4-2015

His-Tagged and Myc-Tagged βCA Fusion Genes May Aid in the Characterization of the Role of Carbonic Anhydrases in Photosynthesis

Dequantarius Speed

His-Tagged and Myc-Tagged βCA Fusion Genes May Aid in the Characterization of the Role of Carbonic Anhydrases in Photosynthesis

By

Dequantarius Speed

Undergraduate Honors Thesis under the direction of

Dr. James Moroney

Department of Biological Sciences

Submitted to the LSU Honors College in partial fulfillment of

the Upper Division Honors Program

April, 2015

Louisiana State University & Agricultural and Mechanical College

Baton Rouge, LA

Table of Contents

Abstract

Carbonic anhydrases (CAs) are a family of enzymes that catalyze the interconversion of CO_2 and HCO_3^-. CAs are necessary in biological systems because the uncatalyzed interconversion between CO_2 and HCO_3^- occurs at too slow a rate to sustain many physiological processes. It has been established that CAs play a critical role in the CCM of cyanobacteria, green microalgae, and C4 plants. However, the role of CA in C3 plant photosynthesis remains to be determined.

In *Arabidopsis thaliana*, there are fourteen carbonic anhydrase genes and there is evidence that several of the CAs function to increase the diffusion of CO_2 through the mesophyll cells to increase the [CO_2] around Rubisco. Due to the competing oxygenase reaction catalyzed by Rubisco, photosynthesis is largely limited by the environmental [CO_2]. Long et. al (Science 312: 5182, 2006) demonstrated a 14% increase in crop yields by increasing the external [CO_2] to 600 ppm around soybean crops. Our laboratory is interested in the factors that regulate the diffusion of CO_2 into and out of the plant mesophyll cells. To this end, we wish to ascertain the function and tissue-specific and sub-cellular localization of each CA isoform. In this thesis, work is presented on the cloning of myc-tagged and his-tagged version of four βCAs. We have generated entry vectors encoding his-tagged fusions of βCA2 and βCA4.1 and myc-tagged fusions of βCA2, βCA4.1, and βCA4.2. We have also generated destination vectors for the expression of the his-tagged βCA2 and the myc-tagged βCA4.1 in *E. coli*. The goal of this work is to use purified his-tagged proteins as antigens to obtain antibodies against the CAs. In addition, if the myc-tagged CAs retain sufficient catalytic activity, they will be used to follow the genes when they are introduced back into higher plants for genetic complementation.

Introduction

The enzyme Rubisco uses CO_2 to catalyze the carboxylation of ribulose-1,5-bisphosphate in the first step of the Calvin-Benson Cycle. Rubisco may also utilize O_2 to oxygenate ribulose-1,5-bisphosphate in a wasteful process leading to photorespiration (Lorimer and Andrews, 1973; Moroney et al., 2013). Photosynthetic organisms living in environments susceptible to low [CO_2] and drought have evolved biochemical and structural changes that ultimately function to increase the internal [CO_2] and water use efficiency (Badger and Price, 1994; Moroney et al., 2007; Von Caemmerer et al., 2004). Examples of this adaptation include the C4 photosynthesis in corn (Ehleringer et al., 1997) and the CAM type photosynthesis in pineapples and cacti (Crayn et al., 2004; Nobel and Hartsock, 1986).

Carbonic anhydrases (CAs) are zinc-containing metalloenzymes that catalyze the reversible hydration of CO_2 to HCO_3^-. CAs have been classified as α, β, γ, or δ types based on their distinct amino acid sequences and each class appears to have evolved independently (Hewett-Emmett and Tashian 1996; So et al. 2004). While CAs are ubiquitous, the four classes are found in specific groups of organisms. To date, the δCAs have been found solely within the marine diatom *Thalassiosira weissflogii* (Park et al., 2007; Roberts et al., 1997) but Soto et al. (2006) have reported preliminary characterizations of a putative δCA cDNA in the marine alga *Emiliania huxleyi*. Animals contain only the α class of CAs but plants contain α, β, and γ CAs (Badger and Price, 2003; Fabre et al., 2007: Hewett-Emmett and Tashian, 1996).

CAs are required for biological systems because the un-catalyzed interconversion of CO_2 and HCO_3^- is slower than the observed rates of CO_2 fixation by Rubisco within living cells (Badger and Price, 1994; Khalifah, 1971). Due to the presence of high-levels of functional CA genes in leaves, it has been long hypothesized that CA plays some role in photosynthesis. Carbon concentrating mechanisms (CCMs) have been discovered in some aquatic photosynthetic

organisms when [CO_2] is limited as the diffusion of CO_2 in water is 10,000 times slower than the diffusion of CO_2 through air. It has been discovered that CAs function in the CCM of cyanobacteria, green microalgae, and C4 plants (Badger and Price, 2003; Moroney et al., 2011). CAs are absolutely necessary for photosynthesis in cyanobacteria, green microalgae, and C4 plants and their expression is highly regulated (Badger and Price, 1994; Burnell and Hatch, 1988; Price et al., 2008; Von Caemmerer et al., 2004).

To compensate for the low dissolved [CO_2] in many aquatic environments, cyanobacteria and several species of green microalgae have evolved a CCM that is heavily dependent on the presence and activity of specific CA isoforms (Badger and Price, 1994; Badger and Price, 2003; Moroney et al., 2007; Moroney et al., 2011; Moroney et al., 2013). Studies of the model microalga *Chlamydomonas reinhardtii* have proven immensely useful in the understanding of CAs and the roles they may play in various CCMs (Badger and Price, 2003; Moroney et al., 2007; Moroney et al., 2011; Spalding et al., 1983).

C4 plants, comprising roughly 3% of terrestrial plant species, have evolved a specialized leaf structure called "Kranz anatomy." By restricting Rubisco to the inner bundle sheath cells and shuttling CO_2 to Rubisco in the form of a 4-carbon (C4) acid such as malate, the Kranz anatomy serves to concentrate CO_2 around Rubisco. CAs are restricted to the mesophyll cells and convert CO_2 to HCO_3^- which is subsequently fixed to phosphoenolpyruvate (PEP) by PEP carboxylase (PEP-C) to generate a C4 acid. The C4 acid is shuttled to the bundle sheath cells where it is decarboxylated to PEP and CO_2. CO_2 is then fixed by Rubisco in the Calvin Cycle. To date, no CCM has been discovered in C3 plants but there is much interest in the genetic engineering of a CCM within some C3 crop species such as rice (Karki et al., 2013; Leegood, 2002).

In *Arabidopsis thaliana*, there are fourteen carbonic anhydrase isoforms that have been identified: eight αCAs and six βCAs (Table 1). The expression patterns of αCA1-3 and βCA1-6 have been partially characterized by Fabre et al. (2007). Functional genes encoding βCA1, βCA2, βCA4, and βCA5 were strongly expressed in aboveground tissues with little expression detected in the roots while βCA3 and βCA6 were expressed in the roots with stronger expression in the aboveground tissues (Fabre et al., 2007). Analyses of *A. thaliana* T-DNA knockouts for single CAs suggest compensatory relationships among some of the proteins (Robert DiMario, unpublished). *βCA5*$^{-}$ mutants are the only single mutants that display a noticeable growth phenotype (Robert DiMario, unpublished). Noticeable growth phenotypes have been observed in double mutants. *βCA1*$^{-}$*/βCA4*$^{-}$ *A. thaliana* double mutants show increased stomatal density and impaired CO_2-regulation of stomatal movements (Hu et al., 2010). Using the subcellular localization and expression data of the various *A. thaliana* CAs, multi-knockout T-DNA lines lacking CAs localized to the same subcellular region were created to eliminate compensation effects; in particular, the *βCA2*$^{-}$*/βCA4*$^{-}$ double mutant displayed reduced growth phenotypes (Robert DiMario, unpublished).

To understand the role each CA isoform may play in photosynthesis, our laboratory has generated and studied the phenotypes of *A. thaliana* mutants deficient in one or more gene(s) coding for several βCA isoforms. While GUS- and GFP-CA gene fusions have been useful in characterizing the subcellular localizations of some αCAs and βCAs (Fabre et al., 2007; Robert DiMario, unpublished), analysis of the complementation of *A. thaliana βCA*$^{-}$ mutants is hindered by the lack of available methods to observe the stable expression and translation of βCAs. We are taking two separate approaches to overcoming this obstacle (Figure 1).

Table 1. A list of the *Arabidopsis* αCAs and βCAs and their subcellular locations.

Name	Gene	Location
αCA1	At3g52720	Chloroplast[3]
αCA2	At2g28210	Cell wall
αCA3	At5g04180	Unknown
αCA4	At4g20990	Unknown
αCA5	At1g08065	Unknown
αCA6	At4g21000	Unknown
αCA7	At1g08080	Unknown
αCA8	At5g56330	Unknown
βCA1	At3g01500	Plasma Membrane[10]/Chloroplast[8]
βCA2	At5g14740	Cytosol[8]
βCA3	At1g23730	Cytosol[8]
βCA4	At1g70410	Plasma Membrane[8]
βCA5	At4g33580	Chloroplast[8]
βCA6	At1g58180	Mitochondria[8]

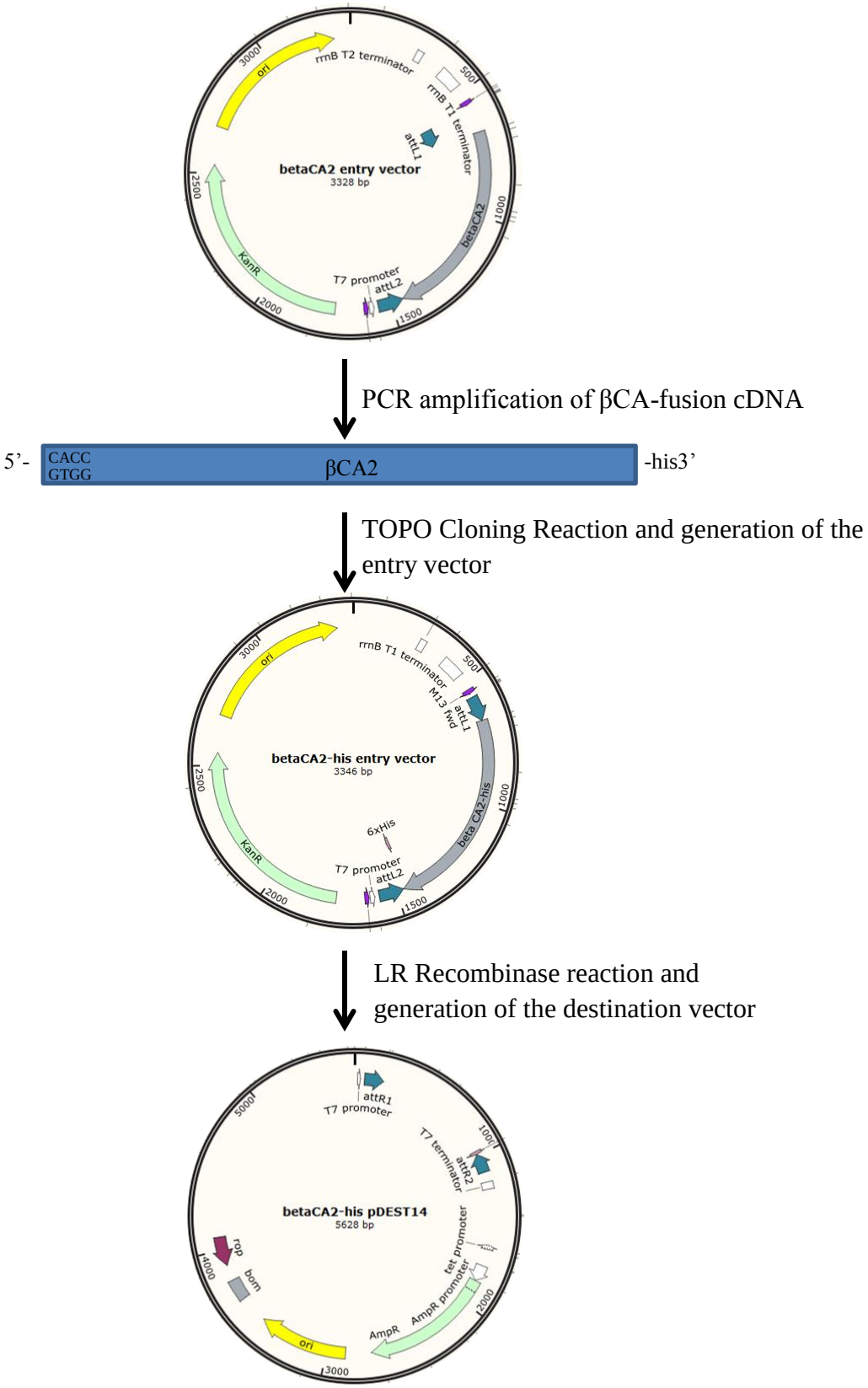


Figure 1. Simplified Overview of the steps leading to the cloning of a βCA-fusion gene into the destination vector for expression. The gene encoding the his-tagged βCA2 is used as an example.

Our first approach aims to use entire βCA proteins as antigens to generate antibodies with high affinity to specific βCAs. To this end, I set out to generate 6xHis-tagged βCA gene fusions (βCA-his). The his-tagged protein can then be transformed into *E. coli* for overexpression. The 6xHis tag will allow purification by Ni^{2+}-column chromatography (Khan et al., 2006). The 6xHis tag can be enzymatically cleaved from the mature protein before further purification by FPLC (Sheehan and Sullivan, 2004). The purified protein can then be sent to an antibody-raising facility to generate antibodies specific to each βCA isoform.

The second approach sought to take advantage of the commercial availability of antibodies to myc-epitope tags to allow Western Blot analysis of the stability of βCA expression for the analysis of the *βCA*$^{-}$ mutant complementation studies. If myc-tagged βCA (βCA-myc) protein fusions retain sufficient enzymatic activity, they may be transformed into *A.thaliana βCA*$^{-}$ mutants for complementation analysis. Before transforming plant lines with the βCA-myc proteins, the enzyme's activity can be measured by transforming each βCA-myc gene into an *E. coli* cell line for overexpression and production of sufficient amounts of βCA-myc for *in vitro* determination of CA activity of crude cell extracts by the electrometric method (Wilbur and Anderson, 1948).

For my thesis project, I set out to generate 6xHis and myc-tagged gene fusions of βCA2, βCA4.1, βCA4.2, and βCA5. The designation βCA4.1 corresponds to the CDS of *Arabidopsis* βCA4 with a leader sequence that directs the protein to the plasma membrane. The designation βCA4.2 corresponds to the CDS of Arabidopsis βCA4 without the leader sequence. Table 2 lists each gene fusion.

Table 2. A list of each of the βCA-fusion genes and their predicted sizes.

βCA-fusion gene	**Expected Fragment Sizes (bp)**
βCA2-myc	814bp
βCA2-his	802bp
βCA4.1-myc	877bp
βCA4.1-his	865bp
βCA4.2-myc	811bp
βCA4.2-his	799bp
βCA5-myc	940bp
βCA5-his	928bp

Materials and Methods

Plasmid Vectors and *E. coli* cell lines

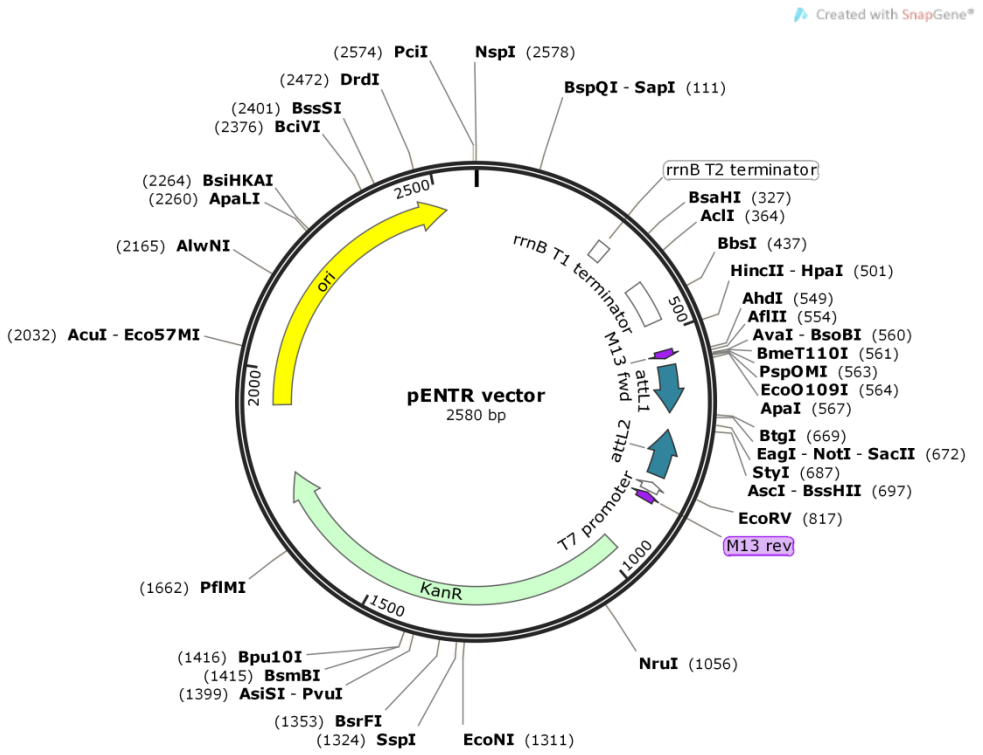
The pENTR/D-TOPO Cloning Kit ordered from Invitrogen contains 20µL pENTR/D-TOPO vector (15-20ng/µL), 10µL 12.5mM dNTPs, salt solution (1.2M NaCl; 0.06M $MgCl_2$), 1mL sterile water, 20µL each of the M13 forward and reverse sequencing primers (0.1µg/µL dissolved in TE Buffer, pH 8.0), OneShot TOP10 chemically competent *E. coli,* S.O.C. medium (2.0% tryptone, 0.5% yeast extract, 10mM NaCl, 2.5mM KCl, 10mM MgCl2, 10mM MgSO4, 20mM glucose dissolved in dH2O), and a pUC19 control plasmid. The pENTR/D-TOPO vector is compatible with Invitrogen's Gateway Cloning System and features M13 forward and reverse priming sites and a kanamycin resistance gene (Figure 2).

The pDEST14 vector was acquired from Invitrogen (Figure 2). The vector is compatible with Invitrogen's Gateway Cloning System. The vector features a T7 promoter and an ampicillin resistance gene coding for β-lactamase.

One ShotTOP10 chemically competent *E. coli* (F- mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 Δ lacX74 recA1 araD139 Δ(araleu)7697 galU galK rpsL (StrR) endA1 nupG) cells were obtained from Invitrogen. hsdR allows efficient transformation of unmethylated DNA from PCR amplifications. mcrA allows efficient transformation of methylated genomic DNA. LacZΔM15 allows blue/white color screening of recombinant clones. endA1 eliminates nonspecific digestion by endonuclease I. recAI reduces non-specific recombination.

The BL21(DE3)pLysS (F- ompT gal dcm lon hsdSB(rB- mB-) λ(DE3) pLysS(cam^R)) *E. coli* cell line was a gift from Dr. John Larkin. The BL21pLysS *E. coli* cell line carries the pLysS cam^R plasmid that expresses T7 lysozyme to inhibit leaky expression of genes under the control of a T7 promoter. The plasmid also carries the T7 RNA polymerase gene under control of the

A



B

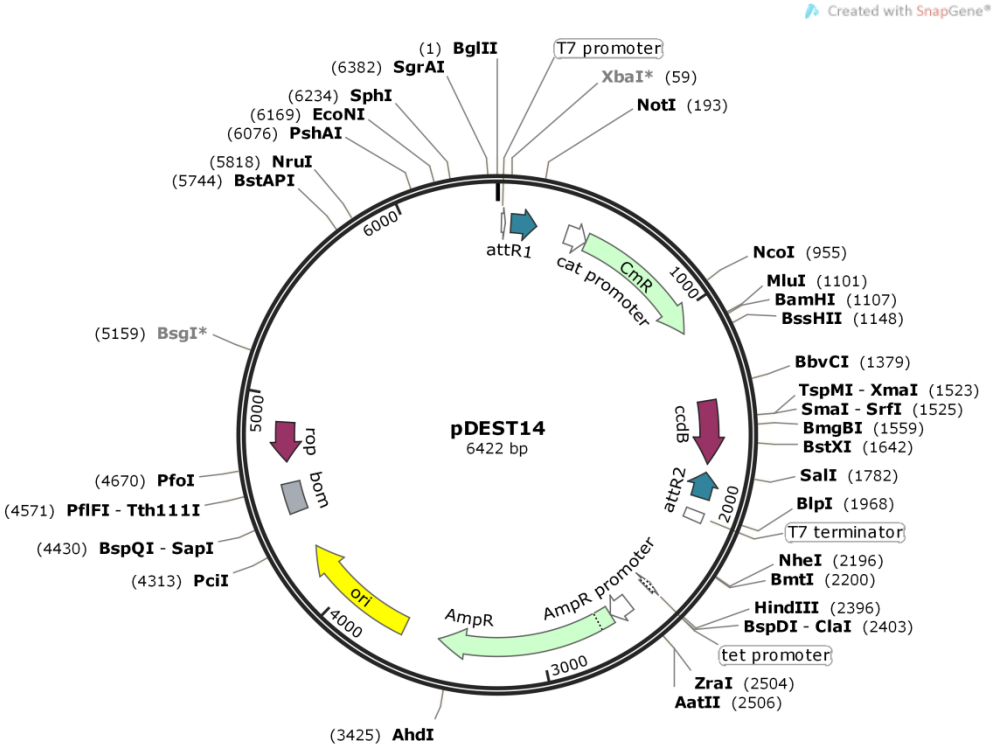


Figure 2. Annotated plasmid vector maps. A: pENTR/D-TOPO vector map. B: pDEST14 vector map.

lacUV5 promoter. T7RNA polymerase-driven expression is repressed until induction by IPTG. camR confers resistance to chloramphenicol. Chloramphenicol is a necessary component of the growth medium in order to maintain the pLysS camR plasmid within the cell line.

Primers used for cloning of βCA c-terminal fusion variants

Primers were designed to complement the 3' terminus of the CDS with the sequence CAG ATC CTC TTC TGA GAT GAG TTT TTG TTC inserted after the initial 5' codon to fuse a myc epitope-tag to the carboxy-terminus of the βCA variants. Primers were designed to complement the 3' terminus of the CDS with the sequence GTG GTG GTG GTG GTG GTG inserted after the initial 5' codon to fuse a 6xHis-tag to the carboxy-terminus of the βCA variants. CACC was attached to the 5' terminus of the forward primers to enable compatibility with the Gateway cloning system (Invitrogen). Primers were acquired from IDT and diluted to a 100μM concentration in 1X TE buffer (10mM Tris-HCl, pH 8.0; 0.1mM EDTA) (Table 3).

PCR Amplification of βCA-fusion constructs

The coding DNA sequence (CDS) for *A. thaliana* βCA2, βCA4.1, βCA4.2, and βCA5 were amplified by the polymerase chain reaction (PCR) from an entry vector obtained from Robert DiMario in the Moroney Laboratory. Phusion (2X) DNA Polymerase Master Mix was acquired from NEB. 50μL reactions were prepared by mixing 21.5μL dH_2O, 1.5μL DMSO, 0.5μL forward primer, 0.5μL reverse primer, and 1μL βCA-fusion PCR product with 25μL Phusion (2X) Master Mix. Reactions were carried out using a Bio-Rad T100 Thermal Cycler. The amplification protocol for βCA2-myc was as follows:

1. Initial denaturation at 98^{o}C for 3 minutes.
2. Cycle #1 – repeat five times.
    a. Denaturation at 98^{o}C for 30 seconds.

Table 3. Primers used in the PCR amplification of the βCA-fusion genes.

Primer Name	**Primers** (5' → 3')
βCA2forward	CACC ATG GGA AAC GAA TCA TAT GAA GAC GCC ATC GAA GCT CTC AAG
βCA2myc	TCA CAG ATC CTC TTC TGA GAT GAG TTT TTG TTC TAT AGA ATG AAC GGG GGA
βCA2his	TCA GTG GTG GTG GTG GTG GTG TAT AGA ATG AAC GGG GGA AAT
βCA4.1forward	CACC ATG GCT CCT GCA TTC GGA AAA TGT TTC ATG TTC TGC TGC
βCA4.1myc	TTA CAG ATC CTC TTC TGA GAT GAG TTT TTG TTC AGA GAA GGC AAA AGC AGG AGT
βCA4.1his	TTA GTG GTG GTG GTG GTG GTG AGA GAA GGC AAA AGC AGG
βCA4.2forward	CACC ATG GCA ACG GAA TCG TAC GAA GCC GCC
βCA4.2myc	TTA CAG ATC CTC TTC TGA GAT GAG TTT TTG TTC AGA GAA GGC AAA AGC AGG AGT GGT
βCA4.2his	TTA GTG GTG GTG GTG GTG GTG AGA GAA GGC AAA AGC AGG
βCA5forward	CACC ATG GCA GCC ACT CCC ACA CAC TTC TCT
βCA5myc	TCA CAG ATC CTC TTC TGA GAT GAG TTT TTG TTC AGA CCA AAC TGA CCG GTC
βCA5his	TCA GTG GTG GTG GTG GTG GTG AGA CCA AAC TGA

b. Re-annealing at 49^{o}C for 30 seconds.

c. Extension at 72^{o}C for 20 seconds.

3. Cycle #2 – repeat thirty times.

a. Denaturation at 98^{o}C for 30 seconds.

b. Re-annealing at 67^{o}C for 30 seconds.

c. Extension at 72^{o}C for 20 seconds.

4. Final extension at 72^{o}C for 5 minutes

The amplification protocol for βCA4.2-myc was as follows:

1. Initial denaturation at 98^{o}C for 3 minutes.
2. Cycle #1 – repeat five times.

a. Denaturation at 98^{o}C for 30 seconds.

b. Re-annealing at 62^{o}C for 30 seconds.

c. Extension at 72^{o}C for 20 seconds.

3. Cycle #2 – repeat thirty times.

a. Denaturation at 98^{o}C for 30 seconds.

b. Re-annealing at 67^{o}C for 30 seconds.

c. Extension at 72^{o}C for 20 seconds.

4. Final extension at 72^{o}C for 5 minutes

The amplification protocol for βCA2-his, βCA4.1-his, and βCA4.2his was as follows:

5. Initial denaturation at 98^{o}C for 3 minutes.
6. Cycle #1 – repeat five times.

a. Denaturation at 98^{o}C for 30 seconds.

b. Re-annealing at 53^{o}C for 30 seconds.

c. Extension at 72^{o}C for 20 seconds.

7. Cycle #2 – repeat thirty times.
    a. Denaturation at 98^{o}C for 30 seconds.
    b. Re-annealing at 67^{o}C for 30 seconds.
    c. Extension at 72^{o}C for 20 seconds.
8. Final extension at 72^{o}C for 5 minutes

The amplification protocol for βCA4.1-myc, βCA5-his, and βCA5-myc was as follows:

1. Initial denaturation at 98^{o}C for 3 minutes.
2. Cycle #1 – repeat five times.
    a. Denaturation at 98^{o}C for 30 seconds.
    b. Re-annealing at 56.5^{o}C for 30 seconds.
    c. Extension at 72^{o}C for 20 seconds.
3. Cycle #2 – repeat thirty times.
    a. Denaturation at 98^{o}C for 30 seconds.
    b. Re-annealing at 67^{o}C for 30 seconds.
    c. Extension at 72^{o}C for 20 seconds.
4. Final extension at 72^{o}C for 5 minutes

The products of the PCR reaction were analyzed by electrophoresis on a 1% (w/v) agarose gel containing 1mg/mL EtBr and the target DNA fragment was purified using the Qiaquick Gel Extraction Kit (Qiagen). The concentrations of the gel-purified PCR products were determined by NanoDrop. The purified products were then stored at 4^{o}C.

Entry vector cloning

The pENTR/D-TOPO vector was supplied at a concentration of 15-20ng/µL and features a kanamycin resistance gene. Roughly 4ng βCA PCR product and 1µL salt solution were brought to a volume of 5µL using sterile water. 1µL of the pENTR/D-TOPO vector was added to the 5µL reaction mix then mixed gently and incubated for 30 minutes at room temperature then placed on ice.

The βCA-fusion/pENTR/D-TOPO was transformed into One Shot Top10 chemically competent *E. coli* cells acquired from Invitrogen. One 50µL vial of chemically competent One Shot Top10 cells was gently mixed with the 6µL pENTR⁄D-TOPO vector reaction mix then incubated on ice for 30 minutes. The cells were heat shocked for 30 seconds at 42^{o}C in a thermal cycler without shaking then immediately transferred to ice. 250µL of room temperature S.O.C. medium was added to the cells. Then the cells were incubated at 37^{o}C for 1 hour while shaking horizontally at ~200rpm. 100µL from each transformation was spread on pre-warmed 2xyt (1.6% tryptone, 1.0% yeast extract, 0.5% NaCl dissolved in dH_2O) agar supplemented with 50µg/mL kanamycin (2xyt+kan) and incubated at 37^{o}C overnight.

Analysis of βCA-fusion/pENTR/D-TOPO Transformants by Restriction Enzyme Digestion

Single colonies were picked from the 2xyt+kan agar plates and cultured in 2xyt+kan. Plasmid DNA was isolated using the Qiaprep Spin Miniprep Kit (Qiagen). Double enzyme restriction digests were carried out by gently mixing 4µL isolated βCA-fusion/pENTR⁄D-TOPO plasmid vector, 2.5µL appropriate NEB Buffer, 0.5µL BSA, 1µL restriction enzyme #1, and 1µL restriction enzyme #2 and brought to a final volume of 20µL with dH_2O (see Table 4 for details). One enzyme was specific to a restriction site within the vector backbone; the other restriction enzyme cleaved within the βCA-fusion cDNA. The reactions were incubated for 3 hours at 37^{o}C. The restriction enzymes were replaced with dH_2O for the control reactions. The digested samples

Table 4. A list of the entry vector constructs and their sizes along with the enzymes and buffers used in the restriction digest reactions and the predicted number and sizes of restriction fragments.

βCA-fusion/pENTR/ D-TOPO Vector	Vector Size (bp)	Enzyme 1	Enzyme 2	NEB Buffer	Expected Fragment Sizes (bp)
βCA2-myc	3358	AclI	AleI	4	590, 2768
βCA2-his	3346	AclI	AleI	4	590, 2756
βCA4.1-myc	3421	EcoRV-HF	XhoI	4	651, 2770
βCA4.1-his	3409	EcoRV-HF	XhoI	4	639, 2770
βCA4.2-myc	3355	EcoRV-HF	XhoI	4	651, 2704
βCA4.2-his	3343	EcoRV-HF	XhoI	4	639, 2704
βCA5-myc	3484	AclI	KpnI-HF	4	1046, 2438
βCA5-his	3472	AclI	KpnI-HF	4	1046, 2426

were loaded onto a 1% (w/v) agarose gel containing 1mg/mL EtBr. Electrophoresis occurred for ~30 minutes at 100V.

βCA-fusion/pENTR Sequencing

Plasmid vector samples that yielded the expected fragment sizes were sequenced using the M13 forward and reverse primers on the ABI 3130XL Genetic Analyzer by the LSU Genomics Facility (https://biosci-batzerlab.biology.lsu.edu/Genomics/genomics_services_ABI-3130XL.php). The reported DNA sequences were aligned against the DNA sequence of the wild-type protein using the ClustalW2 multiple sequence alignment tool (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and the TAIR databank.

Recombination of βCA-fusion cDNA into the pDEST14 and Transformation of OneShot TOP10 Chemically Competent *E. coli*

The cDNA from the βCA2-his/pENTR⁄D-TOPO vector and βCA4.1-myc/pENTR⁄D-TOPO vector was recombined into the pDEST14 vector using the LR Recombination Reaction (Invitrogen). 1µL each of βCA-fusion/pENTR⁄D-TOPO vector and pDEST14 vector were incubated in 4µL 5x LR Recombinase Buffer (Invitrogen) and 9µL 1X TE buffer with 4µL LR Clonase enzyme (Invitrogen) for 1 hour at room temperature. The reaction was terminated by the addition of 2µL Proteinase K solution (Invitrogen) and incubation at 37^{o}C for 10 minutes.

5µL of the βCA-fusion/pENTR⁄D-TOPO vector and pDEST14 vector recombinase reaction were mixed into one 50µL vial of *E. coli* One Shot Top10 cells and incubated on ice for 30 minutes. The cells were heat shocked for 30 seconds at 42^{o}C then immediately returned to the ice. 250µL of room temperature S.O.C. medium was added to the cell mixture and the cells were incubated at 37^{o}C while shaking horizontally at ~200rpm. After 1 hour, 100µL from each

transformation was spread on pre-warmed 2xyt agar plates supplemented with 100µg/mL ampicillin (2xyt+amp) and incubated at 37°C overnight.

Analysis of βCA-fusion/pDEST14 *E. coli* Transformants by Restriction Enzyme Digestion

Single colonies were picked from the 2xyt+amp agar plates and resuspended in 1mL 2xyt medium supplemented with 100µg/mL ampicillin. Cell cultures were incubated overnight at 37°C. Plasmid DNA was isolated using the Qiaprep Spin Miniprep Kit (Qiagen). Enzymatic digestion was carried out by incubating 4µL isolated βCA-fusion/pDEST14 plasmid vector, 2.5µL 10X NEB Buffer, 0.5µL BSA, 1µL restriction enzyme #1, and 1µL restriction enzyme #2 brought to a final volume of 20µL with dH_2O for 3 hours at 37°C (see Table 5 for details). One enzyme was specific to a restriction site within the vector backbone; the other restriction enzyme cleaved within the βCA-fusion cDNA. The digested samples were loaded onto a 1% (w/v) agarose gel containing 1mg/mL EtBr. Electrophoresis occurred for ~30 minutes at 100V.

Overexpression of βCA-fusion gene in *E. coli* BL21pLysS

Chemically competent BL21pLysS cells were thawed on ice then gently mixed. A 110µL aliquot of the competent cells was added to a pre-chilled 14-mL round-bottom falcon tube. 14.3M β-mercaptoethanol (β-ME) was added to each tube to a final concentration of 25mM; the tubes were gently swirled to mix. 50ng of βCA-fusion/pDEST14 was added to each transformation reaction and swirled gently. The transformation mixture was then incubated on ice for 30 minutes. After 30 minutes, the transformation mixture was heat shocked at 42°C for exactly 45 seconds then incubated on ice. After 2 minutes, 0.9mL of SOC medium preheated to 37°C was added to the transformation reaction. The transformation reaction was incubated at 37°C while shaking at ~200 rpm. After 1 hour, 100µL of the transformation reaction was spread on 2xyt+amp agar plates and incubated at 37°C overnight. After the overnight incubation, a

Table 5. A list of the destination vector constructs and their sizes along with the enzymes and buffers used in the restriction digest reactions and the predicted number and sizes of restriction fragments.

βCA-fusion/ pDEST14	**Vector Size (bp)**	**Enzyme 1**	**Enzyme 2**	**NEB Buffer**	**Expected Fragment Sizes (bp)**
βCA2-myc	5640	HindIII	HindIII	2	1376, 4260
βCA2-his	5628	HindIII	HindIII	2	1364, 4260
βCA4.1-myc	5673	XhoI	EcoRI	3	1178, 4490
βCA4.2-myc	5603	XhoI	EcoRI	3	1178, 4421

single colony was inoculated into 5mL 2xyt+amp (100µg/mL) + 200µL of 20% glucose + chloramphenicol (50µg/mL) and incubated overnight at 37oC while shaking at ~200rpm. The overnight culture was diluted in 10mL fresh 2xyt+amp (100µg/mL) + 400µL of 20% glucose + chloramphenicol (50µg/mL) and grown to an O.D. of 0.6. The culture was split into two 4mL cultures; one tube was treated with 25µL 0.1M IPTG to induce expression of the βCA-fusion protein. Both cultures were incubated at 37^{o}C while shaking at 200rpm. 100µL aliquots of each culture were stored at 4^{o}C overnight. 20µL of each induced and non-induced culture were mixed with 20µL 6XSDS Sample Buffer for later analysis by SDS-PAGE.

SDS-PAGE Analysis

The 40µL mix of cell culture and 6XSDS (12% SDS; 0.006% bromophenol blue; 4.7% glycerol; 1.2% 0.5M Tris, pH 6.8; 9.3% DTT dissolved in dH_2O) buffer was heat shocked at 95^{o}C for 5 minutes before loading on the 12% SDS-polyacrylamide gel. Electrophoresis occurred at 80V for 15 minutes then 150V for 1 hour. The gel was stained with Coomassie Brilliant Blue (0.25% Coomassie; 50% MeOH; 7% glacial acetic acid dissolved in dH_2O) overnight then de-stained for several hours to remove excess background stain.

Results

Amplification of βCA-fusion genes

Table 2 lists the predicted size of each βCA-fusion gene following PCR amplification. The annealing temperature in the first five cycles of each PCR was lowered to allow the primers to anneal to the βCA cDNA template without perfect complementarity. This was necessary because the reverse complement primers contained the bases for the myc-tag or his-tag. After the first five cycles of the PCR, the annealing temperature was increased to prevent the primers from annealing to PCR amplicons that did not complement the sequence of the C-terminal 6xHis or myc-epitope tags. This increase in annealing temperature of the last 30 cycles of the reaction should ensure the ratio of untagged PCR product:tagged PCR product was minimal. Figure 3 shows the agarose gel electrophoresis of the gel-purified PCR product from the βCA-fusion amplification reactions. All eight of the βCA-fusion genes appeared to be successfully amplified by PCR. βCA2-myc and βCA2-his migrated to ~800bp. βCA4.1-myc and βCA4.1-his migrated to ~900bp. βCA4.2-myc and βCA4.2-his migrated to ~800bp. The βCA4.2-fusion genes migrated slightly further than the longer βCA4.1-fusion genes. βCA5-myc and βCA5-his migrated to ~1000bp. In all cases, it seemed that the 6xHis-tagged PCR products migrated slightly further than their myc-tagged counterparts.

TOPO Cloning

The pENTR⁄D-TOPO vector enables the directional cloning of a blunt-end PCR product into a vector for entry into the Gateway System or the MultiSite Gateway System (Invitrogen). By adding the four bases "CACC" to the 5' end of the PCR product, "TOPO cloning" takes advantage of the sequence specificity of the *Vaccinia* virus DNA topoisomerase I to enable the cloning of blunt-ended PCR products with >90% efficiency (Invitrogen; Cheng and

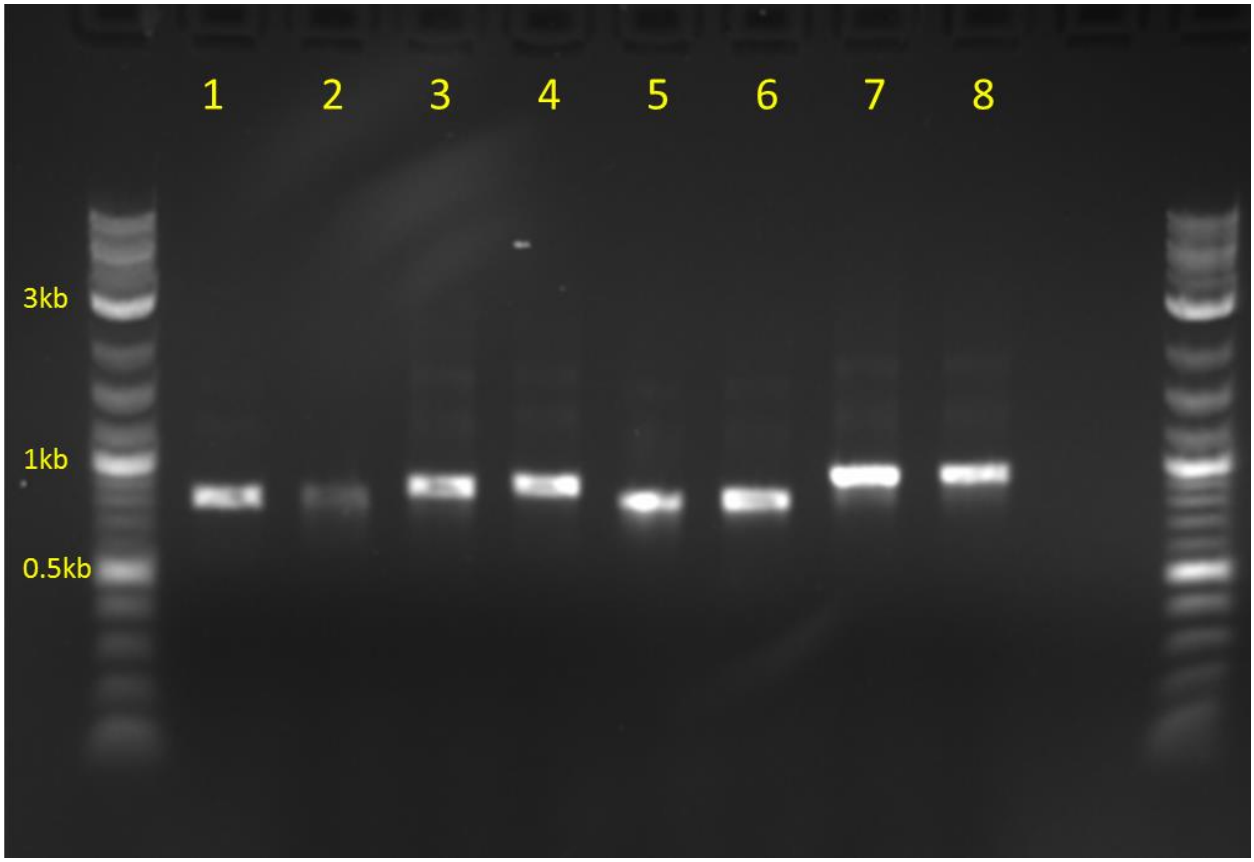


Figure 3. Agarose gel electrophoresis of PCR products using βCA-fusion gene primers. 1µL 6X Dye, 3µL gel-purified βCA-fusion PCR product, and 2µL dH_2O loaded per lane. 2µL of 2-log DNA ladder (100mg/mL) loaded for comparison. Lane 1: βCA2-his; Lane 2: βCA2-myc; Lane 3: βCA4.1-his; Lane 4: βCA4.1-myc; Lane 5: βCA4.2-his; Lane 6: βCA4.2-myc; Lane 7: βCA5his; Lane 8: βCA5myc. Electrophoresis occurred for 30 minutes at 100V.

Shuman, 2000; Shuman, 1991; Shuman, 1994). Invitrogen's pENTR/D-TOPO vectors feature attL1 and attL2 sites derived from bacteriophage λ to allow recombinational cloning of target genes in entry vectors with Gateway destination vectors (Invitrogen; Landry, 1989). The GTGG overhang on the L1 border of the cloning vector invades the 5'CACC of the PCR product, anneals to those bases, and stabilizes the PCR product in the proper orientation (Invitrogen). A kanamycin resistance gene allowed for the selection of transformants in *E. coli.* The pENTR/D-TOPO vectors also feature M13 forward and reverse priming sites to enable the sequencing of the gene insert (Figure 2).

Restriction fragment analysis of βCA-fusion/pENTR/D-TOPO vector

Figure 4 shows the migration pattern of the plasmid vectors isolated from the One Shot Top10 chemically competent *E. coli* transformed with each βCA-fusion/pENTR/D-TOPO vector. Table 4 lists the restriction enzymes used in the double digests of each plasmid vector and the expected sizes of the resulting DNA fragments. The undigested βCA-fusion/pENTR/D-TOPO vectors migrated to slightly >3000bp. The digested βCA2-fusion/pENTR/D-TOPO vectors yielded one ~600bp fragment and one ~2700bp fragment. The digested βCA4.1-fusion/pENTR/D-TOPO vectors yielded one ~600bp fragment and one ~2700bp fragment. The digested βCA4.2-myc/pENTR/D-TOPO vector yielded one ~600bp fragment and one ~2700bp fragment. The restriction digest of βCA4.2-his/pENTR/D-TOPO vector yielded a single fragment instead of the two expected fragments. The digestion of neither βCA5-fusion/pENTR/D-TOPO vector yielded the proper fragment pattern. These results suggest the TOPO cloning reactions were unsuccessful for βCA4.2-his and both of the βCA5-fusion genes.

DNA Sequencing

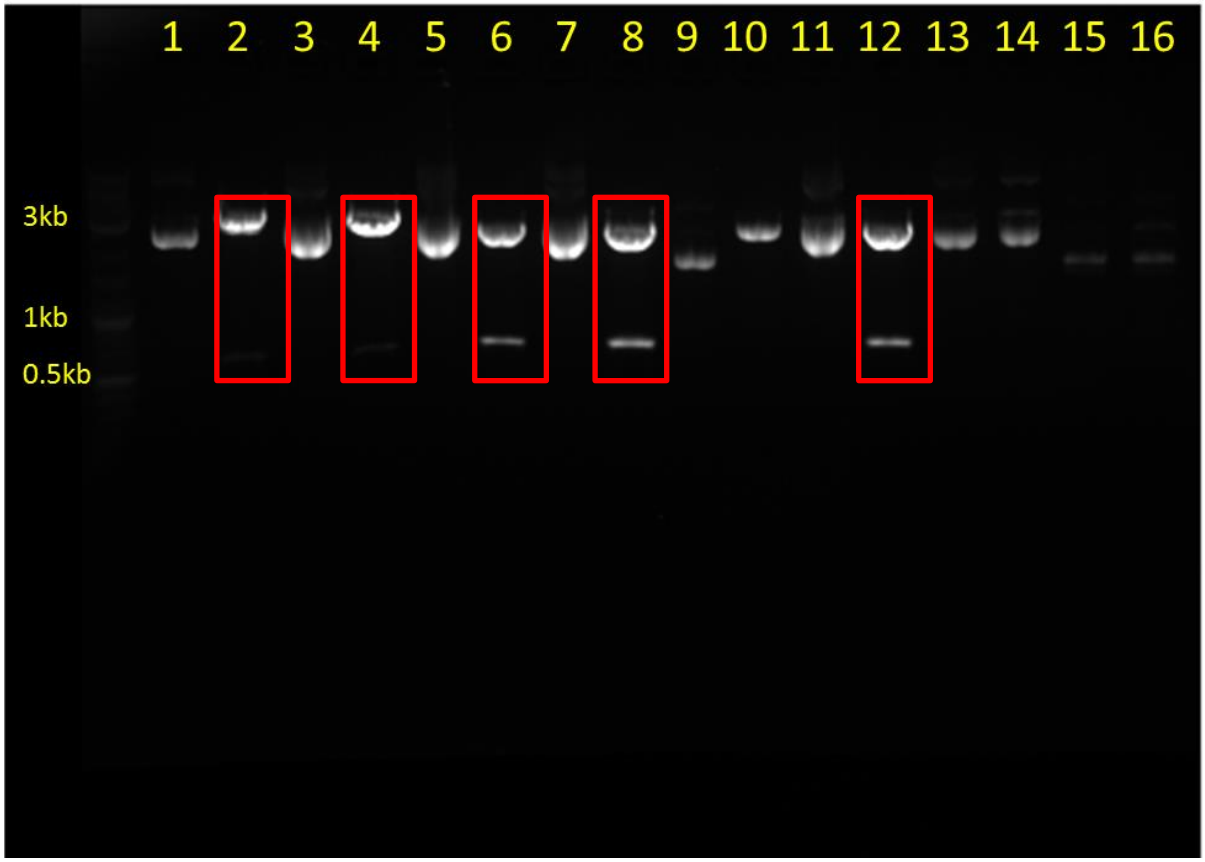


Figure 4. Agarose gel electrophoresis of undigested and digested βCA-fusion/pENTR/D-TOPO vectors. 4µL 6X Dye was added to each sample. 12µL of each sample was loaded per lane. Lane 1: undigested βCA2-his/pENTR/D-TOPO; Lane 2: digested βCA2-his/pENTR/D-TOPO; Lane 3: undigested βCA2-myc/pENTR/D-TOPO; Lane 4: digested βCA2-myc/pENTR/D-TOPO; Lane 5: undigested βCA-4.1his/pENTR/D-TOPO; Lane 6: digested βCA-4.1his/pENTR/D-TOPO; Lane 7: undigested βCA-4.1myc/pENTR/D-TOPO; Lane 8: digested βCA-4.1myc/pENTR/D-TOPO; Lane 9: undigested βCA-4.2his/pENTR/D-TOPO; Lane 10: digested βCA-4.2his/pENTR/D-TOPO; Lane 11: undigested βCA-4.2myc/pENTR/D-TOPO; Lane 12: digested βCA-4.2myc/pENTR/D-TOPO; Lane 13: undigested βCA5-his/pENTR/D-TOPO; Lane 14: digested βCA5-his/pENTR/D-TOPO; Lane 15: undigested βCA5-myc/pENTR/D-TOPO; Lane 16: digested βCA5-myc/pENTR/D-TOPO.

The sequencing results for βCA2-his/pENTR/D-TOPO, βCA2-myc/pENTR/D-TOPO, βCA4.1-myc/pENTR/D-TOPO, and βCA4.2-myc/pENTR/D-TOPO showed the sequences were identical to the corresponding wild-type βCA gene with the myc-tags and his-tags in frame. βCA4.1-his/pENTR/D-TOPO has been submitted to the LSU Genomics Facility for sequencing.

Restriction fragment analysis of βCA-fusion/pDEST14 vector

Table 5 lists the enzymes used in the double enzyme restriction digestion reactions of the βCA-fusion/pDEST14 vectors transformed into One Shot Top10 chemically competent *E. coli*. Figure 5 shows the migration pattern of the plasmid vectors isolated from the One Shot Top10 chemically competent *E. coli* transformed with each βCA-fusion/pDEST14 vector. The undigested βCA-fusion/pDEST14vectors migrated above 3000bp. The digested βCA2-his/pDEST14 vectors yielded one ~1500bp fragment and one >3kb fragment. The digested βCA4.1-myc/pDEST14 vectors yielded one ~1kb fragment and one >3kb fragment. The digested βCA2-myc/pDEST14 and βCA4.2-myc/pDEST14 vectors did not yield the expected fragment patterns.

SDS-PAGE Analysis

Figure 6 displays the results of the SDS-PAGE analysis of *E. coli* BL21pLysS cells transformed with βCA2-his/pDEST14 and βCA4.1-myc/pDEST14. 10µL of each induced and un-induced SDS-gel sample was loaded per lane. After de-staining to remove excess Coomassie Brilliant Blue from the gel, it appeared that the induced and un-induced cell cultures yielded the same banding pattern. There was no expression of the βCA-fusion proteins observed.

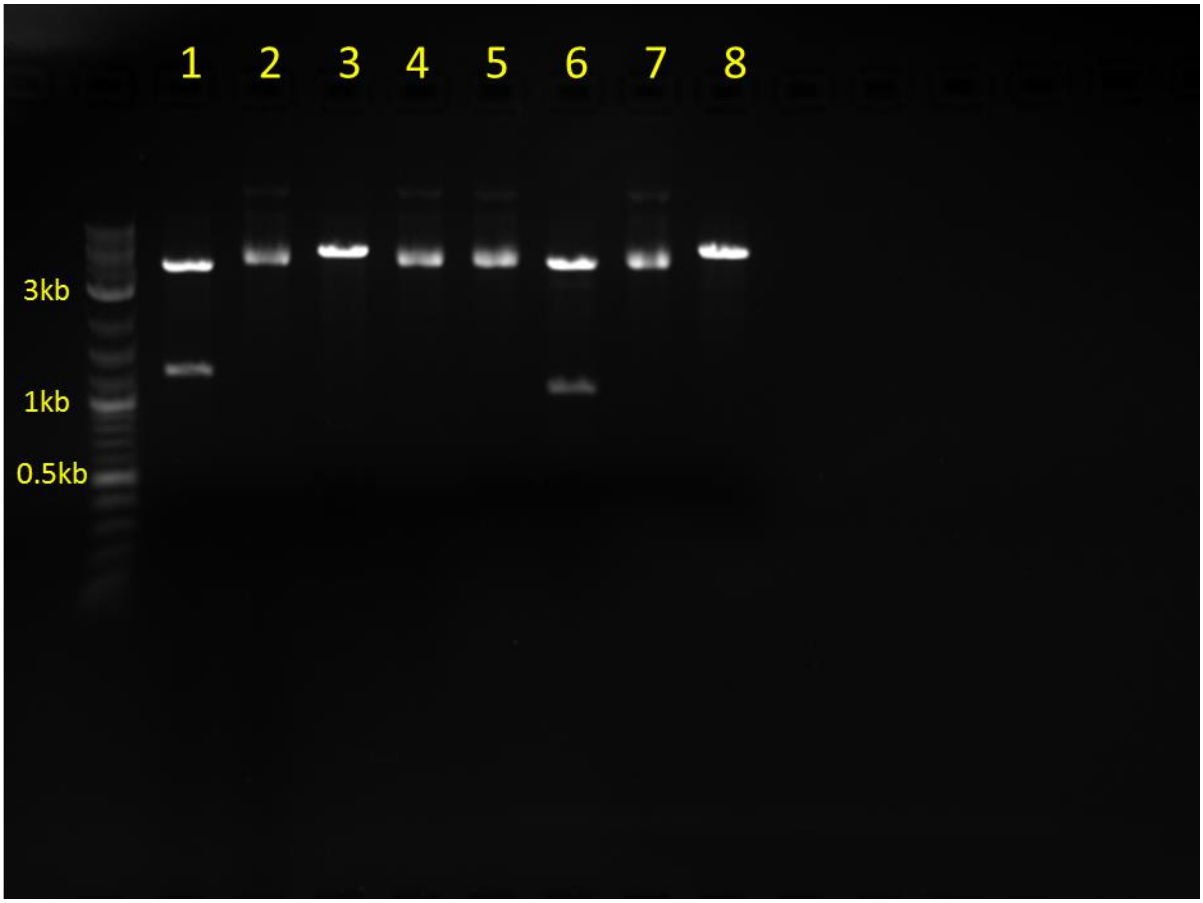


Figure 5. Agarose gel electrophoresis of undigested and digested βCA-fusion/pDEST14 vectors. 4µL 6X Dye was added to each sample. 12µL of each sample was loaded per lane. Lane 1: digested βCA2-his/pDEST14; Lane 2: undigested βCA2-his/pDEST14; Lane 3: digested βCA2-myc/pDEST14; Lane 4: undigested βCA2-myc/pDEST14; Lane 5: undigested βCA4.1-myc/pDEST14; Lane 6: digested βCA4.1-myc/pDEST14; Lane 7: undigested βCA4.2-myc/pDEST14; Lane 8: digested βCA4.2-myc/pDEST14.

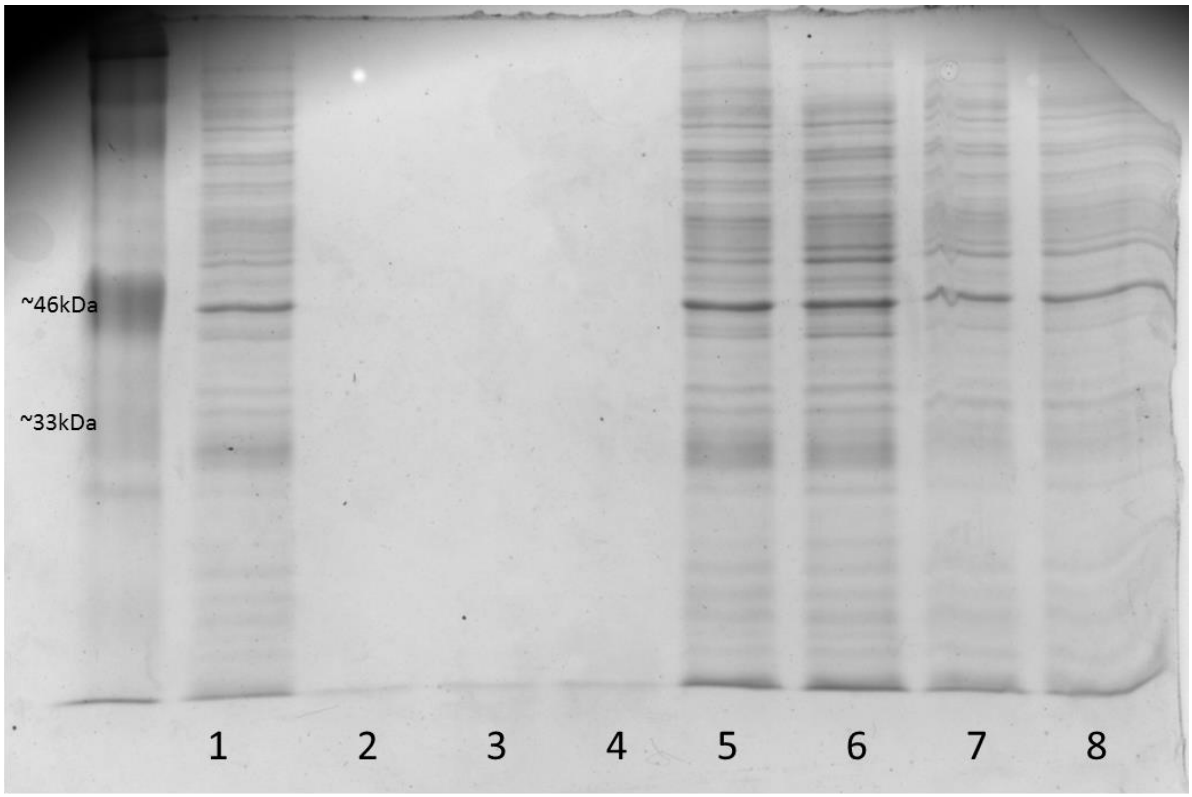


Figure 6. SDS-PAGE analysis of *E. coli* BL21pLysS cells transformed with βCA2-his/pDEST14 and βCA4.1-myc/pDEST14. 10µL *E. coli* BL21pLysS/6X SDS sample mix were loaded per lane. 5µL of Kaleidoscope™ Prestained Standards (Bio-Rad) protein ladder was loaded for comparison. Electrophoresis occurred for 15 minutes at 80V then for 1 hour at 150V. Lane 1: BL21pLysS/βCA2-his/pDEST14 induced with 1mM IPTG; Lane 2: uninduced BL21pLysS/βCA2-his/pDEST14; Lane3: BL21pLysS/βCA4.1-myc/pDEST14 induced with 1mM IPTG; Lane 4: uninduced BL21pLysS/βCA4.1-myc/pDEST14. Lane 5: BL21pLysS/βCA2-his/pDEST14 induced with 1mM IPTG; Lane 6 : uninduced BL21pLysS/βCA2-his/pDEST14; Lane 7: BL21pLysS/βCA4.1-myc/pDEST14 induced with 1mM IPTG; Lane 8: uninduced BL21pLysS/βCA4.1-myc/pDEST14.

Discussion

The electrophoretic analysis of the gel-purified PCR products from the amplification of the βCA-fusion genes suggests that all eight fusion genes (Table 2) were successfully amplified. I performed the TOPO cloning reaction on each of the fusion genes to create an entry vector clone.

After selection on 2xyt+kan(50µg/mL) agar plates, single colonies were picked from the transformation plates and cultured overnight in liquid 2xyt+kan(50µg/mL). Plasmid DNA was isolated using the Qiaquick Miniprep Spin Kit (Qiagen) then enzymatically digested. None of the digestion reactions of the βCA4.2-his/pENTR/D-TOPO or βCA5-fusion/pENTR⁄D-TOPO vectors yielded the expected number and sizes of fragments suggesting the failure of the TOPO cloning reactions for those genes. The double enzyme digestions of βCA2-his/pENTR⁄D-TOPO, βCA2-myc/pENTR⁄D-TOPO, βCA4.1-his pENTR⁄D-TOPO, βCA4.1-myc/pENTR⁄D-TOPO, and βCA4.2-myc/pENTR⁄D-TOPO yielded fragments corresponding to the expected sizes and numbers of fragments (Table 4). The βCA2-his/pENTR⁄D-TOPO, βCA2-myc/pENTR⁄D-TOPO, βCA4.1-his/pENTR⁄D-TOPO, βCA4.1-myc/pENTR⁄D-TOPO, and βCA4.2-myc/pENTR⁄D-TOPO vectors were submitted for DNA sequencing on the ABI 3130XL Genetic Analyzer by the LSU Genomics Facility (https://biosci-batzerlab.biology.lsu.edu/Genomics/genomics_services_ABI-3130XL.php). The sequence reads for βCA4.1-his/pENTR⁄D-TOPO have not been received as of yet. The sequences of the βCA2-his/pENTR⁄D-TOPO, βCA2-myc/pENTR⁄D-TOPO, βCA4.1-myc/pENTR⁄D-TOPO, βCA4.2-myc/pENTR⁄D-TOPO vectors were aligned using ClustalW2 multiple sequence alignment and showed complete DNA sequence identity with the corresponding wildtype genes. The sequences

were also BLASTed against the *Arabidopsis* genome using TAIR. Each of the sequences was identical to the corresponding wildtype genes.

The gene insert from βCA2-his/pENTR⁄D-TOPO, βCA2-myc/pENTR⁄D-TOPO, βCA4.1-myc/pENTR⁄D-TOPO, βCA4.2-myc/pENTR⁄D-TOPO were cloned into the Gateway-compatible pDEST14 destination vectors using Invitrogen's LR Recombination Reaction. The pDEST14 vector features an ampicillin resistance gene to allow for the selection of transformants in *E. coli*. One Shot Top10 chemically competent *E. coli* was transformed with the βCA2-his/pDEST14, βCA2-myc/pDEST14, βCA4.1-myc/pDEST14, and βCA4.2-myc/pDEST14 vectors. Tranformants were selected by overnight incubation at 37^{o}C on 2xyt+amp agar plates. Single colonies were picked and cultured in liquid 2xyt+amp(100μg/mL) overnight at 37^{o}C while shaking at ~200rpm. Plasmid DNA was isolated from each transformant and digested (Table 4). Only the digests of the βCA2-his/pDEST14 and βCA4.1-myc/pDEST14 transformants yielded the predicted number and sizes of restriction fragments.

The isolated βCA2-his/pDEST14 and βCA4.1-myc/pDEST14 vectors were transformed into the *E. coli* BL21pLysS for overexpression of the fusion gene and analysis by SDS-PAGE. The SDS-PAGE results showed no induction of the expression of the fusion gene for either transformant. However, we have discovered that our laboratory stock of chloramphenicol had expired. The selective pressure of chloramphenicol is necessary to maintain the pLysS cam^{R} plasmid necessary for expression transcription factors of T7 RNA polymerase. Since the βCA-fusion genes were under control of a T7 RNA polymerase promoter, the genes would not be expressed in the absence of the pLysS cam^{R} plasmid. It should also be noted that the IPTG used in the experiment was a liquid solution made over one year ago and was likely expired.

The restriction digestions of the βCA4.2-myc/pENTR⁄D-TOPO, βCA5-his/pENTR⁄D-TOPO, and βCA5-myc/pENTR⁄D-TOPO vectors suggest the TOPO cloning reactions were unsuccessful. The results of the restriction digestions and DNA sequencing reactions of entry vectors βCA2-his/pENTR⁄D-TOPO, βCA2-myc/pENTR⁄D-TOPO, βCA4.1-myc/pENTR⁄D-TOPO, βCA4.2-myc/pENTR⁄D-TOPO suggest that the target genes were successfully cloned into the entry vector in the proper orientation. Even though the restriction digestions of βCA4.1-his/pENTR⁄D-TOPO yielded the predicted fragment pattern, more conclusive results await the return of the DNA sequences.

The restriction enzyme digestions of βCA2-his/pDEST14 and βCA4.1-myc/pDEST14 suggest the successful recombination of the target genes into the pDEST14 destination vectors. However, the SDS-PAGE analysis of *E. coli* BL21pLysS cells transformed with βCA2-his/pDEST14 and βCA4.1-myc/pDEST14 revealed no induction of βCA expression in either transformant. It is possible the gene fusions were cloned properly, but no conclusive statement can be made until the expression of βCA-fusion proteins is observed in transformants.

If attempts to generate the βCA4.2-his/pENTR/D-TOPO, βCA5-his/pENTR/D-TOPO and βCA5-myc/pENTR/D-TOPO vectors continue to be unsuccessful, we may employ a different cloning system to generate the vectors. We are still working to generate the βCA2-myc/pDEST14 and βCA4.2-myc/pDEST14 vectors. With new stocks of chloramphenicol and IPTG, we will repeat attempts to induce protein expression in the BL21pLysS *E. coli* cell line.

Future Plans

The 6xHis-tag will allow purification of the βCA-his fusion proteins by Ni^{2+}-column chromatography. Once expression of the βCA-his proteins can be detected in *E. coli*, the protein will be overexpressed in *E. coli* then purified using Ni^{2+}-column chromatography to produce sufficient amounts of the βCA to use as an antigen for raise antibodies against the protein. Once we detect enhanced CA activity in an *E. coli* strain expressing a βCA-myc gene, we will proceed with transforming the protein into *A. thaliana.* We will observe the localization of the gene once reintroduced into higher plants by identifying the protein antibodies again the myc-epitope.

Citations

1. **Badger MR, Price GD** (1994) The role of carbonic anhydrase in photosynthesis. Annual Review of Plant Physiology and Plant Molecular Biology **45**: 369-392
2. **Badger MR, Price GD** (2003) CO_2 concentrating mechanisms in cyanobacteria: molecular components, their diversity and evolution. Journal of Experimental Botany **54**: 609-622
3. **Buren S, Ortega-Villasante C, Blanco-Rivero A, Martinez-Bernadini A, Shutova T, Shevela D, Messinger J, Bako L, Villarejo A, Samuelsson G** (2011) Importance of post-translational modifications for functionality of a chloroplast-localized carbonic anhydrase (CAH1) in *Arabidopsis thaliana*. PLoS One **6**: e21021
4. **Burnell JN, Hatch MD** (1988) Low bundle sheath carbonic anhydrase is apparently essential for effective C4 pathway operation. Plant Physiology **86**: 1252-1256
5. **Cheng C, Shuman S** (2000) Recombinogenic flap ligation pathway for intrinsic repair of topoisomerase IB-induced double-strand breaks. Molecular Cellular Biology **20**: 8059-8068
6. **Crayn DM, Winter K, Smith AC** (2004) Multiple origins of crassulacean acid metabolism and the epiphytic habit in the Neotropical family Bromeliaceae. Proceedings of the National Academy of Sciences of the United States of America **101**: 3703-3708.
7. **Ehleringer JR, Cerling TE, Helliker BR** (1997) C4 photosynthesis, atmospheric CO2, and climate. Oecologia **112**: 285-299
8. **Fabre N, Reiter IM, Becuwe-Linka N, Genty B, Rumeau D** (2007) Characterization and expression analysis of genes encoding α and β carbonic anhydrases in *Arabidopsis*. Plant, Cell, and Environment **30**: 617-629

9. **Hewett-Emmett D, Tashian RE** (1996) Functional diversity, conservation, and convergence in the evolution of the α-, β-, and γ-carbonic anhydrase gene families. Molecular Phylogenetics and Evolution **5**: 50-77
10. **Hu H, Boisson-Dernier A, Israelsson-Nordstrom M, Bohmer M, Xue S, Ries A, Godoski J, Kuhn JM, Schroeder JI** (2010) Carbonic anhydrases are upstream regulators of CO2-controlled stomatal movements in guard cells. Nature Cell Biology **12**: 87-93
11. **Karki S, Rizal G, Quick WP** (2013) Improvement of photosynthesis in rice (Oryza sativa L.) by inserting the C4 pathway. Rice **6**: 28
12. **Khalifah RG** (1971) The carbon dioxide hydration activity of carbonic anhydrase I. Stop-flow kinetic studies on the native human isoenzymes B and C. Journal of Biological Chemistry **246**: 2561-2573
13. **Khan F, Mingyue H, Taussig MJ** (2006) Double-hexahistidine tag with high-affinity binding for protein immobilization, purification, and detection on ni-nitrilotriacetic acid surfaces. Analytical Chemistry **78**: 3072-3079
14. **Landy A** (1989). Dynamic, Structural, and Regulatory Aspects of Lambda Site-specific Recombination. Annual Review of Biochemistry **58**: 913-949
15. **Leegood R** (2002) C4 photosynthesis: principles of CO2 concentration and prospects for its introduction into C3 plants. Journal of Experimental Botany **53**: 581-590.
16. **Long SP, Ainsworth EA, Leakey ADB, Nosberger J, Ort DR** (2006) Food for thought: lower-than-expected crop yield stimulation with rising CO2 concentrations. Science **312**: 1918-1921

17. **Lorimer GH, Andrews TJ** (1973) Plant photorespiration—an inevitable consequence of the existence of atmospheric oxygen. Nature **243**: 359-360.

18. **Moroney JV, Ynalvez RA** (2007) Proposed carbon dioxide concentrating mechanism in *Chlamydomonas reinhardtii*. Eukaryotic Cell **6**: 1251-1259

19. **Moroney JV, Jungnick N, DiMario RJ, Longstreth DJ** (2013) Photorespiration and carbon concentrating mechanisms: two adaptations to high O_2, low CO_2 conditions. Photosynthesis Research **117**: 121-131

20. **Moroney JV, Ma Y, Frey W, Fusilier KA, Pham TT, Simms TA, DiMario RJ, Yang J, Mukherjee B** (2011) The carbonic anhydrase isoforms of *Chlamydomonas reinhardtii*: intracellular location, expression, and physiological roles. Photosynthesis Research **109**: 133-149

21. **Nobel PS, Hartsock TL** (1986) Leaf and stem CO_2 uptake in the three subfamilies of the Cactaceae. Plant Physiology **80**: 913-917

22. **Park H, Song B, Morel FMM** (2007) Diversity of the cadmium-containing carbonic anhydrase in marine diatoms and natural waters." Environmental Microbiology **9**: 403-413

23. **Price GD, Badger MR, Woodger FJ, Long BM** (2008) Advances in understanding the cyanobacterial CO_2-concentrating-mechanism (CCM): functional components, C_i transporters, diversity, genetic regulation and prospects for engineering into plants. Journal of Experimental Botany **59**: 1441-1461

24. **Roberts SB, Lane TW, Morel FMM** (1997) Carbonic anhydrase in the marine diatom *Thalassiosira weissflogii* (Bacillariophyceace). Journal of Phycology **33**: 845-850

25. **Sheehan D, O'Sullivan S** (2004) Fast protein liquid chromatography. Protein purification protocols. Humana Press: 253-258

26. **Shuman S** (1991). Recombination mediated by *Vaccinia* virus DNA Topoisomerase I in *Escherichia coli* is sequence specific. Proceedings of the National Academy of Science USA **88**: 10104-10108

27. **Shuman S** (1994). Novel approach to molecular cloning and polynucleotide synthesis using *Vaccinia* DNA Topoisomerase. Journal of Biological Chemistry **269**: 32678-32684

28. **So AKC, Espie GS, Williams EB, Shively JM, Heinhorst S, Cannon GC** (2004) A novel evolutionary lineage of carbonic anhydrase (ε Class) is a component of the carboxysome shell. Journal of Bacteriology **186**: 623-630

29. **Soto AR, Zheng H, Shoemaker D, Rodriguez J, Read BA, Wahlund TM** (2006) Identification and preliminary characterization of two cDNAs encoding unique carbonic anhydrases from the marine alga *Emiliania huxleyi*. Applied & Environmental Microbiology **72**: 5500-5511

30. **Spalding MH, Spreitzer RJ, Ogren WL** (1983) Carbonic anhydrase-deficient mutant of *Chlamydomonas reinhardii* requires elevated carbon dioxide concentration for photoautotrophic growth. Plant Physiology **73**: 268-272

31. **Von Caemmerer S**, **Quinn B, Hancock NC, Price GD, Furbank RT, Ludwig M** (2004) Carbonic anhydrase and C4 photosynthesis: a transgenic analysis. Plant, Cell, & Environment **27**: 697-703

32. **Wilbur KM, Norman GA** (1948) Electrometric and colorimetric determination of carbonic anhydrase. Journal of Biological Chemistry **176**: 147-154

Acknowledgements

I would like to thank Robert DiMario for his assistance with this work. Very little of this work would have been accomplished without his guidance. I would also like to thank him for providing the CDS for βCA2, βCA4.1, βCA4.2, and βCA5.

I would like to thank the members of my thesis committee: Dr. James Moroney, Dr. David Longstreth, Dr. Steve Pollock, and Dr. Zhi-Yuan Chen. I especially want to thank Dr. Moroney for serving as my research advisor through my undergraduate career. My time in the Moroney laboratory has been an invaluable experience as a student and a peer working in an academic research environment.

Lastly, I would like to thank the other members of the Moroney laboratory for creating a great social environment during my undergraduate research experience.