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Characterization of a Novel Clade of Transporters in *Phytophthora*

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Abstract

The oomycete *Phytophthora parasitica* has a worldwide distribution and is an economically important pathogen of more than 100 species[4]. RNA-seq analysis showed that one gene, PPTG_16698 has the 5th highest level of expression of all transport proteins in the zoospore stage, and is highly conserved throughout *Phytophthora* species. This project attempts to characterize the important biological role that PPTG_16698 plays in *P. parasitica* and other oomycetes. Three strategies have been implemented to accomplish this goal: growth analysis by heterologous expression in yeast, metabolite analysis in yeast, and construction of a GFP fusion protein to enable localization of the gene in oomycete hyphae by confocal microscopy.

Confocal microscopy is expected to confirm the vacuolar localization of this gene. If this gene is localized to a vacuolar membrane, then heterologous expression in yeast should result in differential accumulation of metabolites mobilized by this transporter. In preliminary growth assays, expression of this gene did not inhibit the growth of yeast. Therefore, expression of the gene does not result in sequestering of a growth-limiting metabolite. To determine whether expression of the transporter results in the accumulation of polyamines, which are organic compounds necessary for growth in eukaryotes, polyamine levels will be measured by dansylation of amines and separation by HPLC. Other metabolites will be assayed by liquid chromatography mass spectroscopy analysis.

Introduction

Phytophthora are a genus of water molds that reproduce both sexually and asexually, as do all organisms from the phylum Oomycota[4]. The thallus, or

undifferentiated tissue at the body of the organism is called mycelium. The mycelium contains hyphae, at which growth begins[4]. Under moist and warm conditions, *Phytophthora* produce zoosporangia. These asexual spores are diploid and biflagellate[4]. After swimming for an arbitrary amount of time, the zoospores will encounter a solid barrier to their movement and encyst. The encystment is rapid and is characterized by the formation of a cell wall and the shedding of both flagella. The cysts are the last stage in the asexual reproduction of *Phytophthora*[4].

The root of the word *Phytophthora* comes from the Greek words *phyto*, meaning plant, and *phthora*, meaning destroyer[4]; and these organisms do just that. Although hyphae generally are the main structure involved in fungal and oomycete disease because of their growth pattern, other studies have suggested that the zoospores traveling through soil and water are the main mode of dispersion for *Phytophthora* to infect plants[3].

One species, *Phytophthora parasitica,* has been shown to cause root disease and leaf wilting in an enormous variety of crops and flowering plants worldwide[4]. It is therefore of economic importance to the farming industry. A whole genome shotgun sequence of *Phytophthora parasitica* was released by the Broad Institute in 2011, and since then, researchers have begun working diligently to characterize those genes with hopes of potential application in anti-pathogenic measures[7]. One gene in *P. parasitica*, PPTG_16998, was selected for study for a variety of reasons. This gene is notable because it has high levels of sequence conservation in various species of *Phytophthora*, as shown in Figure 1. However, this gene is not found outside of *Phytophthora*, suggesting its important biological function is specific to the genus.
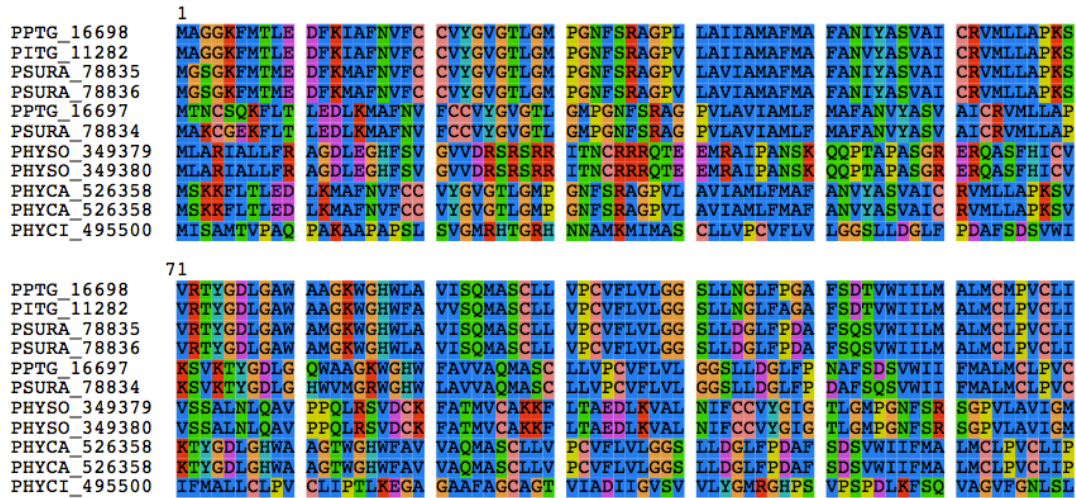
Figure 1. SeaView^{TM} alignment of similar genes in *Phytophthora* species based on FungiDB^{TM} BLAST hits. The letters correspond to amino acids, and the colors indicate the type of amino acid. This shows PPTG_16698 to be highly conserved amongst *Phytophthora* species. Selection pressure has not significantly altered this gene's sequence, which indicates that it has an important biological function.

In addition, in the free-living swimming zoospore stage of this pathogen, PPTG_16998 is predicted to be the membrane transporter gene with the 5th highest level of expression based on RNA-Sequencing data, as shown in Figure 2. This data furthers suggests its significance in the functioning of the organism, as well as its involvement in the transport of some metabolite.

Locus	Description	Zoospore expression (Log 2 transformed data)
PPTG_1857	ABC transporter family	9.12
PPTG_18151	ABC transporter family	6.79
PPTG_10722	ABC transporter family	6.74
PPTG_14680	NH3 transporter	6.28
PPTG_16998	AAA family Unknown function	6.17
PPTG12815	Drug /metabolite transporter family	6.06

Figure 2. RNA-Seq data shows PPTG_16698 to be the transporter with the 5th highest level of expression in the zoospore stage.

RNA-Seq data also shows that this gene is in a superfamily of amino acid and/or polyamine transporters. Polyamines, such as putrescine, spermidine, and spermine are aliphatic organic compounds that are necessary for life in the eukaryotic cell[9]. They play a vital role in many cellular processes, such as cell division and protein synthesis[9]. Dr. Morris's lab has previously shown that the zoospore stage of this organism only transports polyamines[2], and since it is highly expressed in the zoospore stage, it is thus predicted to be a polyamine transporter. This transporter will likely play a role in maintaining homeostasis regarding polyamine levels in the cell.

Three strategies have been implemented to accomplish the goal of characterizing this gene: growth analysis by heterologous expression in yeast, metabolite analysis in yeast, and construction of a green fluorescent protein fusion to enable localization of the gene in oomycete hyphae by confocal microscopy. Growth analysis and metabolite analysis involves heterologous expression in a wild type yeast strain, BY4741, of

PPTG_16698. By inserting the gene into competent yeast cells via an *E. coli* plasmid, any natural regulation mechanisms found in *P. parasitica* cells are not involved in the gene expression, and it is thus expressed constitutively. Any differences in growth and metabolics between the wild type strain and transformant strain are expected to be a result of the transport or sequestering of exogenous materials, such as polyamines. Confocal microscopy is expected to confirm the vacuolar localization of this gene. If this gene is localized to a vacuolar membrane, then heterologous expression in yeast should result in differential accumulation of metabolites mobilized by this transporter.

Part 1: Growth analysis by heterologous expression in yeast

Design: The purpose of this assay is to determine any phenotypic growth difference between wild type yeast strain BY4741 and transformant strain of PPTG_16698 in BY4741. In this case, BY4741 is being interpreted as the control strain. This will be done by transforming PPTG_16698 into BY4741, and measuring growth rates of the two strains of cells under varying conditions by checking the absorbance over 20 hours.

Materials and Methods

Preparation of cells and transformation:

First, the nucleotide sequence of this gene was codon-optimized for expression in yeast and inserted into the pYES-DEST52 vector by GenScript[TM]. The pYES-DEST52 vector was inserted into *Escherichia coli* and contains several important counterparts: a GAL1 promoter so that it is only expressed in the presence of galactose; a selectable marker for the biosynthesis of uracil; and a selectable marker for ampicillin resistance[10].
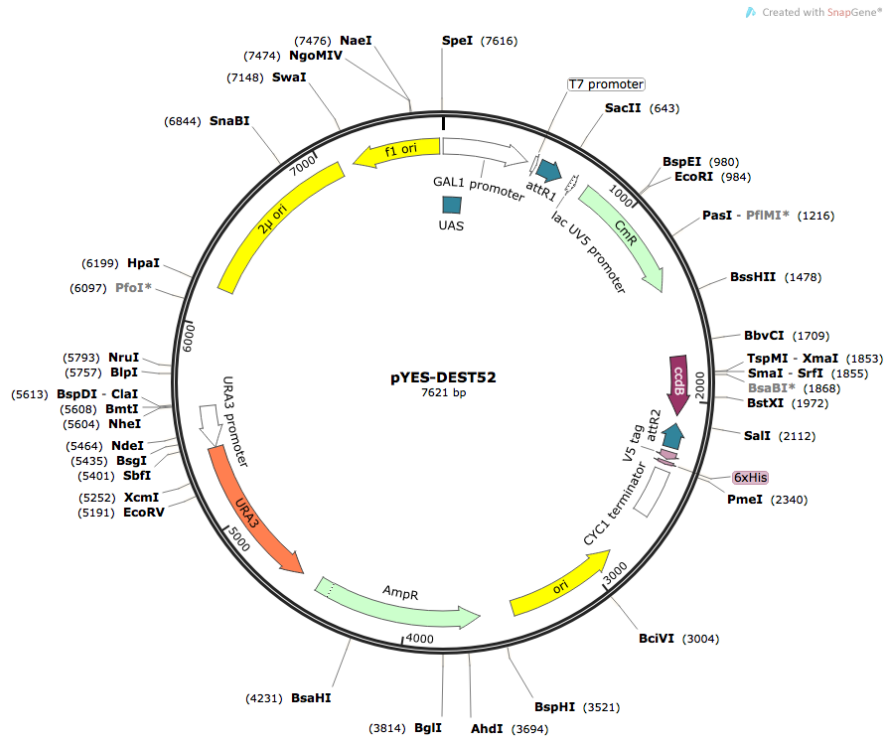
Figure 3. pYES-DEST52 vector containing a GAL1 promoter, URA3 promoter, and AmpR ampicillin resistance sequence[10].

Then, the gene was transformed into a wild type yeast strain, BY4741, which is derived from *Saccharomyces cerevisiae* strain S288C[6]. To complete the transformation, the plasmid *E. coli* cells were grown on Luria broth agar plates with 1 micromolar concentration of ampicillin and incubated overnight in a 37°C chamber. The presence of ampicillin selects against any contamination of outside bacteria cells. Then, a single colony of the selected cells was grown overnight in a flask with liquid Luria broth and 1 micromolar concentration of ampicillin in a shaking 37°C incubator. The plasmid DNA was purified from the liquid culture of cells using the AxyPrep Plasmid Miniprep Kit[TM], following the manufacturer's instructions. This was confirmed by verifying for the DNA concentration and absorbance ratio using the NanoDrop 2000[TM].

Meanwhile, BY4741 yeast cells were grown on yeast peptone dextrose agar plates overnight in a 30°C incubator. A new plate was streaked from a single colony of the original plate. They were made competent by growing to an OD600 of 1.2, spinning them down, and washing the pellet with cold water twice and cold 1M sorbitol twice. 50 μL of these competent yeast cells were mixed with 5 μL of the prepared plasmid.

Then, using the Bio-Rad Gene Pulser Xcell^{TM}, an electrical field of 1500 volts was applied to increase the permeability of the cell membrane so that exogenous plasmid DNA could be inserted into the yeast cell. The cells were treated immediately with a 1:1 ratio of cold yeast peptone dextrose and sorbitol, and allowed to incubate for one hour while shaking at 37°C to recover. Next, they were plated on complete synthetic media with the absence of uracil and allowed to grow overnight in a 30°C incubator. Only the cells that had taken up the plasmid with the gene for the biosynthesis of uracil were capable of growth. The resulting selected cells were streaked for single colony isolates on fresh CSM-uracil plates.

Growth comparison assay:

Twelve single colony isolates of the transformant cells and twelve isolates of the wild type cells were grown for 20 hours in a shaking incubator at 30°C in test tubes containing 1 mL of CSM + 10% glucose. A 10 μL aliquot of each sample was placed in fresh tubes containing 1 mL of CSM + 10% glucose and allowed to grow for 20 hours at 30°C in a shaking incubator. Then, the samples were collected, diluted to roughly 0.200 at OD600, and placed in a Costar^{TM} 24-well plate. Four samples of each were placed in separate wells with 1 mL of CSM + 10% glucose. Another four samples of each were

placed in separate wells with 1 mL of CSM + 10% galactose. The final four samples of each were placed in separate wells with 1 mL of CSM + 10% galactose + 100 μL of 1 millimolar concentration of putrescine. These were allowed to grow in an incubator for twenty hours at 30°C with light shaking, and absorbance reads were collected every 60 minutes.

Results

Because of the GAL1 enhancer in the pDEST-YES52 plasmid, PPTG_16698 is only turned on in the presence of galactose. Correspondingly, there was no significant difference in growth between the wild type and transformant cells that were grown in 1 mL of CSM + 10% glucose. There was also no significant difference in growth between the samples that were grown in 1 mL of CSM + 10% galactose, despite the gene being overexpressed in the transformant strain. Finally, there was also no significant difference in growth between the two strains that were grown in 1 mL of CSM + galactose + 100uL of 1 millimolar putrescine.
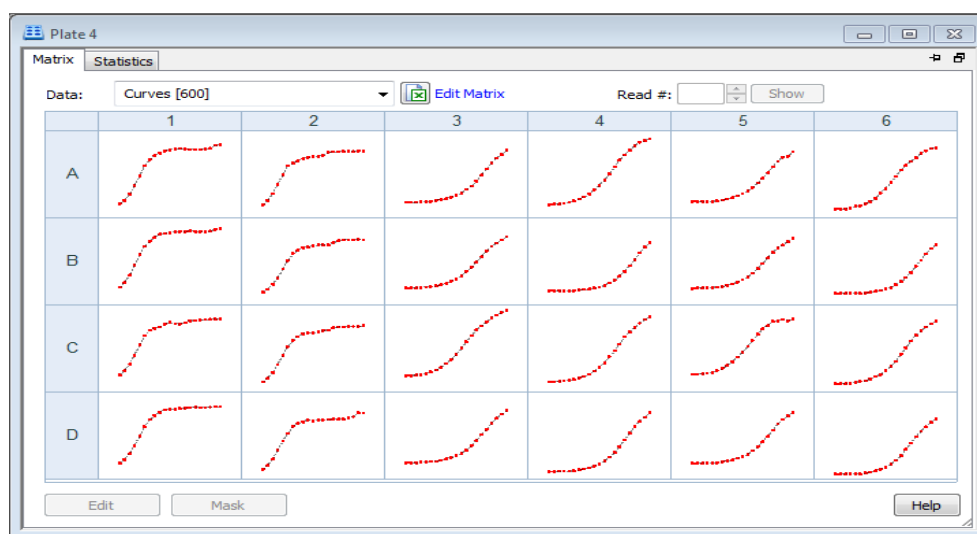
Figure 4. Growth curves for heterologous expression in yeast show no significant difference in growth. Column 1: WT isolates in CSM + Glu. Column 2: Tr isolates in CSM + Glu. Column 3: WT isolates in CSM + Gal. Column 4: Tr isolates in CSM + Gal. Column 5: WT isolates in CSM + Gal + putrescine. Column 6: Tr isolates in CSM + Gal + putrescine
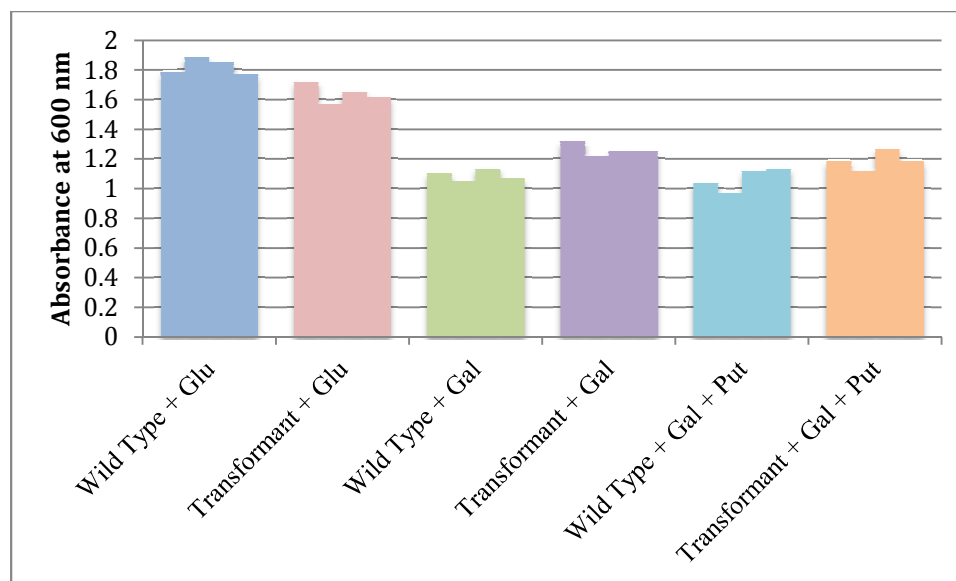

Figure 5. Recorded absorbance of wild type and transformant strains in glucose, galactose, or galactose and putrescine after 20 hours of growth.

Part 2: Metabolite analysis in yeast

Design: No obvious differences in growth due to overexpression of PPTG_16698 were observed in part one, but a difference in metabolites may still be observed in those cells. Menaka Ariyaratne from Dr. Paul Morris's lab developed a high performance liquid chromatography method for quantifying polyamines- namely, putrescine, spermidine, and spermine. She followed the method described in Ariyaratne, (2014) which involves

dansylating the polyamines and separating them with the HPLC method of Smith et al., (1985) using a methanol solvent gradient.

Materials and Methods

Preparation of cells:

Samples from part one were prepared for HPLC analysis of the concentration of putrescine, spermidine, and spermine in the cells. Specifically, the samples of the strains in galactose were chosen as the control and in galactose plus putrescine were chosen as the test samples. Three samples of each were collected and centrifuged for two minutes at 8,000 x *g*. They were then resuspended in 1 mL of sterile distilled water, and spun again for two minutes at 7,000 x *g*. This washing step was repeated. They were then spun down and the water was removed before they were frozen with liquid nitrogen. Because the cells had the same final concentration after growth, they were not diluted and it can be assumed that the same number of cells were in each sample.

HPLC analysis:

Ariyaratne obtained data from the cells via her protocol[1].

Results

The results showed that when grown in the presence of CSM and galactose, the wild type and transformant cells had either approximately the same concentration of polyamines, or the wild type had an increased concentration. For putrescine, average wild type levels were 2.4 μM, and transformant levels were also 2.4 μM. For spermidine, wild

type levels were 16.8 μM compared to transformant levels of 8.5 μM. For spermine, wild type levels were 3.9 μM compared to transformant levels of 1.8 μM.

However, levels were significantly altered when the cells were grown in the presence of exogenous putrescine. For putrescine, the average wild-type levels were 1.4 μM, and the transformant levels were 4.1 μM. For spermidine, the average wild type levels 7.4 μM, while transformant levels were 10.8 μM. For spermine, the average wild-type levels were 1.6 μM, while transformant levels were 2.4 μM.
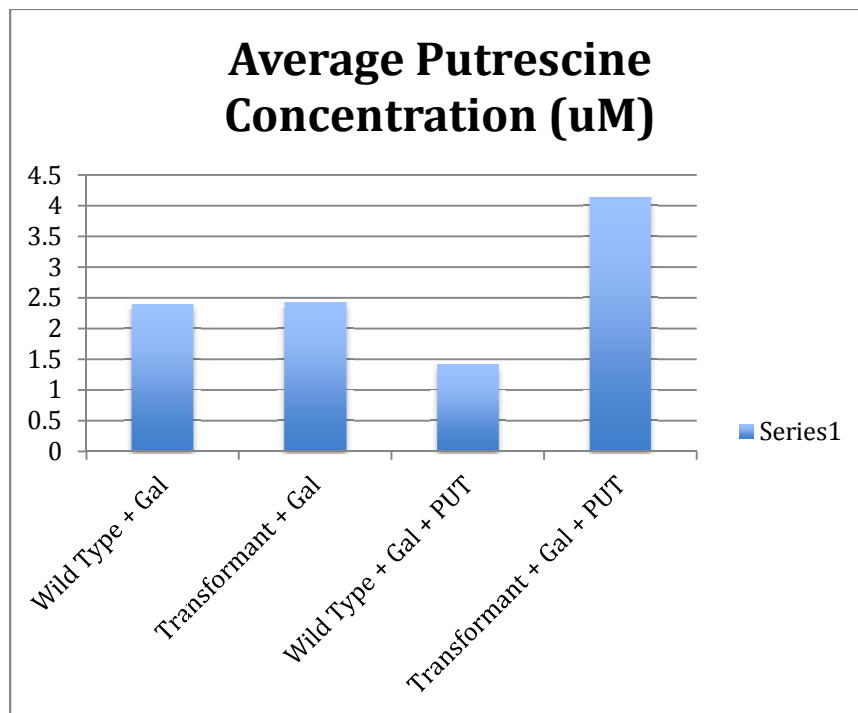

Figure 6. Average putrescine concentrations in in wild type cells and transformant cells in the presence of galactose, or galactose and putrescine.
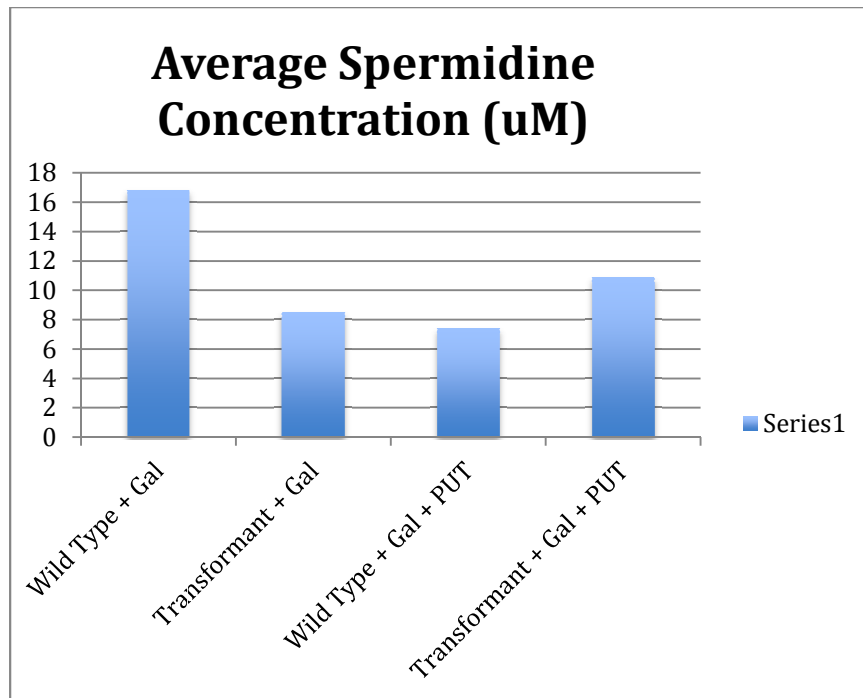
Figure 7. Average spermidine concentrations in in wild type cells and transformant cells in the presence of galactose, or galactose and putrescine.
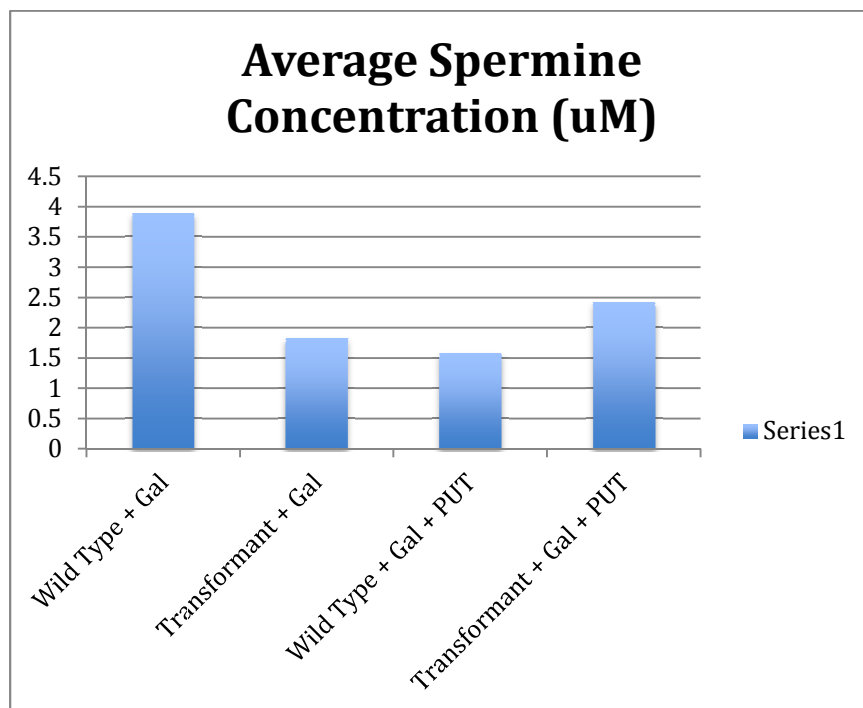
Figure 8. Average spermine concentrations in in wild type cells and transformant cells in the presence of galactose, or galactose and putrescine.

Part 3: Localization of the gene in oomycete hyphae

Design: The gene PPTG_16698 was amplified using a nested polymerase chain reaction, cloned with a green fluorescent protein plasmid, verified for no mutations, and transformed into *Phytophthora sojae* hyphae. It was then viewed under a confocal microscope to determine the localization of the protein.

Materials and Methods

Polymerase Chain Reaction:

A nested PCR was utilized to reduce non-specific binding of DNA sequences. The PCR was aimed at amplifying the PPTG_16698 sequence from the plasmid preparation in part 2. In the first reaction, a gradient PCR was used with annealing temperatures from 58°C to 62°C for 30 cycles at 30 seconds each. There was a 72°C 1 minute and 30 second extension time followed by a final extension at 72°C for 10 minutes. A 1% agarose electrophoresis gel was run, and the strongest band was identified. Then, the PCR product that produced that band was used as template in the second PCR reaction.
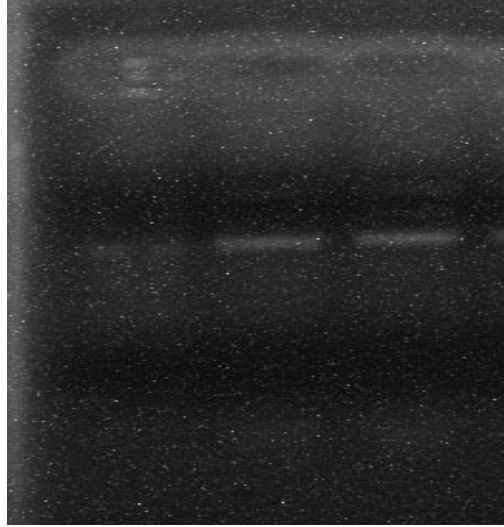
Figure 9. Gel electrophoresis results with correct band size of PPTG_16698.

The primers used in the second PCR, as well as the perimeters for the reaction, is still under design. A 1% agarose electrophoresis gel will run with approximately 100 μL of product, and the correct bands will cleaned using the NucleoSpin Gel and PCR Clean-up kit[TM], following the manufacturer's instructions. Concentration of DNA and the correct absorbance ratio in the gel clean product will be verified using the NanoDrop 2000.

Cloning of pGFPN and PPTG_16698 through homologous recombination:

The plasmid utilized for cloning is the pGFPN plasmid, provided by the Judelson lab[5]. This *E. coli* plasmid has a gene for a green fluorescent protein, an NheI restriction endonuclease cut site at the N-terminus, a neomycin phototransferase marker, and a gene for ampicillin resistance[5]. Andrew Wagner used the AxyPrep Plasmid Miniprep Kit[TM] to prepare the plasmid, following the manufacturer's instructions. Then, a restriction digest using NheI and 10x NEBuffer from New England Biolabs[TM] was conducted following

the manufacturer's instructions. The cut plasmid was run on a 1% agarose electrophoresis gel, and the band was cut and cleaned using the NucleoSpin Gel and PCR Clean-up Kit[TM], following manufacturer's instructions. Concentration of DNA and the correct absorbance ratio in the gel clean product was verified using the NanoDrop2000[TM].
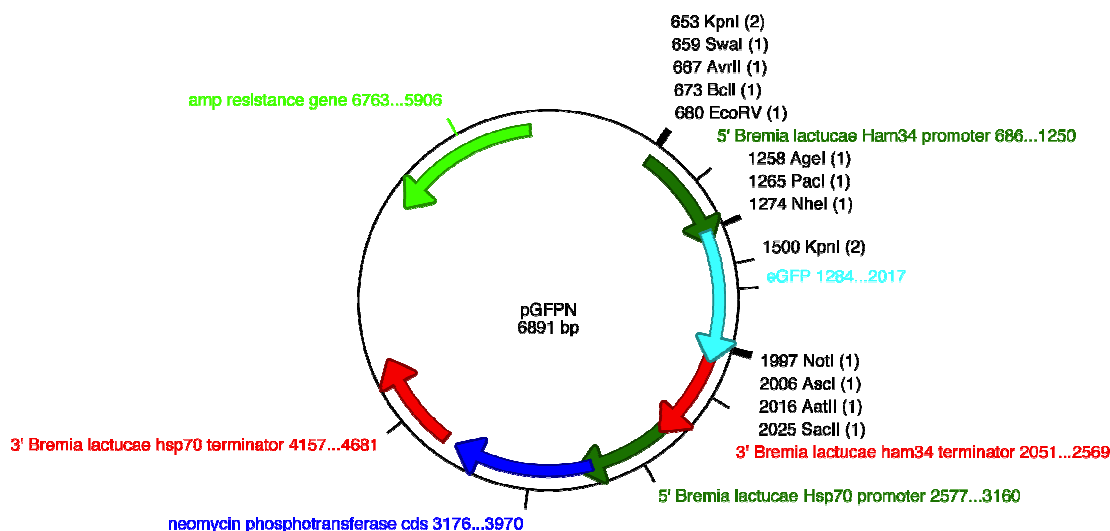

Figure 10. pGFPN plasmid with AmpR ampicillin resistance gene, NheI restriction site, and green fluorescent protein gene[5].

Next, a clonase reaction was set up with the cut and cleaned plasmid, and the cleaned PPTG_16698 PCR product. This was completed using the Clontech Infusion Kit[TM], following the manufacturer's instructions.

Transformation into E. coli*:*

The Morris lab prepared top 10 *E. coli* competent cells previously. 2 µL of the plasmid and insert from the clonase reaction were added to 50 µL of top 10 competent cells. The cells were electroporated using the Bio-Rad Gene Pulser Xcell[TM] with an electrical field of 1800 volts. The cells were allowed to recover by adding cold Super

Optimal Broth media, and then incubating for one hour while shaking at 37°C. The cells

were plated on LB + ampicillin plates. Single colony isolates were selected for and

streaked onto new LB + ampicillin plates. It will be verified that the cells contain the

insert by running the same nested PCR reaction as described previously. The band will be

cut out and cleaned using the NucleoSpin Gel and PCR Clean-up kit[TM], following the

manufacturer's instructions. Then, the product will be sequenced to verify that no

mutations have occurred in these processes.

Transformation into P. sojae*:*

By transforming the green florescent tagged plasmid and PPTG_16698 insert into

another *Phytophthora* species, *P. sojae*, the gene will still be overexpressed, but the

species are similar enough that protein will localize in the same location as it would in *P.*

parasitica. First, the *E. coli* plasmid will be grown and prepared following the same

methods as previously described for preparing the pYES-DEST52 vector. Then,

electroporation at 1500 volts with the Bio-Rad Gene Pulser Xcell[TM] will transform the

plasmid and insert into prepared P. sojae cells, following the same protocol as previously

described for the electroporation of pYES-DEST52 into competent BY4741 cells. The

transformant cells will be selected for on CSM + glucose plates in the absence of uracil.

Viewing under confocal microscope:

Live *P. sojae* cells will be mounted on a slide and viewed under the confocal

scanning light microscope at the BGSU Center for Microscopy and Microanalysis. The

vacuole can be identified as a circle on the image, and the protein will be visible due to the green fluorescent tag. No extra staining will be required to obtain this image.

Discussion

Heterologous yeast expression indicates that while the transformant cells do not uptake a growth-limiting metabolite, they may still be involved in the uptake of polyamines. The HPLC results show that wild-type cells on average have higher levels of the polyamines spermine and spermidine than transformant cells do when both are expressed in the presence of galactose. This difference is probably not significant, and may be due to the fact that wild type cells are lacking the plasmid that transformant cells have. However, the levels observed after the addition of exogenous putrescine are indeed significant. The results show a lower level of polyamines in wild-type cells than in transformant cells. Likely, the wild-type cells are reacting to the putrescine in the environment by either shutting off the mechanism for biosynthesis of polyamines, or the mechanism for uptake of exogenous polyamines. However, the overexpression of the gene prevents the transformant cells from reacting in that way. Because of this, it is likely that the gene is indeed involved in the transport of polyamines into the cell, although not at a level toxic enough to inhibit growth of the cell.

The missing piece of the puzzle in this project is the localization of the protein, which could not be completed in the time allotted. This portion of the project will continue in the coming months. If transient expression of this gene shows that the protein is localized to the vacuole of *P. sojae*, that data will support the evidence that was obtained in the HPLC analysis of polyamine levels.

Because *P. parasitica* is such a devastating pathogen to crops worldwide, any additional information about its functioning can have potential application to farmers. With the growing population and worldwide food crisis, it is unreasonable to think that enough food can be produced without genetic modification of some sort. Consumption of genetically modified crops has no scientific foundation that suggests it is dangerous to humans. Despite that, the stigma persists. It may be more effective for scientists to instead focus on the pathogens and how genetic modification of those can improve crop yields and decrease incidence of disease. Knowing that PPTG_16698 may be involved in the transport of polyamines, which are vital to many processes in cell growth and development, altering the gene to a certain amount could result in poor cell growth, and ultimately, less infection of host crops.

Acknowledgements

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