WORCESTER POLYTECHNIC INSTITUTE

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CAN PLANT CHLOROPLASTS FUNCTION IN MAMMALIAN CELLS?

A Major Qualifying Project
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Report Submitted to:
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Abstract

The goal of this project is to determine if plant chloroplasts isolated from spinach can maintaining active chloroplast gene expression when placed into a mammalian cell environment. Chloroplasts were isolated and purified from fresh spinach leaves and introduced into human fibroblasts. RNA was isolated from chloroplasts, control fibroblasts and chloroplast-incubated fibroblasts at 24, 48, and 72 hours and expression of chloroplast gene *rbcL* quantified by RT-PCR. If functional, chloroplasts can provide a source of energy and metabolism.

1 Introduction

1.1 The Field of Synthetic Biology

Synthetic Biology is an expanding field that has made it possible for the creation of synthetic cells. It can be generally thought about as “the deliberate (re)design and construction of novel biological and biologically based systems to perform new functions for useful purposes, that draws on principles elucidated from biology and engineering” (https://www.erasynbio.eu/about/synthetic-biology). Specifically, it is of interest to use energy metabolism mechanisms from one organism and transfer them to another. The chloroplast organelle could be a focus for synthetic biology. Another goal of synthetic biology is to create a chemically synthesized artificial chromosome that can be put into a cell to produce a synthetic cell.

Recently, synthetic biology principles-- which focus on the design of new biological structures such as enzymes and cells-- have been incorporated into many experiments. Agapakis and colleagues explored introducing photosynthetic bacteria directly into animal cells. One way the researchers explored this was through endocytosis of two strains of macrophages (Agapakis et al, 2011). When introducing the photosynthetic bacteria S. *elongatus* inside a mammalian cell, they found that the cyanobacteria have minimal effects on the host cell and are able to divide in the cytoplasm of the host cell. It is tricky to make sure the phagocytotic entity that is entering the host cell does not break down and digest the entity via its lysosomes. They found that when high numbers of the bacterium remains inside J774 cells (zebrafish cells) for several days, there are minimal effects (Agapakis et al, 2011). This can be

described as a sort of symbiosis between the invading entity and the host cell. Listeriolysin O from L. *monoctogenes* (Ilo) was used as a host cell and was found that foreign entities were able to divide in the cytoplasm of the host cell. They found S. elongatus engineered with both invasin and listeriolysin showed an increase in the red autofluorescence over a two day period post-injection. Schultz et al., reported using ~1-10 mM sodiuM ascorbate to measure organelle intactness. They used Arabidopsis in their study and determined that 50-100 mM of sodium ascorbate in a grinding buffer were successful as a dye and was import-competent.

The idea of a hybrid has been accomplished before. A cybrid is simply a hybrid cell with a nuclear genome of one source and the mitochondrial genome from another source. This fusion shows that it could be possible to create a synthetic cell using chloroplasts. The chloroplast and mitochondria organelles both arose from endosymbiosis. For example, kleptoplastic (sequestered organelles from host organisms) sea slugs studies showed no evidence of horizontal gene transfer in long term function. As reported by Serodio, previous studies have found the performance of kleptoplasts to be superior to chloroplasts within original algal cells. Rumpho et al (2011) showed that many of the 1000 nuclear-encoded plastid-proteins may not be present in animal nuclear genes.

Using these engineered systems can become a way to discover symbiotic relationships in biological life. A syntrophic relationship is used to describe interactions that are specific and beneficial that can be used to discover new metabolic niches in nature (Wintermute and Silver, 2010). With new engineered systems, new models and

interactions can be studied such as the creation of an artificially engineered mammalian chloroplast.

1.2 The Chloroplast Organelle

1.2.1 Historical perspectives

The plant cell chloroplast is an organelle that has been studied for about a century since the discovery by Konstantin Mereschkowski in 1905 and his discovery of symbiogenesis (https://theendosymbiotichypothesis.wordpress.com/history). The chloroplast is the major organelle of plant cells for determining photosynthetic capacity. Photosynthetic capacity is directly related to the overall quantity and distribution of leaf chloroplasts. According to the endosymbiotic theory, the chloroplast has been thought to come from a cyanobacterial cell that have their own DNA.

Multiple views of different models of the chloroplast exist that show how this organelle has been viewed since its discovery. According to the view of the “helical” model (Paolillo, 1970), cylindrical granum are wound around a right-handed helix. They took plastids, which are a group of organelles that function to carry out photosynthesis (Leegold, 2010), from tobacco, spinach, among others and showed that they displayed helices wound in the same direction and are co-directional. Stroma membranes, in the helical model, are wound around granum. Thylakoids, in this view, are tilted at an angle of 22 degrees. The helices are tilted at an angle of 10-25 degrees with respect to the grana stacks. There are multiple disagreements with the helical model. Recent studies (Shimoni, 2005) have doubt over the helical model although some aspects of this model still hold true-- termed the pairwise organization model. Stroma thylakoids in this view

are perpendicular to the axes of the grana ($\alpha = 0$ degrees). Also, grana are constructed with repeating units of two layers. As Shimoni notes, the stroma membranes bifurcate into stacked layers that encloses the lumen in the chloroplast (Shimoni et al, 2005). This is known as bifurcations and comprise a fork model. Despite the continuity of the bifurcation model, Arvidsson and Sunby (1999) found that the thylakoid membrane may be put together by a continuous membrane and the granum are thus formed by piles of three thylakoid. Thus, a bifurcation model is shown.

Mustardy et al (2008) proposed a quasi-helical model to settle these disagreements between the models. Mustardy hypothesized that new layers of a granum can be added independently- providing that they are in a proper angle, avoiding hindrance.

Figure 1: Historical Perspectives of the chloroplast

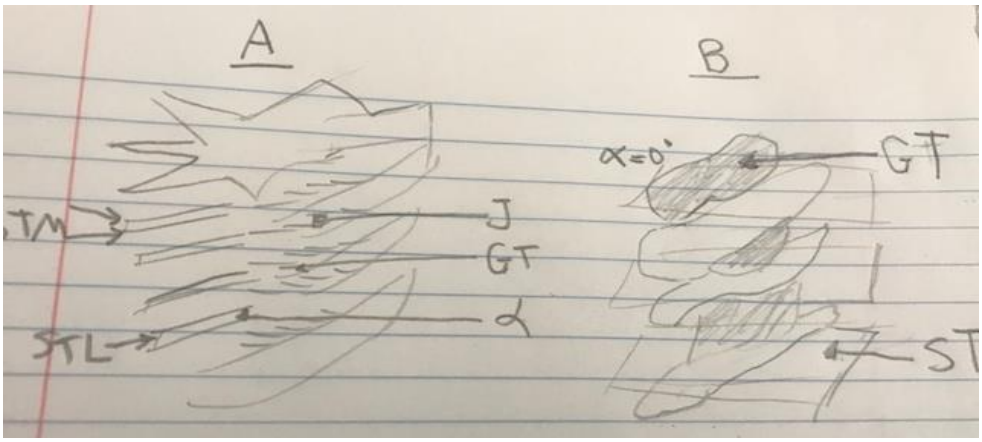
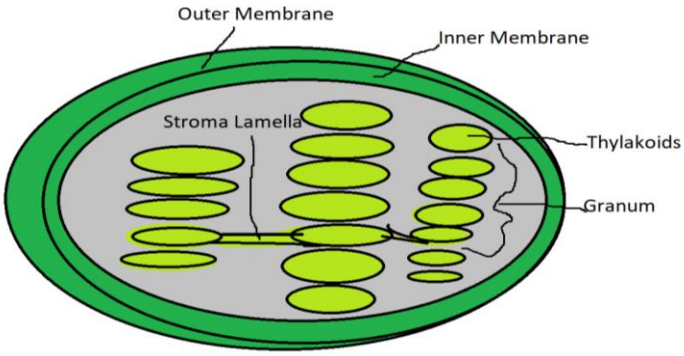


Figure 1: Historical Views of the chloroplast, adapted from Mustardy (2008). A: Paolillo's Helical Model. B: Shimoni's Bifurcation model. Acronyms as such: α: tilt angle between granum and thylakoid membrane, STM: stroma thylakoid membrane, STL: Stroma Thylakoid lumen, J: junction between granum and stroma thylakoids, GT: Granum thylakoid, ST: Stroma thylakoid

1.2.2 Structure

The typical structure of a chloroplast in figure 1 is shown below. The chloroplast has genes organized in operons (linked genes) that makes the organelle ideal for genetic engineering (Jenson, 2014). With a synthetic biology standpoint, molecular mechanisms of the chloroplast that could utilize ATP or NADPH could be still evident in a eukaryotic cell line. Photosynthesis uses carbon dioxide, water, and light to produce glucose and oxygen-- represented by the chemical equation $6CO_2 + 6H_2O \rightarrow C_6H_{12}O_6 + 6O_2$.

Figure 2: Structure of the Chloroplast



The organelle has a complex structure that is the place of photosynthesis in the plant cell.The many interworking parts of the plant cell. In the chloroplast comprises of 10-20 thylakoid disks of 300-600 nm in diameter. The chloroplast has a double membrane that surrounds the organelle and one around the thylakoid stacks. Inside the organelle, there are two types of fluid: stroma that is present outside the thylakoids and lumen inside the thylakoids. Each stack of thylakoids is termed a granum. Connecting each granum are structures of stroma lamella that comprise a design known as a fretwork.

1.2.3 Function

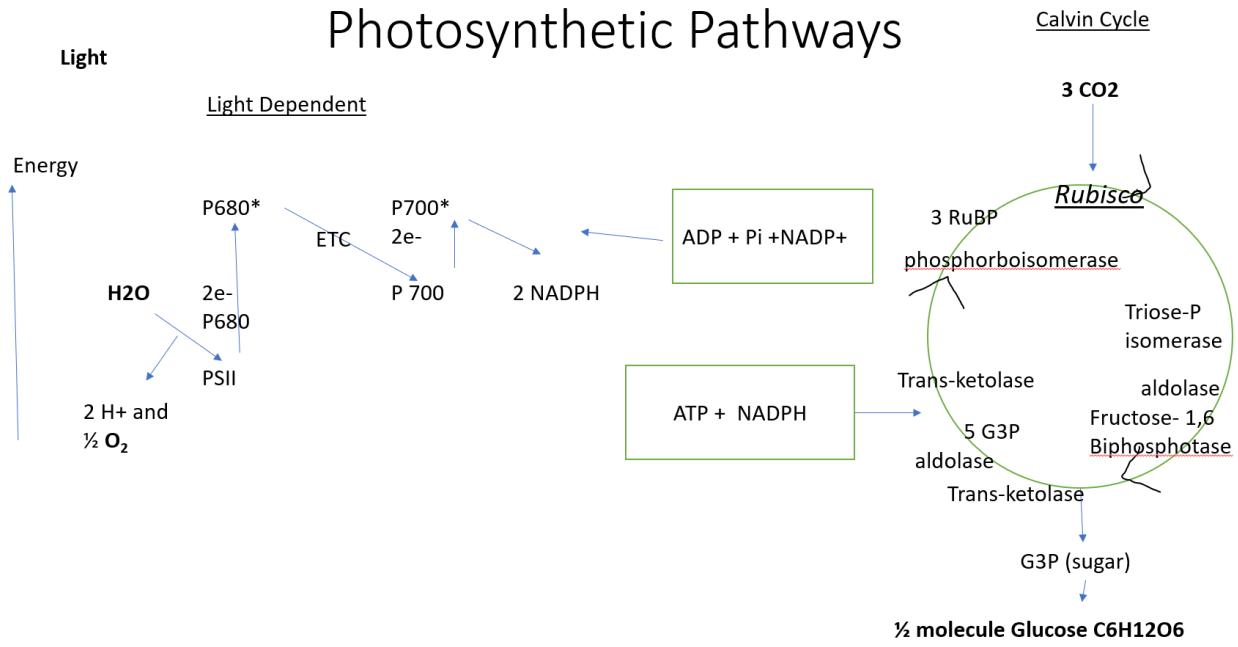
There are two major phases of photosynthesis: the light dependent reactions and the light-independent reactions. The first stage, the light-dependent reactions, takes place in the thylakoid membrane. In the first step of the pathway, water is split. Sunlight drives the formation of ATP and NADPH and oxygen is released as a byproduct. This primarily happens through a linear way through what is known as the Z-scheme using two photosystems of the light-dependent reaction termed PSII and PSI-- with electrons traveling from PSII to PSI. Photosystems (PS) are reaction centers that comprise of pigments intertwined with proteins. Photons excite PSII (700 nm), an electron transport chain occurs, and then the PSI (680 nm) is excited by photons. A complex known as cytochrome b6f is responsible the majority of the proton gradient generated between the two photosystems. PSII oxidizes H_20 and PSI reduces NADP+. An oxygen evolving center in PSII, breaks water into oxygen and protons. ATP and NADPH are the result of this first reaction through which synthetic biology principles could be utilized using the chloroplast machinery.

Light dependent reaction (dark) reaction, or the Calvin Cycle, are the second set of reactions of photosynthesis that takes place in the stroma. This step can take place as long as ATP and NADPH are available and happens in either the light or dark. In this stage, ATP and NADPH are used to reduce carbon dioxide and make sugar.

In this cycle, ATP delivers energy and NADPH delivers hydrogen and electrons to the stroma. Sugar phosphates form and carbon dioxide from the air is affixed to RuBP, which produces an unstable intermediate that splits into 2 PGA molecules. In all, 6

carbon dioxides enter the cycle and 12 PGAL molecules are produced. Two of these PGAL molecules are used to produce a six carbon sugar phosphate and the others are used to regenerate RuBP. The light and dark reaction are shown in figure 2-- with the Rubisco (italic and underlined) being the only plastid-encoded protein.

Figure 3: Simplified diagram of photosynthesis



Researchers have studied two main pathways of photosynthesis. Whatley and colleagues (Whatley, 1963) found flavoprotein TPN reductase of chloroplasts catalyzed the reduction of TPN can be separated into two steps: the light-induced reduction of ferredoxin and a dark reduction of TPN by reduced ferredoxin. They found that in the non-cyclic (linear) electron flow, photoreduction of ferredoxin is a prerequisite for this event-- although only when coupled with TPN reduction. This is the most common route

of photosynthesis. They also note that ferredoxin is required for the cyclic photophosphorylation (Hiura et al, 1981).

Benson et al (1998) saw that chloroplasts display the most chloroplast content in the winter months as compared to the summer months. Benson looked at the life cycle of Codium fragile from 1979 to 1982 and found that the physiology of C. fragile shows that it displays ideal growth strategies in the winter months when there is the most amount of carbon fixation and chloroplast sizes. Benson found that the most chlorophyll content occurred in the months of November through March with the least chlorophyll content in July. This result shows that during the experimental procedures would be best performed when chlorophyll contents are highest in the winter months as evident by the strong seasonal trend.

1.2.4 Chloroplast genome and the plant nuclear genes

The chloroplast genome and the plant nuclear genome send molecular signals to one another in a process known as retrograde signaling. According to Timmis and Scott (1983), the 7.7 kbp PstI fragment of spinach chloroplast DNA are homologous to nuclear DNA sequences. Results from their experiment show that there is a covalent integration of chloroplast DNA and the nuclear DNA. Timmis and Scott were the first to show a shared homology of chloroplast and nuclear DNA. Embryo development has been linked to specific proteins of nuclear genes that encode chloroplast proteins For example, a chloroplast gene interacts with gene products of the GENOMES UNCOUPLED (GUN) loci. This retrograde signalling is seen when photosynthesis is inhibited while *gun* mutants still nuclear-encoded photosynthetic genes. Proteins of the

chloroplast are not only required for proper functioning of the organelle but also cell growth. As an example, biosynthesis of amino acids, vitamins, and minerals are associated with embryo defective proteins. Disruption of biosynthetic pathways could result in altered phenotypes such as reduced seed pigmentation and embryo arrest.

1.3 Mammalian and mitochondria energetics

Mammalian cells are function differently from plant cells. Plant mitochondria produce energy in animals and humans, which form a matrix of an inner and outer membrane. Mitochondria produce energy in the form of ATP through cellular respiration by converting organic molecules into water, ATP, and carbon dioxide. The mitochondria -- an organelle responsible for eukaryotic oxidative metabolism-- genome contains 13 protein coding sequences for respiratory complexes. Oxidation of NADPH and FADH2 are carried out by complexes I, II, III, and IV. Ubiquinone carries electrons from complex I and II to complex III by ubiquinone and complex III to IV by cytochrome c. ATP synthase is responsible for much of the energy production of the mammalian cell.

1.4 Project goals and hypotheses

A primary aim of our research is to investigate whether plant chloroplasts can function in a mammalian cell environment without the plant nuclear genome and act as a glucose generator. Chloroplasts are the plant's energy source for production of sugars, amino acids, fatty acids, and oxygen. We propose to introduce chloroplasts into human fibroblasts and measure expression of chloroplast genes by RT-PCR. The biochemical machinery in the chloroplast organelle has the possibility of functioning inside of a

mammalian cell if accepted. If successful, our results could be used to investigate whether plant chloroplasts can function in a mammalian cell environment without the plant nuclear genome.

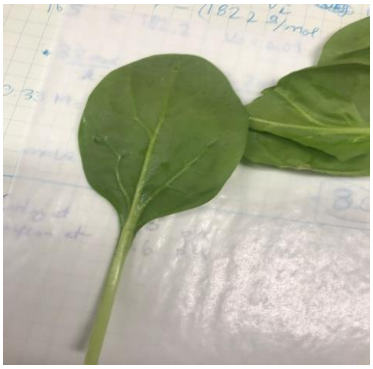
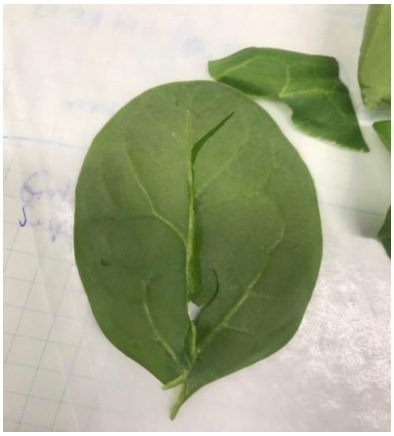
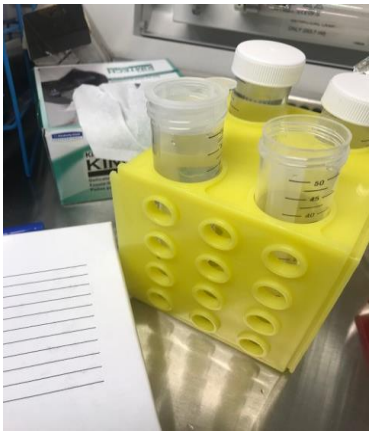
This project makes use of synthetic biology to create photosynthetic mammalian cells. This experimental work will aim to create a photosynthetic cell by combining chloroplasts of spinach leaves (spinacia oleracea) and skin fibroblast mammalian cells. Instead of solely relying on cellular respiration to generate energy, the photosynthetic capabilities could be utilized for energy production. This could have applications in space travel or long submarine travel in which a ‘solar patch’ would supply energy using photosynthetic pathways, something that might be performed in the future.

2 Materials and Methods

2.1 Isolation of Chloroplast organelles

Spinach was used from Holaday, Inc (CA) to isolate chloroplasts. The spinach leaves were larger than what would be normally found in a common grocery store. A procedure of a cell biology laboratory manual from William Heidcamp, a Professor at Gustavus Adolphus College was used. Materials included spinach, mortar and pestle, cheesecloth, and a hemocytometer. Two buffer solutions were made: a grinding solution (0.33 M sorbitol, 10mM sodium pyrophosphate, 4 mM magnesium chloride, 2 mM ascorbic acid) and a suspension solution (0.33 M sorbitol, 2 mM EDTA, 1 mM magnesium chloride, 50 mM HEPES). Using a pH meter, the grinding solution was adjusted to a pH of 6.5 and the suspension solution was adjusted to a pH of 7.6. The procedure was performed in a biosafety hood. Spinach leaves were deveined and

chopped with a fine blade. Leaves were put into a mortar along with 15 mLs of the grinding solution. Once ground using a mortar and pestle, the solution was filtered through two layers of cheesecloth into a 50 mL conical centrifuge tube and centrifuged at 300xg for 1 minute at 4 degrees Celsius. The cheesecloth separated larger sediment. Supernatant was placed into 2 mL eppendorf tubes and spun at 1000*g for 7 minutes. Chloroplasts were suspended in 5 mLs of cold suspension solution for further study

A 	B 	C 
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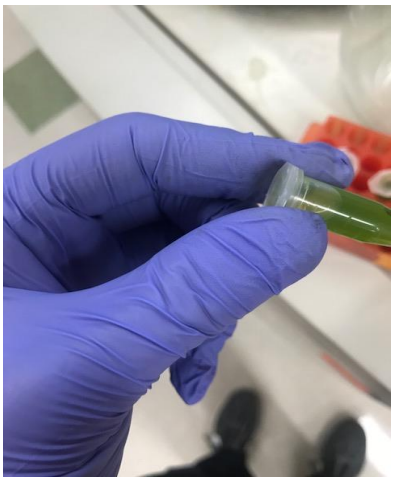

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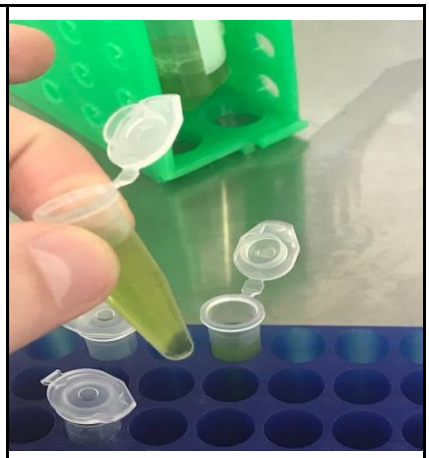

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Figure 4: Isolation of chloroplasts

A: Spinach leaf, B: Leaf deveined, C: Grinding solution through filter, D: Ground leaf in mortar, E: Paste with grinding solution, F: Cheesecloth photo, G:50 mls centrifuge tube, H: supernatant moved to Eppendorf tube, I: Supernatant at bottom of Eppendorf tube

As outlined in figure 4, the process first began with deveining a spinach leaf (fig. 4A, 4B) using a fine blade and a petri dish. The grinding solution buffer was put into a 50 mL centrifuge tube filter (fig. 4C) and a blade was used to chop the spinach (fig. 4D). Afterwards, a paste was created (fig. 4E) with 15 mls of the grinding solution and the chopped spinach leaves and the paste was put through a double-layer cheesecloth (fig. 4F). The subsequent filtered solution was collected in a 50 mL centrifuge tube and centrifugation was performed for 300*g from 1 minute at 4 degrees Celsius (fig. 4G). The paste had to be pressed through the cheesecloth and excess spinach was discarded that did not flow through the cheesecloth. A hemocytometer and a

microscope was used in this procedure. The upper, aqueous supernatant layer was transferred to 10 eppendorf tubes (fig. 4H) and spun at 1000*g from 7 minutes (fig. 4I). The chloroplast sediment was collected on the bottom of the eppendorf tube and put into the refrigerator. As an example count, an average of 474,000 cells/mL was determined by averaging the counts by using the formula:$cells/mL = (sum\ of\ counts/number\ of\ boxes) * 10^4 * Dilution\ Factor$. A dilution factor of 1 was used in this formula. A sample calculation from the data below in table 1 is ((40+45+55+48+49)/5)*10^4*1)= 474,000 cells/mL.

Table 1: Example of counts using a hemocytometer

Box of hemocytometer	Count
1	40
2	45
3	55
4	48
5	49

2.2 Cryopreservation of Chloroplasts

Cells were frozen using a freezing media. The cryopreservation media was utilized to freeze cells using 10% DMSO in media. Cell suspensions were pipetted into 2 mL vials and stored at a $-20°$ Celsius freezer.

2.3 Visualizing of chloroplasts using Qtracker ® Cell Labeling Kits

Cells were dyed using a cell tracker kit. A 10 nM solution was prepared using 1 uL of a Qtracker ® component “A” and “B” and incubated at room temperature for 5 minutes. Growth media (0.2 mL) was added and vortexed for 30 seconds. Cells ($1*10^6$ cells/ml) were added to the tube of the labelling solution and incubated for 50 minutes at 37 degrees Celsius.After incubation, the eppendorf tube was spun down. Para-formaldehyde (1 mL) was added to the pellet. After 30 minutes, the eppendorf tube was spun down. A 10 uL pellet was put on a slide and *nail polish* was used to bind the circular glass on the microscope slide. As a control, a DNA label Hoechst dye was used.

2.4 Cell Culture of Mammalian Cells and Introduction of chloroplasts

A cell line of 2097s human fibroblasts were used in this experiment according to a previous procedure outlined by Nass (1969). Proper cell culture techniques were used in the lab to maintain the cell line. Cells were co-cultured with isolated chloroplasts at 37 degrees Celsius. Nass found that chloroplasts were present for five generations or five days. The co-incubation procedure was performed at a ratio of 25 chloroplasts per cell.

A ratio of one cell per twenty-five chloroplasts was used-- similar to that of Nass (1969) that was used for the subsequent gel electrophoresis. Two grown media were made: one without HEPES and another with HEPES (49 mLs media and 1 mL HEPES). Three mLs of growth media were placed into two 6-well plates along with a chloroplast-fibroblast mixture. One 6-well plate was placed in the incubator and another plate was left outside exposed to sunlight. The equation that was used is below with “s”

representing cells and “c” representing chloroplasts for 25 times the chloroplasts for every cell.

$$25[C_S]V_S = [C_C][V_C]$$

2.5 RNA Isolation with TRIzol

A TRIzol procedure was used to isolate RNA with two parts: lysing and isolation of RNA. For the lysing, 1 mL of TRIzol Reagent was used per 50-100 mg of tissue and then the suspension was incubated for 5 minutes. Then, 0.2 mL of chloroform was added per 1 mL TRIzol and then incubated for another 2-3 minutes. The mixture was separated into three phases: phenol-chloroform, an interphase layer, and an aqueous phase. The suspension was centrifuged at 12,000g at $4°$ C. The aqueous phase, which contained the RNA, was transferred to a new tube with a pipette. For the isolation, 0.5 mLs of isopropanol was used for every 1mL of TRIzol reagent used in the lysis, incubated for 10 minutes, and centrifuged for 10 minutes at 12,000g, and supernatant discarded. The pellet was resuspended in 1 mL 75% ethanol per 1 mL of TRIzol reagent, vortexed, and centrifuged for 5 minutes at 75,000g. The supernatant was discarded and air dried for 5-10 minutes.The pellet was resuspended in 20 uL of RNA-ase free water and incubated in a hot plate at 55-60 Celsius for 10-15 minutes. RNA concentration was calculated by the formula A260 * dilution * 40= ug RNA/mL, with a ratio of A260/A280 of ~2 was considered pure. Concentrations were determined by using a Nanodrop spectrophotometer.

The TRIzol experiment with three sample collected was performed at times post 24, 48, and 72 hours post incubation with chloroplasts for both the incubator and

outside samples. After removing cell culture medium and washing with PBS, the TRIzol procedure was performed. At the end of this procedure, a total of six TRIzol samples would be collected-- two for each time point for each of the two by three well plates.

2.6 Synthesis of cDNA and Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) for rbcL

RNA primers were chosen based on having a similar melting temperature (Tm) (Haider, 2015). Primer design was as follows: 5'-ATATCTTGGCAGCATTCCGAG-3' as the forward primer and 5'-TCTCTCCAGCGCATAAATGG-3' as the reverse primer. Stock solutions were made using 228 uL TrisHCL for the forward primer and 311 uL TrisHCl for the reverse primer.

Eight samples were run on agarose gel: three for for the incubator samples at each of the time points, three for the samples placed outside with HEPES at each of the time points, one for a positive control of isolated chloroplasts, and one for a negative control of just fibroblast RNA. Complementary DNA was synthesized using Quanta BioSciences cDNA Synthesis Kit according to manufacturer's protocol. According to Quanta BioSciences, a thermal cycler is programmed for 3 cycles: 1 at 22 degrees Celsius for 5 minutes, 1 cycle at 42 Celsius for 30 minutes, and 1 cycle at 85 degrees for 5 minutes and then the cycler will be held at 4 degrees Celsius (Quanta Bioscience) cDNA would be amplified using PCR according the GoTaq DNA polymerase (Promega). There are three components to this procedure: Qscript Reaction Mix (5x), qScript Reverse Transcriptase at 20x concentration, and Nuclease free water. Table 2, below, shows primer design for the RT-PCR procedure. Samples would be held at 95 degrees

Celsius for 5 minutes, at an annealing temperature (54-60 degrees Celsius) for 15 seconds, and 72 degrees Celsius for 30 seconds. This cycle would be repeated 29 times. The Gotaq PCR procedure included a 2-minute denaturation step at 95 degrees, annealed at 5 degrees below the melting temperature of primers for 30 seconds to 1 minute, and a final extension step at 72 degrees Celsius for 1 minute for every 1 kb of DNA amplified for 5 minutes at 72 degrees Celsius. The samples were run on 1.5% agarose gel in TAE buffer with ethidium bromide at 100 volts. The gel was imaged with BioRad Image Station.

Table 2: Primer Design for RT-PCR

Primer	**Forward sequence of primer 5'-3'**	**Reverse sequence of primer 5'-3'**	**Annealing temperature (degrees C)**
rbcL.1	ATA TCT TGG CAG CAT TCC GAG	TCT CTC CAG CGC ATA AAT GG	55

Table 3 shows the cDNA set-up for 1 ug final concentration of RNA. The Quanta procedure was followed according to manufacturer's instructions. Concentrations were read on the Nanodrop and Volume of RNA and water was determined. The Promega procedure was used for PCR amplification for the gel electrophoresis. The annealing temperature of the gene of interest, rbcL is 55 degrees Celsius and has a length extension product of 1206 nucleotides (Hasebe et al, 1994).

Table 3: cDNA synthesis outline (Quanta ®)

sample	RNA (ng/uL)	RNA mass (ng)	Volume RNA (uL)	Quanta	RNA-ase free water	Total
A	8.4	134.4	16	4	0	20
B	36	134.4	3.73	4	12.27	20
C	109.291	134.4	1.23	4	14.77	20
D	47.978	134.4	2.8	4	13.19	20
E	24.309	134.4	5.52	4	10.47	20
F	100.171	134.4	1.34	4	14.65	20
Cells	43.288	134.4	3.1	4	12.89	20
chloroplast	148.544	134.4	0.9	4	15.09	20

In table 4, below, shows the reaction volumes that will used for RT-PCR according to the supplied protocols.

Table 4: Reaction Volumes for RT-PCR for a 25 uL reaction volume

Component	**Volume**	**Final concentration**
GoTaq ® Green Master Mix, 2x	12.5 ul	1X
Upstream primer, 10 uM	2.5 ul	0.1 -1.0 uM
Downstream primer, 10 uM	2.5 ul	0.1- 1.0 uM
DNA template	4 ul	<250 ng
Nuclease-Free water to	25 ul	N/A

The protocol for RT-PCR is outlined in table 5. The initial cycle went for 1 cycle. Denaturation, annealing, and extension went for 30 cycles. Final extension went for 1 cycle and held at 4 degrees. cDNA amplified product was run on an agarose gel for analysis.

Table 5: RT-PCR protocol for RT-PCR rbcL

Steps	Number Cycles	Temp (Celsius)	Duration (min)
Initial cycle	1	95	2
Denaturation	30 (denaturation, annealing, and extension)	95	1
Annealing	---	52	2
Extension	---	72	40 seconds
Final extension	1	72	5
Hold	1	4	*Infinity*

3 Results

As preliminary results, spinach was chopped with a fine blade and PBS solution was added. The solution was placed under an inverted microscope an image is shown in figure 5 below. The small circular dots that surround the larger connected cells shows individual floating chloroplasts surrounding larger unbroken cells. Each chloroplast is approximately 150 um in length.

Figure 5 Microscope imaging of intact floating chloroplasts from spinach leaves and larger unbroken cells

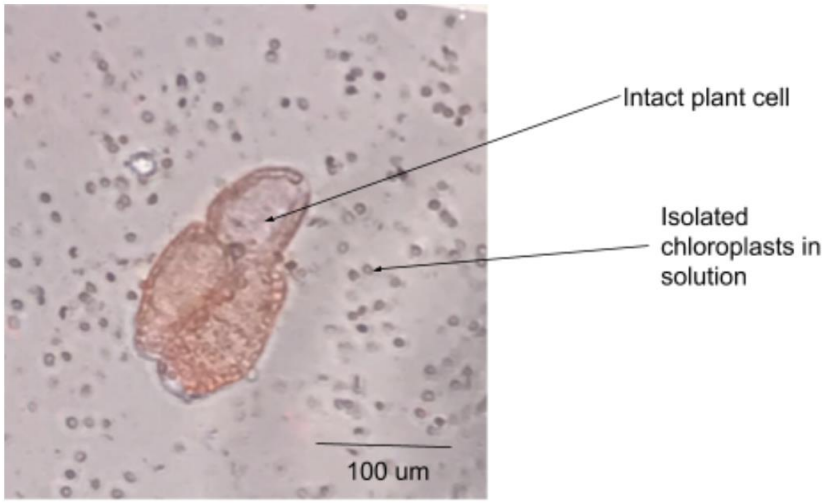

Table 4 shows the results of the Trizol experiment for isolation of RNA. Absorbancy measurements were recorded using a NanoDrop. The RNA was able to be quantified by the equation A260 * dilution * 40= ug RNA/mL. As reported in table 4, the spectrophotometer gave a read-out in units of ng/uL. The amplified PCR product were run a gel. RNA concentration results are below in table 4 as determined by Nanodrop.

Table 4: RNA from Nanodrop

sample	RNA (ng/uL)
A	8.4
B	36
C	109.291
D	47.978
E	24.309
F	100.171
Cells	43.288
chloroplast	148.544

Amplified PCR products were run a gel. The RT-PCR results are shown below in figure 6. This first lane was the 2-log ladder, the next three lanes, were samples at 24, 48, and 72 hours for the incubated samples. The following three lanes at 24, 48, and 72 hours were the experimental samples that were left outside and exposed to light. The final two lanes acted as negative and positive controls, respectively-- with cell as the negative control and chloroplasts as the positive control. The expected size of the rbcL product would be 524 base pairs. The agarose gel of the amplified rbcL gene is shown below in figure 6. Amplified PCR product can be seen in lane 9 from the positive control. Fibroblasts acted as a negative control in lane 8 and chloroplasts acted as a positive control in lane 9.

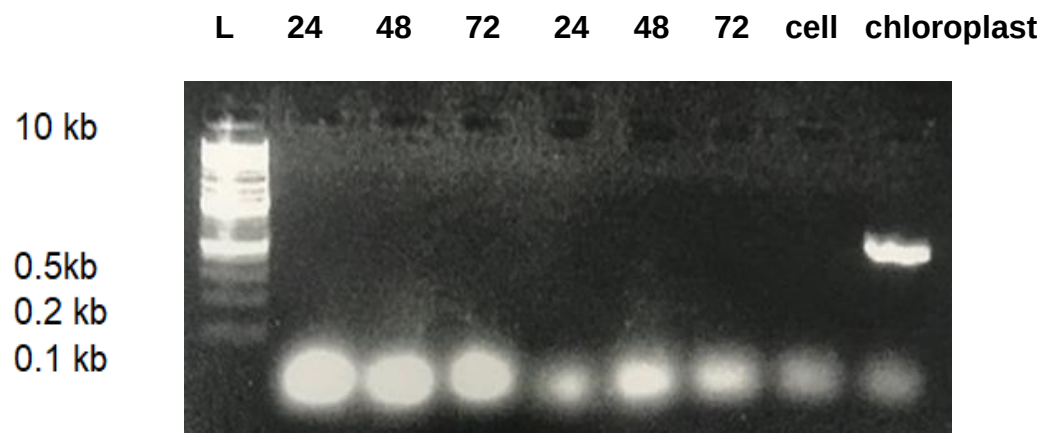

Figure 6: Agarose gel for rbcL forward and reverse primers

Legend: lane 1: ladder; lanes 2- 4: co-incubation of cells and chloroplasts at 24, 48, and 72 hours inside incubator without HEPES; lanes 5-7: co-incubation of cells and chloroplasts at 24, 48, and 72 hours with HEPES; lane 8: cell fibroblasts negative control; lane 8: chloroplasts positive control

Figure 7, below shows a representative picture of fibroblasts that were used in this study. The cells were stained with DAPI.

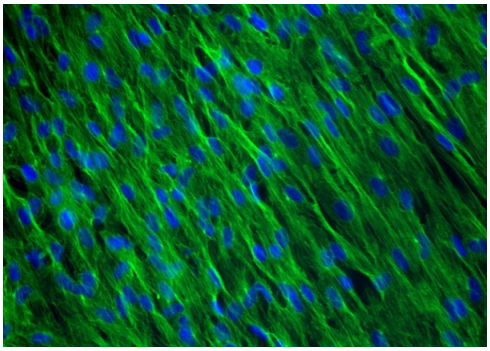

Figure 7: Fibroblast 2097s stained with DAPI and x-actin conjugated to AlexaFluor 488 (green)

Table 5 shows the time course of the sample purity. The purity of the experimental samples, positive control, and negative control are shown as the A260/280 value. A range of 1.8-2.0 is expected for high purity of samples

Table 5: Time Course of purity of samples

Sample	Average A260/A280	Sample	A260/A280 (purity)
Incubator	1.52	Cells	1.98
Outside	1.64	Chloroplasts	1.62

4 Discussion

The isolation of chloroplasts from spinach was successful. After the conclusion of the procedure, isolated chloroplasts were detected from centrifugation. A chloroplast pellet, as shown in figure 4I, can be seen. This procedure was repeated multiple times and chloroplasts were isolated each time following appropriate aseptic techniques from a procedure that was followed each time. During the procedure, an average of 300,000 cells/mL were determined by using a hemocytometer and a microscope. Live cells were counted and averaged for a total average live cells per mL. When chloroplasts were put together with 10% PBS solution and put under an inverted microscope, there were many small chloroplasts floating around were shown in figure 5. Nass et al's (1969) protocol was used to introduce chloroplasts into mammalian cells. Gene expression was present for chloroplast control sample only although not for experimental samples.

Somewhere in the course of the project, nucleic acid samples were lost although the project was deemed a success as RNA isolation yielded gene expression as seen in the agarose gel for the positive control. Future experiments could be conducted similar to this project. The RNA that was isolated showed to be moderately pure. One way to increase purity of RNA is to add a chelating agent in order to protect against RNA extraction by addition of RNAases that degrades RNA. Another was to optimize purity is to make sure the sample is thoroughly homogenized.

Fibroblast cells are a common cell line and play a fundamental role in the human body. These cells also play a crucial role in wound healing. The fibroblast also plays a critical role in wound healing as they are a part of the major connective tissue in the human body. They play a role in the maintenance of the extracellular matrix- producing glycoproteins and polysaccharides. Fibroblasts have an abundance of the rough endoplasmic reticulum and are large in size.

One method of accomplishing introduction of the chloroplast organelle would be phagocytosis of the chloroplast organelle or possibly a high-speed centrifugation to transfer the organelle into the skin cell. A staining step would also be used to stain the chloroplast organelle with carboxyflorescein diacetate or another commonly used dye and sodium ascorbate. Combination of fibroblast cell culture with isolated chloroplasts could make it possible for successful introduction of foreign organelles.

Currently, high performance kleptoplasts have been attributed to increased light exposure or improved photoprotection. This experiment aimed to corroborate this finding by showing expression of the gene rbcL through expression of RT-PCR. Further research has found an alternative explanation to heightened photosynthetic abilities: an

increased production of inorganic carbon (Serodio et al, 2014). Some parameters of the rbcL includes that the transcript length is 1428 basepairs, has a translation length of 475 residues, and one coding exon.

Some of the difficulties of this project include maintaining a suitable environment for the co-incubation procedure. This would be considered a prominent issue in the project's experimental design. When co-incubating cells and chloroplasts, the culture was contaminated after the start of the experiment for some iterations of the start of the co-incubation procedures. Extra caution needed to be taken to ensure suitable environmental conditions for proper growth of chloroplasts and fibroblast mammalian cells. A limitation in isolating chloroplasts is that at the conclusion of the isolation procedure, the chloroplast suspension is only viable for a few days. It would be important to work with a fresh batch of isolated chloroplasts.

RT-PCR in figure 6 shows the amplified rbcL gene. Unfortunately, even though that it was promising that some cells might have taken up chloroplasts, no expression was found on the agarose gel experimental groups. In this experiment, rbcL was able to be successfully amplified following a TRIzol RNA protocol and cDNA synthesis and subsequent amplification for the positive control. For the experimental groups, rbcL gene expression did not produce amplified PCR products on an agarose gel. This result raised a few key points to review at the conclusion of the projection such as using using time points closer together for TRIzol RNA extraction. Using closer time points than 24-hour intervals might be worthwhile in future research as chloroplast uptake and subsequent rbcL gene expression might be possible at 6, 12, and 18-hour time

intervals. Future work might also look into controlling oxygen conditions to promote successful uptake of chloroplasts and healthy functioning of mammalian cells.

The expression of the gene rbcL was determined by real-time polymerase chain reaction, or RT-PCR. rbcL may be one of the most abundant proteins on Earth and has been used to determine phylogenetic relationships. This gene, which is about 55,000 Daltons, encodes for the large subunit of the RuBisCo enzyme which converts CO_2 in the air into sugars and carbohydrates. rbcL has a high universality but low resolution (Wattoo et al, 2016). Another gene, rbcS, encodes for the small subunit of the Rubisco enzyme. RuBisCo is responsible for the catalysis of carboxylation, which is the first step in carbon fixation. In addition, RuBisCo is responsible for catalyzing the introduction of oxygen and RuBP. This gene is encoded by plant chloroplasts. Recently, rbcS and rbcL have been used in DNA barcoding which is a way to establish DNA-based identification of species that makes use of short genetic markers. Specialists and non-specialists alike can use barcodes obtained from tiny amounts of a tissue sample. One real world application of rbcL is using it for DNA barcoding to identify organisms by their DNA. rbcL has been approved for DNA barcoding (Little and Jeanson, 2013). The standard barcode is mitochondrial cytochrome c oxidase 1 gene (CO1). Barcoding has four components: specimens (repositories of biological material), laboratory analysis (protocols), use of databases (such as the International Nucleotide Sequence Database Collaborative and the Barcode of life Database), and data analysis (identifying and finding closest matches in known database). There has been some evidence in the past about oxygen and decreases in gene expression in modulating rbcL expression. Figure 8 shows a logo of DNA barcoding.

Figure 8: DNA barcoding

https://www.dnabarcoding101.org/lab/

Following the expression of rbcL could be useful in determining the success of introducing chloroplasts into mammalian cells. Gene expression analysis would be able to highlight the potential expression of chloroplast genes. Further work could be performed on other cell lines in addition to 2097 fibroblasts and varying oxygen content, or the effects of transcription factors on expression. Future experiments might broaden the scope of this project to more of an engineering or biochemical approach than that was performed in this project. This project shows promise for future similar experiments using a similar methodology.

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