) and 1μl of Apa I (10μg/μl) restriction enzymes, 2μl of dH$_2$O and 1μl of 10 X promega buffer A were mixed and

incubated at 37°C for 4 hours. Aliquots of 5µl of PY and PZ miniprep samples were also digested. For PY, 1µl of Kpn I (10µg/µl) and 1µl of Xba I (10µg/µl) was used while for the PZ samples, 1µl of Xho I (10µg/µl) and 1µl of Xba I (10µg/µl) was used. To ensure complete digestion, 1µl of each enzyme was added to each digestion reaction after 2 hours and then incubated again for another 2 hours. Digestion reactions were terminated by incubation of the reactions at 65°C for 5minutes. The digested samples were then analyzed on a 0.8% agarose gel containing EtBr as previously described to confirm presence of PGT 9 inserts in the pPICZA and pPICZAα vectors and if they corresponded to the right sizes. A 30µl aliquot of each of the 4 PX samples was sent for DNA sequencing as were PY A and C samples and PZ 1 and 9 samples. Samples were sequenced at University of Tennessee, Knoxville Sequencing Facility. P.X 8 S. C 6 (X6), PY A, and PZ 1 were selected for transformation into yeast.

Transformation of Recombinant PGT 9 and Empty Vectors into *Pichia pastoris*

Plasmid Midiprep of pPICZA, pPICZAα, and Recombinant Vectors Containing PGT 9

For transformation into yeast, 5-10µg of DNA is required in a maximum volume of 10µl according to the Easy select Pichia manual (Invitrogen). Selected positive colonies were grown overnight in 50ml of selective low salt $LB_{zeocin\ (25mg/L)}$ media at 37°C while shaking at 250 rpm. A midiprep was carried out using 40ml of the overnight culture using the Quantum Prep Plasmid Midiprep Kit according to manufacturer's instructions except that 30s spins were extended to 60s to ensure complete elution. Elution was performed using 600µl of sterile dH_2O. A 2µl and 5µl aliquot of each midiprep was analyzed on a 0.8% get containing EtBr as previously described. Samples were run alongside quantitative DNA markers as previously described. The intensities

of the midiprep bands were compared with that of the intensities of the bands of the 1kb plus

exACTGene DNA ladder to determine the concentration of DNA in each sample (Table 9).

Table 9. DNA Concentration from Quantified Midipreps of Recombinant PGT 9 and Vectors

Vector	µg/600µl	PGT 9 Insert	(µg/600µl)
pPICZA	16.89	X6	18.0
pPICZAα	10.8	Y2	14.4
		Z13	12.3

Linearization, Purification, and Concentration of Plasmid DNA

A 300µl aliquot of each plasmid was linearized using Sac I. The Sac I restriction enzyme

cuts once in the 5' AOX 1 region of the vector to linearize the empty and recombinant vectors

prior to transformation into yeast. Sac I was selected based on the restriction mapping of PGT 9

that confirmed there are no internal Sac I sites within the PGT 9 sequence. Each digestion

reaction consisted of 300µl of midiprep, 30.3µl of buffer J, and 3µl of Sac I. The digestion

reaction was incubated at $37^{o}C$ for 2 hours after which an additional 3µl of Sac I was added and

incubated for another 2 hours to ensure complete digestion. Digestion was terminated by

incubating the digestion reaction at $65^{o}C$ for 5 minutes. A 2µl aliquot was analyzed on a 0.8%

agarose gel containing EtBr to confirm complete linearization of each construct.

Phenol: chloroform extraction and ethanol precipitation were performed to get the

required concentration of DNA and also to get rid of contaminating proteins in the sample.

Phenol:chloroform was prepared in a ratio of 1:1 as described in the appendix and stored at $4^{o}C$.

An equal volume (320µl) of the phenol: chloroform was added to each of the linearized plasmid

samples and vortexed for 10s. It was then centrifuged for 60s at 10,000 X g in microcentrifuge at room temperature. The top (aqueous) phase was collected and transferred to a new microcentrifuge tube and an equal volume of chloroform added. It was vortexed again and centrifuged for 1minute and the top (aqueous) phase was collected and transferred into a new tube. After the phenol:chloroform extraction, ethanol precipitation of DNA was carried out. First, 1/4 volume of 7.5M ammonium acetate was added to the aqueous phase and then 2 volumes of ice cold 100% ethanol was added, the sample gently mixed and incubated at -80°C for 15 minutes. The sample was centrifuged at 10,000 X g for 15 minutes at 4°C. The ethanol was removed and the DNA resuspended in 250µl of ice cold 80% ethanol. The sample was centrifuged again at 10,000 X g at 4°C for 10 minutes. The orientation of the tube in the centrifuge was noted in order to keep track of the DNA pellet. The ethanol was decanted gently, the DNA pellet dried in a speed vac, and the pellet resuspended in 10µl of sterile dH$_2$O.

In order to determine the concentration of DNA, a 1:100 dilution using 0.5µl of each concentrated DNA sample was carried out. A 2µl and a 5µl aliquot were analyzed alongside quantitative markers on a 0.8% agarose gel as previously described. This also helped to confirm the sizes of the empty vectors and the vectors containing the PGT 9 inserts. When desired concentrations of 5-10ug/5-10ul of water were not achieved, more plasmid DNA was linearized, purified (phenol:chloroform), and concentrated (ethanol precipitation). The sample that did not have the required DNA concentration for transformation was used to resuspend the newly concentrated sample to raise the concentration of the DNA sample.

Transformation into *Pichia pastoris* by Electroporation

Competent yeast cells were prepared for this process. The *Pichia pastoris* strain X33 was grown overnight in YPD medium (see appendix) at 30°C, 250rpm. A 0.5ml aliquot of the overnight culture was used to inoculate 500ml of freshly prepared YPD medium. The cells were grown overnight at 30°C to reach an OD_{600} of 1.309. The culture was centrifuged 4°C for 5 minutes in a Sorvall RC-5B refrigerated super speed centrifuge (Fisher Scientific) at 1500 X g. The supernatant was discarded and the cells resuspended in 500ml of ice cold sterile water and centrifuged again to pellet the cells. This was followed by resuspending the cells in 250ml of ice cold water, centrifuged and the supernatant discarded. The pellet obtained from the previous step was resuspended in 20ml of ice cold 1M sorbitol and centrifuged. The pellet obtained was resuspended in 1ml of 1M sorbitol to get a final total volume of 1.5ml. The competent cells were saved on ice and used on the same day.

For the electroporation procedure, 0.2cm cuvettes were used as recommended by the Easy Select^{TM} Pichia manual (Invitrogen). A total of 5 cuvettes were incubated on ice, 3 of them to transform the different linearized vectors with the PGT 9 sequence and the two others for transforming empty vectors pPICZA and pPICZAα to serve as negative controls for subsequent rPGT 9 expression. For each transformation, a total of 8μg of DNA was used as shown in Table 10.

Table 10. DNA Concentration from Phenol:choroform Extraction and Ethanol Precipitation

Construct/Vector	Concentration (µg/µl)	Volume (µl) Used for Electroporation	Total DNA (µg)
X6	1.1	7.3	8µg
PY A	1.55	5.2	8µg
PZ1	1.55	5.2	8µg
pPICZA	1.15	7.0	8µg
pPICZAα	1.3	6.2	8µg

For each sample, 8µg of linearized DNA was mixed with 80µl of freshly prepared competent cells and transferred to the cold 0.2cm micropulser cuvette. This was incubated on ice for 5 minutes and then pulsed using a current of 1.5kV. Following the pulse, 1ml of ice cold 1M sorbitol was added to the cells and the mixture transferred into a sterile 15ml tube. The cells were then incubated at 30°C for 2hours without shaking for the cells to recover. Once cells had recovered, 25µl, 50µl, 100µl, and 200µl aliquots were evenly spread on selective low salt YPDS$_{zeocin (50mg/l)}$ plates (appendix). Competent cells acquire zeocin resistance as the vector contains the zeocin resistance gene. A negative control was done by transforming competent yeast cells with water and no vector. The plates were incubated at 30°C until colonies grew, which was on the 4th day. The plates with colonies were stored at 4°C and PCR screens were carried out to confirm that the transformation process was successful.

PCR Screening of Pichia Transformants

To confirm the insertion of PGT 9 constructs and vectors into the yeast genome, 17 colonies were selected at random for each strategy and PCR screened using clone-specific primers. The PCR screens were carried out as previously described but with some modifications.

Using a sterile toothpick, colonies were selected and inoculated in 5µl of sterile dH$_2$O in a 1.5ml centrifuge tube. After inoculation, the sterile toothpicks were used to streak another YPDS$_{zeocin (50mg/l)}$ plate to save replicates. Yeast cells were not lysed by boiling as was the case with the *E.coli* cells. To lyse the yeast cells, a 1.5µl aliquot of 6U/µl solution of lyticase was added to the resuspended yeast cells and incubated in a 30°C water bath for 10 minutes. The samples were then frozen at -80°C for 10 minutes. A 1µl aliquot of each sample was used as template in the PCR screens. A master mix was prepared as previously described and 9µl of the mix was placed into 18 different PCR tubes. For each tube, 1µl of the template was then added. One tube received 1µl of water to serve as a negative control. The PCR cycles used was as previously described (Table 3). PCR reactions were analyzed on a 0.8% agarose get stained with ethidium bromide as previously described.

Positive colonies were T-streaked on YPDS$_{zeocin (50mg/l)}$ plates to get single colonies that were then grown overnight in YPDS$_{zeocin (50mg/l)}$ medium and 500µl of culture was saved at -80°C in 50% glycerol. Five colonies were grown and saved for each cloning strategy as follows: for PGT 9 in pPICZA the samples were labeled X6[1] - X6[5] and their duplicates labeled X6[1]^o- [5]^o; for PGT 9 in pPICZAα to be expressed and secreted with 14 extra amino acids the samples were labeled PY A[1]- PY A[5] and their duplicates labeled PY A[1]^o- [5]^o; for PGT 9 in pPICZAα to be expressed and secreted with a native N-terminal the samples were labeled PZ [1]-[5] and their duplicates labeled PZ [1]^o- [5]^o; for yeast cells transformed with just the empty

pPICZA vector the samples were labeled pPICZA [1]-[5] and their duplicates pPICZA [1]^o-[5]^o; for yeast cells transformed with empty pPICZAα vector the samples were labeled pPICZAα [1]-[5] and their duplicates pPICZAα [1]^o-[5]^o.

Expression of Recombinant PGT 9 in *Pichia pastoris*

Overnight culture from the glycerol stock of the positively transformed yeast cells for strategy 1 (PGT 9 in pPICZA) was carried out using the stock labeled X6[1]. A sterile toothpick was used to obtain a sample from the stock and inoculated in 250ml freshly prepared BMGY media (see appendix) and incubated at 30°C overnight at 250rpm to generate biomass for induction of rPGT 9. Biomass for yeast transformed with pPICZA (pPICZA [1]) vector was also generated to act as a negative control for the expression of rPGT 9. Once the culture reached an OD$_{600}$ of 2.023 for X6 and 2.67 for the negative control (25hours), the cells were centrifuged in a Sorvall RC-5B refrigerated superspeed centrifuge at 3000 X g for 5 minutes at room temperature to pellet the cells. The cells were resuspended in BMMY media (see appendix) to an OD$_{600}$ of 1.0 and 1ml aliquots collected from both samples as preinduction samples were centrifuged at 10,000 X g for 3 minutes and saved at -80°C in a 1.5ml microcentrifuge tube. The remaining sample was treated by adding 100% methanol to reach a final concentration of 0.5% to induce protein expression. For example, cells for expression of rPGT 9 (X6) were resuspended in a total volume of 125ml and 625µl of 100% methanol was added. The negative control cells were resuspended in a total volume of 250ml of BMMY and 1.25ml 100% methanol was used for induction. Each induction was carried out in a 1000ml baffled flask that was closed with a double layer of cheesecloth. The 1000ml flask containing the induced cells was incubated at 30°C for 4 days while shaking at 250rpm. Samples (1ml) were collected every 6 hours for the

first 24 hours. Induction was maintained by adding 100% methanol to a final concentration of

0.5% every 24 hours. After 24 hours, samples were then collected every 12 hours until the fourth

day. Thus 1ml samples were collected at 6, 12, 18, 24, 36, 48, 60, 72, 84, and 96 hours

postinduction. The 1ml samples were centrifuged at 10,000 X g and the cells stored at -80^0C for

subsequent analysis.

Sample Preparation for Analysis

       The preindution and postinduction samples collected at the different time intervals were

prepared for analysis by SDS PAGE and Western blotting. Cells were thawed and resuspended

in 100µl of breaking buffer (50mM sodium phosphate, 1mM PMSF, 1mM EDTA, 5% glycerol,

5mM pH 7.4). Cells were broken using acid washed beads. To the cells resuspended in 100µl of

breaking buffer, an equal volume of 0.5mm acid washed beads was added. The sample was then

vortexed for 30s, incubate on ice for 30s and the cycle was repeated 8 times. The samples were

centrifuged at 4^oC for 10 minutes at 10,000 X g. The supernatant, which should contain soluble

rPGT 9, was transferred into a new 1.5ml microcentrifuge tube and kept on ice.

SDS-PAGE and Western Blot Analysis of Samples

       A total of 50µl of each sample was used for this analysis. The 50µl samples were

transferred into new microcentrifuge tubes and 50µl of 2X dye was added to the samples. The

samples were incubated in a boiling water bath for 10 minutes and centrifuged at 10,000 X g for

1 minute. Samples prepared consisted of both X6 transformed yeast cells and pPICZA

transformed yeast cells (negative control). Samples were then analyzed by SDS PAGE as

previously described with slight adjustments. From the prepared sample, 20µl was obtained and

run on the gel alongside 5µl of the broad range marker. Samples were run alongside the negative control samples corresponding to the times postinduction. Two gels were prepared: one gel was stained with coomassie blue stain to observe and see if there was expression of the rPGT 9 in the yeast cells transformed with the PGT 9 sequence against the negative control lanes, and one gel was used for Western blot analysis.

Western blot analysis was carried out as previously described with slight adjustments. After transfer of proteins from the gel to the nitrocellulose membrane, the membrane was blocked for 1 hour using blocking buffer prepared with 1X PBS (see appendix). Washes were carried out with 20ml of 1X PBST and antibodies were also prepared using 1X PBST. The primary antibody used was anti c-myc monoclonal antibody directed against the c-myc epitope at the C-terminal of rPGT 9. It was prepared by adding 2µl of antibody to 10ml of PBST (1:5000). Incubation with primary antibody was carried out at room temperature for 2 hours. Anti-goat antimouse IgG AP was used as secondary antibody diluted 1X PBST. A 1:10000 dilution (1µl of antibody in 10ml of 1X PBST) was used and incubation was for 1 hour. Development of nitrocellulose membrane for AP was done as previously described.

Scale-Up of Expression, Desalting, and Enrichment of rPGT 9

Based on the test inductions, 6 hours postinduction with methanol was chosen as the optimal time for sample collection. Cells were collected 6 hours postinduction, divide into 2 samples and centrifuged at 3000xg for 5 minutes using the Sorvall RC-5B refrigerated super speed centrifuge at 25°C. The supernatant was discarded and the cell pellet was kept on ice. Cells were resuspended in 6ml of breaking buffer (50mM sodium phosphate, 1mM PMSF, 1mM EDTA, 5% glycerol, 5mM βME, pH 7.4).

Cells were lysed using French press (3 times at 2000psi) and centrifuged in a Sorvall RC-5B refrigerated super speed centrifuge at 13000xg for 20 minutes at 4°C. The supernatant was collected and kept on ice and the pellet was discarded.

A pre-packed PD-10 column was equilibrated with cold 25 ml of equilibration/wash buffer (50mM sodium phosphate, 300mM sodium chloride, 5mM BME, pH 7.5) at 4°C. The column was not allowed to run dry by capping it when there was about 2ml of equilibration wash buffer left at the top of the column. The remaining equilibration buffer was run out immediately before the sample was applied to the column.

A total sample volume of 2.5ml was applied to the column and the first 2.5ml of flow-through was collected and discarded. Wash buffer (50mM sodium phosphate, 300mM sodium chloride, 5mM BME, pH 7.5) was added to the column and the first 3.5ml of eluate was collected and saved on ice. The column was rinsed with 25ml of wash buffer (50mM sodium phosphate, 300mM sodium chloride, 5mM BME, pH 7.5) and then with 25ml of water. The column was saved at 4°C for future use. A total of 3.5ml desalted eluate sample was obtained from the PD-10 column.

A 4 ml bed volume immobilized metal affinity column (IMAC) was used for rPGT 9 enrichment. The packed column was equilibrated with 40 mls of equilibration buffer (50mM sodium phosphate, 300mM sodium chloride and 5mM BME, pH 7.5). The eluate sample from the desalting step was then run through the IMAC column adjusting the flow rate so that it took 15 minutes (minimum) for the sample to pass through the column (approximately one drop every 10s) to allow the rPGT 9 to bind to the column. Four fractions were collected and labeled flow-through (FT 1-4). A 1ml sample was collected for the first 3 fractions and 500μl for the fourth fraction. The column was then washed with wash buffer (50mM sodium phosphate, 300mM

sodium chloride, and 5mM BME, pH 7.5), and 2ml fractions were collected while monitoring the A_{280} until it approached zero (12 fractions were collected and labeled EW 1-12). Tightly bound rPGT 9 was eluted with 150 mM imidazole buffer (150mM imidazole, 50mM sodium phosphate, 300mM sodium chloride, 5mM BME, pH 7.5) and 2ml fractions were collected. A total of 10 fractions were collected and labeled HF 1-10.

Highly enriched 150mM fractions were collected and pooled together (fractions 1-4). The combined samples were transferred into a Centricon-30 and centrifuged in a Sorvall RC-5B super speed centrifuge using a swinging bucket at 4000 X g at 4°C until sample reached 500μl (sample was centrifuged for about 10 minutes). The sample was then resuspended in 2ml of 50mM phosphate buffer containing 14mM βME (pH 7.5) and centrifuged at 4000 X g at 4°C for 10 minutes. The sample was then resuspended in 2ml of 50mM phosphate buffer containing 14mM βME (pH 7.5) and transferred into a 2ml microcentrifuge tube on ice to be used for screening for GT activity. A 400μl aliquot of the desalted and concentrated rPGT 9 was saved in 20% glycerol (final concentration) at -20°C to test for the stability of the enzyme if activity was found. All fractions were analyzed by SDS-PAGE and Western blot after the enzyme assay had been carried out to confirm that the correct fractions (containing enriched rPGT 9) were used for the assay.

Screening Recombinant PGT 9 for Glucosyltransferase Activity

Substrate and ^{14}C-UDP Glucose Preparation

      The aglycone substrates to be tested were prepared to a stock concentration of 50nmols/5µl in ethylene glycol monomethylether. The substrates used, their molecular weights, and amount used are listed in Table 11. Once each substrate was used, the remainder was tightly capped, sealed with parafilm, and stored at -20°C in a non-self-defrosting freezer. The molecular weight of each compound used is shown in Table 11.

Calculation:

    50nmols x molecular weight of substrate = mass required/ 5µl of 100% ethylene glycol

                              monomethylether.

      UDP-^{14}C glucose was diluted to 20,000cpm/10µl in 50mM phosphate buffer of pH 7.5 from a stock of 50µCi/2.5ml. A 0.451µl aliquot UDP-^{14}C glucose stock was added to 9.55µl buffer to obtain 20,000cpm/10µl.

Table 11. Flavonoid Substrates Used for Recombinant PGT 9 Enzyme Assay and their Molecular weights.

Class of flavonoid	substrate	Molecular Weight
Chalcone	2',4,4',6' tetrahydroxychalcone	272.25
Flavanone	Naringenin	272.25
	Hesperitin	302.2
	Eriodictyol	288.25
	Isosakuranetin	286.28
	Prunin (Naringenin 7-O-glucoside)	434.4
Flavone	Apigenin	270.25
	Luteolin	286.28
	Diosmetin	300.26
	Scutallerein	286.24
	Luteolin-7-O- glucoside	448.39
Flavonols	Kaempferol	286.24
	Quercetin	338.3
	Fisetin	286.24
	Gossypetin	318.14
	4' methoxyflavonol	268.27
Isoflavone	4'-acetoxy-7-hydroxy-6-methoxy isoflavone	326.31
Phenolics (Coumarins)	Umbelliferone	162.15
	Esculetin	178.15
Simple Phenolics	2',4' dihydrobenzaldehyde	138.12

<u>Glucosyltransferase Screening Assay for rPGT 9</u>

GT activity was screened adapting the method of McIntosh et al. (1990). For the enzyme reaction, 5µl containing 50nmoles substrate, 10µl of UDP-[U-^{14}C] glucose (20,000cpm/10µl), 10µl of concentrated rPGT 9 and 50µl of 50mM phosphate buffer (pH 7.5) containing 14mM βME were combined in a microcentrifuge tube to make a final reaction volume of 75µl. This was carried out in duplicates. Each reaction was incubated at 37°C for 15 minutes. Each reaction was terminated by adding 15µl 6M HCl.

Two negative controls were carried out. One set was run without rPGT 9 and 1 with denatured rPGT 9. rPGT9 was denatured by boiling for 10 minutes in a water bath. The negative control with denatured rPGT 9 had 15µl of 6M HCl added before adding 10µl of rPGT 9 to the reaction. A positive control was carried out by using young grapefruit leaf extract as an enzyme source because young leaves have been shown to be a rich source of GT activity. The leaves were ground in 50mM phosphate buffer (pH 7) containing 14mM BME. A 50µl aliquot of the extract was added to 10µl of 50mM phosphate buffer, 10µl of UDP-[U-^{14}C] glucose (20,000cpm/10µl) and 5µl of naringenin (50nmol/5µl). To test if the concentrated enzyme fraction from yeast had any inhibiting effects on the reaction, another positive control was carried out with grapefruit leaf extract and 10µl of rPGT 9 in place of the 50mM phosphate buffer. After stopping the reaction as previously described, 250ul of ethyl acetate was added and vortexed vigorously for 10 seconds. A 150ul aliquot of the organic phase (upper phase) was collected, 2ml of cytoscint scintillation fluid was added and the samples counted using a Beckman LS 6500 scintillation counter. As only 150µl of the ethyl acetate layer was counted, the formula below was used to calculate the total cpm of UDP-^{14}C glucose incorporated in 250µl:

Measured CPM/150µl = Xcpm /250µl to get the total CPM in 250µl.

For the water soluble simple phenolics, reverse-phase (RP) HPLC was used. The reaction was set up with 35µl of the concentrated rPGT 9, 20µl of cold UDP-glucose (100nmols), and 5µl of aglycone substrate (50nmols). The reactions were incubated in a 37°C water bath for 30 minutes. The reaction was stopped by adding 200µl of methanol to the reaction. The samples were then centrifuged at 10,000 X g for 15 minutes to precipitate the proteins. The supernatant for each sample was transferred into a new tube and dried using a Speedvac concentrator from Thermo Electron Corporation (Waltham, MA). The pellets obtained were resuspended in 40µl of milli Q water and analyzed by C-18 RP HPLC with isocratic elution. Flow rate was 1ml/min. using Waters 1525 Binary HPLC Pump ,Waters 2487 Dual λ Absorbance Detector, and Waters Symmetry® C18 column (5µm, 4.6 × 150 mm) from Waters (Milford, MA). Elution strategy used for each substrate is shown in Table 12. The wavelengths used for detection correspond to the λmax of the aglycones.
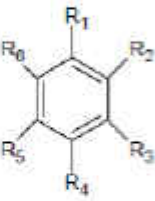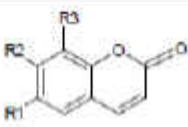
<u>Cloning of PGT 11 into pPICZA for Transformation into *Pichia pastoris*</u>

Primer Design and Cloning Strategy

PGT 11 consensus sequence was also identified from the harvEST database and successfully amplified from a young grapefruit leaf cDNA library (Mallampalli, 2009). It was cloned into TOPO vector and transformed into *E. coli* (Mallampalli, 2009). In this current study, primers were designed to modify the ends of PGT 11 to introduce restriction sites present in the multiple cloning sites of pPICZA. Two primers were designed for this purpose. The sense primer was designed to introduce the yeast consensus sequence and the Kpn I restriction site. In order to

introduce the yeast consensus sequence, 2 amino acids would have to be added to the N-terminus

of the rPGT 11 after it is expressed (Figure 16).

Table12. Structures of Aglycones and HPLC Conditions Used for Analysis of Different Simple Phenolic Substrates (adapted from Ahmad and Hopkins 1993; Lin 2011 and ref. therein).

Compound	Structure	Substitution Pattern	Detection Wavelength (nm)	Mobile Phase
Gentisic acid		R1=COOH R2=R5=OH	330	10% ACN
Vanillic acid		R1=COOH R3=OCH3 R4= OH	262	
Vanillin		R1=CHO R3=OCH3 R4=OH	273	
Catechol		R2=R3=OH	280	15% ACN
P-Hydroxybenzoic acid	R_1, R_6, R_2, R_5, R_3, R_4	R1=COOH R4=OH	255	15% MeOH
P-hydroxyphenyl-pyruvic acid		R1=CH3COCOOH R4=OH	235	
P-hydroxyphenyl-acetic acid		R1=CH3COOH R4=OH	215	
Caffeic acid		R1= -CH=CHCOOH R5=R6=OH	322	30% MeOH
Ferulic acid		R1= -CH=CHCOOH R3=OCH3 R4=R5=OH	324	
O-Coumaric acid		R1= -CH=CHCOOH R3=OH	325	
P-Coumaric acid		R1= -CH=CHCOOH R5=OH	308	
Scopoletin	R3, R2, R1	R1=OCH3 R2= OH	340	

Note. Mobile phases were diluted in 0.1M sodium phosphate buffer (pH 3.0). ACN: Acetonitrile. MeOH: Methanol.
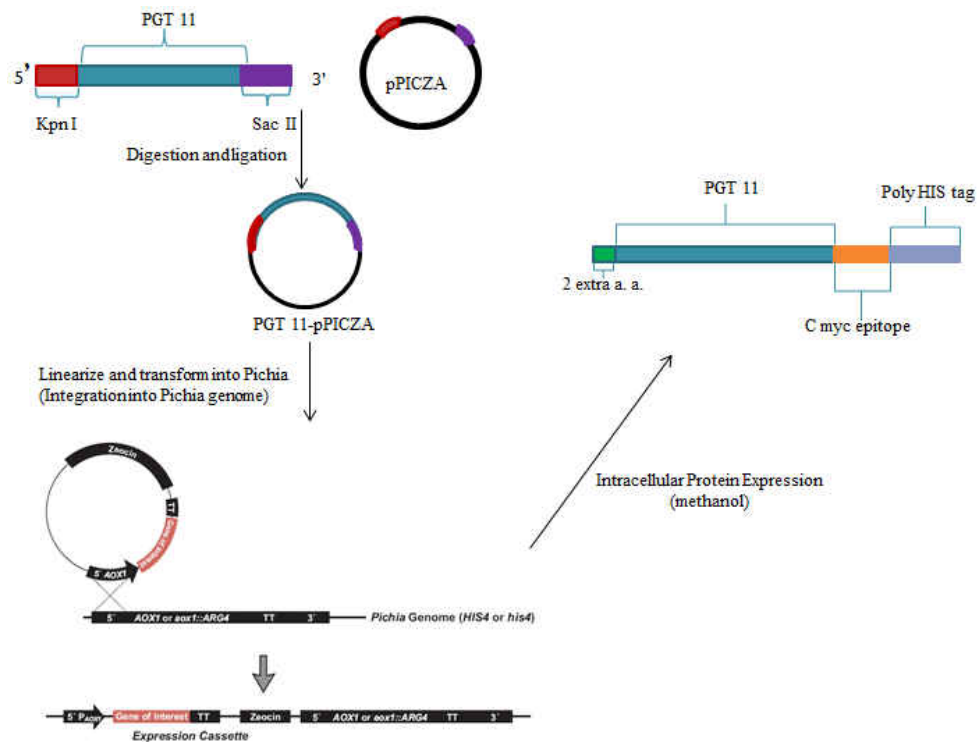
Figure 16. Cloning Strategy for PGT 11 into pPICZA Vector. Recombinant PGT 11 will be expressed and secreted with 2 extra amino acids at the N-terminus (adapted from Invitrogen manual).

The antisense primer was designed to introduce the SacII restriction site and also to take off the stop codon of PGT 11 such that it would be recombinantly expressed with a c-myc epitope for detection and a polyhistidine tag (6X His tag) for purification. Primer designs are shown in Table 13.

Table 13. Primers, Tm, and Restriction Sites Introduced for Cloning PGT 11 into pPICZA.

Sense primer	Antisense primer
(CSP138F) (50^{0}C)(KpnI restriction site) 5' CGGGGTACCGCCATG GCCATGAGCAGTCCCCATGTTGTG 3'	(CSP139R)(52^{0}C)(SacII restriction site) 5' CCTCCGCGGTGCGGGCCATTGCTTG 3'

Green=linkers, purple=restriction sites, red= original PGT 11 start codon, Blue= bases introduced to obtain the yeast consensus sequence [(G/A) NNATGG] as instructed by the user manual. NB. Blue sequence introduces a new start codon adding 2 amino acids to the N-terminal of PGT 11 when expressed.

Plasmid (PGT 11-TOPO) DNA Isolation

In order to modify ends of PGT 11, Top 10 *E. coli* cell containing PGT 11 in pCR4 TOPO were cultured in 10ml of LB $_{amp(100mg/L)}$ [Luria-Broth media containing ampicillin at 100 milligrams/liter] overnight at 37°C, 250 rpm. The next morning, a plasmid miniprep was carried out following the QIAprep Spin Miniprep kit protocol. A total of 500ml of low salt LB$_{zeo\ (25mg/l)}$ (see Appendix B) was also prepared for culture of TOP 10 cells containing pPICZA. A 10ml aliquot of this media was inoculated with the TOP 10 *E. coli* cells and cultured overnight at 37°C, 250rpm and plasmid miniprep was carried out the following morning. A 2µl aliquot of each miniprep sample was mixed with 3µl of water and 1µl of 6X dye. The sample was run on a 0.8% agarose gel at 100V for 30 minutes to confirm successful isolation.

PCR Modification of 5' and 3' Ends of PGT 11

A stock concentration of 200µM for each primer was prepared by centrifuging the tubes containing the primers in an eppendorf centrifuge for 1minute at 10,000 X g and then adding the appropriate amount of sterile distilled water. A 20µM working solution (final volume of 50µl) was prepared by adding 5µl of stock solution to 45µl of sterile distilled water. The 200µM stock and 20µM working solutions of primers were stored at -20˚C.

Isolated plasmid DNA (PGT 11-TOPO) was used as the template for PCR amplification using clone-specific primers as shown in Table 12. PCR tubes contained 2.5µl of 20µM solution of sense and antisense clone-specific primers (CSP), 2.5l of a 1:1000 diluted template, 1ul of Taq polymerase, 10µl of 5X colorless buffer and 3lµl of sterilized deionized water. PCR steps, temperatures, and cycles are shown in Table 14. A 2µl aliquot of PCR products were analyzed using a 0.8% DNA agarose gel stained with ethidium bromide.

Table 14. PCR Steps Used in the Modification of 5' and 3' ends of PGT 11.

Step	Temperature (^{o}C)	Time (seconds)	# of Cycles
Initiation	94	60	1
Denaturation	94	30	30
Annealing	50	30	
Elongation	72	120	
Final Elongation	72	300	1

TOPO Cloning of PCR Modified PGT 11

The rPGT 11 PCR product was cloned into pCR®4-TOPO vector using the TOPO TA Cloning Kit (Invitrogen) according to manufacturer's instructions. The TOPO cloning reaction consisted of 4μl aliquot of modified PGT 11 PCR products, 1μl of salt solution, and 1μl of TOPO vector and the reaction was incubated for 30 minutes at room temperature. The TOPO cloning reaction was subsequently transformed into One-Shot Top 10 competent *E. coli* cells as follows: a 2μl aliquot of the TOPO cloning reaction was transferred into tube containing 50μl competent cells. The procedures for transformation were carried out following the manufacturer's instructions (Invitrogen).

For the positive ligation control, 1μl of pUC19 was added to 50μl of One-Shot competent cells. After transformation, 250μl; of S.O.C medium was added to each transformation reaction. The reactions were then incubated at 37°C at 225 rpm for 1hour and 100μl of culture containing transformation reaction was spread onto each LB$_{amp(100mg/L)}$ plate. For the pUC19 control, the

transformation was diluted 1:10 into LB$_{amp(100mg/L)}$ liquid media and 100µl was spread on each LB$_{amp(100mg/L)}$ plate. Plates inoculated with the transformation samples were incubated overnight at 37°C.

Individual colonies from the PGT 11-pCR4 TOPO transformed plates were carefully picked using sterile toothpicks and streaked onto plates containing LB$_{amp(100mg/L)}$ media to make replicates. Each toothpick was then placed into a sterile 0.5ml eppendorf tube containing 100µl of sterile distilled water. The samples were boiled for 5 minutes to lyse the cells and 1µl of cell lysate was added to 9µl of PCR master mix. The PCR master mix contained the following: 4µl of 10mM dNTPs, 10µl of 20µM T3 sense primer, 10µl of 20µM T7 antisense primer, 40µl of 5 X green Go-Taq buffer, 94µl of sterile dH20, and 2µl of Taq polymerase. The conditions for the PCR reaction were as previously described. Sterile dH$_2$O was used in place of cell lysate as a negative control. The PCR products were analyzed using a 0.8% DNA agarose gel stained with ethidium bromide.

Three positive colonies from each plate were T-streaked on selective LB$_{amp(100mg/L)}$ agar plates to obtain single colonies. Single colonies were selected from the streaks, inoculated in 25ml of LB$_{amp(100mg/L)}$, and grown overnight at 37°C at 250 rpm. From the overnight culture, 500µl was collected and added to 500µl of sterile glycerol and stocks were stored at -80°C. From the remaining culture, midipreps were carried out using the Quantum Prep Plasmid Midiprep Kit according to manufacturer's instructions except that 30s centrifugations were carried out for 60s to ensure complete elution. Elution of DNA sample was carried out with 600µl of sterile dH$_2$O. A midiprep for the pPICZA vector was carried out from TOP 10 competent *E. coli* cells that had been transformed with the vector and stored at -80^{0}C.

Restriction Digest of Insert and Vector

Digestion was also carried out using the Kpn I and Sac II restriction enzymes to confirm the presence of PGT 11 in the TOPO vector. A 2μl aliquot of each midiprep from sample positive colonies (1, 2, and 3) was digested. The digestion mix was made up of 2μl of plasmid, 1μl of buffer A, 1μl of each restriction enzyme, and 5μl of distilled water. This was incubated at 37°C for 2hours. Each digest was then analyzed on a 0.8% agaroase gel stained with ethidium bromide. A 2μl aliquot of the pPICZA vector was also digested using the same set of restriction enzymes. Once plasmids were confirmed to have the modified PGT 11, a 40μl aliquot of each sample was sent to be sequenced at University of Tennessee Knoxville Sequencing Facility. Sequencing results confirmed the presence of modified PGT 11 in TOPO vector. Colonies 1and 2 were selected for further work on PGT 11.

In order to carry out ligation reactions, larger scale restriction digest of the modified PGT 11 sequences and pPICZA were carried out (Table 15). From the midipreps, 16μl aliquots of modified PGT 11 in TOPO and pPICZA vector were digested with Kpn I/ Sac II restriction enzymes to obtain sticky ends for ligation. After incubating the digestion mix at 37°C for 2hours, 1μl of each enzyme was added to the reaction and incubated for another 2hours at 37°C. This was to ensure complete digestion of the inserts and vectors.

Table 15. Restriction Digest Set-Up for Modified PGT 11 and pPICZA

Insert/Vector (16μl of each)	Restriction Enzymes (1μl of each)	Buffer (2μl of each)
Colonies 1 and 2 (PGT 11-TOPO)	Kpn I/Sac II	A
pPICZA	Kpn I/Sac II	A

The digested samples were then run on a 0.8% agarose gel stained with ethidium bromide as previously described. PGT 11 and pPICZA vector digested bands were cut out of the agarose gel and gel- purified following the Promega Wizard SV Gel and PCR clean-up system. From the gel-purified bands, 2μl and 5μl of each insert and vector were loaded on an agarose gel as previously described alongside quantitative Lambda/Hind III and 1kb plus exACTGene DNA markers to determine the concentration of each and also to confirm the size of the insert and vectors. The concentrations of each gel purified digest were determined and recorded in Table 16. Colony 1 was selected for ligation reactions.

Table 16. Concentration of Digested pPICZA and PGT 11 Used for Ligation as Determined Using Quantitative DNA Marker.

Vector/ Insert	Concentration (ng/μl)
Colony 1 (PGT 11)	18.0
Colony 2 (PGT 11)	18.0
pPICZA	16.5

Ligation of PGT 11 with pPICZA

After determining the concentration of the vectors and insert, ligation were carried out using different molar ratios (insert:vector) of 1:1 and 3:1. The ligation mix was incubated at 15°C for 4hours. The different volumes used for the different ratios are recorded in Table 17.

The different volumes used in the ligation procedure were as previously described. A total of 100ng of vector was used (Promega catalog, Part# 9PIM180). The volume corresponding to this was calculated by simply dividing the required amount (100ng) by the concentration of the vector.

Table 17. Reaction Mixtures for Ligation of pPICZA Vector and PGT 11 Insert.

Molar Ratio (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	1.2	13.2	2.2	2	3.4	22
3:1	3.7	13.2	2.2	2	0.9	22

After the ligation reactions were incubated, 2μl of the ligation products were transformed into Top10 *E. coli* cells to serve as a holding cell line. The procedure for transformation was as previously described. After adding 2μl of the cloning product to the 50μl of Top10 cells, it was incubated on ice for 30 minutes. The cells were then heat shocked at 45°C for 30 seconds and incubated on ice again for 2 minutes. Plain low salt LB $_{zeocin(25mg/l)}$ was used instead of SOC media due to high salt concentration in the SOC media which could inhibit the antibiotic zeocin leading to false positives. Cells were resuspended in 250ml of plain low salt LB media and incubated at 37°C in a vertical position for 1 hour while shaking at 225 rpm for the cells to recover. For the negative control, 1μl of pUC 19 was transformed into 50μl of Top10 cells and the heat shock process followed as described. After 1 hour of recovery, 100μl of the cells were evenly plated on pre-warmed LB$_{zeocin (25mg/L)}$ agar plates and incubated overnight at 37°C. A 1:10 dilution for the cells transformed with pUC 19 was carried out and a 100μl aliquot was evenly plated on low salt LB$_{zeocin (25 mg/L)}$ to serve as the negative control.

Single colonies were selected from the plate on which cells transformed with the 3:1 ligated product were grown. Six colonies were selected and a miniprep carried out as previously described. Samples were labeled Colony A-F. The miniprep samples were sent to be sequenced

at University of Tennessee Knoxville Sequencing Facility to confirm the presence of PGT 11 in pPICZA.

CHAPTER 3

RESULTS

<u>Optimization of Soluble Recombinant PGT 9 Expression in *E.coli*</u>

The expression of rPGT 9 in *E. coli* resulted in most of rPGT 9 being packed in inclusion bodies as an insoluble fraction (Mallampalli, 2009). Several conditions were varied such as media, temperature, and time of sample collection postinduction but insufficient soluble protein was obtained for GT activity screening (Figure 17). Osmotic stress has been shown to increase the amount of soluble protein expressed in *E. coli* for some proteins (Arakawa and Timasheff, 1985; Oganesyan et al, 2006). For example, betaine and sorbitol have been used as osmotic reagents to improve on the amount of soluble proteins produced (Blackwell and Horgan, 1991; Arakawa and Timasheff, 1985). These osmolytes were used in LB $_{amp(100mg/L), chlr (34 mg/L)}$ in an attempt to increase the amount of soluble rPGT 9. As a control, induction for the expression of rPGT 9 was also carried out in the absence of betaine and sorbitol. More soluble rPGT 9 was observed when induction was carried out with betaine and sorbitol (Figures 17-18). Therefore, subsequent inductions of rPGT 9 expression were carried out in the presence of betaine and sorbitol. However, most of the rPGT 9 was still trapped in inclusion bodies (Figure 17).

The effect of temperature on soluble rPGT 9 expression in the presence of betaine and sorbitol was also investigated. The time of sample collection postinduction was also varied. Results showed using media containing betaine and sorbitol at 25°C to be the optimum condition for the expression of soluble rPGT 9 in the *E. coli* system (Figure 18). The optimum time for sample collection was 6 hours postinduction. Results obtained for different expression experiments were not consistent with respect to the amount of soluble rPGT 9. Under optimum expression conditions, most of the rPGT 9 was still found in inclusion bodies (Figure 18).
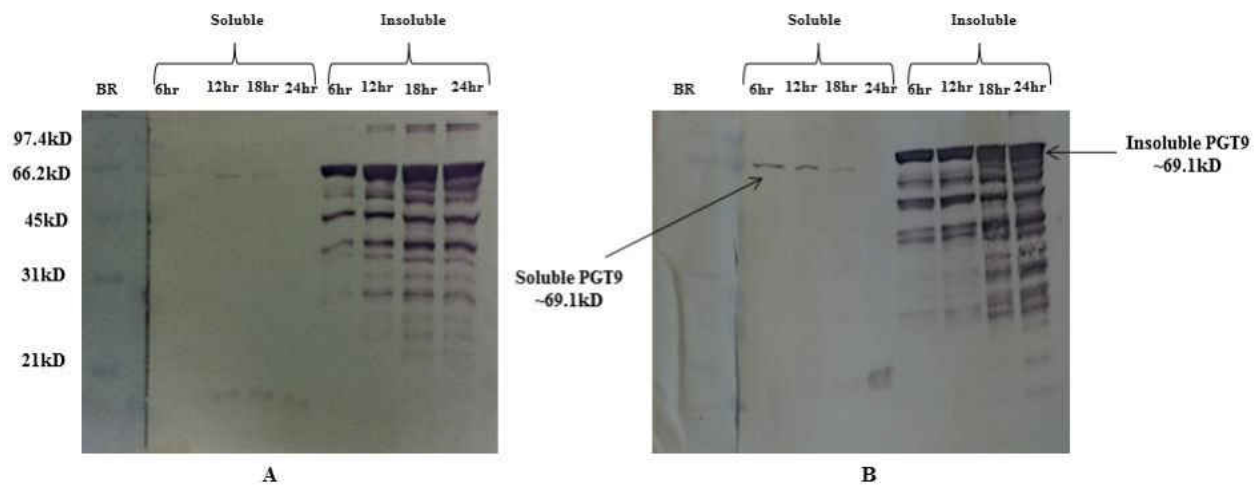
Figure 17. Western Blot Analysis of Samples Collected at Different Times Postinduction from *E. coli*. A. Induction in the absence of betaine and sorbitol. B. Induction in the presence of betaine and sorbitol. Both inductions were carried out at 25^0C. NB. Blot had to be over-developed to see soluble rPGT 9 bands hence the background noise seen on the blot. Most of rPGT 9 protein was trapped in inclusion bodies. BR. Broad Range Marker.

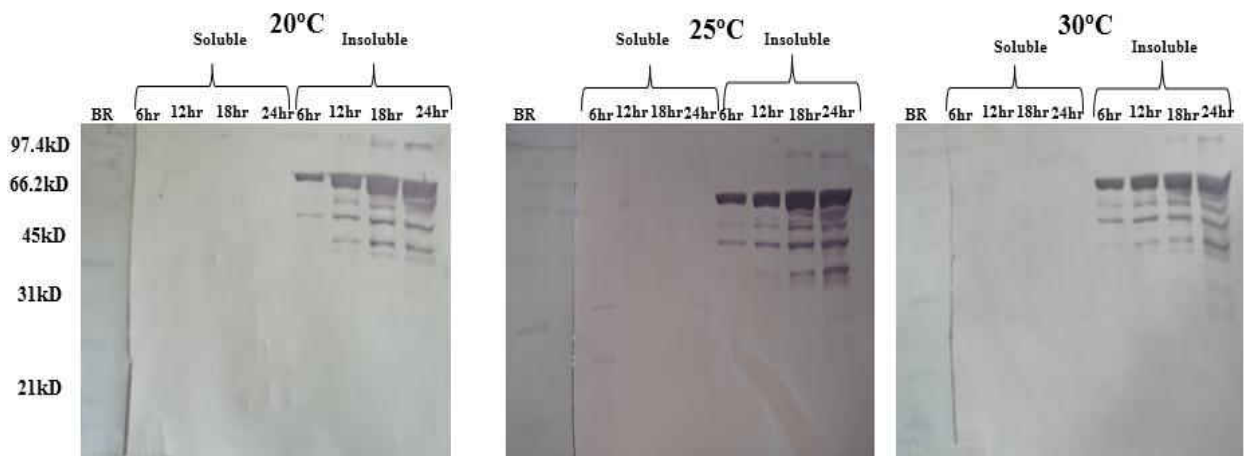Figure 18. Variation of Temperature and Time of Sample Collection Postinduction in the Presence of Betaine and Sorbitol in *E. coli*. From analysis, optimum condition for expression of soluble rPGT 9 was 6 hours postinduction at 25^oC in the presence of betaine and sorbitol. NB. Gels were over developed to see soluble rPGT 9 bands hence sufficient soluble rPGT 9 was not obtained. BR. Broad Range Marker.

Enrichment of Soluble Recombinant PGT 9 by IMAC

The optimal expression conditions for expression of soluble rPGT 9 and a larger cell

culture were used to obtain a larger sample of soluble protein for enrichment by immobilized

metal affinity chromatography (IMAC). Column fractions were analyzed by silver stained SDS-

PAGE gels and by Western blot analysis (Figure 19). Results show that there was still

insufficient soluble rPGT 9 obtained from this procedure to be able to screen for GT activity

(Figure 19 B). There also were many contaminating bands in the fractions in which soluble rPGT

9 was detected (Figure 19 A).
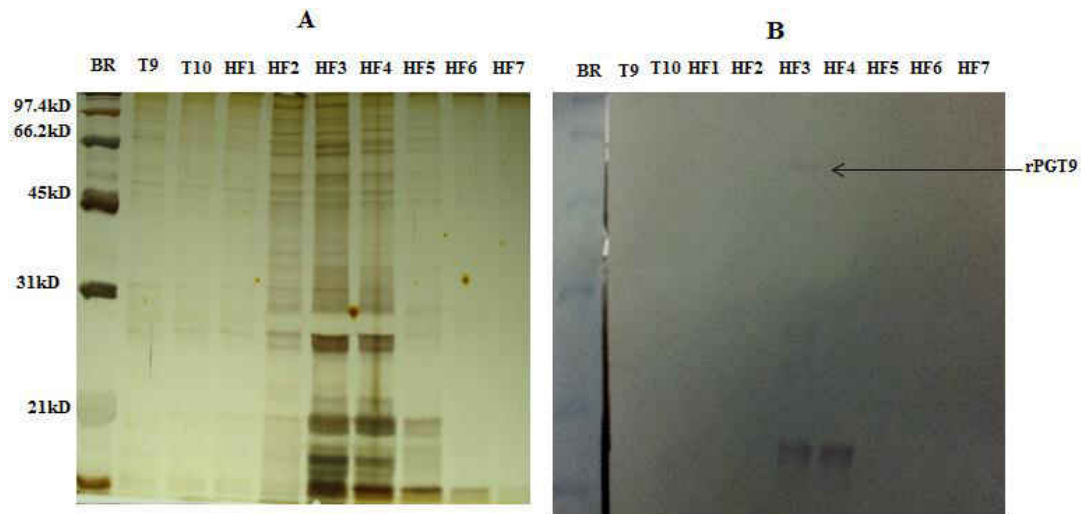
Figure 19. Enrichment of Soluble rPGT 9 by Immobilized Metal Affinity Chromatography. **A**.
Silver stained gel of fractions eluted with a 150mM (HF) imidazole buffer. T9-T10. Last two
fractions eluted with 10mM (T) imidazole. **B**. Western blot analysis of HF fractions. Soluble
rPGT 9 protein was detected in fraction HF3.

Plasmid DNA Extraction and PCR Modification of PGT 9

Because of issues with inclusion bodies when expressing rPGT 9 in *E. coli,* rPGT 9 was

cloned into pPICZA and pPICZAα vectors for transformation and expression in yeast. For

cloning of PGT 9 into pPICZA and pPICZAα vectors, unmodified PGT 9 in pCR®4-TOPO

vector was isolated from Top10 competent *E. coli* cells. PGT 9 was modified to have Kpn I/

ApaI restriction sites to be cloned into the pPICZA vector. PGT 9 also was modified to have Kpn

I / Xba I sites to be cloned into pPICZAα vector and PGT 9 was modified to have Xho I/ Xba I

sites for cloning into the pPICZAα vector using a different cloning strategy. The PCR modified

PGT 9 products were analyzed on a 0.8% agarose gel and the modified PGT 9 PCR product

corresponded to the expected size of 1470bp (Figure 20).
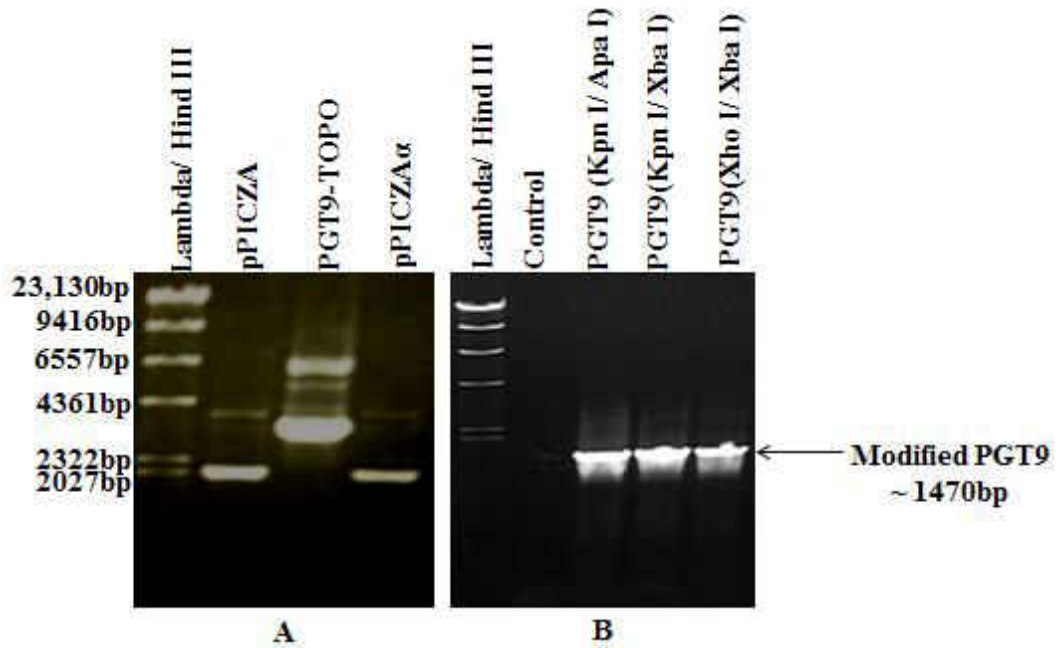
Figure 20. Agarose Gel Analysis of Miniprep of Unmodified PGT 9 and Vectors and PCR
Modified PGT 9. **A**. Miniprep of plasmid DNA (PGT 9 in TOPO vector), pPICZA and pPICZAα
vectors. A 2μl aliquot of each sample was loaded alongside Lamba/Hind III marker. **B**. PCR
modification of PGT 9 for cloning into pPICZA and pPICZAα vectors.

The modified PGT 9 sequences were then cloned into pCR®4-TOPO vector for

amplification and transformed into one-shot Top10 *E. coli* competent cells to serve as a holding

cell line. Single colonies obtained from the selective LB$_{amp(100mg/L)}$ plates were screened by PCR

reactions using the modified PGT 9 sequences as templates for the reaction as described in

methods. PCR screens of single colonies selected from selective LB$_{amp(100mg/L)}$ plates for each

modification strategy showed positive bands for all colonies (Figure 21). The amplified bands corresponded to the ~1470bp band of PGT 9 sequence that was expected (Figure 21). The plate with cells transformed with PGT 9 with Kpn I and ApaI restriction sites was labeled X, that with PGT 9 with Kpn I and Xba I was labeled Y, and the last plate having cells transformed with PGT 9 modified with Xho I and Xba I, was labeled Z. Only 7 colonies grew on plate Y and all were PCR screened. All colonies screened showed amplification of the PGT 9 gene. This suggests that ligation with TOPO vector and transformation into *E. coli* was successful.
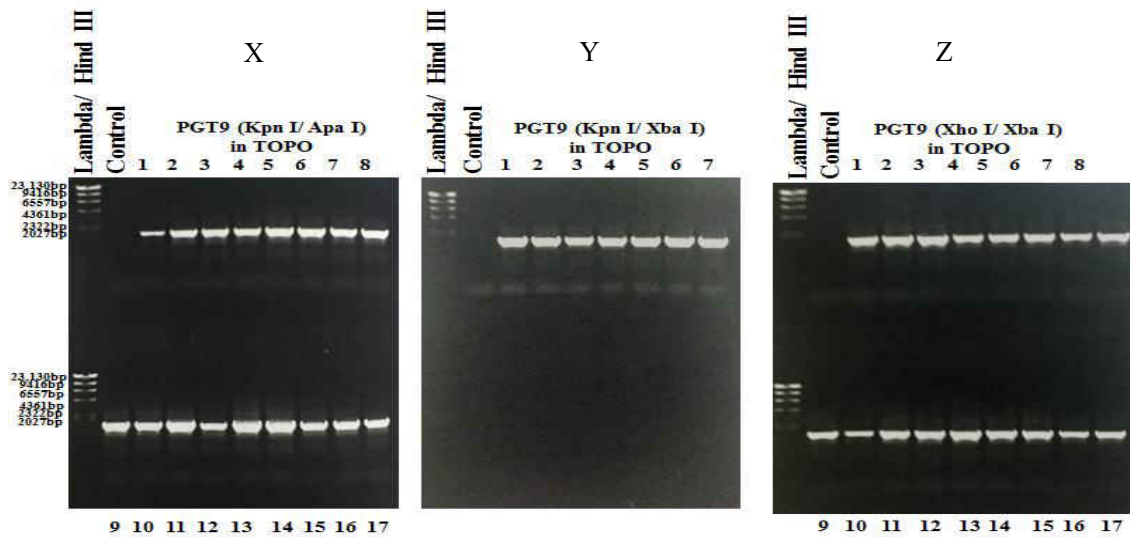
Figure 21. PCR Screen Results of *E. coli* cells Transformed with PGT 9 Modified to Have Different Restriction Sites. PGT 9 band of ~1470bp was amplified for all the different transformation.

Restriction Digests, Ligations, and Transformations of rPGT 9 into Top 10 Competent *E. coli*

In order to further confirm cloning of rPGT 9 into TOPO for subsequent cloning into pPICZA and pPICZAα vectors, *E.coli* colonies with positive PCR screen results were grown overnight in LB$_{amp(100mg/l)}$ media and the plasmid DNA isolated as previously described. Colony X6 (PGT 9 modified to have Kpn I/Apa I restriction site) was selected to be cloned in pPICZA. Colony Y2 (PGT 9 modified to have Kpn I/Xba I restriction sites) was selected to be cloned in

pPICZAα for extracellular secretion of rPGT 9 protein with a nonnative N-terminal (14 extra amino acids). Colony Z13 (PGT 9 modified to have Xho I/Xba I restriction sites) was selected to be cloned into pPICZAα for extracellular secretion of PGT 9 with a native N-terminal. Once the plasmid for each modified PGT 9 sequence was isolated, a restriction digestion reaction was set up as described in the materials and method section. Restriction enzymes with sites incorporated into PGT 9 were used in the reaction.

Results show the ~1470bp band corresponded to the PGT 9 sequence and the larger bands of 3956bp corresponded to the linearized TOPO vector (Figure 22 A). The linearized pPICZA and pPICZAα band corresponded to sizes of the linearized vectors which are 3329bp and 3593bp respectively (Figure 22 B). A 2µl and 5µl aliquot of each gel purified sample was run on an agarose gel and the concentration determined using 1kb plus exACTGene DNA quantitative ladder and Lambda/ Hind III marker (Figure 23). Concentrations are in Table 5.
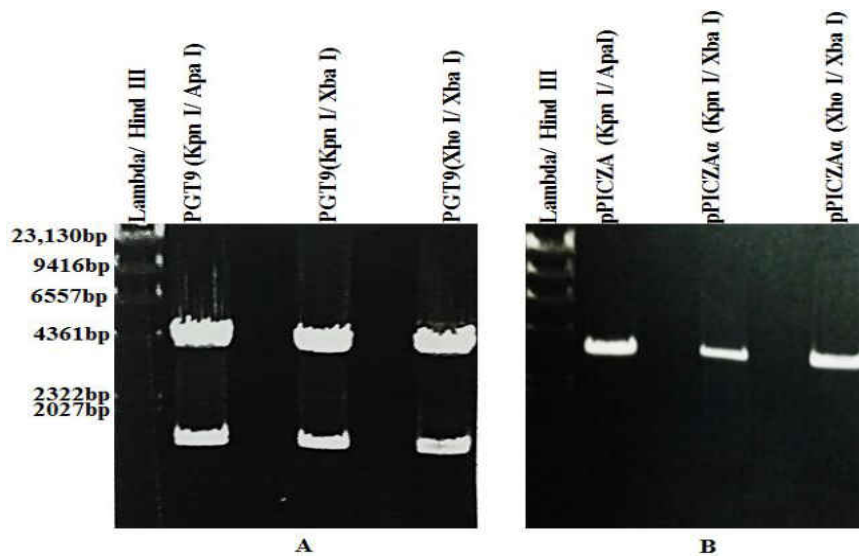

Figure 22. Restriction Digest of PGT 9 in TOPO and Empty pPICZA and pPICZAα Vectors. A. Complete Restriction Digest of Modified PGT 9 Sequences from TOPO Vector. B. Complete restriction digests of pPICZA and pPICZAα vectors for ligation with the corresponding digested PGT 9 sequences. Restriction enzymes used for each digestion are indicated.
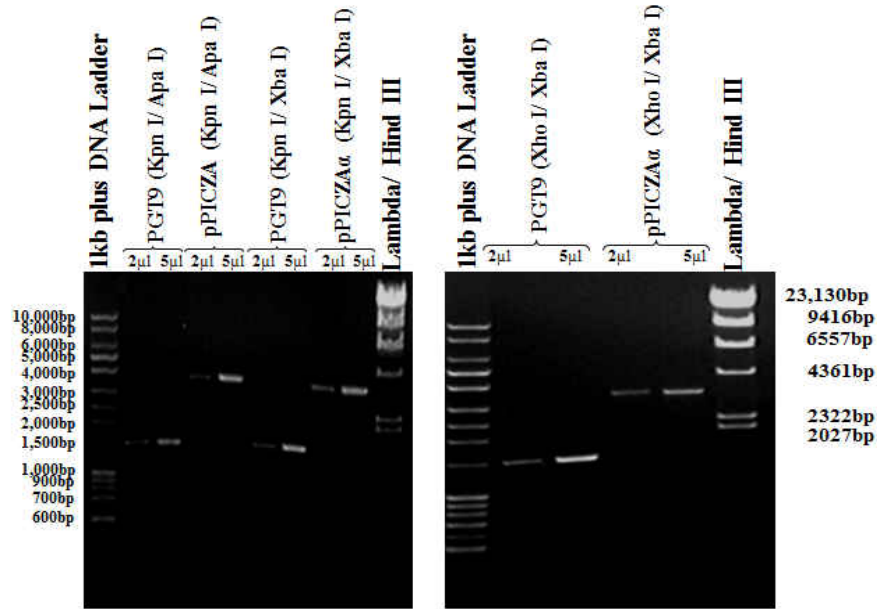
Figure 23. Digested and Gel Purified PGT 9, pPICZA and pPICZAα Vectors. Concentrations of each sample were determined by comparing the band intensities with that of the quantitative markers.

After determining the concentration of each sample, ligation was carried out at 15°C for 4 hours. The ligated products were then transformed into One Shot Top 10 *E. coli* cells. Successful ligation was confirmed first by PCR reaction using CSPs that were used for each modification. Bands corresponding to PGT 9 were observed for each cloning strategy (Figure 24). Miniprep was also carried out and the isolated plasmid digested with the corresponding pair of restriction enzymes. After digestion, the ~1470bp band was observed (Figure 25). Band sizes of 3,329bp and 3,593bp corresponding to pPICZA and pPICZAα, respectively, were also observed on the gel (Figure 25).
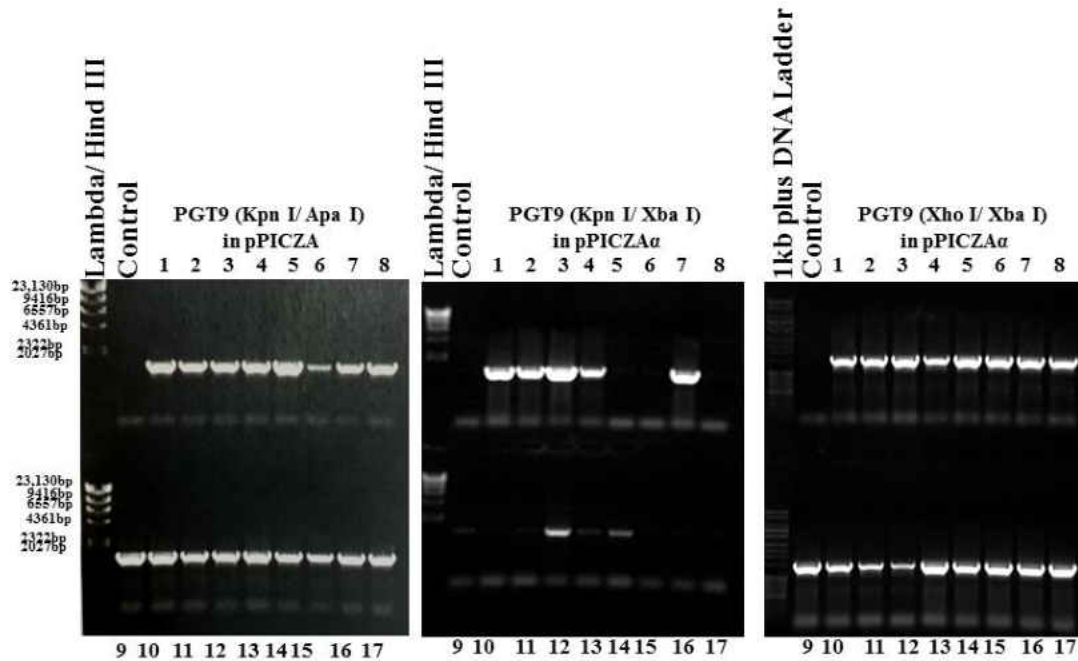
Figure 24. PCR Screen Results of Selected *E.coli* Colonies Transformed with Modified PGT 9 in pPICZA and pPICZAα Vectors. Clone specific primers used for each modification were used in the PCR screens. Ligation into pPICZA and pPICZAα as well as transformation into *E. coli* were successful.
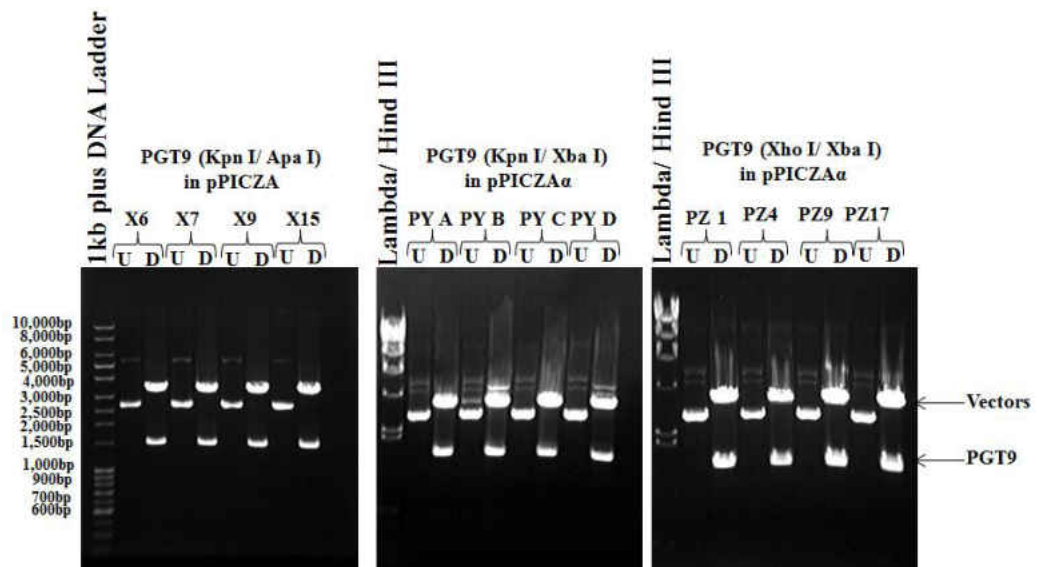
Figure 25. Digestion of Plasmid DNA Isolated from Positive PCR Screened Single *E. coli* Colonies for Each Modification Strategy. Results confirm the presence of PGT 9 in each vector. Bands resulting from digestion corresponded to pPICZA (3329bp), pPICZAα (3593bp) and PGT 9 (~1470bp).

Sequencing results confirmed the presence of PGT 9 sequence in both vectors in-frame with the c-myc epitope (for detection) and the 6X His tag (for purification). X6, PY A, and PZ1 were selected as colonies to be used for further work. Representative sequence alignments with original PGT 9 sequence for all three cloning strategies are shown in Figures 26-28.

Linearization, Purification, Concentration, and Transformation of rPGT 9 into *Pichia pastoris*

For transformation into yeast, 5-10µg of linearized DNA plasmid in 5-10µl of sterile deionized water was required. The pPICZA and pPICZAα vectors that contained the PGT 9 sequences were linearized using Sac I as described in the materials and methods section. Complete lineariz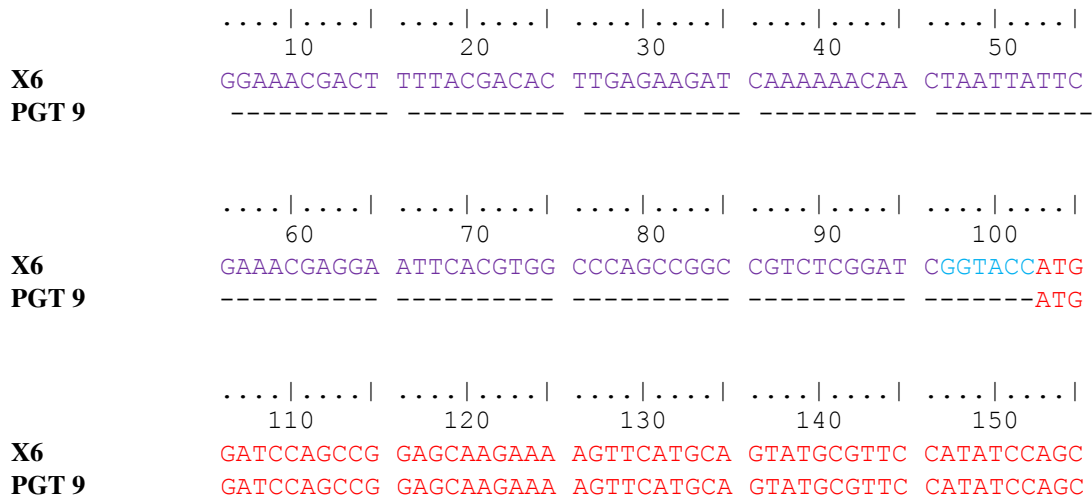ation was confirmed as previously described. Band sizes corresponded to the size of rPGT 9 as well as to the empty vectors (Figure 29). rPGT 9 from strategy 1 (PGT 9 in pPICZA) has an expected size of ~4799bp while rPGT 9 from strategies 2 and 3 (PGT 9 in pPICZAα vector) had expected sizes of ~5062bp. Once complete linearization was confirmed, the rest of the digest was treated with phenol: chloroform extraction and ethanol precipitation to remove contamination proteins and concentrate the sample as described in the materials and methods. Each sample was run on an agarose gel to confirm that the sample had been concentrated (Figure 29).

A 2µl and 5µl aliquot of each 1:100 diluted sample was run on the gel to determine the final concentration of DNA in the samples obtained after the phenol: chloroform extraction and ethanol precipitation (Figure 30). After the concentration was determined (Table 10), a volume corresponding to 8µg of DNA was used for the yeast transformation process. The transformed cells were then grown on selective YPDS$_{zeocin \ (50mg/L)}$ agar plates and single colonies were selected and PCR screened.

A

```
....|....| ....|....| ....|....| ....|....| ....|....|
     10        20        30        40        50
```
X6   GGAAACGACT TTTACGACAC TTGAGAAGAT CAAAAAACAA CTAATTATTC
PGT 9 ---------- ---------- ---------- ---------- ----------

```
....|....| ....|....| ....|....| ....|....| ....|....|
     60        70        80        90        100
```
X6   GAAACGAGGA ATTCACGTGG CCCAGCCGGC CGTCTCGGAT CGGTACCATG
PGT 9 ---------- ---------- ---------- ---------- -------ATG

```
....|....| ....|....| ....|....| ....|....| ....|....|
     110       120       130       140       150
```
X6   GATCCAGCCG GAGCAAGAAA AGTTCATGCA GTATGCGTTC CATATCCAGC
PGT 9 GATCCAGCCG GAGCAAGAAA AGTTCATGCA GTATGCGTTC CATATCCAGC

B

```
....|....| ....|....| ....|....| ....|....| ....|....|
     1410      1420      1430      1440      1450
```
X6   TAATTTTGAC AGATTAGTTA AGATGGTTCT TCAGCAAGGA AATTGGACCG
PGT 9 TAATTTTGAC AGATTAGTTA AGATGGTTCT TCAGCAAGGA AATTGGACCG

```
....|....| ....|....| ....|....| ....|....| ....|....|
     1460      1470      1480      1490      1500
```
X6   GAACCGAAAC CCTTCACGGG CCCGAACAAA AACTCATCTC AGAAGAGGAT
PGT 9 GAACCGAAAC CCTTCACTAG ---------- ---------- ----------

```
....|....| ....|....| ....|....| ....|....
     1510      1520      1530
```
X6   CTGAATAGCG CCGTCGACCA TCATCATCAT CATCATTGA
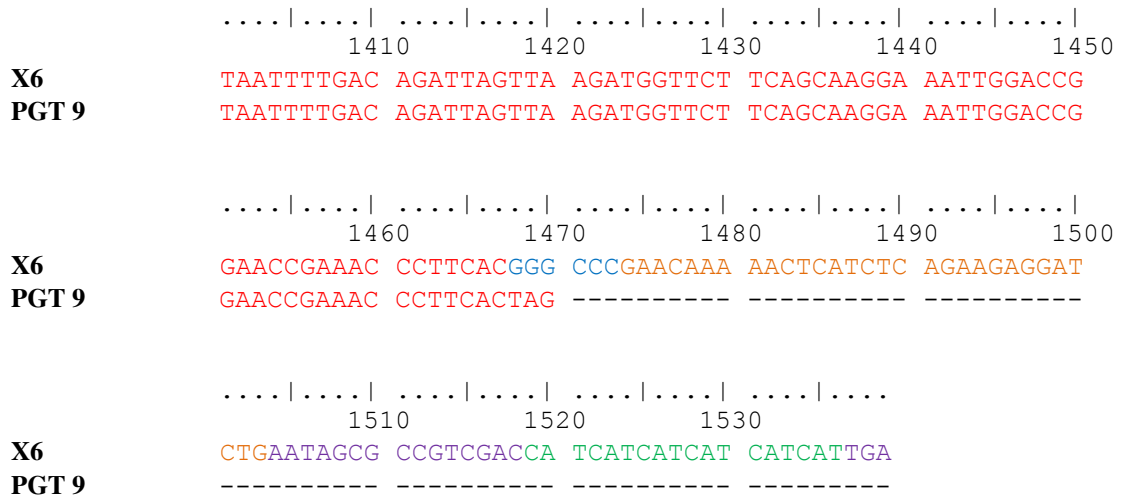PGT 9 ---------- ---------- ---------- ---------

Figure 26. DNA Sequencing Analysis of Recombinant PGT 9 (X6) in pPICZA with Original PGT 9 Sequence. A. 5' sequence alignment of rPGT 9 with original PGT 9 sequence. B. 3' sequence alignment. Red=PGT 9; Blue= restriction site; Purple= pPICZA. Green= 6X His tag. Orange=C-myc epitope.

A

```
    ....|....| ....|....| ....|....| ....|....| ....|....|
         310        320        330        340        350
PY A   CGAGAAAAGA GAGGCTGAAG CTGAATTCAC GTGGCCCAGC CGGCCGTCTC
PGT9   ---------- ---------- ---------- ---------- ----------


    ....|....| ....|....| ....|....| ....|....| ....|....|
         360        370        380        390        400
PY A   GGATCGGTAC CATGGATCCA GCCGGAGCAA GAAAAGTTCA TGCAGTATGC
PGT9   ---------- -ATGGATCCA GCCGGAGCAA GAAAAGTTCA TGCAGTATGC


    ....|....| ....|....| ....|....| ....|....| ....|....|
         410        420        430        440        450
PY A   GTTCCATATC CAGCACAAGG CCATGTAAGT CCCTTGATGC AAGTAGCCAA
PGT9   GTTCCATATC CAGCACAAGG CCATGTAAGT CCCTTGATGC AAGTAGCCAA
```

B

```
    ....|....| ....|....| ....|....| ....|....| ....|....|
        1410       1420       1430       1440       1450
PY A   ACAATAATTT TGACAGATTA GTTAAGATGG TTCTTCAGCA AGGAAATTGG
PGT 9  ACAATAATTT TGACAGATTA GTTAAGATGG TTCTTCAGCA AGGAAATTGG


    ....|....| ....|....| ....|....| ....|....| ....|....|
        1460       1470       1480       1490       1500
PY A   ACCGGAACCG AAACCCTTCA CCGTCTAGAA CAAAAACTCA TCTCAGAAGA
PGT 9  ACCGGAACCG AAACCCTTCA CTAG------ ---------- ----------


    ....|....| ....|....| ....|....| ....|....| ...
        1510       1520       1530       1540
PY A   GGATCTGAAT AGCGCCGTCG ACCATCATCA TCATCATCAT TGA
PGT 9  ---------- ---------- ---------- ---------- ---
```

Figure 27. DNA Sequencing Analysis of Recombinant PGT 9 (PY A) in pPICZAα with Original PGT 9 Sequence. A**.** 5' sequence alignment of rPGT 9 (PY A) with original PGT 9 sequence. B. 3' sequence alignment. Red=PGT 9; Blue= restriction site; Purple= pPICZA. Green= 6X His tag. Orange=C-myc epitope.

A

```
             ....|....| ....|....| ....|....| ....|....| ....|....|
                 210        220        230        240        250
PZ 1         CGATGTTGCT GTTTTGCCAT TTTCCAACAG CACAAATAAC GGGTTATTGT
PGT 9        ---------- ---------- ---------- ---------- ----------


             ....|....| ....|....| ....|....| ....|....| ....|....|
                 260        270        280        290        300
PZ 1         TTATAAATAC TACTATTGCC AGCATTGCTG CTAAAGAAGA AGGGGTATCT
PGT 9        ---------- ---------- ---------- ---------- ----------


             ....|....| ....|....| ....|....| ....|....| ....|....|
                 310        320        330        340        350
PZ 1         CTCGAGAAAA GAATGGATCC AGCCGGAGCA AGAAAAGTTC ATGCAGTATG
PGT 9        ---------- --ATGGATCC AGCCGGAGCA AGAAAAGTTC ATGCAGTATG
```

B

```
             ....|....| ....|....| ....|....| ....|....| ....|....|
                 1410       1420       1430       1440       1450
PZ 1         TAATTTTGAC AGATTAGTTA AGATGGTTCT TCAGCAAGGA AATTGGACCG
PGT 9        TAATTTTGAC AGATTAGTTA AGATGGTTCT TCAGCAAGGA AATTGGACCG


             ....|....| ....|....| ....|....| ....|....| ....|....|
                 1460       1470       1480       1490       1500
PZ 1         GAACCGAAAC CCTTCACCGT CTAGAACAAA AACTCATCTC AGAAGAGGAT
PGT 9        GAACCGAAAC CCTTCACTAG ---------- ---------- ----------


             ....|....| ....|....| ....|....| ....|....
                 1510       1520       1530
PZ 1         CTGAATAGCG CCGTCGACCA TCATCATCAT CATCATTGA
PGT 9        ---------- ---------- ---------- ---------
```

Figure 28. DNA Sequencing Analysis of Recombinant PGT 9 (PZ 1) in pPICZAα with Original PGT 9 Sequence. A**.** 5' sequence alignment of rPGT 9 (PZ 1) with original PGT 9 sequence. B. 3' sequence alignment. Red=PGT 9; Blue= restriction site; Purple= pPICZA. Green= 6X His tag. Orange=C-myc epitope.
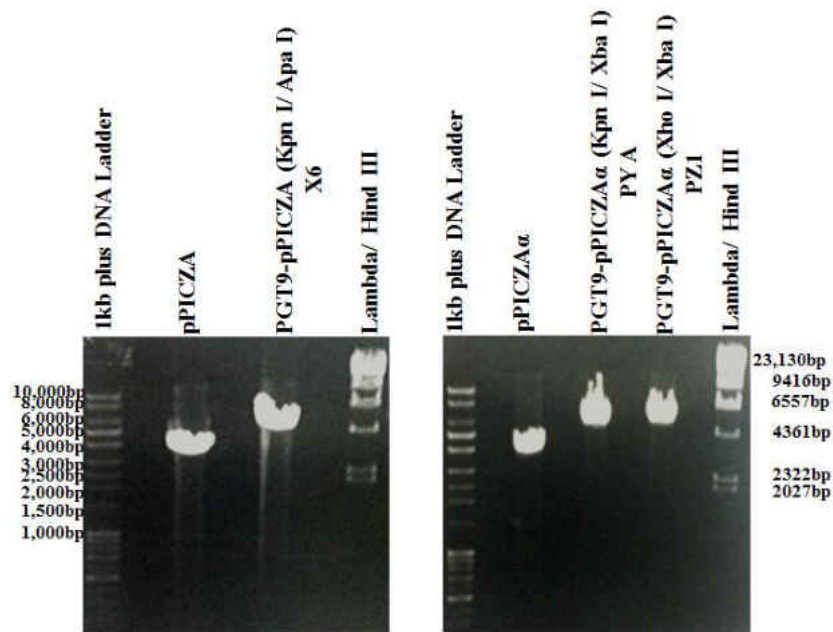
Figure 29. Completely Linearized Empty Vectors and Vectors Containing PGT 9 Insert. Linearization was carried out using Sac I restriction enzyme. Linearized plasmid bands corresponded to empty vector sizes and sizes of vector containing PGT 9 insert for the different cloning strategies as expected. Bands confirm that DNA samples were successfully concentrated.
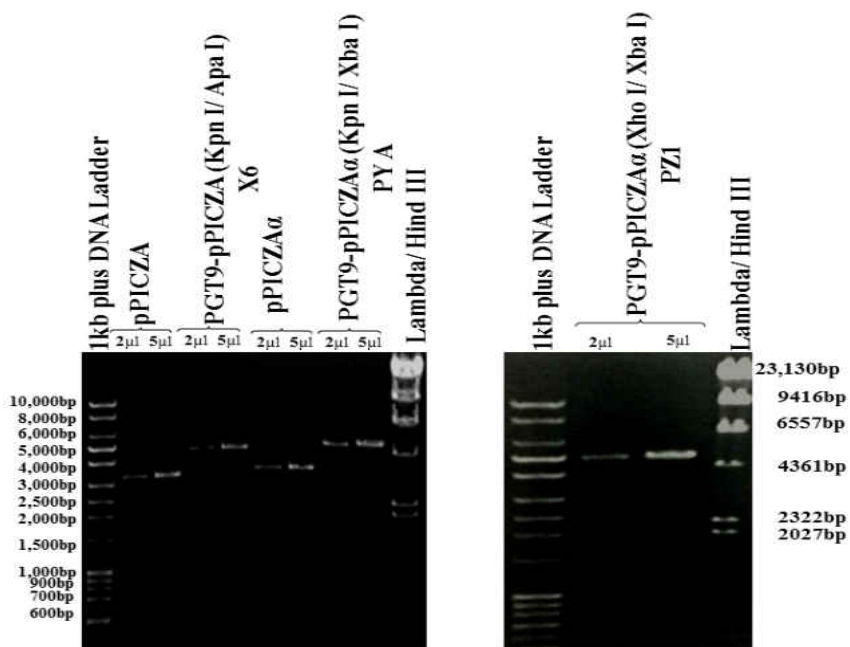

Figure 30. Diluted DNA Samples (1:100) of Phenol:chloroform and Ethanol Precipitated samples Used to Determine Concentration. Band intensities of each sample were compared to that of the quantitative markers.

Some of the single colonies for each strategy were positive (Figure 31). This confirms transformation of PGT 9 into the yeast genome. Yeast cells were also transformed with empty pPICZA and pPICZAα vectors to serve as negative controls for expression of rPGT 9 (Figure 31).
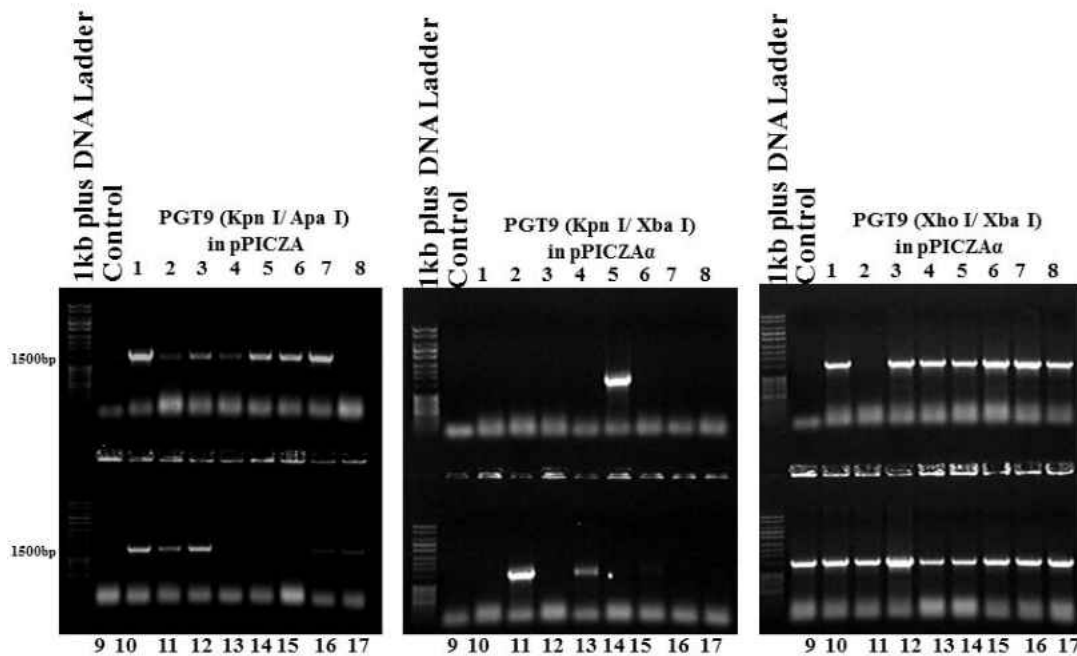

Figure 31. PCR Screen of Single Yeast Colonies After Transformation and Selection on the Low Salt LB$_{zeocin (50mg/L)}$ Agar Plates. Yeast genome was used as template and PGT 9 band (~1470bp) was amplified.

Expression of Recombinant PGT 9 in *Pichia pastoris*

For test inductions of rPGT 9, the positive colony labeled X6[1] was selected. Cells were inoculated in BMGY to generate biomass for induction. The cells from the BMGY culture were then transferred into BMMY media for induction with methanol. Samples were collected every 6 hours for the first 24 hours and then every 12 hours for the next 3days postinduction. The

negative control, which consisted of yeast cells transformed with the empty vector, was also

treated. Because of the small volume of test induction cultures, cells collected at different times

postinduction were lysed using beads and analyzed by SDS PAGE and Western blot using

antibodies against the c-myc epitope of rPGT 9. rPGT 9 was detected for all the samples except

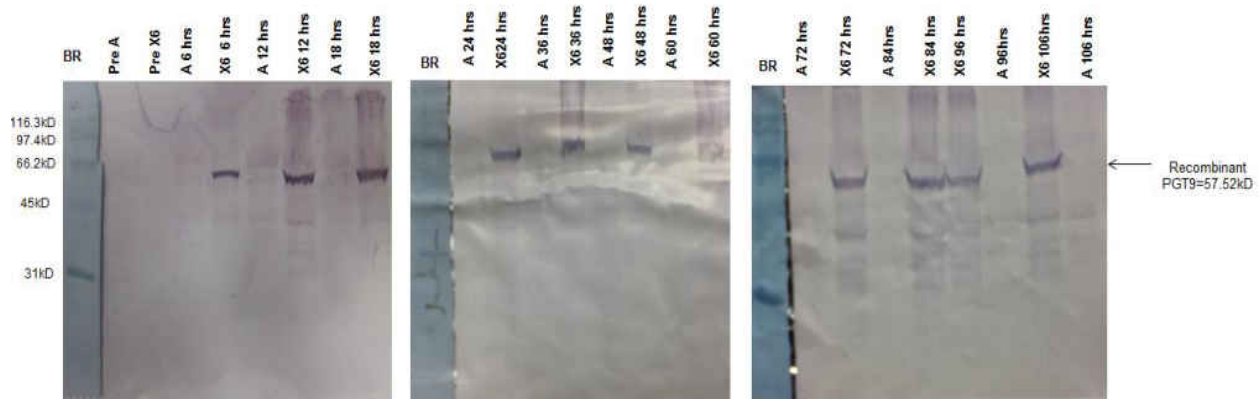for the negative controls (Figure 32).

Figure 32. Representative Western Blot Analysis of Test Inductions for Recombinant PGT 9 in *Pichia pastoris* at 30°C Using Methanol. Samples were collected at different times post induction. Detection was carried out using anti c-myc antibodies. A. Yeast cells transformed with empty vector. X6. Yeast cells transformed with PGT 9 sequence.

A protein microassay was performed on the samples that were analyzed by Western blot.

This was done in order to determine the protein concentration of each sample. Based on the

concentrations obtained for each sample (Table 18), and the Western blot analysis (Figure 32),

the optimal time for sample collection was determined to be 6 hours postinduction.

Table 18. Concentration and Volumes of Samples Used in Western Blot Analysis for Test-Induction of Recombinant PGT 9 in Yeast.

Post-Induction Time (hours)	Protein concentration (µg/µl)	Volume loaded on gel (µl)
6	2.2	5
12	2.4	5
18	3.9	5
24	4.1	5
36	2.5	5
48	2.4	5
60	2.7	5
72	3.15	5
84	1.8	5
96	2.4	5
108	2.7	5

Scale-Up and Enrichment of Recombinant PGT 9

Induction of rPGT 9 expression in yeast was carried out and all the cells were collected at 6 hours postinduction, lysed and IMAC carried out to enrich soluble rPGT 9. The fractions from the column were analyzed by silver stained SDS PAGE gels. rPGT 9 was detected in the first four 150mM eluted fractions (Figure 33 A). These enriched fractions were pooled, desalted, and concentrated to further enrich rPGT 9. Western blot results showed that enrichment of rPGT 9 was achieved (Figure 33 B).
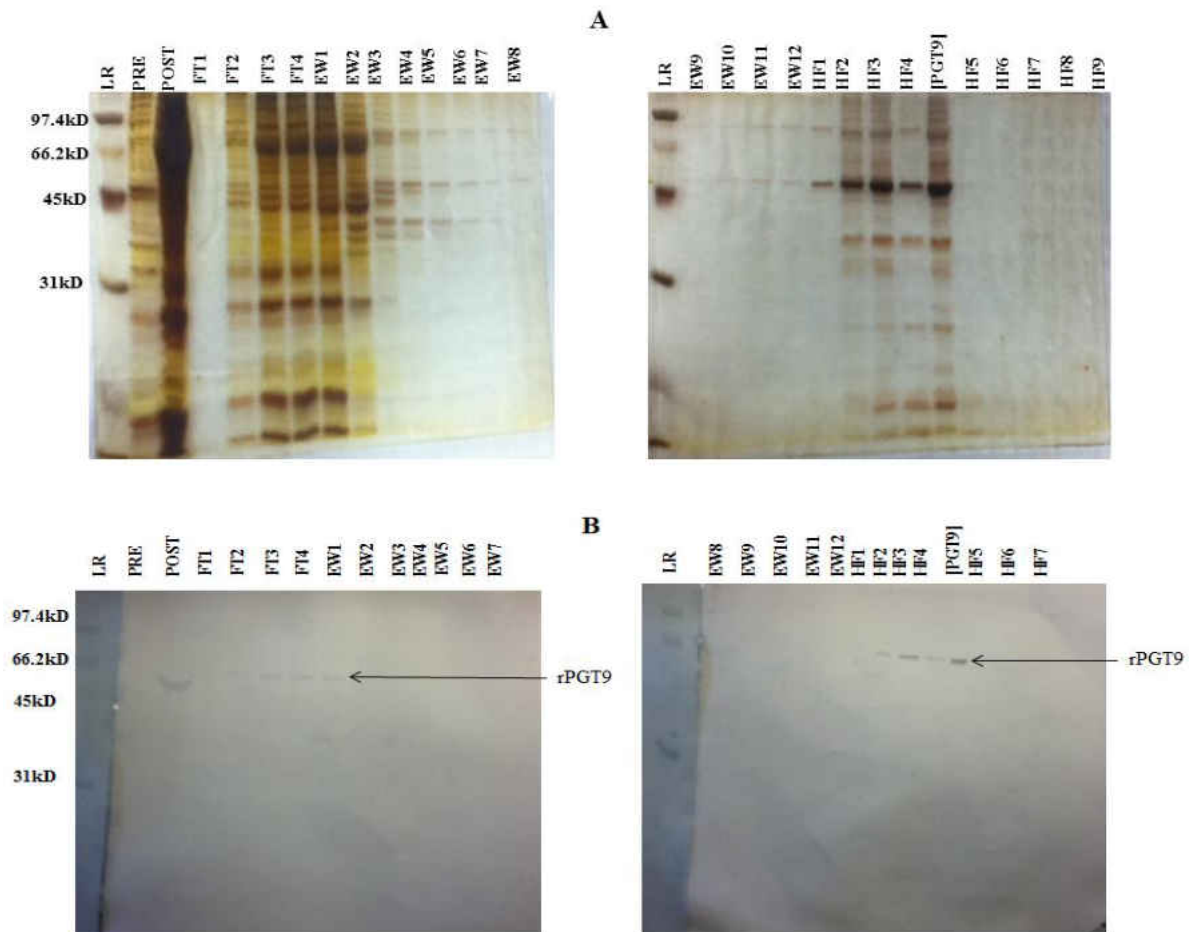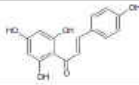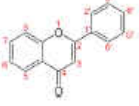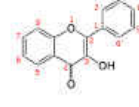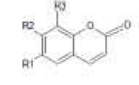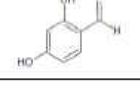
Figure 33. Analysis of Fractions Obtained from Immobilized Metal Affinity Chromatography. **A**. Silver stained gels of fractions obtained from IMAC column. First four 150mM (HF) imidazole fractions were pooled, desalted and concentrated. **B**. Western blot analysis of fractions from IMAC. Blot confirms the concentration of rPGT 9 from the first four HF fractions. LR, Low range marker; FT, Flow through samples; EW, Washes with wash buffer; T, 10mM imidazole washes; HF, 150mM imidazole washes; [rPGT 9], Pooled, desalted and concentrated fraction.
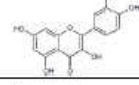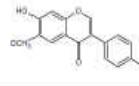
Screening Recombinant PGT 9 for GT Activity

The pooled and concentrated fraction from the enrichment step was used to screen for activity of rPGT 9 using different flavonoid substrates and some simple phenolic substrates. No activity was detected for rPGT 9 for the 18 flavonoid substrates used in the assay (Table 19).

Table 19. Results from Screening Recombinant PGT 9 for GT Activity. No significant GT activity detected for substrates used.

Group	Class of Substrate	Structure	Substrate	Average CPM (n=2) Incorporated
Flavonoid	Chalcone		2'4,4',6' tetrahydroxychalcone	0
	Flavanone		Naringenin 4'=5=7=OH	20
			Hesperitin 3'=5=7=OH, 4'= OCH3	8
			Eriodictyol 3'=4'=5=7=OH	8
			Isosakuranetin 5=7=OH, 4'=OCH3	17
			Prunin 4'=5=OH, 7= -O-glucose	22
	Flavone		Apigenin 4'=5=7=OH	0
			Luteolin 3'=4'=5=7=OH	8
			Diosmetin 3'=5=7= OH, 4'=OCH3	36
			Scutallerein 4'=5=6=7=OH	0
			Luteolin-7-O- glucoside 3'=4'=OH, 7= -O-glucose	11
	Flavonol		Kaempferol 5=7= 4'=OH	12
			Quercetin 3'=4'=5=7= OH	0
			Fisetin 4'=5'=7= OH	2
			Gossypetin 3'=4'=5=7=8= OH	0
			4' methoxyflavonol 4'= OCH3	0
	Dihydroflavonol		Dihydroquercetin	0
	Isoflavone		4'-acetoxy-7-hydroxy-6-methoxy isoflavone	0
Phenolic	Coumarins		Umbelliferone R1=R3=H, R2=OH	0
			Esculetin R1=R2=OH, R3=H	0
	Simple Phenolics		2', 4' -dihydrobenzaldehyde	14

A positive control for the reaction was carried out using crude protein extract from young grapefruit leaf (Table 20). Quercetin was used as the substrate in the positive control reaction. In one of the positive control reactions, 10µl of the concentrated rPGT 9 fraction was added to determine if the reaction would be inhibited compared to the positive control that did not have the concentrated fraction of rPGT9. There was incorporation of radioactive counts in both controls which indicate that there was no inhibitory effect of the concentrated rPGT 9 fraction.

Table 20. Results Obtained from Positive Control Reactions Using Grapefruit Leaf Extract with and without the Enriched rPGT 9 Fraction. Quercetin was used as substrate for controls.

| Positive Control	Enzyme Source(s)	Average CPM (n=2) Incorporated
A	Grapefruit Leaf Extract only	1330
B	Grapefruit Leaf Extract + [rPGT 9] fraction	1294

Initial screens did not determine the substrate for rPGT 9. Other phenolic compounds found in grapefruit (Table 12) in glucosylated forms were also screened as potential substrates for rPGT 9 (Fieldman and Hanks 1965; Harborne 1983; Gardana et al. 2008). Because of their water soluble nature, HPLC was used for analysis (Table 12). The reactions were carried out in duplicates. The standard for each substrate was analyzed once and the retention time was noted (Table 21). The experimental samples were then analyzed and the retention time from the duplicates compared with that of the standards (Table 21). Representative HPLC chromatograms for rPGT 9 reactions are shown in Figure 34. Positive controls were carried out using grapefruit young leaf crude extract as enzyme source. One positive control contained concentrated rPGT 9 fraction while the other had just grapefruit leaf crude extract. A negative control was carried out

using denature enzyme with one of the potential substrate. Formation of new peak(s) with shorter

retention time(s) as well as decrease in the aglycone peak is indicative of activity. This was

observed for catechol, *p*-hydroxyphenylacetic acid, vanillin, vanillic acid, and gentisic acid

(Table 21 and Figure 34).

Table 21. Summary of Retention Times of Major Peaks for Standards, Positive Control, Negative
Control, and Experimental Groups for Each Substrate.

Compound	Retention Time (RT) of Standard (minutes)	Retention Time (RT) of Experimental. MEAN±SD (minutes)	Retention Time of New Peak(s) Formed MEAN±SD (minutes)
Catechol	2.70 (1)	2.82 ± 0.05 (2)	i) 2.02 ± 0.05 (2) ii) 1.44 ± 0.06 (2)
Caffeic acid	6.61 (1)	n. d.	6.01 ± 0.07 (2)
p-Coumaric acid	12.03 (1)	11.92 ± 0.07 (2)	n. d.
O-Coumaric acid	26.14 (1)	26.59 ± 0.09 (2)	n. d.
Ferulic acid	12.684 (1)	12.83 ± 0.03(2)	n. d.
Gentisic acid	12.888 (1)	13.68 ± 0.06 (2)	n. d.
Vanillic acid	10.253 (1)	10.01 ± 0.17 (2)	i) 1.70 ± 0.06 (2) ii) 9.07 ± 0.03 (2)
Vanillin	17.60 (1)	17.21 ± 0.61 (2)	i) 1.75 ± 0.003 (2) ii) 9.11 ± 0.007 (2)
Scopoletin	9.90 (1)	9.91 ± 0.02 (2)	n. d.
p-Hydroxyphenylacetic acid	16.43 (1)	16.06 ± 0.02 (2)	i) 1.68 ± 0.04 (2) ii) 2.30 ± 0.03 (2) iii) 7.38 ± 0.02 (2)
p-Hydroxyphenylpyruvic acid	4.66 (1)	4.49 ± 0.002 (2)	n. d.
p-Hydroxybenzoic acid	13.74 (1)	13.62 ± 0.03 (2)	i) 1.67 ± 0.06 (2) ii) 7.46 ± 0.01(2)
p-Hydroxybenzoic acid (Negative control with Denatured Enzyme)	13.74 (1)	13.57 ± 0.10 (2)	i) 1.65 ± 0.09 (2) ii) 7.41 ± 0.03 (2)
Quercetin (Positive control with Grapefruit leave extract only)	26.77 (1)	28.47 ± 1.58 (2)	Multiple peaks
Quercetin (Positive control with grapefruit leaf extract + concentrated rPGT9 fraction)	26.77 (1)	30.71 ± 0.03 (2)	Multiple peaks

n = number of injections. n.d. = none detected.
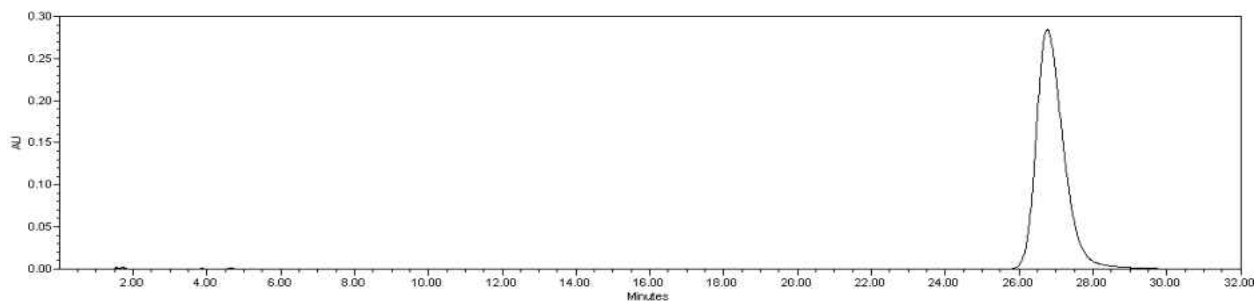
A1. Quercetin Standard.
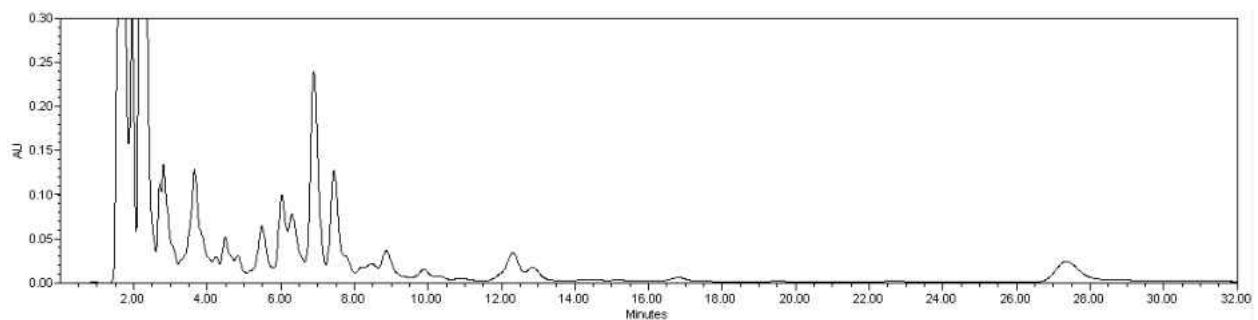
A2. Positive Control Reaction 1 Using Quercetin as Substrate (Grapefruit Leaf Extract only).
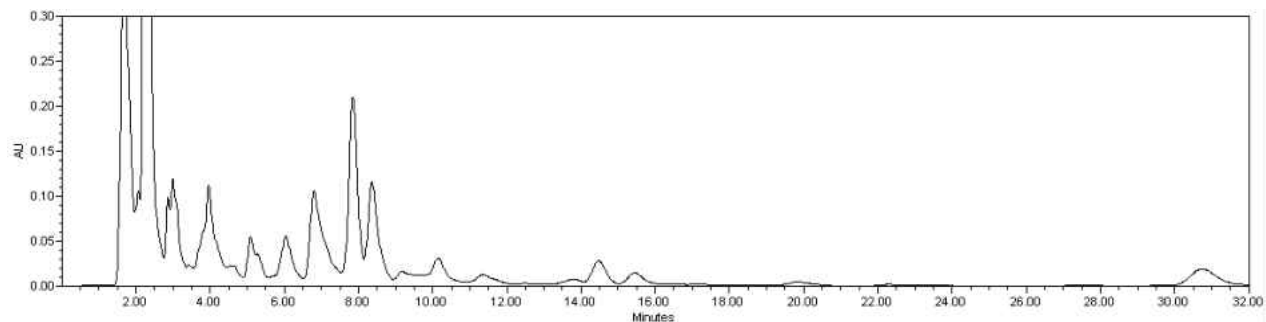
A3. Positive Control Reaction 2 Using Quercetin as Substrate (Grapefruit leaf extract + rPGT 9).
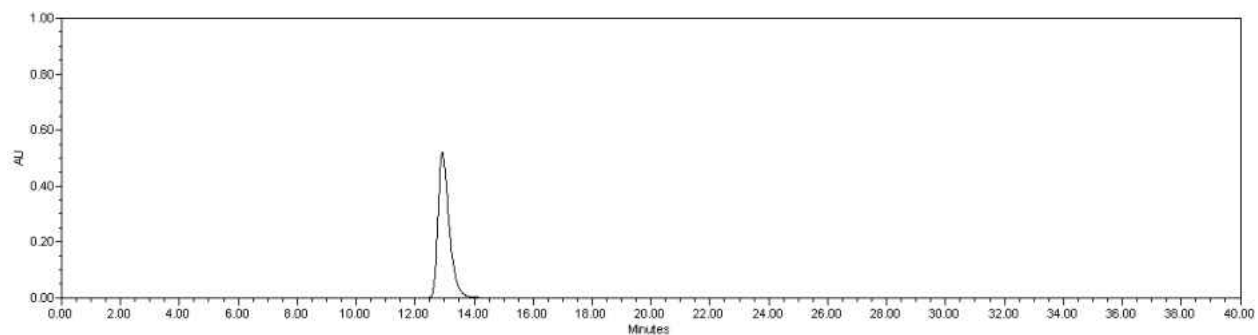
B1. *p*-Hydroxybenzoic Acid Standard.
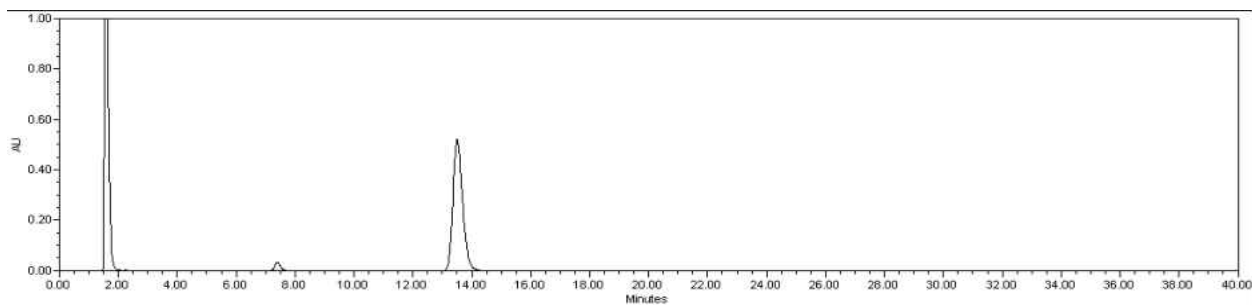
B2. Negative Control Using Denatured Enzyme and *p*-Hydroxybenzoic as Substrate.
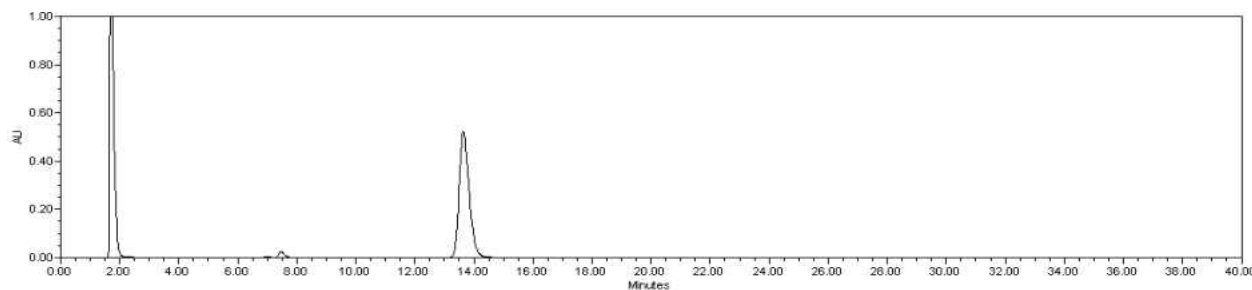
B3. *p*-Hydroxybenzoic Experimental.
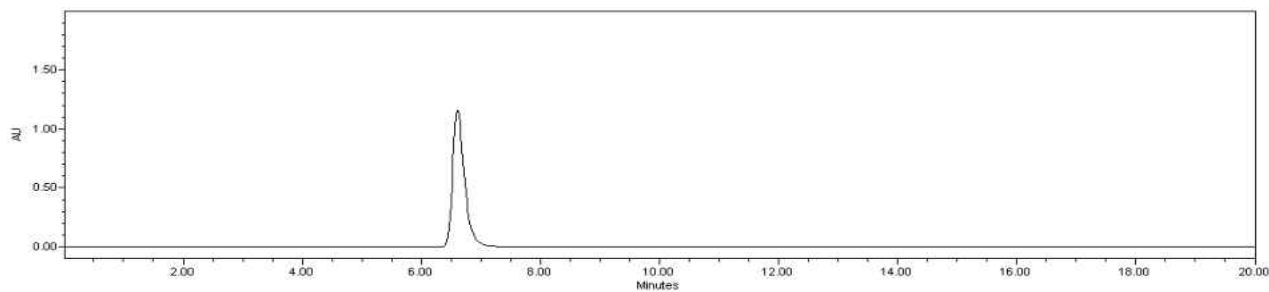
C1. Caffeic Acid Standard.
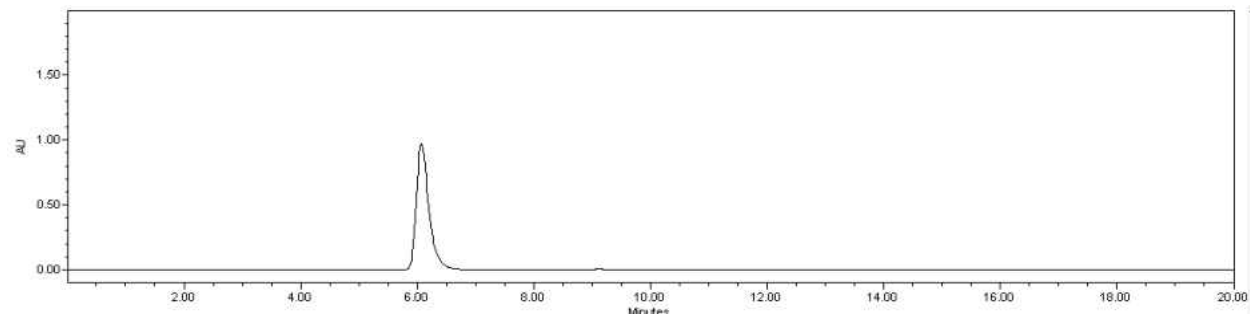
C2. Caffeic Acid Experimental.
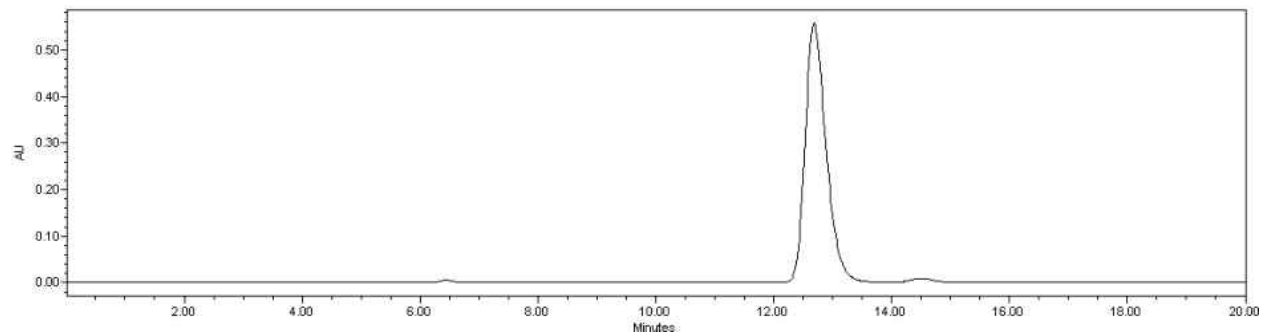
D1. Ferulic Acid Standard,
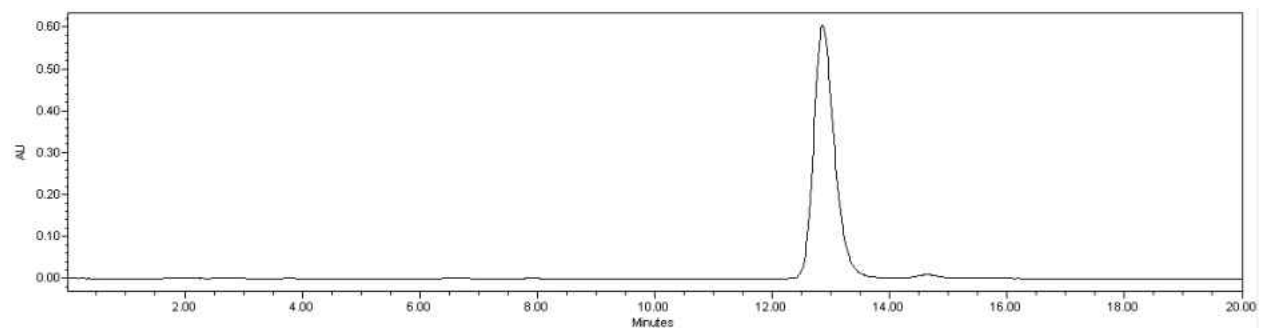
D2. Ferulic Acid Experimental.
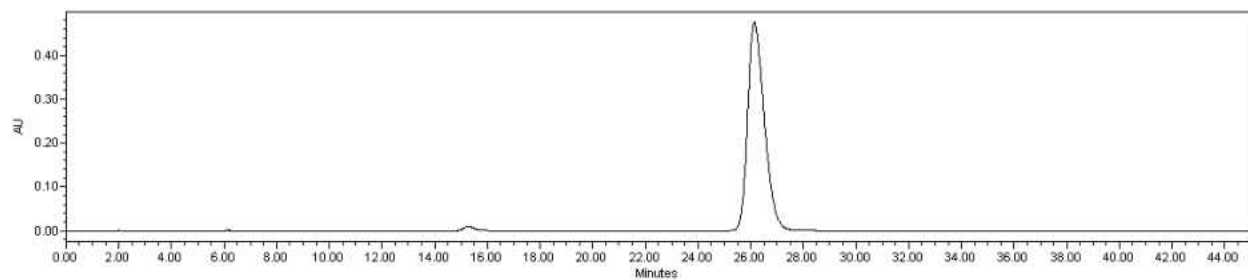
E1.O-coumaric Standard
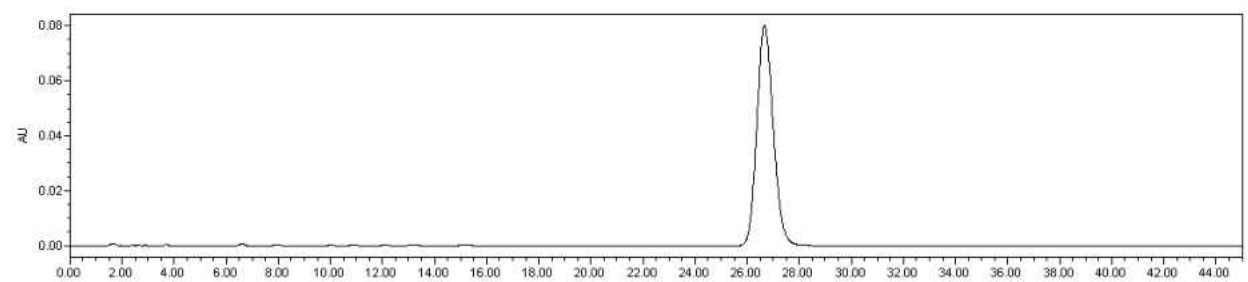
E2. O-coumaric Experimental
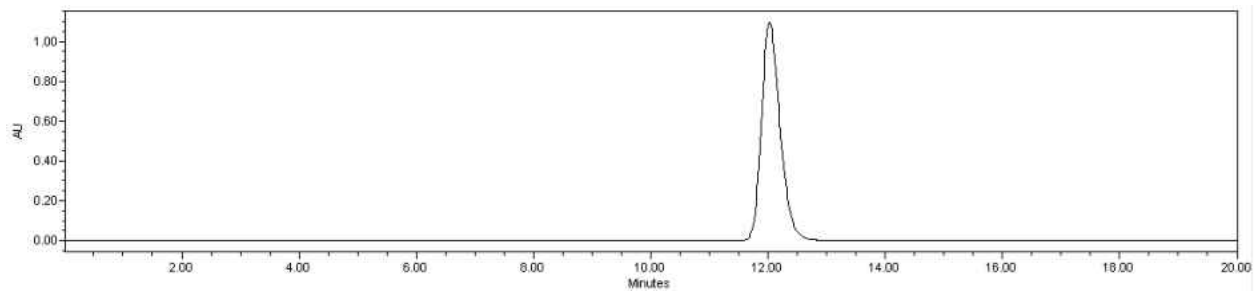
F1. *p*-Coumaric Acid Standard
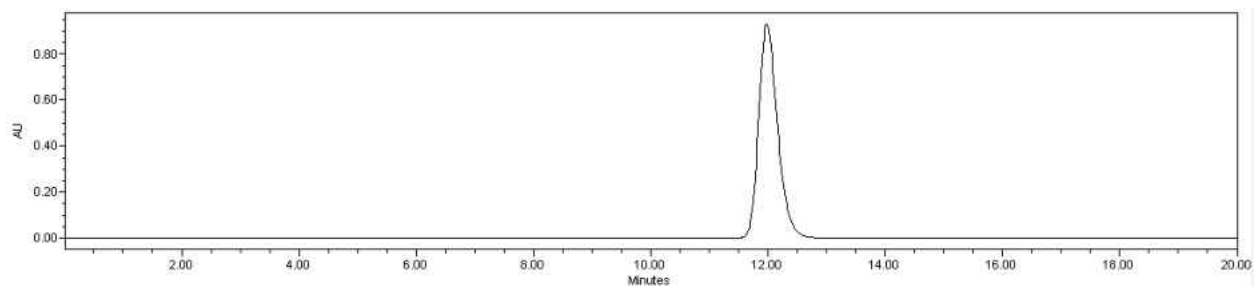
F2. *p*-Coumaric Acid Experimental
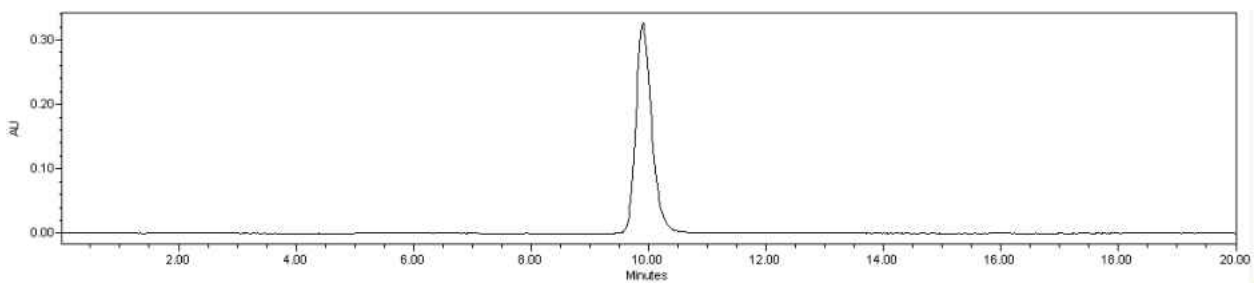
G1. Scopoletin Standard.
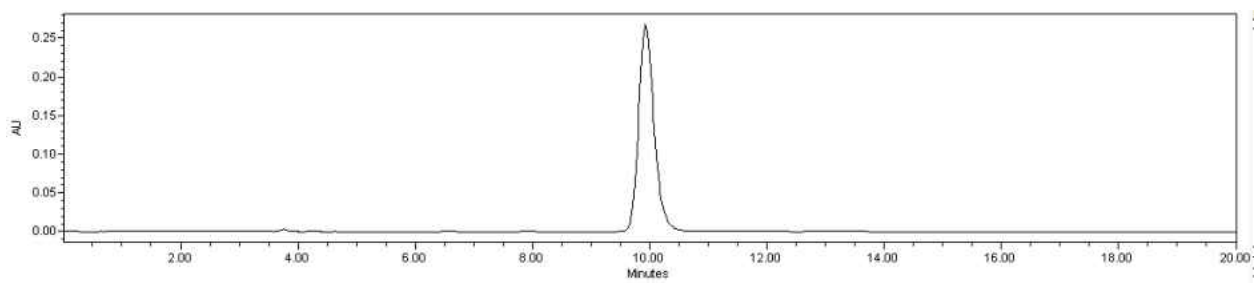
G2. Scopoletin Experimental
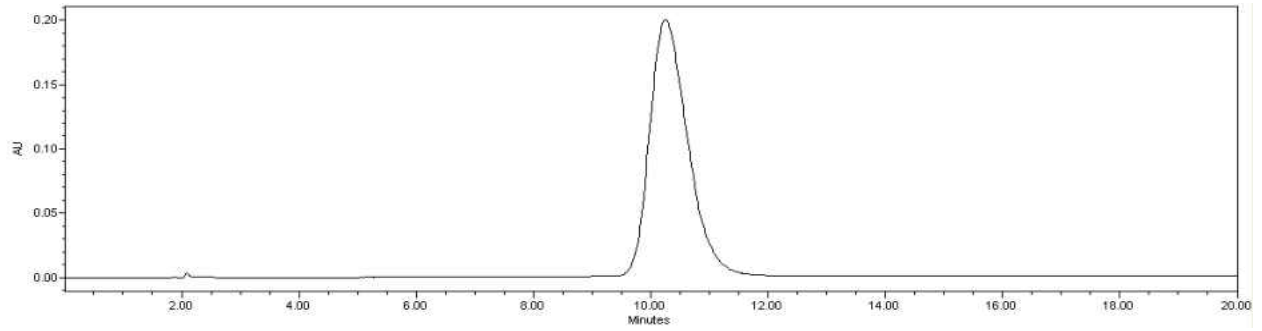
H1. Vanillic Acid Standard
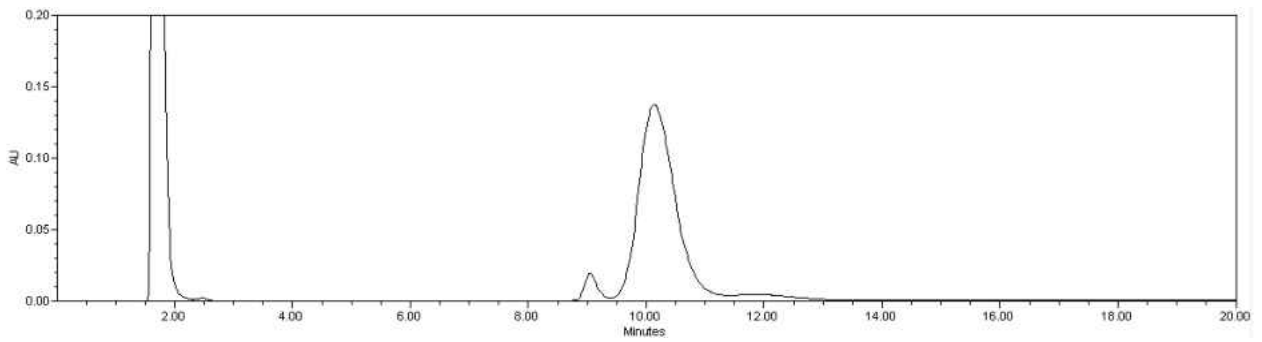
H2. Vanillic Acid Experimental
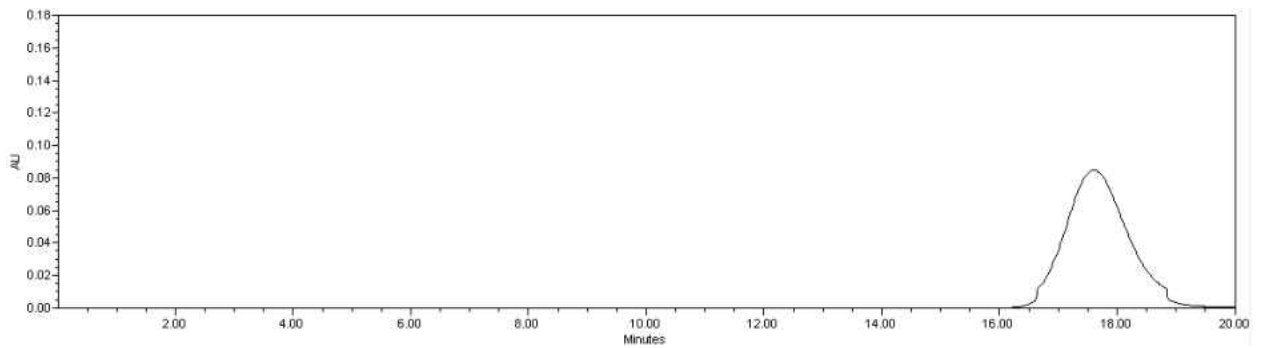
I1. Vanillin Standard.
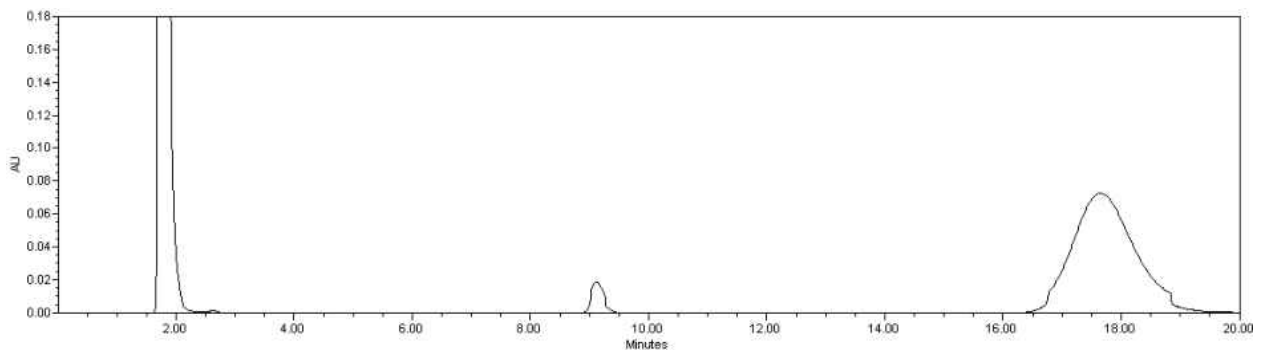
I2. Vanillin Experimental.
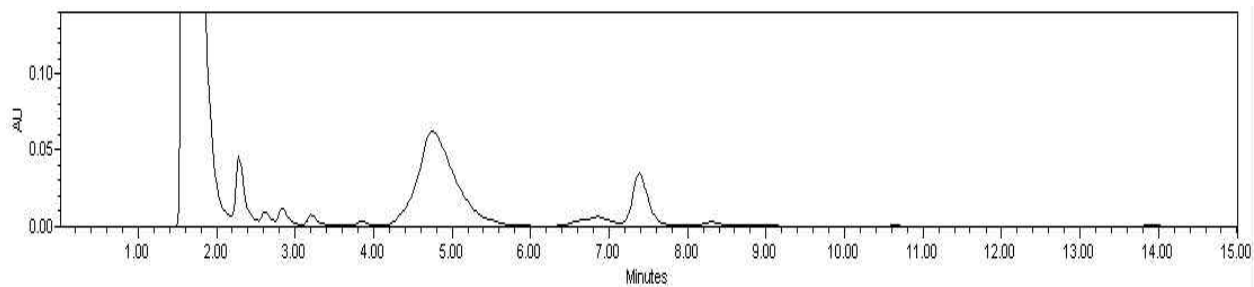
J1. *p*-hydroxyphenylpyruvic Acid Standard
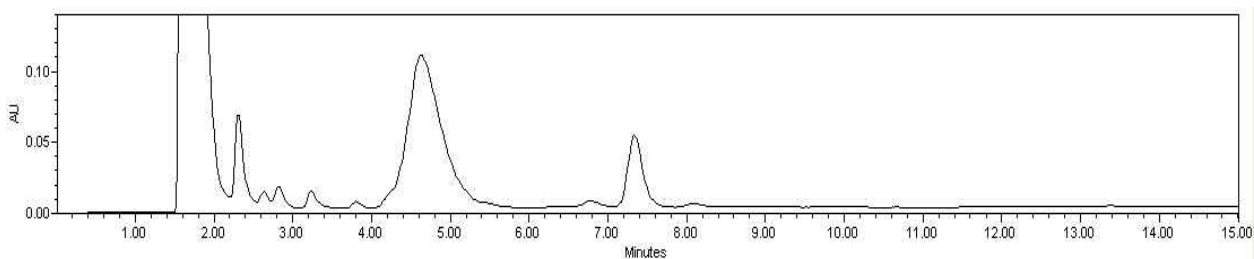
J2. *p*-hydroxyphenylpyruvic Acid Experimental
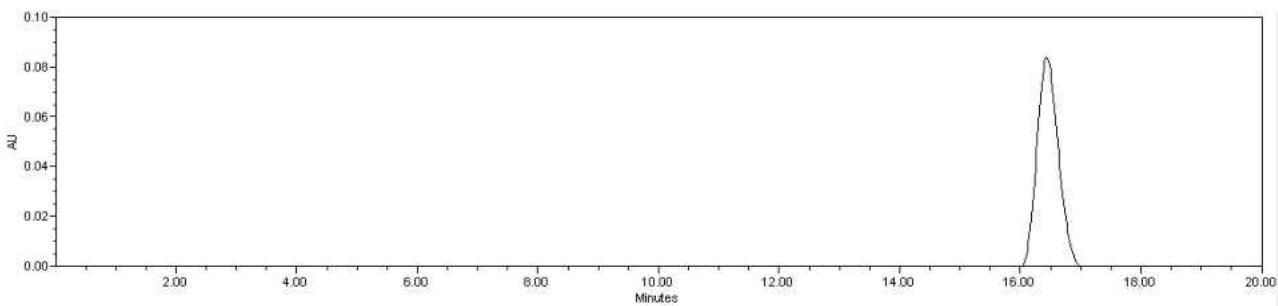
K1. *p*-hydroxyphenylacetic Acid Standard
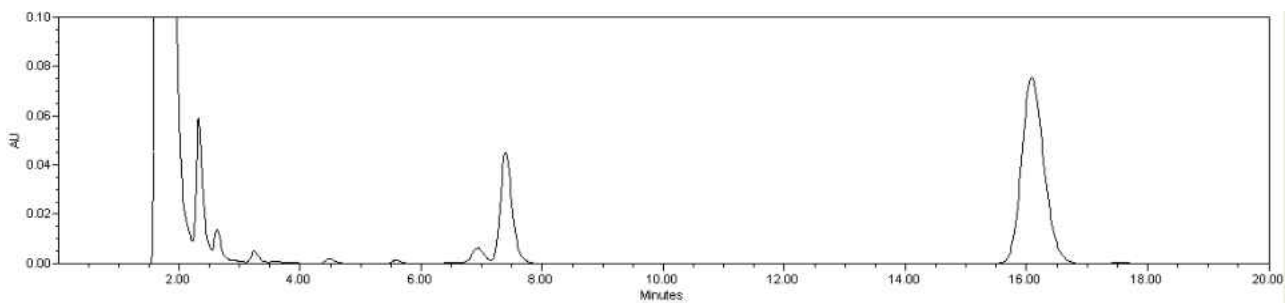
K2. *p*-hydroxyphenylacetic Experimental
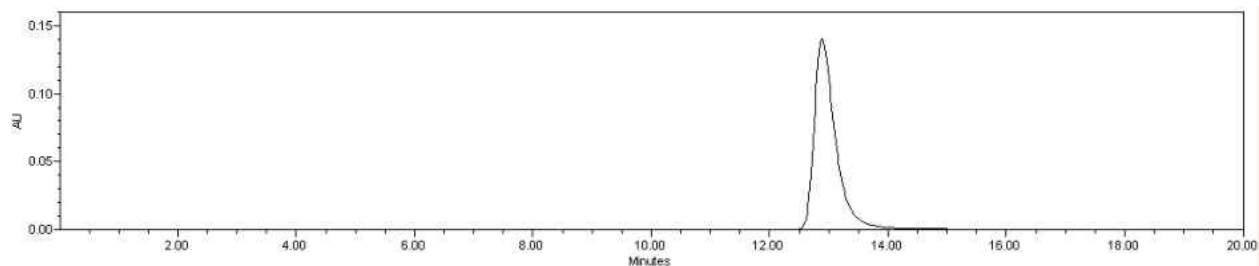
L1. Gentisic Acid Standard.
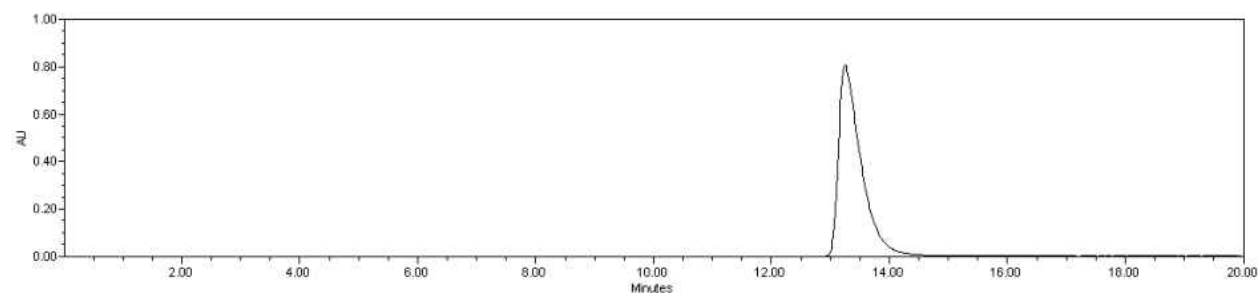
L2. Gentisic Acid Experimental
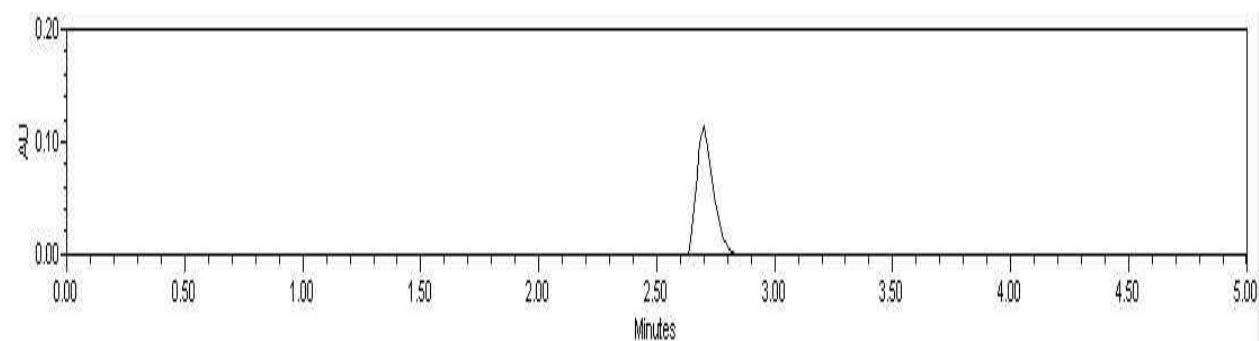
K1. Catechol Standard.
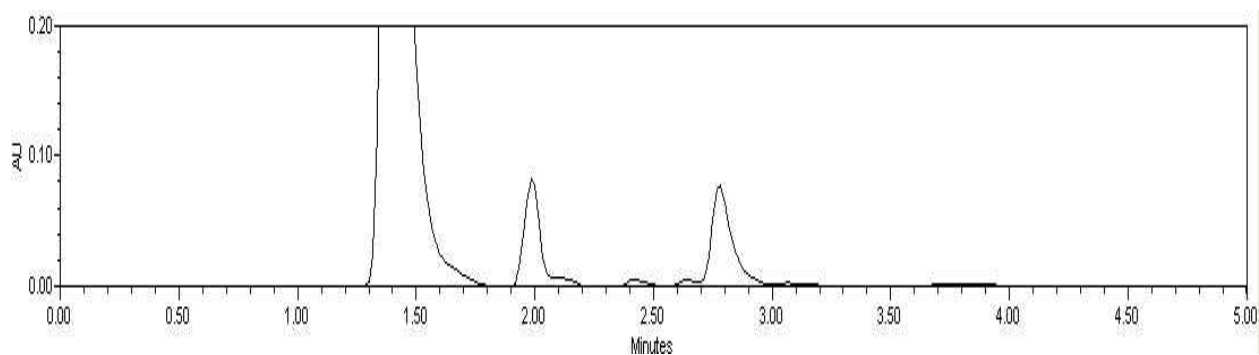
K2. Catechol Experimental

Figure 34. Representative HPLC Chromatograms for rPGT 9 Assays with 12 Phenolic Compounds. Reduction of substrate peak height in the experimental chromatograms or appearance of new peaks suggests formation of a product.

Cloning of PGT 11 into pPICZA for Transformation into *Pichia pastoris*

Plasmid DNA (PGT 11-TOPO) Isolation and PCR Modification

For cloning of PGT 11 in to pPICZA vectors, unmodified PGT 11 initially cloned into pCR®4-TOPO vector was isolated from Top10 competent *E. coli* cells. PGT 11 was modified to have Kpn I/ Sac II restriction sites to be cloned into the pPICZA vector. The PCR modified PGT 11 product was analyzed on a 0.8% agarose gel alongside the unmodified PGT 11 sequence in TOPO vector. Modified PGT 11 sequence corresponded to the expected 1374bp (Figure 35).
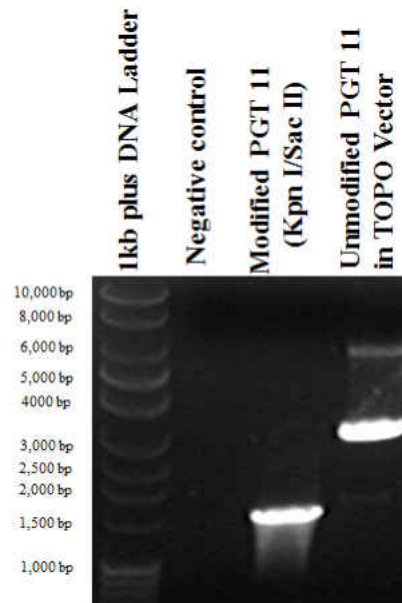
Figure 35. Agarose Gel Analysis of Miniprep of Unmodified PGT 9 in TOPO Vector and PCR Modified PGT 11.

The modified PGT 11 sequence was cloned into pCR®4-TOPO vector for amplification and transformed into one-shot Top10 *E. coli* competent cells to serve as a holding cell line. PCR screens of single colonies selected from selective LB$_{amp(100mg/L)}$ plates for each modification strategy showed positive bands for all colonies and the amplified bands corresponded to the ~1374bp band of PGT 11 sequence that was expected (Figure 36). This suggests that ligation with TOPO vector and transformation into *E. coli* was successful.
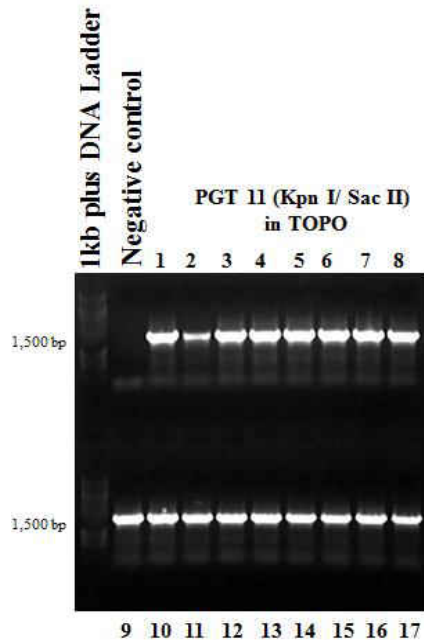
Figure 36. PCR Screen Results of *E. coli* Cells Transformed with PGT 11 Modified to Have Kpn I and Sac II Restriction Sites. PGT 11 band of ~1374bp was amplified from each sample.

Colonies 1, 2, and 3 were selected and midiprep was carried out to isolate plasmid DNA. A midiprep of pPICZA vector from Top 10 competent *E. coli* cells was also carried out. A 2μl aliquot of each isolated plasmid was digested with Kpn I and Sac II restriction enzymes. After digestion, the ~1374bp band corresponding to PGT 11 and the larger bands of 3956bp corresponding to the linearized TOPO vector were observed (Figure 37). A band size of ~3,329bp corresponding to the digested pPICZA vector was also observed (Figure 37). Sequencing results confirmed the presence of PGT 11 sequence in the TOPO vector for all samples. Colonies 1 and 2 were selected for further work.
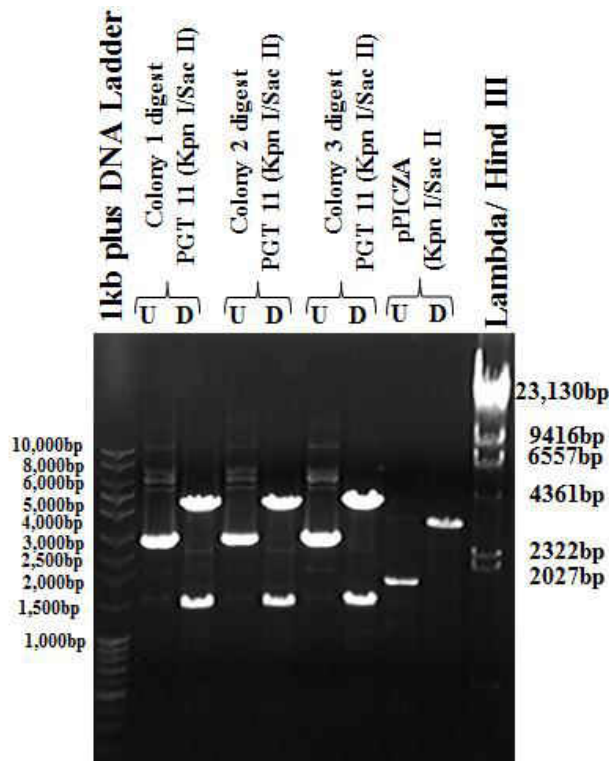
Figure 37. Digestion of pPICZA and Plasmid DNA Isolated from Positive PCR Screened Single *E. coli* Colonies.

Restriction Digests, Ligations, and Transformation of Recombinant PGT 11 into Top 10 *E. coli*

A 16µl aliquot of each plasmid DNA (modified PGT 11 in TOPO and pPICZA) were

digested with Kpn I/ Sac II restriction enzymes and run on a 0.8% agarose DNA gel (Figure 38

A). A 2µl and 5µl aliquot of each gel purified sample was run on an agarose gel and the

concentration determined using 1kb plus exACTGene DNA quantitative ladder and Lambda/

Hind III marker (Figure 38 B and Table 16). After determining the concentration of each

sample, ligation reactions were set up as described in the materials and methods section.

Successful ligation was confirmed by sequencing the recombinant plasmid isolated from 6

colonies (Colonies A-F). Sequencing results confirmed the presence of PGT 11 in pPICZA and a

representative sequence alignment with original PGT 11 sequence is shown below in Figure 39.
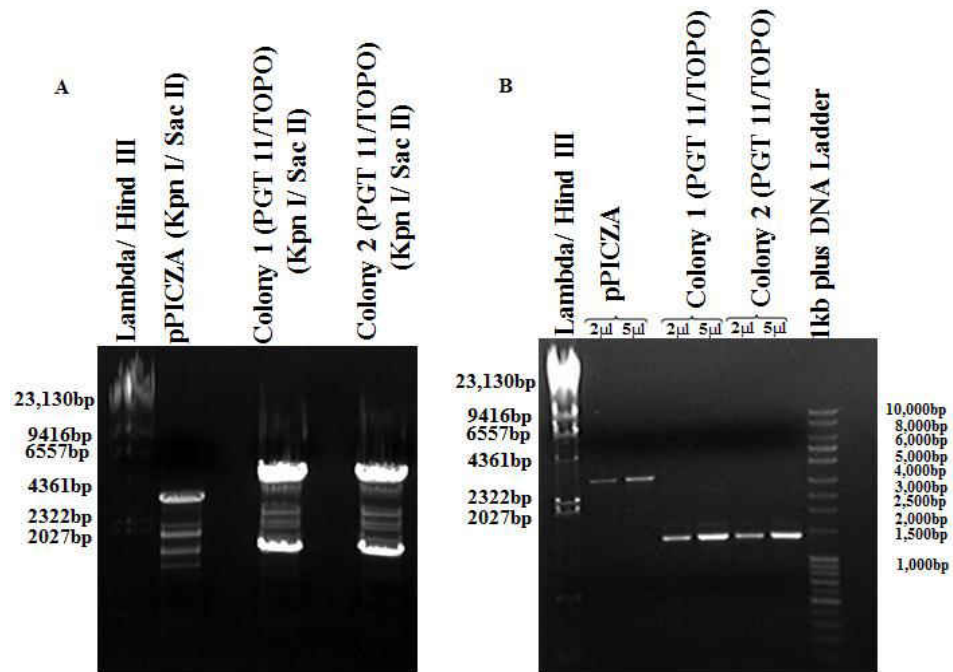
Figure 38. Digested and Gel Purified PGT 11and pPICZA Vector. A. Restriction digests of pPICZA and colonies A and B plasmids. B. Gel purified pPICZA and PGT 11 for ligation. Concentrations of each sample were determined by comparing the band intensities with that of the quantitative markers.

A

```
        ....|....| ....|....| ....|....| ....|....| ....|....|
                10        20        30        40        50
D-pPICZA_5  GGGTACGACT TTTACGACAC TTGAGAAGAT CAAAAAACAA CTAATTATTC
PGT 11      ---------- ---------- ---------- ---------- ----------


        ....|....| ....|....| ....|....| ....|....| ....|....|
                60        70        80        90        100
D-pPICZA_5  GAAACGAGGA ATTCACGTGG CCCAGCCGGC CGTCTCGGAT CGGTACCGCC
PGT 11      ---------- ---------- ---------- ---------- ----------


        ....|....| ....|....| ....|....| ....|....| ....|....|
                110       120       130       140       150
D-pPICZA_5  ATGGCCATGA GCAGTCCCCA TGTTGTGGTA ATACCGAATC CAGAACAAGG
PGT 11      ------ATGA GCAGTCCCCA TGTTGTGGTA ATACCGAATC CAGAACAAGG
```

B

```
        ....|....| ....|....| ....|....| ....|....| ....|....|
                1360      1370      1380      1390      1400
D-pPICZA_3  TCCATCAAGC AATGGCCCGC ACCGCGGCGG CCGCCAGCTT GGGCCCGAAC
PGT 11      TCCATCAAGC AATGGCCCGC ATGA------ ---------- ----------


        ....|....| ....|....| ....|....| ....|....| ....|....|
                1410      1420      1430      1440      1450
D-pPICZA_3  AAAAACTCAT CTCAGAAGAG GATCTGAATA GCGCCGTCGA CCATCATCAT
PGT 11      ---------- ---------- ---------- ---------- ----------


        ....|....| ....|....| ....|....| .
                1460      1470      1480
D-pPICZA_3  CATCATCATT GA
PGT 11      ---------- ---------- ---------- -
```

Figure 39. DNA Sequencing Analysis of Recombinant PGT 11 with Original PGT 11 sequence.
A**.** 5' sequence alignment of rPGT 11 with original PGT 11 sequence. B. 3' sequence alignment.
Red=PGT 11; Blue= restriction site; Purple= pPICZA.Tan=introduced yeast consensus sequence.
Green= 6X His tag. Orange=C-myc epitope.

CHAPTER 4

DISCUSSION

Plant secondary metabolites play several roles in plants and have also been shown to have positive impacts on human health (Zhao et al. 2005 and ref. therein; Martens et al. 2010 and ref. therein; Kennedy and Wightman 2011). Flavonoids are a class of plant secondary metabolites and constitutes the third largest class of secondary metabolites with the terpenoids and alkaloids occupying the first and second positions, respectively (Zwenger and Basu 2008 and ref. therein; Martens et al. 2010 and ref. therein). Flavonoids play vital roles such as pigmentation, antibacterial and antifungal agents, UV protection, allelopathy, flavor, feeding deterrents, and feeding attractants, pollen germination, plant-plant interactions, and plant-insect interactions amongst other roles in plants (Li et al. 1993; Miller et al. 1999; Iwashina 2003 and ref. therein; Bylka et al. 2004; Cushnie et al. 2005). Several studies have also shown flavonoids to play vital roles in human health such as coronary heart diseases, strokes, cancer, and wound healing amongst others (Cook and Sammons 1996 and ref. therein; Brusselmans et al. 2005, Sen et al. 2002, Vaya et al. 2003; Shetty et al. 2007). The remarkable properties of this class of compounds have drawn significant attention and a lot of research work on these compounds is being carried out.

Once flavonoids are synthesized in plants, they undergo several different modifications and one of the prominent modifications is glucosylation. Glucosylation of these compounds changes their chemical properties as well as their biological activities. Glucosylation has been shown to impact the stability and transport of these compounds (Fukuchi-Mizutani et al. 2003; Zhao and Dixon 2009). Flavonoids are also involved in plant pigmentation and glucosylation has been shown to be important for this property (Fukuchi-Mizutani et al. 2003). Glucosylation, in

part, has also been shown to have an impact on the taste properties of these compounds (Mansell and McIntosh 1990). As mentioned earlier, these compounds play several roles in plants and most of the active forms required to perform the functions are glucosides (Iwashina 2003 and ref. therein; Solovchenko and Schmitz-Eiberger 2003). Glucosylation is therefore a very important modification for this class of compounds.

The glucosylation of flavonoids is catalyzed by glucosyltransferase (GT) enzymes. These enyzymes bind a UDP-activated glucose and transfer the glucose moiety to its substrate (Owens and McIntosh 2009). These enzymes are therefore very important as they catalyze the modification of some of these flavonoids to their biologically active forms. Naringin, a bitter tasting flavanone, is highly accumulated in leaf tissues of grapefruit and makes up to 40-70% dry weight of young leaves and fruits (Jourdan et al. 1985; McIntosh and Mansell 1990). Naringin is a diglycoside and one of the sugar moieties is glucose. The enzyme that catalyzes the glucosylation of naringenin to yield prunin in grapefruit has been identified and biochemically characterized (McIntosh and Mansell 1990; McIntosh et al. 1990). Prunin is eventually rhamnosylated on the glucose moiety to yield naringin (Bar-Peled et al. 1991). Other subclasses of flavonoids such as flavones and flavonols are also accumulated in high levels in the glucoside form (McIntosh and Mansell 1990; Owens and McIntosh 2011 and ref. therein). A flavonol 3-O-GT (GQ141630) clone from grapefruit was identified and biochemically characterized (Owens and McIntosh 2009). Other plant secondary metabolites and phenolics are also present in grapefruit as glucosides (Berhow et al. 1998, Fieldman and Hanks 1965). Due to the high accumulation of flavonoid and other plant secondary metabolite glucosides in grapefruit, it is likely to be a rich source of these diverse GT enzymes.

The homology between different flavonoid GT sequences outside the PSPG box is very low. For example, the PSPG box of PGT 1 had up to 60% identity when compared with some other GTs but this identity decreased when the entire sequences were compared (RoySarkar et al. 2007). Also, results from aligning individual pairs of a total of 88 GT sequences from *Arabidopsis thaliana* showed substantial differences between these sequences some lower than 30% (Li et al. 2001). This makes it difficult to predict the function of these enzymes based on their sequence alone. GTs with very low sequence identity (20%) were shown to catalyze the glucosylation of the same set of molecules (Osmani et al. 2008 and ref. therein). Two other GT enzymes from the same class showed different levels of substrate specificity, one having a narrow specifity while the other having broad acceptor specificity (Osmani et al. 2008 and ref. therein). It is therefore necessary to clone, express, and screen for GT activity against a variety of substrates from different classes.

After PGT 9 and PGT 11 were identified from the harvEST database as Cit. 1322.1 and Cit. 6237, primers were designed and the sequences were successfully amplified from young graprefruit leaf cDNA (Mallampalli 2009). The sequence amplified from the young grapefruit leaf cDNA (PGT 9) had a single base difference at position 1263 when compared to the consensus sequence that was obtained from the database (Cit. 1322.1) as shown in Figure 11. This single base, however, did not lead to any amino acid change. This strongly suggests that PGT 9 could be evolutionarily conserved within citrus as the harvEST database consists of expressed sequence tags from different Citrus species. The PGT 9 sequence contained the PSPG box and was therefore hypothesized to be a plant secondary product glucosyltransferase. Based on previous biochemical characterizations of flavanone 7-O-GT and flavonol 3-O-GT (McIntosh and Mansell 1990; McIntosh et al. 1990; Owens and McIntosh 2009), the hypothesis that rPGT 9

is a flavonoid GT and is biochemically regulated was also tested. The same hypotheses were also tested for rPGT 11. To test these hypotheses, rPGT 9 and 11 were cloned into yeast for heterologous expression and biochemical characterization.

Expression of rPGT 9 in *E. coli*

Heterologous expression of rPGT 9 was first carried out using the *E. coli* expression system. The *E. coli* expression system offers a lot of advantages over other eukaryotic expression systems such as simplicity, well known genetics, fast high-density cell cultivation, as well as being inexpensive (Sorenson and Mortensen 2005). The vector used was pCD1, a modified version of the pET vector that has a thrombin cleavage site where the tags can be cleaved off after expression of the recombinant protein (Owens and McIntosh 2011 and ref. therein). It was cloned such that rPTG9 was expressed with an N-terminal thioredoxin tag and polyHis tag. Initial attempts to express rPGT 9 in *E. coli* yielded very little soluble rPGT 9 while the most of it was packed in inclusion bodies (Mallampalli 2009). The reasons why rPGT 9 may be packed in inclusion bodies include: over-expression, misfolding, and because it is a foreign protein. Several attempts to increase the amount of rPGT 9 such as varying media, temperature, and time of sample collection post induction did not yield any fruitful results (Mallampalli 2009).

Another factor that has been shown to increase the amount of soluble protein expression in *E. coli* is osmotic stress (Arakawa and Timasheff 1985; Blackwell and Horgan 1991). Blackwell and Horgan (1991), on using sorbitol and glycine betaine, reported a dramatic increase in the amount of soluble protein as well as the disappearance of the protein from the insoluble fraction. This strategy was employed in order to attempt to increase the amount of soluble rPGT 9 being expressed. Based on Western blot analysis of samples induced in the presence and

absence of osmotic stress, a slight increase in the amount of soluble rPGT 9 was observed (Figure 16). Expression in the presence of betaine and sorbitol while varying temperature and time of sample collection postinduction was carried out in an attempt to determine the optimum conditions that yielded the most soluble rPGT 9. As shown in Figure 17, 25°C was still the optimum temperature while 6 hours was best time for sample collection. Even though this was the case, the amount of soluble rPGT 9 observed while expressing rPGT 9 under the same conditions was not repeatable. In an attempt to increase the amount of soluble rPGT 9 that could be isolated, the total volume of the cell culture was increased to 1 liter and the sample was processed and IMAC performed. Despite this, enough rPGT 9 was not obtained to test for activity and there was a lot of contaminating proteins in the fraction that contained the rPGT 9 protein (Figure 19).

Even though some other clones yielded sufficient soluble protein when expressed in *E.coli* to test for activity (Owens and McIntosh 2009; Lin 2011) this was not the case with rPGT 9. Significant effort was put into expressing rPGT 9 in *E. coli* because the rPGT 9 was expressed such that it had a thrombin cleavage site at which the N-terminal tags could be cleaved (Owens et al. 2008; Owens and McIntosh 2009). This would have permitted the testing of activity of rPGT 9 with and without the tags to determine if the tags affected the activity of the rPGT 9. Due to the unsuccessful expression of sufficient amounts of soluble rPGT 9 in *E. coli*, another expression system was used.

<div align="center">

Expression of rPGT 9 in *Pichia pastoris*

</div>

Being a eukaryotic protein, expressing rPGT 9 in a eukaryotic expression system offers more advantages than a prokaryotic system. *Pichia pastoris*, a yeast strain, was selected as the

eukaryotic expression system. The advantages offered by using yeast for heterologous expression include: protein processing, folding and posttranslational modifications, higher protein production levels both intracellularly and extracellularly as well as the availability of the expression system as a commercially available kit (Cereghino and Cregg 1999; Invitrogen Manual Cat. No. K1740-01 2010 and ref. therein). PGT9 is a eukaryotic protein and may therefore fold better in an environment that is similar to its natural source than when expressed in a prokaryotic system. The major problem of inclusion bodies that was faced in *E. coli* is avoided by using a eukaryotic system and, as such, successfully expressed rPGT 9 will be found in the soluble fraction that can be used to test for activity. The Pichia expression system when compared to other eukaryotic systems is faster, easier, and less expensive (Invitrogen Manual Cat. No. K1740-01 2010). Unlike the pCD1 vector, the pPICZ vectors have not been modified such that the tags can be cleaved off after recombinantly expressing PGT 9. It will not be possible to test the effect of the tags on the activity of rPGT 9 if activity is found. One option would be to clone PGT 9 into the vectors with its stop codon before the tags, but this will result in losing the tags which are used to confirm expression and also enrich rPGT9. Another option would be to modify the vectors such that the protein is expressed with a cleavage site between rPGT 9 and its tags. As such the tags can be cleaved off.

pPICZA and pPICZAα were used as vectors for cloning rPTG 9 into Pichia. pPICZAα contains an α-secretory signal that drives the secretion of the expressed recombinant protein into the media. Expression of rPGT 9 in pPICZA does not lead to the secretion of rPGT 9 into the media and the cells have to be lysed to obtain the expressed protein. Both strategies were employed in cloning PGT 9 into *Pichia*. Successful cloning was confirmed by PCR using the yeast genome as a template and primers specific to rPGT 9 (Figure 31).

Secretion of rPGT 9 into the media serves as a purification step as *Pichia* secretes low levels of native proteins and as such, the major protein component of the media will be the secreted recombinant protein. Despite this apparent advantage, *Pichia* transformed with rPGT 9 using pPICZA was used for expression over pPICZAα. The reason is that during the process of secreting the expressed recombinant protein from the *Pichia* cells, proteins with glycosylation site (Asn-X-Ser/Thr) get glycosylated (Invitrogen Manual Cat. No. K1740-01 2010). There were a couple of these sites found in the PGT 9 sequence and glycosylation would likely have a negative effect on the activity of the protein. The large volume of the media used to carry out induction is another obstacle as this needs to be concentrated before analysis. Given the low stability of flavonoid glucosyltransferases, secretion of the rPGT 9 in the media for up to 6 hours at 30°C could also lead to significant loss of activity before screening assays are performed. Despite this, an attempt was made to take advantage of the purification that is achieved after secretion of rPGT 9 into the media.

rPGT 9 expression was induced with methanol. The rPGT 9 was cloned into pPICZA and pPICZAα to be under the control of the AOX1 promoter that is turned on in the presence of methanol. So, in the presence of methanol, the AOX1 promoter is turned on and the expression of rPGT 9 is driven. Successful expression was confirmed by Western blot analysis for rPGT 9 cloned in pPICZA. rPGT 9 was not detected in the media and so the secretion strategies with pPICZAα did not work. 6 hours post induction was observed to be the best time for sample collection (Figure 32). Immobilized metal affinity chromatography was used to enrich rPGT 9 and the enriched fraction was used to test screen for GT activity (Figure 33).

Due to the presence of the PSPG box, it was hypothesized that rPGT 9 was a plant secondary product glucosyltransferase, specifically a flavonoid GT. As such, some plant

secondary metabolites which are found in grapefruit were used to screen for GT activity (Berhow et al.1998; Fieldman and Hanks 1999; Owens and McIntosh 2011 and ref. therein). As glucosylated flavonoids are highly accumulated in *Citrus paradisi,* representative members of different classes of flavonoids were screened as the first potential substrates for rPGT 9. No significant activity was detected for the initial screens (Table 19). Therefore, results obtained with flavonoids do not support the hypothesis that PGT 9 codes for a flavonoid GT.

A few potential simple phenolic substrates were also tested. As shown in Figure 34, some of the simple phenolics did show new peaks for the experimental. This suggests that these compounds could be potential substrates for rPGT 9. There was reduction in the peak height of quercetin aglycone and appearance of several peaks in the experimental. Crude leaf extract was used and as such there are several enzymes present that could have used up quercetin to form multiple products. Also the presence of multiple peaks can be associated with the presence of other compounds in the crude extract. Because the quercetin aglycone was consumed significantly, it confirms that the assay conditions used were good.

Based on the chromatogram of caffeic acid (Figure 34 C), the aglycone peak was not observed in the experimental and this suggests the complete conversion of the aglycone to product. To observe how the caffeic acid aglycone is converted to product over time, then a time course reaction would be needed. For some of the substrates such as catechol, vanillin, vanillic acid, gentisic acid, *p*-hydroxyphenylacetic acid, and *p*-hydroxybenzoic acid, there was a slight reduction in the aglycone peak and/or the appearance of new peaks. The appearance of a new peak with a shorter retention time is indicative of the formation of a more polar compound which could be the glucoside. These compounds may be potential substrates for rPGT 9 protein. Some GTs are known to use several substrates and this could be the case for rPGT 9. A negative

control was carried out just for *p*-hydroxybenzoic acid and the new peaks observed in the experimental were also observed in the negative control sample (Figure 34 B). This may be because the enzyme was not completely denatured. The peak could also be as a result of some other component of the reaction. These experiments need to be repeated to confirm the results.

The λmax used for the analyses were those of aglycones. If there is a considerable difference between the λmax of the aglycone and glucosides of these compounds, then it may not be possible to fully detect the glucoside peaks for the some of the compounds. It is for this reason that a reduction in peak height of the standard aglycone in the experimental is considered as an indication of activity. The use of several controls and possible the λmax of the aglycone and glucoside of these compounds will be necessary for interpretation.

If the results with these simple phenolics are not reproducible, then the substrate of rPGT 9 remains elusive. As such additional potential substrates found in grapefruit should be screened. There is also a possibility that rPGT 9 may have been denatured in the process of lysing the cells and during the process of enrichment even though similar methods were used for the biochemical characterization of rPGT 7 (GQ141630) (Owens and McIntosh 2009). Another possibility could be that PGT 9 uses a different sugar donor other than UDP-glucose. Even though most plant GTs use UDP-glucose as a sugar donor, there are other possible UDP-sugar donors such as UDP-xylose, UDP-galactose, UDP-rhamnose, UDP-glucuronic acid and UDP-mannose (Osmani et al. 2008). When a BLAST search was done using rPGT 9 protein sequence, it showed similarity to glucosyltransferases (data not shown) but, as mentioned earlier, function cannot be predicted by sequence alone and testing needs to be done. The C-terminal tag could also interfere with the 3-D structure of the enzyme and, because there is no cleavage site to cut off the tag, the effect of the tag on the rPGT9 sequence will have to be tested at a later date.

<u>Directions for Future Research</u>

The first logical step will be to retest the simple phenolic substrates that seemed to have products and/or a decrease in the peak of the aglycone. A time course experiment will confirm the conversion of substrate to product or simply the disappearance of the substrate that can be monitored by HPLC. This time course experiment is also necessary to determine the initial velocity of the reaction which is important for further characterization of rPGT 9. If one or all of these simple phenolics are substrates, then the kinetics of rPGT 9 can be studied in order to determine which of the substrates is the preferred substrate for rPGT 9. The optimum pH, buffer, and temperature will need to be determined before studying the kinetics of rPGT 9.

So far, not all the potential substrates found in grapefruit have been screened. If the simple phenolic experiments are not reproducible, the next logical step will be to continue screening for substrates until the PGT 9 activity is determined. Several other simple phenolics found in grapefruit that are not currently available in the lab such as sialicylic acid, phloretin, m-coumaric among others should be acquired and tested (Fieldman and Hanks 1965). For example, there are other classes of plant secondary metabolites that have also been shown to exist in the glucosylated form such as terpenoids. Limonoate A-ring monolactone, a triterpenoid compound in grapefruit has also been shown to be glucosylated and would be a potential substrate to be tested (Hasegawa et al. 1997). The hypothesis that this is a plant secondary metabolite GT cannot be ruled out until other potential substrates are screened.

The presence of the C-terminal tag could also affect the activity of PGT 9. The PSPG box that confers recognition and binding to the UDP activated sugar is situated near the C-terminal. There is a possibility that the tag could influence the 3-D structure of the rPGT 9 protein and

hence its ability to bind to the sugar donor or possibly the substrate as well. When PGT 7 (GQ141630) was tested for activity with and without the N-terminal tags under optimal conditions (40°C, phosphate buffer, pH 7.5), there was little difference between the activities (Owens and McIntosh 2009) but when Tris HCl (pH 7.5) was used in place of phosphate buffer (pH 7.5), there was a 70% reduction in activity for the recombinant GQ121630 (McIntosh and Owens 2009). This shows that the the tag had an effect on the enzyme activity and this should be tested for rPGT 9. GQ141630 was expressed in *E. coli* with an N-terminal tag as opposed to a C-terminal tag for rPGT 9. Therefore, the effect of the C-terminal tag on activity remains to be tested. Using a modified expression system that could result in the cleavage of the tags after expression of rPGT 9 could reveal the effect of the tag on the activity of the protein as screens can be carried out with and without the tag. The pPICZA vector should be modified to have a cleavage site such that when PGT 9 is expressed, the tags could be cleaved off.

Also, as GQ141630 showed activity against flavonols, this clone will be heterologously expressed in yeast with a C-terminal tag and the activity compared with that expressed in *E. coli* with an N-terminal tag to compare the effect of both tags on GQ141630 activity (Devaiah and McIntosh Personal communication). If there is a significant decrease or total loss of GQ141630 acitivity with C-terminal tags, then it is possible that the C-terminal tag could be interfering with the binding of the enzyme to the UDP-glucose since the UDP-glucose binding motif (PSPG box) for the enzyme is situated near the C-terminal.

The reaction conditions used such as phosphate buffer of pH 7.5 as well as the temperature of 37°C are based on other experiments that have been conducted so far on other glucosyltransferases, most of which seemed to work best at these conditions (McItosh and Mansell 1990; McIntosh et al. 1990; Owens and McIntosh 2009). The optimum temperature for

flavonol 3-O-GT was 40°C (Owens and McIntosh 2009) while that if the flavanone 7-O-GT was 37°C (McIntosh and Mansell 1990). Once activity of rPGT 9 is found, then the temperature and buffer pH would be varied to determine the optimal conditions required for its activity.

PGT 11 is currently in the process of being transformed into *Pichia*. So far, PGT 11 has been successfully cloned into pPICZA vector. Once the desired linearized PGT 11-pPICZA construct is obtained from phenol: chloroform extraction, it should be transformed into *Pichia*. Optimization of expression and enrichment should be carried out as described for rPGT 9. The rPGT 11 protein should be screened for GT activity following the same procedures as described for rPGT 9 and for the flavonol 3-O-GT clone (Owens and McIntosh 2009).

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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 with sorbitol

APPENDIX B: Stock Solutions and Media Recipes

10X YNB:

134 grams of yeast nitrogen base (YNB)

Add 1000ml of distilled water

Heat solution to dissolve completely

Filter sterilize the solution and store at 4°C

500X B (0.02% Biotin):

20mg biotin

Add 100ml dH$_2$O

Filter sterilize the solution and store at 4°C

10X D (20% Dextrose):

200 grams D-glucose

Add 1000ml dH$_2$O

Filter sterilize and store at room temperature

10X M (5 % Methanol):

5ml methanol

Add 95ml dH$_2$O

Filter sterilize and store at 4°C

10X GY (10 % Glycerol):

100ml glycerol

Add 900ml dH$_2$O

Filter sterilize and store at room temperature

Plain Liquid LB Medium:

25 grams LB broth powder

Dissolve in 900ml of dH_2O

Adjust pH to 7.5

Make up volume to 1000ml

Autoclave and store at 4^oC

Liquid LB Medium with Antibiotics

25 grams LB broth powder

Dissolve in 900ml of dH_2O

Adjust pH to 7.5

Make volume up to 1000mL

Autoclave for 20minutes and allow to cool to about 55^oC

Add 0.1 gram of Amplicillin and/or without 0.034 gram of Chloramphenicol

Store at 4^oC

LB-Agar Plate with Antibiotics:

12.5grams of LB broth powder

Add 900ml of dH_2O

Adjust pH to 7.5

Add 7.5 grams of agar

Make up volume to 1000ml

Autoclave for 20minutes and allow to cool to about 55^oC

Add 0.1 gram Ampicillin and/or 0.034 gram Chloramphenicol

Pour media into plates and allow to solidify

Store at 4^OC.

Plain Liquid low Salt LB medium:

10 grams tryptone

5 grams yeast extract

5 grams NaCl

Dissolve in 900ml of dH_2O

Adjust pH to 7.5

Add dH2O to 1000ml

Autoclave for 20 minutes

Store at 4^oC

Liquid Low Salt LB Medium with Antibiotics:

10 grams tryptone

5 grams yeast extract

5 grams NaCl

Dissolve in 1000ml of dH_2O

Autoclave for 20 minutes

Allow to cool to about 55^oC

For low salt LB with Zeocin, add 25mg of zeocin

Store at 4^oC. If media contains Zeocin store in the dark

Low Salt LB Agar Plates:

To a liter of plain low salt liquid LB media, add 15g agar

Autoclave for 20 minutes

Allow medium to cool to about 55^oC

Add desired antibiotics

Pour into plates, allow medium to solidify

Invert plates and store 4^oC (If zeocin is added, store in the dark).

Plain liquid YPD medium:

10 grams yeast extract

20 grams peptone

Add sterile dH2O to 900ml

Autoclave for 20 minutes

Add 100ml 10X

Liquid YPD Medium with Zeocin:

10 grams yeast extract

20 grams peptone

Add sterile dH2O 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°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°C

Add 25 mg of Zeocin

Pour medium in plates and allow to solidify

Store plates containing zeocin in the dark at 4°C

Liquid YPDS Medium with Zeocin:

10 grams yeast extract

182.2 grams sorbitol

20 grams peptone

Add sterile dH2O to 900ml

Autoclave for 20 minutes

Add 100ml 10X D

Cool solution to $\approx 60^oC$

Add 100 mg of Zeocin and store medium at 4^oC 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 dH2O to 900 ml

Autoclave for 20 minutes

Add 100 ml 10X D

Cool solution to $\approx 60^oC$

Add 100 mg of Zeocin and pour into plates.

Store plates in the dark at 4^oC

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 sterile solutions:

100ml 1M potassium phosphate buffer, pH 6

100ml 10X YNB

2ml 500X B

100ml 10X GY

Store media at 4°C

BMMY Medium:

10 grams yeast extract

20 grams peptone

Dissolve above in 700ml distilled water

Autoclave 20 minutes

Cool to room temperature and add the following sterile solutions:

100ml 1M potassium phosphate buffer, pH 6

100ml 10X YNB

2ml 500X B

100ml 10X M

Store media at 4°C

APPENDIX C: Staining Solution Recipes

Ponceau S staining solution:

0.5 grams Ponceau S

10ml acetic acid

Add dH$_2$O to 100ml

Amido Black Stain B:

0.1 gram Amido Black 10B

10ml acetic acid

Add dH$_2$O to 100ml

Coomassie Blue Staining Solution:

0.006 grams Coomassie Brilliant Blue G250

10ml acetic acid

40ml methanol

Add ddH$_2$O to 100ml

Destaining Solution

300ml Methanol (30%)

100ml Acetic acid (10%)

Make up volume to 1000ml with dH$_2$O

APPENDIX D: Gel Recipes

<u>SDS-PAGE Gel (10 %)</u>

Separating Gel Composition:

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

1.25ml 4X running buffer

2.33ml distilled water

50µl 10% SDS

50µl APS

10µl TEMED (add last)

Stacking Gel composition:

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

500µl 4X stacking gel buffer

1.84ml distilled water

20µl 10% SDS

20µl APS

5µl TEMED (add last)

<u>0.8/% Agarose Gel</u>

0.24 grams Agarose powder

Add 30 ml of 1X TAE buffer

Heat until agarose melts

APPENDIX E: Buffer Recipes

4X SDS-PAGE Seperating Gel Buffer:

36.5 grams (1.5 M) Tris-base

Adjust pH to 8.8

Add dH$_2$O to 200ml

4X SDS-PAGE Stacking Gel Buffer:

3 grams (1.5 M) Tris-base pH 6.8

Adjust pH to 8.8

Add ddH$_2$O to 200ml

SDS-PAGE Running/ Tank Buffer:

30 gramsTris Base

144 grams Glycine

10 grams SDS

Add dH$_2$O to 1000ml

Adjust pH to 8.3

10% SDS Solution:

10 grams SDS

Add ddH$_2$O to 100ml

2X SDS-PAGE Sample Buffer:

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

4 grams SDS

20ml glycerol

Add dH$_2$O to 100ml (To every 1ml of the mixture, add 20μl βME before use).

4X SDS-PAGE Sample Buffer:

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

2ml 20% SDS Solution

4ml 50% glycerol

0.2mg Bromophenol Blue

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

10X TBS-T Buffer:

8.76 grams sodium chloride

10ml of 1M Tris-base solution pH 8.0

500μl of tween-20

Add ddH$_2$O to 1000ml

10X PBS Buffer:

76 grams NaCl (1.3M)

10 grams Na$_2$HPO$_4$ (70mM)

4.1 grams NaH$_2$PO$_4$ (30mM)

Adjust pH to 7.2

Add ddH$_2$O to 1000 ml

1X PBS-T Buffer:

100ml 10X PBS

Add ddH$_2$O to 1000ml

Add 0.05 % of tween 20 and stir to mix completely

10X Western Blot Transfer Buffer:

30 grams Tris base

144 grams glycine

Add dH_2O to 1000 ml

Alkaline Phosphatase Buffer:

5.8 grams of NaCl

1.02 grams of $MgCl_2$

100ml 1M Tris base solution

Dissolve in 900ml of dH_2O

Adjust pH to 9.5

Make up volume to 1000ml

50mM Phosphate Buffer (pH 7.0):

0.2918 grams of Monosodium Phosphate

0.7733 grams of Disodium Phosphate

Dissolve in 900ml of dH_2O

Adjust pH to 7

Make up volume to 1000ml

IMAC Equilibration/ Wash Buffer (pH 7.5):

0.2918 grams of Monosodium Phosphate

0.7733 grams of Disodium Phosphate

17.532 grams of NaCl

Dissolve in 900ml of dH_2O

Adjust pH to 7.5

Make up volume to 1000ml

IMAC Elution Buffer (pH 7.5):

0.2918 grams Monosodium Phosphate

0.7733 grams Disodium Phosphate

17.532 grams NaCl

10.21g Imidazole

Dissolve in 900ml of dH_2O

Adjust pH to 7.5

Make up volume to 1000ml

IMAC MES Buffer (pH 5.0):

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

Adjust pH to 5.0

Add dH2O to 1000ml

50X TAE Buffer:

50ml EDTA (pH 8.0)

28.6ml Glacial Acetic Acid

121 grams Tris Base

Add ddH2O to 1000ml

Breaking Buffer:

6 grams sodium phosphate (monobasic)

372mg EDTA

Dissolve in 850ml of dH_2O

Add 50ml glycerol

Adjust pH to 7.4

Add dH2O to 1000ml

Store at 4^oC

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

1 M Potassium Phosphate Buffer, pH 6:

132ml 1 M K_2HPO_4

868ml 1M $KHPO_4$

Adjust pH to 6.0

Autoclave for 20 minutes and store at room temperature

Phenol Equilibration:

Measure desired volume of phenol (25ml)

Add 0.1% 8-hydroxyquinoline 2-carboxylic acid (25mg)

Add equal volume (25ml) of 0.5M Tris-HCL (pH 8.0)

Stir above mixture for 15 minutes

Remove the upper-most layer of the mixture and discard
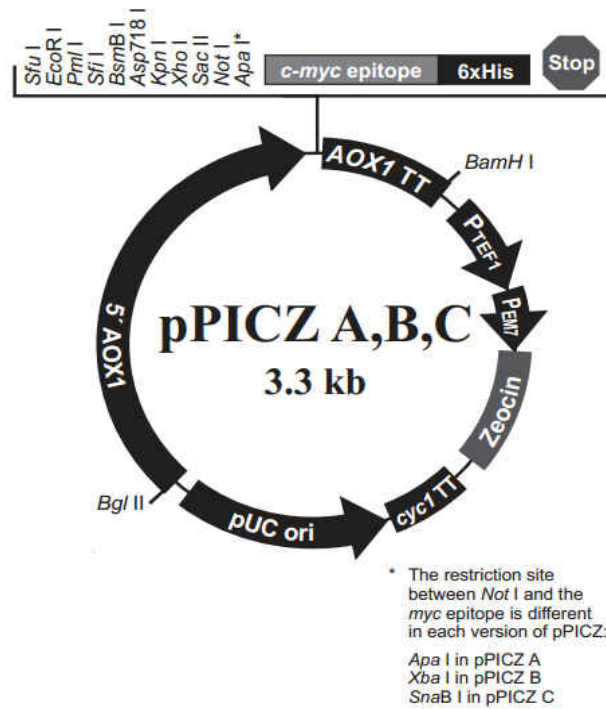
Add an equal volume (25ml) of 0.1M Tris-HCl (pH 8) to the lower layer

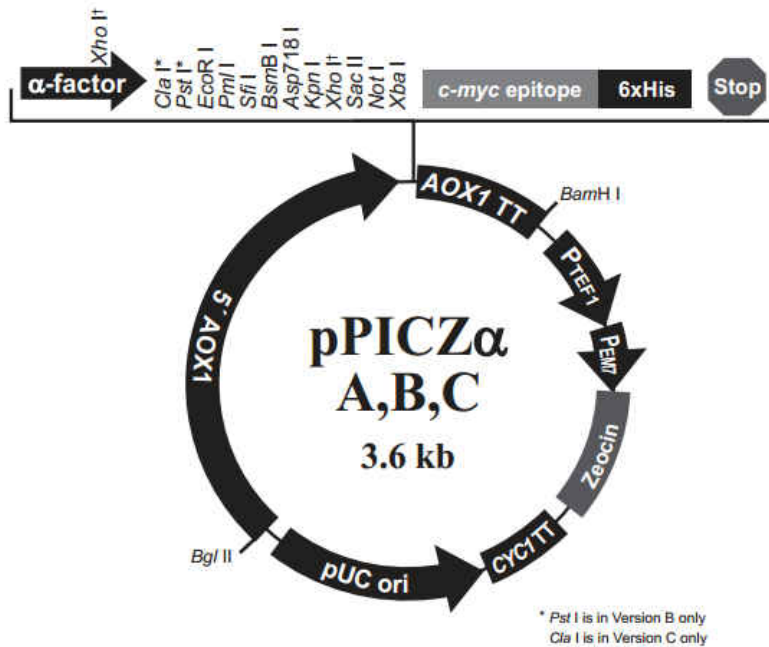Stir for 15 minutes, measure pH using pH paper

Repeat steps 3-7 until pH of phenol >7.8

APPENDIX F. Vector Maps

pPICZA Vector Map (Adapted from Invitrogen Manual)

pPICZAα Vector Map (Adapted from Invitrogen Manual)

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