Site-Directed Mutagenesis of GPR1 in 
*Saccharomyces cerevisiae*

an Honors Thesis submitted by

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**Introduction**

The organism *Saccharomyces cerevisiae*, more commonly known as Brewer’s yeast, is used for the everyday functions of making bread and brewing beer. In addition to these practical functions, *Saccharomyces cerevisiae* is also used as a model organism, allowing scientists to learn more about the human genome and physiology through the study of yeast cells. *Saccharomyces cerevisiae*, just like other organisms, is able to sense changes in its environment and respond to those changes by a series of biochemical chain reactions. The first component of any pathway is the signaling and receiving of the message that is to be transmitted. Thus, receptors are a very important part in signal transduction pathways. *S. cerevisiae* has three main G-protein coupled receptors (GPCRs), and the focus of this study is on one of these, the GPR1 receptor. One of the receptors, STE2, is involved in sensing pheromones that stimulate mating. GPR1, on the other hand, is involved in detecting and metabolizing glucose. In the absence of glucose, however, the STE2 pheromone-sensing pathway will not be activated. Much is known about the STE2 pathway, but little is known about the components and series of the GPR1 pathway.

Whereas the human genome contains genes for over three hundred G-protein coupled receptors, the *S. cerevisiae* genome contains only three. G-protein coupled receptors such as STE2, STE3 and GPR1 are transmembrane domain receptors consisting of seven helices (Oliveira et al., 1993). These G-protein coupled receptors have an extracellular component to which the ligand (either glucose or the pheromone) binds, and an intracellular component, which transmits the signal to a G protein and then on to the next messenger in the pathway. In the case of STE2, the pheromone ligand binds to the receptor and the receptor then sends the signal into the cell, eventually causing the cell to
manner. The GPR1 receptor senses glucose, which binds to the receptor and is transmitted into the cell.

Much information is known about the sequence and components involved in the mating pathway in *S. cerevisiae*, but knowledge is limited as to how this pathway responds to the presence or absence of nutrients such as glucose. The ongoing research done by faculty and staff at Carson-Newman College has shown that in the absence of the protein (Gpr1p), which senses glucose, pheromones are more weakly detected, and thus yeast cells are less likely to undergo mating activity. Because of this knowledge, as well as knowledge about how the pathways work, it has been hypothesized that the mating and glucose sensing pathways are interwoven and depend upon one another in some form. However, the effect of the absence of the STE2 gene on the sensing of glucose is not yet known.

The GPR1 gene codes for the Gpr1 protein, which consists of 961 amino acids. Of the seven loops that make up the total Gpr1p, the third loop is the largest, consisting of approximately 346 amino acids (Xue et al., 1998). Within the third loop of the protein lies a short, basic sequence, two copies of which are also found at the termini of the protein, one at the N terminus and the other at the C terminus. This third loop was determined to be vital to the function of the Gpr1 receptor. Xue and colleagues (1998) discovered that after mutating the sequence that encodes the third loop of the protein, the protein coupled receptor lost function. Without the third loop, the protein is unable to bind to the G protein, and thus unable to send the signal down the rest of the pathway.

G protein coupled receptors are located on the cell surface. Since Gpr1 is a member of the GPCR family, it should also be located on the cell surface. In order to determine the location of the Gpr1p receptor, scientists Xue and colleagues (1998) added a green
fluorescent protein (GFP) tag to the gene that codes for the receptor. When the cell transcribed the gene to RNA and then translated it to protein, the GFP tag was also expressed. Everywhere the Gpr1p receptor was located the GFP tag was visible under ultraviolet light. By performing this experiment, the scientists found that the Gpr1p receptor was indeed localized to the cell surface.

Xue et al. (2001) have also introduced several mutations in the GPR1 gene and found that in the third cytoplasmic loop of Gpr1p membrane-proximal regions there are sequences near the N-terminal end that are necessary for function. When these sequences were deleted, the protein was found to be dysfunctional.

Xue, Battle, and Hirsch (2001) also state that many GPCRs contain a set of highly conserved amino acid sequences. The high degree of conservation suggests that these sequences are necessary for the function of the GPCR. These conserved sequences include: the alanine at position 193, the phenylalanine at position 262, the tryptophan at position 634, and the tyrosine at position 676 as are highlighted in Figure 1.
Figure 1. Sequence of GPR1 gene. Target deletions of amino acids that were deleted are outlined by red boxes.

Shi and colleagues (2009) recently determined the importance of the N-terminus of Ste2p for function. In their study, they performed target mutations involving the N-terminus and then performed several assays to test the function of the mutant cells. They found that deletion of the first twenty amino acids of Ste2p yielded a decrease in mating efficiency. Therefore, they concluded that the N-terminus is necessary for adequate function of the Ste2 protein (Shi et al., 2009). However, the specific significance of the N-terminus in the function of Gpr1p is not known. Therefore, the aim of this study was to delete sequences of the N-terminus of GPR1 to monitor the effects on function and cell viability.

The Gpr1 receptor acts as a part of the Ras (Rat sarcoma)/cAMP pathway to sense glucose and other carbon sources so that the yeast cell can be nourished, carry out essential cell activities, and grow. In the presence of a carbon source such as glucose, the C terminus of the Gpr1 receptor binds to the N terminus of the Gpa2 protein. Gpa2 protein forms an unusual complex with "mimic proteins" (proteins that mimic the structure and
function of native proteins) Gpb1 and Gpb2. These two mimic proteins inhibit Gpa2, preventing its binding to the receptor on the membrane’s surface. In the presence of glucose, Gpa2 can bind to the Gpr1p receptor, thus releasing Gpb1 and Gpb2 (Harashima and Heitman, 2005). After binding to the receptor, Gpa2 signals the cell to synthesize cAMP and activate Protein Kinase A (Kraakman, et al., 1999). The activation of PKA causes growth stimulation, pseudohyphal differentiation, and loss of stress resistance yielding a decreased amount of growth under stress (Versele et al., 2001).

The contributions of this study to the ongoing research on the interdependence of the two pathways, the nutrient sensing and the pheromone sensing GPCRs in yeast, were to create mutant forms of the GPR1 gene. Four individual target mutations as well as C terminus truncations (deletions of a series of amino acids at the beginning of the protein sequence) were introduced in the GPR1 gene to determine which specific regions of the gene code for the specific portions of the protein are necessary for function. If the target mutations resulted in a loss of function of the receptor, then that segment of nucleotides is necessary to make the receptor function.
Methods and Materials

Strains Used

TM24 cells: *S. cerevisiae*, Mat-A, ste2D, ade1, trp1:kan, ura3-52, leu2-3, leu2-112, Dmfa1 fus1-HIS3, sstD, ste3::URA3, Dmfa2::fus1-lacZ. Other cells were of the same genotype but were also gpa2:hyg.

Liquid Cultures

In the series of experiments, MLT liquid media was used for many of the liquid cultures. One liter of MLT media was made by mixing 6.7 grams of YNB (yeast nitrogen base), 1.8 grams of trp DO, 5 grams of casamino acid (CAA), and 20 grams of dextrose. The mixture was then autoclaved to sterilize. Colonies from various strains were then taken from an MLT plate using a sterile toothpick, placed in the liquid media, and vortexed.

Lysogeny Broth ampicillin (LB amp) plates and liquid media were also utilized in these series of experiments. The LB amp plates were made by adding 250 ml of deionized water to 20 g of LB agar. The mixture was autoclaved for 45 minutes, cooled, and 250 μl of ampicillin was then added. The mixture was thoroughly swirled to equally distribute the ampicillin in the solution, and the solution was poured into plates. The LB amp liquid media was made by mixing 300 ml of deionized water with 6 grams of LB broth and 300 μl of ampicillin.

Promega Bacterial Transformation Quick Protocol

Competent Nova Blue *E. coli* cells were chilled on ice until thawed, and mixed by gently flicking the tube. One μl of the DNA being used to transform the cells was then added. Tubes were then quickly flicked to mix and immediately returned to ice for ten minutes. After the ten minute ice incubations, the cells were heat shocked in a 42°C water
bath for 60 seconds. The tubes were then placed on ice for two minutes. Seven hundred µl of SOC medium was then added, and the cells were incubated at 37° for 60 minutes, with shaking. During the 60-minute incubation, the tubes were opened two to three times for a few seconds to aerate. Seventy µl of cells were then plated on LB amp plates and incubated overnight.

**Quick and Easy TRAFO Yeast Transformation Protocol**

For this transformation method, single stranded carrier DNA was boiled for five minutes then chilled in ice water. Yeast TM24 cultures were inoculated into a Yeast Peptone Dextrose (YPD) liquid media for twelve hours at 37 degrees Celsius. Cells were centrifuged, and the supernatant was discarded. A transformation mix consisting of 240 µl of PEG, 36 µl lithium acetate, 50 µl of boiled, cooled, and vortexed SS-carrier DNA, and 34 µl of plasmid DNA plus water was added, and the cells were resuspended. This mixture was incubated in a water bath at 42°C for 50 minutes, after which the tubes were microcentrifuged and the supernatant discarded. One ml of sterile water was added into the tube to resuspend the cells by pipetting up and down. Ten µl and 100 µl samples of each transformation were plated on appropriate media and allowed to grow.

**Generation of GFP Epitope Tag**

A Green Fluorescent Protein (GFP) tagged GPR1 version was generated by using Polymerase Chain Reaction (PCR). The primers used for this PCR reaction were: 5’-AGTGAGTACGCAAACAATAGGA-3’ being the forward primer and 5’-CAATTTTTTTAAGGTTTTGCTG-3’ being the reverse primer. Plasmid DNA taken from bacterial colonies transformed with a plasmid obtained from Mark Rosenthal and purified using the StrataPrep® Plasmid Miniprep Kit (Genomics). Polymerase Chain Reaction
(PCR) was then performed using the PerfectTaq™ 5 PRIME Mastermix (5 PRIME) protocol and using a gradient thermocycler. Analysis of the PCR products by agarose gel electrophoresis showed the MR/GFP construct was successfully amplified. The PCR product was then used to transform Nova Blue *E. coli* cells, and the MR/GFP plasmid construct was then used to transform *S. cerevisiae* cells by the lithium acetate based Quick and Easy TRAFO protocol (Gietz and Woods, 2002). The viability of the transformed yeast cells was tested using a heat shock and Fus1/lacZ fluorescence assay.

**Site-directed Mutagenesis**

Mutants of the GPR1 gene were made by performing site-directed mutagenesis. These mutations included: deletion of amino acids 10-15, 15-20, and 20-25 of the GPR1 sequence, deletions of the amino acid alanine at position 193, deletion of tryptophan at position 634, and deletion of tyrosine at position 676. Primers were generated using the PrimerX website (http://www.bioinformatics.org/primerx/cgi-bin/DNA_1.cgi). Forward and reverse primers can be seen in Table 1.
Table 1. Forward and reverse primers used for site-directed mutagenesis.

<table>
<thead>
<tr>
<th></th>
<th>Forward Primer (5’ to 3’)</th>
<th>Reverse Primer (5’ to 3’)</th>
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<tr>
<td>Δ15-20</td>
<td>AACGCGTTGAAAGAAAAGAGAGTTG</td>
<td>CAACTCTCTTTTCTTTCAACGCCTT</td>
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<tr>
<td>Δ20-25</td>
<td>'GTCATCCTTACTATCTCTCGACAG</td>
<td>CTGTGGAGAGATAGTAAGGATGAC</td>
</tr>
<tr>
<td>ΔA at position 193</td>
<td>GTACCTGCACTTTAAAGCTTAGCCTC</td>
<td>GAAGGCTAAGCTTAAATGGCGAGGTAC</td>
</tr>
<tr>
<td>ΔW at position 634</td>
<td>GTGCTATATTGGGATACCTTCCCCCATTTG</td>
<td>CAATGATGGGGAAAAGTATCCCAATATACGAC</td>
</tr>
<tr>
<td>ΔY at position 676</td>
<td>CGTCGACGTCATTGTTTCTGTCAGAAGAAAAC</td>
<td>G T T T T C C T T GA C A C G A A C A T G A C G T C G A C G</td>
</tr>
</tbody>
</table>
**Fus1-LacZ Fluorescence Assay**

A TM-24 strain of yeast containing deletions in the STE2, GPA1, FAR1, and containing a FUS1/lacZ construct was grown in liquid YPD media for one day at 37°C. The truncation of the C-terminus of Gpr1 as well as the amino acid deletions were inoculated and grown in liquid MLT media for one day with incubation at 37°C. Cells were then aliquotted into a 96-well microtiter plate in volumes of 50 µl. The cell density was determined by measuring the absorbance at 600 nm using an BIO RAD iMark ™ Microplate Absorbance Reader. Twenty µl of a substrate containing 60 µl of 10mM fludeoxyglucose (FDG) and 1.3 ml of 250 mM 3-(N morpholino) propanesulfonic acid (MOPS) with a pH of 7.2 and 5% Triton-X-100 were added to each of the wells. For one run, the cells were left in a dark drawer for approximately fifteen hours and the fluorescence was then read on a Tcam plate reader (BioRad). For the second run, the plates were only left in the dark drawer for five hours and then read on the Tcam plate reader. The relative fluorescence activity was calculated by dividing the fluorescence by the cell count to normalize the results.

**Heat Shock Assay**

Cells were depleted of glucose by growing in liquid YPD or media lacking tryptophan (MLT) for two days with incubation at 37°C. Two hundred µl of each culture were taken and distributed into appropriately labeled epitubes. Into the original cultures, 600 µl of a 20% glucose solution was added and was incubated for four hours at 37°C. After the incubation, 200 µl of the remaining cultures were distributed in the remaining epitubes. Each of the epitubes was centrifuged for two minutes, and the supernatant was discarded. 200 µl of a solution with a pH of 5.5 was added to each of the epitubes of
colonies that were not being heat shocked. To the cells that were being heat shocked, 200 µl of a solution with a pH of 8 were added. Each of the cells was resuspended in these solutions. The cells that were being subjected to heat shock were then placed in the epitube heater at 55°C for 30 minutes. Upon completion of the heat shock, the cells were centrifuged and the supernatant was discarded. The cells were then resuspended in liquid YPD or MLT media, respectively. A serial-5 fold dilution was performed using a 96 well microtiter plate. Ninety µl of water were placed in each well, with only 10 µl of cells being transferred to each well. Three µl of each well were then plated on MLT or YPD plates into correctly labeled columns. The plates were left to grow at room temperature for three days.
Results

The initial goal of this project was to generate a version of Gpr1p with a GFP tag on it so it could be tracked within the cell. This was to be done by adding DNA sequences to either end of the gene for GFP by PCR and then recombining that with GPR1 carried on a plasmid by in vivo ligation. After several failed attempts at obtaining a PCR product, the emphasis of this project was switched from adding an epitope tag of GFP to the N-terminus of GPR1 to introducing more subtle changes via site-directed mutagenesis of the GPR1 gene. The mutations that were chosen included: truncation of the C terminus, deletion of the alanine at position 193, the phenylalanine at position 262, the tryptophan at position 634, and the tyrosine at position 676. The effects of the mutations on the phenotypes of the yeast organisms were measured using two different assays, the Fus1/lacZ assay and a modification of the heat shock assay mentioned by Xue et al. (2001). The modification to the heat shock assay was developed by Freeman (2011).

The concentrations and A260/A280 ratios of the plasmids that were purified and used to transform yeast cells are shown in Table 2. The A260/A280 ratio measures the purity of the DNA and protein with a value of between 1.4 and 1.9 demonstrating good quality DNA.
Table 2. Concentrations and A260/A280 ratios of plasmids to determine DNA purity.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Concentration</th>
<th>A260/A280</th>
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<tbody>
<tr>
<td>Deletion of 10-15</td>
<td>171.8643 µg/ml</td>
<td>0.9951</td>
</tr>
<tr>
<td>Deletion of 15-20</td>
<td>187.4094 µg/ml</td>
<td>0.9967</td>
</tr>
<tr>
<td>Deletion of 20-25</td>
<td>60.6447 µg/ml</td>
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<tr>
<td>Deletion of A</td>
<td>126.5367 µg/ml</td>
<td>1.0682</td>
</tr>
<tr>
<td>Deletion of W</td>
<td>58.8916 µg/ml</td>
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<tr>
<td>Deletion of Y</td>
<td>6.803 µg/ml</td>
<td>1.6301</td>
</tr>
<tr>
<td>GFP</td>
<td>5.2828 µg/ml</td>
<td>1.4713</td>
</tr>
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</table>

Fus1/lacZ Assay

In the TM24 strains that were used in the fluorescence assay, the Fus1/lacZ construct produces an enzyme called β-galactosidase. In the absence of the GPA1, a gene that is deleted in the TM24 strain, this pathway is constitutively active. In the absence of the GPA2, the pathway shows diminished activity. If any of the target mutations of the wild type TM24 cells showed less fluorescence than regular wild type cells, and thus diminished activity, the mutation was a dominant mutation that overrode the original mutation. In the Gpa2 deleted cells in which the target mutations were introduced, if the fluorescence was greater than that of normal GPA2 delete cells, the mutation had a positive impact on the pathway and restored function, indicating this gene acts upstream of GPA2.

As shown in Figure 2, the truncations of the C-terminus of the GPR1 gene had an effect on the relative fluorescent activity relative to the wild type cells. Each series represents one of the many clones appearing on the plate of selective media following
transformation. Non-mutant wild type cells showed relative fluorescent activity of 22000 to 59000 depending on the clone. It should be noted that these wild type cells were not mock transformed, and what effects the transformation process may have on this activity are not known. Each of the mutants showed a relative fluorescent activity of less than 20000. The deletions of nucleotides 10-15 showed the largest decrease in both runs of the assay, with a relative fluorescent activity (RFA) of approximately 8000. Each of the clones had relatively the same RFA. The deletions of nucleotides 15-20 had approximately the same RFA for each clone as the 10-15 delete cells on the first run, but showed a slightly higher RFA (10000) for the second run. Clones one, four, and seven of the 20-25 delete wild type cells had a relative fluorescent activity of 12000, whereas clones two, three, five, six, and eight showed the lowest RFA of all the mutants at 2000.
Figure 2. Effects of the truncation of the C-terminus of GPR1 on the amount of relative fluorescence activity (fluorescence divided by cell count) shown by different clones of wild type cells. (WT=wild type; WT Δ10-15=wild type cells with amino acids 20-25 deleted; WT Δ15-20=wild type cells with a deletion of amino acids 15-20; WT Δ20-25=wild type cells with deletion of amino acids 20-25).

The deletion of specific amino acids had a similar effect on wild type cells to the effect of the C-terminus truncation, as can be seen in Figure 3. The wild type ΔY cells had a RFA range of 2000-8000, much less than the 23000-59000 RFA of the normal wild type
cells. The range of RFA of wild type ΔW cells was less broad than the range of RFA for wild type ΔY cells. The clone with the least RFA was clone one at 2000, and the clones with the most RFA were clones three and four at 6000.

Figure 3. Relative fluorescence activity of mutant cells (WT ΔY and WT ΔW) and wild type cells (WT) are shown. Four individual clones were used.
As was seen in the previous mutations of wild type cells, the addition of a GFP epitope tag to the GPR1 gene diminished the fluorescence of the cells, shown in Figure 4. Clone four, run two of the GFP tagged cells, had the highest fluorescence of any of the tagged clones at 10000. Clones one and two of the second run had the smallest fluorescence of any of the GFP clones at 2000. Both of these values, as well as the values that fall within that range, are smaller than the fluorescence of the wild type cells.

Figure 4. Relative fluorescence activity of cells with a green fluorescent protein (GFP) epitope tag as compared with the relative fluorescence activity of wild type cells. (WT=wild type; GFP=wild type cells with an added GFP epitope tag).

Whereas most gpr1 mutant cells showed a lower RFA than wild type cells, the effects of the mutations of the gpr1 gene in GPA2Δ cells were sporadic, which can be seen in Figure 5. The GPA2Δ cells with a deletion of nucleotides 10-15 had a higher relative
fluorescent activity than that of the second run’s normal GPA2Δ cells. Clone two had a RFA of approximately 7000, whereas the second run’s GPA2Δ cells had a RFA of approximately 3000. However, the relative fluorescent activity of no mutants was higher than the RFA of GPA2Δ cells of the first run. Clone three of the GPA2Δ with deletions of nucleotides 15-20 showed the highest RFA of its clones at over 6000, which is much higher than the regular GPA2Δ cells. However, clones one and four did not have a higher RFA than the regular GPA2Δ cells. For the Δ20-25 cells, clones one and two of the first run had a slightly higher RFA than the normal second run GPA2Δ cells. The other clones had a lower RFA than either set of the normal GPA2Δ cells.
Figure 5. Effects of the truncation of the C-terminus of the GPR1 gene on the relative fluorescent activity of GPA2Δ cells. (GPA2Δ=TM24 cells with a deletion of GPA gene; GPA2Δ Δ10-15=GPA2Δ cells with a deletion of amino acids 10-25 of GPR1 gene; GPA2Δ Δ15-20=GPA2Δ cells with a deletion of amino acids 15-20 of the GPR1 gene; GPA2Δ Δ20-25=GPA2Δ cells with a deletion of amino acids 20-25 of GPR1 gene).

In addition to testing the effects of C-terminus truncation on the RFA of GPA2Δ cells, the effects of deletions of three different amino acids on the RFA of the GPA2Δ cells were also tested. As is shown in Figure 6, each clone of the first run of GPA2Δ ΔY cells showed a higher relative fluorescent activity than the second run of normal GPA2Δ cells. However, every clone of the second run of GPA2Δ ΔY cells had a lower RFA than the second run of GPA2Δ cells. The same was true for the GPA2Δ ΔW cells and GPA2Δ ΔA. The first run had a higher RFA, and the second run had a lower RFA than normal GPA2Δ cells. The first run of
GPA2Δ ΔW cells’ RFA was not quite as high as that of the first run of GPA2Δ ΔY cells or GPA2Δ ΔA cells.

![Graph of Effects of Amino Acid Deletion of Gpr1 on the Pheromone Pathway in GPA2Δ Cells]

**Figure 6.** Effect of specific amino acid deletions on the relative fluorescent activity of GPA2Δ cells. (GPA2Δ=TM24 cells with a deletion of GPA2 gene; GPA2Δ ΔY=GPA2Δ cells with a deletion of the tyrosine at position 676 of the GPR1 gene; GPA2Δ ΔW=GPA2Δ cells with a deletion of tryptophan at position 634 of the GPR1 gene; GPA2Δ ΔA=GPA2Δ cells with a deletion of alanine at position 193 of the GPR1 gene).

After measuring the effects of mutations of the gpr1 gene on the pheromone pathway, the effects of the addition of a GFP plasmid on this pathway were also measured.
As is shown in Figure 7, the relative fluorescent activity of the GPA2Δ cells in the first run was 8000-10000. In the second run, the RFA of the GPA2Δ cells was much lower, around 2200 and 2400. Each of the clones containing the GFP tag had a RFA of slightly less than 4000, which was more than the RFA of the second run normal GPA2Δ cells, but lower than the RFA of the first run normal GPA2Δ cells. However, in the second run, the RFA of cells containing the GFP tag was lower than both runs of normal GPA2Δ cells.

**Figure 7. Effect of Addition of GFP Plasmid on Pheromone Pathway in GPA2Δ Cells**

**Heat Shock Assay**

The modification of the heat shock assay was used to test the activity of GPR1 in the mutated yeast cells. The size and density of the colonies reflect the number of starting cells. If the cells were plated directly without being depleted of glucose, they would have
grown less than cells containing glucose. When heat is added to the mixture, it diminishes growth in wild type cells. However, as Xue and colleagues (2001) have shown, cells without GPR1 or Gpa2 are more resistant to heat shock and grow more than normal wild type cells. If the growth of wild type cells containing the mutation in GPR1 were more resistant to heat shock than wild type cells containing the normal sequence for GPR1, it would suggest that the mutation is a null mutation that diminishes the activity of the Gpr1 protein. On the other hand, if they grew the same amount as normal wild type cells, it would suggest that the mutation did not affect the activity of the Gpr1 protein.

As for the Gpa2 delete cells, if the mutations in the GPR1 gene decreased the amount of growth as compared to Gpa2 delete cells with a normal GPR1 sequence, the activity of the pathway might have been restored, causing them to act more like wild type cells. If the opposite were true, and the Gpa2 with the mutations grew the same amount or more than the normal Gpa2 delete cells, the activity of the pathway would not be restored.
As can be seen in both Figures 8A and B, wild type cells that had glucose added to them but were not heat shocked (Lane 3, Figure 8A) showed the most cell growth, both in the number of colonies and the density of those colonies present. The cells that were not given glucose, both the ones that were heat shocked and the ones that were not, (Lanes 1 and 2, Figure 8A) grew approximately the same amount. The cells that were heat...
shocked and had glucose added (Lane 4, Figure 8A) grew more than the ones without glucose, but less than the cells that had glucose added but were not heat shocked (Lane 3, Figure 8A).

<table>
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<tr>
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<td><strong>Heat Shock</strong></td>
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**Figure 9A**: Amount of growth for TM24 GPA2Δ cells. Rows represent serial 5-fold dilutions. [(+) represents the presence of heat shock or glucose; (-) represents the absence of heat shock or glucose.]

**B**: Amount of growth of GPA2Δ cells after being heat shocked. [(-,-)=no glucose, no heat shock; (+,-)=glucose, no heat shock; (-,+)=no glucose, heat shock; (+,+)=glucose, heat shock.]

In contrast, for GPA2Δ cells, the amount of growth was approximately the same for the positive control (Lane 4, Figure 9A), the negative control (Lane 1, Figure 9A), and the cells that had glucose added but were not heat shocked (Lane 2, Figure 9A). The cells that were heat shocked but did not receive glucose (Lane 3, Figure 9A) showed double the amount of growth of the other cells.
Figure 10A: Growth of wild type ΔW cells after heat shock. Six serial 5-fold dilutions are shown in rows of cells plated on MLT plates. [(+) denotes presence of glucose or heat shock; (-) denotes absence of heat shock or glucose.] B: Cell count and amount of growth of TM24 wild type ΔW cells after being heat shocked. Numerical values are based on the equation used by Freeman (2011). [(-,-)=no glucose, no heat shock; (+,-)=glucose, no heat shock; (-,+)=no glucose, heat shock; (+,+)=glucose, heat shock.]

It can be seen both from Figures 10A and B that the cells with the ΔW grew more after being heat shocked but not having glucose added (Lane 2, Figure 10A). The negative control (Lane 1, Figure 10A) had the second largest amount of growth, while the cells with only glucose added (Lane 3, Figure 10A) and the cells that had both glucose and heat added grew the same amount (Lane 4, Figure 10A).
After measuring the effects of heat shock and glucose on normal wild type and wild type cells with a deletion of the tryptophan at position 634 of the gpr1 gene, the effects of heat shock on cells with a C-terminus truncation of the gpr1 gene were also measured. The amount of growth for the TM24 wild type cells with the deletion of amino acids 20-25 of the GPR1 gene that had glucose added to them but were not heat shocked (Lane 3, Figure 11A) showed a slightly higher growth than those that were not heat shocked and did not have glucose added (Lane 1, Figure 11A). The cells that were heat shocked without glucose

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<td>1</td>
<td>WT</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>WT Δ20-25</td>
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<td>+</td>
</tr>
<tr>
<td>3</td>
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<tr>
<td>4</td>
<td>WT Δ20-25</td>
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**Figure 11A:** Growth of wild type cells with deletion of amino acids 20-25 of GPR1 gene after heat shock and glucose addition. Eight serial 5-fold dilutions are represented by rows of cells. ([+] denotes presence of glucose or heat shock; [-] denotes absence of glucose or heat shock.)

**B:** Effect of heat shock on TM24 wild type Δ20-25 cells. The values shown are the amount of growth of colonies based on the Freeman Scale (2011). [(-,-)=no heat shock, no glucose; (+,-)=glucose, no heat shock; (-,+)=no glucose, heat shock; (+,+)=glucose, heat shock.]
(Lane 2, Figure 11A) showed the smallest amount of growth, and the cells that were subjected to both (Lane 4, Figure 11A) had a slightly larger growth than the former (Lane 2, Figure 11A), but a lesser amount of growth than either of the cell types that were negative (Lane 1 and Lane 3, Figure 11A) for the heat shock.

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</table>

Figure 12A: Amount of growth of GPA2Δ Δ15-20 cells after being shocked. Seven serial dilutions were plated on MLT plates. (Some dilutions showed no growth as opposed to the next dilution. This could be due to pipetting errors). [(+) denotes presence of glucose or heat shock; (-) denotes absence of glucose or heat shock.]

B: Amount of growth in units based on the Freeman Scale of GPA2Δ Δ15-20 cells after being heat shocked. [(−,−)=no glucose, no heat shock; (+,−)=glucose, no heat shock; (−,+)=no glucose, heat shock; (+,+)=glucose, heat shock.]

As seen in Figures 12A and B, the GPA2Δ Δ15-20 cells that had glucose added but were not heat shocked (Lane 3, Figure 12A) grew the most of any cells. The cells that were heat shocked without having glucose added to them (Lane 2, Figure 12A) showed the least amount of growth, while the positive (Lane 4, Figure 12A) and negative (Lane 1, Figure
controls showed approximately the same amount of growth, between the two extremes.

<table>
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<tr>
<td>4</td>
<td>Gpa2Δ Δ20-25</td>
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Figure 13A: Amount of growth of GPA2Δ Δ20-25 cells after being exposed to glucose and heat shock. Seven 5-fold serial dilutions were performed and plated on MLT plates. Dilutions are represented by rows of cell colonies. [(+) denotes presence of glucose or heat shock; (-) denotes absence of heat shock or glucose.]

B: Effect of heat shock on GPA2Δ Δ20-25 cells. Amount of growth is given in units of the Freeman Scale (2011). [(-,-)=no glucose, no heat shock; (+,-)=glucose, no heat shock; (-,+)=no glucose, heat shock; (+,+)=glucose, heat shock.]

In Figures 13A and B the effects of heat shock on GPA2Δ Δ20-25 cells can be seen. The cells that were exposed to neither glucose nor heat (Lane 1, Figure 13A) showed the largest amount of growth, while those cells that were exposed to both variables (Lane 4, Figure 13A) showed the least amount of growth. Both the cells that were negative for heat shock but positive for glucose (Lane 3, Figure 13A) and the cells that were positive for heat
shock but negative for glucose (Lane 2, Figure 13A) showed the same amount of growth, giving a value of sixty according to the Freeman Scale (2011).

Results Overview

The wild type cells with a deleted tryptophan but were neither heat shocked nor had glucose added to them (Lane 1, Figure 10A) grew the same amount as normal wild type cells. The ones that were subjected to heat shock but no glucose grew more than normal wild type cells (Lane 1, Figure 8A). The mutant cells that had glucose added but were not heat shocked (Lane 3, Figure 10A) grew significantly less than the normal cells (Lane 3, Figure 8A). When subjected to both heat shock and glucose (Lane 4, Figure 10A), the cells with the tryptophan deletion grew less than normal wild type cells (Lane 4, Figure 8A).

Wild type cells with deletions of amino acids 20-25 of the Gpr1 (Figure 11A and B) gene showed different responses than those with the deleted tryptophan. The negative control cells (Lane 1, Figure 11A) grew more than normal wild type cells (Lane 1, Figure 8A). The mutants that were heat shocked but did not have glucose added (Lane 2, Figure 11A) also showed more growth that normal wild type cells (Lane 2, Figure 8A). The mutants with glucose but no heat shock (Lane 3, Figure 11A) grew less than normal cells (Lane 3, Figure 8A), and the mutants that were subjected to both glucose and heat shock (Lane 4, Figure 11A) grew more than normal (Lane 4, Figure 8A).

Gpa2Δ cells with deletions of amino acids 15-20 of the Gpr1 gene that were neither heat shocked nor had glucose added (Lane 1, Figure 12A) grew more than normal Gpa2Δ cells (Lane 1, Figure 9A). The mutants that were shocked with heat but not glucose (Lane 2, Figure 12A) grew less than normal (Lane 2, Figure 9A). The mutants with glucose added that were not shocked (Lane 3, Figure 12A) grew the same amount as normal (Lane 3,
Figure 9A). The mutants that were shocked with both heat and glucose (Lane 4, Figure 12A) grew more than normal Gpa2Δ cells (Lane 4, Figure 9A).

The deletion of Gpr1 amino acids 20-25 had different effects on cell growth than deletion of amino acids 15-20. The mutants with a deletion of amino acids 20-25 that were also negative controls (Lane 1, Figure 13A), showed more growth than normal Gpa2Δ cells (Lane 1, Figure 9A), and the same amount of growth as the Gpa2Δ cells with the deletion of amino acids 15-20 (Lane 1, Figure 12A). However, for the cells that were heat shocked but did not have glucose added (Lane 2, Figure 13A) the deletion of amino acids 20-25 yielded more growth than both normal Gpa2Δ cells (Lane 2, Figure 9A) and Gpa2Δ cells with the deletion of Gpr1 amino acids 20-25 (Lane 2, Figure 12A). The mutants with glucose added but no heat shock (Lane 3, Figure 13A) grew less than both previously mentioned sets of cells (Lane 3, Figure 9 and Lane 3, Figure 13A). Lastly, the cells positive for both heat shock and glucose (Lane 4, Figure 13A) grew less than both Gpa2Δ cells (Lane 4, Figure 9A) and Gpa2Δ with deletion of amino acids 15-20 (Lane 4, Figure 12A).
Discussion

When exposed to elevated temperatures, cells activate heat shock proteins as well as stress response elements that increase cell proliferation. Since proteins become denatured at higher temperatures, the proteins become dysfunctional. The role of the heat shock proteins and stress response elements is to continue growth of the cells despite the denaturing of proteins.

In order to test the effects of the mutations of the GPR1 gene, a heat shock assay was done to test the response of cells to heat. When gpr1 deleted cells are heat shocked, heat shock proteins are activated and the cells show an increased growth when compared to wild type cells. When the cells with the mutant GPR1 gene were exposed to heat shock, if the mutations yielded a decrease in function of the GPR1 gene, the cells would be expected to show an increased growth when heat shocked due to the activation of heat shock proteins. This effect was observed in the deletion of the tryptophan at position 634. When heat shocked, the cells that lacked the tryptophan grew more than wild type cells. This suggests that the deletion of the tryptophan caused a decrease in functionality of the GPR1 gene, causing it to respond more like gpr1 delete cells. This decrease in functionality was also shown by the Fus1/lacZ assay. The cells with the deleted tryptophan showed a decrease in the production of the β-galactosidase enzyme as evident by the lower relative fluorescence compared to the relative fluorescence of wild type cells.

However, when the wild type TM24 cells with a truncation of amino acids 20-25 were heat shocked, they showed less growth than when they were not heat shocked. This behavior is like wild type cells rather than GPR1 delete cells.
By viewing these results, it can be concluded that the deletion of these amino acids has a minimal effect on the function of the Gpr1 protein. These mutations do not render the protein completely dysfunctional. When the function of the Gpr1 protein was analyzed by the Fus1/lacZ assay, the truncation of these amino acids showed a minimal effect on the function of the protein. The mutated cells showed lower fluorescence than wild type cells, but more fluorescence than cells with other mutations, again suggesting that the deletion of amino acids 20-25 does not have as large an effect on the Gpr1 protein.

After analyzing the results relevant to gpa2Δ cells, inconsistency was found in the fluorescence of runs 1 and 2, probably due to a mishap in procedure. Thus, based on the fact that run 1 values fall far higher than the standard deviation of all other cells tested, the comparative discussion is best directed toward run 2. Experimentation where GPR1 truncation occurred led to a vast array of results. In gpa2Δ 10-15 and 15-20, a consistent increase in RFA was found, and it can be inferred that the pheromone pathway experienced a positive mutation that helped restore functionality. Conversely, gpa2Δ 20-25 displayed a similar and slightly lower RFA than the control, meaning the mutation was not beneficial to functionality of the pathway and possibly deleterious to function. As can be seen from Figure 5, no real implications follow the deletion of amino acids in regard to the pheromone pathway of gpa2Δ cells. Since functionality was seen both above and below the RFA value of the control in each amino acid deletion, the results are inconclusive.

In the heat shock assay, GPA2Δ cells were compared to other cells that experienced truncation or amino acid deletion. In both GPAΔ 15-20 cells, it is evident that the addition of glucose or heat shock did have an effect on growth and, thus, functionality of the pathway. When neither glucose nor heat shock was administered, the cells grew more than
the control. This implies that the pathway experienced a decrease in functionality. In the presence of glucose, we noticed that functionality of the pathway remained lower in relation to the control, despite the addition of heat shock. The decreased growth of cells in a heat shock only experiment can lead to the conclusion that heat shock is directly related to the increase in functionality of the pathway. Possible explanations include the idea that heat shock proteins are activated and facilitate the reconstruction of mutations and truncations in cells to benefit the functionality of the pathway.

Likewise, from the GPA2Δ 20-25 cells’ results, a similar emphasis on the positive impact of heat shock on the functionality of the pathway can be seen. Here the negative control and addition of glucose only yielded results representative of decreased pathway function. These results suggest that neither the mutation nor the addition of glucose was beneficial. On the other hand, both experiments where heat shock was administered revealed an increase in functionality of the pathway. Thus, the heat shock caused the cell to react in some way that reversed the effects of the truncations of the C-terminus. Finally, when comparing the GPA2Δ cells to wild type TM24 cells without truncation, we notice that glucose has a negative effect on pathway functionality for wild type cells, yet no effect on the GPA2Δ cells. Similarly, heat shock seems to produce a negative effect on pathway functionality in non-truncated GPA2Δ cells, yet has no effect on wild type cells. This leads to the conclusion that heat shock is only directly beneficial to the cell when a truncation is present and may give insight into the function heat shock proteins have when activated. When no truncation was present, the excessive temperature was simply a hindering factor toward pathway functionality because it put stress on the cell, and the resulting actions the cell took were not relevant to increasing pathway functionality.
One other way of interpreting the results includes comparison of both assays. The most obvious conclusion that can be drawn from the results is that amino acid deletion does not seem to show relevance to functionality of the pheromone pathway. Also, the truncation of GPA2Δ 15-20 must either prove beneficial or ineffective in relation to the pheromone pathway’s functionality. The truncation of GPA2Δ 20-25 was found to be harmful to the functionality of the pheromone pathway. One consistency can be seen: the fact that heat shock seems to improve functionality in the presence of truncation.

The mutant cells reacted in various ways to the heat shock and glucose. While some mutants seemed unchanged by heat shock or glucose, still others grew significantly more or less than the non-mutated cells. This assay was only done once. Therefore, in order to attain more accurate results, more assays should be run and the results averaged. This would give more accurate results to determine the effects of each specific mutation of Gpr1 on the resistance to heat shock. Once sequencing has been completed, the mutations can be verified.
Acknowledgements

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Works Cited


