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# Searching for potential binding partners of arabidopsis $\beta$ -amylase9 using yeast 2-hybridization

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Searching for Potential Binding Partners of *Arabidopsis*  $\beta$ -amylase9 Using Yeast 2-Hybridization

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An Honors Program Project Presented to  
the Faculty of the Undergraduate  
College of Science and Mathematics  
James Madison University

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by Sheikh Rubana Hossain

May 2018

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Accepted by the faculty of the Department of Biology, James Madison University, in partial fulfillment of the requirements for the Honors Program.

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PUBLIC PRESENTATION

This work is accepted for presentation, in part or in full, at Biosymposium on April 13, 2018 .

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## Abstract

In plants, starch is a major carbon and energy storage compound. Starch is made as a product of photosynthesis while plants are in light and is degraded at night. Our lab is interested in the cellular mechanism of starch degradation in plants and for our studies we use *Arabidopsis thaliana* as a model.  $\beta$ -amylases are primarily responsible for the hydrolysis of starch in plants and a total of nine  $\beta$ -amylases genes are encoded in *Arabidopsis thaliana*. These nine genes are identified as *BAM1-9*. BAM9 is located in the chloroplast where starch is located, and is present in all flowering plants but it is not catalytically active. It was published that BAM9 was very strongly expressed in the transition between nighttime and daytime. BAM9 may have an altered activity as a regulatory protein. Since regulatory proteins function by interacting with other proteins, we used the yeast two-hybrid system to attempt to identify interacting protein(s). As a first step, yeast cells were transformed with a BAM9 bait plasmid. Next, a cDNA library screen was performed where yeast cells with the bait were transformed with a library that encodes for potentially interacting proteins. The expression of reporter genes was observed. Next, sequencing and bioinformatics analysis were used to identify the potentially interacting proteins(s). Twenty one unique hits were detected from both screens. Lastly false positives were then eliminated by a series of control experiments. Partial but overlapping sequences of AMY3 were identified four times in the bioinformatics analysis and a conserved coil region of AMY3 was identified in each of the prey plasmids. Obtaining more comprehensive information about protein-to-protein interactions will aid our understanding of BAM9's function.

## Introduction

In almost all plants, starch is a major carbon and energy storage compound. Starch is synthesized in the chloroplasts found in the leaves of plants and starch is also synthesized in amyloplasts found in the starch-storing tissues of many crops (Pfister et al., 2016). Not only is starch important to plants, starch is also extremely valuable for humans as it is a primary carbohydrate and sustainable raw material source for humans (Pfister et al., 2016). Almost half of our daily energy uptake comes from starch (Busi et al., 2015). Other uses of starch by industries include using it for cosmetics, paper, plastics and textile where starch granules are modified to fit the specific uses of starch (Ellis et al., 1998).

Starch is comprised of two polymers made of glucose: amylose and amylopectin (Lloyd et al., 2005). Amylopectin is composed of  $\alpha$ -1, 4 linkages with  $\alpha$ -1, 6 branches making it a large highly branched molecule, whereas amylose is only composed of  $\alpha$ -1, 4 glycosidic linkages (Zeeman et al., 2010). Both of these molecules make up semicrystalline particles but amylopectin is about 80% of starch granules (Lloyd et al., 2005). During the day, some photosynthate remains in the chloroplasts and is stored as starch instead of being converted to sucrose for the use of energy for growth. This starch is known as transient starch, which is broken down at night to provide leaves and the rest of the plant with adequate substrates for respiration and sucrose synthesis (Lloyd et al., 2005). *Arabidopsis* that is not able to make starch and does not have transient starch as a reserve has lower growth rates, suggesting that the breakdown of starch is important to plants for their survival (Smith et al., 2007). Thus, one cellular mechanism of special

interest is starch degradation in plants; in particular we use *Arabidopsis thaliana* as a model for its practicality in lab settings.

The majority of the starch created during the light phase in *Arabidopsis* leaves is broken down during the night and a number of enzymes work together to accomplish this. Enzymes such as glucan, water dikinase (GWD1),  $\alpha$ -amylase,  $\beta$ -amylase and other enzymes work together in a complex set of reactions to degrade starch (Streb et al., 2012).  $\beta$ -amylase seems to be an important enzyme employed in degrading starch as evident by high levels of starch accumulation in mutants lacking  $\beta$ -amylase (Daniel et al., 2008).

A total of nine  $\beta$ -amylases genes are encoded in *A. thaliana* identified as *BAM1-9*. *BAM5* codes for a catalytically active cytosolic enzyme (Monroe and Preiss, 1990; Monroe *et al.*, 1991; Wang et al., 1995). *BAM8* and *BAM7* are also not plastidic and act as transcription factors in the nucleus (Reinhold et al., 2011). *BAM1*, *BAM2*, *BAM3* and *BAM6* code for catalytically active, plastid-localized enzymes (Lao *et al.*, 1999; Kaplan and Guy, 2005; Sparla *et al.*, 2006; Fulton et al., 2008; Monroe *et al.*, 2014). Mutants lacking *BAM3* resulted in higher starch levels and lower maltose levels during the dark phase in comparison to wild type (Fulton et al., 2008). Double mutants lacking both *BAM1* and *BAM3* resulted in an ever-higher starch levels and even lower maltose levels compared to *bam3* mutant. This indicates the importance of the *BAM1* and *BAM3* to starch breakdown (Fulton et al., 2008). *BAM4* and *BAM9* code for plastid-localized proteins; however, no catalytic activity has been observed for either of these proteins (Fulton et al., 2008; Li et al., 2009; Monroe, unpublished). Compared to the other BAMs, *BAM9* is poorly understood.

BAM9 is of interest because its amino acid sequence is more divergent than any of the other *Arabidopsis* BAMs. BAM9 is located in the chloroplast where starch is located, however its function is not documented (Daniel et al., 2008). BAM9 orthologs not being clustered with the other BAM orthologs indicates that BAM9 function is distinctive from the other  $\beta$ -amylases. There is evidence that *BAM9* mRNA expression is at its highest at the end of the dark phase, suggesting that BAM9 may be present during the light phase while starch is being made (Chandler et al., 2001). This pattern of protein expression is in contrast with the expression of the other eight  $\beta$ -amylases (Smith et al., 2004). There are also other differences between BAM9 and the other  $\beta$ -amylase. When BAM9's amino acid sequence is compared to the active  $\beta$ -amylases, some amino acids that are conserved in the active  $\beta$ -amylase are missing in the BAM9 active site (Fulton et al., 2008). However, BAM9 does have a unique region of conserved amino acids, which are not present in the other  $\beta$ -amylase (Monroe, unpublished). This region of conserved amino acids creates a conserved patch on the protein surface, which could indicate that BAM9 is binding to something else to accomplish its function.

There is evidence that BAM9 has a function in starch metabolism. Plants lacking BAM9 and BAM3 have an increased starch buildup compared to those lacking just BAM3 suggesting that BAM9 is aiding in the starch break down somehow (Steidle, 2010). Since BAM9 is proposed to have an altered activity and is present across flowering plants, acquiring more information about BAM9 may help us obtain some understanding about its function in plants. Protein-protein interactions are vital for organisms as they take part in almost all cellular activities. Acquiring more information about proteins that interact with BAM9 will provide us with more information about

BAM9's function and starch metabolism. I used the yeast two-hybrid system to identify proteins that may interact with BAM9.

The yeast two-hybrid system can be applied to learn more about protein–protein interactions. This application uses a protein encoded by the gene *GAL4*, which is found in the yeast *Saccharomyces cerevisiae*. This protein is unique as it contains two functional domains, a DNA-binding domain and an activation domain, which help initiate the transcription of genes (Gietz et al., 1997). This particular system can be used to screen for any proteins that interact with a particular protein of interest (Gietz et al., 1997). The protein of interest is referred to as the “bait”, and this protein is expressed as a fusion to the *GAL4* DNA-binding domain. On the other hand, the prey proteins, which come from a chosen cDNA library, are attached to the *GAL4* activation domain (Tian et al., 2012). When the bait interacts with one of the prey proteins it brings the two *GAL4* domains together and transcription is activated at the *GAL4*-binding site, which is present in the promoters of two reporter genes, *HIS3* and *lacZ*, that have been added to the *Saccharomyces cerevisiae* genome (Tian et al., 2012).

Currently, not a lot of information has been attained for BAM9 and its function. Attaining more information about which protein binds with BAM9 will aid in getting a better understanding on how BAM9 functions in the starch degrading process. Using the yeast two hybrid technique we obtained evidence that BAM9 may be binding to a chloroplast-located  $\alpha$ -amylase, *AMY3*, which is also reported to be involved in starch metabolism (Seung et al., 2013). Starch breakdown is a very important process for any plant as starch supplies the plant with both a carbon source and energy source. Starch is

also a large part of the human diet and various humans' industrials. Therefore having a fundamental understanding of the process that breaks down starch is important for multiple fields.

## Materials and Methods

### *Transformation of Bait Vector into Y190-Strain Yeast*

BAM9-pAS1 was a bait plasmid constructed previously by Dr. Amanda Storm. BAM9-pAS1 was transformed into Y190 yeast by following a modified version of the protocol described by Tian et al. (2012). Yeast cells (*Saccharomyces cerevisiae*) were grown in 2 mL yeast extract peptone dextrose (YPD) media and the tube was placed in a 30 °C incubator overnight. The next morning the culture was diluted by taking 0.5 mL of the cells and adding 4.5 mL YPD. Next the diluted cells were separated into two tubes and allowed to grow for another 2.5 hours. The cells were then pelleted for 5 minutes at 3000 rpm at room temperature (RT). The supernatant was carefully poured out and the pellet was resuspended using 4 mL sterile DI water. The cells were again pelleted as described above. The pellet was resuspended in 200 µL Solution I (100 mM LiAc, 10 mM Tris-HCl [pH 7.5], 1 mM EDTA) and the resuspension was set aside for 10 min at RT. To this tube 100 µl of the Y190 cells, 2 µL of BAM9-pAS1 DNA, 10 µL of boiled calf thymus DNA (10 mg/ml), and 700 µL of Solution II (100 mM LiAc, 10 mM Tris-HCl [pH 7.5], 1 mM EDTA, and 50 % PEG-3350) was added and the mixture was pipetted up and down to mix (Tian et al., 2012). The tubes were placed into a 30 °C water bath for 30 min and next placed into a 42 °C water bath for 15 min. The tubes were then centrifuged at 3000 rpm for 2 min and the supernatant was carefully removed. To the remaining pellet 200 µL of DI water was added and the cells were resuspended. The cells were serially diluted and each dilution was plated on Complete Media (Difco Yeast Nitrogen Base without amino acids, 2% glucose, 4 mM serine, 0.1 mM adenine hemisulfate salt, 0.15 mM lysine HCl, 0.3 mM phenylalanine, 0.15 mM tyrosine, 0.1 mM

arginine HCl, 0.15 mM methionine, 1.5 mM threonine, 0.75 mM leucine, and 0.1 mM histidine) missing tryptophan (CM-Trp) plates. The plates were placed in a 30 °C incubator for 3 days.

#### *Validation of Bait Vector for the Utilization of Yeast Two-Hybrid Screen*

In order to determine if the bait vector was suitable for further experimentation, the BAM9-pAS1 vector was tested for self-activation to detect the level of basal expression of the reporter genes. The basal expression of the HIS3 reporter gene was established by transforming the BAM9-pAS1 bait vector into the Y190 strain yeast which was streaked onto CM –Trp -His plates with concentrations of 3-amino-1,2,4-triazole (3-AT), a competitive inhibitor of histidine expression, ranging from 0 – 75 mM as 100 mM or higher is not suitable to use (Tian et al., 2012). For a control an already verified bait vector, pSEIII2-pAS1 was also plated CM –Trp -His plates with varying 3-AT concentrations. Plates were left in a 30° incubator for seven days after which the plates were observed for the lowest concentration of 3-AT with no growth.

#### *cDNA Library Screen*

Once the yeast strain containing BAM9-pAS1 bait vector was validated, the bait vector in Y190 yeast was screened with prey vectors from the cDNA library. Prey vectors were generated from an Arabidopsis cDNA library (CD4-22) obtained from the Arabidopsis Biological Resource Center at Ohio State University. cDNAs from the library were fused with the activation domain of the GAL4 transcription previously by Dr. Amanda Storm. First, three plates with BAM9-pAS1 lawns were created by taking BAM9-pAS1 yeast colonies and suspending them into 300 µL of sterile DI water. Then

100  $\mu$ L was pipetted onto each CM-Trp plates and incubated in 30 °C for four days. Then, 1 mL sterile DI water was pipetted onto each plate until majority of the lawn was suspended in liquid and these suspended cells were placed into 1.5 mL tubes. The suspended cells were added to 500 mL YPD media in intervals of 800  $\mu$ L until the optical density at 600 nm ( $OD_{600}$ ) was close to 0.1. The inoculated media was shaken in a 30 °C incubator and the  $OD_{600}$  was monitored until it was close to 0.4. The cells were pelleted for 5 minutes at 3000 rpm in increments of 250 mL. The supernatant was decanted and the pellet was resuspended in 100 mL sterile DI H<sub>2</sub>O. The resuspended cells were centrifuged for 5 minutes at 3000 rpm and the supernatant was decanted again. The pellet was then resuspended in 50 mL Solution I (100 mM LiAc, 10 mM Tris-HCl [pH 7.5], 1 mM EDTA). The cells were pelleted as above and resuspended in 2.5 mL of Solution I. To this suspension of pellet, 30  $\mu$ g of the prey vectors containing the cDNA library, 125  $\mu$ L of boiled calf thymus DNA, (10 mg/mL) and 15 mL of Solution II (100 mM LiAc, 10 mM Tris-HCl [pH 7.5], 1 mM EDTA, and 50% PEG-3350) were added. This total solution was aliquotted into 23 1.5 mL tubes, with 700  $\mu$ L of solution in each. The tubes were placed into a 30° C water bath for 30 minutes and next placed into a 42° C water bath for 15 minutes. The tubes were centrifuged at 6000 rpm for 1 minute and the supernatant was carefully pipetted out. To the remaining pellet 400  $\mu$ L of DI water was added and resuspended. For each of the 23 tubes, the 400  $\mu$ L resuspension was equally plated onto two CM -Trp -Leu -His +25 mM 3-AT plates, producing a total of 46 plates. These plates were placed in the 30 °C incubator and the growth was monitored over 1-3 weeks. Once the colonies had grown, they were streaked onto newly made CM -Trp -Leu plates to keep as stocks. One 400  $\mu$ L resuspension was used to create a serial dilution and

200  $\mu$ L of each dilution was plated on a CM -Trp -Leu +25 mM 3-AT plate. These plates were also placed in the 30° C incubator and the growth was monitored for 5 days in order to see if the transformation efficiency is appropriate.

### *X-gal Assay*

For colonies that grew on CM -Trp -Leu -His +25 mM 3-AT plates, indicating expression of the *HIS* reporter gene, an X-gal Assay was done to establish expression of the reporter gene, *lacZ*, following a similar procedure dictated by Tian et al. (2012). Colonies were streaked onto newly made CM -Trp -Leu plates from the stock plates and placed in the 30° C incubator for 4 days. A nitrocellulose membrane, cut in the size of the Petri dish, was positioned on top of the media surface for each plate, with marks to indicate orientation, and using a gloved hand the membrane was pressed against the surface of the media to make sure that the membrane was in contact with the colonies. This membrane was left for two minutes, after which the membrane was slowly removed using forceps and placed colony side up in a foil boat. Fresh liquid nitrogen was obtained and the foil boat was placed on the liquid nitrogen for 20 seconds. After the 20 seconds the boat was submerged into the liquid nitrogen for two minutes. The boat with the membrane was taken out of the liquid nitrogen and set aside to thaw. In a sterile Petri dish with nothing in it, 20  $\mu$ L of X-gal (20 mg/mL in DMF) and 1.5 mL Z buffer (60 mM Na<sub>2</sub>HPO<sub>4</sub>, 40 mM NaH<sub>2</sub>PO<sub>4</sub>, 10 mM KCl, 1 mM MgSO<sub>4</sub>, pH 7.0) was added and the Petri dish was gently moved to mix the two solutions. A Whatman filter paper was added to the same dish and allowed to absorb the liquid. Next the nitrocellulose membrane was positioned above the Whatman filter making sure the colonies were not facing the

Whatman filter. The dish was covered with Parafilm and placed in the 37 °C incubator for 24 hours, after which the dish was observed for blue colonies.

### *Isolation of Prey Vector*

Prey vectors were then isolated from colonies that turned blue indicating the presence of *lacZ* expression. These colonies were inoculated into 2 mL of CM -Leu liquid broth and the tubes were placed in a 30 °C shaking incubator overnight. The inoculated media was transferred to a 1.5 mL tube and allowed to pellet for 1 minute in a centrifuge. The supernatant was discarded and the cells were resuspended in 67 mM KH<sub>2</sub>PO<sub>4</sub> and 10 µL of Zymolyase solution (50:49:1, glycerol:Zymolyase [G-Biosciences]: 1M Tris). The tubes were allowed to incubate one hour in a 37 °C incubator. After the hour the tubes were spun down and the supernatant was discarded. Next the miniprep protocol to attain yeast DNA as described in the typical alkaline-lysis method was followed, with the final isolated plasmid DNA being resuspended in 25 µL of DI H<sub>2</sub>O (Joly, 1996). Next the DNA was transformed into competent DH5α *E. coli* cells following an altered version of the high efficiency transformation of *E. coli* with plasmids as dictated by Inoue *et al* (1990). Changes made include using 200 µL competent cells with 10 µL of miniprepped prey vector DNA and using 800 µL SOC media instead of LB media in the recovery step. 100 µL of the transformed cells were spread on LB-Carb plates and placed overnight in a 37 °C incubator. Colonies which successfully grew were grown up 3 mL LB-Amp broth overnight in a 37 °C shaker and the cells were miniprepped as described in the alkaline-lysis method. The miniprepped DNA was dissolved in DI H<sub>2</sub>O to reach a concentration of 150 ng/µL and then each sample was shipped for sequencing by Eurofins Genomics. The standard Gal4AD primer (TACCACTACAATGGATG) was utilized to sequence

each sample. The prey plasmid insert sequence was entered into BLASTn to identify the gene encoded and the sequences were also entered into the UniProt database to identify aspects of the identified protein such as location, family and function.

#### *Elimination of False Positives*

False positive interactions need to be eliminated before further experimentation, thus multiple transformations of the potentially interacting prey plasmids were done. Some of the potentially interacting prey plasmids were transformed into just Y190 yeast, into BAM9-pAS1 yeast, into empty-pAS1 yeast, and into pSEIII2-pAS1 yeast using the same protocol as explained above. The prey vector transformed into empty Y190 yeast was plated on CM -Leu -His +3-AT while the other three transformations were plated on CM -Leu -Trp -His +3-AT. Next, the colonies that grew were used to perform X-gal assays following the protocol stated above. Colonies that grew and turned blue on plates other than the one where the prey vector was transformed into BAM9-pAS1 yeast were deemed a false positive.

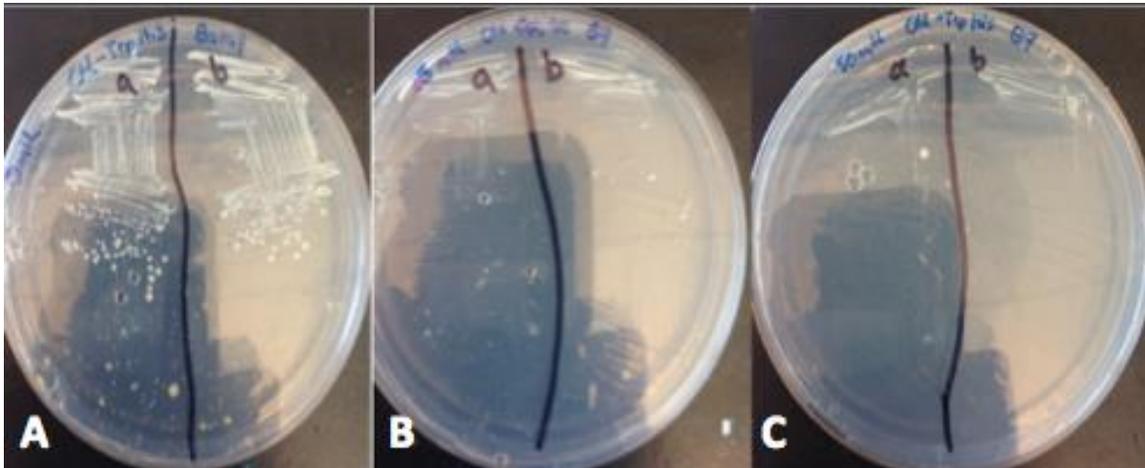
## Results

BAM9 is unique compared to the other  $\beta$ -amylases found in *A. thaliana*. BAM9's sequence is not conserved in certain regions where the other  $\beta$ -amylase are and its mRNA expression is at its highest at the end of the dark phase, which is unusual compared to the other  $\beta$ -amylases (Smith et al., 2004). BAM9 does have a region of conserved amino acids clustered on the surface of the protein. While mutant *bam9* does not show starch buildup, *bam3bam9* plant mutants result in an increased starch buildup compared to just *bam3* mutants (Steidle, 2010). BAM9 is also present in all flowering plants, thus this fact and the build up of starch in double mutants suggests the importance of BAM9. BAM9 may function as a regulatory protein and binds with another protein to complete its function. A yeast two-hybrid experiment was conducted to try to identify potential binding partner(s).

### *Validation of Bait Vector for the Utilization of Yeast Two-Hybrid Screen*

The yeast transformed with the BAM9-pAS1 bait vector were tested for self-activation to identify the level of basal expression of the reporter genes to see if the bait vector was suitable for further experimentation. If the fusion of BAM9 to Gal4BD activated the expression of *HIS3* on its own, then the bait vector would not be suitable for rest of the rest of the experiment. Also, the basal expression of *HIS3* had to be identified before moving to the next stage. The basal expression of the *HIS3* reporter gene was established by streaking transformed BAM9-pAS1 onto CM -Trp -His plates with

increasing concentrations of 3-amino-1,2,4-triazole (3-AT), ranging from 0 – 75 mM. Growth on plates with 75 mM 3-AT or higher would indicate that the bait vector was not suitable to use (Tian et al., 2012). Noteworthy growth was seen on the plate without 3-AT and a few colonies were seen on the plate made with 10 mM 3-AT, however, no colonies were seen growing on the plate made with 25 mM 3-AT or any concentration above 25 mM (Fig. 1). Therefore, this bait vector was validated and suitable for further experimentation.



**Figure 1.** BAM9-pAS1 transformed yeast colonies plated on varying concentrations of 3-AT media. (A) Shows colonies plated on 0 mM 3-AT media, (B) shows colonies plated on 25 mM 3-AT media, and (C) shows colonies plated on 50 mM 3-AT media. The two halves of each plate are two different strains of yeast transformed with the BAM9-pAS1 bait vector.

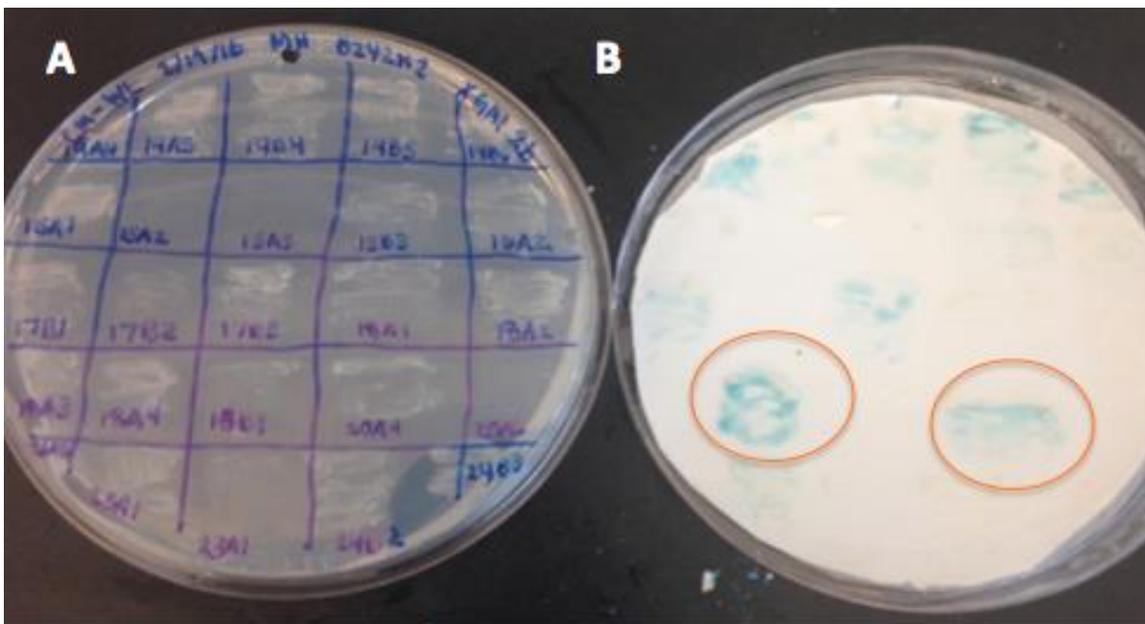
#### *cDNA Library Screen*

After the BAM9-pAS1 vector was validated, BAM9-pAS1 lawns were made and cells from these lawns were transformed with 30  $\mu$ g of prey vectors containing the cDNA library obtained from the roots and leaves of *A. thaliana*. These transformed cells were plated on CM -Trp -Leu -His +25 mM 3-AT plates and the plates were monitored for 1-3 weeks. The screen was conducted twice and the number of colonies that grew on the

selective plate was noted. Thirty colonies grew on the selective media for the first cDNA Library Screen (Table 1). A second cDNA Library Screen was completed where 75 colonies grew on the selective media (Table 1).

### *X-gal Assay*

Colonies that grew on the selective media without histidine from the initial selection were used to conduct X-gal assays to establish expression of the reporter gene, *lacZ*. The change of color of the colonies from white to blue in the presence of the X-gal substrate specified that *lacZ* gene was being expressed (Fig. 2). From the first screen, of the 30 colonies that grew, 12 turned blue when the X-gal Assay was conducted. From the second screen, of the 75 colonies that had grown on the selective media with histidine, 62 colonies turned blue (Table 1).



**Figure 2.** The master plates from the first screen and respective X-gal assays. (A) Depicts master plate with colonies that grew on selective media while (B) shows change of color (indicated by circles) or no change of color after X-gal assay performed. The colonies that turned blue detect expression of the *lacZ* gene.

**Table 1.** Summaries of reporter gene expression: the number of colonies that grew on media lacking Histidine and the number of colonies that turned blue.

	Screen 1	Screen 2
Number of colonies that grew on CM-Trp,-Leu,-His + 25 mM 3-AT	30	75
Number of colonies positive growth colonies that turned blue in X-gal assay	12	62

#### *Isolation of Prey Vector*

Seventy four colonies that had grown on the selective media without histidine and had also turned blue in the X-gal assay were identified and used for further experimentation. Liquid cultures of each of the colonies were grown and alkaline-lysis method was used to isolate the prey plasmids from the yeast cells. Once the plasmid DNA was obtained, it was transformed to competent DH5 $\alpha$  *E. coli* cells and then minipreped again to obtain enough high quality DNA for sequencing. BLASTn was then used to identify the gene that was encoded by the prey plasmid. For the first screen, eight unique insertions were identified and for the second screen, fourteen unique insertions were identified. UniProt was utilized to identify and characterize each possible interacting protein and the data attained, including protein name, function and location, are summarized in Table 2. Some of the proteins had characteristics that were unknown,

which was also noted in the Table 2. Twenty one unique hits were detected from both screens. Eight of the unique sequences were from proteins identified as located in the chloroplast. The number of hits of each isolate was also examined carefully. Among the unique hits, five Arabidopsis genes were represented more than once, while others were singletons.

#### *Elimination of False Positives*

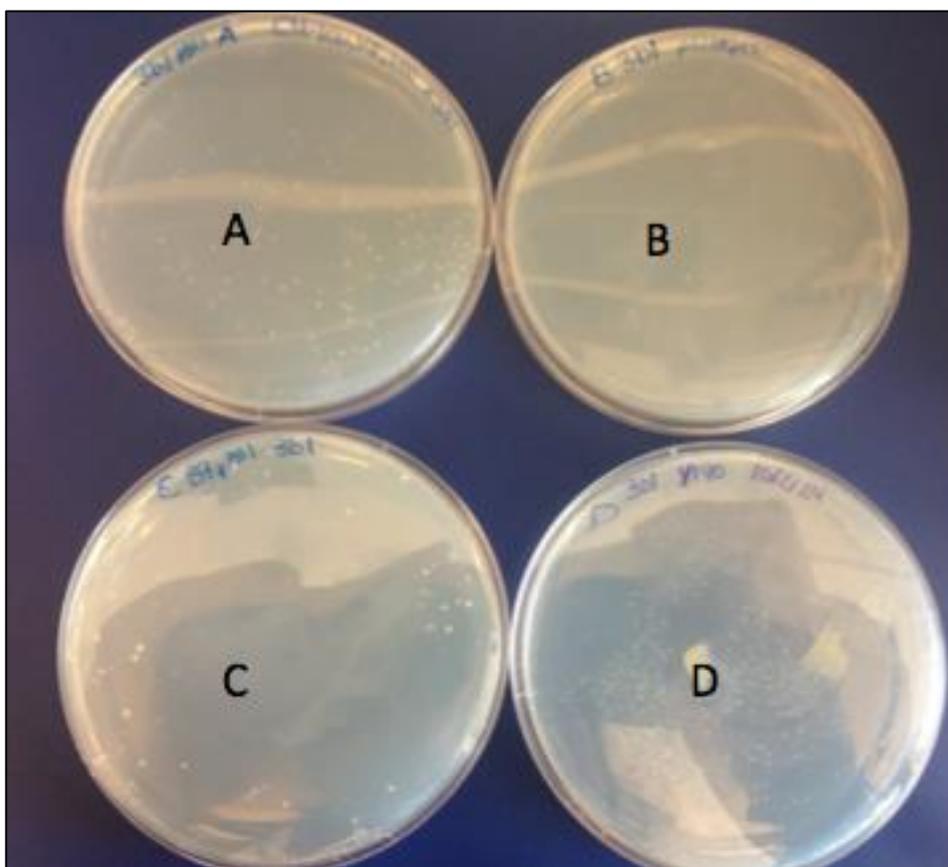
For this portion of the experimentation only those prey plasmids encoding proteins that are located in the chloroplast were used as BAM9 is also located in the Chloroplast (Table 2.) The number of hits was also considered due to limited time and resources. Alpha-amylase 3 (AMY3) was identified in four different prey vectors: 1A1, 7A2, 6A3, 8A2. Thus these isolates were the only clones tested for false positives. This was done by transforming each prey vector plasmid into Y190 yeast by itself, or into yeast containing the BAM9-pAS1 bait vector, the pAS1 empty vector, or the pSEIII2-pAS1 control vector. The prey vector transformed into Y190 yeast by itself was plated on CM -Leu -His +3-AT while the other three transformations were plated on CM -Leu -Trp -His +3-AT. For all four isolated prey vectors 1A1, 7A2, 6A3, 8A2, substantial growth of the colonies was only seen when the prey vector was transformed into BAM9-pAS1 yeast indicating that they were not false positives (Fig. 3). The colonies that grew were then used to perform X-gal assays and only the prey vectors that were transformed into BAM9-pAS1 yeast turned blue indicating the expression of the *lacZ* gene (Fig. 4).

**Table 2.** Summary the Prey Plasmid characteristic and localizations

Location	(Screen #) and Isolate Label	Number Of Hits	UniProt identifier	Protein Name	Gene Number	Function
Chloroplast	(1) 9B4	1	Q9SKI3	Expressed protein	AT2G06520	Protein domain specific binding, Photosynthesis
	(1) 10A2	1	O80601	Protein prenyltransferase superfamily protein	AT1G10095	Protein prenylation
	(1)14A3	1	P04778	Chlorophyll a-b binding protein 1	At1g29930	Photosynthesis, light harvesting in photosystem I
	(1) 17A4	1	O49675	Probable carotenoid cleavage dioxygenase 4	At4g19170	May be involved in carotenoid cleavage.
	(2) 1A1, 7A2, 6A3, 8A2	4	Q94A41	Alpha-amylase 3	At1g69830	May be involved in the determination of the final structure of glucans
	(2) 1B2,	2	Q9FV52	Methionine aminopeptidase 1B	At1g13270	Removes the N-terminal methionine from nascent proteins
	(2) 20B1	1	Q9M1X3	AT3g63160/F16M2_10	AT3g63160	Unknown
	(2) 5A2	1	Q8RWT8	Serine-tRNA ligase	A0A1P8ASH4	Catalyzes the attachment of serine to tRNA (Ser)
Mitochondrion	(1) 9B3	1	P93303	ATP synthase protein YMF19	At2g07707 backwards	one of the chains of the nonenzymatic component of the mitochondrial

						ATPase complex.
<b>Plasma membrane</b>	(2) 8B1	1	P43286	Aquaporin PIP2-1	At3g53420	Water channel required to facilitate the transport of water across cell membrane
	(2) 1A3	1	O82462	Glutamate--tRNA ligase	At5g26707	Catalyzes the attachment of glutamate to tRNA(Glu)
	(2) 6A1	1	Q38900	Peptidyl-prolyl cis-trans isomerase CYP19-1	AT2G16600 .1	PPIases accelerate the folding of proteins
	(2) 18B1	1	Q9SYM5	Trifunctional UDP-glucose 4,6-dehydratase	At1g78570	Trifunctional enzyme involved in UDP-beta-L-rhamnose biosynthesis
<b>Location</b>	<b>(Screen #) and Isolate Label</b>	<b>Number Of Hits</b>	<b>UniProt identifier</b>	<b>Protein Name</b>	<b>Gene Number</b>	<b>Function</b>
<b>Nucleus</b>	(2) 6A2,12B2, 12B3	3	Q8L3X8	Serine/arginine-rich SC35-like splicing factor SCL30	At3g55460	Involved in intron recognition and spliceosome assembly
	(1)12A2	1	Q8RWF0	26S proteasome non-ATPase regulatory subunit 13 homolog A	AT5G45620 backward	Acts as a regulatory subunit of the 26S proteasome
<b>Unknown</b>	(2) 1B1, 5A3	2	O82388	At2g29670/T27A1 6.23	At2g29670	RNA processing
	(2) 2A1	1	<u>D7LZY9</u>	Uncharacterized protein	ARALYDR AFT_48892 4	Unknown
	(2) 3A1, 19A1	2	Q9LYP4	BAG family molecular chaperone regulator 3	At5g07220	Co-chaperone that regulates diverse cellular pathways

	(1) 9B8	1	Q9SI59	Uncharacterized protein	At2g16590 backwards	Unknown
	(1) 6A3	1		Unknown	At3g41950 backwards	Unknown
	(2) 22A2	1	Unknown	Unknown	AF506713.1	Unknown



**Figure 3.** Growth of colonies transformed with prey vector plasmid 6A3 into (A) empty-pAS1 yeast, (B) pSEIII2-pAS1, (C) BAM9-pAS1 yeast and (D) Y190 yeast. The prey vector transformed into empty Y190 yeast was plated on CM -Leu -His +3-AT while the other three transformations were plated on CM -Leu -Trp -His +3-AT.

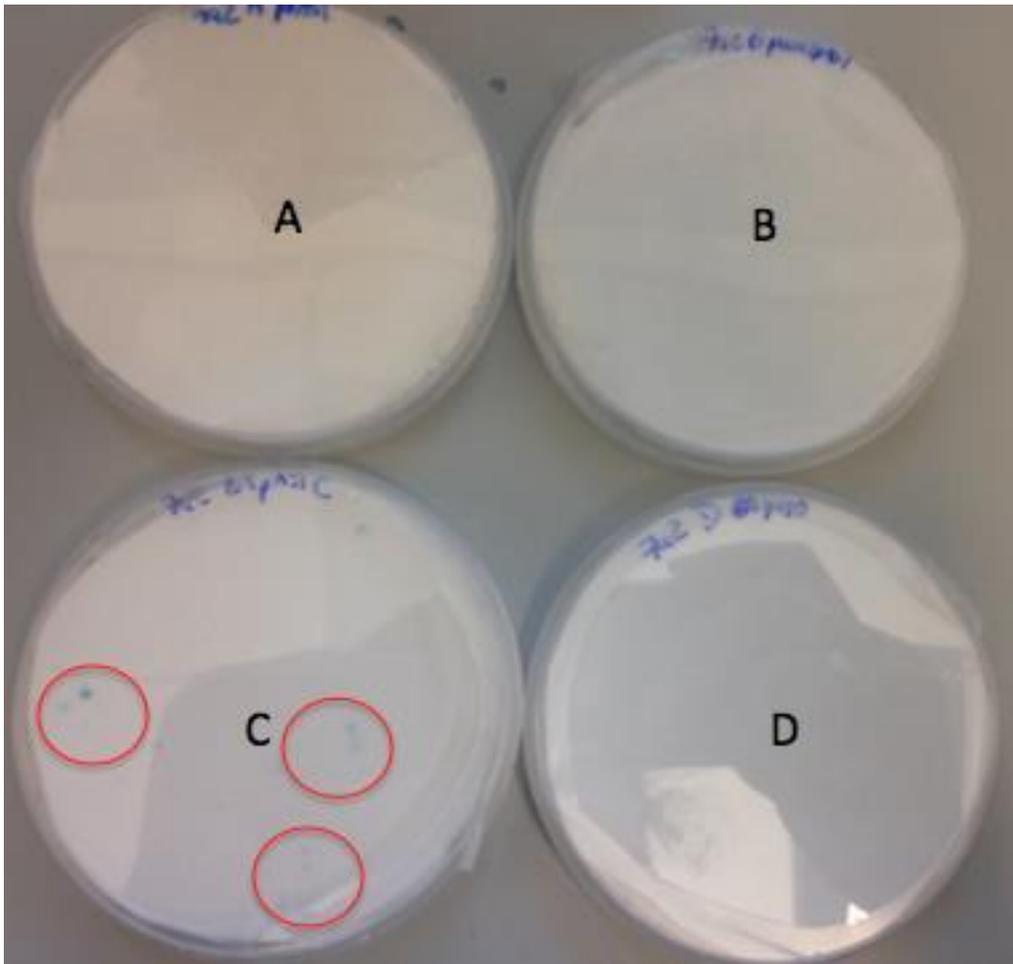


Figure 4. X-gal assay performed with prey vector plasmid to eliminate false positives. Shows the appearance of blue color (indicated by circles) or no color after X-gal assay performed with prey vector plasmid 7A2, which was transformed into (A) empty- pAS1 yeast, (B) pSEIII2-pAS1, (C) BAM9-pAS1 yeast and (D) Y190 yeast

#### *Analysis of Prey Vector Plasmid Sequence*

Once it was verified that the prey vectors 1A1, 7A2, 6A3, 8A2 were not false positives, then the DNA sequences they contained were further analyzed. All four contained sequences from the AMY3 gene, but because they were variable in length, they represented unique isolates (Fig 6). These four sequences were aligned with the DNA

sequence of AMY3 in *A. thaliana* (AtAMY3). This was done using the alignment tool of ApE (A plasmid Editor). The prey vectors 1A1, 7A2, 6A3, 8A2 all had the same 337 nucleotides in common:

```
TTGAAACTGAAGCTGCCCAAGTGAGTAAACCCAAACGAAAAACAGATAAAGAAGTGTCT
GCTAGTGGATTTACTAAAGAAATCATCACGGAGATAAGGAACTTGGCAATTGACATTTTC
CTTCATAAGAATCAGAAGACAAACGTCAAAGAAGTGCAGGAAAACATTCTACAAGAAA
TTGAGAAACTGGCTGCGGAGGCATATAGCATATTTAGAAGCACAACTCCAGCTTTTTCC
GAGGAAGGTGTTTTAGAAAGCAGAGGCTGACAAGCCTGACATTTAAAATCTCCTCAGGAAC
CGGCTCGGGATTTGAGATATTATGCCAAGGTTTCAACTGGGA
```

The AMY3 amino acid sequence was then obtained from five other flowering plants (*Ricinus communis* (XP\_002520134.1), *Vitis vinifera* (XP\_002270049.1), *Solanum tuberosum* (XP\_006357265.1), *Beta vulgaris* (XP\_010683251.1), and *Amborella trichopoda* (XP\_011622500.1)) An amino acid sequence alignment was made using Clustal Omega and formatted using BoxShade version 3.21 (Fig. 5) The alignment was used to divide the protein into four regions (Chloroplast Transit Peptide, Starch Binding Domain 1, Starch Binding Domain 2 and Catalytic Domain) based on conserved regions in all the six flowering plants (Glaring et al., 2006). The amino acid sequence for the inserts of each of the four prey vectors isolates was obtained and used to see what regions of the AMY3 protein they contained. The protein sequence encoded in all four isolates overlapped with the last part of the Starch Binding Domain 2 and the first part of the Catalytic domain (Fig. 6).

→ Chloroplast Transit Peptide

Amborella 1 MATRIRKPSLHHHTKW-NPFSN----QKLR-NYSNNPRLNHKLRNSFSGLNCTYKRFDD  
 Beta 1 MSTAAEPLDLHLALRQPTPKIQPKSRKNLFFSLNFSRAS-----KIRGSFSS---N-  
 AtAMY3 1 MSTVPTESLLHHSYLHNSKVNRR-GNRSFTPIISLNLRSHTSNKLLHSTCKKSVG--VSS-  
 Solanum 1 MSTVTIEPLGHYL-RRSPKLYP-NPKKTSQFSLNYSRR-----PLSGIATLRFCDYRRR-  
 Ricinus 1 MSTVTVEPLLRFSG-REKSLPIG-SRKILPSSSLNFSK-----ILLSN-GSSFCNEKRS-  
 Vitis 1 MSTVCIIEPLFQRCR-RENPRFRL-KSLATKPSLNMSPK-----P-LRN-GGSFCNEKSL-

Starch Binding Domain 1

Amborella 55 IRSFSIKPGVVIRASSTN-TSVEAVASDVIFETTFQIKRSEKVEGKISVRDHO-KDD  
 Beta 51 -----SIQKSVSAHCLATITLAQISEPELVVYSEFEIPRLNKVEGKICVRLDQ--KDG  
 AtAMY3 57 -----MNKSFVAIRASSDTAVVETAQSDVIFKEIFPQRIEKAEKGIYVRLKE--VKR  
 Solanum 54 -----RTVP---IRASSTPAAVIETSEQLDVVFETFSERPEAEKGISRLDVG-KDE  
 Ricinus 53 -----PPTSHTVRASSITDTALLETFKSADVIFKETFSLSRIETTEGKIFVRLDKEEKDQ  
 Vitis 52 -----HGMRPLGAA--SID---TALFETDVFVKETFLIKRIEVEGKISRRLDPG-KNG

Amborella 113 DKSQVAIGCNLPGKWLHWGVHYDQDVSEWDQPPDMRPPDSIAIKDYAIETPLKKSPL  
 Beta 104 DDWQLIVGCNLPKWLHWGVHYVGDVGSEWDQPPNEMRPPGSAIKDYAIETPLKQSSS  
 AtAMY3 110 KNWELSVGCSLPGKWLHWGVSYVGDVGSEWDQPPDMRPPGSAIKDYAIETPLKKL--  
 Solanum 105 ENWELSVGCSLPGKWLHWGVHYTDDTGVSEWDQPPNEMRPPGSAIKDYAIETPLQO---  
 Ricinus 108 QRWQLSVGCSLPGKWLHWGVSYVGDVGSEWDQPPKNMRFRGSAIKDYAIETPLKKS--  
 Vitis 101 ENWQLIVGCNLPKSWLHWGVSYDVGSEWDQPPNEMRPPGSAIKDYAIETPLKLLSS

Amborella 173 AVEENSLMEVQIDKVNHSVGALEFVFLKDEETGAWYQHGRDFVCLLEDLQENDKVGDD  
 Beta 164 SEGEDCLHEVNINFNCSNIAAINFVLKDEEAGSWYQHGRDFVPLVDNLRDGDNVIG-  
 AtAMY3 168 -SEEDSFEVAINNLESSAAAFVFLKDEETGAWYQHGRDFKVLVDVDPDNLNIGGA  
 Solanum 162 ----FAFQEVKIDISSKWSIAAINFVLKDEETGVYQHGRDFKPLVDCLEDDANIVGV  
 Ricinus 166 -SEADMFEVKIDDPNNSIAAINFVLKDEETGAWYQHGRDFKVLVDVYLLGDNVVGGA  
 Vitis 161 ASERDILHEVTIDFSPNSIAAIRFVLKDEEYGAWYQHGRDFEVLIDVYLCGENTVGA

Starch Binding

Amborella 233 KKSFSLWPGDFVKPEVLLTAIKREANGQPNGGKDARKKALHEEYIYEIFVKEKMV  
 Beta 223 KKGLSWPGNLD---SHMLTECEAPQSVAECSGEASPPPTLANLLEGFYEEQPIVKEVPV  
 AtAMY3 227 KKGEG---ALGQLSNIPKQDKSAET-----DSIEERKGLQEFYEEMPTSKRVAD  
 Solanum 218 KKEENIWSGLKLSNILLNPEASPSKGESSSNIGSS--AKNRHLEGFYEEHVTVKETLV  
 Ricinus 225 KRGFSIWPGSL--LSNMLLKTETTLFSKDEENNSTKDVKQDSGOLKGFYEEQPIKQVTV  
 Vitis 221 KEGFCIWPGLGQLSNMLLKAEESHKQGLSSS-----VSGDLITGFYEEHSIVKEVPV

Domain 2

Amborella 293 GNYLTVSVQENEEKNKALVLFDTDLPGNVLHWGVCRDNGKKWEIPOASHPPSINLFRKK  
 Beta 280 CNSLTVSVKCPETDKNLVSEVETDLPGDVVLHWGVCRDNPKEWEVPAAPHPPEVTVFKNK  
 AtAMY3 275 DNSVSVTAKCPETSKNIVSIVETDLPGDVTVHWGVCRNCKKKWEIPESEVYEETSIFKNK  
 Solanum 276 DNLTVSVKCPETAKNIVCIVDTDLPGNVLHWGVCRDCKGDKKIWEIPAMEVPAETVTVFKNK  
 Ricinus 283 QNSATVSVKCPKTAKYLYVETDLPGEVVLHWGVCRDDAKNWEIPESSPHPPETVTVFKNK  
 Vitis 275 DNSVTVSVKCPETANLYVETDLIGVVVHWGVCRDDEKTWEIPEAPHPPETKIFKKN

Amborella 353 ALQTSIQFKENGGSSGLFTLDKELAGLFLVKIDGYTWLNNSDFYPLSAEIGTSSV  
 Beta 340 ALRTRKIQTKEDGFGSKGLFTLDNEITGFEVFLKLNDDKWLNRGNDYFPLSGVHNKGGSS  
 AtAMY3 335 ALRTRLQKEDGNGSGFLFLDGLKLEGLCFVLKLNENTWLNRCEDFYVFLTSSSSPVE  
 Solanum 336 ALRTRLQKEDGNGSSGLFTLDGGLSGFVFKLDENMNLNCRGDFYPLSNGVHLHLEE  
 Ricinus 343 ALQTNLQPNVGGNGCSGLFLDDEFAVGLFLVKLNENTWLNRCGNDFYPLSTSSSLPTQ  
 Vitis 335 ALRTRLQSKEDGNGSGGLFTLDELEGLFLVKLNENTWLNRCMNDFYPLLSSSLPBAQ

Amborella 413 RPTKINAP**EG**HKEEDIS**NDV**KNDTWTIEE**SGSSQ**LEKSQSGAN**SPVSRVSYTDEIITNET**  
 Beta 400 **SAE**---IEK--N---V**SS**-----EVL**SGPIRDE-NPPVENQEAHTIPPTDITIKET**  
 AtAMY3 395 **TEA**---**AV**-----**SKPKRKTDK**EV**SA**SG**FTKEIITET**  
 Solanum 396 **RKQ**---NEESNS-----**SQ**LANRSP**EIQEGSVY**TDEI**IKET**  
 Ricinus 403 **PGQ**---**QSE**GV**L**-----**ASGKDAEGN**EEV**SR**AY**TDEI**IDE**T**  
 Vitis 395 **SRC**---**QSE**GV**L**-----**RVVSV**PT**EISGKT**AGENE**IVSDA**Y**T**DE**IGTIN**ET

**Coil Region**

Amborella 473 **RSLVSDISSE**RSAN**MKSK**ARE**SILQ**EIEK**LAAEAYSIFR**SS**IP**TF**LKELVSEPEI**-**EKP**  
 Beta 442 **RNLV**NG**ISSE**TK**WKTKSKEA**QED**ILQ**EIEK**LAAEAYGIFR**SS**MTLSE**AVAG**FE**-**TEL**  
 AtAMY3 425 **RNLAI**DIS**SHKNQ**KT**NVKEVQEN**IL**Q**EIEK**LAAEAYSIFR**SS**IT**EA**FSE**EV**L**EA**A-DKP**  
 Solanum 430 **RSLVSDISSE**K**SRKTKN**KE**TQE**IL**Q**EIEK**LAAEAYGIFR**SS**IP**TF**EA**SE**EV**-**LOP**  
 Ricinus 438 **RNLV**NG**ISSE**K**VROTK**KE**AQES**IL**Q**EIEK**LAAEAYSIFR**SS**IP**TF**ES**SV**L**E**VE**KA**P**  
 Vitis 441 **RNLVSDISSE**K**RQTKTK**Q**AES**IL**Q**EIEK**LAAEAYSIFR**SS**IP**TF**SE**AV**L**E**---**L

**Catalytic Domain**

Amborella 532 **QPKICS**GTGT**GYE**IL**CO**GF**NWES**H**KSGR**W**YSEL**Y**E**KA**ADIV**SL**GFT**VI**WL**PP**P**TES**SVS**PE  
 Beta 501 **EVEI**CSGTGT**GYE**IL**CO**GF**NWES**H**KSGR**W**YME**L**Q**K**V**SEL**SSL**G**FT**VI**WL**PP**P**TES**SVS**PE  
 AtAMY3 484 **DIK**IS**SGT**C**GF**EIL**CO**GF**NWES**H**KSGR**W**Y**EL**Q**E**K**A**DEL**AS**L**G**FT**VI**WL**PP**P**TES**SVS**PE  
 Solanum 489 **DVK**IS**SGT**C**GF**EIL**CO**GF**NWES**H**KSGR**W**YK**EL**E**KA**EL**SSL**G**F**VI**WL**PP**PT**SVS**AE  
 Ricinus 498 **PAKICS**GTGT**GYE**IL**CO**GF**NWES**H**KSGR**W**Y**ME**L**E**K**AA**E**SSL**G**F**VI**WL**PP**PT**SVS**PE  
 Vitis 498 **BEK**IS**SGT**C**GF**EIL**CO**GF**NWES**H**KSGR**W**Y**ME**L**SK**V**AE**SSL**G**FT**VI**WL**PP**P**TES**SVS**PE

Amborella 592 **GYMPK**DLYNLNSRY**GT**EL**K**TL**V**RF**F**HE**V**G**I**K**V**L**G**DA**V**LN**H**RC**A**H**Y**KN**Q**NG**V**WN**I**FG**G**R  
 Beta 561 **GYMPK**DLYNLNSRY**GT**DEL**K**T**V**K**S**F**H**Q**V**G**I**K**V**L**G**DA**V**LN**H**RC**A**H**SK**N**Q**NG**V**WN**I**FG**G**R  
 AtAMY3 544 **GYMPK**DLYNLNSRY**GT**DEL**K**D**T**V**K**F**H**Q**V**G**I**K**V**L**G**DA**V**LN**H**RC**A**H**KN**Q**NG**V**WN**I**FG**G**R**  
 Solanum 549 **GYMP**DLYNLNSRY**GS**DEL**K**V**T**V**K**F**H**Q**V**G**I**K**V**L**G**D**V**LN**H**RC**A**SE**N**Q**NG**V**WN**I**FG**G**R**  
 Ricinus 558 **GYMPK**DLYNLNSRY**GS**DEL**K**D**V**K**S**L**H**Q**V**G**I**K**V**L**G**DA**V**LN**H**RC**A**H**Q**N**Q**NG**V**WN**I**FG**G**R  
 Vitis 558 **GYMP**DLYNLNSRY**GS**DEL**K**V**L**V**K**S**F**H**Q**V**G**I**K**V**L**G**D**V**LN**H**R**C**A**Q**Y**Q**N**Q**NG**V**WN**I**FG**G**R**

Amborella 652 **LNWDD**RA**V**AD**DP**H**FQ**GR**NKSS**GD**NF**HA**AP**NI**DHSQ**D**FVR**ND**KEW**L**N**W**L**R**NE**I**G**Y**D**GW  
 Beta 621 **LNWDD**RA**V**AD**DP**H**FQ**GR**NKSS**GD**NF**HA**AP**NI**DHSQ**D**FVR**ND**KEW**L**C**W**L**R**K**E**I**G**Y**D**GW**  
 AtAMY3 604 **LNWDD**RA**V**AD**DP**H**FQ**GR**NKSS**GD**NF**HA**AP**NI**DHSQ**D**FVR**ND**KEW**L**C**W**ME**E**I**G**Y**D**GW**  
 Solanum 609 **LNW**D**RA****V**AD**DP**H**FQ**GR**NKSS**GD**NF**HA**AP**NI**DHSQ**D**FVR**ND**KEW**L**W**L**R**E**I**G**Y**D**GW**  
 Ricinus 618 **LNWDD**RA**V**AD**DP**H**FQ**GR**SKSS**GD**NF**HA**AP**NI**DHSQ**D**FVR**ND**KEW**L**C**W**L**R**E**I**G**Y**N**GW  
 Vitis 618 **LNWDD**RA**V**AD**DP**H**FQ**GR**NKSS**GD**NF**HA**AP**NI**DHSQ**D**FV**RED**KEW**L**C**W**L**R**K**E**I**G**Y**D**GW**

Amborella 712 **RLDFV**RGF**WGGY**V**KDY**DA**EP**Y**F**AV**G**E**Y**W**D**SL**S**Y**T**Y**G**EM**D**HN**Q**DA**H**R**Q**RI**D**W**I**N**A**T**NG**  
 Beta 681 **RLDFV**RGF**WGGY**V**KDY**DA**SK**P**Y**FA**V**G**E**Y**W**D**SL**S**Y**T**Y**G**EM**D**YN**Q**DA**H**R**Q**RI**D**W**I**N**A**T**CG  
 AtAMY3 664 **RLDFV**RGF**WGGY**V**KDY**MD**AK**P**Y**FA**V**G**E**Y**W**D**SL**S**Y**T**Y**G**EM**D**YN**Q**DA**H**R**Q**RI**D**W**I**N**A**T**SG  
 Solanum 669 **RLDFV**RGF**WGGY**V**KDY**DA**EP**Y**F**AV**G**E**Y**W**D**SL**S**Y**T**Y**G**EM**D**HN**Q**L**PH**R**Q**RI**D**W**I**N**A**T**NG**  
 Ricinus 678 **RLDFV**RGF**WGGY**V**KDY**ME**AE**P**Y**FA**V**G**E**Y**W**D**SL**S**Y**T**Y**G**EM**D**HN**Q**DA**H**R**Q**RI**D**W**I**N**A**T**NG  
 Vitis 678 **RLDFV**RGF**WGGY**V**KDY**MD**AE**P**Y**FA**V**G**E**Y**W**D**SL**S**Y**T**Y**G**EM**D**HN**Q**DA**H**R**Q**RI**D**W**I**N**A**T**NG

Amborella 772 **TAGAF**D**V**TT**K**G**IL**HS**AL**GR**CE**Y**W**RL**S**D**Q**K**G**PP**G**V**V**GW**W**PS**RA**V**T**FI**EN**H**D**T**G**S**T**Q**G**H**WR**  
 Beta 741 **SAGAF**D**V**TT**K**G**IL**HS**V**L**R**CE**Y**W**RL**S**D**P**K**G**K**PP**G**V**V**GW**W**PS**RA**V**T**FI**EN**H**D**T**G**S**T**Q**G**H**WR**  
 AtAMY3 724 **AAGAF**D**V**TT**K**G**IL**HS**AL**Q**CE**Y**W**RL**S**D**P**K**G**K**PP**G**V**V**GW**W**PS**RA**V**T**FI**EN**H**D**T**G**S**T**Q**G**H**WR  
 Solanum 729 **TAGAF**D**V**TT**K**G**IL**HS**AL**R**CE**Y**W**RL**S**D**Q**K**G**K**PP**G**V**V**GW**W**PS**RA**V**T**FI**EN**H**D**T**G**S**T**Q**G**H**WR  
 Ricinus 738 **TAGAF**D**V**TT**K**G**IL**HS**AL**R**CE**Y**W**RL**S**D**Q**K**G**K**PP**G**V**V**GW**W**PS**RA**V**T**FI**EN**H**D**T**G**S**T**Q**G**H**WR  
 Vitis 738 **AAGAF**D**V**TT**K**G**IL**HS**AL**GR**CE**Y**W**RL**S**D**Q**K**R**K**PP**G**V**V**GW**W**PS**RA**V**T**FI**EN**H**D**T**G**S**T**Q**G**H**WR

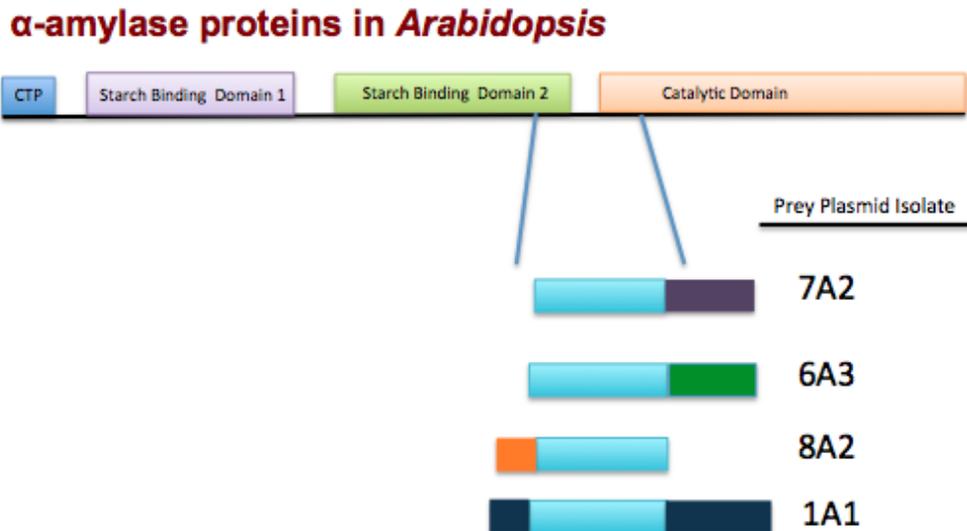
Amborella 832 **FPS**G**K**EM**Q**GY**AI**L**TH**PG**T**PA**V**F**D**H**I**F**S**HY**R**DE**I**S**A**L**I**CL**R**HR**R**K**K**IN**C**RS**I**VE**I**R**K**AE**R**  
 Beta 801 **FPG**K**E**M**Q**GY**AI**L**TH**PG**T**PA**V**F**D**H**I**F**S**NY**HE**I**S**TL**S**Y**R**NR**R**K**K**IN**C**RS**I**V**I**N**H**K**A**E**R**  
 AtAMY3 784 **FPE**G**K**EM**Q**GY**AI**L**TH**PG**T**PA**V**F**D**H**I**F**S**D**Y**H**S**E**I**A**L**SL**R**NR**R**K**K**IN**C**RS**E**V**I**D**K**S**E**R  
 Solanum 789 **FPG**K**E**M**Q**GY**AI**L**TH**PG**T**PA**V**F**D**H**I**F**S**GY**Q**PE**I**CL**N**LS**L**R**K**R**K**IN**C**RS**M**V**V**I**T**K**A**E**R**  
 Ricinus 798 **FPN**G**K**EM**Q**GY**AI**L**TH**PG**T**PA**V**F**D**H**I**F**S**HY**R**SE**I**AS**L**IS**L**R**K**R**NE**I**H**CR**S**SV**K**I**T**K**A**E**R**  
 Vitis 798 **FPG**K**E**M**Q**GY**AI**L**TH**PG**T**PA**V**F**D**H**I**F**S**HY**R**SE**I**AS**L**IS**L**R**NR**NE**I**H**CR**S**T**I**Q**I**T**M**A**E**R**

```

Amborella 892 DVYAAITIDDRVTIKIGPGHYEPPSGSQNWSLIAQCQDYKWEVL
Beta 861 DVYAAIIDEKVKAMKIGPGHYEPPSSQWSLAIEGNDYKWEAL
AtAMY3 844 DVYAAIIDEKVKAMKIGPGHYEPPNGSQNWSVAIEGRDYKWEETS
Solanum 849 DVYAAVIDKTAIKIGPGHYEPPICQQRKVAIEGNDYKWEELS
Ricinus 858 DVYAAIIEKVKAMKIGPGHYEPPSGK-NWSVAIEGKDYKWEAS
Vitis 858 DVYAAIIDEKVKAMKIGPGHYEPPKQQRWVAIEGKDYKWEETS

```

**Figure 5.** Amino acid sequence alignment of six AMY3 proteins from flowering plants. Sequences from *Arabidopsis thaliana* (AtAMY3, NP\_564977.1), *Ricinus communis* (XP\_002520134.1), *Vitis vinifera* (XP\_002270049.1), *Solanum tuberosum* (XP\_006357265.1), *Beta vulgaris* (XP\_010683251.1), and *Amborella trichopoda* (XP\_011622500.1) were aligned using Clustal Omega and formatted using BoxShade version 3.21.



**Figure 6.** Map of the domains of AtAMY3 aligned with sequences from the four clones identified in the BAM9 yeast two-hybrid screen. The four regions of AtAMY3: Chloroplast Transit Peptide (CTP), Starch Binding Domain 1, Starch Binding Domain 2 and Catalytic Domain are based on Glaring et al. (2006). The amino acid sequences from the four prey plasmid isolates (1A1, 7A2, 6A3, 8A2) are aligned below the map.

## Discussion

Compared with the other  $\beta$ -amylases from Arabidopsis, BAM9 has some unique features. The BAM9 sequence is not conserved in several regions where all of the catalytically active  $\beta$ -amylases have conserved residues (Fulton et al., 2008). BAM9 also has an mRNA expression pattern, which is unlike the other  $\beta$ -amylases where mRNA expression peak is highest at the end of the dark phase (Chandler et al., 2001). Since BAM9 is found in all flowering plants and since *bam3bam9* mutant plants accumulate more starch than just *bam3* plants, it is likely that BAM9 plays an important role in starch breakdown even though it has no known catalytic activity (Chandler et al., 2001). BAM9 also has a region of conserved amino acids on the protein surface, which is not seen in the other  $\beta$ -amylases (Monroe unpublished). These facts together suggest that BAM9 may act as a regulatory protein involved in starch degradation. The yeast 2-hybrid protocol was used to identify its potential binding partners.

First the BAM9 bait vector, BAM9-pAS1, was made and validated to see if it was suitable for further experimentation. BAM9-pAS1 was transformed into Y190 yeast cells; these cells were plated on selective media without the amino acids, tryptophan and histidine, and with various concentrations 3-AT. A range of 3-AT concentrations were used because BAM9-pAS1 may self-active and this way we are able to detect and control the level of basal expression of the *HIS3* reporter gene. The BAM9-pAS1 vector's product by itself can cause leaky expression of *HIS3* reporter gene and to control for this leaky expression a competitive inhibitor of histidine expression, 3-AT, was used in the media (Gietz et al., 1997). If colonies were able to grow on this selective media then it

told us that BAM9-pAS1 was transformed in the yeast cells accurately and that concentration of 3-AT was not enough to inhibit the basal expression of the *HIS3* reporter gene. Significant growth was seen on media without any 3-AT and this indicated that the transformation process was successful (Fig. 1). There was some growth on the plate with 10 mM 3-AT suggesting that 10 mM was not enough to control for the basal expression. However, no growth was seen in media made with 25 mM 3-AT and above (Fig. 1). This suggested that 25 mM was enough to inhibit the basal expression of the *HIS3* reporter gene and BAM9-pAS1 was suitable to use for the remaining steps.

Once the bait vector was deemed suitable for use, two separate screens were carried out with prey vectors containing the cDNA library obtained from roots and leaves of Arabidopsis. For the first screen 30 colonies grew while for the second screen 75 colonies grew on the selective media without tryptophan, leucine, and histidine but with 25 mM 3-AT (Table 1). The lack of tryptophan in the media selected for the BAM9-pAS1, whereas omitting leucine in the media selected for the prey plasmid. Not having histidine in the media selected for potential interactions between BAM9 and another protein and having 25 mM 3-AT inhibited basal expression of *HIS3* gene. Therefore, colonies that grew on this selective media had both bait and prey vectors transformed accurately and the proteins they encoded were potentially interacting, as cells were able to express *HIS3* reporter gene. Next, the expression of a second reporter gene was investigated by performing X-gal assays. X-gal assays detect the expression of the *lacZ* gene by turning blue as expression of *lacZ* allows the cells to make a protein that breaks down X-gal into a blue product (Fig 2). Of the 30 colonies that grew in the first screen, 12 produced blue color, and in the second screen, 62 of the 75 colonies turned blue

(Table 1). The change of color of the colonies to blue indicated the expression of a reporter gene, *lacZ*. The expression of *lacZ* suggested potential interaction between BAM9 and another prey protein.

The 74 colonies that grew on selective media and expressed *lacZ* were then minipreped to isolate the prey plasmids and these plasmids were transformed into DH5 $\alpha$  *E. coli* cells. The plasmids were then isolated and sequenced. Eight unique prey insertions were detected from the first screen and fourteen unique prey insertions were detected from the second screen. Each potential interacting protein encoded in the prey plasmids was characterized and arranged in Table 2 based on where the protein is localized. The proteins identified were predicted to be localized in the chloroplast, mitochondria, nucleus, and plasma membrane, while some localizations were unknown. Proteins that were predicted to be located in the chloroplast were of special interest because BAM9 is also plastid localized. Therefore, if BAM9 does bind with a protein to act as a regulatory protein, its binding partner would have to be in the same cellular compartment. The number of hits was also important as that indicated the protein had a higher potential of being a true binding partner. Therefore, identifying  $\alpha$ -amylase3 (AMY3) as a prey plasmid was noteworthy, as not only is AMY3 located in the chloroplast, but it was identified in four independent isolates: 1A1, 7A2, 6A3, 8A2. Due to these observations, and lack of time and resources, only these four isolates were used for further experiments. AMY3 is one of three proteins in Arabidopsis belonging to the alpha-amylase family, and the only one that is located in chloroplasts (Glaring et al., 2006). AMYs are endoamylolytic enzymes that breakdown the  $\alpha$ -1,4-glucosidic linkages of starch. The function of AMY3 in Arabidopsis is unclear since mutants lacking this

gene did not have a starch accumulation phenotype indicating that AtAMY3 may not be that important in starch breakdown or that another enzyme may be taking over its function (Yu et al., 2005).

Each prey plasmid (1A1, 7A2, 6A3, 8A2) was tested to eliminate false positives by transforming them into Y190 yeast, BAM9-pAS1 yeast, empty-pAS1 yeast, and pSEIII2-pAS1 yeast. Ideally, only the transformation with AMY3 prey plasmids into BAM9-pAS1 yeast should grow on selective media and turn blue in the X-gal assay in order for us to determine that these are not false positives. Positive growth and *lacZ* expression for an AMY3 prey plasmid transformed into just Y190 yeast would suggest that AMY3 was activating itself and was a false positive. Likewise, growth of the AMY3 prey plasmid transformed into yeast containing empty pAS1 would suggest that some protein product translated from the vector itself was possibly interacting with AMY3 resulting in a false positive. Finally, if the AMY3 prey plasmid transformed into pSEIII2-pAS1 yeast, which expresses an unrelated control protein, resulted in the expression of both reporter genes, that would indicate that a protein product of the bait vector, pSEIII2-pAS1, was also capable of interacting with AMY3 and that it does not have to be just BAM9. This would indicate that AMY3 could be interacting non-specifically if it can interact with an unrelated random protein. When 1A1, 7A2, 6A3, 8A2 were transformed into the four cell types, only the prey isolates which were transformed into BAM9-pAS1 were able to grow on selective media. This indicated that only when BAM9 and AMY3 are expressed together and potentially interacting do we see the expression of *HIS3* reporter gene (Fig 3). These colonies also turned blue in X-gal assays indicating that

these colonies, from the transformation of prey isolates into BAM9-pAS1, are also expressing the second reporter gene, *lacZ* (Fig. 4).

After we determined that the plasmids were not false positives, sequences of the four prey plasmids (1A1, 7A2, 6A3, 8A2) were analyzed to see where they overlap with the *Arabidopsis thaliana* AMY3 amino acid sequence. Previous studies have shown that *AtAMY3* has a chloroplast transit peptide, which signals the protein to enter the chloroplast and remain there (Dutta et al., 2014, Glaring et al., 2011). *AtAMY3* also has three other domains, one C-terminal catalytic domain and two starch-binding domains at the N-terminus (Cantarel et al., 2009). The AMY3 amino acid sequence was attained from five other flowering plants *Ricinus communis*, *Vitis vinifera*, *Solanum tuberosum*, *Beta vulgaris*, and *Amborella trichopoda*. All six of the AMY3 amino acid sequences were aligned and the four regions stated above were identified (Fig 6). The particular amino acid sequences encoded by the four prey plasmids (1A1, 7A2, 6A3, 8A2) all partially overlapped with each other as depicted in Figure 5. The common sequence overlapped with the *AtAMY3* amino acid sequence between the last segment of the Starch Binding Domain 2 and the first part of the Catalytic domain (Fig. 5). The fact that all of the four isolates had the same overlapping region with the *AtAMY3* amino acid sequence points to the importance of this region in the potential interaction of BAM9 and AMY3.

We chose to identify where in the 3D structure of *AtAMY3* this particular interaction region, where all four isolates overlapped, was located to get a better

understanding of how BAM9 might bind. The AMY3 sequence was submitted to I-Tasser (<https://zhanglab.ccmb.med.umich.edu/I-TASSER/>) and a homology model was generated (Figure 7). Information from the alignment in Figure 5 was used to label each of the three domains on the 3D protein structure of *At*AMY3 (Fig. 7).

We made the model and found the region of overlap with the four clones (last part of SBD2). We also used COILS ([https://embnet.vital-it.ch/software/COILS\\_form.html](https://embnet.vital-it.ch/software/COILS_form.html)) and identified a coil motif in this region (Figure 8). The coil is on the surface of the protein (Figure 7), and is perfectly conserved in AMY3 proteins from various plants (Figure 5). Interestingly, the interaction region overlaps with the coil region of *At*AMY3 depicted in Figure 7. This coiled region is also on the surface of the protein and is not hindered by any other portion of the protein. These types of coil regions are seen in any many proteins, and are often involved in binding to other proteins (Lupas, 1996). Functions such as signaling and regulation are often accomplished through coiled regions. There are many benefits of having a coiled region such as it gives the protein a wider range of motion, the protein can reposition the coil region when its binding partner is near, and these regions also aid in dimerization (Assmann *et al.*, 2006).

All four prey isolates contained sequences overlapping with the coil region of *At*AMY3 indicating that BAM9 could potentially bind with this coiled region on the surface of the protein. To further confirm the potential interaction between BAM9 and *At*AMY3, affinity chromatography pull-down experiments need to be conducted. Obtaining more comprehensive information about protein-protein interactions will help us to understand the processes or functions in which these proteins take part. Since BAM9 is encoded in all currently sequenced flowering plant species, having more

information about how and to what BAM9 may interact will give us a more comprehensive understanding of BAM9's role in the regulation of starch metabolism and its presence in the chloroplast.

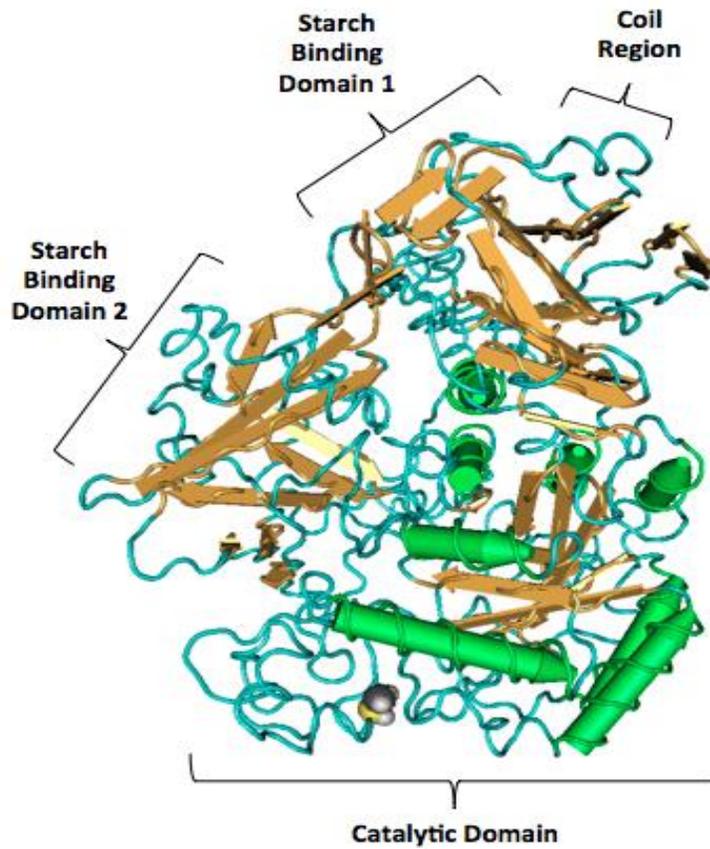


Figure. 7. Homology model of AMY3 from Arabidopsis. The two predicted starch binding domains and the catalytic domain are indicated. A coiled coil motif in the region identified from the four clones isolated in the yeast two-hybrid screen is also labeled.

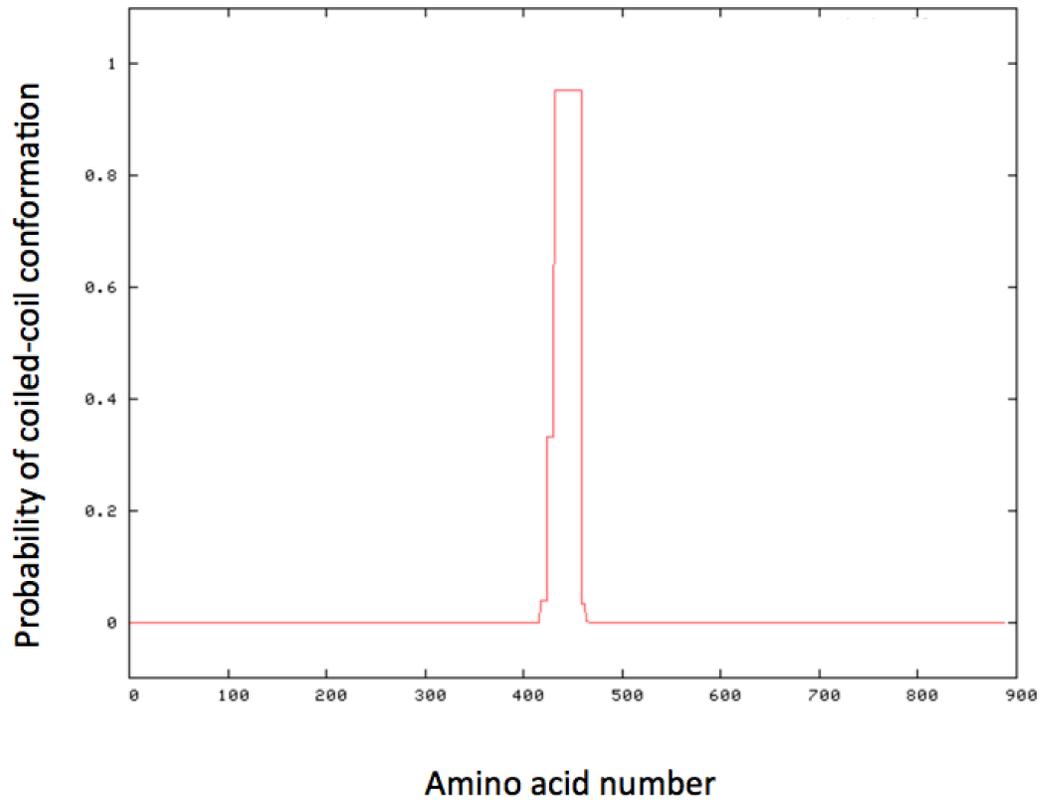


Figure 8. Output from the ExPASy program COILS ([https://embnet.vital-it.ch/software/COILS\\_form.html](https://embnet.vital-it.ch/software/COILS_form.html)) using AtAMY3 as the input amino acid sequence.

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