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INTRODUCTION

Chlorophyll is the light harvesting molecule for many photosynthetic organisms. As a result, chlorophyll is an extremely important molecule, directly or indirectly, to nearly all life. The production of chlorophyll involves a long and complex pathway. The conversion of protochlorophyllide to chlorophyllide is one of the final steps in chlorophyll biosynthesis. Some of the factors affecting the regulation of this process were the focus of this project.

Currently, there are seven nuclear loci known to affect the function of protochlorophyllide oxidoreductase[1], the enzyme which catalyses the conversion of protochlorophyllide to chlorophyllide, in the alga *Chlamydomonas reinhardtii*. The chloroplast genome of *C. reinhardtii* contains three genes coding for the catalytic subunits of protochlorophyllide oxidoreductase[2]. Yet, disruption of the nuclear loci is believed to result in the inhibition of chloroplast production of the enzyme. This evidence may suggest that nuclear loci play a vital role in the regulation of chlorophyll levels in the cell by regulating the products or expression of chloroplast genes, a possible example of nuclear-chloroplast communication.

The strain of the alga *C. reinhardtii* that was studied is a transformed strain, TY 5, probably containing a mutation in one of the seven known nuclear loci affecting protochlorophyllide oxidoreductase function. This mutation was created in the laboratory of Weiyeh Wang (University of Iowa) by randomly inserting a functional Arg7 gene, which allows cells to produce the important amino acid arginine, (causing the phenotype Arg7+) into the genome of a strain of algae that contains a defective Arg7 gene (causing the phenotype Arg7-). In some of the transformed cells, the Arg7+ gene inserts into one of the seven known nuclear loci affecting protochlorophyllide oxidoreductase function, rendering that locus inoperative. The mutation causes a yellow phenotype in the dark in place of the normal green phenotype.

A normal cell is able to produce chlorophyll by both light independent and light dependent pathways. The disruption of protochlorophyllide function eliminates the cells ability to produce chlorophyll by the light independent pathways. Therefore, the yellow

phenotype in the dark is indicative of the strains inability to produce chlorophyll by light independent pathways. The inability to produce chlorophyll is most likely the result of the disruption of a nuclear encoded regulatory protein that affects the function of protochlorophyllide oxidoreductase. The success of the insertion can then be determined by screening for cells possessing the Arg7+ phenotype and that are yellow in the dark.

The specific goals for the project included the determination of which nuclear locus was disrupted, followed by the isolation and sequencing of the gene disrupted in the TY5 strain. The determination of which nuclear locus is mutated in the TY5 strain was attempted by mating the strain with other strains containing known mutations. At a low frequency, only 1%-5%, a mating will result in the formation of a diploid colony[3]. A green diploid colony is produced when the TY5 strain is crossed with another strain containing a mutation in a different locus, but a yellow diploid colony is formed when TY5 strain is crossed with a strain containing a mutation in the same locus. Using this method, the nuclear locus that has been mutated can be determined.

The isolation of the nuclear locus will be accomplished by creating a size-selected genomic library of the TY5 strain. A genomic library is formed by digesting genomic DNA with a restriction endonuclease, ligating the fragments into a vector made of plasmid DNA, and then packaging the resulting vector into phage particles. This phage then inserts the vector into a bacterium, which results in fragments of the DNA stored within bacterial cells. Each bacterium contains only one fragment of algal DNA and the entire genome should be represented by the library. The genomic library can be screened to find a clone containing the Arg7+ gene. Since the insertion of the Arg7+ gene caused the mutation in the nuclear locus, isolating a fragment of the genome containing the Arg7+ gene should contain the desired nuclear locus in the flanking DNA. Once isolated, the DNA can be sequenced and analyzed.

It is suspected that the sequencing of the gene will lead to the identification of a regulatory protein important in chlorophyll biosynthesis, specifically affecting the function of protochlorophyllide oxidoreductase. Isolation of this protein may provide significant insight into how the nucleus regulates the chloroplast and the mechanism by which the cell

regulates levels of chlorophyll. This information will help our understanding of the complex process of nuclear-chloroplast communication.

MATERIALS AND METHODS

COMPLEMENTATION TESTS:

The following procedure was used to cross strains containing a known mutation with the TY5 strain. All strains were maintained on plates containing HSA medium and grown at 25° C. The test strain and the TY5bc1 strain were placed in separate tubes containing 2 mL of N(-) liquid medium to induce gamete formation. These cultures were incubated for 24 hours. Next, 250 μL of cells from each tube were added to a sterile tube. The mixture was allowed to incubate for 4-6 hours. The culture was then plated onto an HSA plate, which was incubated for 24 hours in the light followed by 72 hours in the dark. The plates were then analyzed for the color and formation of diploid colonies. The colonies thought to be diploid were tested for the presence of quadriflagellate cells by staining the cells with iodine followed by examination with a microscope. The second test to determine if the mating test was succesful was to examine the liquid culture after mating. The presence of a pellicle, a dense mat of mating cells found on the surface of a liquid culture, is indicative of a succesful mating. (Procedure adapted from complementation test protocol pg 437-438 of The *Chlamydomonas* Source Book, by Elizabeth Harris)

GENOMIC DNA ISOLATION:

The TY5bc1 strain was placed in a liter of HSA growth medium until a dense culture was observed, normally seven days. The cells were harvested by centrifugation at 8000 rpm for 10 minutes. The cells were resuspended in 15 mL of STE(TEN) buffer. Next, 4 mL of a solution containing 1.5 mL 20% SDS, 1.5 mL 20% sarkosyl, 0.6 mL staph protease(25 mg/mL, available from Sigma) was added slowly to the solution and

incubated overnight with gentle shaking on a tilted shaker at 4.0° C. The solution was incubated with gentle shaking with 10 mL of phenol, 9.6 mL of chloroform, and 0.4 mL of iso-amyl alcohol for 30 minutes. The mixture was centrifuged at 10,000 rpm for 10 minutes. The upper phase was collected and the phenol-CIA extraction was repeated. The DNA was precipitated by adding 0.1 volumes of 3 M sodium acetate, 0.1 volumes of 0.5 M magnesium chloride, and 2 volumes of cold ethanol. The solution was incubated at -20° C for 30 minutes, followed by centrifugation at 10,000 rpm for 10 minutes. The resulting pellet was resuspended in 20 mL of TE buffer. The enzyme RNase A was added to a final concentration of 100 micrograms/mL and the solution incubated at 37° C for 45 minutes. A third phenol-CIA extraction was performed. The DNA in the upper phase was precipitated by the addition of 0.1 volumes of 3M sodium acetate and 2 volumes of cold ethanol. The solution was placed at -20° C for 30 minutes, followed by centrifugation at 10,000 rpm for 10 minutes. The supernatant was removed and the pellet washed in 70% ethanol. The pellet was then placed in a dessicator for five minutes after which it was resuspended in 500 µL of TE buffer. The DNA was quantified by measuring the absorbance of the solution at 260 nm and 280 nm in a UV spectrometer. The above protocol was developed from a procedure given by Weiyeh Wang (personal communication). (Note: wide-bore pipettes were used when transferring solutions containing DNA)

PURIFICATION OF GENOMIC DNA:

The isolated genomic DNA of the strain TY5bc1 was further purified by ultra-centrifugation. To accomplish this, 3.9 mL of 10 mM Tris pH 7.5 containing 500 µL of the isolated DNA(435 µg) was added to a Beckman centrifuge tube containing 0.52 mL of 10 X TEN, 0.65 mL of Ethidium Bromide (in buffer), and 7.8 mL of saturated Sodium Iodide. This mixture was centrifuged in a Beckman near-vertical ultracentrifuge at 44,000 rpm for 44 hours. The tube was examined under UV light. The large bottom layer, the purified genomic DNA, was isolated by puncturing the side of the tube with a needle and drawing the band carefully into a syringe. An equal volume of iso-amyl alcohol was added

and the solution was gently mixed. The top layer was then removed. Seven Iso-amyl extractions were conducted. The DNA was precipitated by diluting the mixture to a total volume of 5 mL with TE buffer, to prevent the Sodium Iodide from interfering with the precipitation reaction. Next, 0.1 volumes of Sodium Acetate and 2 volumes of cold ethanol were added to the mixture. The tube was placed at -20° C for 1 hour. The sample was centrifuged at 10,000 rpm for 10 minutes to pellet the DNA. The pellet was washed with 70 % ethanol and placed in a dessicator for five minutes. The DNA was resuspended in 300 μL of TE buffer and the yeild of DNA was quantified by determining the absorbance of the solution at 260 nm and 280 nm in a UV spectrometer. (Procedure adapted from Preparation of Nucleic Acids, pg. 610-613 of The *Chlamydomonas* Source Book by Elizabeth Harris)

SOUTHERN BLOT PROTOCOL:

The isolated genomic DNA was digested with a restriction endonuclease (Kpn I or BamH I) overnight at 37° C. The DNA fragments were separated by agarose gel electrophoresis in TBE buffer. The DNA was transferred to Zeta-probe nitrocellulose filter (Bio-Rad) by either the Capillary Transfer method or the Alkaline Transfer method.

CAPILLARY TRANSFER PROTOCOL:

The gel was immersed in 1.5 M sodium chloride and 0.5 M sodium hydroxide solution for 30 minutes with gentle shaking to denature the DNA. The gel was rinsed 3 times with doubly deionized water, followed by two, 20 minute washes in 1.0 M Tris-Cl (pH 7.5) and 1.5 M sodium chloride solution. The DNA was transferred to a Zeta-probe filter by the following apparatus (from bottom to top); a tray containing 500 mL of 10X SSC, a glass plate, a Whatman paper wick (cut to width of gel), the gel, Zeta-probe filter, a piece of Whatman paper, a stack of paper towels 2.5 inches tall, a second glass plate, and a flask containing 500 mL of water to provide weight. The capillary transfer was allowed to proceed overnight. The Zeta-probe filter was then washed for five minutes in

2X SSC. The filter was baked at 80° C for one hour to permanently fix the DNA to the filter.

ALKALINE TRANSFER PROTOCOL:

The gel was gently shaken in 0.25 M hydrochloric acid for 8 minutes, followed by two, 1 minute washes in doubly deionized water. The gel was placed in a 0.4 M sodium hydroxide solution for 30 minutes. The gel was immersed in a 3 M sodium chloride and 0.5 M Tris-Cl (pH 7.0) solution for 15 minutes. A "gel sandwich" was made by the following setup (from bottom to top); a 1 inch stack of paper towels, 3 pieces of Whatman paper, 1 piece of Zeta-probe filter, the gel, a second Zeta-probe filter, 3 pieces of Whatman paper, 1 inch of paper towels, a glass plate, and a flask containing 500 mL of water. The Whatman paper and Zeta-probe filters were placed in doubly deionized water for 5 minutes, followed by soaking for 1 minute in 20X SSC before use. The alkaline transfer was allowed to proceed overnight. The Zeta-probe filters were placed in a 5X SSC solution for 30 minutes. The filters were air dried between two pieces of Whatman paper for 2 hours. The DNA was then bound to the filter by baking at 80° C for 1 hour. (Southern Blot Analysis protocol adapted from Molecular Genetics Laboratory Manual by Farah Sogo and David Bollivar)

DIGOXIGENIN (DIG) LABELED PROBE HYBRIDIZATION PROTOCOL:

The filter was washed in hybridization buffer(5X SSC, 0.1% sarkosyl, 0.02% SDS, 1% blocking reagent(Boehringer-Manheim)) for 30 minutes with gentle agitation at 68° C or 45° C, depending upon the probe used (See below descriptions of probes used). The DIG labeled probe was denatured by boiling for 5 minutes, followed by rapid cooling in ice water. The probe (15 ng/mL) was added to hybridization buffer and mixed well. The filter was incubated with the hybridization buffer containing probe overnight at 68° C or 45° C. Next, post hybridization washes were conducted. The filter was washed in 2X SSC, 0.1% SDS solution at room temperature twice for 5 minutes each. The filter was

then placed in a 0.1X SSC, 0.1% SDS solution at 68° C or 45° C twice for 15 minutes each. (Procedure adapted from kit purchased from Boehringer-Manheim)

PROBE FORMATION:

Probes were produced from three different templates, the plasmid pSK.S180, the plasmid pArg2, and the linear pARG gene, for use in Southern Blot analysis.

Linear pARG is a copy of the Arg7 gene that was inserted to cause the mutation. The plasmid pSK.S180 is a more specific probe than the linear pARG. It contains a small fragment(180 bp) of the Arg7 gene that was formed by digestion of the Arg7 gene with Sau3 restriction endonuclease. Multiple copies of the Arg gene fragment have been cloned into the pUC.19 plasmid to produce the pSK.S180 plasmid. (pSK.S180 provided by Dr. Fitnat Yildiz)

The plasmid pJDG7 was constructed by digesting the pArg7.8 gene with the restriction endonuclease Nru I. Blunt ends of the resulting fragments were produced by the addition of T4 DNA polymerase, followed by digestion with the restriction endonuclease Hind III. These fragments were ligated into Blue Script KS+ vector digested with the restiction endonucleases Hind III and Sma I, forming the plasmid pArg2. (pArg2 provided by Dr. Fitnat Yildiz)

The Standard Labeling Protocol from Boehinger-Manheim and Probe Formation using a Thermolyne Amplitron® I thermocycler are two different methods for manufacturing the probe. Either of the two templates can be used to produce a probe by either of the two probe formation procedures.

STANDARD LABELING PROTOCOL:

A random primed probe containing DIG labeled nucleotides was formed by adding 1 µg of template DNA to 16 µL of sterile doubly deionized water. The DNA was denatured by boiling for 10 minutes and quickly chilling in a ice/ethanol bath. Next, 4 µL of DIG High Prime was added and mixed. The labeling reaction was allowed to proceed for 15 hours at 37° C. The reaction was stopped by heating the solution to 65° C for 10 minutes. (Procedure adapted from kit purchased from Boehringer-Manheim)

THERMOCYCLER AMPLIFIED PROBE FORMATION:

A probe that incorporates DIG labeled nucleotides was produced by adding 1 μL of template DNA, 5 μL of 10X PCR buffer, 4 μL of DIG High Prime Mix, 5 μL of primer(2.5 μL of AW1, 2.5 μL of AW2), and 0.5 μL of TAQ DNA polymerase. On top of this solution was added 25 μL of mineral oil. The mixture was placed into a Thermolyne Amplitron® I thermocycler for 35 cycles of 1 minute at 55° C, 2 minutes at 72° C, and 1 minute at 94° C. The mineral oil was removed and the probe transferred to a clean microfuge tube. (Procedure adapted from Weiyeh Wang, personal communication)

STRIPPING OF THE ZETA-PROBE™ FILTER:

The hybridized probes from the Zeta-Probe™ filter were removed by the following protocol to allow a second hybridization to occur. The filter was immersed in a 1 mM EDTA(pH 8.0), 1 mM Tris-Cl (pH 8.0), and 0.1x Denhardts solution at 75° C for 2 hrs. (Procedure adapted from kit purchased from Boehringer Manheim)

DETECTION PROTOCOLS:

Either the Chemiluminesence or the Color Substrate Detection methods were employed to reveal the location of the probe hybridization on the filter.

CHEMILUMINESENCE DETECTION PROTOCOL:

The filter was placed in washing buffer[Maleic acid buffer(100 mM Maleic Acid, 150 mM Sodium Chloride, 50 mM Magnesium Chloride at pH 7.5), 0.3% Tween 20] for 3 minutes. Next, the filter was incubated for 30 minutes in 100 mL of blocking solution (1X blocking reagent in maleic acid buffer). The membrane was incubated for 30 minutes in 20 mL of antibody solution (Blocking solution, anti-DIG-AP conjugate diluted 1:10,000). The filter was washed twice for 15 minutes each in 100 mL of washing buffer. The filter was equilibrated in 20 mL of AP-buffer (0.1M Tris-Cl, 0.1M sodium chloride, 50 mM magnesium chloride, pH 9.5). Next, 1 mL of CSPD solution was added to the filter. The filter was sealed in a seal-a-meal bag, and the air bubbles were removed. The bag was incubated for five minutes at room temperature, after which the excess CSPD

solution was removed. The bag was resealed and incubated at 37° C for 15 minutes. The filter was then exposed to X-ray film for 45 minutes, 2 hours, and 12 hours. (Chemiluminesence kit purchased from Boehringer-Manheim)

COLOR SUBSTRATE DETECTION PROTOCOL:

The filter was equilibrated in 20 mL of wash buffer for 1 minute. The filter was incubated for 45 minutes in 30 mL of blocking solution. Next, the filter was transferred to the antibody solution (6 µL anti-DIG-AP conjugate/ 30 mL blocking solution) for 30 minutes with gentle shaking. The filter was washed twice 15 minutes each in 100 mL of wash buffer. The membrane was equilibrated in 20 mL of detection buffer (0.1 M Tris-Cl, 0.1 M Sodium Chloride, pH 9.5) for 2 minutes. The membrane was placed in a seal-a-meal bag along with 10 mL of detection buffer containing 45 µL of NBT(4-Nitro Blue Tetrazolium Chloride, purchased from Boehringer-Manheim) and 35 µL of x-phosphate (5-Bromo-4-chloro-3-indolyl-phosphate, purchased from Boehringer-Manheim). The bag was incubated in the dark for 12 hours. (Procedure adapted from protocol developed by Boehringer-Manheim)

LIBRARY CONSTRUCTION PROTOCOL:

Isolated DNA from the TY5 bc1 strain was digested overnight at 37° C with Bgl II or BamHI restiction endonnuclease. The DNA was precipitated with 0.1 volume of 3 M sodium acetate and 2 volumes of cold ethanol. The solution was placed at -20° C for 20 minutes and then centrifuged for 10 minutes at 10,000 rpm. The DNA was ligated into the Lambda Dash II vector by incubating with T4 DNA ligase overnight at 4° C. The vector was packaged by adding 4 µL of ligated DNA to the packing extract. The packaging reaction was incubated at 37° C for 2 hours. Next, 500 µL of SM buffer and 20 µL of chloroform were added. The solution was centrifuged for 2 minutes and the top layer was kept and stored at 4° C.

TITERING THE PACKAGING REACTION:

The XL1-Blue MRA (P2) or XL1-Blue MRA strain of bacteria was grown on an LB plate overnight at 37° C. A single colony from the plate was used to inoculate 50 mL of LB supplemented with 10 mM magnesium sulfate and 0.2% maltose. The culture was grown 3-5 hours at 37° C. The culture was then centrifuged at 500x g for 10 minutes to pellet the bacteria. The pellet was resuspended in half the original volume with sterile 10 mM magnesium sulfate solution. The solution was diluted to an optical density of 0.5 at 600 nm. Next, 1 μL of the packaging reaction was added to 200 μL of the bacterial solution, and a 1:10 dilution of the packaging reaction was added to 200 μl of the bacterial solution. The mixture was incubated for 20 minutes with gentle shaking, followed by the addition of 2 mL of NZY top agar (at 48° C). This solution was immediately poured onto an LB plate. After the plate was incubated at 37° C overnight, the number of plaques were counted and the plaque forming units/mL were determined.
(Library Construction protocols adapted from Stratagene Lambda Dash II™ Packaging Kit)

CONSTRUCTION OF A SIZE-SELECTED LIBRARY:

The process of constructing the size-selected library began by digesting TY5bc1 strain genomic DNA and the Blue-script plasmid pBSKS (+) with the restirction endonuclease BamH I. In a microfuge tube, 1 μL of BamH I enzyme was added to 17 μL of ultra-purified TY5bc1 strain genomic DNA and 2 μL of Enzyme Buffer B. In a second microfuge tube, 1 μL of BamH I enzyme was added to 6 μL of pBSKS (+), 2 μL of Enzyme Buffer B, and 11 μL of doubly deionized water. Both tubes were incubated overnight at 37° C. The digested DNA was run on a 0.3% agarose gel with a 1.0% agarose gel support for 50 minutes at 95 volts.

ELUTION OF DNA:

The DNA was eluted from the gel by making an incision in the appropriate lane on the side closest to the wells just above the desired fragment. The incision was made just below the 2000 bp mark, for the pBSKS (+) the incision was made at the 2750 bp mark. A

small circular piece of Whatman (DE81) filter paper was then inserted into the incision. A current of 95 volts was applied in the opposite direction then previously applied for 15 minutes. The filter paper was then removed and stuffed into a 0.5 mL microfuge tube containing a hole in the bottom from a sterile needle. The 0.5 mL microfuge tube was inserted into a 1.5 mL microfuge tube and 100 µL of TE (Tris-EDTA, pH 8.0) was added to the 0.5 mL microfuge tube. The tubes were placed into a microfuge for 5 seconds. The flow-through was discarded. Next, 60 µL of elution buffer (2X TE) was added to the 0.5 mL microfuge tube and both tubes were centrifuged for 5 seconds. The flow-through was transferred to another microfuge tube. The addition of 60 µL of elution buffer and the transfer of the flow-through was repeated a four more times, so that 300 µL of flow-through was collected. The eluted DNA was precipitated by the additon of 900 µL of ethanol, followed by incubation of the solution at -70° C for 30 minutes. The solution was centrifuged at 13,000 rpm for 10 minutes at 4° C and the supernatant was removed. The resulting pellet was washed with 70% ethanol and placed in a dessicator for five minutes. Both pellets were resuspended in 10 µL of doubly distilled water. (Protocol adapted from Molecular Genetics Laboratory Manual, by Farah Sogo and David Bollivar)

LIGATION:

The size selected fragments from the TY5bc1 strain were ligated into the bluescript vector plasmid pBSKS (+) by the following reaction: 10 µL of TY5bc1 DNA, 5 µL of pBSKS (+), 2 µL of doubly distilled water, 2 µL of 10X ligase buffer, 1 µL of DNA ligase enzyme were placed in a microfuge tube and incubated overnight at 16° C. (Protocol adapted from Molecular Genetics Laboratory Manual, by Farah Sogo and David Bollivar)

TRANSFORMATION INTO DH5α:

The ligated plasmid was transformed into freshly made competent cells of the DH5α strain of *E. coli*. First, 20 µL of the ligated plasmid were added to 200 µL of competent cells kept on ice. The solution was then heat-shocked by incubation at 42° C

for 2 minutes. Next, 2 mL of LB broth was added and the tube was incubated at 37° C for 20 minutes. Ten LB + Ampicillin (60 mg/mL) agar plates, on which 200 μL of X-gal solution had been spread, were incubated at 37° C. Finally, 200 μL of the transformation was spread onto each of the ten LB + Ampicillin plates, followed by overnight incubation at 37° C. (Protocol adapted from Molecular Genetics Laboratory Manual, by Farah Sogo and David Bollivar)

Note: All restriction endonucleases were purchased from American Allied Biochemicals, Inc. and the T4 DNA Ligase was purchased from Promega.

Abbreviations:

A-P buffer	-	Alkaline phosphatase enzyme buffer
bp	-	base pairs
CIA	-	Chloroform, Iso-Amyl Alcohol (49:1)
DIG	-	Digoxygenin
DNA	-	Deoxyribonucleic acid
gDNA	-	genomic DNA
pDNA	-	plasmid DNA
EDTA	-	disodium ethylenediamine tetra-acetate
HSA medium	-	High Salt, Acetate growth medium
kb	-	kilobase pairs
LB medium	-	Luria-Broth growth medium
NZY medium	-	NZ amine, Yeast-extract growth medium
N(-) medium	-	Growth medium lacking nitrogen
SDS	-	Sodium Dodecyl Sulfate
SSC	-	Sodium Chloride, Sodium Citrate
STE(TEN)	-	Sodium Chloride, Tris, EDTA
TE	-	Tris-EDTA buffer
UV	-	Ultra-violet
X-gal	-	5-bromo-4-chloro-3-indoyl-β-D-glacactoside

RESULTS AND DISCUSSION

The long-range goal of this project was to isolate and sequence a nuclear locus important to protochlorophyllide oxidoreductase function. First, a number of Southern Blot analyses were conducted to ensure the presence of the inserted Arg7 gene was the cause of the mutation leading to the observed yellow phenotype in the dark.

In the first trial, isolated genomic DNA (gDNA) from the TY5bc1 strain was digested with Kpn I restriction endonuclease and transferred to Zeta-probe™ filter using the capillary transfer method. The probe for the Arg7 gene was created using a thermocycler and linear pArg as the template. The thermocycler amplified large segments of the linear pArg DNA forming a probe having a complementary sequence to the Arg7 gene. The probe was then hybridized with the Zeta-probe™ filter containing electrophoretically separated gDNA at a temperature of 65° C. In order to visualize if the probe had successfully hybridized to the Arg7 gene, a secondary hybridization reaction using an oligo-alkaline phosphatase conjugate containing a complementary sequence to the universal primer sequence found on the Arg7 probe was performed. As a result of this process the alkaline phosphatase enzyme becomes selectively associated with the Arg7 gene. The chemiluminescent substrate CPD-Star™ (Boehringer-Manheim), when acted upon by the alkaline phosphatase enzyme, emits light detectable on X-ray film. If the procedure was successful, dark bands indicating the position of the Arg7 gene are visible on X-ray film. In the first Southern Blot attempt the filter was exposed to X-ray film for 30 minutes and 24 hours, but no bands were visible on either exposure.

A second Southern Blot analysis was attempted using the same conditions, except that a randomly primed linear pArg probe was prepared using the standard labeling procedure. A probe formed in this manner contains complementary sequences of the Arg 7 gene in a wide-range of lengths and from various starting positions on the linear pArg template. Exposures of 24 hours, and 2 hours were developed. No bands were visible on any exposure.

It was suspected that the failure of the second trial was caused by the addition of insufficient primer. This error would have resulted in low levels of probe being produced. In light of this, a third Southern Blot analysis was conducted using the same conditions as

the second trial, except a new probe was formed by the standard labeling procedure of the linear pArg probe. Exposures of 24 hours, 2 hours, and 30 minutes were developed. Two faint bands were present on the 30 minute exposure, which are believed to correspond to the non-functional Arg7 gene and the inserted Arg7 gene.

One of the observed bands is the inserted Arg7 gene. The presence of the second band was expected because the TY5bc1 strain contains a non-functional copy of the Arg 7 gene. Enough sequence homology exists between the probe and the non-functional copy to result in the presence of a band. The two bands confirmed the presence of the inserted Arg7 gene and, more importantly, that only one copy of the inserted Arg7 gene was present, and leads to the conclusion that this insertion is the likely reason for the mutation causing the observed phenotype of the TY5bc1 strain.

Construction of the genomic library:

Since the successful insertion of the Arg7 gene had been confirmed, work on the next phase of the project was begun. In order to isolate the locus the Arg7 gene had disrupted, construction of a library containing the entire genome of the TY5bc1 strain was attempted.

The formation of a genomic library containing the entire genome of the TY5bc1 strain, within a 99% probability, requires (Equation 1) that 3.38 $x10^5$ recombinant phage plaques have to be screened. In other words, to ensure that the desired fragment is contained within the genomic library, the packaging reaction would have to result in a mixture having a value of 3.38 x 10^5 plaque-forming units(pfu, defined as the total plaque forming ability of a given packaging reaction)

Equation 1[4]:

$$\frac{\ln(1 - \text{Probability})}{\ln[1 - (\text{Insert Fragment Size} / \text{Total Size of TY5bc1 Genome})]} = \#\text{ of recombinant plaques to be screened}$$

$$\frac{\ln(1-.99)}{\ln[1-(12{,}000\text{ bp}/1.1 \times 10^8\text{ bp})]} = 3.38 \times 10^5 \text{ plaques}$$

The first step in the construction of a genomic library is the digestion of the TY5bc1 genome with a restriction endonuclease. After ligation of the resulting fragments into the vector Lambda Dash II™, the newly-formed recombinant phage DNA was packaged into phage particles. The phage was then allowed to infect a bacterial strain, thus inserting the vector carrying a fragment of the TY5bc1 genome into the bacterial genome. The bacteria acts as a stable carrier and amplifier of the TY5bc1 fragment. The library can be screened and phage plaques isolated that contain the inserted Arg7 gene. The gDNA from the TY5bc1 strain flanking the Arg7 insert would likely be one of the seven loci affecting the function of protochlorophyllide oxidoreductase. Despite numerous attempts and many changes and improvements to the original protocol, a library that contained the entire genome of the TY5bc1 strain, within a 99% probability, eluded construction.

In the first set of attempts, the restriction endonuclease BamH I was used to digest the genome of the TY5bc1 (Table 1). Under ideal conditions (contaminant free insert, high number of ligatable ends) the expected amount of recombinant plaques per μg of insert is 1.0×10^6 -1.5×10^7. The average pfu value of 7.9×10^5 from the control reaction was close to the expected, indicating that the procedure was successful using the provided control DNA insert. The packaging reaction using TY5bc1 as the DNA source was titered with the bacterial strains XL1-Blue MRA, which allows both recombinant and non-recombinant phage inoculation growth, and XL1-Blue MRA (P2), which only allows recombinant phage inoculation growth. The observed pfu value of 8.8×10^4 for recombinant plaques, i.e. plaques seen when XL1-Blue (P2) was transformed, was far from the needed 3.85×10^5 pfu.

Table 1:
Results of the first trial.

Trial	Bacterial Strain	DNA Source	Dilution	# of plaques/μL	pfu	avg. pfu
1	XL1-Blue MRA	Control	1:10	2200	1.1×10^6	7.9×10^5
1	XL1-Blue MRA	Control	1	960	4.8×10^5	
1	XL1-Blue MRA	TY5bc1	1:10	530	2.7×10^5	1.4×10^4
1	XL1-Blue MRA	TY5bc1	1	4	2.0×10^3	
1	XL1-Blue MRA(P2)	TY5bc1	1:10	30	1.5×10^4	8.8×10^4
1	XL1-Blue MRA(P2)	TY5bc1	1	5	2.5×10^3	

Two more attempts of the experiment under the same conditions as Trial 1 yielded similar results. One likely reason for the low titer might be a result of an inefficient ligation of the insert into the Lambda Dash II vector™. This vector optimally accepts fragments ranging from 9 kb to 23 kb. An experiment was conducted to test whether the fragments generated from the digestion of TY5bc1 fell into this range. This experiment revealed that the majority of the fragments generated by the restriction endonuclease BamH I were smaller than 9 kb, suggesting this was a probable contributor to the inefficiency of the ligation.

It was discovered that the digestion of the TY5bc1 genome by the restriction endonuclease Bgl II resulted in a wider range of larger fragments. As a result, the construction of the library was attempted using fragments generated by the restriction endonuclease Bgl II (Table 2). Trial 2 showed a 5.3% increase in the average pfu value compared to Trial 1, but still fell 2.9×10^5 recombinant plaques short of the required pfu value to construct a complete library. A second trial using fragments generated from the restriction endonuclease Bgl II, Trial 3, was attempted. Trial 3 showed a dramatic increase, nearly 51%, over Trial 2 in the ability to form recombinant plaques. Unfortunately, the average pfu value for Trial 3 of 1.9×10^5 provided only 49% of the needed recombinant plaques to confidently form the genomic library.

Table 2:
Trials 2 and 3

Trial	Bacterial Strain	DNA Source	Dilution	#of plaques/μL	pfu	avg. pfu
2	XL1-Blue MRA(P2)	TY5bc1	1:10	290	1.4×10^5	9.3×10^4
2	XL1-Blue MRA(P2)	TY5bc1	1:10	200	1.0×10^5	
2	XL1-Blue MRA(P2)	TY5bc1	1	68	3.4×10^4	
3	XL1-Blue MRA(P2)	TY5bc1	1:10	560	2.8×10^5	1.9×10^5
3	XL1-Blue MRA(P2)	TY5bc1	1	203	1.0×10^5	

There are many possible reasons for the observed low pfu value. First, a large increase in the average pfu value was seen in the trials using the restriction endonuclease Bgl II(Trials 2 and 3) over the restriction endonuclease BamH I(Trial 1). This indicates that the restriction endonuclease Bgl II resulted in more fragments within the optimal range of the vector, causing a significant increase in the average pfu value. Although an increase in the average pfu value was seen with the restriction endonuclease Bgl II, the

increase in the number of fragments generated within the required range was not high enough for the formation of a library containing the entire genome of the TY5bc1 strain.

A second possible cause of the low average pfu value seen in all the trials may be the result of the isolation of unpure gDNA. The isolated DNA most likely contains not only genomic DNA, but also chloroplast DNA. The presence of the chloroplast DNA and other contaminants could have decreased the efficiency of the ligation into the vector. The unpure DNA also lowered the amount of gDNA actually being added, thus decreasing the amount of genomic DNA actually used in experiments.

A third possible reason for the problems in constructing the library may lie in the ligase reaction itself. The efficiency of the T4 DNA ligase enzyme used in the ligation was called into question during the construction of the size selected genomic library and will be discussed in more detail in the following section.

Although it is probable that the problems could have eventually been overcome, it was decided due to financial and time constraints to try a different approach to isolate the inserted Arg7 gene and flanking DNA.

Construction of a size selected genomic library:

Since the construction of a library containing the entire genome of the TY5bc1 strain proved to be inefficient, the new goal was the formation of a library containing only fragments that are within the same size range of the desired fragment. In order to build the size-selected library, it was crucial to determine the exact size range of the fragment containing the inserted Arg7 gene. This information was determined using Southern Blot analyses.

The gDNA from the TY5bc1 strain was digested using the restriction endonuclease BamH I. The resulting fragments were separated using gel electrophoresis, and the DNA transferred to a Zeta-probe™ nitrocellulose filter by the capillary transfer method. In Trials 1 and 2 the probe used to hybridize to the Arg7 gene was formed using the template pArg2. This plasmid was formed by the digestion of the Arg7 gene with the restriction endonucleases Pst I and Sal I. The resulting ≈300 bp fragment was ligated into a Blue Script KS+ vector. This plasmid served as the template for the formation of the

probe by the randomly-primed method. In Trial 1 the probe was hybridized to the Zeta-probe™ filter at a temperature of 65° C. After the chemiluminescent detection procedure there were no visible bands on exposures to X-ray film of 5 hours and 13 hours.

In Trial 2 two changes to the protocol were made. First, the lack of signal strength in Trial 1 may have been the result of insufficient amount of the primary randomly-primed probe. In Trial 2 the amount of the primary probe was double that of the previous attempt. The second change was to optimize the probe hybridization temperature. Using Equation 2, the optimal hybridization temperature was determined to be 54.7° C. After stripping the Zeta-probe™ filter of the probes from Trial 1 both of the above changes were instituted, but after exposures to X-ray film of 4 hours, 5 hours, and 12 hours there were no conclusive bands present. On all three exposures there are possible bands, but because these are vague and undefined, the results proved to be inconclusive.

Equation 2[5]:

Tm - 20° C = optimum hybridization temperature

Tm = 81.5° C + 16.6(log [Na+]) + 0.41(fraction Guanine + Cytosine) - 0.63(% formide) - (600/L)

Tm = 81.5° C + 16.6(log 0.5102) + 0.41(0.58) - 0.63(0) - (600/300) = 74.7° C

74.7° C - 20° C = 54.7° C optimum hybridization temperature

Tm = melting temperature L = hybrid length in bp

Trial 3 was conducted using the same protocol as Trial 2 except the hybridization temperatures were lowered to 45° C in the hope that the low temperature would result in more hybridization to the Arg7 gene. Unfortunately, no bands were observed when the filter was exposure to X-ray film.

In Trial 4 a new probe was used because of the inability of the pArg2 probe to hybridize with the Arg7 gene at any of the above conditions. This probe was made by the formation of fragments complementary to the template plasmid pSK.S180 using a thermocycler. The plasmid pSK.S180 is a very specific probe for the Arg7 gene because it contains multiple copies of a 180 bp segment from the Arg7 gene ligated into a pUC19

vector. The pSK.S180 probe was hybridized to the Zeta-probe filter containing the electrophoretically separated gDNA at a temperature of 45° C. The relatively low temperature was selected because of the high specificity of the probe. Detection of the bands was attempted using the chemiluminesence method, but exposures of 30 minutes and 4 hours to X-ray film did not reveal the presence of any bands.

In Trial 4 the probe had been formed using a thermocycler, but in Trial 5 the probe was formed by the random priming of the pSK.S180 plasmid. This process results in a probe of varying length and origin from the template DNA and is consequently less specific. This probe was hybridized to electrophoretically separated gDNA bound to a Zeta-probe™ filter at a temperature of 45° C. Using the chemiluminesence detection method, no distinct bands were visible despite exposures of 40 minutes and 4 hours to X-ray film.

After five unsuccessful attempts using the Southern Blot analysis, a new approach was implemented. Southern Blots failed using probes derived from two templates, formed in two different manners, and hybridization of the probe to the target DNA was attempted under various conditions, the isolated DNA may not only contain gDNA but also chloroplast DNA and other contaminants. In order to ensure that contaminated DNA would not be a source of error for future Southern Blot attempts, the gDNA was carefully re-isolated by the original protocol and further purified by ultra-centrifugation. The genomic DNA was purified by centrifugation at 44,000 rpm for 44 hours in a solution high in Sodium Iodide. The centrifuging created salt gradients and the contents were separated based upon density. Two distinct bands were observed and the large lower band containing the purified gDNA was isolated. This highly purified gDNA was used for the remainder of the project.

Another reason for the unsuccessful Southern Blots may lay in the failure of the chemiluminescent detection method. Possible bands were seen in two of the trials, indicating that some primary hybridization had occurred, but the failure to visualize distinct bands may have been the result of poor resolution of the chemiluminescent method in conjunction with the probes and conditions used. Since the resolution capability of the chemiluminescent method was in doubt, an alternative detection method was employed.

The manner in which the color substrate detection method acts is very similar to the detection procedure used in Western(protein) Blots. Nucleotides labeled with DIG were incorporated into the probe during its formation. After the probe has hybridized to the target DNA, an antibody conjugated with the Alkaline Phosphatase enzyme is applied to the Zeta-probe™ filter. The antibody selectively binds to nucleotides labeled with DIG, causing the alkaline phosphatase enzyme to become associated with the target DNA. Two compounds, NBT and X-phosphate, produce purple bands at the site of the target DNA when acted upon by the Alkaline phosphatase enzyme.

The first attempt using the color-substrate system, Trial 6, and the ultra-purified gDNA from the TY5bc1 strain was disappointing. The probe was formed by the random-priming of the pSK.S180 plasmid and the hybridizations were conducted at 68° C.

A second attempt using the randomly-primed pSK.S180 probe and color substrate system was attempted, but in Trial 7 the hybridizations were conducted at 45° C. The lower temperature decreases the specificity of the probe, but also increases the level of hybridization. The greater amount of hybridization should cause a strong enough signal to be resolved. Unfortunately, Trial 7 failed to yield any visible bands.

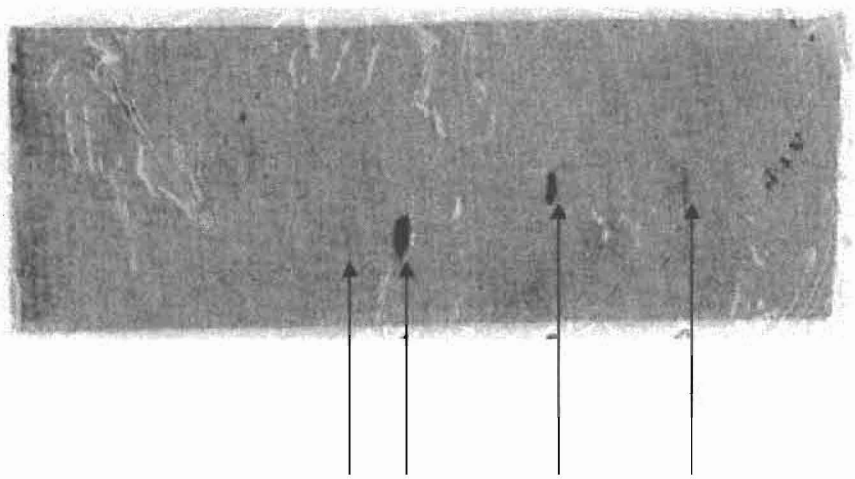
Trial 7 made it apparent that the probe was the probable reason for the failures of the attempts with the color substrate system. Due to its purity, it was known that the gDNA was not the cause of the failed attempts and there was no reason to suspect the color substrate system was at fault. Therefore, a new probe was made using a thermocycler to form large fragments complementary to the linear pArg template. This probe was less specific than the pSK.S180 and would have greater primary hybridization, hopefully resulting in a visible signal. The linear pArg derived probe was hybridized to the Zeta-probe filter at 65° C. Finally, after 7 trials, a successful Southern Blot was obtained (Figure 1). Three distinct bands are visible; the control of linear pArg DNA, and two bands in the lane corresponding to the digested gDNA from the TY5bc1 strain. The control shows that primary probe hybridization occurred with DNA containing the Arg7 sequence. The other two bands, corresponding to fragments sized 2000 bp and 1000 bp, are the non-functional copy of the Arg7 gene and the inserted Arg7 gene of the TY5bc1 strain. (For summary of Southern Blot Analyses, see Table 3)

Table 3:
Southern Blot Analyses of TY5bc1

Trial	gDNA Source	Probe Template and Method of Formation	Hybridization Temperature	Detection Method	Results
1	TY5bc1	pArg2, random-priming	65 °C	Chemi-luminescence	No bands
2	TY5bc1	pArg2, random-priming	54.7 °C	Chemi-luminescence	No bands
3	TY5bc1	pArg2, random-priming	45 °C	Chemi-luminescence	No bands
4	TY5bc1	pSKS.180, thermocyler amplification	45 °C	Chemi-luminescence	No bands
5	TY5bc1	pSKS.180, random priming	45 °C	Chemi-luminescence	No bands
6	TY5bc1 (ultra-purified)	pSKS.180, random priming	68 °C	Color Substrate	No bands
7	TY5bc1 (ultra-purified)	pSKS.180, random priming	45 °C	Color Substrate	No bands
8	TY5bc1 (ultra-purified)	linear pArg, thermocyler amplification	65 °C	Color Substrate	Two bands (2000 bp, 1000 bp)
9	Arg7 mt+	linear pArg, thermocyler amplification	65 °C	Color Substrate	One band (2000 bp)

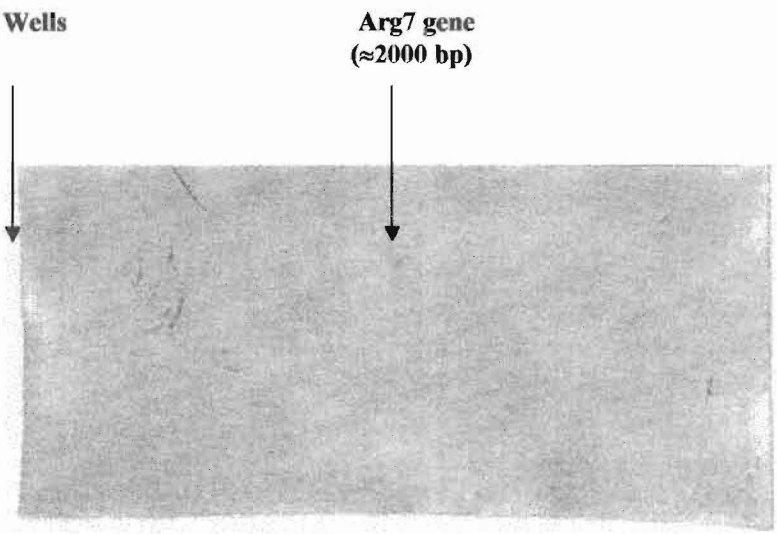
It is impossible to determine which band is the inserted Arg7 and which is the non-functional copy from this Southern Analysis. Therefore, gDNA from the Arg7+ strain (a strain differing only in the absence of the inserted Arg7 gene) was isolated. A Southern Analysis using the same conditions as Trial 8 revealed a single band (Figure 2). This band represents the location of the non-functional Arg7 gene with a fragment size of 2000 bp. Based on this information the fragment containing the inserted Arg7 gene is 1000 bp when digested with the restriction endonuclease BamH I.

Figure 1:
Southern Blot Analysis (Trial 8) of TY5bc1 gDNA.



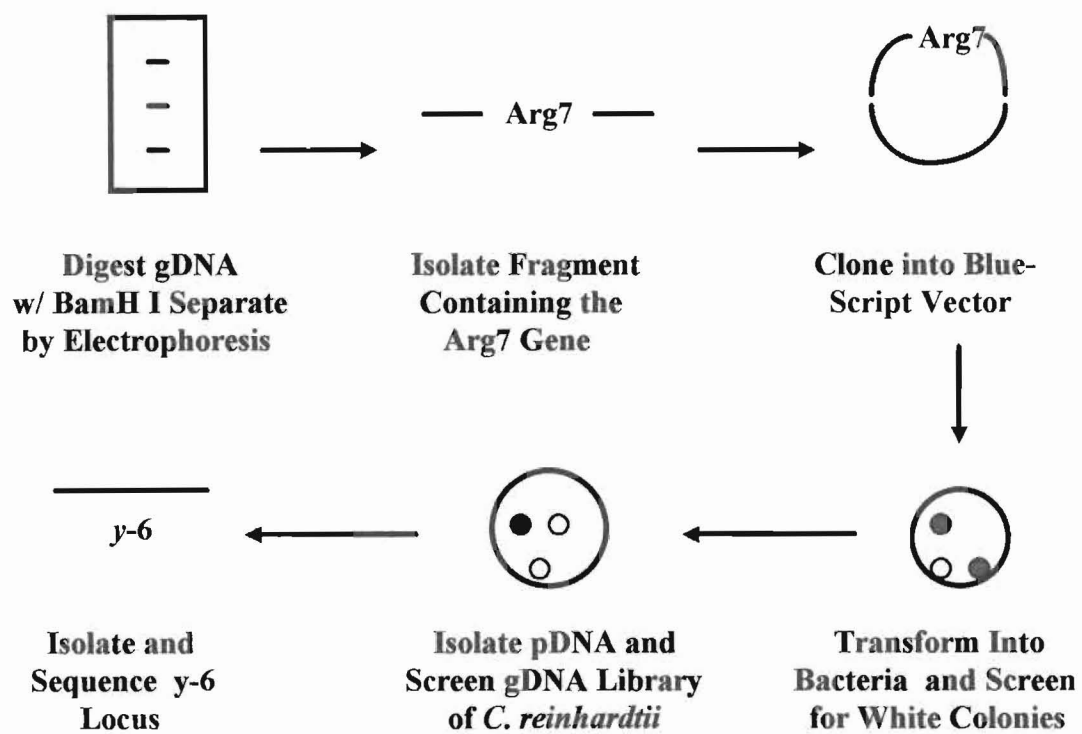
Note: Although faint, fragment 2 was clearly visible on the actual filter.

Figure 2:
Southern Blot Analysis (Trial 9) of Arg7+ strain gDNA



The next step in the formation of the library was to isolate the fragments within the range known to contain the inserted Arg7 gene. This was accomplished by separating the fragments produced by the restriction endonuclease BamH I by gel electrophoresis. The fragments in the desired range were run onto a piece of filter paper having a positive charge. The negatively charged DNA binds to the filter paper, followed by elution of the DNA from the filter paper using high salt concentrations. Next, the isolated fragments were ligated into a Blue Script vector that allows blue-white colony selection. Blue-white colony selection allows the colonies containing recombinant plasmids to be differentiated from those where recombination was unsuccessful. A blue colony is indicative of no recombination occurring because the plasmid still has a functional Lac-Z gene, which produces the β-galactosidase enzyme that acts on the substrate X-gal, resulting in the blue appearance of the colony. In a white colony recombination must have occurred because the recombinant insert has disrupted the Lac-Z gene, causing the white phenotype of the colony (Figure 3).

Figure 3:
Construction of a Size-Selected Library



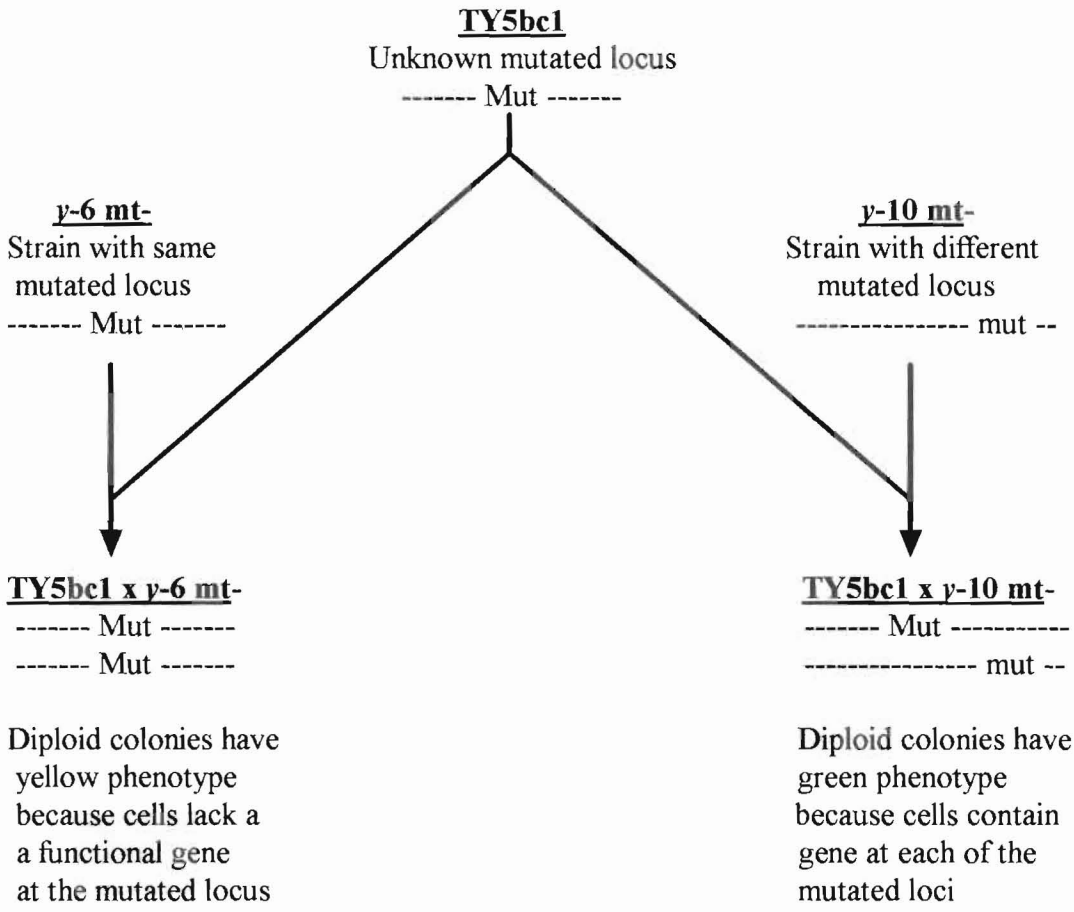
The construction of the size-selected library using the Blue-script vector was attempted twice. Both trials resulted in a lack of any colonies when grown on LB+Amp agar plates for 24 hours at 37° C. The absence of any colonies raised doubts about the efficiency of the ligation reaction. In order to test the efficiency of the ligation, the Blue-script vector was digested with the restriction endonuclease BamH I. The re-ligation of the Blue-script vector, which should result in large numbers of blue colonies, yielded no bacterial colonies. The most likely cause for the failure of the library construction, was due to either contaminated or inefficient ligase enzyme. Although much progress was made towards the goal of making the size-selected library, further work on the project ended after the second attempt of the library formation.

Complementation Tests:

The Arg7 gene was randomly inserted into the genome of the TY5 strain; as a result, it was necessary to determine exactly which locus had been disrupted. Disruptions of any of the seven nuclear loci known to affect the function of the enzyme protochlorophyllide oxidoreductase, can lead to a yellow phenotype in the dark. Complementation tests that would reveal which locus had been mutated in the TY5bc1 strain were conducted.

The alga *C. reinhardtii* is haploid for the majority of its life cycle, becoming diploid only by mating. In a complementation test, haploid individuals are induced to mate and the phenotype of the diploid cells are examined. The strain with the unknown mutated locus, TY5bc1, is induced to mate with strains containing a known mutation in one of the seven nuclear loci affecting protochlorophyllide oxidoreductase function. If the resulting diploid cells are green in the dark, the TY5bc1 strain must have a different mutated locus than the test strain because the diploid cells must contain at least one functional copy of the genes affecting the conversion of protochlorophyllide to chlorophyllide. If the diploid colonies are yellow in the dark, this indicates both the TY5bc1 strain and the test strain have a mutation in the same locus because the diploid cells lack a functional copy of a gene required for light independent chlorophyll biosynthesis (see Figure 4).

Figure 4:
Explanation of Complementation Tests;



Preliminary evidence suggests that the gene at the locus *y*-6 is the disrupted locus in the TY5 strain. A yellow colony was observed when the strain TY5 was crossed with the strain *y*-6 mating type minus (mt-), indicating that the two strains have the same mutated locus and the strains are of opposite mating types. These data are considered preliminary as a result of not being able to determine the presence of quadriflaggelate cells, an indicator of diploid cells. The strain TY5bc1 was crossed with *y*-1 mt+, *y*-1 mt-, *y*-5 mt+, *y*-5 mt-, *y*-6 mt+, *y*-6 mt-, *y*-7 mt+, *y*-7 mt-, *y*-8 mt+, *y*-8 mt-, *y*-10 mt+, and the *y*-10 mt- strain. The results of these crosses appear in Table 4.

Table 4
Results of Complementation Tests

Strains crossed	*Results*
y-6 mt- x TY5bc1	Yellow colonies
y-5 mt- x TY5bc1	Green colonies
y-6 mt- x *y*-7 mt+	Green colonies

From these results it is clear that the TY5 strain is mating type + and it is likely the mutated locus in the TY5 strain is the *y*-6 gene. The cross of the strain *y*-6 mt- with the strain *y*-7 mt+ served as a control reaction. Crossing the *y*-6 mt- strain with *y*-7 mt+, produced the expected green colonies. This evidence confirms that a cross with a strain of opposite mating type and different mutated locus does not produce yellow colonies, and supports the preliminary conclusions.

SUMMARY AND FUTURE WORK

Significant progress towards the goal of isolating and determining the nature of a mutated locus of the TY5bc1 strain has been made. The first hurdle overcome was confirming that the inserted Arg7 gene was the source of the mutation in the TY5bc1 strain, and that only one copy was present in the strains genome. This goal was accomplished by Southern Blot analysis of gDNA digested with the restriction endonuclease Kpn I.

The second significant finding was to determine the identity of the disrupted locus using complementation tests. It was found that the TY5bc1 strain is mating type + and most likely has a mutation in the *y*-6 locus.

The actual isolation of the mutated locus was first attempted by the construction of a library containing the entire genome of the TY5bc1 strain. Although much improvement was observed in the formation of this library, this approach proved to be unfeasible.

The construction of a size-selected library showed more promise. Southern Blot analysis using gDNA digested with the restriction endonuclease BamH I revealed that the inserted Arg7 gene is part of a 1000 bp fragment. It is also worth noting that probe which was constructed using a thermocycler and linear pArg as the template hybridized to the

Arg7 gene and was detected efficiently using the color substrate detection method. This combination proved successful while many other probes and the chemiluminescent detection method all failed to work.

Unfortunately, attempts at cloning these fragments into a Blue Script vector were unsuccessful. Since the control ligation showed that the ligase enzyme was inefficient, the failure to construct the size-selected library was probably the result of contaminated or inefficient ligase enzyme. Because this project has laid the groundwork for the construction of a size-selected library, future trials should result in the formation of this library. The library can be screened to locate colonies containing the fragment of gDNA with the Arg7 gene. From these colonies, the fragment containing the Arg7 gene can be isolated and used to screen a genomic library of *C. reinhardtii*. Screening this library using the isolated fragment as a probe will result in finding colonies containing the locus, likely the *y*-6 locus, that had been mutated. The gDNA from these colonies can be isolated and sequenced. The sequence of this locus affecting protochlorophyllide oxidoreductase function can be compared against a database of known proteins. This search will determine whether an analogous protein is already known or whether a novel protein has been discovered. Regardless, it is likely this protein is a regulator of chlorophyll biosynthesis and the information gained from the sequence of the locus may provide important insights into the nature of nuclear-chloroplast communication.

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- Angie Piper backcrossed the TY 5 strain to form the TY 5bc1 strain used in the project.

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