Recombination and Screening of Putative Grapefruit Glucosyltransferase 4 Expressed in Pichia pastoris.

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Recombination and Screening of Putative Grapefruit Glucosyltransferase 4
Expressed in *Pichia pastoris*

Thesis submitted in partial fulfillment of Honors

By

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ABSTRACT

Flavonoids are a group of plant secondary metabolites that are vital to the cell systems of plants. The intake of these chemicals is advantageous to animals for their antioxidant properties that affect the function of immune and inflammatory cells. The bitter taste of grapefruit (*Citrus paradisi*) and other citrus species is caused by the accumulation of glycosylated flavonoids. Glucosyltransferases (GTs) are enzymes that add glucose moieties to a carbon or hydroxyl group of natural products. The function of a putative secondary product GT clone was tested. In previous research, putative GT 4 was cloned into a pCD1 modified pET expression system, heterologously expressed in *E.coli*, and screened for activity with a few substrates; little GT activity was found. Issues of protein localized to inclusion bodies in bacteria were addressed. PGT 4 is being heterologously expressed in yeast (*Pichia pastoris*) to allow for protein production and analysis. PGT 4 was screened for GT activity with different flavonoid subclass representatives and simple phenolics.
ACKNOWLEDGEMENTS

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INTRODUCTION

I. Plant Metabolism

The complex system of plant metabolism is a popular subject of academic and industrial research. The intricate pathways and products of plants have been investigated for reasons of pure academic knowledge to applied research in genetic engineering with economic implications (Harborne and Simmonds, 1964; Traka and Mithen, 2009; Kiplimo et al., 2012; and ref. therein). The plant is a self-sufficient organism comprised of mechanisms to feed, hydrate, and protect itself while maintaining a semi-static location in its environment. The primary metabolic pathways of plants, concerning their nutrition and growth, have been elucidated with the study of plants’ primary metabolites such as carbohydrates, lipids, proteins, and nucleic acids, while secondary metabolites and their enzymatic pathways are still undergoing much research (Ribéreau-Gayon, 1972; Harborne, 1993; Vaistij et al., 2009; He et al., 2012; and ref. therein). The complex role of secondary metabolites in plants is still being investigated. Regarded as “waste products” less than fifty years ago, more recent research has found that these chemicals help plants interact with their environment and significantly impact a plant’s health and defense (Springob and Kutchan, 2009; and ref. therein).

Adapting over generations by natural selection, plants and their metabolic pathways have evolved to better reproduce and protect against the environment and potential predators (Wink, 2003; and ref. therein). For these reasons, plants contain pathways to produce plant secondary products which directly help to increase reproductive success by flavor and pigment production and UV petal patterning (Harborne and Boardley, 1983; Owens and McIntosh, 2011; Scora and Kumamato, 1983; Kesterson and Hendrickson, 1957; and ref. therein). Secondary plant
metabolites are compounds found in all higher plants that also function as a defense against herbivores, microbes, viruses, and other harmful entities (Harborne, 1964; Vogt and Jones, 2000; Wink, 2003; Ferreyra et al., 2012; and ref. therein). The accumulation of secondary metabolites, such as phenylpropanoids, terpenoids, betacyanins, and flavonoids, improves the overall survival and reproductive fitness of plant species (Harborne, 1993; Wink, 2003; Vaistij et al., 2009; Ferreyra et al., 2012; and ref. therein).

II. The Flavonoids

The McIntosh lab focuses its research primarily on one group of secondary metabolites, the flavonoids. Flavonoids are derived from the amino acid phenylalanine which is formed via the Shikimate pathway. The flavonoids are subsequently formed via the phenylpropanoid pathway to produce a fifteen carbon structure (reviewed in Harborne and Simmonds, 1964; Ribéreau-Gayon, 1972; Winkel-Shirley, 2001; Owens and McIntosh 2011). These carbons are arranged by a multi-enzyme pathway into two benzene rings connected by a three carbon bridge (Figure 1). This basic structure of flavonoids is further modified by enzymes to create the subclasses of flavonoids which include chalcones, aurones, flavanones, flavones, isoflavones, dihydroflavonols, flavonols, leucoanthocyanidins, and anthocyanidins (reviewed in Harborne and Simmonds, 1964; Owens and McIntosh, 2011).

These subclasses differ by substitutions in ring composition. For example, flavonoid 3 hydroxylase (F3H) adds a hydroxyl group to the position 3 of the flavonoid skeleton of dihydroflavonols, and flavonol synthase (FLS) creates a double bond on the carbon bridge to

Figure 1. Flavonoid Skeleton (Celestino et al., 2003)
produce flavonols. The phenylalanine and flavonoid biosynthetic pathways can be seen in Figure 2 (Winkel-Shirley 2002).

![Flavonoid Biosynthetic Pathway](image)

**Figure 2. Flavonoid Biosynthetic Pathway** (Adapted from Winkel-Shirley 2002)

All of the subclasses have different functions within the plant and are found in an array of different plant species (Harborne and Simmonds, 1964; Springob and Kutchan, 2009; Harborne, 1993; Winkel-Shirley 2002; and ref. therein). Flavonoids, such as anthocyanidins, are known to help in pigment production and therefore have been found most abundantly in the outer coverings of fruits or petals of plants (Forkman et al., 1980; Modolo et al., 2009). However
other subclasses can be found in more abundance in the fruit tissue and leaves of plants which are consumed by animals (Harborne, 1993; Scora and Kumamato, 1983; Berhow, 1998). Quercetin, a flavonol, is one of the most widely distributed flavonoids in the world and can be found in many edible plants such as apples and onions (Ribéreau-Gayon, 1972).

III. Benefits of Flavonoids on Human Health

The flavonoids contain many properties that benefit the health of the plants as well as an organism that consumes it (Springob and Kutchan, 2009; and ref. therein). The presence of flavonoids in a healthy diet has made them a popular subject for possible pharmaceuticals or medical treatments. The benefits of these naturally-occurring aromatic secondary plant metabolites are extensive and include antioxidant, antiviral, antiallergic, antidiabetic, cardioprotective, and anti-carcinogenic characteristics (Rice-Evans et al., 1995; Caltagirone et al., 2000; Traka and Mithen, 2009; Toumi, 2009). Thus, the study of the synthesis and regulation of flavonoids is vital for economic and clinical reasons.

Several clinical trials have been conducted to test possible advantageous effects of flavonoids on heart health. The Zutphen Elderly Study, conducted in the Netherlands starting in 1960, found that flavonoid intake was significantly inversely associated with mortality from coronary heart disease in elderly men, ages 65-84 (Hertog and Feskens, 1993). Their flavonoid intake was controlled through a diet of tea, onions, and apples, which all contain different subclasses of flavonoids. Overall, it was found that flavonoids inhibit the oxidation of low-density lipoprotein and reduce thrombic tendency (Hertog and Feskens, 1993). Epidemiology studies reported a high dietary intake of flavonoids can also reduce the risk of heart disease (Knekt et al., 2002). Flavonoids were also found to increase vascular relaxation in porcine coronary arteries (Xu et al., 2007). These findings have furthered flavonoid synthesis research to
discover compounds that could be used in pharmaceuticals or in a dietary treatment plan (Vastij et al., 2009).

Due to their antioxidant effects, flavonoids are used in the production of many medicines and supplements (Traka and Mithen, 2009; and ref. therein). Free radicals are produced by normal metabolic processes and/or exposure to other sources such as ultra-violet light or smoke. The unpaired electrons on free radical species can cause considerable cell damage and can lead to cancer development or other chronic diseases (So et al., 1997; Middleton and Kandaswami, 1990; and ref. therein). Flavonoids are found to have free radical scavenging abilities due to their hydroxyl group substitutions about the flavonoid skeleton (Amic et al., 2003; Rice-Evans et al., 1995).

Flavonoids can also help control the proliferation of carcinogenic cells (So et al., 1997; and ref. therein). For example, baicalein, galagin, hesperetin, naringenin, and quercetin were all found to have antiproliferative activity against breast cancer cells (So et al., 1997; Caltagirone et al., 2000). Flavones and isoflavones have also been found to prevent cancer by maintaining a rich diet of these flavonoid compounds (Birt et al., 2001). Intake of flavonoids has been found to reduce many types of cancer including lung and breast cancer (Zava and Duwe, 1997; Le Marchand et al., 2000; and ref. therein).

Flavonoids have also been found to exhibit anti-HIV activity as well as inhibit HIV-I reverse transcriptase, protease, and integrase (Wang et al., 2004; Xu et al., 2000). One study found baicalin, a flavonoid compound from a Chinese herbal medicine, inhibited HIV infection upon viral entry (Li et al., 2000). Some flavonoids have even been used to create drugs for chemotherapy of the HIV infection (Vlietinck et al., 1998). These examples only shed a small light on the plethora of flavonoid natural products that are used against the HIV infection. Again,
this shows that the flavonoids are a popular subject for pharmaceutical research to treat many different diseases.

Evidence has been found to suggest that flavonoids regulate cell function. This leads to a significant immune and inflammatory response by flavonoids that aid in secretory processes, mitogenesis, cell-cell interactions, and regulation of homoeostasis of the immune system (Middleton and Kandaswami, 1992; Traka and Mithen, 2009; and ref. therein). The flavonoids will continue to be studied for their health benefits to humans to further elucidate any other medicinal implications.

IV. **Glucosylation of Flavonoids**

Over 7,000 naturally-occurring flavonoids have been discovered to date (Harborne and Simmonds, 1964; Harborne, 1993; Andersen and Markham, 2006). The basic structure lends itself to substitutions from many types of molecules. Flavonoids can readily go under the processes of hydroxylation, acylation, glucosylation, and many other substitutions (Harborne and Simmonds, 1964; Ribéreau-Gayon, 1972; Winkel-Shirley, 2001). *In vivo*, flavonoids are found to be highly substituted; the majority of compounds are present in glycosylated forms (Andersen and Markham, 2006). Each substitution of the flavonoid skeleton changes the chemical properties of the compound.

One of the final steps of flavonoid biosynthesis, glucosylation, is a significant process because the substitution of hydrogen from the ring or hydroxyl group for a glucose molecule can affect its toxicity, regulate homeostasis, and enhance the solubility of the compound (McIntosh and Mansell, 1990; Bowles, 2006; Traka and Mithen, 2009; and ref. therein). The enzyme responsible for the transfer of the glucose moiety is called a glucosyltransferase (GT). Over
12,000 putative glucosyltransferase sequences have been collected and appear in various
databases from different organisms including but not limited to *Vitis vinifera*, *Arabidopsis thaliana*, and *Citrus paradisi*, but only around 40 GT sequences have had biochemical confirmation of function (Owens and McIntosh, 2009; Masada et al., 2009; Vaistij et al., 2009; and ref. therein). GTs that glucosylate plant natural products and small lipophilic molecules belong to the GT 1 family (Vogt and Jones, 2000; Vaistij et al., 2009). Many different types of sugars can be transferred by GTs; however, many characterized flavonoid GTs have been found to prefer uridine diphosphate glucose (UDP-glucose) for transfer (McIntosh and Mansell, 1990; McIntosh et al., 1990; Owens and McIntosh, 2009; and ref. therein). GTs that are selective for UDP-glucose are known as UGTs, and most characterized UGTs have been found to have further substrate selectivity to prefer just one subclass of flavonoids (Li et al., 2007; Modolo et al., 2007; Owens and McIntosh, 2009; and ref. therein).

V. Grapefruit Model System

The model plant system used for this research is *Citrus paradisi*, or grapefruit. The grapefruit plant is an ideal subject because it is known to contain many glucosylated flavonoids (Scora and Kumamato, 1983; Dugo et al., 2005; Berhow, 1998). Grapefruit is especially rich in flavanones and flavones (Owens and McIntosh, 2009; and ref. therein). These subclasses are known as early flavonoids as they are produced upstream in the flavonoid biosynthesis pathway (Harborne and Simmonds, 1964; Dean, 2003). Different amounts of flavonoids are found throughout all different types of grapefruit tissue and stages (McIntosh and Mansell 1990; Jourdan et al 1985; Berhow, 1998; Scora and Kumamato, 1983). In young grapefruit leaves, 40-70% of the dry weight was found to be the bitter compound naringin (McIntosh and Mansell, 1990; Jourdan et al., 1985; Kesterson and Hendrickson, 1957). Some speculate that this
compound in the young grapefruit tissue has a bitter taste for defense (Del Rio et al., 1998). It has been discovered that when a rhamnose sugar is added to naringenin 7-O-glicoside, based on the substitution site, it can change the flavor composition to bitter naringin or tasteless narirutin. Tasteless linkages are found in non-bitter species, such as *Citrus reticulata* and *Citrus sinensis*. Both linkages, bitter and tasteless, are found within *Citrus paradisi* (Frydman et al., 2004; Jourdan et al., 1985; Kesterson and Hendrickson, 1957, Barthe et al., 1987, Berhow 1998). The effect of glycosylation on taste makes it a popular subject of research for the citrus industry (Horowitz and Gentili, 1969; Shaw et al., 1984; Drewnowski et al., 2001; Mansell and McIntosh, 1991; Mansell et al., 1983; Jourdan et al., 1985; Barthe et al., 1987).

VI. Putative Glucosyltransferase 4

Various putative glucosyltransferase clones studied in our lab, in particular the one presented in this research, were obtained from a young grapefruit leaf cDNA library (Roy Sarkar et al., 2007). Degenerate primers were designed against the PSPG (Plant Secondary Product Glucosyltransferase) box, a forty-four amino acid sequence unique to glucosyltransferases. Smart Race RT-PCR was used to amplify 5’ clones, and then clone specific primers were designed to “walk out” the sequences and find the 3’ ends (Sibhatu, 2003; Roy Sarkar, 2004; Roy Sarkar et al., 2007; Strong, 2005). All clones had a high identity with other glucosyltransferases within the PSPG box region and a low identity outside the PSPG box region (Roy Sarkar et al, 2007).

Figure 3 shows the PGT 4 with the start and end of PSPG box region marked with arrows.

| 1     | ATG GCA TCC GAA GCC AGC CAG GTT CAC TTT CTC TTG CTT CCT TAC | 45    |
| 1     | Met Ala Ser Glu Ala Ser Gln Val His Phe Leu Leu Leu Pro Tyr | 15    |
| 46    | TTG GCT CCA GCC CAC TGTT ATT CCC ATG TTT GAC ATT GCT AGA TTG | 90    |
| 16    | Leu Ala Pro Gly His Leu Ile Pro Met Phe Asp Ile Ala Arg Leu | 30    |
| 91    | CTT CGG CAG CAT GGA GCT ATT GTT ACT ATT GTC ACC ACC CCA GTT | 135   |
| 31    | Leu Ala Gln His Gly Ala Ile Val Thr Ile Val Thr Thr Pro Val | 45    |
Figure 3. Amino Acid Sequence of PGT 4 with arrows denoting the start and end of the Plant Secondary Product Glucosyltransferase (PSPG) Box.

The focus of this research is the study of one putative glucosyltransferase clone, PGT 4 (Figure 3). The PGT 4 sequence consists of 1467 base pairs, and the PGT 4 protein has a predicted molecular weight of 54 kD. This size is within the normal range observed for the majority of plant GTs (McIntosh and Mansell, 1990; McIntosh et al., 1990; Durren and McIntosh, 1999; Owens and McIntosh, 2009; and ref. therein).

Once the 5’ and 3’ ends of the PGT 4 clone had been determined, it was then cloned into a bacterial vector, pCR® 4-TOPO® (Invitrogen) by previous researchers, Christy Strong and Leslie Epling (Strong, 2005; Epling, 2007). Josephat Asiago cloned PGT 4 into the pCD1 vector, a modified version of the pET-32A cloning vector with an engineered thrombin cleavage site to remove the tags for better purification upon expression (Winkel, VA Tech). Once the presence of PGT 4 was confirmed within pCD1, Asiago screened for flavonoid glucosyltransferase activity of PGT 4 expressed in *E.coli*, but little to no activity was found. Inclusion bodies, vacuoles in bacteria that store expressed proteins, rendered much of the expressed protein insoluble and unable to be active. Thus, PGT 4 has now been cloned into the pPICZA vector (Invitrogen;
Sanje Roje, WSU) and transformed into *Pichia pastoris*, a eukaryotic system, to eliminate the problems with inclusion bodies and insoluble protein and to again test for flavonoid glucosyltransferase activity. This research was conducted with the hypothesis that PGT 4 would glucosylate flavonoid substrates, acting as a flavonoid glucosyltransferase.
MATERIAL AND METHODS

I. Materials

Chemicals and Reagents

Agarose, agar, yeast extract, tryptone, yeast nitrogen base powder, and sodium chloride were used in making liquid media (Fisher Scientific, Fairlawn, NJ). Ampicillin (Fisher Scientific) and zeocin™ (Research Products Int. Corp., Mt. Prospect, IL) were needed to make media and agar plates with antibiotics. Ethidium bromide, EtBr (10 mg/mL), was used to visualize DNA in gel electrophoresis. One kb DNA Ladder (Fisher Scientific) was utilized to verify DNA band lengths. Go Taq DNA Polymerase, 5 X green/colorless buffer, and deoxyribonucleotide triphosphates (dNTPs) were used for polymerase chain reactions (Promega Madison, WI). T4 DNA ligase enzyme, ligation buffer, restriction enzymes Apa I, Kpn I, EcoRI, Not I (Promega, Madison, WI), and clone specific primers (Integrated DNA Technology, Coralville, Iowa) were utilized for clone reconstruction and DNA cloning. Tris base, tetramethylethelynediamine (TEMED, electrophoresis grade), ammonium persulfate (APS), 40% acrylamide solution (acrylimide:bis-acrylamide, 19:1), coomassie brilliant blue, and glacial acetic acid were purchased from Fisher Scientific. Nitrocellulose membrane (0.45 μm pore size) was bought from Thermo Fisher Scientific, Waltham, MA. Whatman chromatography paper (3MM CHR), 5-bromo-4-chloro-3’-indolyphosphate p-toludine salt (BCIP) and nitro-blue tetrazolium chloride (NBT), (Fisher Scientific) were used for protein gels and Western blots. Monoclonal mouse c-myc antibody and goat, anti-mouse IgG alkaline phosphatase (AP) conjugates were purchased from Novagen (Madison, WI). The Mini-PROTEIN Tetra gel electrophoresis system, micropulser cuvettes, and Silver Stain Kit were purchased from Bio-rad
Lyticase enzyme (from Arthobacter leutus) and acid-washed glass beads (pore size 0.5 mm) were purchased from Sigma Aldrich (St. Louis, MO). Coomassie® R-250 for protein gel staining was purchased from Life Technologies™ (Grand Island, NY).

**Cells and Vectors**

The pCD1 vector was obtained from Brenda Winkel of the Virginia Polytechnic Institute and State University (Blacksburg, VA). The pCR® 4-TOPO® vector and One Shot® Top Ten Chemically Competent Cells were purchased from Invitrogen (Carlsbad, CA). The pPICZA vector and X33 parent strain of Pichia pastoris were a gift from Sanja Roje of Washington State University (Pullman, WA). Competent yeast cells were prepared following the protocol from the Invitrogen Easy Select™ Pichia Expression Kit.

**Kits**

The QIAdprep® Spin Miniprep Kit was purchased from Qiagen (Valencia, CA). The Wizard® SV Gel and PCR Clean-Up System was purchased from Promega. The Quantum Prep® Plasmid Midiprep Kit was purchased from BIO-RAD. The TOPO TA Cloning® Kit was purchased from Invitrogen.

**Buffers**

All of the following buffers were prepared by recipes in Appendix A. TAE (Tris-Acetate EDTA) was prepared for DNA electrophoresis. One molar potassium phosphate (pH 6) was prepared for growth media. TANK Buffer (pH 8.3), TBS buffer, AP buffer (pH 9.5), 1 mM PMSF (phenylmethylsulfonyl fluoride), 1 mM EDTA (ethylenediaminetetraacetic acid), breaking
buffer transfer buffer, blocking solution, developing solution, and stop buffer were all prepared for SDS-PAGE and Western blot analysis.

**Culture Media**

Low salt LB (Luria-Bertani) liquid media and LB agar plates were prepared as described in Appendix B. YPD (yeast, peptone, dextrose) liquid media and YPDS (yeast, peptone, dextrose, and sorbitol) agar 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.

**Flavonoids and Phenolic Substrates for Enzymes Assays**

Naringenin, hesperitin, eriodictyol, isosakuranetin, apigenin, luteolin, diosmetin, scutallerein, kaempferol, quercetin, fisetin, gossypetin, 4’-methoxyflavonol, 4’-acetoxy-7-hydroxy-6-methoxy isoflavanone were purchased from Sigma and Indofine.

**II. Methods**

**Clone Specific Primer Design**

Based on the BioEdit (Ibis Biosciences, Carlsbad, CA) file received from Dr. Daniel Owens, clone specific primers for putative glucosyltransferase 4 were designed in order to amplify the sequence by polymerase chain reaction (PCR). The primers were designed as shown below (Table 1).

**Table 1. PGT 4 Clone Specific Primer Sequences for PCR Amplification.** The bolded nucleotides correspond to the restriction enzyme cutting site in each primer.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Rest. Enzyme</th>
<th>Annealing Temp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSP 144 F</td>
<td>5’ CAT GGG TAC CAT GGC ATC CGA AGC CAG CCA 3’</td>
<td>Kpn I</td>
<td>62.3 °C</td>
</tr>
<tr>
<td>CSP 145 R</td>
<td>5’ CGC GGG CCC AAT CAC TTC TGA GCT CGG CTG 3’</td>
<td>Apa I</td>
<td>64.3 °C</td>
</tr>
</tbody>
</table>
The restriction enzymes chosen were to help facilitate cloning into the pPICZA vector as Apa I and Kpn I are located within the vector’s multiple cloning site (Invitrogen). The primers were ordered from Integrated DNA Technology through the ETSU Quillen College of Medicine Molecular Biology Core Facility (Johnson City, TN).

Low Salt LB Liquid Media Culture

E.coli cultures were grown from Josephat Asiago’s glycerol stocks of PGT 4 stored at -80°C. Various glycerol stocks were chosen to verify the sequence of PGT 4. Low salt LB liquid media was made according to the recipe in Appendix B. The glycerol stocks were saved in the pCR® 4-TOPO® vector (Invitrogen) which has an ampicillin resistance. Ampicillin was added to the LB media to produce a final concentration of 100 mg/L. LB liquid media with antibiotic was inoculated with a dab of glycerol stock using an autoclaved toothpick or pipet tip. Cultures were inoculated overnight, 12-16 hours, in a New Brunswick Shaker Incubator at 250 rpm at 37 °C.

Isolation of DNA Plasmid

The QIAprep® Spin MiniPrep Kit was used to isolate DNA plasmid from the LB liquid media cultures. Five milliliters of culture were used for each MiniPrep. The cells were centrifuged in autoclaved centrifuge tubes (Fisher Scientific) in a Sorvall® Biofuge pico microcenterfuge, and the Qiagen protocol was followed to isolate the DNA plasmid. Fifty μL of DNA was isolated from each MiniPrep and was labeled and stored at -20 °C.

DNA Gel Electrophoresis

Thirty mL 0.8% agarose gels were made to verify the presence of plasmid DNA in the MiniPreps and to verify the vector length by comparison to the 1 kb DNA ladder (See recipe in
Appendix C). Two and a half μL ethidium bromide (10 mg/mL) were added to each gel before solidification in order to view the DNA with a UV illuminator. The gel was cooled in an electrophoresis cell (Owl Separation Systems, Inc., Portsmouth, NH) and each well was loaded with 6μL of a mixture including 2μL DNA, 1μL 6Xdye, and 3μL distilled water in each lane. The electrophoresis cell was run for one hour at 100 volts. The gels were viewed in an Epi Chem II Darkroom from UVP Bioimaging Systems and images saved as computer files for later use.

**DNA Sequencing**

Once the presence of DNA plasmid in the MiniPreps was confirmed, an aliquot was sent to the Molecular Biology Resource Facility of the University of Tennessee in Knoxville, TN for sequencing. The DNA sequencing results were sent via electronic mail in BioEdit files. The files were compared with the PGT 4 sequence obtained from Dr. Daniel Owens. Upon alignment of all the glycerol stocks sent for sequencing, it was found that not one of the glycerol stocks contained the full length PGT 4 sequence. All of the stocks were partial clones.

**Reconstruction of Full-length PGT 4**

Different glycerol stocks from Josephastrasiago were grown. DNA plasmid was isolated and sent for sequencing to try to find a stock of the full length PGT 4 clone. After sequencing several glycerol stocks and finding only partial clones, glycerol stocks from Christy Strong were grown, and the isolated DNA was sequenced. The glycerol stocks labeled 181.2A and 155.1A contained partial PGT 4 clones in the pCR® 4-TOPO® vector, but each contained significant
pieces. A strategy was devised to cut and ligate the pieces together. To perform a directed ligation, it is necessary to have a unique cutting site. However, it was discovered that only one enzyme could cut the two partial clones in the desired range to ligate them together. A challenge arose. *EcoRI* was needed to internally cut the partial clones, but it is located on both sides of the pCR® 4-TOPO® vector multiple cloning site (Figure 4).

**Primer Design**

With the multiple *EcoRI* cutting sites within the pCR® 4-TOPO® vector multiple cloning site, the PGT 4 partial clones (181.2A and 155.1A) needed to be amplified outside the pCR® 4-TOPO® vector. Thus, primers were designed to PCR amplify the partial clones out of their vector, and restriction enzymes were chosen to easily clone PGT 4 into pPICZA after the reconstruction. Figure 5 below shows the schematic for the reconstruction of PGT 4 with the designed primers. Table 2 shows the clone specific primer sequences designed to PCR amplify the pieces out of the pCR® 4-TOPO® vector.

![Figure 5. Plan for the Reconstruction of PGT 4](image)

Four clone specific primers were designed against the partial clones to amplify them out of the pCR® 4-TOPO® vector. The internal *EcoRI* site was used to ligate the two pieces together within an empty pCD1 vector.
Table 2. Primer Design for PCR Amplification out of pCR® 4-TOPO® vector.
Bolded bases represent restriction enzyme cutting sites for each primer.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Rest. Site</th>
<th>Annealing Temp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSP 144 F</td>
<td>5' CATG GGTACC ATG GCC TGG GAA GCC AGC CA 3'</td>
<td>KpnI</td>
<td>62.3 °C</td>
</tr>
<tr>
<td>CSP 149 R</td>
<td>5' GAT GAATTC CAG CCA TGG TGC AAG AAC CCT 3'</td>
<td>EcoRI</td>
<td>64.8 °C</td>
</tr>
<tr>
<td>CSP 150 F</td>
<td>5' CTG GAATTC ATC TCT GGA AGG GAT ATC AGC TGG GTT TCA 3'</td>
<td>EcoRI</td>
<td>65.8 °C</td>
</tr>
<tr>
<td>CSP 151 R</td>
<td>5' CGC GCGGCCGC AAT CGC TTC TGA GCT CGG CTG TTG 3'</td>
<td>NotI</td>
<td>61.6 °C</td>
</tr>
</tbody>
</table>

The strategy to reconstruct the PGT 4 clone was to PCR amplify the two partial clones and insert the digested, gel purified PCR product into an empty pCD1 vector one at a time to reconstruct the PGT 4 clone successfully. Below is a flow chart of the plan used to reconstruct the PGT 4 clone (Figure 6).

Figure 6. Plan for the Reconstruction of PGT 4. The flow chart shows the process used to reconstruct the PGT 4 clone from the two partial clones in an empty pCD1 vector.
PCR Amplification

To amplify the 181.2A and 155.1A partial clones via PCR, the following contents were added into a PCR tube: 2.5 μL of 1000 fold dilution of MiniPrep DNA of 181.2A or 155.1A (0.001μg/μL), 10μL of 5X green Go-Taq buffer, 2.5 μL of 20μM sense primer, 2.5 μL of 20μM antisense primer, 1.0 μL of Go-Taq polymerase, 31μL of distilled, sterile water to total a 50 μL PCR reaction. The annealing temperature varied with the primers, which have relatively close annealing temperatures to avoid PCR mistakes. The PCR reaction was carried out in the MyCycler thermocycler (Bio-Rad), which was set up with the protocol below (Table 3).

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature (°C)</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>94</td>
<td>1 minute</td>
</tr>
<tr>
<td>2</td>
<td>94</td>
<td>30 seconds</td>
</tr>
<tr>
<td>3</td>
<td>60</td>
<td>30 seconds</td>
</tr>
<tr>
<td>4</td>
<td>72</td>
<td>2 minutes</td>
</tr>
<tr>
<td>5</td>
<td>72</td>
<td>3 minutes</td>
</tr>
</tbody>
</table>

Gel Extraction and Purification

After the PCR reaction was complete, the PCR products were loaded into lanes of a 0.8% agarose gel, as previously described. The gel electrophorised for an hour at 100 volts and was viewed on a Spectroline® Transilluminator with a 302 nm UV light (Westbury, NY). A sterilized metal razor was used to excise the desired band out of the agarose gel. The desired 181.2A product was 1100 base pairs, while the 155.1A piece was around 350 base pairs. Their lengths were determined in comparison to the 1 kb ladder, run in the first lane of the gel. The bands were put into separate sterile micro centrifuge tubes, labeled, and DNA extracted using the protocol of the Wizard® SV Gel and PCR Clean-Up System (Promega Madison, WI). The DNA was eluted into 50 μL of sterile, distilled water and stored at -20 °C.
**Digestion of PCR Product and pCD1 vector**

The DNA concentrations of the purified gel bands were tested using the NanoDrop spectrophotometer (Thermo Scientific). For each digestion, 1 µg of DNA was used. The 181.2A PCR product and empty pCD1 vector were both digested with *KpnI* and *EcoRI*. To set up a digestion, 1 µg of DNA, 1µL of each enzyme, 2 µL enzyme-specific 10Xbuffer, and 0.2 µL BSA for stabilization was added to a varied amount of sterile, distilled water to a total reaction volume of 20 µL. Digestions were incubated at 37 ºC in a water bath or incubator for three hours. The digestion was halted by putting the reactions in the 65 ºC water bath for five minutes. The digestion products were combined with 4 µL of 6Xdye and added to the lanes of an agarose gel. The desired bands were extracted and purified as discussed previously.

**Ligation of 181.2A into the pCD1 vector**

The concentration of the purified, digested DNA was tested using the NanoDrop. The insert, 181.2A, had a concentration of 8.1 ng/µL, and the vector, pCD1, had a concentration of 17.9 ng/µL. These concentrations were used to setup a ligation of the two pieces to make a circular DNA plasmid. The following calculations were made using an online ligation calculator (Collins, 2011: http://django.gibthon.org/tools/ligcalc/). The three ratios were used to optimize ligation conditions. Having a higher concentration of insert:vector is useful when performing a ligation. The ligation mixtures were added to sterile PCR tubes and left at 4 ºC overnight. For each ligation, the insert and vector had varying concentrations thus the ratios shown may seem skewed.
Table 4. Ligation Ratios for Insertion of 181.2A into pCD1

<table>
<thead>
<tr>
<th>Ratio (Insert:Vector)</th>
<th>1:1</th>
<th>3:1</th>
<th>6:1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insert DNA (µL)</td>
<td>4.1</td>
<td>8.2</td>
<td>10</td>
</tr>
<tr>
<td>Vector DNA (µL)</td>
<td>10</td>
<td>6.8</td>
<td>4.1</td>
</tr>
<tr>
<td>Ligase (µL)</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Ligase Buffer (µL)</td>
<td>4.0</td>
<td>4.0</td>
<td>4.0</td>
</tr>
<tr>
<td>Sterile, distilled water (µL)</td>
<td>0.9</td>
<td>0.0</td>
<td>0.9</td>
</tr>
</tbody>
</table>

Transformation of Ligated 181.2A and pCD1

Once the ligated product had been at 4 °C for at least twelve hours, a transformation was performed. Following the Invitrogen protocol for One Shot® Top Ten Chemically Competent Cells, the cells were thawed and 10 µL of ligated product of each ratio was added to a different tube of Top 10 cells. A control of undigested vector plasmid was also inoculated into a tube of competent cells. The cells were incubated on ice for 30 minutes and then heat shocked for 30 seconds at 42 °C. Then, 250 µL of low salt LB media was added to each tube, and they were shaken for an hour at 225 rpm at 37 °C. One hundred µL of each tube was plated with a sterile tool onto LB agar plates with an ampicillin concentration of 100mg/L. (See Appendix B for LB agar plate recipe.) The LB agar plates were kept at 37 °C overnight and checked for colonies the next day.

PCR Check for Insertion of 181.2A

A test was performed to confirm the presence of 181.2A in pCD1 before isolated DNA plasmid was sent off for sequencing. Four colonies were found on the 3:1 ratio plate the next day. An autoclaved pipet was used to collect cells from each colony and diluted into 10 µL to use as DNA template for the PCR reaction. A PCR reaction was setup by loading a PCR tube with 1 µL of 1000 fold dilution of MiniPrep DNA of pCD1+181.2A (0.001µg/µL), 2 µL of 5X green
Go-Taq buffer, 0.5 μL of 20μM sense primer, 0.5 μL of 20μM antisense primer, 1.0 μL of Go-Taq polymerase, 5 μL of distilled, sterile water to total a 10 μL PCR reaction. The PCR reaction was run with an annealing temperature of 60 °C, and 2 μL of 6X dye was added to the PCR product and loaded into lanes of a 0.8% agarose gel. Illumination of the gel showed bands at 1100 bp in two of the four colonies suggesting the presence of 181.2A insert in pCD1. These colonies were inoculated into LB liquid media with amp (100mg/L) and grown overnight at 37 °C and 250 rpm. Plasmid DNA was isolated the next day and sent to UTK for sequencing.

Insertion of 155.1A into pCD1+181.2A vector

Upon confirmation of the first insert, the 155.1A PCR product and the pCD1+181.2A vector were digested with EcoRI and NotI to insert the second piece and reconstruct the PGT 4 clone. The same digestion protocol was followed as discussed previously. The 20 μL digestion was loaded into a well of a 0.8% agarose gel. After running the gel at 100 volts for an hour, correct bands were extracted and purified. The concentrations of the vector and 155.1A insert were found using the Nano Drop method. The partial clone, 155.1A, was 7.3 ng/μL, and the vector+181.2A was 6.1 ng/μL. A ligation was set up between the two pieces using an online ligation Calculator (Collins, 2011; http://django.gibthon.org/tools/ligcalc/). To a sterile PCR tube the following was added: 4 μL ligase buffer, 1 μL ligase, 10 μL vector, 1.1 μL insert, and 3.9 μL distilled water. The ligation was incubated overnight at 4°C. A transformation was performed the next day following the same protocol as discussed above. LB agar plates with transformants were left at 37 °C overnight for colonies to grow. Nine colonies were found the next day and were tested to confirm the insertion of the 155.1A piece. The same protocol for a PCR was used as before. When the gel with PCR products was illuminated one colony had the full 1467 base
pair sequence of PGT 4. This colony was inoculated into LB amp (100mg/L) media and grown overnight. The DNA plasmid was isolated the next day and sent for sequencing at UTK. The sequencing results confirmed the reconstruction of full-length PGT 4 in the pCD1 vector.

**Cloning into the pPICZA vector**

Upon the reconstruction of PGT 4, the clone was able to be cut out of the pCD1 vector and inserted into the pPICZA vector to be transformed into yeast. The restriction enzymes chosen to create the primers were chosen with the pPICZA multiple cloning site in mind. Thus, the sense and antisense primers of the newly reconstructed PGT 4 sequence included KpnI and Ntl sites. The same sites were also found in pPICZA in a manner in which PGT 4 could be directionally cloned into pPICZA without having to design new primers.

To clone into pPICZA, the same processes were used as in the case of pCD1. The pCD1 vector containing the PGT 4 sequence was digested with KpnI and Ntl to isolate the PGT 4 band. Empty pPICZA vector was also digested with KpnI and Ntl. The vector and PGT 4 insert bands were extracted and purified. Then, an online ligation calculator was used to setup the three ratios for ligation (Collins, 2011; http://django.gibthon.org/tools/ligcalc/). The ligation set up is detailed in Table 5.

<table>
<thead>
<tr>
<th>Table 5. Ligation of PGT 4 and pPICZA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ratio (Insert:Vector)</strong></td>
</tr>
<tr>
<td>Insert DNA (µL)</td>
</tr>
<tr>
<td>Vector DNA (µL)</td>
</tr>
<tr>
<td>Ligase (µL)</td>
</tr>
<tr>
<td>Ligase Buffer (µL)</td>
</tr>
<tr>
<td>Sterile, distilled water (µL)</td>
</tr>
</tbody>
</table>

The next day, the ligation products were transformed into One Shot® Top 10 Chemically Competent Cells. After incubating for 24 hours at 37 °C, there were 12 colonies on the three
plates. Following a PCR Check, it was found that five colonies had the PGT 4 sequence successfully inserted into the pPICZA vector. These five colonies were inoculated into LB _zeocin_ (25mg/L) media and grown overnight. The DNA plasmid was isolated by MiniPrep the next day and sent to UTK for sequencing. The sequencing results showed that the PGT 4 sequence had been inserted correctly; however, the myc epitope and polyhistidine tags of the pPICZA vector, directly following the 3’ end of the PGT 4 insertion, were out of frame. The myc epitope and polyhistidine tags are essential for enzyme purification, thus it was imperative that the codons encoding the amino acids to make these tags were in frame. A single base pair insertion was needed to bring the tag sequences in frame. To keep the tags in frame, the clone specific primer needed to be a multiple of three to not frameshift the tags in the pPICZA vector sequence following PGT 4. Figure 7 shows the sequence before and after the single insertion.

**Primer Modification for Addition of Base Pair**

Two new primers were designed to amplify the PGT 4 sequence to be in frame within pPICZA. The 5’ primer sequence (CSP 163F) was the same as the original CSP 144F with a few changes. The beginning first three base pairs were altered to correspond with the pPICZA vector to help anneal the primer to the sequence for amplification. Also, additional base pairs were added at the end of the 5’ primer sequence to match the melting temperature of the 3’ primer and increase probability of correct PCR amplification. Only one base had to be added to the 3’ primer sequence (CSP 164R) to frameshift the tags (Table 6). Thus, using the original primer design of CSP 151 R, one “G” was added directly after the final codon of the PGT clone, an isoleucine, and directly before the Not I restriction site of the clone specific primer. Figure 7 shows the sequence before and after the single insertion.
Figure 7. Frameshift in purification tag sequences due to clone specific primer.
A. This figure shows the sequence with out-of-frame tags. The presence of multiple isoleucines in the second line instead of the histidine tag is seen. B. This figure shows the sequence with the insertion of a “G” at base pair 1465. This insertion caused the tags to be in frame and the histidine tag is clearly seen on the second line followed by the stop codon.

Table 6. This table displays the newly designed primers to amplify PGT 4 to be in frame within pPICZA. The bolded base pair shows the insertion. The 3’ primer is designed as the complementary sequence. Thus, the addition of a “C” within the primer sequence will result in the addition of a “G” into the sequence to frameshift the sequence following the 3’ end that codes for vital tags to aid in identification and purification of the protein.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Rest. Enzyme</th>
<th>TM (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSP 163 F</td>
<td>5’ATC GGT ACC ATG GCA TCC GAA GCC AGC CAG GTT 3’</td>
<td>KpnI</td>
<td>62.3</td>
</tr>
<tr>
<td>CSP 164 R</td>
<td>5’ CTG GCG GCC AAT CAC TIC TGA GCT CGG CTG TTG CA 3’</td>
<td>NotI</td>
<td>64.3</td>
</tr>
</tbody>
</table>

PCR Amplification of Recombinant PGT 4

PCR amplification was performed with the newly designed primers (Table 6). Three PCR tubes were loaded with: 12.5 μL 2 X PCR Master Mix (see Appendix B for recipe), 0.5 μL CSP 163 F, 0.5 μL CSP 164R, 1000X isolated PGT 4 in pPICZA plasmid (the correct but out of
frame sequence), and 11.25 µL sterile, distilled water. The reactions were inserted into the Eppendorf thermocycler and run with the same protocol as in Table 3. The PCR products were loaded onto a 0.8% agarose gel and run at 100 volts for 1 hour. The bands were visualized with UV light and extracted from the gel using a sterile blade. The PCR product was purified using the protocol of the Wizard® SV Gel and PCR Clean-Up System (Promega). The DNA was eluted into 50 µL of sterile, distilled water and stored at -20°C.

**TOPO TA Cloning of PGT 4**

The PCR product was used for TOPO TA Cloning® following the Invitrogen protocol: 4 µL of PCR product was mixed with 1 µL empty TOPO® vector and 1 µL TOPO® salt solution and incubated at room temperature for 30 minutes. Two µL of this solution were added to a thawed tube of One Shot® Top 10 Chemically Competent Cells. The cells were incubated on ice for 5 minutes, heat shocked for 30 seconds in a 42 °C water bath, and then incubated on ice for 2 minutes. With this protocol, the PCR product is rapidly ligated into the vector and does not need an extended incubation period. After adding 250 µL LB media, the cells were shaken for an hour at 225 rpm and 37 °C. Varying amounts of the sample (25, 50, 100, 200 µL) were plated onto LB amp (100mg/L) agar plates and incubated at 37 °C overnight. A positive control was also conducted using 1 µL PUC19 in an additional tube of One Shot® Top 10 Chemically Competent Cells. The next day, four colonies were found on the selective LB plates. The insertion of the PCR product was confirmed by a PCR screen using the new clone-specific primers, CSP 163F and CSP 164R (Table 6).

**Cloning into pPICZA with PGT 4 Base Insertion**

A digestion of PGT 4 in TOPO and empty PICZA plasmid was conducted with KpnI and NotI. With the same setup as mentioned before, the digestions were left at 37 °C for three hours,
and then placed in the 65°C water bath for five minutes to stop the reaction. The digestion products were loaded onto a 0.8% agarose gel and run at 100 volts for an hour. The desired bands were extracted and purified using the protocol of the Wizard® SV Gel and PCR Clean-Up System (Promega). The DNA was eluted into 50 μL of sterile, distilled water and stored at -20 °C. The concentrations of the pPICZA vector and PGT 4 insert were measured using the NanoDrop and found to be 11.9 ng/μL and 2.9 ng/μL, respectively. A ligation reaction was set up using an online ligation calculator (Collins, 2011; http://django.gibthon.org/tools/ligcalc/) and incubated at 4°C overnight (Table 7).

<table>
<thead>
<tr>
<th>Table 7. Ligation of pPICZA and PGT 4 with base insertion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ratio (Insert:Vector)</td>
</tr>
<tr>
<td>Insert DNA (μL)</td>
</tr>
<tr>
<td>Vector DNA (μL)</td>
</tr>
<tr>
<td>Ligase (μL)</td>
</tr>
<tr>
<td>Ligase Buffer (μL)</td>
</tr>
<tr>
<td>Sterile, distilled water (μL)</td>
</tr>
</tbody>
</table>

The ligated products were transformed into OneShot® Top 10 Chemically Competent Cells using the manufacturer’s protocol (Invitrogen). Each plate had a large number of colonies. Sixteen were chosen at random; this is the number that can fit in the lanes of one agarose gel with a negative and positive control and two lanes of 1 kb ladder. Of these sixteen colonies tested with PCR, only two had a band at around 1500 signifying the presence of the PGT 4 insert. The colonies were inoculated into LBZeocin (25mg/L) media and grown overnight. The DNA plasmid was isolated by MiniPrep and sent for sequencing. The results from UTK showed that the insertion was successful and that the myc epitope and polyhistidine tags of pPICZA were in frame. The full PGT 4 sequence with tags in frame is shown in Figure 8.
**Isolation of DNA Plasmid for Linearization**

To transform PGT 4 into yeast, 5-10 μL of linearized DNA is required (EasySelect™ Pichia Expression Kit). To obtain a sufficient amount of DNA to linearize, a Quantum Prep® Plasmid Midiprep was performed following the manufacturer’s protocol (BIO-RAD). A 5 mL culture of a single colony of in-frame PGT 4 pPICZA was grown in LB\textsubscript{zeocin} (25mg/L) at 37°C and 250 rpm overnight. The small culture was then inoculated into 45 mL more of selective media and grown accordingly. The 50 mL culture was harvested using a Sorvall® RC-5B centrifuge. The BIO-RAD protocol was followed to isolate the plasmid, and 600 μL eluted DNA was stored at -20°C.
Figure 8. DNA and translated amino acid sequence of PGT 4 in frame with the identification and purification tags within pPICZA. The last line shows the His tag and stop codon confirming the correction.
**Linearization of Plasmid DNA**

The Invitrogen manual recommends the use of three restriction enzymes: *Sac I, Pme I,* or *BstXI* to linearize the pPICZA vector. These enzymes only cut the vector once. A restriction enzyme map was made for the PGT 4 sequence to see if it contained any internal cutting sites for any of these three enzymes. PGT 4 had an internal cutting site for *Sac I.* PGT 4 did not contain internal cutting sites for *Pme I* or *Bst XI.* Either could have been used; however, *BstXI* was used because it was in stock in the lab. In a sterile 1.5 mL centrifuge tube, 300 μL Midiprep DNA, 34 μL 10X Buffer D, 3 μL 100X BSA, and 3 μL *BstXI* were added. The reaction was placed in a 37°C water bath for four hours. After four hours, an additional 4 μL of *BstXI* was added to the reaction. The digestion was continued at 37°C overnight. A 0.8% agarose gel was made to run a sample containing: 1 μL digestion product, 4 μL distilled water, and 1 μL 6X dye. The gel confirmed complete linearization of the plasmid DNA. Once confirmed, the sample was placed in the 65°C water bath to stop the digestion. The digested DNA was stored at 4°C until the phenol:chloroform extraction and ethanol precipitation could be performed.

**Concentration of Linearized DNA**

To concentrate the linearized DNA to sufficient amounts for electroporation, a phenol:chloroform extraction was performed. The phenol: chloroform solvent was prepared as described in Appendix A. First, 350 μL phenol:chloroform solvent was added to the linearized DNA in a microfuge tube. The tube was vortexed and then centrifuged at 13,000 x g for 2 minutes. Second, 300 μL of the upper aqueous phase was extracted and transferred to a new, sterile microfuge tube. After transfer, 30 μL of 7.5 M sodium acetate and 750 μL 100% ethanol were added to the sample. The sample was either incubated on ice for two hours or stored at -20 °C overnight to
precipitate the DNA. After this time, the microfuge tube was centrifuged for 10 minutes at 14,000 X g at 4°C. The supernatant was discarded, and the DNA pellet was resuspended in 1 mL 80% ethanol. The microfuge tube was centrifuged again for 10 minutes at 14,000 X g at 4°C. The supernatant was discarded. The tube was inverted to dry for 30 minutes. For best results, the tube was placed in a SpeedVac Concentrator (Thermo Electron Corporation) for 15 minutes. The DNA was resuspended in sterile, distilled water, 5 to 20 μL for best results. The concentration of DNA was determined using the NanoDrop. A 20:1 diluted sample was used to test the concentration so as not to waste any DNA for electroporation. The linearized DNA, ready for electroporation, was stored at -20 °C.

**Yeast Harvest for Electroporation**

With linearized DNA prepared for transformation, competent yeast cells were grown. An overnight 5 mL culture of *Pichia pastoris* strain X33 was grown at 30°C and 250 rpm in YPD (yeast, peptone, dextrose) media. (See Appendix B for recipe) Next, 100 μL of the yeast culture was inoculated into 50 mL YPD media and grown overnight at 30°C and 250 rpm until reaching the recommended OD_{600} of 1.3-1.5. The density was testing using a spectrophotometer against a blank of YPD media. The optimal density was reached after approximately 13.5 hours. The yeast cells were harvested at 4°C in a Sorvall® RC-5B super speed centrifuge (Fisher Scientific) at 1,500 X g for 5 minutes. The cells were washed with 50 mL ice cold, sterile distilled water and centrifuged at the same rate and time. The cells were then washed with 10 mL ice cold, sterile distilled water and centrifuged at the same rate and time after which the cells were washed with 10 mL ice cold, sterile 1 M sorbitol and centrifuged at the same rate and time. The cells were
then resuspended in 1 mL ice cold, sterile 1 M sorbitol. The yeast cells were stored on ice until subsequent use for electroporation.

**Electroporation of PGT 4 into Yeast**

Following the Invitrogen EasySelect™ *Pichia* manual, a 0.2 cm micro pulser cuvette (Bio-Rad) and the sample of concentrated, linearized DNA were incubated on ice for 5 minutes. From the yeast harvest, 80 μL of yeast cells were transferred to the linearized DNA sample and incubated on ice for 5 minutes. The mixture was then added to the cold electroporation cuvette and incubated on ice an additional 5 minutes. The cuvette was placed in a micropulser and pulsed once at 1.5 kV. Immediately after the pulse, 1 mL ice cold 1M sorbitol was added to the cuvette. The contents of the cuvette were transferred to a sterile 15 mL tube and incubated at 30°C for one hour without shaking after which the transformants were spread evenly over YPDS<sub>zeocin</sub> (100mg/L) plates (See Appendix B for recipe) in 25, 50, 100, and 200 μL aliquots. The plates were incubated at 28°C for 2-3 days until colonies were observed. After initial growth, the plates were stored at 4°C.

**PCR Screen of Yeast Transformants**

To confirm transformation of PGT 4 into the yeast genome, a PCR screen using AOX1 primers was conducted. Colonies were chosen at random to test. Each chosen colony was inoculated into 10 μL sterile, distilled water in a PCR tube. To isolate the DNA from the yeast cells, 5 μL of 5U/μL lyticase was added to each PCR tube. The samples were incubated at 30°C for 10 minutes and then at -80°C for 10 minutes. The cell lysates were then thawed and used as template for PCR. A negative control of empty pPICZA vector and a positive control of Miniprep plasmid of PGT 4 in pPICZA were also used as control templates. In each PCR
reaction the following was added: 5 μL 2X PCR Master Mix (See Appendix B for recipe), 0.5 μL 10 mM 5’ AOX1 primer, 0.5 μL 10 mM 3’ AOX1 primer, 3 μL sterile water, and 1 μL DNA template. The PCR was run in a thermocycler with the following protocol (Table 8).

**Table 8. Temperature Protocol for PCR with AOX1 primers**

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature (°C)</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>95</td>
<td>5 minutes</td>
</tr>
<tr>
<td>2</td>
<td>95</td>
<td>1 minute</td>
</tr>
<tr>
<td>3</td>
<td>54</td>
<td>1 minute</td>
</tr>
<tr>
<td>4</td>
<td>72</td>
<td>1 minute</td>
</tr>
<tr>
<td>5</td>
<td>72</td>
<td>7 minutes</td>
</tr>
</tbody>
</table>

To confirm insertion, the 10 μL PCR products were loaded onto a 0.8% agarose gel and run for 1 hour at 100 volts. The gel was visualized to verify positive bands corresponding to the 1467 bp PGT 4 sequence plus 300 bp of AOX1 priming sequence. Colonies found to be positively transformed were freshly streaked on YPDS<sub>zeocin</sub> (100mg/L) plates. Also, cultures of each positive colony were grown overnight in YPDS<sub>zeocin</sub> (100mg/L) media, saved in 50% glycerol, and stored at -80°C.

**Test Inductions for Expression of PGT 4**

From each of the freshly streaked plates of positively transformed colonies, a single colony was inoculated into 25 mL BMGY (See Appendix B for recipe) in a 250 mL baffled flask. The culture was grown overnight at 30°C and 250 rpm to generate biomass. The culture was grown until it reached an OD<sub>600</sub> of 2-6 (approximately 22 hours) (Invitrogen). The yeast cells were harvested by centrifugation in a Sorvall® RC-5B at 4°C for 5 minutes at 3,000 X g. The cells were resuspended in 30 mL BMMY (See Appendix B for recipe) and centrifuged again for 5 minutes at 3,000 X g. The cells were resuspended in 50 mL BMMY and transferred to sterile 500 mL baffled flasks. Additional aliquots of BMMY were added to the cultures to reach
an OD$_{600}$ of 1. The flasks were covered with two layers of cheesecloth to allow aeration for proper culture growth. The flasks were shaken at 30°C and 250 rpm. Every 24 hours, 100% methanol was added to the cultures to a final concentration of 0.5% methanol to maintain induction. Samples were taken out every six hours on the first day and every 12 hours on days 2-4. A 1 mL sample was taken from each culture at the following time points (hours): 0, 6, 12, 24, 36, 48, 60, 72, 84, and 96. Each 1 mL sample was centrifuged at 13,000 X g for 3 minutes. The supernatant was discarded, and the cell pellet was stored at -80°C until SDS-PAGE analysis.

**Preparation of Samples for SDS-PAGE Analysis**

Cell pellets were thawed on ice. One hundred μL Breaking Buffer with 1% PMSF (See Appendix B for recipe.) and an equal amount of acid-washed glass beads were added to each sample. The samples were vortexed for thirty seconds and placed on ice for thirty seconds for eight cycles. After lysing the cells, samples were centrifuged for 10 minutes at 14,000 x g at 4°C. The supernatant was transferred to a new, sterile centrifuge tube. Concentrations of protein within the samples were measured with the Nano Drop. Fifteen microliters was prepared for each well containing 5 μL loading dye and 15 μL mixture of sample and distilled water. To accurately analyze the amount of activity at different time intervals, an equal amount of protein from each sample was loaded onto the protein gel. The amount of protein in 15 μL of the sample with the lowest concentration was calculated and the same amount of protein was loaded from each sample. Distilled water was used to dilute the more concentrated samples to 15 μL. The samples were boiled for ten minutes and centrifuged for 1 minute at 13,000 x g before loading.
SDS-PAGE Electrophoresis

A protein electrophoresis cell from BioRad was used. Two glass plates were secured in clamps. Ten percent separating gel and fifteen percent stacking gel solutions were mixed in separate beakers following recipe in Appendix C. The 10% separating gel was poured by pipet in between the glass plates until reaching 2 cm from the top and allowed to solidify for 25 minutes. To level off the top of the separating gel, 400 μL water-saturated butanol was poured between the plates. After five minutes, the butanol was lifted off the gel using Whatman paper. The 15% stacking gel was poured by pipet to the top of the glass plates, eliminating any bubbles between the plates. A comb was inserted in between the plates and allowed to solidify for 25 minutes. The plates were placed within the electrophoresis cell, and 1 X TANK Buffer was added to the middle and outer compartments (See Appendix B for recipe). The wells were loaded with the 20 μL prepared samples using flat pipet tips. The cell was run for 15 minutes at 85V and 1.5 hours at 100V.

Staining and Destaining of Protein Gels

Protein gels were stained with Coomassie® Brilliant Blue R250 and destained with gel destaining solution (See Appendix B for recipes). Staining solution was added to protein gels in a dish and heated in microwave for 10 seconds, not allowing the solution to boil. The gels were then shaken for 15 minutes. The solution was decanted, and the protein gels were rinsed with distilled water. Destaining solution was added to the gels, and they were heated in the microwave for 10 seconds. Gels were shaken until desirable background was achieved.

Protein gels were also silver stained according to the manufacturer’s instructions in the Bio-Rad Silver Stain Kit. Developed gels were photographed and archived.
Electro-blotting of SDS-PAGE

While the protein gel was running, pieces of Whatman paper (3MM CHR) and nitrocellulose membrane were cut to the size of the protein gel. The nitrocellulose membranes were moistened in distilled water for 5 minutes. Electro-blotting cassettes, porous pads, and filter were moistened in a glass dish in 1 X Transfer Buffer (See Appendix B for recipe). The electro-blotting cassettes were assembled as follows without air bubbles in between the layers: anode side, porous pad, filter paper, protein gel, nitrocellulose membrane, filter paper, porous pad, and cathode side. The cassettes were fastened and loaded into the cell. A stirrer bar gently stirred the buffer in the cell when connected to a voltmeter at 65V for 1 hour.

Western Blot Analysis

After electro blotting, the protein gel was placed in a tray of Coomasie BrilliantBlue G250 and gently shaken overnight to be destained later (See Appendix B for Coomasie staining and destaining solutions). The nitrocellulose membrane was immediately placed into a tray of blocking solution (Appendix B) and gently shaken for 2 hours. The blocking solution was poured off, and the membrane was washed by gently shaking with 25 mL TBS with 0.02% sodium azide for 10 minutes. The wash was poured off, and the primary antibody, a monoclonal antibody c-myc from mouse, was added in a 30 mL 1:2500 solution of TBS with 0.02% sodium azide and gently shaken for 2 hours. The primary antibody solution was poured off, and the membrane was washed three times for 10 minutes each with 50 mL TBS without sodium azide. The membrane was transferred to a new tray, and the secondary antibody, a goat, anti-mouse immunoglobulin G conjugate to alkaline phosphatase (IgG-AP), was added in a 30 mL 1:10000 solution of TBS without sodium azide and gently shaken for 2 hours. The secondary antibody was poured off and the membrane was washed as before. The membrane was then added to a tray of developing
solution (See Appendix B for recipe) for up to 1 minute until bands were visible. The membrane was immediately transferred to the stop buffer (50 mL 1X TBS, 200 µL 0.5M EDTA, pH 8). After 5 minutes in the stop buffer, the membrane was dried on a paper towel. All Western blots were scanned and originals archived into a lab notebook.

**Scale-Up Expression of PGT 4**

Western blot analysis found optimum post-induction times for PGT 4. Twelve hours post-induction was chosen due to darkness of band on Western blot and convenience of experiment timing for purification. A scale-up of expression was conducted by growing a 100 mL yeast culture to induce with methanol for protein production. A fresh yeast colony was inoculated into 25 mL BMGY for 18 hours at 37°C and 250 rpm until OD$_{600}$ of 2-6. A 1 mL aliquot of the 25 mL culture was inoculated into 50 mL of BMGY and grown 18 more hours at 37°C and 250 rpm to an OD$_{600}$ of 2-6. The cells were harvested by centrifugation at 3,000xg and 4°C for 5 minutes. The cells were washed with 20 mL BMMY, containing methanol, and centrifuged at 3,000xg and 4°C for 5 minutes. The cells were resuspended in BMMY, containing methanol, to an OD$_{600}$ of 1. The yeast culture was split into 250 mL centrifuge tubes. The cells were harvested by centrifugation at 3,000xg and 4°C for 5 minutes. The cell pellets were saved at -80°C until enzyme purification and assay.

**Purification and Concentration of PGT 4**

Cell pellets were thawed on ice. To each pellet, 3 mL of Breaking Buffer with 1 mM PMSF was added (See Appendix A for recipe). The suspended pellet and buffer (6 mL) was poured into a French press (Thermo Electron Corporation). The cells were lysed three times at 2000 psi. The cell lysate was centrifuged at 13,000xg and 4°C for 20 minutes. The supernatant was decanted into a new test tube and stored on ice, and the cell pellet was discarded.
A PD-10 column was equilibrated with 25 mL of Column Buffer (See Appendix A for recipe). A 2.5 mL aliquot of cell lysate was applied to the equilibrated PD-10 column to remove any salt from the protein. The flow through was discarded, and 3.5 mL Column Buffer was applied to the column to elute the protein. The flow through was collected in a 50 mL centrifuge tube and labeled PD-10 elution. Another 2.5 mL aliquot of cell lysate was desalted in the same manner. The PD-10 was re-equilibrated with 25 mL Column Buffer to restore the column for further use.

The protein was further purified using an immobilized metal affinity chromatography (IMAC) column from TALON™ Resins. All purification techniques were done at 4 °C to maintain stability of the enzyme. The histidine tag on the recombinant protein allows for purification through metal affinity by binding the protein to the column and eluting it with imidazole. The 4 mL bed IMAC column was equilibrated with 40 mL Column Buffer. The PD-10 elution was applied slowly to the column. The flow through was collected in 2 mL centrifuge tubes in 1 mL fractions. The IMAC column was washed with column buffer until all non-bound proteins had been removed, leaving only tagged protein. The wash was collected in 2 mL centrifuge tubes in 2 mL fractions. When protein concentration was below 0.1 mg/mL, Column Buffer with 150 mM imidazole was applied to the column to elute the tagged protein. Ten each 2 mL fractions were taken and tested for protein concentration using the NanoDrop at 280 nm. Four 2 mL elution fractions with the highest concentration of protein were added to a Amicon® Ultra-15 and centrifuged at 4,000xg and 4°C for 10 minutes. Centrifugation was continued until the sample was concentrated to 500 µL. To remove imidazole from the purified protein, the concentrated sample was resuspended to a final volume of 2 mL with 50 mM phosphate buffer with 14 mM βME (See Appendix A for recipe) and centrifuged again at the same rate and time.
until concentrated to 500 µL. The concentrated sample was resuspended to a final volume of 2 mL with 50 mM phosphate buffer with 14 mM βME and stored on ice for enzyme assay. After enzyme assay, the enzyme was stored at 4°C for SDS-PAGE and Western blot analysis. The IMAC column was equilibrated with 20 mL MES buffer, 20 mL milliQ water, and 25 mL 20% ethanol with 0.1% sodium azide for storage and reuse.

**Preparation of 14C-UDP-glucose and Flavonoid Substrates for Enzyme Assay**

To test for enzyme activity, 14C-UDP-glucose was used as the substrate donor. A working solution of 14C-UDP-glucose was prepared by mixing 50 µL 14C-UDP-glucose (261 mCi/ mmol) with 950 µL 50 mM phosphate buffer to reach 20000 cpm/10µL. Various flavonoids were used as the substrate acceptors (Table 9). A working solution of each flavonoid substrate was dissolved into 100% ethylene glycol monomethylether to a concentration of 50nmole/5µL and stored at -20 °C.

**Table 9. Flavonoid Substrates Screened with PGT 4 for Activity**

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<td>Isoflavone</td>
<td><img src="image5" alt="Isoflavone Structure" /></td>
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Screening of PGT 4 for Flavonoid Glucosyltransferase Activity

A total of 14 flavonoids were used as potential acceptor substrates to screen for GT activity with PGT 4. Each test reaction was performed in duplicate with each flavonoid substrate. A negative control using enzyme denatured with HCl were also performed in duplicate with each flavonoid substrate. This negative control served as a background reading of activity with each substrate. The test results were corrected against these background readings to verify positive activity. A second negative control was conducted with quercetin using denatured PGT 4 that had been boiled for ten minutes. A positive control using young grapefruit leaf extract as the GT enzyme source was performed with quercetin. A second positive control combining young grapefruit leaf extract and PGT 4 was performed to test for possible inhibiting effects of rPGT 4 on glucose incorporation.

The screening assay was adapted from McIntosh et al. (1990). For each test reaction the following contents were added in respective order: 50 µL 50 mM phosphate buffer with 14 mM βME, 5 µL flavonoid substrate (50 nmol), 10 µL \(^{14}\)C-UDP-glucose working solution (20,000 cpm), and 10 µL purified enzyme. For each negative control reaction, 15 µL 6M HCl was added before the enzyme. The second negative control used 10 µL boiled enzyme. For the first positive control, 60 µL young grapefruit leaf extract, suspended in 50 mM phosphate buffer with 14 mM βME, 5 µL quercetin, and 10 µL \(^{14}\)C-UDP-glucose working solution was added to a reaction tube. The young grapefruit leaf extract was prepared by crushing a young grapefruit leaf with mortar and pestle in 2 mL 50 mM phosphate buffer with 14 mM βME and pH 7.5. For the second positive control, 50 µL young grapefruit leaf extract, 5 µL quercetin, 10 µL \(^{14}\)C-UDP-glucose, and 10 µL purified enzyme was loaded into a reaction tube. Each control was performed in duplicate.
Each reaction was incubated for 5 minutes at 37°C. After 5 minutes, 15 µL 6M HCl was added and vortexed for 10 seconds to stop the reaction. To separate any radiolabeled flavonoid glucosides from unincorporated $^{14}$C-UDP-glucose, 250 µL ethyl acetate was added to the reaction tube, vortexed for 10 seconds, and separated into organic and aqueous phases. A volume of 100 µL was pipetted from the top organic phase and added to 2 mL scintillation fluid. The amount of radiolabeled $^{14}$C-UDP-glucose transferred to flavonoid substrates was analyzed using a Beckman LS 6500 scintillation counter and counts corrected to determine cpm in 250 µL of ethyl acetate.
RESULTS AND DISCUSSION

Confirmation of PGT 4 for Transformation

PGT 4 had been previously tested for flavonoid GT activity in *E.coli*. Limited substrates were used and little activity was found. One challenge that arose was the presence of inclusion bodies within the prokaryotic system. The inclusion bodies were found to store the induced protein, and substantial soluble protein was not present for analysis. Thus, PGT 4 was to be transformed into a eukaryotic system, *Pichia pastoris*, in order to obtain more soluble protein for analysis.

PGT 4 glycerol stocks from Josephat Asaigo were used to confirm the sequence of PGT 4 before insertion in pPICZ A and transformation into yeast. Primers were designed based on previous sequencing results. A previously compiled Bioedit sequence of PGT 4 was obtained from Dr. Daniel Owens. Clone specific primers were designed based on the information from this sequence. See Table 1 for the sequences of primers CSP 144 F and CSP 145 R. Cultures of the glycerol stocks were made in LB media, and MiniPreps were conducted to isolate DNA plasmid. The newly designed primers were used in a polymerase chain reaction with plasmid DNA as template and with a $T_M$ of 64 °C to confirm the presence of the full length PGT 4 sequence within the glycerol stocks. However, PCR product was not found (Figure 9). PCR was repeated with several different stocks with no resulting band at the expected size of full-length PGT 4, 1467 base pairs.
**Figure 9.** PCR of glycerol stocks of PGT 4. Lane M is 1 kb ladder. Lanes 2-6 are PCR product of 5 different PGT 4 glycerol stocks. No PCR product at 1467 bp was found.

PCR was repeated with a lower $T_M$, $59^\circ C$. It was hypothesized that the primers could not anneal properly to the template because their sequences were not completely conserved to that of PGT 4. The agarose gel of the second PCR reaction had identical results. It was hypothesized that the full length was not present within the stocks. Thus, DNA from MiniPreps of several different glycerol stocks was sent to UTK for sequencing. The results showed that no glycerol stock contained the full length sequence; however, each one had a partial PGT 4 clone.

**Reconstruction of PGT 4**

Before transformation into yeast could occur, full-length PGT 4 had to be reconstructed. Two partial clones were found, that when digested and ligated together, could reconstruct the full length PGT 4 sequence. The partial clones, 181.2A and 155.1A, were archived by a previous researcher, Christy Strong. After receiving the sequencing results of these 2 partial clones, a cloning strategy was designed. The single internal restriction site within the overlapping region of the 2 partial clones that cut PGT 4’s sequence only once was *EcoRI*. However, both partial
clones were saved within the pCR® 4-TOPO® vector, whose multiple cloning site contains multiple sites for EcoRI (See Figure 4). With EcoRI sites on either side of the partial clone inserts, the desired pieces of the clones could not be directly digested and ligated. A new strategy was designed.

The partial clone sequences could be amplified out of the pCR® 4-TOPO® vector by PCR. To PCR amplify, more clone specific primers were made (Figure 10). To be transformed into yeast, PGT 4 was to first be cloned into the pPICZ A cloning vector. Thus, primers to amplify the partial clones were designed with this in mind. The 5’ and 3’ end restriction enzyme sites were chosen to be Kpn I and Not I to allow for directional cloning into the pPICZA vector (Figure 11). The 1100 bp piece of Clone 181.2A and 300 bp piece of Clone 155.1A were to be PCR amplified out of the pCR® 4-TOPO® vector and ligated into the pCD1 cloning vector one at a time (Figure 12).

Figure 10. Reconstruction of PGT 4. This diagram shows the cloning strategy of the 2 partial clones to successfully reconstruct PGT 4.
**Figure 11.** Vector Map of pPICZ A. Restriction enzymes *Kpn I* and *Not I* were chosen for the 5’ and 3’ primer restriction enzyme sites, respectively. The map shows these enzymes directionally in its multiple cloning site (Invitrogen).

**Figure 12.** This pCD1 vector map shows the restriction cloning sites directionally (5’ to 3’). This vector was a gift from Brenda Winkel of Virginia Tech. The restriction enzymes chosen digest pCD1 a single time. The partial clones were inserted directionally one at a time.
**PCR Amplification of 181.2A piece**

The strategy to PCR amplify the partial clones out of the pCR® 4-TOPO® vector and directionally clone the pieces into the pCD1 vector was successful. To reconstruct PGT 4, Clone 181.2A was amplified by PCR with clone specific primers, CSP 144F and CSP 149R. See Table 2 for details. Figure 13 shows the 1100 bp piece of Clone 181.2A that was PCR amplified from MiniPrep DNA of Clone 181.2A.

The 181.2A bands at around 1100 bp were extracted and gel purified. Multiple bands were excised and purified to obtain a good DNA concentration for digestion. The purified DNA was used for digestion with *KpnI* and *EcoRI*. The pCD1 vector was also digested with *KpnI* and *EcoRI* in order for the insert and vector to be ligated together (Figure 14).

![Figure 13. PCR Amplification of 181.2A piece. Lane M is the 1 kb ladder. Lane 1 is pCD1 vector MiniPrep DNA. Lanes 2-5 are PCR products with CSP 144F and CSP 149R. The band around 1100 bp suggests the desired 181.2A piece.](image-url)
Figure 14. Digestion of 181.2A piece and pCD1 vector. M represents 1 kb ladder. A. Lanes 1-3 are 181.2A digestion with KpnI and EcoRI. B. Lanes 1-4 are pCD1 vector digested with KpnI and EcoRI. All wells were loaded with 15 µL from digestion and 3 µL 6x loading dye. Bands were excised and gel purified for ligation.

Ligation and Transformation of 181.2A into pCD1

The digested 181.2A piece bands and the digested pCD1 bands were extracted and gel purified. The digested DNA of the insert and the vector were ligated together. The ligated product was transformed into OneShot® Top 10 Cells. Colonies were grown on selective LB plates with ampicillin (100 mg/L). Four transformant colonies grew overnight on the plates at 37°C. A PCR screening was conducted with CSP 144F and CSP 149R to verify the presence of the 181.2A piece within the pCD1 vector. The ligation ratio of 3:1 insert:vector resulted in colonies which successfully included the 181.2A insert. Two colonies showed a positive band at 1100 bp, suggesting the 181.2A piece was successfully ligated in the vector (Figure 15).
Figure 15. PCR Screen for Confirmation of 181.2A Ligation. M represents 1 kb ladder. Lanes 1-4 are PCR product from transformed colonies. The band around 1100 bp indicates the presence of the 181.2A piece within pCD1.

The colonies containing the insert, Colonies 3 and 4 were inoculated into LB media with ampicillin (100 mg/L). DNA plasmid was isolated to send for sequencing. Once the sequence was confirmed, more DNA plasmid was isolated to insert the second partial clone, 155.1A.

PCR Amplification of 155.1A

Clone specific primers were designed to amplify 155.1A out of the pCR® 4-TOPO® vector. CSP 150F and CSP 151R were used in the PCR reaction (See Table 2 for details). The PCR products showed a band around 300-400 bp, the desired size of the 155.1A piece (Figure 16).
Figure 16. PCR Amplification of 155.1A piece. M is 1 kb ladder. Lanes 1-5 are PCR product of 155.1A piece. Bands were excised, gel purified, digested with *EcoRI* and *NotI* and ligated into pCD1.

These five bands were excised and gel purified for digestion. To reconstruct PGT 4 directionally, the pCD1 vector with the 181.2A insert and the 155.1A piece were both digested with *EcoRI* and *NotI*. Figure 17 shows an agarose gel of a digestion of pCD1 + 181.2A piece and of 155.1A piece. The 155.1A piece bands were extracted and gel purified. The pCD1 + 181.2A piece digestion was not successful and was repeated, Figure 18. The bands were extracted and gel purified for ligation.

Figure 17. Digestion of pCD1 vector and 155.1A piece. M is 1 kb ladder. Lanes 1-2 are pCD1 digested with *EcoRI* and *NotI*. Lanes 3-5 are 155.1A piece digested with *EcoRI* and *NotI*. Digestion of pCD1 was repeated for proper bands. Bands from Lanes 3-5 were used for ligation.
Figure 18. Repeated digestion of pCD1. M is 1 kb ladder. Lanes 1-3 are pCD1 digested with EcoRI and NotI. Bands were excised and gel purified for ligation.

Ligation and Transformation of 155.1A piece into pCD1 + 181.2A piece

The DNA from gel purification was used to set up a ligation. The ligation product was transformed into OneShot® Top 10 Cells. Colonies were grown on selective LB plates with ampicillin (100 mg/L). Nine transformant colonies grew overnight on the plates at 37°C. A PCR screening was conducted with CSP 144F and CSP 151R to verify the presence of the 155.1A piece within the pCD1 vector. One colony showed a positive band at around 1500 bp, suggesting the 155.1A piece was successfully ligated in the vector. This band suggests the full length reconstruction of PGT 4 (Figure 19).
Figure 19. PCR Confirmation of PGT 4 Reconstruction. M is 1 kb ladder. Lanes 1-11 are PCR product from each respective colony found on selective plates after transformation. Colony 4 shows a band at 1500 bp suggesting presence of full length PGT in plasmid DNA.

The colony containing full length PGT 4, Colony 5, was inoculated into LB media with ampicillin (100 mg/L). DNA plasmid was isolated to send for sequencing. The sequencing results confirmed the successful reconstruction of PGT 4 (Figure 20).
<table>
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<th>Sequence</th>
<th>Position</th>
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<tbody>
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<td>810-850</td>
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<td>1160-1200</td>
<td>CGGCTGGAAT TCATCTCTGG AAGGGATATC AGCTGGGGTT CAAATGCTTA</td>
</tr>
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</table>
When designing primers to amplify the partial clones, restriction enzymes were chosen that existed directionally within the pPICZ A multiple cloning site (Figure 11). To clone PGT 4 into the pPICZ A vector, full-length PGT 4 in pCD1 and empty pPICZ A vector were both...
digested with *KpnI* and *NotI*. Figure 21 shows both digestions. The bands were excised and gel purified. The purified DNA was used for a ligation.

**Figure 21. Digestion of pPICZ A vector and PGT 4 in pCD1.** M is 1 kb ladder. Lanes 1-2 are pPICZ A vector digested with *KpnI* and *NotI*. Lanes 3-5 are PGT 4 in pCD1 digested with *KpnI* and *NotI*. Bands from Lanes 1-2 and bands at 1500 bp from Lanes 3-5 were excised, gel purified, and used for ligation.

The ligation product was transformed into OneShot® Top 10 Cells. Colonies were grown on selective LB plates with zeocin (25 mg/L). Twelve transformant colonies grew overnight on the plates at 37°C. A PCR screening was conducted with CSP 144F and CSP 151R to verify the presence of the PGT 4 within the pPICZ A vector. Five colonies showed a positive band at around 1500 bp, suggesting PGT 4 was successfully ligated in the pPICZ A vector (Figure 22).
Figure 22. PCR Screening of Transformed ligated pPICZ A and PGT 4. M is 1 kb ladder. Lanes 1-13 are PCR product from respective colonies. Lane 13 is a positive control. Lane 14 is a negative control. Colonies 2, 3, 8, 9 and 10 showed bands at 1500 bp suggesting presence of PGT 4 within plasmid DNA.

The colonies containing full length PGT 4, Colonies 2,3,8,9, and 10, were inoculated into LB media with zeocin (25 mg/L). The isolated DNA plasmid was sent for sequencing. The sequencing results confirmed the successful insertion of PGT 4 into the pPICZ A vector (Figure 23).
Frame shifting PGT 4 in pPICZ A

Upon alignment of the PGT 4 sequencing results, it was found that the c-myc epitope and histidine tags on the 5’ end of the pPICZ A vector, immediately following the PGT 4 sequence, were out-of-frame. These tags are essential for identification and purification of the enzyme. Without these tags in frame, the presence of PGT 4 would not be visible on a Western blot. Thus, the frame shift was studied. It was found that only one base pair needed to be added to the 3’ primer to successfully frame shift the sequence. The challenge arose because the 3’ primer base pair length was not a multiple of three. This fact led to a frame shift of the tags which were designed with specific amino acid codon sequences which are in multiples of three. See Figure 7 for more details.
A new 3’ end primer, CSP 164R was designed inserting one guanine base pair to the primer sequence. A new 5’ end primer, CSP 163F, was designed to more closely match the melting temperature of the new primer sequence to ensure proper annealing and amplification by PCR. Amplification by PCR resulted in a desired band of around 1500 bp (Figure 24). PCR product was rapidly cloned into the pCR® 4-TOPO® vector, digested with \textit{KpnI} and \textit{NotI}, and ligated into pPICZ A vector also digested with \textit{KpnI} and \textit{NotI}.

![Figure 24. Digestion of pPICZ A vector and PGT 4 in pCR® 4-TOPO®.](image)

The ligation product was transformed into OneShot® Top 10 Cells. Colonies were grown on selective LB plates with zeocin (25 mg/L). Several transformant colonies grew overnight on the plates at 37°C. Seventeen colonies were chosen at random for PCR screening. A PCR screening was conducted with CSP 163F and CSP 164R to verify the presence of the PGT 4 within the pPICZ A vector. Colonies 5 and 13 showed a very light positive band at around 1500 bp, suggesting PGT 4 was successfully ligated in the pPICZ A vector (Figure 25).
Figure 25. PCR Screening of Transformed ligated pPICZ A and PGT 4. M is 1 kb ladder. Lanes 1-13 are PCR product from respective colonies. Lane 19 is a positive control. Colonies 5 and 13 showed faint bands at 1500 bp suggesting presence of PGT 4 within plasmid DNA.

The colonies containing full length PGT 4, Colonies 5 and 13, were inoculated into LB media with zeocin (25 mg/L). DNA plasmid was isolated and sent for sequencing. The sequencing results confirmed the insertion of PGT 4 into the pPICZ A vector with the c-myc epitope and histidine tags in frame (Figure 26).

Figure 26. Frameshift of pPICZ A tags for identification and purification. PGT 4 sequencing results of Colonies 5 and 13 showed tags in frame allowing for identification and purification when transformed into yeast (Invitrogen).
Transformation of PGT 4 into *Pichia pastoris*

X-33 strain yeast cells were grown in YPD media and harvested for electroporation. The pPICZ A vector storing the PGT 4 sequence was linearized with *Bst*XI for electroporation. The Invitrogen manual suggests the use of *SacI*, *Bst*XI, and *PmeI*. A restriction map of the PGT 4 sequence revealing an internal cutting site of *SacI*. Thus, *Bst*XI or *PmeI* had to be used. *Bst*XI was chosen due to its availability in lab stocks. Isolation of plasmid was conducted via MidiPrep, and DNA was digested overnight with *Bst*XI. To confirm complete linearization, a 2 μL sample from the digestion was run on an agarose gel. The results confirmed the complete digestion. (Figure 27).

![Figure 27. Linearization of PGT 4 in pPICZA with BstXI.](image)

The digested DNA was concentrated by the processes of phenol: chloroform extraction and ethanol precipitation. The DNA was resuspended in 20 μL distilled water for electroporation. Electroporation with concentrated DNA and yeast cells was conducted as described and sampled were spread on selective YPDS plates (zeocin 100 mg/L). After incubation for three days, six colonies were found and PCR screened with CSP 163F and CSP 164R to verify the presence of the PGT 4 within the yeast genome (Figure 28).
Figure 28. PCR Screening of Transformants of Electroporation I. M is 1 kb ladder. Lanes 1-6 are PCR product from respective colonies with CSP 163F and CSP 164R. Lane 7 is positive control. Lack of bands suggests absence of PGT 4 in yeast genome of Colonies 1-6.

When the presence of PGT 4 was not found, it was hypothesized that the yeast cells were not properly lysed when isolating DNA for PCR amplification. Presence of the colonies on the selective plate indicated that the colonies should contain the pPICZ A vector sequence with zeocin resistance. Cell lysing and PCR screens were repeated several times with identical results. A second electroporation with new linearized DNA and yeast cells was conducted. PCR screening of nine transformed colonies still found no presence of PGT 4 in the yeast genome (Figure 29).

Figure 29. PCR Screening of Transformants of Electroporation II. M is 1 kb ladder. Lanes 1-10 are PCR product of respective colonies with AOX1 primers. Lane 11 is a positive control. Lane 12 is a negative control. Lack of bands suggests absence of PGT 4 in yeast genome of Colonies 1-10.
Possible solutions to optimize transformation success were studied. The Invitrogen manual recommends electroporation with 1µg/µL and to use 5-10 µL. In previous electroporations, the recommended amount of DNA was used but not within the recommended volume due to insufficient drying after ethanol precipitation. To increase concentration of linearized DNA, a Speed Vac Concentrator (Thermo Electron Corporation) for fifteen minutes. This process allowed all remaining ethanol to be evaporated and the DNA pellet was resuspended in 5 µL.

A third electroporation was conducted preparing the linearized DNA as described. The transformation product was plates on selective YPDS plates (zeocin 100mg/L). After incubation at 28°C for three days, eleven colonies were found and screened by PCR for the presence of PGT 4. Three colonies showed PCR product just above 1500 bp. These bands were the desired length representing the 1467 bp PGT 4 sequence and the 300 bp AOX1 priming site sequence (Figure 30).

**Figure 30. PCR Screening of Transformants of Electroporation III.** M is 1 kb ladder. Lanes 1-11 are PCR product of respective colonies with AOX1 primers. Lane 12 is a positive control. Lane 13 is a negative control. Bands around 1500 bp suggest presence of PGT 4 in yeast genome of Colonies 7-9.
These bands confirmed the sequence of PGT 4 within the yeast genome in Colonies 7, 8, and 9. These yeast colonies were restreaked on fresh YPD plates, grown at 28°C for 2 days, and saved at 4°C.

**Western Blot Analysis of Test Inductions**

Test inductions of Colonies 7, 8, and 9 were conducted. Samples were taken at 0, 6, 12, 24, 36, 48, 60, 72, 84, and 96 hours. Cell pellets were saved at -80°C for SDS-PAGE and Western Blot analysis.

Samples were lysed and resuspended in Breaking Buffer (Appendix A). Protein aliquots from all samples were used for SDS-PAGE, electro-blotted onto a nitrocellulose membrane, and shaken with appropriate antibodies for Western blot analysis. Development of the Western blot showed protein production was optimum at 6-12 hours post-induction. Figure 31 shows the Western blots of all test induction samples of each colony.
A. Western Blot Analysis of Test Inductions of PGT 4 Expression

Bands were found around 54 kD, the hypothesized protein size of PGT 4. The heaviest bands occur at 6 and 12 hours suggesting optimum protein production post-induction.

B. Western Blot Analysis of Test Inductions of PGT 4 Expression

Lighter bands found at later times post-induction. Results suggest longer incubation time decreases protein production.

Figure 31. Western Blot Analysis of Test Inductions. A. Bands were found around 54 kD, the hypothesized protein size of PGT 4. The heaviest bands occur at 6 and 12 hours suggesting optimum protein production post-induction. B. Lighter bands found at later times post-induction. Results suggest longer incubation time decreases protein production.
On the two Western blots, bands were found around 54 kD, the hypothesized protein size of PGT 4. The darkest bands occurred at 6 and 12 hours suggesting optimum protein production post-induction. Much lighter bands were found at later times post-induction. These results suggest a longer incubation time decreases protein production.

Purification of PGT 4

To test for specific protein function, PGT 4 was isolated from all other proteins produced within yeast. The C-terminal His-tag within the pPICZ A vector allowed PGT 4 to be purified using metal affinity chromatography for purification. Yeast cells were grown to optimum post-induction time of 12 hours and harvested. The cell pellet was resuspended in Breaking Buffer with βME to prevent the formation of disulfide bridges and PMSF to prevent protease activity in the protein. The cells were lysed by French press, and the cell lysate was centrifuged. The supernatant was desalted with a PD-10 column to remove any salt that could interfere with binding to the metal affinity column. The desalted elution was subsequently applied to an IMAC column, and fractions were saved at 4°C to be analyzed by SDS-PAGE, Silver Staining, and Western blot. The Silver Staining shows three dark lanes which are the washes from the IMAC column eluting protein without a His tag. The next four lanes show the elutions of tagged PGT 4 which were concentrated for enzyme assay. The product of the concentration, the purified enzyme, is shown in the last lane (Figure 32). The Western Blot shows the lack of tagged enzyme in the washes, the tagged enzyme in the elutions, and the final purified enzyme used for assay (Figure 33). The final concentration of purified enzyme used for assay was 0.91 mg/mL.
Figure 32. Silver Stain of IMAC Purification for PGT 4 Screening. M represents low range protein ladder. W1-3 represent wash fractions collected from IMAC column. The bands represent all proteins washed away to purify PGT 4. E1-4 signify elutions from IMAC after addition of imidazole. These four fractions were concentrated for protein analysis. PE represents purified PGT 4 used for enzyme screening.

Figure 33. Western Blot of IMAC Purification for PGT 4 Screening. M represents low range protein ladder. W1-3 represent wash fractions collected from IMAC column. The bands represent all proteins washed away to purify PGT 4. E1-4 signify elutions from IMAC after addition of imidazole. These four fractions were concentrated for protein analysis. PE represents purified PGT 4 used for enzyme screening.
Screening of PGT 4 for Flavonoid Glucosyltransferase Activity

A total of 14 flavonoids were used as potential acceptor substrates to screen for GT activity with PGT 4. Each test reaction was performed in duplicate with each flavonoid substrate. A negative control using enzyme denatured with HCl were also performed in duplicate with each flavonoid substrate. This negative control served as a background reading of activity with each substrate. The test results were corrected against these background readings to verify positive activity. A second negative control was conducted using denatured PGT 4 that had been boiled for ten minutes with quercetin. A positive control using young grapefruit leaf extract as the GT enzyme source was performed with quercetin. A second positive control combining young grapefruit leaf extract and PGT 4 was performed to test for inhibiting effects of glucose incorporation. The amount of radiolabeled $^{14}$C-UDP-glucose transferred to flavonoid substrates was analyzed using a Beckman LS 6500 scintillation counter to determine cpm in 250 µL.

Results show the average cpm incorporation of the substrates tested with PGT 4 (Table 10).

**Table 10. Screening of PGT 4 for Flavonoid Glucosyltransferase Activity**
(Note: 20000 cpm $^{14}$C-UDP-glucose was used per reaction)

<table>
<thead>
<tr>
<th>Substrate Name</th>
<th>Substrate Structure</th>
<th>Average cpm incorporation (n=2, 250 µL ethyl acetate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naringenin</td>
<td><img src="image" alt="Naringenin Structure" /></td>
<td>n.a.</td>
</tr>
<tr>
<td>Hesperitin</td>
<td><img src="image" alt="Hesperitin Structure" /></td>
<td>10</td>
</tr>
<tr>
<td>Eriodictyol</td>
<td><img src="image" alt="Eriodictyol Structure" /></td>
<td>n.a.</td>
</tr>
<tr>
<td>Flavones</td>
<td>Isosakuranetin</td>
<td>n.a.</td>
</tr>
<tr>
<td>-------------</td>
<td>----------------</td>
<td>------</td>
</tr>
<tr>
<td></td>
<td>Apigenin</td>
<td>n.a.</td>
</tr>
<tr>
<td></td>
<td>Luteolin</td>
<td>n.a.</td>
</tr>
<tr>
<td></td>
<td>Diosmetin</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>Scutellerin</td>
<td>n.a.</td>
</tr>
<tr>
<td>Flavonols</td>
<td>Kaempferol</td>
<td>n.a.</td>
</tr>
<tr>
<td></td>
<td>Quercetin</td>
<td>n.a.</td>
</tr>
<tr>
<td></td>
<td>Fisetin</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>Gossypetin</td>
<td>n.a.</td>
</tr>
</tbody>
</table>
According to these results, no significant GT activity was found with PGT 4. All tests were conducted in duplicate, and the results were corrected against duplicate background controls and for 250 μL ethyl acetate. The positive control with crude extract showed activity as several GTs are found within young grapefruit leaf tissue. The second positive control, using crude extract and purified enzyme, showed increased activity levels compared to the crude extract. This result could suggest that PGT 4’s preferred substrate acceptor is found within the crude extract. Also, this finding could be explained by PGT 4 being a part of a multi-enzyme complex. The increased activity of the control could be a result of PGT 4’s interaction with other necessary enzymes to exhibit glucosyltransferase activity.
SUMMARY and FUTURE WORK

PGT 4 was successfully reconstructed to its full-length of 1467 base pairs. The c-myc epitope and histidine tags were shifted into frame allowing for identification and purification of PGT 4 for flavonoid GT analysis. PGT 4 was found to have predicted size of around 54 kD. Optimum post-induction time was determined to be 12 hours by Western blot analysis. SDS-PAGE, Silver staining, and Western blot analysis were conducted to ensure presence of purified protein. PGT 4 was screened against 14 flavonoid substrates for flavonoid glucosyltransferase activity. No significant GT activity was found with PGT 4. Repeats of the screening with these flavonoids and the controls could be done to ensure accurate results. If the results of the positive controls are duplicated, the possible implications could be explored. A compound profile could be created to narrow down potential substrate acceptors of PGT 4.

Further screening with representatives from other flavonoid subclasses not previously tested could be conducted to find PGT 4’s preferred substrate acceptor. Although PGT 4 did not exhibit any flavonoid glucosyltransferase activity with tested flavonoids, the enzyme could be further tested to reveal activity with other secondary metabolites such as simple phenolic compounds. Previous work has shown GT activity with some phenolic compounds using reverse high performance liquid chromatography (Wamucho 2012, Hayford 2012).

If no glucosyltransferase activity is found, it may be necessary to use a yeast expression vector that can cleave the fusion tag after purification to rule out any interference of the extra sequence on the GT activity of the enzyme.

This work has helped to narrow down the list of possible substrate acceptors of PGT 4. With this knowledge, further work, including the suggested methods, will elucidate the preferred substrate of PGT 4 and help to distinguish its role in grapefruit metabolism.
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APPENDIX A
Buffer Recipes

50X TAE Buffer
- 50 mL EDTA (pH 8.0)
- 28.6 mL glacial acetic acid
- 121 grams Tris Base
- Add dH$_2$O to 1000 mL
- Add 20 mL of 50X TAE to 980 mL dH$_2$O to dilute to 1X TAE for agarose gels

1 M Potassium Phosphate Buffer, pH 6
- 132 mL 1 M K$_2$HPO$_4$
- 868 mL 1 M KH$_2$PO$_4$
- Adjust pH to 6 with KOH if needed.
- Autoclave for 20 minutes and store at room temp

Breaking Buffer, pH 7.4
- 6 grams monobasic sodium phosphate
- 372 mg EDTA
- 50 mL glycerol
- Dilute to 900 mL
- Adjust pH to 7.4 with NaOH
- Dilute to 1 Liter
- Store at 4°C
- Add desired protease inhibitors
  - 50 µL of 0.1 M PMSF was added to 5 mL BB before lysing cell pellets.

5X SDS-PAGE TANK Buffer
- 15.1 grams Tris base
- 94 grams glycine
- Dilute to 900 mL with distilled water
- Add 50 mL 10% SDS
- Dilute to 1 Liter with distilled water

10X Western Blot Transfer Buffer
• 30.3 grams Tris base
• 144.1 grams glycine
• Dilute to 1 Liter
• Store at 4°C
• 100 mL 10X stock
• 700 mL distilled water
• 200 mL methanol

5X Tris-Buffered Saline (TBS)
• 40 grams NaCl
• 1 gram KCl
• 15 grams Tris base
• Dilute to 800 mL with distilled water
• Adjust pH to 7.4 with HCl
• Dilute to 1 Liter with distilled water

Alkaline phosphatase buffer
• 1.17 grams NaCl
• 203 mg MgCl$_2$ – H$_2$O
• 2.42 grams Tris
• Dilute to 80 mL with distilled water
• Adjust pH to 9.5 with HCl
• Dilute to 200 mL with distilled water

Blocking Solution
• 2.5 grams milk powder
• 40 µL sodium azide
• Dilute to 50 mL with 1X TBS

Developing Solution
• 45 mL alkaline phosphatase buffer
• Add following immediately before developing Western blots
• 180 µL NBT in 0.083 g/mL in 70% N,N-dimethylformamide
• 180 µL BCPIP in 0.42 g/mL in 100% N,N-dimethylformamide

50 mM Phosphate Buffer (pH 7.5)
- 0.2918 grams monosodium phosphate
- 0.7733 grams disodium phosphate
- Add 900 mL distilled water
- Adjust pH to 7.5
- Dilute to 1 Liter

**Column Buffer (pH 7.5)**

- 0.2918 grams monosodium phosphate
- 0.7733 grams disodium phosphate
- 17.532 grams NaCl
- Add 900 mL distilled water
- Adjust pH to 7.5
- Dilute to 1 Liter

**IMAC Elution Buffer (pH 7.5)**

- 0.2918 grams monosodium phosphate
- 0.7733 grams disodium phosphate
- 17.532 grams NaCl
- 10.21 grams imidazole
- Add 900 mL distilled water
- Adjust pH to 7.5
- Dilute to 1 Liter

**IMAC MES Buffer (pH 5)**

- 3.9046 grams 2-(N-morpholine)-methanesulfonic acid (MES)
- Add 900 mL distilled water
- Adjust pH to 5
- Dilute to 1 Liter

**APPENDIX B**
Media and Stock Solution Recipes

Low Salt LB Media

- 10 grams tryptone
- 5 grams yeast extract
- 5 grams sodium chloride
- Add 450 mL dH₂O and stir with magnetic stirrer
- Adjust pH to 7.5 using 6M NaOH
- Fill up to 500 mL with dH₂O
- Autoclave for 20 minutes
- Cool to room temperature
- Store at 4 °C
- Add antibiotic as necessary
  - Ampicillin – 100mg/L
  - Zeocin -25mg/L

LB Agar Plates

- Low Salt LB media
- Agar (15 grams/L)
- Add agar to desired amount of Low Salt LB media and autoclave for 20 minutes
- Cool at 42°C
- Add desired antibiotic (see above concentrations)
- Pour into sterile plates
- Allow to harden and cool.
- Store inverted at 4°C
- Cover LB Zeocin plates with aluminum foil

Phenol:Chloroform Solvent

- Combine equal parts of phenol and 0.5 M Tris-HCl, pH 8
- Add 0.1% 8-hydroxyquinoline 2-carboxylic acid
- Stir mixture for 15 minutes.
- Remove upper layer of the mixture
- Add an equal volume of 0.1 M Tris-HCl to the mixture
- Stir for 15 minutes
- Repeat above steps until pH of phenol: chloroform is above 7.8

10 X YNB (pH 5.4)
• 134 grams of yeast nitrogen base (YNB)
• 100 grams ammonium sulfate
• Add 900 mL distilled water
• Adjust pH to 5.4
• Dilute to 1000 mL with distilled water
• Heat solution to dissolve completely
• Filter sterilize and store solution at 4°C

**500 X B (0.02% Biotin)**

• 20 milligrams biotin
• Dilute to 100 mL with distilled water
• Filter sterilize and store solution at 4°C

**10 X D (20% Dextrose)**

• 200 grams of D-glucose
• Dilute to 1000 mL distilled water
• Filter sterilize and store at room temp

**10 X M (5% Methanol)**

• 5 mL methanol
• Add 95 mL distilled water
• Filter sterilize and store at 4°C

**10 X GY (10% Glycerol)**

• 100 mL glycerol
• Dilute to 1000 mL with distilled water
• Filter sterilize and store at room temperature

**YPD Liquid Media**

• 10 grams yeast extract
• 20 grams peptone
• Dilute to 900 mL with distilled water
• Adjust pH to 7.5 using 6M NaOH
• Autoclave for 20 minutes
• Add 100 mL 10 X D

**YPD Agar Plates**
• Desired amount of liquid YPD media
• Add 15 grams agar/ liter
• Autoclave for twenty minutes
• Add antibiotic, Zeocin (100 mg/L)
• Store in dark at 4°C

YPDS Media and Plates

• 10 grams yeast extract
• 20 grams peptone
• 182 grams sorbitol
• Dilute to 900 mL distilled water
• Adjust pH to 7.5 using 6M NaOH
• For plates, 15 grams agar / liter
• Autoclave for 20 minutes
• Add 100 mL 10 X D
• Cool solution to 42°C in water bath
• Add antibiotic, Zeocin (100 mg/L)
• Store plates in dark at 4°C

BMGY / BMMY Media

• 10 grams yeast extract
• 20 grams peptone
• Dilute to 700 mL with distilled water
• Adjust pH to 7.5 using 6M NaOH
• Autoclave for 20 minutes
• Cool to room temp and add:
  - 100 mL sterile 1M potassium phosphate buffer, pH 6
  - 100 mL sterile 10 X YNB
  - 2 mL sterile 500 X B
  - 100 mL sterile 10 X GY for BMGY
  - 100 mL sterile 10 X M for BMMY
• Store at 4°C

2X PCR Master Mix

• 200 µL 5X GoTaq Reaction Buffer
• 2 µL 100 mM dNTP Mix
• 5 µL GoTaq enzyme of 5 w/ µL stock
• Add sterile, distilled water to 500 µL

Coomassie Blue staining solution
• 6 mg Coomassie Brilliant Blue G250
• 10 mL acetic acid
• 40 mL methanol
• Dilute to 100 mL with distilled water

Destaining solution

• 300 mL 30% methanol
• 100 mL 10% acetic acid
• Dilute to 1 L with distilled water
APPENDIX C

Gel Recipes

0.8% Agarose Gel

- 0.24 grams of agarose
- Add 30 mL 1X TAE (pH 8.2)
- Heat for 1 minute in microwave
- Add dH₂O to fill back up to 30 mL
- Add 2.5 μL of EtBr (10mg/mL)

SDS-PAGE

<table>
<thead>
<tr>
<th></th>
<th>10 % Separating Gel</th>
<th>5 % Stacking Gel</th>
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<tbody>
<tr>
<td>Distilled water</td>
<td>2.3 mL</td>
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<td>40% Acrylamide and</td>
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<td>bis-acrylamide</td>
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<td>solution, (19:1)</td>
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<tr>
<td>1.5 M Tris, pH 8.8</td>
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<td>1.0M Tris, pH 6.8</td>
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<td>TEMED*</td>
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<tr>
<td>10% APS</td>
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<td>20 μL</td>
</tr>
<tr>
<td>* add last</td>
<td>Total</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5 mL</td>
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