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Identifying Myosin Class XI Globular Tail Interactions With Plant Organelle Proteins

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Abstract:

Cytoplasmic streaming, rapid movement of cytosol and organelles moving throughout the plant cell, raises questions such as how/why these organelles are being moved and how much this affects plant growth. It is believed that the organelles are being moved via different myosin proteins by attaching to their globular tail via ATP hydrolysis. Therefore, this project aimed to explore myosin globular tail interactions with plant organelle-associated proteins. These interactions are interesting because different myosins are binding to different organelles. Myosin class XI in *Arabidopsis thaliana* was examined specifically because of its similarity to class V myosin in animals. To examine these interactions, yeast-two hybrid assays were used with bait and prey plasmids. In order to identify specific interactions, many yeast-two hybrid assays were performed between different myosin proteins and proteins associated with plant organelles. Additionally, a coiled-coil region (*KCA1CC* and *GCN4CC*) was inserted into the bait and prey plasmid, respectively, to promote dimerization, which can enhance protein interactions. There were fewer protein interactions in

the matings without a coiled-coil region. Additionally, the fact that some interactions don't depend on the coiled-coil domain suggests that these interactions are not relevant for the binding of organelles to myosins.

Introduction:

The observation of cytoplasmic streaming, which is organelles, such as peroxisomes and Golgi stack, moving in an organized fashion throughout the cytoplasm in plant cells leads one to certain questions (Shimmen 2007). For instance, why and how are these organelles being moved? They appear to be moving along actin filaments in the cell by class V myosin motor proteins in animals and fungi and class XI myosin motor proteins in plants (Li and Nebenführ 2008). This system is referred to as the actomyosin system, and it often moves plant organelles at speeds up to 10 μm/s (Peremyslov et al. 2008). Myosin utilizes a large amount of energy from ATP hydrolysis to move the organelle/cargo molecule along actin filaments (Shimmen 2007), and therefore, the cargo molecule's movement must be important. Myosin-driven movements seem to be responsible for organelle distribution and shape, polarity, cell growth, and actin dynamics and organization (Madison and Nebenführ 2013). The myosin moves the cargo by attaching its head to the actin filament, and the myosin's globular tail domain is what appears to actually be binding to the cargo

molecule. The globular tail domain at the C-terminus end lets these myosin motor proteins bind to different cargo molecule's proteins (Li and Nebenführ 2008), but what decides which and how many cargo molecules each myosin can and will interact with? Motor-cargo interactions seem to differ even within an individual organism due to the differing makeup of protein domains and proteins associated with each cargo molecule (Li and Nebenführ 2008). It has been observed that class XI myosins can target several different organelles. Therefore, the identification of these binding partners is key to understanding different myosins' functions (perhaps many) in cytoplasmic streaming (Li and Nebenführ 2007).

The purpose of this experiment was to explore myosin globular tail interactions with organelle-associated proteins and identify binding partners to different myosin isoforms. There are 13 different class XI myosins in *Arabidopsis thaliana* (Reddy 2001). Six of these, XI-A, XI-B, XI-C, XI-D, XI-E, and XI-J, are located in the male reproductive tissue (pollen and stamen), and the other seven of these, XI-1, XI-2, XI-F, XI-G, XI-H, XI-I, and XI-K, are expressed throughout the plant (Peremyslov et al. 2008). Three of the thirteen myosin isoforms (Myo2p, Myo4p, and Myosin XI) have been positively identified so far for intra-tail interactions in *Arabidopsis* (Li and Nebenführ 2008). These discoveries were made using yeast two-hybrid assays, and this experiment will be using yeast two-

hybrid assays as well. Yeast two-hybrid assays also have been used to identify interacting partners for specific myosins, such as RabC2a and RabD1 for *Arabidopsis* Myo11B2 (Hashimoto et al. 2008), and the goal is to identify more interacting partners for more myosin isoforms. Yeast two-hybrid assays use the separate domains of GAL4, the DNA binding domain (BD) and the activation domain (AD), to examine protein-protein interactions (Amberg and Botstein 1997). This is done by using two plasmids, which encode the BD attached to a protein of interest and the AD attached to another protein of interest, respectively. The BD and AD domains will only come together if the bait and prey proteins interact (Amberg and Botstein 1997). The yeast two-hybrid assay provides a way to see if the protein of interest encoded by the bait plasmid is interacting with the protein of interest encoded by the prey plasmid because they must interact for reporter gene expression to occur. One can see this schematic in Figure 1 below:

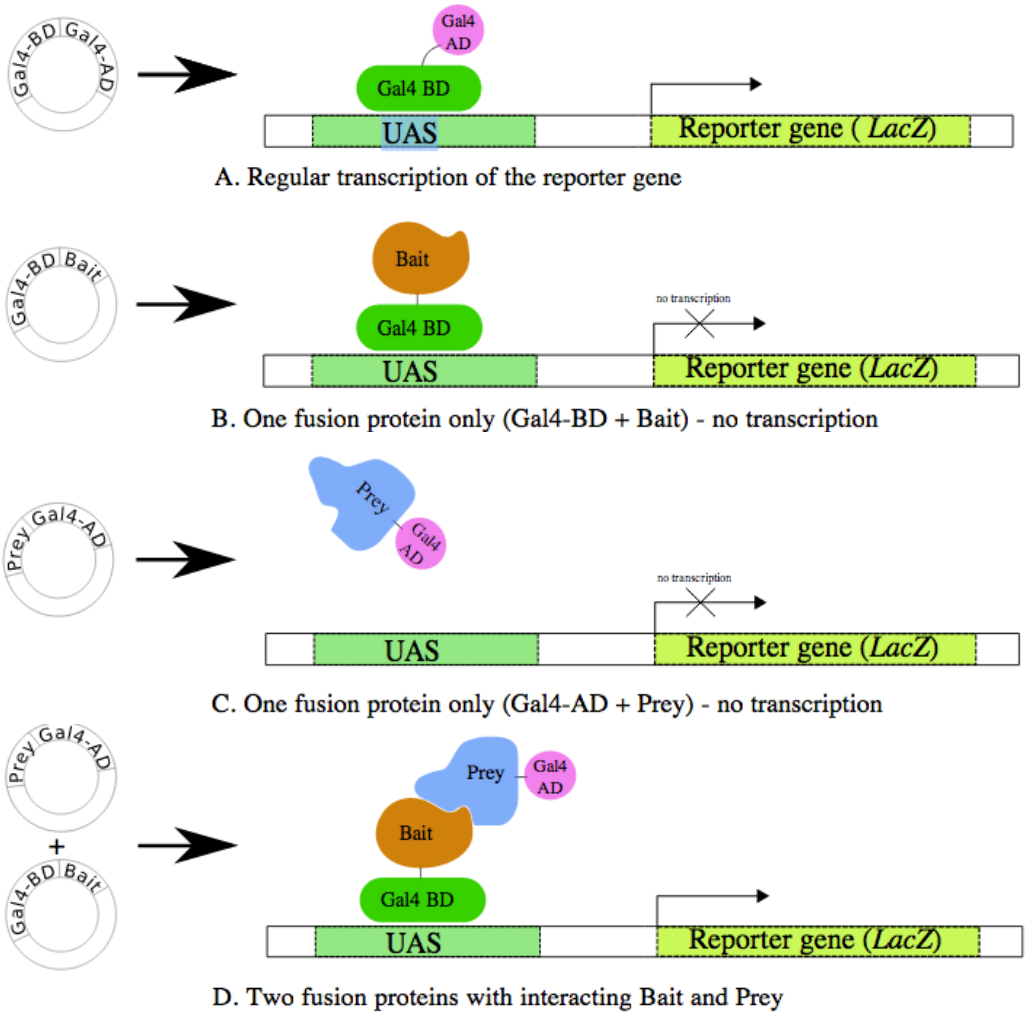

Figure 1. Example of a yeast-two hybrid. (http://en.wikipedia.org/wiki/File:Two_hybrid_assay.svg)

It is believed that dimerization helps to stabilize the interaction between the myosin globular tail and the cargo protein (Li and Nebenführ 2008). Therefore, a coiled-coil coding region is being inserted into each bait plasmid (*KCA1CC*) and into each prey plasmid (*GCN4CC*) for dimerization. Some bait plasmids are being made that do not contain a coiled-coil coding region as well so that the results of binding with and without dimerization can be examined.

So, more myosin globular tail-binding partners will be able to be discovered via inserting a coiled-coil coding region into both individual bait plasmids and individual prey plasmids, allowing dimerization to occur.

Methods:

Primers

The following primers were designed based on the cDNA (or mRNA) sequence from National Center for Biotechnology Information (NCBI) databases and the known amino acid alignments to identify the globular tail domains of MYA2, XIB, and XI-I. The sequence in parentheses is the globular tail domain, and the underlined sequence identifies the restriction site for cloning.

- *MYA2GT*-NcoI-Fwd: (64° C annealing temperature)
    - CGA CCA TGG GC (GGC AAG GCT GGG AAA TCT GC)
- *MYA2GT*-Stp-XmaI-Rev: (62°C annealing temperature)
    - GCA CCC GGG GC (GTG CAA GAA TAC AAA TGC TGG A)
- *XIBGT*-NcoI-Fwd: (62°C annealing temperature)
    - CGA CCA TGG GC (GGA AAT GCT GGG AAA TCT GCT)
- *XIBGT*-Stp-XmaI-Rev: (64°C annealing temperature)
    - GCA CCC GGG GC (GTG CAA GAA TAC GAA TTC TGG G)
- *XI-IGT*-NcoI-Fwd: (64° annealing temperature)

- CGA CCA TGG GC (TCG CGT AGA TCC AAA TTA ACC G)

- *XI-IGT*-Stp-XmaI-Rev: (64° annealing temperature)
  - GCA CCC GGG GC (AAT GAT CTG CTT TGA GGT TGA AG)

The annealing temperatures were determined by the 2+4 rule: every A or T contributes 2°, and every G or C adds 4°; one adds up all the temperature values of only the bases that will pair up with the given template, in example those given in parentheses above.

Overnight Cultures

Liquid cultures were made of *E. coli* of strains EST11 (containing *MYA2GT*), EST12 (containing *XI-IGT*), and pAN928 (pGBKT7-*KCA1CC*, the vector). After inoculation, the test tubes were placed in a roller drum at 37°C over night to allow the cultures to grow. The next morning, a Zyppy Plasmid Mini Prep Kit was used to isolate the plasmid from the *E. coli* cultures. A Nanodrop Spectrophotometer was used to quantify the amount of DNA present.

PCR

Polymerase Chain reaction (PCR) was run for *MYA2GT*, *XIBGT*, *XI-IGT*, *MYA1GT*, and *XIKGT*. PCR is used to amplify a DNA sequence of interest. This involved placing a total of 20 μl of fluid into a mini-micro centrifuge tube for each reaction and then placing each mini-micro centrifuge tube into the PCR machine. The makeup of the *MYA2GT* reaction included: 1 μl of template

(EST11), 4 µl 5X Buffer, 2 µl dNTP, 0.8 µl *MYA2GT*-NcoI-Fwd, 0.8 µl *MYA2GT*-XmaI-Rev, 11.2 µl dH2O, and 0.2 µl Phusion Polymerase. The makeup of the *XIBGT* reaction included the same concentrations and ingredients as stated before but used *Arabidopsis* flower seedling cDNA as the template and *XIBGT*-NcoI-Fwd and *XIBGT*-XmaI-Rev as the primers. The makeup of *XI-IGT* used EST 12 as the template and *XI-IGT*-NcoI-Fwd and *XI-IGT*-XmaI-Rev as the primers. The PCR program used was B62E45D, and the conditions for B62E45D are as follows: 1. 98°C for 30 seconds 2. 98°C for 10 seconds 3. 62°C for 30 seconds 4. 72°C for 45 seconds 5. Repeat 2-4 29 more times 6. 72°C for 5 min 7. Hold at 4°C.

DNA Purification

The PCR DNA was purified using the Wizard SV Gel and PCR Clean-Up Kit. The DNA was eluted with 30 µl ddH_2O and stored at -20°C. DNA purification was done for *MYA2GT*, *XIBGT*, *XI-IGT*, *MYA1GT*, and *XIKGT*.

Restriction Digest

Restriction enzymes *Nco*I-HF (20 U/µl) and *Xma*I (10 U/µl) were used to cut both the bait plasmid backbones, pAN928 and GBKT7, and the purified PCR products (*MYA2GT*, *XIBGT*, *XI-IGT*, *MYA1GT*, and *XIKGT*) for 2 hours at 37°C. pAN928 was the bait plasmid backbone used for MYA2GT, XIBGT, and XI-IGT, and it contains the coiled-coil coding region (*KCA1CC*). GBKT7 was used for

MYA1GT and XIKGT, and it also contained the coiled-coil coding region (*KCA1CC*). The reactions for *MYA2GT*, *XIBGT*, *XI-IGT*, *MYA1GT*, *XIKGT*, and pAN928 included the following: 14 µl PCR product or pAN928, 2 µl 10x Buffer 4, 2 µl BSA, 0.6 µl *Nco*I-HF (20 U/µl), and 1.4 µl *Xma*I (10 U/µl), which came to a total of 20 µl. 1 µl of calf-intestinal phosphatase (CIP) was next added to the solution and the solution incubated at 37°C for 1 hour. CIP was added to remove the phosphate groups at the ends of the vector (pAN928), commonly referred to as "sticky ends."

Gel Electrophoresis

Digested PCR and plasmid fragments were purified by gel electrophoresis.

The gel was run at 100 V for 1 hour. The gel was placed into an TAE + ethidium bromide solution for approximately 15 minutes to stain the DNA. A UV light was used to show the DNA fragments on the gel, and a picture was taken. DNA fragments of the right length in bp were cut out of the gel and purified with the wizard kit for *MYA2GT*, *XIBGT*, *XI-IGT*, *MYA1GT*, *XIKGT*, and pAN928 the same as before.

Ligation

Ligation was performed to place each digested PCR product (*MYA2GT*, *XIBGT*, and *XI-IGT*) into the vector plasmid (pAN928) and ligate the sticky ends

together of the insert and vector for each insert separately. Similarly, *MYA1GT* and *XIKGT* were placed into the vector plasmid pGBKT7 ("B") without the coiled-coil domain. These included: pAN928 Nil (no PCR product added), pAN928 + *MYA2GT*, pAN928 + *XIBGT*, pAN928 + *XI-IGT*, "B" Nil, B + *MYA1GT*, and B + *XIKGT*. The vector: insert molar ratio for each reaction was 1:3. The amount of vector to add into each reaction was 100 ng (converted to μl). For the amount of each insert to add to each reaction, it was 50 ng (converted to μl). Each microcentrifuge tube was incubated at room temperature for 20 minutes, then 4°C overnight, and then 1 hour at room temperature the next day.

Transformation of *E. coli*

Transformation of *E. coli* with plasmids was performed after ligation, and this was done with all 7 ligation reactions (pAN928 Nil, pAN928 + *KCA1CC-MYA2GT*, pAN928 + *KCA1CC-XIBGT*, pAN928 + *KCA1CC-XI-IGT*, "B" Nil, B + *MYA1GT*, and B + *XIKGT*). The plasmids were transformed into *E. coli* TOP 10 cells by heat shock method. The remaining 100 μl of solution for each tube was each spread on LB plates that contained kanamycin (50 μg/mL). The plates were incubated over night at 37°C.

Colony PCR

Colony PCR was performed to see if the insert-vector ligation was successful

and in the correct orientation for *KCA1CC-MYA2GT*, *KCA1CC-XIBGT*, *KCA1CC-XI-IGT*,

MYA1GT, and *XIKGT*. Sterilized toothpicks were used to pick up colonies on each plate, inserted into the PCR tube and then dropped into the corresponding test tube containing LB media for overnight cultures. The PCR tubes were placed in the PCR machine and the program COOKBUGS was run. A master mix was made for *KCA1CC-MYA2GT*, *KCA1CC-XIBGT*, *KCA1CC-XI-IGT*, *MYA1GT*, and *XIKGT* that totaled 60 µl for each. 10 µl of each master mix was pipetted into each PCR tube after they were done with the COOKBUGS program, and the conditions are as follows: 1. 98°C for 10 minutes 2. 4°C for 10 minutes. For 5 tubes of *KCA1CC-MYA2GT*, the master mix included the following: 3 µl *KCA1CC*-NdeI-F, 3 µl *MYA2GT*-Stp-XmaI-Rev, and the common ingredients in every tube were 3 µl dNTP, 12 µl Taq Buffer, 1.2 µl Taq Polymerase, and 37.8 µl dH2O. The master mix was the same for each one except for the reverse primer, which was *XIBGT*-Stp-XmaI-Rev for *KCA1CC-XIBGT*, *XI-IGT*-Stp-XmaI-Rev for *KCA1CC-XI-IGT*, *MYA1GT*-Stp-XmaI-Rev for *MYA1GT*, and *XIKGT*-Stp-XmaI-Rev for *XIKGT*. However, the forward primer for both *MYA1GT* and *XIKGT* was SEQ T7 instead of *KCA1CC*-NdeI-F. The program for the PCR was A62E120D, and the conditions are as follows: 1. 95°C for 30 seconds 2. 95°C for 30 seconds 3. 62°C for 60 seconds 4. 68°C for 2 minutes 5. Repeat 2-4 29 more times 6. 68°C for

5 min 7. Hold at 4°C. Another gel was then run to check for the right size bands. The bands for each were expected to be approximately 1900 bp. If the gel produced expected results, the test tubes that matched the positive PCR reactions were placed into the 37°C roller drum to incubate over night and to allow the culture to grow. The Zyppy Plasmid Mini-Prep Kit was used the next day as before with each overnight culture. The DNA concentration was determined with the Nano Drop Spectrophotometer.

DNA Sequencing

The bait plasmids of *KCA1CC-MYA2GT*, *KCA1CC-XI-IGT*, *MYA1GT*, and *XIKGT*

were all sequenced to check if they had the correct DNA sequences for each. The DNA sequence for each was then compared to the known sequence.

Making *E. coli* Stock

E. coli stock cultures were prepared of *KCA1CC-XI-IGT*, *KCA1CC-MYA2GT*, *MYA1GT*, and *XIKGT*. Bacterial stocks were made by pipetting 300 μL of 50% glycerol and 700 μL of the desired bacteria culture into a freezer tube. The final percent of glycerol was 15%. The stock was stored in the -80°C freezer.

Yeast (*Saccharomyces cerevisiae*) Transformation

The manufacturer's protocol was followed for the yeast transformations of *KCA1CC-XI-IGT*, *MYA1GT*, and *XIKGT* (Clontech 2010). This protocol is a

polyethylene glycol/lithium acetate based method, which acts to disrupt the membrane, allowing for the uptake into yeast. Each yeast transformation was plated similarly to the *E. coli* transformations.

Yeast Matings

One mL of 2X YPDA was first pipetted into individual microcentrifuge tubes

before the colonies were placed into the microcentrifuge tube via sterile toothpick.

One colony of yeast transformed with bait plasmid and one colony of yeast transformed with prey plasmid were taken each from fresh plates (no more than 1 month old) via sterile toothpick and put into a microcentrifuge tube. The microcentrifuge tubes were vortexed to break up the colonies and sufficiently mix the solution. The microcentrifuge tubes were incubated at 30°C with 200 rpm shaking for approximately 20-24 hours. A list of all the bait and prey plasmids that were mated together can be seen in Table 1 and Table 2 below in results.

Plating

The yeast cultures were spread on different selection-plates to see if there were indeed bait and prey plasmids present, and to see if the encoded proteins actually interacted with each other. At 20-24 hours, 1/10 dilutions are made of all matings. 100 μL of each 1/10 dilution was plated onto a DDO (-Trp/-Leu) plate

and onto a DDOXA (-Trp/-Leu/+Xαgal/+AbA) plate. DDO plates check to see if the bait and prey plasmids are indeed present in the same yeast cell. Prey plasmids encode for leucine synthesis, and bait plasmids encode for tryptophan synthesis. DDOXA plates check to see if the bait and prey plasmids actually interacted in the yeast cells; if there is an interaction, α-galactosidase is produced which cleaves the α linkage of X-α-gal and produces a blue colony (similar to β-galactosidase reporters). So, if there are blue colonies on the plate, those are where the prey and bait proteins interacted with each other. AbA is short for Aureobasidin A, an antibiotic that is deadly to *S. cerevisiae*. Because one of the reporter genes in the yeast strain Y2HGold (the strain into which we have transformed the bait plasmids) is *AUR1-C*, any protein-protein interaction that turns on these reporter genes ("positive interaction") will express this gene. This gene encodes for the enzyme inositol phosphoric ceramide synthase, which causes the cell to be resistant to AbA (Clontech 2010). The plates were then incubated at 30°C for 3-5 days. After 3-5 days is up, the plates were taken out and analyzed.

Results:

In our experiment, the bait plasmid contains the globular tail domain of one of various myosins, and the prey plasmid contains any potential interacting

protein. In this case an inserted cDNA library from seven-day-old *Arabidopsis* seedlings will be used in the prey plasmid. The bait constructs were made first, specifically those which encode DB-*KCA1CC*-MYA2GT, DB-*KCA1CC*-XIBGT, DB-*KCA1CC*-XI-IGT, DB-MYA1GT, and DB-XIKGT (MYA1GT and XIKGT plasmids that do not contain a coiled-coil region). The goal was to test different myosin isoforms and whether or not they're globular tail domains bind to different proteins on organelle surfaces. The globular tail domains may be anchors for myosin on organelle surfaces.

PCR was used first to amplify the DNA sequences of interest. A gel was run to check for the right band size and see if the PCR worked. Restriction Digest was performed to cut the PCR products and the vector (pAN928) that they will be placed into. A gel was run to make sure the Digest worked. Ligation was performed to place the PCR product into the vector and attach the *KCA1CC* region. The bait plasmids were transformed into *E. coli* cells, and then they were transformed into yeast. Yeast matings were then performed between the yeast bait plasmids and yeast prey plasmids to check for specific protein-protein interactions. The results can be seen

in the figures and tables below:

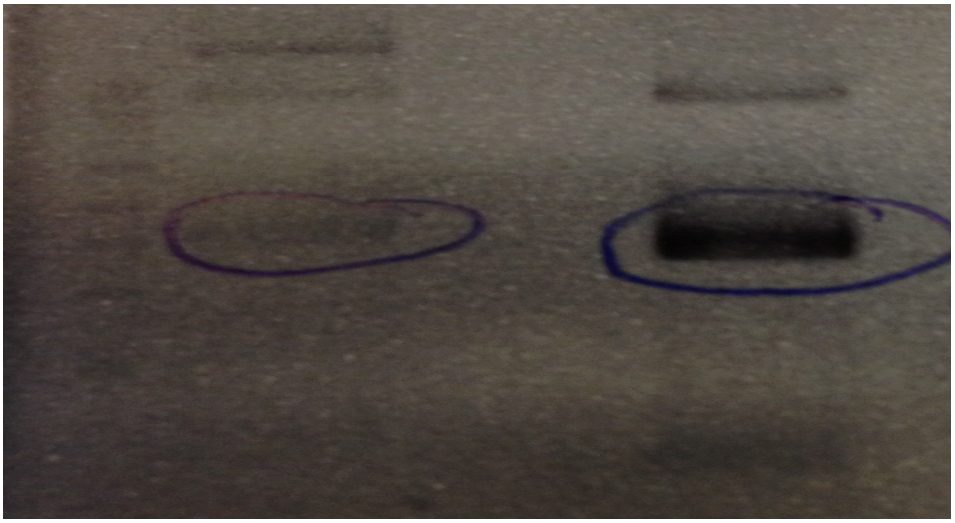

Figure 2. Gel electrophoresis run after digest for *MYA2GT, XIBGT, XI-IGT*. Ovals indicate correct band sizes of digested PCR products. No product was obtained for *XIBGT*

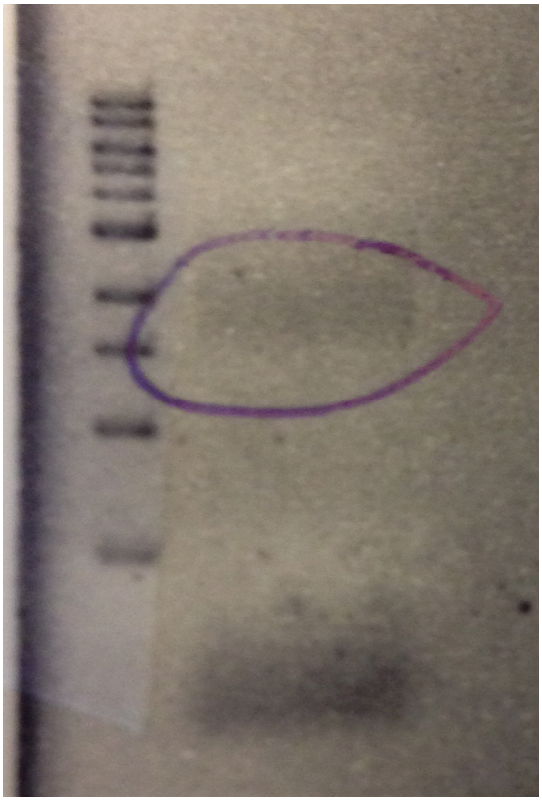

Figure 3. Gel electrophoresis run after digest for *XIBGT*. Oval indicates the correct band size of digested PCR product.

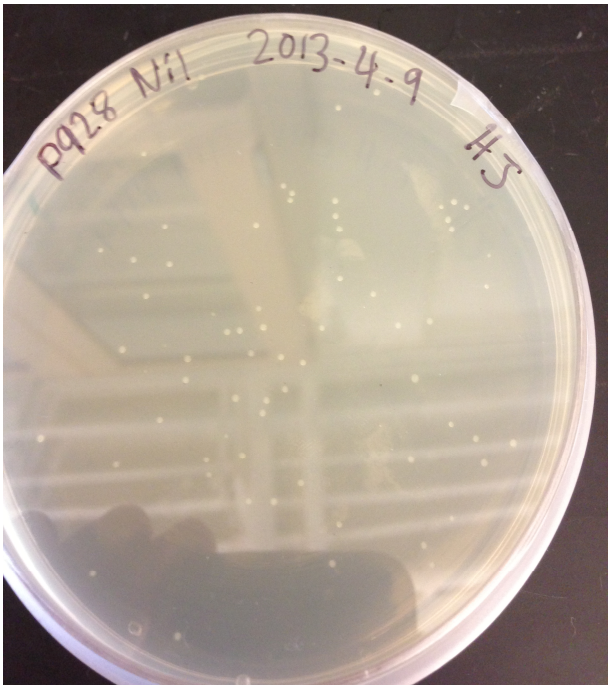

Figure 4. pAN928 Nil plate. The Nil plate does not have a PCR product inserted into the pAN928 plasmid. Little to no growth was expected compared to the other plates.

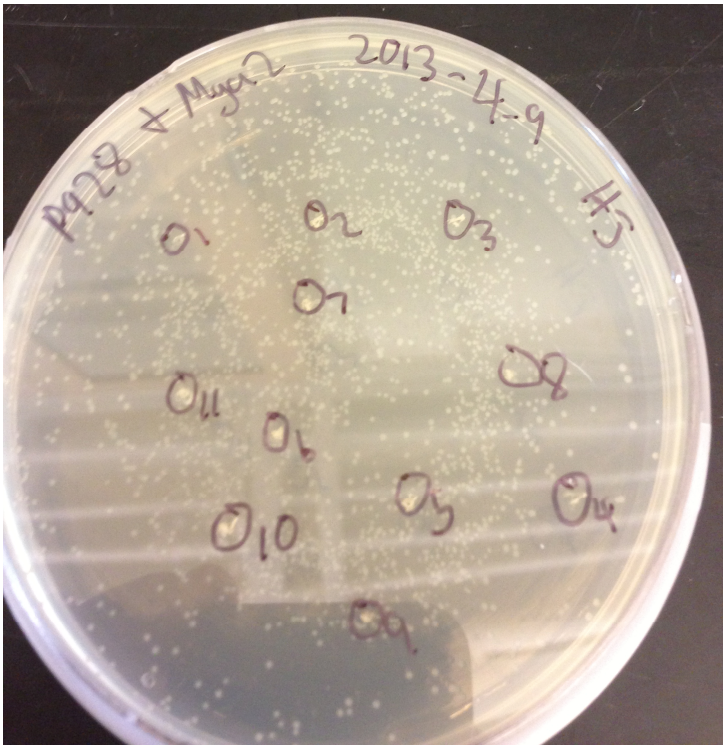

Figure 5. pAN928+*MYA2GT* plate after colony PCR. Circles with numbers identify specific colonies used in colony PCR.

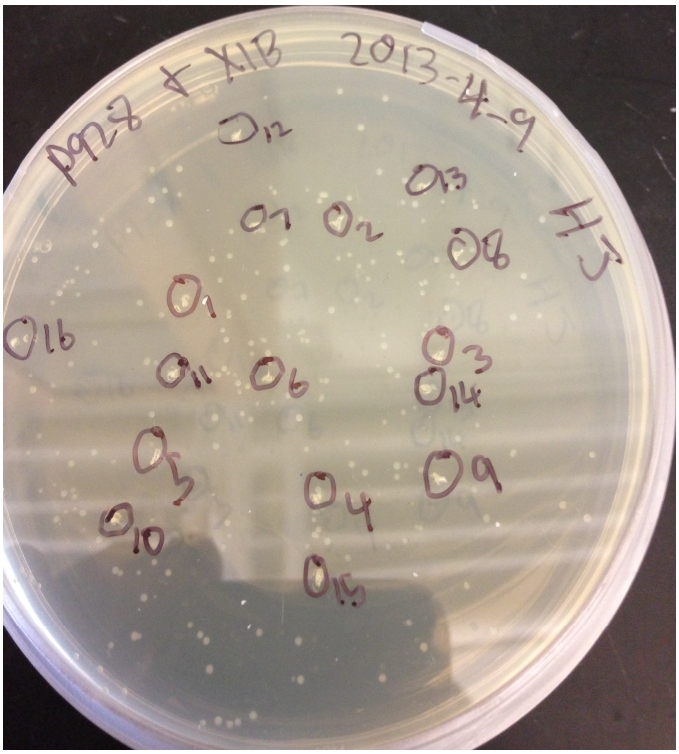

Figure 6. pAN928+*XIBGT* plate after colony PCR. Circles with numbers identify specific colonies used in colony PCR.

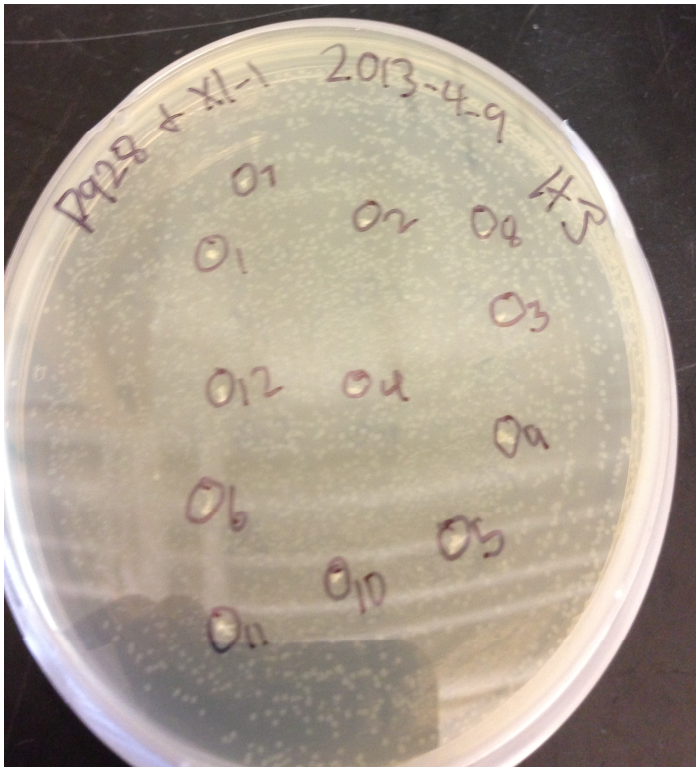

Figure 7. pAN928+*XI-IGT* plate after colony PCR. Circles with numbers identify specific colonies used in colony PCR.

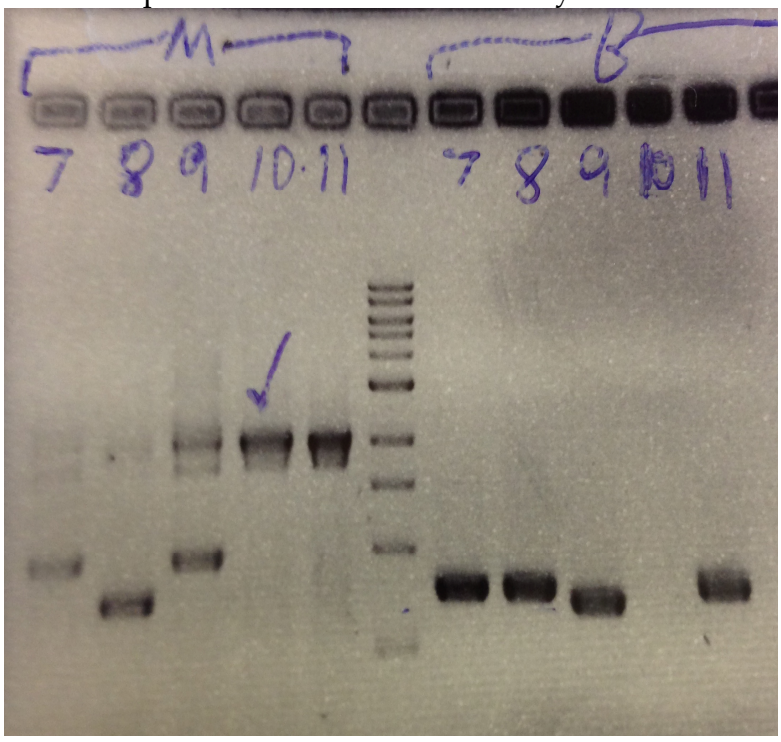

Figure 8. Gel electrophoresis run after colony PCR for *KCA1CC-MYA2GT* (left) & *KCA1CC-XIBGT* (right). Checkmark indicates correct size of band.

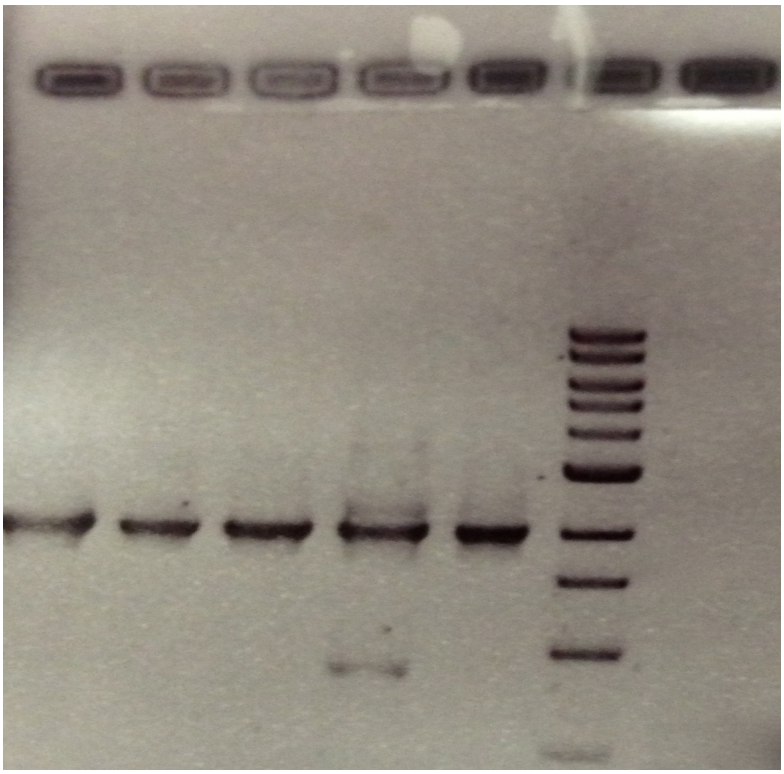

Figure 9. Gel electrophoresis run after colony PCR for *KCA1CC-MYA2GT*.

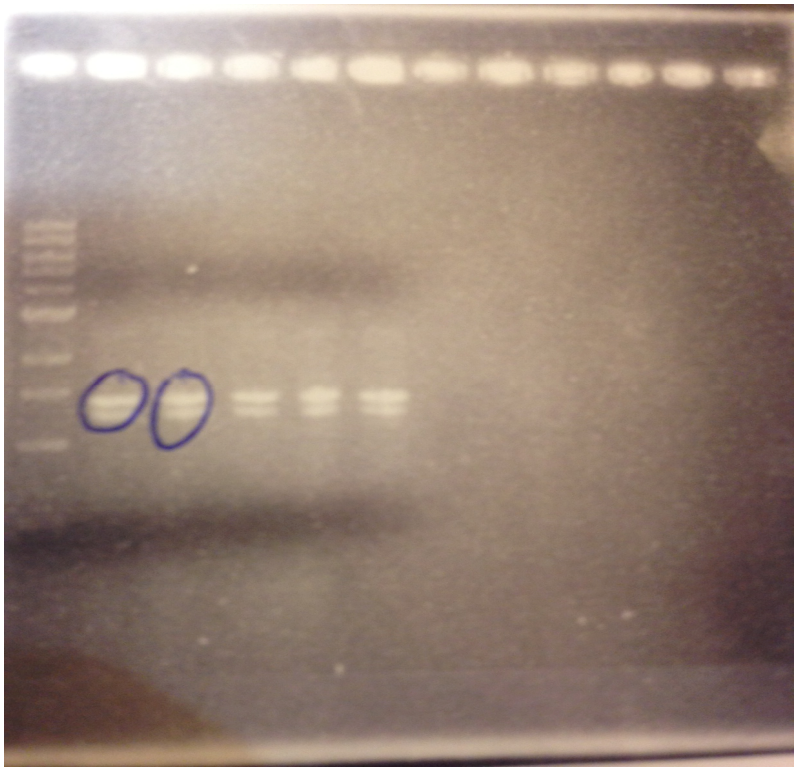

Figure 10. Gel Electrophoresis run after colony PCR for *MYA1GT*. Circles indicate correct band sizes.

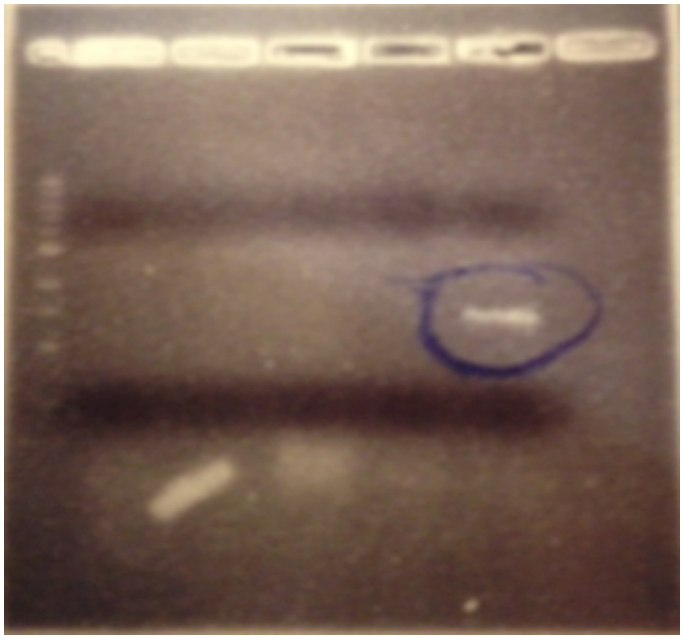

Figure 11. Gel Electrophoresis run after colony PCR for *XIKGT*. Circle indicates correct band size.

Candidate	Gene	Bait Plasmids				
		BK	BK1	BKK	BKI	B-1
		KCA1CC	KCA1CC-MYA1GT	KCA1CC-XIKGT	KCA1CC-XI.IGT	MYA1GT
A9	ptrotein exordium like 4	yes	yes	no	few	
A11	SLAC homologue 3	yes	yes	few	yes	few (1-3)
A67	GMP-synthase-C & glutamine anidotransferase domain-containing protein	yes	yes	yes (med)	yes	yes
E78	putative phosphoethanolamine N-methyltransferase 3	yes	yes	yes	yes	yes (med)
K20	nucleic acid-binding, OB-fold-like protein	yes	yes	yes	yes (med)	yes (med)
O21	actin depolymerizating factor 3	yes	yes	yes	yes (med)	yes
O28	40S ribosomal protein S7-3	yes	yes	yes	yes (med)	no
O66	putative plastid-lipid-associated protein 5	yes	yes	few (1/2 DDO)	yes	few (4)
P7	adenosylhomocysteinase	yes	yes	few (1; ~15 DDO)	yes	few (1; 10 DDO)

Table 1. Results from the first round of BK1 Candidate matings with bait plasmids. Yes, no, and few all signify if there were blue colonies and the number of colonies on the DDOXA plates (successful protein-protein interaction). "Yes" indicates numerous colonies, and "No" indicates zero colonies. Only references to DDO plates (presence of proteins) are if it says so specifically.

Candidate	Bait Plasmids				
	BK	BK1	BKK	BKI	B-K
	KCA1CC	KCA1CC-MYA1GT	KCA1CC-XIKGT	KCA1CC-XI.IGT	XIKGT
1	yes	yes	yes (med)	yes (med)	1; ~50 DDO
3	yes	yes	few (2; ~20 DDO)	few (2)	3; 6 DDO
4	yes	yes	yes (med)	yes	7; ~80 DDO
6	yes	yes	yes	yes	Yes
9	yes	yes (moldy)	yes	yes	Yes (1/2 DDO)
10	yes	yes (moldy)	yes (1/2 DDO)	few (3; ~40 DDO)	No
12	yes	yes (moldy)	yes	yes	Yes
13	yes	yes	yes	yes	Yes (1/5 DDO)
14	yes	yes	yes	yes	1; 13 DDO
15	yes (1/3 DDO)	yes (few)	yes	few	1; ~80 DDO
16	yes	yes	yes (1/5 DDO)	yes	No
19	yes	yes	yes (1/2 DDO)	yes	Yes (1/2 DDO)
20	yes	yes	yes (1/3 DDO)	few (9)	Yes (1/2 DDO)
22	yes	yes (moldy)	yes (med: 12)	few (9)	Yes (1/4 DDO)
25	yes	yes	few (4)	yes (1/4 DDO)	1; ~80 DDO

Table 2. Results from the first round BKK Candidate matings with bait plasmids. Yes, no, and few all signify if there were blue colonies and the number of

colonies on the DDOXA plates. “Yes” indicates numerous colonies, and “No” indicates zero colonies. Only references to DDO (presence of proteins)plates are if it says so specifically.

		Bait Plasmids							
		BDB	BK	BK1	B-1	BKK	B-K	BKI	BK2
Prey Candidate	Gene		KCA1CC	KCA1CC-MYA1GT	MYA1GT	KCA1CC-XIKGT	XIKGT	KCA1CC-XI.IGT	KCA1CC-MYA2GT
AG-A9	protein exordium like 4		+++	+++		-		+	
AG-A11	SLAC homologue 3		+++	+++	(+)	+		+++	
AG-A67	GMP-synthase-C & glutamine anidotransferase domain-containing protein		+++	+++	+++	++		+++	+++
AG-E78	putative phosphoethanolamine N-methyltransferase 3		+++	+++	++	+++		+++	+++
AG-K20	nucleic acid-binding, OB-fold-like protein		+++	+++	++	+++		++	
AG-O21	actin depolymerizating factor 3		+++	+++	+++	+++		++	+++
AG-O28	40S ribosomal protein S7-3		+++	+++	-	+++		++	+++
AG-O66	putative plastid-lipid-associated protein 5		+++	+++	(+)	++		+++	+++
AG-P7	adenosylhomocysteinase		+++	+++	(+)	(+)		+++	++
AD-A11		-	-	-		++	(+)	+	++
AD-A67		-	-	-	-	-	(+)	+	++
AD-028		-	-	-	-	(+)	(+)	++	++
AD-066		-	-	+		+	(+)	++	++
AD-P7		-	-		-	(+)	+	+	++

Table 3. Results from the second round of BK1 Candidate matings with bait plasmids. “+” indicates positive colonies, but the number is less than ¼ the DDO plate. “++” indicates positive colonies in the range between ¼ and ½ the DDO plate. “+++” indicates positive colonies numbering more than ½ of the DDO plate. “(+)” indicates only between 1-3 positive colonies on the DDOXA plate (colonies present on DDO plate). “–“ indicates no positive colonies on the DDOXA plate (colonies present on DDO plate). Highlighted cells indicate no growth on DDO plate.

	Bait Plasmids							
	BDB	BK	BK1	B-1	BKK	B-K	BKI	BK2
Prey		KCA1CC	KCA1CC-MYA1GT	MYA1GT	KCA1CC-XIKGT	XIKGT	KCA1CC-XI.IGT	KCA1CC-MYA2GT
AG-K1		+++	+++		++	(+)	++	+++
AG-K3		+++	+++		(+)	+	(+)	++
AG-K4		+++	+++		++	+	+++	+++
AG-K6		+++	+++		+++	+++	+++	+++
AG-K9		+++	+++		+++	++	+++	+++
AG-K10		+++	+++		++	-	(+)	-
AG-K12		+++	+++		+++	+++	+++	+++
AG-K13		+++	+++		+++	+	+++	+++
AG-K14		+++	+++		+++	(+)	+++	+++
AG-K15		++	+		+++	(+)	+	++
AG-K16		+++	+++		+	-	+++	++
AG-K19		+++	+++		++	++	+++	+++
AG-K20		+++	+++		++	++	+	+++
AG-K22		+++	+++		++	+	+	++
AG-K25		+++	+++		(+)	(+)	++	++
AD-K4	-	+		-	(+)	+	+	++
AD-K6	(+)	-			(+)	+	++	+++
AD-K12	-	-			(+)	+	+	++

Table 4. Results from the second round of BKK matings with bait plasmids. "+" indicates positive colonies, but the number is less than ¼ the DDO plate. "++" indicates positive colonies in the range between ¼ and ½ the DDO plate. "+++" indicates positive colonies numbering more than ½ of the DDO plate. "(+)" indicates only between 1-3 positive colonies on the DDOXA plate (colonies present on DDO plate). "–" indicates no positive colonies on the DDOXA plate (colonies present on DDO plate). Highlighted cells indicate no growth on DDO plate.

Figure 2 shows the restriction digest was successful with *MYA2GT* and *XI-IGT*; there was no band for *XIBGT*. Figure 3 was the gel that was successful for *KCA1CC-XIBGT*. One can see in Figures 4, 5, 6, and 7 the transformed plates for pAN928 Nil, pAN928+*MYA2GT*, pAN928+*XIBGT*, and pAN928+*XI-IGT*. The pAN928 Nil plate was the one that did not have the PCR product inserted into the plasmid and was expected to have zero to few colonies compared to the other plates; it had fewer colonies than the other 3 transformed plates that had PCR

products ligated into pAN928. The pAN928+*MYA2GT* plate and pAN928+*XI-IGT* plate had about the same number of colonies, and the pAN928+*XIBGT* had fewer colonies but still more than the Nil plate. Colony PCR was then performed to check if the transformation was successful and followed by a gel. The different colonies picked on each plate are labeled in Figures 5, 6, and 7. Figure 8 shows a gel that was run and produced the expected size band for *KCA1CC-MYA2GT*; M10 and M11 were the expected size bands on the gel, and there were not any bands of the expected size for *KCA1CC-XIBGT*, suggesting that this fusion construct was not created.

Colony PCR for the KCA1CC-MYA2GT fusion gene identified several positive candidates, ~1900 bp bands (Figure 9). M14 was analyzed further, and it was found to match the expected sequence of *KCA1CC-MYA2GT*. Figure 10 shows the gel run after a successful colony PCR for *MYA1GT*; M1 in the second well was analyzed further and sequenced; M1 matched the known sequence of *MYA1GT*. Figure 11 shows the gel run after a successful colony PCR for *XIKGT*; B-K 5 in the sixth well was analyzed further and sequenced, and B-K 5 matched the known sequence for *XIKGT*.

Tables 1, 2, 3, and 4 show all of the bait and prey yeast matings that have been performed thus far. Bait plasmids were mated with both BK1 candidates and BKK candidates. The BKK candidates (prey plasmids picked from a

KCA1CC-XIKGT screening by another researcher in the lab-Anna Vick) have not been sequenced, and they are therefore unidentified. However, they all had differentiating band sizes when run on a gel and appear to be different protein products. This is important so that the same matings are not being performed with the thought that they are different prey plasmids. Prey candidates with "AG" in front of the name indicates a prey plasmid with an inserted *GCN4CC* region, and candidates with "AD" in front of the name indicates a prey plasmid without a coiled-coil region inserted into the plasmid. The prey plasmids were all constructed by another researcher in the lab (Anna Vick). One can see from all 4 tables that BK (*BD-KCA1CC*) had protein-to-protein interactions with every prey plasmid (BK1 or BKK candidates) it was mated against. *KCA1CC-MYA1GT* (BK1) also had successful protein interactions with every prey plasmid it was mated against. *KCA1CC-XIKGT* (BKK) and *KCA1CC-XI-IGT* (BKI) both had successful protein interactions with most prey candidates they were mated with (at least a few colonies) except with prey candidate A9, which encodes for protein exordium-like 4. As for *MYA1GT* without the coiled-coil region (B-1), it interacted with most of the prey candidates it was mated against but there tended to be fewer colonies. B-1 had no interaction at all with AG-O28 and AD-K4 though, where BK1 did. *XIKGT* without the coiled-coil region (B-K) successfully interacted with most of the prey candidates it was mated with, but

the colonies were fewer on average compared to BKK. *KCA1CC-MYA2GT* had protein interactions with almost every prey candidate it was mated against.

Discussion:

We were not able to obtain a *KCA1CC-XIBGT* bait plasmid. The experiment began by using *Arabidopsis* seedling cDNA in the PCR with *XIBGT* primers, and then it was changed to flower cDNA as *XIB* is more highly expressed in flowers than in seedlings, which helped initially with getting the expected size band after the digest. However, transformation of *KCA1CC-XIBGT* into *E. coli* has failed to produce expected band size when run on a gel after colony PCR. The reason for this is unknown, and more investigation needs to be done. The following bait plasmids were all successfully made: *KCA1CC-MYA2GT*, *KCA1CC-XI-IGT*, *MYA1GT*, and *XIKGT*. Each was sequenced, and the sequence matched the known sequence.

It was unexpected that BK (BD and *KCA1CC*) by itself interacted with all of the prey candidates it was paired with since it does not encode any part of a myosin protein. However, just because there is an interaction does not mean that BK could function as a myosin protein in moving the cargo molecule. More yeast-two hybrid assays need to be done with BK, testing it with more prey candidates. One of the biggest reasons that the candidate proteins associated

with prey plasmids were tested against different myosin isoforms was to compare the protein interactions, or lack there of, between bait plasmids and prey plasmids that have the *KCA1CC* region and between bait plasmids and prey plasmids without the *KCA1CC* region associated with them. Having the coiled-coil region promotes dimerization. So, it can be seen how much of a factor dimerization is playing in the protein interactions. Overall, there appeared to be more protein interactions when the coiled-coil regions were present compared to when they were not in the Yeast-two hybrid assays. However, sometimes there was the same number of interactions in bait plasmids with and without the *KCA1CC* region. When *KCA1CC-MYA1GT* was compared to *MYA1GT*, the results showed that there were more interactions with prey plasmids when the coiled-coil region was present overall. AG-O28 was particularly interesting because B-1 had no interaction at all, and BK1 had strong protein interactions, indicating that the coiled-coil coding region was vital to the interaction occurring. However, B-1 had the same degree of protein interactions on multiple plates as BK1 such as AG-A67 and AG-O21, which shows the dimerization was not a factor in whether or not the proteins interacted in these 2 instances. *KCA1CC-XIKGT* and *XIKGT* did not show a direct correlation between the number of protein interactions the bait plasmid had with the prey plasmid and whether or not the coiled-coil regions influenced the interactions or not; *XIKGT*

had numerous successful protein interactions by itself. BKK interacted with AG-K10, and B-K did not interact with that particular prey plasmid at all, but this was one of the only instances where the coiled-coil region may have made a difference.

KCA1CC-XI-IGT simply showed that it had many protein interactions with all of the prey candidates it was mated with, but it still needs to be compared to matings between *XI-IGT* without the *KCA1CC* region and the same prey candidates that *KCA1CC-XI-IGT* was mated against. The *XI-IGT* bait plasmid has not been made yet, but this plasmid will be very useful in comparing matings with prey plasmids to *KCA1CC-XI-IGT*. Similarly, *KCA1CC-MYA2GT* interacted with many of the prey plasmids it was mated with, but there is not a *MYA2GT* bait plasmid without the coiled-coil region to mate with prey plasmids and compare results. Once *XI-IGT* and *MYA2GT* bait plasmids have been constructed, dimerization with the coiled-coil regions can be examined more closely with these two myosin proteins to see how much of a difference dimerization is making in whether or not the proteins interact with prey plasmid proteins.

Another reason that the candidate proteins associated with prey plasmids were tested against different myosin isoforms was to see whether or not specific plant organelles could interact with only one specific myosin isoform or many.

The candidates from the BK1 and BKK screenings were expected to interact with the BK1 and BKK bait plasmids, respectively. BKK candidates interacted with BKK bait plasmids, and BK1 candidates interacted with BK1 bait plasmids as expected. However, BK1, BKI, and BK2 bait plasmids all interacted with the BKK candidates as well (they all interacted with BK1 candidates too). This shows that certain prey candidates are not limited to only one specific myosin isoform, but instead, can interact with multiple myosin isoforms.

There were many prey candidates that interacted with both bait plasmid constructs, one with the *KCA1CC* coding region and one without. However, there was never a Yeast-two hybrid assay where there were only interactions with the bait plasmid without the *KCA1CC* region and no interactions with the bait plasmid with the *KCA1CC* region. Therefore, dimerization of the coiled-coil regions in the bait (*KCA1CC*) and prey (*GCN4CC*) plasmids seems to be important and a factor in whether or not certain myosin proteins can successfully interact with cargo proteins.

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Figure 1: http://en.wikipedia.org/wiki/File:Two_hybrid_assay.svg