UNIVERSITY OF CALIFORNIA, SAN DIEGO

A Tool for Visualization of Recombinant Protein Expression in *Chlamydomonas reinhardtii* with a Self-Cleaving FMDV 2A Peptide

A Thesis submitted in partial satisfaction of the requirements

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in

Biology

by

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Professor Eric Allen

2017

The Thesis of Emily Noelle Schmidt is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Co-Chair

Chair

University of California, San Diego

2017

DEDICATION

This thesis is dedicated to everyone I've badgered for scientific and life advice, but most importantly a huge thanks to the largest ears of all: Koopa my Pembroke Welsh corgi.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

GFP—Green fluorescent protein

OFP—Orange fluorescent protein

FMDV—Foot and Mouth Disease Virus

CHO cell—Chinese hamster ovary cell

GRAS—generally regarded as safe

ER—endoplasmic reticulum

FACS—fluorescent activated cell sorting

UTR—Untranslated Region

ars1—arylsulfatase

PCR—polymerase chain reaction

TAP—algal growth media comprising Tris base, acetic acid, EDTA, and trace elements

LIST OF FIGURES

ACKNOWLEDGEMENTS

I would like to acknowledge Professor Stephen Mayfield and Doctor Anthony Berndt, as well as the members of the Mayfield lab for their mentorship and guidance throughout this project, as well as a special thank you to Joseph Ostrand for operating the FACS on my behalf.

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ABSTRACT OF THE THESIS

A Tool for Visualization of Recombinant Protein Expression in *Chlamydomonas reinhardtii* with a Self-Cleaving FMDV 2A Peptide

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Recombinant proteins are widely used for industrial purposes including manufacturing, pharmaceuticals, and beauty. Using microalgae as a biomanufacturing platform has piqued research interests due to their photosynthetic or heterotrophic capabilities and cellular machinery

that has the ability to correctly fold and secrete many recombinant proteins. However, several obstacles remain, such as low heterologous protein expression and a lack of strong expression and secretion vectors specific to microalgae. Here we made progress toward a molecular biology tool to help overcome these challenges by easing the quantification of a secreted recombinant protein. This was achieved by fusing the recombinant protein to a self-cleaving viral peptide from the Foot and Mouth Disease Virus (FMDV), a secretion peptide, and a GFP fluorescent marker. By establishing the ratio of the GFP to the protein of interest, a quantification can be obtained of the secreted recombinant protein of interest by measuring GFP fluorescence. This work presents the basis for a polycistronic tool that offers a quick method of quantifying recombinant protein in the microalga *Chlamydomonas reinhardtii* for biotechnological and industrial purposes.

Chapter 1: Introduction

1.1 Algae as a Biomanufacturing Platform

Recombinant proteins have greatly enhanced modern biotechnology and its practices by making manufacturing processes cheaper and more efficient, especially in the field of enzymes, therapeutic proteins, pharmaceuticals, and nutraceuticals (Kirk, Borchert, and Fuglsang 2002; Almaraz-Delgado et al. 2014). Recombinant proteins and enzymes widely used in industrial settings are expressed in organisms such as yeast, Chinese hamster ovary (CHO) cells, and bacteria, yet the resource inputs of sugar feedstocks and nutrient-dense media required for these organisms to produce at high yields is expensive (Rasala and Mayfield 2015). Yeast have the advantage of eukaryotic post-translational modification, but have been shown to hyper-glycosylate proteins and potentially cause an immune response in humans (Rasala and Mayfield 2015). CHO cells can express complex therapeutic proteins, but only at low levels (Rasala and Mayfield 2015; Corchero et al. 2013). Bacteria, especially the widely used *E. coli*, present challenges when used as an expression system for recombinant proteins due to high purification costs, optimal temperature requirements, complex protein folding complications (Rasala and Mayfield 2015), and possible cell toxicity for some recombinant proteins. Interestingly, microalgae such as the model alga *Chlamydomonas reinhardtii* has been shown to correctly fold and secrete complex proteins such as antibodies and human proteins that may not be able to be produced in prokaryotic organisms due to the specificity of their cellular machinery (Specht, Miyake-Stoner, and Mayfield 2010; Rasala and Mayfield 2015).

In addition to the laboratory benefits of using *Chlamydomonas reinhardtii* for photosynthetic expression, the model alga has received the denotation of "generally regarded as safe" (GRAS) for human consumption which reduces the need for downstream protein purification. Each product is evaluated on a case by case basis to receive GRAS status from

FDA's Center for Food Safety and Applied Nutrition (http://www.cfsan.fda.gov). This status has been given to many green algae species including but not limited to: *Chlorella*, *Scenedesmus*, *Dunaliella*, *Spirulina*, and *Schizochytrium*, and more recently *Chlamydomonas*. Due to these advantages, microalgae are a competitive alternative to classic systems and have considerable potential to be cheaper and more efficient for industrial use (Scranton et al. 2015; Rasala and Mayfield 2015).

1.2 Genetic Modification of *C. reinhardtii* for the Purpose of Protein Secretion

Optimization and engineering of the nuclear genome expression vectors could improve protein folding, stability, and accumulation in the cytosol, as well as greatly reduce the cost of production by secretion of the desired protein through the ER (Rasala et al. 2012; Rasala et al. 2014; Specht, Miyake-Stoner, and Mayfield 2010). It has been shown in *Chlamydomonas reinhardtii* that up to 95% of a protein of interest can be secreted into the culture media using an endogenous *ars1* secretion peptide, which could improve protein isolation techniques by avoiding costly centrifugation, cell lysis, and filtration, thus simplifying downstream processing (Rasala et al. 2012). Another benefit of *C. reinhardtii* is that it has the potential to be mated to introduce new alleles through independent assortment (Rasala et al. 2014) and has the ability of genetic modification by transformation in all three genomes: nuclear, chloroplast, and mitochondrial (Scranton et al. 2015; Rasala et al. 2012; Specht, Miyake-Stoner, and Mayfield 2010; Rasala and Mayfield 2015).

These genetic modification and protein secretion advantages are, however, accompanied by a few challenges. Low nuclear expression has been observed in *C. reinhardtii* and transformation location specificity of the construct in the nucleus is impeded by random integration (Rasala et al. 2012). Poor expression of nuclear encoded recombinant proteins has

previously been reported; though many solutions have been proposed to overcome low expression such as synthetic promoters, expression vectors, and transcriptional fusion techniques, challenges still persist (Rasala et al. 2012). During *Chlamydomonas* nuclear transformation, the construct randomly inserts into a location of the nuclear genome that leads to variable protein expression due to a positional effect in the genome (Scranton et al. 2015; Rasala and Mayfield 2015; Rasala et al. 2012). In this project, individual transformants were hypothesized to express differing levels of protein due to the positional effects (Scranton et al. 2015; Rasala and Mayfield 2015); however, the relative levels of the fused fluorescent protein would remain proportional.

Here we propose an expression tool to overcome the unpredictable transcriptional productivity and variability of random integration by screening with a transcriptionally fused fluorescent marker. The fluorescence allows us to isolate transgenic cells with preferable phenotypes (in this case high GFP expression) using high throughput screening techniques such as Fluorescent Activated Cell Sorting (FACS) and 96 well format fluorescence plate reader. Cells with high GFP expression are hypothesized to also have high levels of secondary protein (in this case xylanase or OFP) due to the transcriptional fusion. This new expression tool that can visualize real-time protein expression in live cells would allow microalgae to become an even better recombinant protein production platform for use in industry.

1.3 Co-electroporation

Regulatory rulings from the United States Department of Agriculture (USDA) limits the outdoor growth of plants with the transgene for antibiotic resistance. Similarly, the United States Environmental Protection Agency (EPA) limits outdoor use of antibiotics when making a product for market. In order to create an expression construct for outdoor photosynthetic growth,

the organism must not contain elements for antibiotic resistance. Antibiotic resistance from Hygromycin was intentionally not incorporated in-line with the construct, but rather incorporated separately into the genome using co-electroporation. This strategy was chosen with the intention of getting high yields of products, then using mating to cross out the resistance elements while preserving the gene. Further research needs to be done to accomplish these goals and create a market-ready algal strain.

1.4 A Tool to Quantify Protein Production

The aim of this project was to create a tool to visualize and quantify the expression of the recombinant protein xylanase using fluorescent proteins. Xylanase is a widely used enzyme in the paper, pulp, and food industries, and its applications are steadily increasing. Previously, the xylanase gene has been fused to antibiotic resistance and expressed and correctly folded in *Chlamydomonas reinhardtii* (Rasala et al. 2012). To achieve quantification of fluorescence relative to the amount of recombinant protein, the two genes were fused together by inserting between them the Foot and Mouth Disease Virus self-cleaving 2A peptide (Ryan, King, and Thomas 1991; Rasala et al. 2012). Previous experiments have used a self-cleaving 2A peptide mechanism from the FMDV1 and a marker, most commonly an antibiotic resistance marker, for selection (Scranton et al. 2015; Rasala et al. 2013; Franklin et al. 2002), but this FMDV 2A peptide has not been shown to be effective in C. reinhardtii when not associated to antibiotic resistance. To reduce or remove the need for linked antibiotic resistance, one possible way to visualize efficiency of protein expression is by transcriptionally fusing the recombinant xylanase to a reporter *Chlamydomonas* optimized GFP gene (Fuhrmann, Oertel, and Hegemann 1999) using the FMDV self-cleaving 2A peptide (Figure 1).

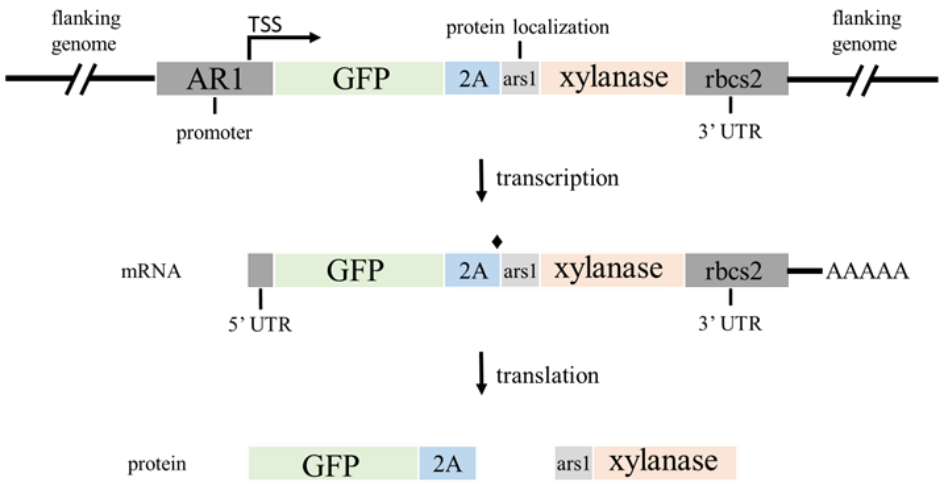

Figure 1: Expression cassette of secreted xylanase in the nuclear genome of C. reinhardtii that produces two proteins. Transcription of the construct is driven by the AR1 promoter, consisting of the HSP70A promoter and rbcs2 5' UTR. ♦ denotes the cleavage site of the 2A peptide during translation, yielding two protein products. ars1 designates an arylsulfatase presequence, which will allow for ER localization and extracellular secretion of xylanase.

The 2A peptide has a " self-cleaving" proteolytic processing mechanism which leaves the resulting protein product with one 3' proline tail as a result of the fusion and cleavage (Ryan, King, and Thomas 1991). In this experiment, the xylanase –a non-fluorescent enzyme—was transcriptionally fused to the fluorescent marker GFP via the 2A self-cleaving peptide. A second construct was created utilizing the fluorescent protein OFP which replaced the xylanase for ease of screening and quantification. An endogenous secretion peptide was also employed for ease of harvesting and purification in an industrial setting (Rasala and Mayfield 2015; Rasala et al. 2012) to make a final construct of AR1 promoter-GFP-2A-secretion peptide-xylanase-3' Untranslated Region (UTR) (Figure 1) or AR1 promoter-crGFP-2A-secretion peptide-OFP-3' UTR. Herein these constructs will be referred to as GFP-2A-Xylanase or GFP-2A-OFP and are assumed to have been co-electroporated into *Chlamydomonas reinhardtii* with the hygromycin resistance cassette unless otherwise noted.

Previous studies have been successfully conducted that employ the 2A peptide in *Chlamydomonas* for the purpose of expressing recombinant protein when fused to an antibiotic resistance marker (Rasala and Mayfield 2015; Scranton et al. 2015; Rasala et al. 2012; Rasala et al. 2014; Rasala et al. 2013), but the 2A peptide has not yet to our knowledge been used to fluorescently visualize a secondary protein in *Chlamydomonas*.

1.5 Ble-2A-GFP-2A-OFP

In order to overcome difficulties encountered during screening, an alternative construct was built which utilized a bleomycin resistance cassette fused to GFP, with the same 2A OFP 3'UTR following the GFP as seen in the hygromycin gene cassettes (Figure 2). A FLAG tag was added to OFP, as well as a 2 codon insertion at the C terminus of the 2A peptide to increase the cleavage efficiency (Kim et al. 2011). The bleomycin-driven expression had no need to be co-electroporated with hygromycin resistance, thereby eliminating the need to screen out colonies which received the Hyg gene but not the GFP-2A-OFP or GFP-2A-Xylanase construct.

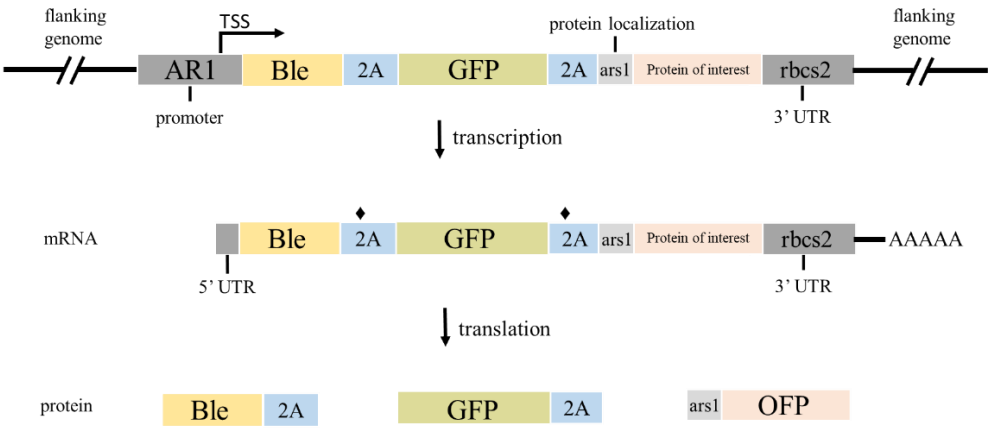

Figure 2: Expression cassette of polycistronic Ble-2A-linked secreted OFP in the nuclear genome of *C. reinhardtii*. Expression is driven by the AR1 promoter, followed by the gene for bleomycin resistance. ♦ denotes the cleavage site of the 2A peptides during translation, yielding three protein products.

A shortcoming of this construct was the long term viability of a marketable secreted protein product in which the organism contained an antibiotic resistance marker. This Ble-2A-linked expression construct does not currently qualify for EPA and USDA approval because of the antibiotic resistance, however it was made for the purpose of scientific exploration of the GFP transcriptional fusion construct design. Herein this construct will be referred to as “Ble-2A-GFP-2A-OFP”.

Chapter 2: Experimental Results and Discussion

2.1 Cloning

Genes for AR1 GFP, 2A OFP, Flag tagged xylanase 3'UTR and the secretion peptide *ars1* were PCR amplified from four existing lab plasmids with adapters for SLiCE cloning (Zhang, Werling, and Edelmann 2012). Restriction sites were not used due to the lack of compatible sites and need for multiple fragment assembly. Unfortunately, the SLiCE extract used was not of acceptable quality and failed to recombine the homologous DNA fragments to create a recombinant plasmid.

Splicing by Overlap Extension (SOE) PCR (Badalà, Nouri-mahdavi, and Raoof 2008) was the next method attempted to assemble the constructs. No colonies were successfully grown that contained the correct plasmid.

Ultimately, a Gibson Assembly kit was purchased and yielded gene positive colonies upon sequencing. A premature stop codon was found during sequencing, so Site Directed Mutagenesis (New England Biolabs) was used to delete the accessory three base pairs. Two constructs were made: AR1-GFP-2A-*ars1*-OFP-3'UTR and AR1-GFP-2A-*ars1*-Xylanase-FLAG-3'UTR, both in a pUC19 vector backbone (Figure 1). The resulting plasmids were sequenced and the construct was restriction digested with AatII restriction enzyme to linearize the plasmid for electroporation.

The gene for hygromycin resistance was PCR amplified from a lab stock containing the pBR2 plasmid. The PCR was screened on an agarose gel and column purified before electroporation.

2.2 Acquiring the Transformants

Hygromycin electroporation efficiency was calculated to be optimal at 300 ng of DNA which yielded the most desirable amount of colonies per electroporation. A total of 1 μg of construct and antibiotic resistance cassette DNA was used per $7.5*10^7$ cells electroporated in a cuvette.

From the co-electroporation, the GFP-2A-OFP construct was sorted on the FACS, greatly reducing the number of transformants which only received the gene for hygromycin resistance and not the gene cassette for fluorescence (Figure 3). Gating and execution of the flow cytometer was performed by Joseph Ostrand. Sorted populations were plated on hygromycin TAP agar, and 384 colonies were picked and grown in deep well plates for further screening. 21 out of 384 unique transformants were chosen for further study based on GFP data from the plate reader.

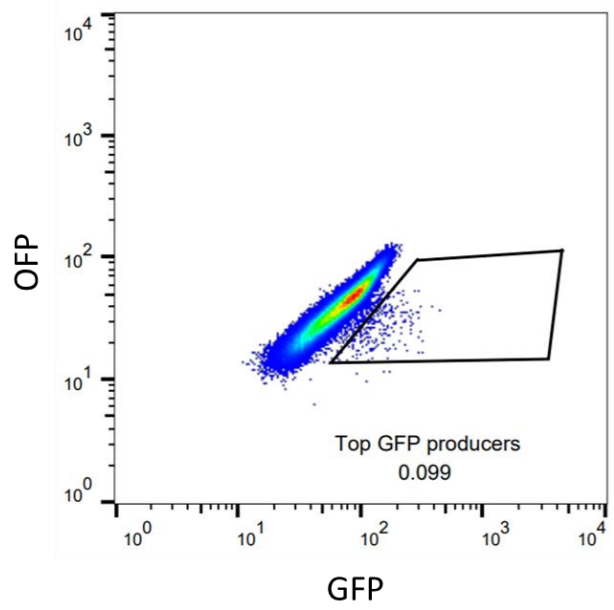

Figure 3: Fluorescent Activated Cell Sorting (FACS) to isolate the top 1% of GFP producing cells. Flow cytometry was performed to isolate the GFP producing population (boxed) for further screening and quantification.

The GFP-2A-Xylanase co-electroporation transformants were not sorted due to FACS maintenance issues at the time of use. Transformants were instead picked into deep well plates

and screened for GFP on the plate reader for expression. Favorable GFP expressing colonies were condensed for further screening.

Algal colonies were screened for incorporation of the expression construct by PCR amplification and visualization via agarose gel electrophoresis (Figure 4). DNeasy DNA prep (Qiagen) was found to be necessary to visualize specific amplicons from genomic-incorporated DNA.

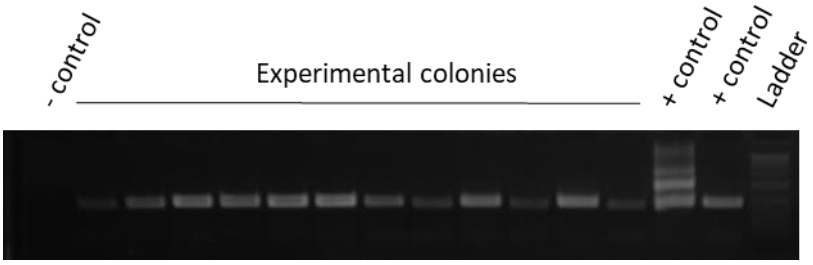

Figure 4: Gene screen for incorporation of the DNA construct into the *Chlamydomonas reinhardtii* genome. Negative control is WT algal strain, positive controls are plasmid and a previously sequenced algal colony.

Three electroporation replicates of gene positive algal colonies were sent for sequencing. The results from the genomic incorporation matched the intended reference sequence for the expression construct.

2.3 Correlation of GFP and Protein of Interest

The effectiveness of the tool was tested using a microplate reader to measure GFP fluorescence compared to the protein of interest. For this experiment, the construct containing OFP as the protein of interest was used in order to have similar units to quantify the correlation. Relative fluorescence units are vastly variable due to the gain setting on the microplate reader. For this reason, the gain settings for each fluorescent protein were conserved over each of the trials to obtain consistent and comparable results.

Xylanase was measured using the EnzChek Ultra Xylanase Assay Kit (ThermoFisher) to detect enzymatic activity. The hydrolysis of the hemicellulose by xylanase results in an unquenching of an attached fluorescent dye that has an excitation/emission maxima of ~358/455 nm. Fluorescence of the dye and intracellular GFP were read on a microplate reader. Reproducible results were not obtained, subsequent experiments yielded different expression trends for the same algal cultures (Figure 5).

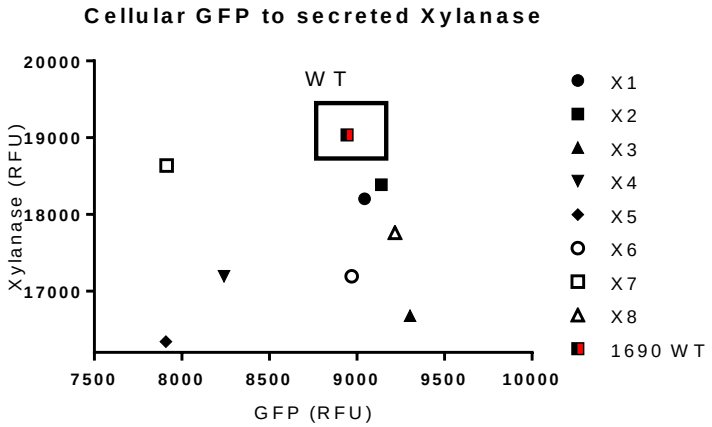

Figure 5: Xylanase measured by enzymatic assay compared to cellular GFP. No clear correlation was found between the enzymatic activity and GFP fluorescence. Data was measured in triplicate and averaged.

These inconsistent data are likely due to either low expression of the xylanase enzyme or a loss of activity in the dye from prolonged (two day) storage at room temperature during shipping. This method was discontinued and Western blotting was employed.

2.4 Correlation of GFP and OFP

Of the 21 unique transformants chosen for screening, a positive correlation is seen between GFP and OFP (Figure 6Figure 6).

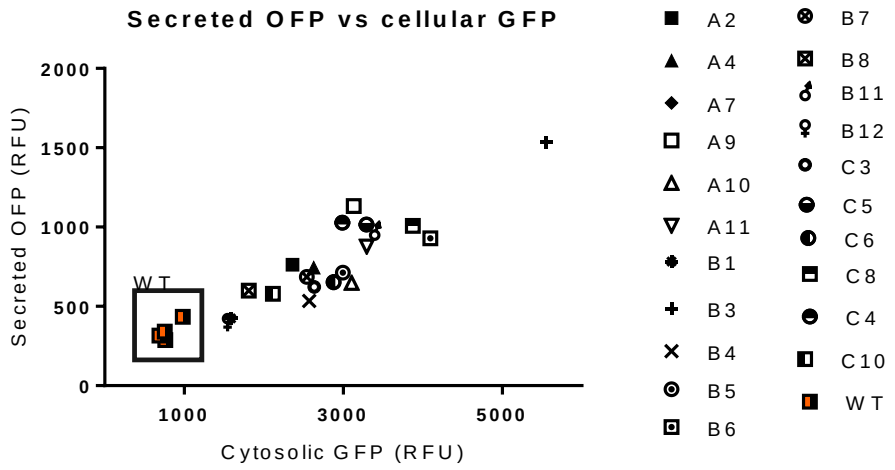

Figure 6: Secreted OFP vs cellular GFP. Microplate fluorescent readings of cellular GFP and secreted OFP were graphed based on relative fluorescence units (RFU). A general linear trend was found: higher GFP correlated to higher OFP. Individual colony names can be located using the legend to the right.

This suggests that as the cell transcribes GFP, a similar amount of OFP is transcribed as well. These data correlate to previous findings using the FMDV 2A peptide in algae (Rasala et al. 2013). Presumably, other proteins will perform in the same fashion. The original purpose of the GFP-2A-Xylanase construct was to confirm these data; however, more investigation is needed into the accuracy and repeatability of using an enzyme substrate to measure the translation correlation of GFP and xylanase.

2.5 Stability of Phenotype for OFP

Phenotype and OFP stability was tested after ~25 generations grown under typical lab growth conditions. The amount of secreted OFP at day one was statistically similar to the amount of secreted OFP at day 13 (~25 generations of time). The wildtype background strain (WT) was statistically lower than the expressors (Figure 7).

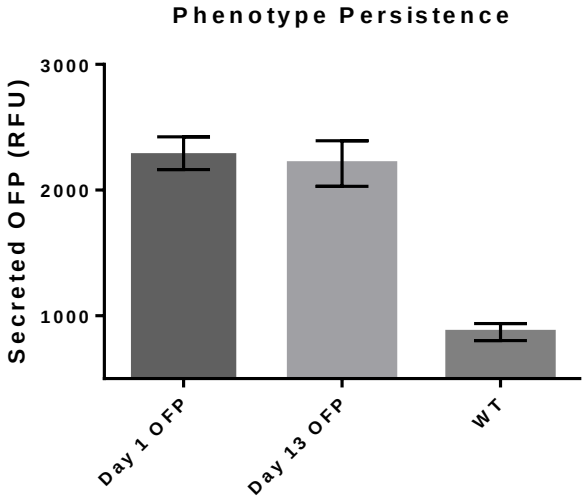

Figure 7: Phenotype persistence. Secreted OFP phenotype remains over successive (~25) generations. Day 1 and day 13 OFP data is not statistically different; OFP secretion is significantly different than WT by a one-way ANOVA. The p-value was calculated to be $p < 0.001$. Error bars represent the standard deviation.

2.6 UV Mutagenesis

UV mutagenesis was performed on the GFP-2A-OFP construct, and a kill curve was observed. The optimal mutagenesis time was 90 seconds, with 90-100% of colony death at three minutes. This experiment was not continued due to sub-par fluorescence seen on the plate reader of the P1 generation. Efforts were directed to creating a construct which expresses the proteins at an appreciable amount.

2.7 Western blotting

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to visualize protein production. Culture cell pellets were concentrated by resuspension in a smaller volume of TBST, lysed by sonication, and boiled in Laemmli and BME to denature and reduce the protein. Cultures were prepared in parallel to a known GFP producing algal strain as well as a purified GFP standard. Coomassie staining was used on a second identically loaded gel to visualize protein movement (Figure 8).

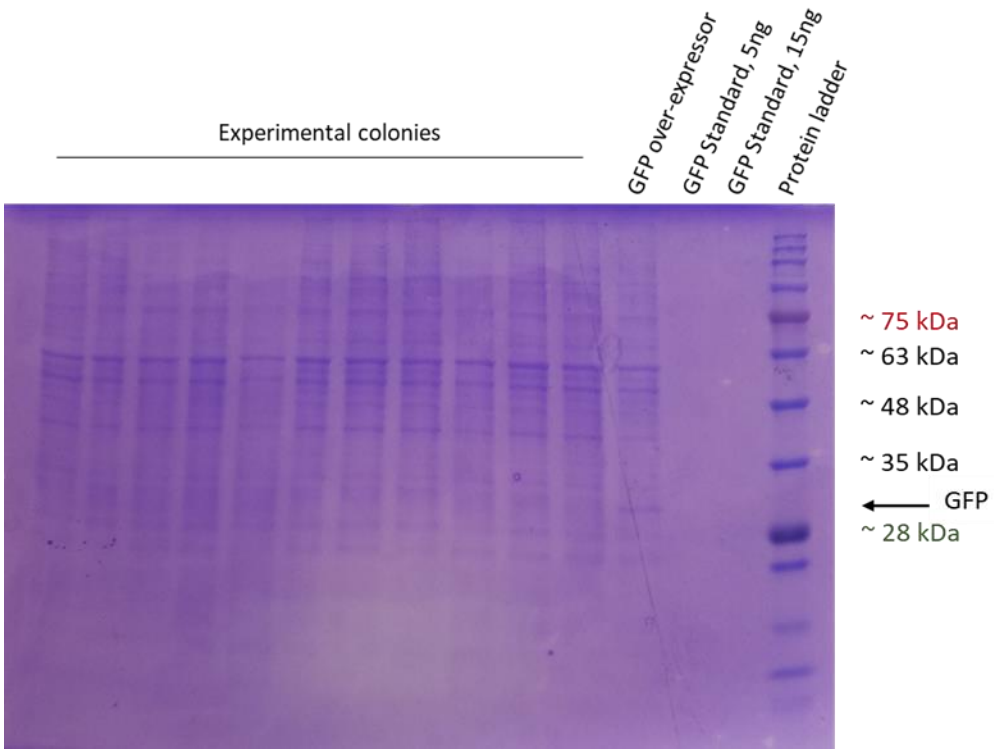

Figure 8: Coomassie staining to confirm proteins ran through SDS PAGE. Lysed cell proteins appear to have successfully ran through the gel. Standards are purified protein and do not contain the cellular proteins of the experimental lanes, seen by the absence of stain.

It was hypothesized that the proteins retained charge-charge interactions even after denaturation, and did not transfer onto the nitrocellulose properly. Ponceau S stain was used to confirm protein transfer to the nitrocellulose membrane before probing with antibody (Figure 9).

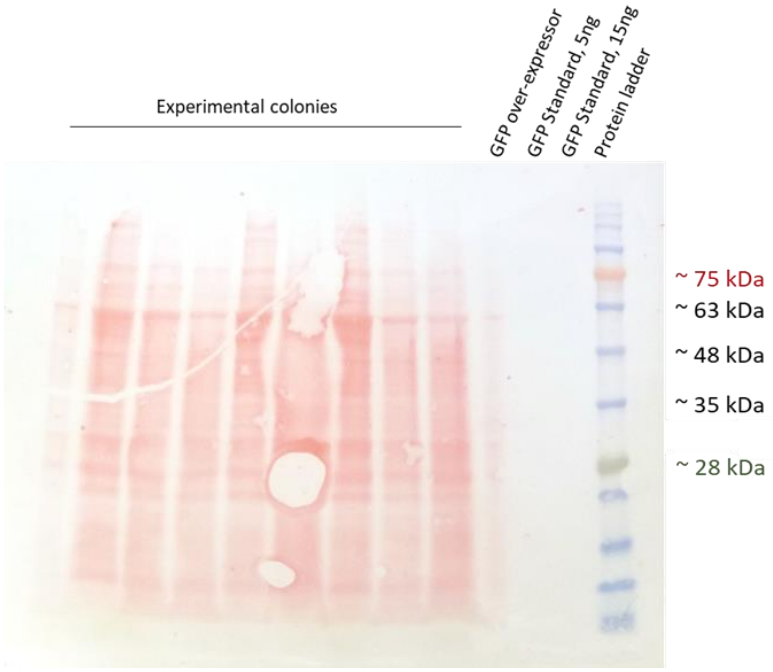

Figure 9: Ponceau S staining to visualize protein transfer to nitrocellulose. Despite air bubbles during transfer, lysed cell proteins transferred to the nitrocellulose membrane before antibody probing.

Anti-GFP and anti-FLAG antibodies were utilized to probe for the expressed fluorescent proteins seen on the microplate reader. No bands were seen in the experimental lanes using only a primary antibody (Figure 10).

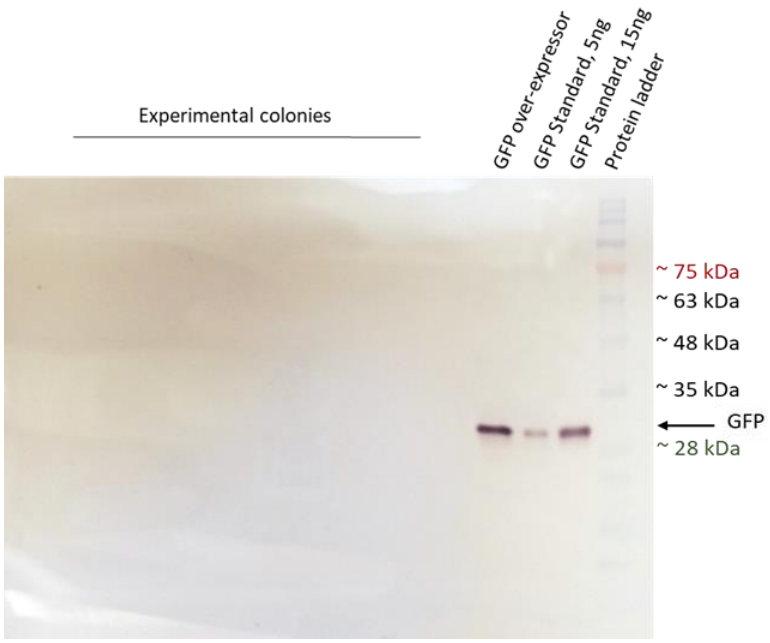

Figure 10: Western Blot of GFP-2A-OFP. Anti-GFP antibody and AP staining with NBT/BCIP was used for development. Expression of GFP was not detected despite controls performing as expected.

After Ponceau S confirmation that the cellular proteins were present, yet no experimental bands appeared on a western blot, secondary antibody amplification of the signal was employed. A secondary donkey antibody to the goat anti-GFP was used, and the membrane was blocked in donkey serum. Very dark bands were observed for the standards and positive control, however the experimental lanes remained inconclusive (data not shown).

2.8 Low Expression

The lack of western results could have stemmed from very low expression of recombinant protein from the cells, or from an underlying problem with the screening of the transformants. On reviewing the FACS data, it appeared that there could have been insufficient GFP expression, and the gates were set on the outer edge of the wildtype population to be able to gather the sorted cells back into culture. It was also hypothesized that the OFP filter overlaps slightly with the auto-fluorescence of dead cells. It is possible that the majority of the sorted

population was dying or dead cells, with a minimal amount of expressing live cells which led to the investigation into the cross-talk of fluorophores.

According to flow cytometry studies of heat killed *Chlamydomonas reinhardtii* with a blue 488 nm laser, auto-fluorescence at 530/30 nm channel with a 505 long pass filter is suitable for GFP emission spectra, and detects low amounts of auto-fluorescence compared to other excitation / emission channels (Kay et al. 2013). Similarly, a violet 405 nm laser, auto-fluorescence at 585/15 nm channel with a 575 long pass filter is suitable for OFP emission, suggesting that dead cells only slightly contribute to the GFP and OFP emission data.

It could be the case the majority of gene positive cells sorted that day were subject to transgene silencing due to the gene being incorporated into a poor location in the genome, resulting in low expression.

Further investigation into the origins of this poor expression revealed the Rasala 2011 paper suggesting advantages of reducing the amount of non-native sequences incorporated into the genome (Rasala et al. 2011). In order to increase protein expression, subsequent gene insertions were performed by incorporating smaller amounts of non-native sequences into the genome. The DNA was prepped again, and double restriction digested directly flanking the expression cassette of GFP-2A-OFP, and co-electroporated with the hygromycin resistance gene into *Chlamydomonas reinhardtii*. 960 transformants were picked and grown up in deep well plates to screen for expression on the plate reader.

Initial readings from the plate reader suggested that a higher percent of the double digested DNA recipient colonies express GFP than the singly digested recipient colonies. This replicates the findings in Rasala 2011, suggesting that the gene was originally being silenced or

down-regulated. However, the baseline of background fluorescence could be skewed due to growth cycle and culture density differences in the day the colonies were screened. Ultimately, the hygromycin co-electroporated construct experiments were discontinued in favor of a more promising expression construct linked to bleomycin resistance.

2.9 Designing an Antibiotic Resistant Linked Construct

The previous vectors may have had low expression due to the possible transgene silencing and rare events of co-transformation. Therefore, a new construct was cloned which contained a 2A-linked antibiotic resistance gene, bleomycin (Stevens, Rochaix, and Purton 1996) (Figure 2). Multiple 2A peptides pose a unique opportunity to express tri-cistronic constructs, and have been seen to successfully express multiple fluorescent proteins in human cell lines (Liu et al. 2017). A benefit to the design of this construct is the use of *sh-ble* bleomycin/zeocin-resistance gene that confers higher expression levels in *Chlamydomonas* than those with hygromycin resistance due to the need to translate a 1:1 molar ratio of ble protein to prevent double strand DNA breaks induced by the zeocin (Dumas et al. 1994; Stevens, Rochaix, and Purton 1996).

During electroporation, the Ble-2A-GFP-2A-OFP plasmid was linearized using two restriction enzymes to reduce the transgene silencing seen in singly-digested DNA incorporated into the nuclear genome (Rasala et al. 2011).

Results from the Tecan microplate reader suggested that GFP is correlated to OFP (Figure 11), similar to the correlation with the Hygromycin co-electroporated GFP-2A-OFP

construct (Figure 6).

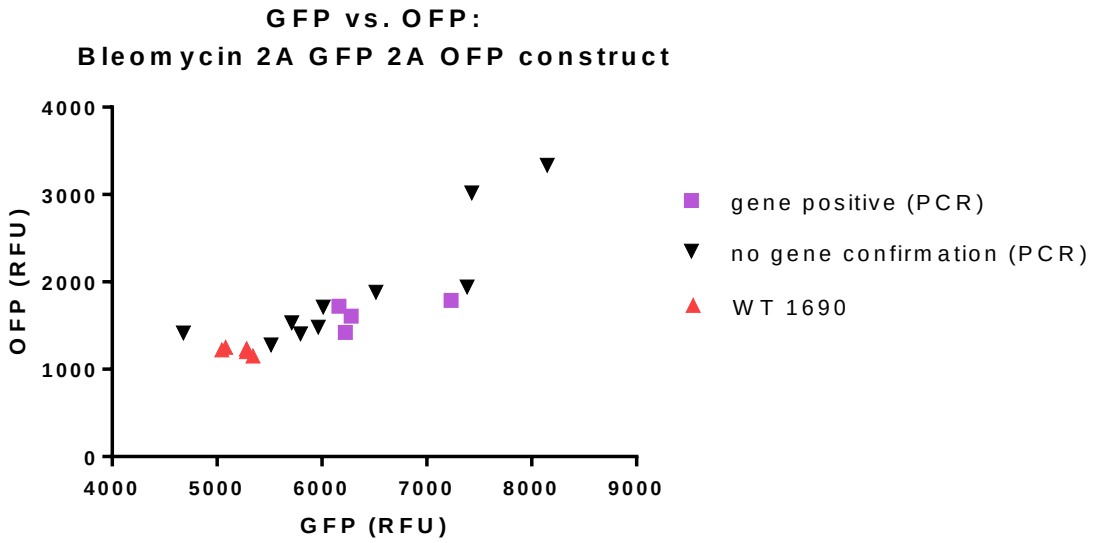

Figure 11: Bleomycin linked expression. Individual transformants with the Ble-2A-GFP-2A-OFP construct were read on a microplate reader at an excitation/emission maxima of 488/522 nm and 548/583 nm for GFP and OFP, respectively.

Gene screens were performed that confirmed gene incorporation for some but not all of the colonies (Figure 12). This suggests that the plate reader data is insufficient as a preliminary screen of colonies to check for fluorescence, or that the gene screen off of genomic DNA is unreliable and does not give consistent results for the primer set chosen.

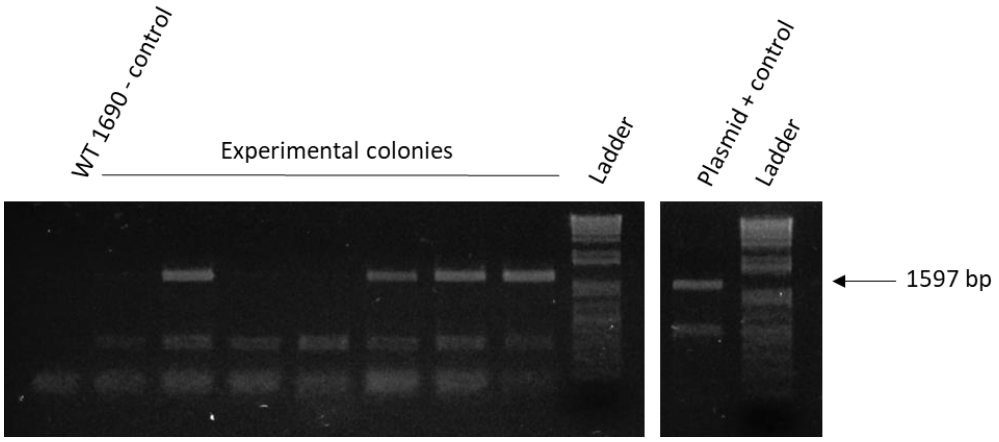

Figure 12: Gene screen of Ble-2A-GFP-2A-OFP colonies. Negative control is WT algal strain, positive control is a previously sequenced algal colony.

Western blotting of the fluorescent colonies revealed that an insufficient level of protein was accumulated in the soluble fraction of cell lysis and the cell pellet fraction after sonication or the use of BugBuster (Millipore #70921) cell lysis reagent (Figure 13).

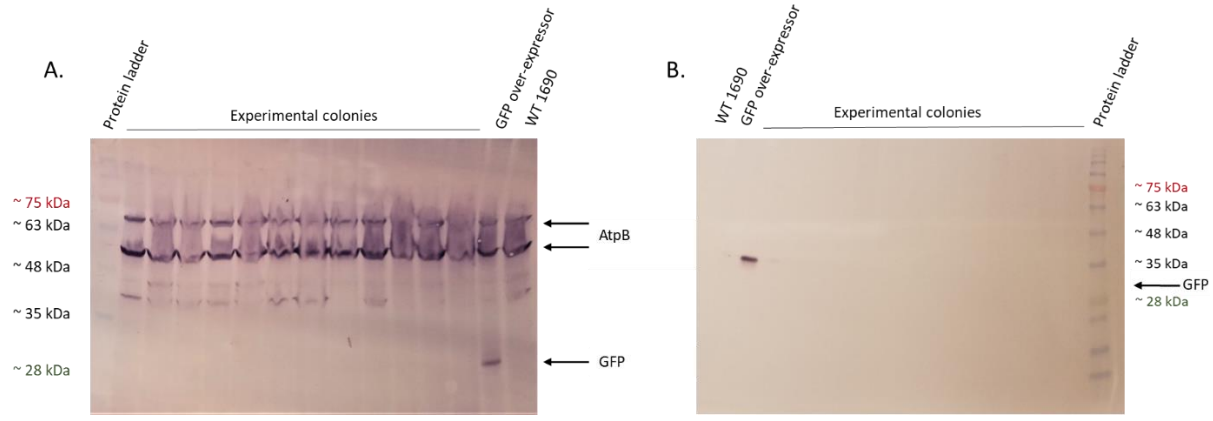

Figure 13: Western blot of Ble-2A-GFP-2A-OFP with loading control. A. Cells were prepped using Bug Buster, the soluble protein fraction was isolated by centrifugation and boiled in 1X Laemmli/BME and loaded onto a 12% SDS-PAGE gel. Electrophoresis was applied for a longer amount of time to let any doublet bands separate from background. AP conjugated anti-GFP and anti-AtpB were used to visualize protein expression. B. Cells were prepped using Bug Buster, the insoluble protein fraction (cell pellet) was boiled in 1X Laemmli/BME and loaded onto a 12% SDS-PAGE gel. AP conjugated anti-GFP was used to visualize protein expression. Only the positive control showed accumulation of GFP in both blots.

2.10 Future Directions

Further experiments are needed to determine the large scale functionality of the hygromycin resistant GFP-2A-protein constructs, and the achievability of using mating to cross out the gene for hygromycin resistance while maintaining high levels of expression.

Ble-2A-GFP-2A protein constructs have a promising future for quantifying protein expression utilizing multiple FMDV 2A peptides to create polycistronic expression systems. Further investigation is needed to locate the transcriptional or translational error occurring in the cells. An area of concern is the possible down regulation of the mRNA when transported from the nucleus to the cytoplasm due to the abundance or orientation of the viral FMDV peptide. Future constructs could employ the use of different 2A peptides to determine if an arrangement of proteins and various 2A peptide combinations is superior to another (Liu et al. 2017).

Additional research needs to be completed to determine the efficacy of this system for use in the production of recombinant proteins. Further experiments need to be done to quantify the protein expression with alternate arrangements of the 2A peptide, and increase expression to adequate levels for bioreactor or large cultivation system growth.

Large scale bioreactor or open-pond growth necessitates a high expressing secretion strain design. It is hypothesized that the current strains may aid in the future creation of a design for biotechnology use.

Chapter 3: Methods

3.1 Expression vectors

Ultramer oligos (Integrated DNA technologies, Eton Biosciences) were synthesized, and existing vectors from the Mayfield lab were used as template DNA in PCR reactions to amplify the genes of interest. Gibson assembly (New England BioLabs) was ultimately used to create the construct GFP-2A-*ars1*-OFP-3'UTR and GFP-2A-*ars1*-Xylanase-3'UTR, driven by the AR1 promoter consisting of the HSP70A promoter and rbcs2 5' UTR. Both gene constructs were assembled into the pUC19 vector backbone (New England BioLabs, courtesy of Lindsey Spiegelman). After sequencing (Genewiz), the coding sequence was found to contain an in-frame stop codon, causing premature mRNA termination. Q5 site-directed mutagenesis (New England BioLabs) was performed according to the manufacturer's protocol on both expression constructs to remove this codon.

Ble-2A-GFP-2A-OFP vector was cloned by Gibson assembly into the pBR9 expression vector with assistance from Anthony Berndt, Ph.D.

3.2 Co-electroporation into *Chlamydomonas reinhardtii*

Chlamydomonas reinhardtii was grown in TAP media on shaking flasks to a mid-log phase (~3 x 10^6 cells/mL). The culture was centrifuged and resuspended in MAX Efficiency Transformation Reagent for Algae (Invitrogen) to a concentration of $3x10^8$ cells/mL. Purified plasmids (QIAprep Spin Miniprep Kit) were linearized with the restriction enzyme AatII (New England BioLabs) or a XbaI-FD/KpnI-HF double digest fragment, and the restriction enzymes were heat inactivated for 20 minutes at 80°C or gel purified. After co-electroporation was performed, gene screens via DNeasy (Quiagen) genomic DNA prep and PCR amplification were conducted to ensure the target gene was incorporated into the genome.

3.3 Fluorescence Quantification

Fluorescence of the proteins was quantified using a microplate reader (Tecan) in clear 96 well plates with a culture volume of 100 μL in each well. Microsoft Excel, Powerpoint, and GraphPad Prism were used to render the data and figures.

3.4 Cell Preparation for Protein Quantification

Sonication at 25% amplification or BugBuster (Millipore) was used to lyse cells and release proteins. Total cellular proteins were concentrated by resuspending the cell pellet in a smaller volume of TBST. Laemmli buffer and β-mercaptoethanol (BME) were used to denature and reduce the proteins for SDS PAGE.

3.5 Western Blotting

Western blots were performed using protein ran on SDS-PAGE transferred to a nitrocellulose membrane. The membrane was blocked with 5% milk or donkey serum and probed with polyclonal alkaline phosphatase conjugated goat anti-GFP and anti-FLAG. Secondary staining was used to amplify the anti-GFP signal, for which a donkey anti-goat AP was employed. Anti-AtpB (Agrisera) was used as a loading control, for which an anti-rabbit AP conjugated secondary was used for visualization. Antibodies were used in various titers between 1:5000 and 1:10,000 to achieve optimal darkness of the blot. NBT and BCIP were used to develop the blots in AP buffer.

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