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Searching for Potential Binding Partners of *Arabidopsis* β -amylase2 using Yeast 2-Hybridization

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

by Megan E. Hines

May 2016

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 15, 2016 .

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Acknowledgements

I would like to thank first and foremost Dr. Jon Monroe for taking me on as an undergraduate researcher in his lab and for encouraging me to undertake this thesis when I doubted myself. I would also like to thank him for being so excited about every aspect of my research. I would like to thank Dr. Amanda Storm for teaching me the ways of the lab and always answering my questions. I would also like to thank her for her inexhaustible patience and encouragement and her flexibility to work with my writing and editing style. I thank Rubana Hossain for helping me with some of the transformations and minipreps near the end so that the process ran smoother, and the other members of the lab—Merwice Baray, Catherine Torres, Matt Kohler, and Jillian Breault—for their support. I would like to thank Dr. Kim Slekar for her endless wisdom in the ways of the yeast. Furthermore, I would like to thank Dr. Steve Cresawn and Dr. Slekar for being my readers for this thesis. Funding for this project was provided by a grant from the National Science Foundation and the support from the Department of Biology at James Madison University.

Abstract

BAM2 is a chloroplast-targeted member of the β -amylase gene family that currently has an unknown function in starch hydrolysis. Previous research indicated that BAM2 did not have significant catalytic activity, but, because the gene is highly conserved and there is a starch-excess phenotype in older plants lacking BAM2, it was hypothesized that BAM2 may instead have a regulatory function. Many regulatory proteins function by interacting with other proteins, so we wanted to test for potential protein binding partners for BAM2 using the yeast two-hybrid system. A plasmid containing the *BAM2* gene from *Arabidopsis thaliana* was co-transformed into Y190-strain yeast (*Saccharomyces cerevisiae*) along with a second plasmid from a library of plasmids containing cDNA of mRNA isolated from mature *Arabidopsis* leaves and roots. If BAM2 interacts with any of the proteins encoded within the cDNA library, transcription in that co-transformed yeast is activated for certain reporter genes whose expression is monitored by growth on selective media and a colorimetric assay. We observed 23 colonies with expression of both reporter genes from the first cDNA library screen and 55 colonies from a second screen. Plasmids containing cDNA were extracted from these colonies and are being tested further for false positives. The cDNA insert in plasmids from 45 potential positive results were sequenced and analyzed to provide information about the validity of interaction with BAM2. One putative positive was identified as an uncharacterized chloroplastic protein, which, after further validation, may represent a previously unidentified protein involved in starch metabolism and may reveal the function of BAM2 in the process of starch hydrolysis.

Introduction

During the day, plants undergo photosynthesis, generating sugars. Of these sugars, about half are used immediately to provide the plant with energy while photosynthesis is occurring; the other half are stored as starch granules in the chloroplast. Starch granules are composed of a mixture of glucose polymers, amylose and amylopectin, which differ in the absence or presence of branching respectively (Zeeman et al., 2010). This starch is broken down at night, when photosynthesis is not occurring, to give the plant a continuous supply of energy in the form of sugar. Starch is primarily hydrolyzed to maltose by β -amylase (BAM) enzymes, the major family of hydrolytic enzymes involved in starch metabolism (Scheidig et al., 2002).

In *Arabidopsis thaliana*, the β -amylase family of genes includes nine members, known as BAM1-9. BAM7 and -8 are targeted to the nucleus and act as transcription factors (Reinhold et al., 2011; Soyk et al., 2014). BAM5 is extrachloroplastic, expressed in phloem tissue, where its function is not known (Lin et al., 1988; Monroe and Preiss, 1990). BAM1, -2, -3, -4, -6, and -9 are all predicted or known to be chloroplast-localized and, therefore, are situated to be involved in starch degradation (Lao et al., 1999; Fulton et al., 2008; Li et al., 2009; Scheidig et al., 2002; Sparla et al., 2006; Zeeman, personal communication; our unpublished results).

Of the chloroplast-targeted BAMs, BAM1 and -3 are the most catalytically active, with BAM1 primarily active during the day (Sparla et al., 2006; Fulton et al., 2008; Valerio et al., 2011) and BAM3 active primarily at night (Smith et al., 2004; Fulton et al., 2008). BAM6 was previously found to have low expression in leaves of young plants (Fulton et al., 2008), but our recent work indicates that BAM6 may function in older plants (Monroe et al., 2014). On the other hand, previous work with BAM4, -9, and -2 indicated these proteins have little or no catalytic activity, and their functions are still unknown. Although BAM4 is catalytically inactive,

it still binds starch and is required for normal starch metabolism (Fulton et al., 2008; Li et al., 2009). BAM9 is non-catalytic likely due to absence of some key amino acids that are conserved in active BAMs (Chandler et al., 2001). The function of BAM9 in starch metabolism remains unclear, although it is conserved among all flowering plants and its expression peaks at the transition between night and day (Chandler et al., 2001; Smith et al., 2005; Mockler et al., 2007). BAM2 was previously shown to have low expression in young leaves and to have low catalytic activity (Fulton et al., 2008), but our lab detected a phenotype in older plants. Because BAM4, -9 and -2 all appear to lack significant catalytic activity and yet produce phenotypes when knocked-out, it was hypothesized that these BAMs may be serving a regulatory function.

The current research on BAM2 led us to believe that this BAM could have a regulatory role in starch degradation. Experiments conducted by Fulton et al. (2008) showed that starch hydrolysis activity of BAM2 was optimal at pH 6, but the specific activity was 25 times lower than that of BAM3 and 50 times lower than that of BAM1. Loss of BAM2 alone or along with other BAMs did not affect starch or maltose levels in young plants (Fulton et al., 2008). Due to the lack of phenotypic effect from the mutation of *BAM2*, Fulton et al. (2008) concluded that BAM2 had no function in young plants. However, our work has shown that there is an excess starch phenotype in older plants lacking BAM2, indicating that BAM2 may function in leaves of older plants or in other tissues (Monroe et al., 2014), and our most recent work has found that the β -amylase activity of BAM2 is salt-dependent (Monroe, unpublished). While we have recently found that BAM2 has some catalytic activity, it may still have a regulatory role.

Phylogenetic analysis of the *BAM2* gene grouped it with the nuclear-targeted *BAM* genes, *BAM7* and -8, each having a core nine-exon structure (Fulton et al., 2008; Li et al., 2009; Monroe, unpublished). Moreover, our analysis suggests that the BAM7 and BAM8 proteins were

derived from BAM2 at about the time of the origin of seed plants. Further analysis indicates that *BAM2*-like sequences that are predicted to be targeted to plastids occur throughout land plants (Monroe, unpublished). The *BAM2* gene also appears to have a conserved region of amino acids on the surface of the protein that could serve as a binding site for another protein. The fact that *BAM2* is widely conserved implies that there is selective pressure for organisms to keep the gene, which also implies that BAM2 has a necessary function.

BAM2 is the focus of this project because of its apparent involvement but unknown function in starch metabolism. The excess starch phenotype seen in leaves of older plants lacking BAM2 indicates that BAM2 is required for the normal functioning of the plant. Because BAM2 was originally observed to have low catalytic activity, its phenotypic importance implied that BAM2 may instead have a regulatory function. Many regulatory proteins function by forming interactions with other proteins (Adrain and Freeman, 2012). For example, recently it was found that PTST (protein targeting to starch) functions by interacting with granule-bound starch synthase (GBSS) to localize it to the starch granule for elongation of amylose (Seung et al., 2015). If BAM2 serves a regulatory function in which it binds to another protein, we are interested in identifying potential binding partners. Even if BAM2 proves to have a catalytic function in plants, as is possible given the recent activity assay results, BAM2 could still form protein interactions of interest. The yeast two-hybrid system was chosen to detect potential binding partners for BAM2.

The classical yeast two-hybrid system is a method used to determine which protein(s) from a cDNA library interact with the protein of interest (Causier and Davies, 2002). One strength of the yeast two-hybrid method is that it allows for the screening of a large number of potential interaction partners from a cDNA library. Yeast cells, *Saccharomyces cerevisiae*, have

transcription factors with DNA-binding and transcription activation domains that can function independently. In yeast two-hybrid systems, the DNA-binding domain of a transcription factor is fused to the gene for the protein of interest in one plasmid, the “bait” vector, and the activation domain is fused to unknown proteins encoded in the library of cDNAs, generating a library of “prey” vectors (Causier and Davies, 2002) (Figure 1).

These two plasmids are transformed into a yeast strain containing mutations in various amino acid biosynthetic pathways (Causier and Davies, 2002). The mutations allow for selection of transformant yeast because the bait and prey vectors contain genes that complement the mutations, allowing only the cells that have taken up the plasmids to grow on certain minimal media (Causier and Davies, 2002). The bait vector and the library of cDNA prey vectors are transformed into yeast cells on a large scale to promote the bait vector being co-transformed with each of the prey vectors. Resulting colonies are screened to find those that have interacting bait and prey proteins (Causier and Davies, 2002). If any of the unknown proteins from the cDNA library interact with the protein of interest, then the two domains of the transcription factor come close enough to stimulate transcription and expression of certain reporter genes (Causier and Davies, 2002) (Figure 2). Expression of reporter genes is detected by growth on selective media or presence of specific catalytic activity. In our BAM2 yeast two-hybrid experiment, the bait and prey genes are fused with the DNA-binding and activation domains of the GAL4 transcription factor, respectively (Causier and Davies, 2002) (Figure 2). The two reporter genes used are *HIS3*, a gene encoding a protein involved in histidine synthesis, and the *lacZ* gene. Expression of these reporter genes is detected by growth on media lacking histidine and presence of beta-galactosidase activity against X-gal substrate, respectively.

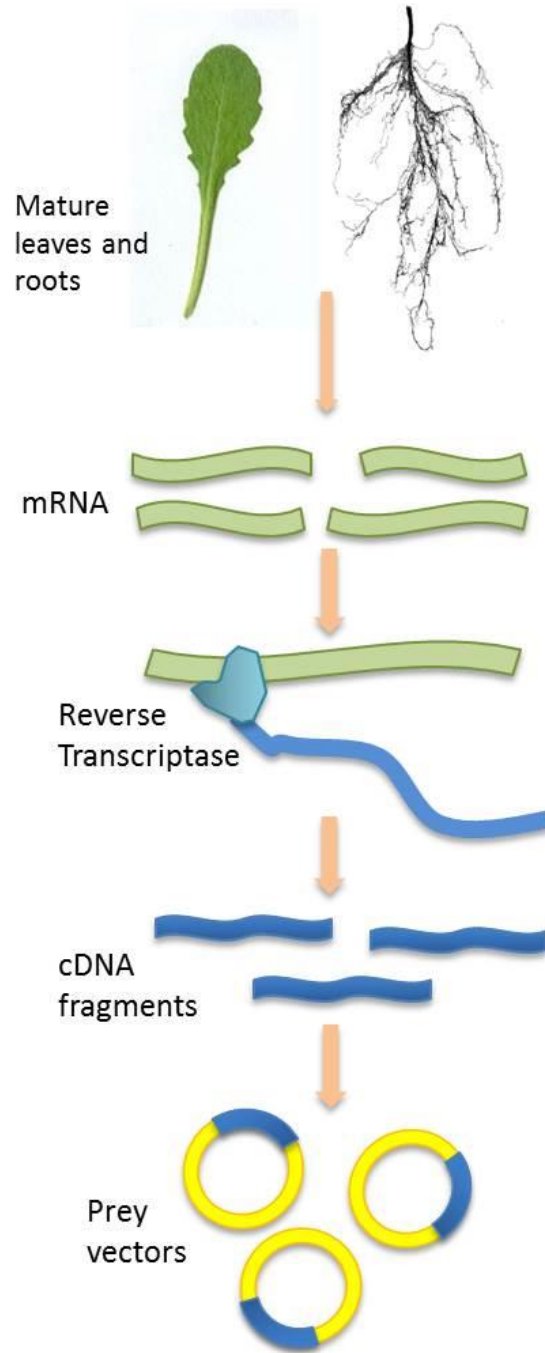


Figure 1. Generation of cDNA library of prey vectors. cDNA created from the mRNA from mature *Arabidopsis* leaves and roots is inserted into prey vectors to generate a cDNA library of prey vectors.

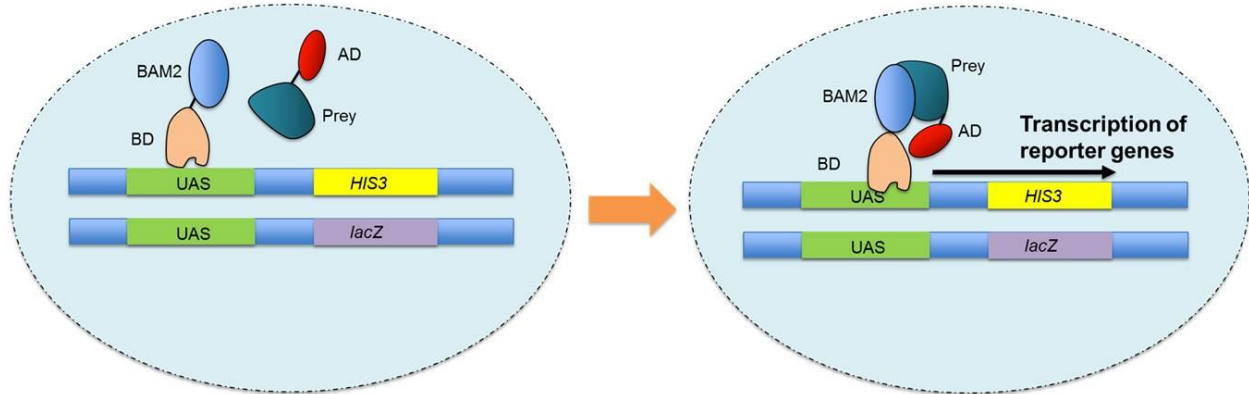


Figure 2. Illustration of the yeast two-hybrid mechanism. BAM2- bait protein; BD- DNA binding domain of the transcription factor; AD- activation domain of the transcription factor; UAS- upstream activation sequence; reporter genes- *HIS3* and *lacZ*.

Currently, studies on BAM2 are limited, and its function is unknown. Our results with *bam2* knockouts in older plants as well as *BAM2*'s widespread presence in land plant genomes caused us to be interested in the protein, and the objective of this study was to determine if BAM2 interacts with another protein, potentially as part of a regulatory function. Determining which protein(s) interact(s) with BAM2 will further our knowledge of the function of BAM2, thereby helping us understand how BAM2 contributes to starch degradation in plants. It could also identify the involvement of as-yet unknown proteins in starch metabolism. As starch is essential to maximal plant growth and is an important food and fuel source, understanding of starch degradation and the regulation of this process in plants may also apply on a grander scale to human diet and biofuels. Because plants are an important source of food for humans and a potential fuel source, it is important to know about the proteins involved in starch metabolism in order to maximize human benefit from plants.

Materials and Methods

Generation of the Prey Vectors

A cDNA library constructed using mRNA extracted from mature *Arabidopsis* leaves and roots was purchased from the Arabidopsis Biological Research Center (ABRC). cDNA was excised from phage into plasmids according to the protocol provided by ABRC.

Construction of the Bait Vector

The sequence encoding the mature form of BAM2, lacking the chloroplast transit peptide, was amplified by PCR from an existing expression vector with Deep Vent DNA Polymerase (New England Biolabs) using forward primer 5'-AACATATGGCAGAGAGTACTGAGGAAGATCGAGTTCC-3' and reverse primer 5'-TTGGATCCTCACTCGGGGTTGGTCTCTTG-3'. The reactions were cycled at 94 °C for 30 seconds, 54 °C for 30 seconds, and 72 °C for 2 minutes, repeated ten times, followed by 25 cycles of 94 °C for 30 seconds, 58 °C for 30 seconds, and 72 °C for 2 minutes, with a final extension of 72 °C for 6 minutes. The PCR product was purified by gel extraction using the Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare) according to the manufacturer's instructions. The *BAM2* insert was then ligated into the pMOS vector using the pMOS*Blue* Blunt Ended Cloning Kit (GE Healthcare). BAM2-pMOS ligations were transformed into competent *Escherichia coli* cells provided with the kit according to the kit's instructions. The transformants were plated on Luria-Bertani medium (LB) plates containing Carbenicillin (Carb), Tetracycline (Tet), Isopropyl β -D-1-thiogalactopyranoside (IPTG), and X-gal and incubated at 37 °C for 24 hours. White colonies, indicating insertion, were chosen from the transformation plates and tested using colony PCR using the same amplification primers used to

amplify *BAM2* from the existing expression vector to confirm insertion of *BAM2* into the pMOS plasmid by presence of a band around 1.5 kb on an agarose gel. The plasmid was minipreped using the alkaline lysis protocol (Bimbiom and Doly, 1979). *BAM2*-pMOS was then digested using *NdeI* and *BamHI* to remove the insert. The pAS1 vector was digested with *BamHI*. The digested products *BAM2* and linearized pAS1 were purified by gel extraction as described above. *BAM2* and pAS1 fragments were ligated using the Roche rapid DNA ligation kit according to the manufacturer's instructions. Ligations were transformed into competent *E. coli* cells and grown overnight. Resulting colonies were minipreped according to the alkaline lysis protocol, but modified to resuspend the plasmid DNA in 25 μ L of sterile dH₂O. The minipreped DNA was digested with *BamHI* and *NdeI* and run on an agarose gel to confirm insertion of *BAM2* into pAS1. Upon gel confirmation of *BAM2* insertion, the *BAM2*-pAS1 bait plasmid was sequenced by Eurofins Genomics using nested primers to obtain the entire *BAM2* sequence. The sequencing primers used were 5'-GACAGCATAGAATAAGTGCG-3', 5'-CAGGCGTAACACTGAATGCC-3', and 5'-ATGTTTAAGAAACATGATGC-3'.

Transformation of Bait Vector into Y190-Strain Yeast

To insert the *BAM2*-pAS1 vector into the yeast, a transformation was conducted using a modified protocol from Tian et al. (2012). Y190-strain yeast, obtained from ABRC, was grown at 30 °C overnight in 2 mL of yeast extract peptone dextrose (YPD) media. The overnight culture was diluted with 4.5 mL YPD and 0.5 mL culture, then aliquoted into two tubes of 2.5 mL each and grown for an additional 2.5 hours. The solutions were transferred to a 15 mL tube and pelleted at 3000 rpm for 5 minutes at room temperature. The cells were resuspended in 4 mL sterile dH₂O, then pelleted at 3000 rpm for 5 minutes at room temperature. The cells were

resuspended in 0.2 mL of Solution I (100 mM lithium acetate [LiAc], 10 mM Tris-HCl [pH 7.5], 1 mM ethylenediaminetetraacetic acid [EDTA]) and incubated at room temperature for ten minutes. To a microcentrifuge tube, 10 μ L of denatured calf thymus DNA (10 mg/ml), 2 μ L of BAM2-pAS1 DNA, 100 μ L of yeast cells, and 700 μ L of Solution II (100 mM LiAc, 10 mM Tris-HCl [pH 7.5], 1 mM EDTA, and 50% polyethylene glycol [PEG]-3350) were added and mixed well (Tian et al., 2012). The tube was incubated at 30 °C for 30 minutes, heat-shocked at 42 °C for 15 minutes, and then pelleted at 3000 rpm for 2 minutes (Tian et al., 2012). The supernatant was discarded, and the pellet was resuspended in 200 μ L of dH₂O. The suspension was serially diluted (1:4 and 1:100) and 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, 0.1 mM tryptophan, 0.75 mM leucine, and 0.1 mM histidine) lacking tryptophan (CM-Trp) plates. The plates were incubated at 30 °C for 3 days.

Validation of the Bait Vector for Use in the Yeast Two-Hybrid Screen

Y190 yeast transformed with the BAM2-pAS1 vector (BAM2-pAS1 yeast) was tested for self-activation and basal expression of the reporter genes to validate the vector's use in the yeast two-hybrid screen. To confirm that the BAM2-pAS1 vector does not self-activate expression of the *lacZ* gene, an X-gal assay was conducted. To test for basal, leaky expression of the *HIS3* reporter gene, BAM2-pAS1 yeast was streaked on CM-Trp,-His plates containing varying concentrations of 3-amino-1,2,4-triazole (3-AT), a competitive inhibitor of the HIS3 enzyme. Concentrations of 3-AT tested were 0, 10, 25, 50, 75, and 100 mM. For comparison, yeast containing pSEIII2-pAS1, a verified bait vector provided by ABRC, were also tested. After

incubation at 30 °C for seven days, growth on the plates was noted. The concentration of 3-AT at which no growth was observed (25 mM) was determined as the appropriate inhibitor concentration to use because it was below the recommended threshold level (100 mM).

cDNA Library Screen

Upon validation of the bait vector as suitable to use in a yeast two-hybrid screen, the BAM2-pAS1 yeast was screened against a cDNA library of prey plasmids using a modified procedure from Tian et al. (2012). Four CM-Trp plates were used to grow up lawns of BAM2-pAS1 yeast. The plates were incubated at 30 °C for five days. Sterile dH₂O was added in 1 mL increments to make an emulsion with the cells, which were added in 800 µL increments to 500 mL of YPD until the media had an optical density at 600 nm (OD₆₀₀) near 0.1. The culture was shaken at 30 °C overnight, until it reached an OD₆₀₀ near 0.4. The cells were pelleted at 3000 x g for 5 minutes and the supernatant was discarded. The pellet was resuspended in 100 mL dH₂O and then pelleted again and the supernatant discarded. Next, the cells were resuspended in 50 mL Solution I (100 mM LiAc, 10 mM Tris-HCl [pH 7.5], 1 mM EDTA), pelleted, and then resuspended in 2.5 mL Solution I. To the 2.5 mL cell solution, we added 125 µL of denatured calf thymus DNA (10 mg/ml), 30 µg of the cDNA library, and 15 mL of Solution II (100 mM LiAc, 10 mM Tris-HCl [pH 7.5], 1 mM EDTA, and 50% PEG-3350). The solution was mixed well and then aliquoted into 26 tubes of 700 µL each. The tubes were incubated in a 30 °C water bath for 30 minutes, then heat-shocked in a 42 °C water bath for 15 minutes, then pelleted at 6000 x g for 1 minute and the supernatants were carefully discarded. Each pellet was resuspended in 400 µL of dH₂O, from which 200 µL was then plated onto each of two CM-Trp,-Leu,-His + 25 mM 3-AT plates. One aliquot from the initial transformation was resuspended,

and serial dilutions were prepared. To determine the transformation efficiency, 200 μL of each dilution was plated on CM-Trp,-Leu plates. The efficiency was calculated by multiplying the number of colonies by the dilution factor, and dividing by the volume plated, then converting from μL to mL. Dilution plates were incubated at 30 °C for 5 days, the resulting numbers of colonies were counted, and the transformation efficiency was calculated. The Screen plates were incubated at 30 °C. At various points from 10 to 20 days after the initial transformation, growing colonies were streaked onto fresh CM-Trp,-Leu plates to create master stocks for further testing. The master stock plates were allowed to grow for four days at 30 °C and then transferred to 4 °C.

X-gal Assay

To determine expression of the *lacZ* reporter gene, an X-gal assay was performed on yeast colonies according to Tian et al. (2012). Colonies to be tested were grown up on media lacking the appropriate amino acids for four days at 30 °C. A nitrocellulose membrane was placed on top of the plate and pressed down to ensure contact between the membrane and the colonies on the media. After two minutes, the membrane was carefully removed from the media surface and transferred to a foil boat with the colony side facing up. The foil boat with the membrane was floated on liquid nitrogen for 20 seconds, then sunk in the liquid nitrogen for two minutes. The boat and membrane were removed, and the membrane was allowed to thaw. In an empty Petri dish, 1.5 mL Z buffer (60 mM Na_2HPO_4 , 40 mM NaH_2PO_4 , 10 mM KCl, 1 mM MgSO_4 , pH 7.0) and 20 μL of X-gal (20 mg/mL in DMF) were mixed, and a Whatman filter paper was placed on top of the solution. The thawed nitrocellulose membrane was placed on top of the Whatman filter with the colony side facing up. The Petri dish was wrapped in Parafilm and incubated at 37 °C for 24 hours; blue colonies were noted.

Isolation of the Prey Plasmid

Prey vectors were isolated from yeast colonies that were able to grow on CM-Trp,-Leu,-His+3-AT and tested positive in the X-gal assay. Colonies were grown up in 2 mL CM-Leu to encourage maintenance of the prey plasmid but not the bait plasmid. The liquid culture was spun down in a microcentrifuge tube for 1 minute, and the supernatant was discarded. The pellet was resuspended in 67 mM KH₂PO₄. To break down the yeast cell wall, 10 µL of Zymolyase solution (50:49:1, glycerol:Zymolyase [G-Biosciences]:1M Tris) was added, and the tube was incubated at 37 °C for 1 hour. The resulting cells were miniprepped according to the alkaline-lysis method, modified to resuspend the plasmid DNA in 25 µL of dH₂O.

The miniprepped DNA from the yeast was used to transform competent DH5α *E. coli* cells using a modified heat shock method in that 10 µL of miniprep DNA was used with 50 µL competent cells for the transformation (Inoue et al., 1990). The transformed cells were plated on LB+Carb and incubated overnight at 37 °C. Resulting *E. coli* transformants were miniprepped using the alkaline-lysis method, and then the DNA was diluted with dH₂O to a concentration of 150 ng/µL and sent for sequencing by Eurofins Genomics using the standard Gal4AD primer, 5'-TACCACTACAATGGATG -3'. The sequence of the cDNA insert in the prey plasmid was submitted to BLAST-n to identify the gene encoded in the prey plasmid. Sequences were analyzed using the UniProt database (<http://www.uniprot.org/>) to determine the subcellular location, functions, and family of the proteins encoded by the sequences.

Elimination of False Positives

To identify false positive interactions, a series of retransformations were conducted with potential interacting prey plasmids. The prey plasmid was transformed into yeast alone, empty-

pAS1 yeast, BAM2-pAS1 yeast, and pSEIII2-pAS1 yeast using the yeast transformation protocol previously described. Transformations with empty-pAS1 yeast, BAM2-pAS1 yeast, and pSEIII2-pAS1 yeast were plated on CM-Leu,-Trp,-His+3-AT, and transformations with yeast alone were plated on CM-Leu,-His+3-AT. Resulting colonies were tested with an X-gal assay to determine expression of the *lacZ* reporter gene. If blue colonies resulted from the X-gal assay in any prey plasmid transformation except the transformation into BAM2-pAS1 yeast, then the interaction was determined to be a false positive, and that prey plasmid was eliminated. Blue colonies resulting from yeast transformed only with the prey plasmid indicated that the prey plasmid was unsuitable for use in the screen because its encoded protein self-activates expression of the *lacZ* gene. If transformation of empty-pAS1 yeast with the prey plasmid resulted in blue colonies, then the protein encoded by the prey plasmid interacted with the products encoded by the pAS1 vector—perhaps the HA-tag—not BAM2 from the bait vector. The transformation with pSEIII2-pAS1 yeast served to demonstrate whether the protein encoded in the prey vector was interacting specifically with BAM2 or was a sticky protein interacting with any bait protein.

Results

Currently, there is little known about BAM2 and its function. Its catalytic activity was found to be negligible (Fulton et al., 2008), but our recent data suggest that it may be catalytically active in the presence of salts. Plants with a *bam2* knockout show an excess-starch phenotype, suggesting that BAM2 is necessary for the proper breakdown of starch. Also suggesting BAM2's importance is that it is conserved among most land plants. Its conservation indicates a selective pressure on the organism to keep the gene. Because BAM2 is conserved and knockout studies show starch buildup, we investigated whether BAM2 might interact with another protein, playing a regulatory role in starch metabolism. To identify potential interaction partners, a yeast two-hybrid experiment was conducted with BAM2.

Construction of the Bait Vector

BAM2 was amplified by PCR from an existing expression vector and cloned into pMOS. Resultant *BAM2*-pMOS colonies were tested with PCR to confirm insertion of *BAM2*. *BAM2*-pMOS was digested using *NdeI* and *BamHI* and the *BAM2* fragment was purified by gel extraction. Purified *BAM2* was ligated with linearized pAS1 vector. To validate insertion of the *BAM2* coding sequence into the pAS1 bait vector, the vector was digested with *NdeI* and *BamHI* and the products were run on an agarose gel. There was a band around 1.5 kb in size, which was the expected size for the *BAM2* insert (Figure 3). The vector was then sent for sequencing (Eurofins Genomics) using primers designed to read the entire *BAM2* gene. The sequencing results were aligned with the known gene sequence, and the alignment indicated that *BAM2* had been inserted into the pAS1 vector in frame with the Gal4 DNA-binding domain (BD).

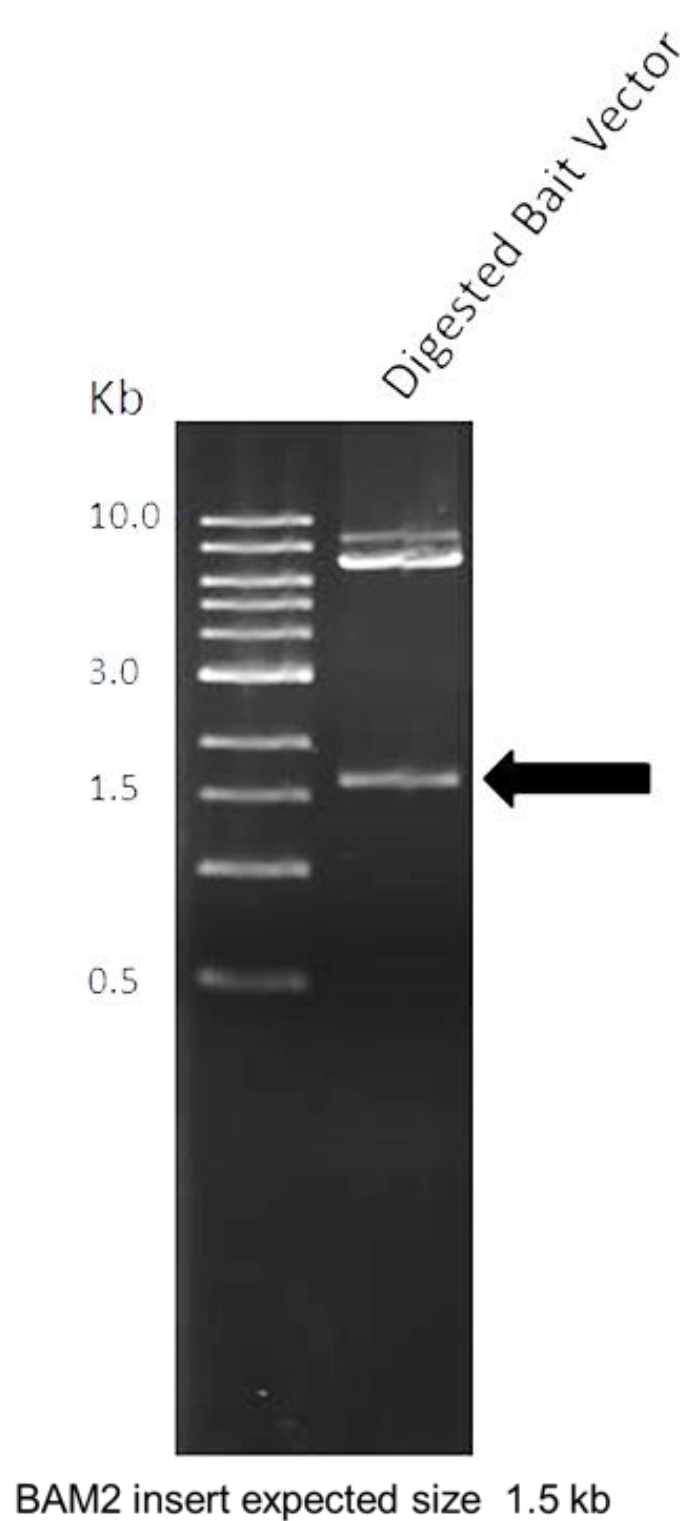


Figure 3. Agarose gel of BAM2-pAS1 digested with *NdeI* and *BamHI*. The arrow indicates the presence of the band at 1.5 kb.

Transformation of Bait Vector into Y190-Strain Yeast

In order to use the BAM2-pAS1 vector in the yeast two-hybrid screen, the vector had to be transformed into Y190-strain yeast. Miniprep BAM2-pAS1 from *E. coli* was transformed into Y190 yeast and plated on media lacking tryptophan to select for transformant yeast containing the BAM2-pAS1 plasmid. On the 1:4 dilution plate, 50-100 transformants resulted, and 5 colonies grew on the 1:100 dilution plate. This growth indicated the successful transformation of BAM2-pAS1 into Y190 yeast with a transformation efficiency of 2.5×10^3 CFU/mL.

Validation of the Bait Vector for Use in the Yeast Two-Hybrid Screen

The BAM2-pAS1 yeast were tested for self-activation and basal expression of the reporter genes *HIS3* and *lacZ* in order to validate the vector for use in the yeast two-hybrid screen. It is important that the fusion of BAM2 to Gal4BD does not activate expression of reporter genes and basal expression of reporter genes needs to be determined for each system, especially for the *HIS3* gene, which has leaky expression. BAM2-pAS1 yeast were grown on media lacking tryptophan and histidine with varying concentrations of 3-AT, an inhibitor of the *HIS3* enzyme, to determine the appropriate concentration of the inhibitor to use in the screen to inhibit the basal expression of the *HIS3* gene. If a bait vector stimulates growth on plates containing 100 mM 3-AT, that bait is considered inappropriate for use in the yeast two-hybrid screen (Tian et al., 2012). The BAM2-pAS1 yeast showed good growth on the plate with no 3-AT and some growth on the plate with 10 mM 3-AT, but there was no growth of the BAM2-pAS1 yeast on plates with 25, 50, 75, or 100 mM 3-AT, so the BAM2-pAS1 vector is suitable to use.

After confirmation that the leaky expression of *HIS3* could be negated with less than 100 mM 3-AT, the BAM2-pAS1 yeast were subjected to an X-gal assay to verify that the bait vector alone does not self-activate expression of the *lacZ* reporter gene. The BAM2-pAS1 colonies were not able to breakdown the X-gal substrate and remained white, indicating that the vector did not self-activate expression of *lacZ*.

cDNA Library Screen

BAM2-pAS1 yeast were transformed with 30 µg of the *Arabidopsis* leaf and root cDNA library of prey vectors and plated on media with 25 mM 3-AT and lacking tryptophan, leucine, and histidine. Colonies that grew on this media were known as “His-positive” because they synthesized histidine in order to grow on the minimal media. This transformation protocol was conducted twice. The first transformation resulted in a transformation efficiency of $\sim 1 \times 10^3$ CFU/mL (Table 1). Three rounds of colonies were streaked onto master plates, yielding 42 total His-positive colonies (Table 1). The master stocks of the yeast died before the colonies could be completely analyzed, so a second screen was conducted. The second transformation resulted in an efficiency of $3\text{-}4 \times 10^4$ CFU/mL and two rounds of master plates with 82 total His-positive colonies (Table 1).

X-gal Assay

Colonies that grew on media lacking histidine, indicating expression of the *HIS3* reporter gene, were also subjected to an X-gal assay. A blue coloration of the colonies was used as an indicator of expression of the *lacZ* reporter gene (Figure 4). From the first screen, 23 of the 42 His-positive colonies also expressed the *lacZ* reporter gene (Table 1). Of the 82 total colonies

Table 1. Number of potentially positive colonies from screens as indicated by reporter gene expression.

	Screen 1	Screen 2
Number of colonies that grew on media lacking histidine	42	82
Number of His-positive colonies that became blue in X-gal assay	23	55
Transformation efficiency	$\sim 1 \times 10^3$ CFU/mL	$3-4 \times 10^4$ CFU/mL

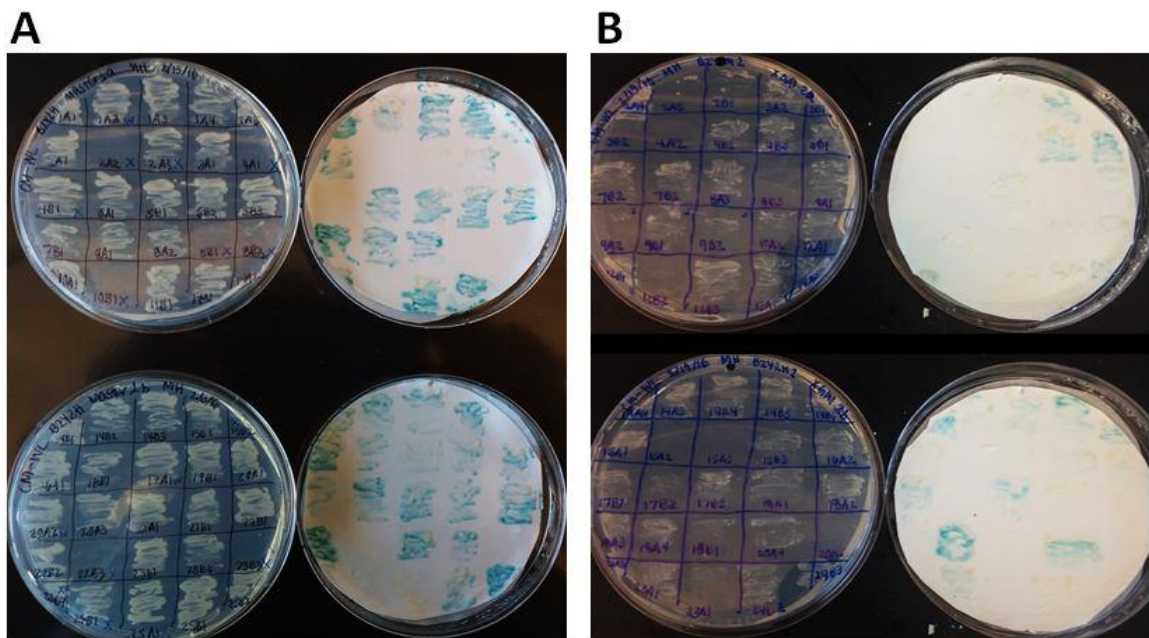


Figure 4. Pictures of the master plates from the second screen aligned with their respective X-gal assays for master plates 1a and 1b (A) and 2a and 2b (B). Blue colonies indicated expression of the *lacZ* reporter gene.

from the second screen that expressed the *HIS3* reporter gene, 55 colonies also expressed the *lacZ* reporter gene (Table 1).

Isolation of the Prey Plasmid

Once colonies expressing both the *HIS3* and *lacZ* reporter genes were identified, their prey plasmids were isolated for further testing and analysis. Initially, several different methods were attempted for isolating the prey plasmid from colonies showing putative positive interactions. Plasmid extraction methods involving glass beads, sonication, and phenol:chloroform did not yield transformable DNA. Ultimately, Zymolyase digestion followed by the alkaline-lysis miniprep method garnered successful isolation of the prey plasmid. The isolated plasmids were retransformed into *E. coli*, miniprepped, and the sequence of the cDNA insert was determined. The sequences were submitted to BLAST-n to identify the gene encoded in the prey plasmids. From the first screen, six plasmids were successfully miniprepped and sequenced, resulting in six individual sequences. From the second screen, 38 prey plasmids were miniprepped and sequenced, and BLAST searches of the sequences revealed 19 unique genes (Table 2). Each unique potential interacting protein identified was analyzed using the UniProt database and sorted according to subcellular location as shown in Table 2.

Elimination of False Positives

After the prey plasmids were isolated and sequenced, a series of transformations was conducted with potential interacting prey plasmids. To evaluate whether the observed reporter gene expression in each of the potential positive colonies was due to a false positive, the isolated prey plasmid was transformed into BAM2-pAS1 yeast, pSEIII2-pAS1 yeast, and empty-pAS1

Table 2. Genes isolated from prey plasmids and their cellular localizations. ^ indicates that the function is predicted based on similarity; * indicates that there is experimental evidence for the function.

Subcellular location	(Screen #) and isolate identifier	Number of hits	UniProt identifier	Gene number	Gene name	Protein name	Family	Function
Chloroplast	(1) 2c1	1	P04778	At1g29930	LHCB1.3	chlorophyll a-b binding protein 1	LHC protein family	Photosynthesis* (Silva et al., 2015)
	(1) 40d1	1	P0CJ48	At1g29920	LHCB1.1	chlorophyll a-b binding protein 2	LHC protein family	Photosynthesis* (Silva et al., 2015)
	(2) 14B6	1						
	(2) 9A1	1	Q8VZ87	At1g29910	LHCB1.2	chlorophyll a-b binding protein 3	LHC protein family	Photosynthesis* (Silva et al., 2015)
	(2) 20A2	1	Q987N7	At1g55670	PSAG	Photosystem I reaction center subunit V	PsaG/PsaK family	Photosynthesis* (Jensen et al., 2002)
	(2) 3A2	1	P10795	At1g67090	RBCS-1A	Ribulose biphosphate carboxylase small chain 1A	RuBisCO small chain	Photosynthesis* (Izumi et al., 2012)
	(1) 40a (2) 1A4, 1A5, 14B1, 14B3, 19B1, 21A1, 22B1, 25B2	1 8	O82230	At2g24020	At2g24020	Nucleoid-associated protein At2g24020	YbaB/EbfC family	DNA binding^
Proteasome	(1) 7aM1	1	Q9SGW3	At1g64520	RPN12A	26S proteasome non-ATPase regulatory subunit 8 homolog A	Proteasome subunit S14 family	Protein catabolic process* (Fu et al., 2001)
Mitochondrion	(1) 15aL1	1	Q93VV9	At3g59280	TXR1	Thaxtomin resistance protein TXR1		Protein import into mitochondrial matrix^
	(2) 1A2	1	Q98ZJ5	At4g37930	SHM1	Serine hydroxymethyltransferase 1	SHMT family	Transferase* (Engel et al., 2011)
Nucleus	(2) 3A1	1	P0CH33	At4g05050	UBQ11	Polyubiquitin 11	Ubiquitin family	Protein catabolic process* (Norris et al., 2006)
	(2) 22A1	1	Q6EWX1	At2g01120	ORC4	Origin of replication complex	ORC4 family	DNA replication^
	(2) 22B2	1	Q9FIW9	At5g39760	ZHD10	Zinc-finger homeodomain protein		Transcription factor* (Tan and Irish, 2006)
Endoplasmic reticulum	(2) 6B1, 14A3, 14B5, 18A4, 20A3	5	Q38798	At5g07340	At5g07340	Calnexin homolog 2	Calreticulin family	Protein folding, chaperone^
	(2) 20A4	1	O04023	At1g09070	SRC2	Protein SRC2 homolog		Stress response* (Kawarazaki et al., 2013)
Plasma membrane	(2) 12A1	1	Q9C8S1	At1g29980	T1P2.9	Putative uncharacterized protein T1P2.9		
	(2) 15B3	1	Q8LPT1	At2g39480	ABCB6	ABC transporter B family member 6	ABCB family	ATPase; transport^
Cytosol	(2) 5A1, 14B2	2	O82462	At5g26710	At5g26707	Glutamate--tRNA ligase	Class-I aminoacyl-tRNA synthetase family	Protein biosynthesis^
	(2) 4B3, 7B1, 8A1, 8A2,14A1, 16B1, 17A1, 23B2	8	O03983	At1g01470	LEA14	Probable desiccation-related protein LEA14	LEA type 2 family	Response to fungus* (Huibers et al., 2009), high light intensity* (Dunaeva and Adamska, 2001), dessication^, and wounding* (Cheong et al., 2002)
Apoplast	(2) 17B3	1	Q9MA41	At1g05850	CTL1	Chitinase-like protein 1	Glycosyl hydrolase 19 family	Developmental protein* (Schneider et al., 1997)
Other/unknown	(2) 17B1	1	Q9SI59	At2g16590	At2g16590	Putative uncharacterized protein At2g16590		
	(2) 25B1	1		At3g41950			Backwards in the vector	
	(1) 5aR1	1		At3g41768			Backwards in the vector	
	(2) 14A4, 16A2	2					Chloroplast genome	

yeast and plated on complete media lacking tryptophan, leucine, and histidine with 25 mM 3-AT. The prey plasmid was also transformed into yeast without any bait vector and plated on complete media lacking leucine and histidine with 25 mM 3-AT. Growing colonies from the transformations indicated expression of the *HIS3* reporter gene, and blue colonies after performing an X-gal assay indicated expression of the *lacZ* reporter gene. If colonies from yeast transformed with only the prey plasmid turned blue from the X-gal assay, then the protein encoded by the prey plasmid self-activated expression of the reporter genes and thus was a false positive interaction with BAM2. If transformation of empty-pAS1 yeast with the prey plasmid resulted in blue colonies, then the protein from the prey plasmid interacted with a product of the pAS1 vector, perhaps the HA-tag, instead of BAM2. The transformation with pSEIII2-pAS1 yeast served to demonstrate whether the protein from the prey vector was interacting specifically with BAM2 or was a sticky protein interacting with any bait vector.

From the first screen, 7aM1, 15aL1, and 40aM1 were successfully isolated and retransformed to test for false positives. The 7aM1 transformation resulted in one colony when transformed with pSEIII2-pAS1 yeast, but there was no growth when retransformed with BAM2-pAS1 yeast, empty-pAS1 yeast, or yeast alone. The transformations for 15aL1, on the other hand, resulted in 40 colonies with BAM2-pAS1, 195 colonies with pSEIII2-pAS1, and 30 colonies with empty-pAS1. When tested with an X-gal assay for expression of the *lacZ* reporter gene, all colonies turned blue meaning that the protein encoded by 15aL1 was a sticky protein, interacting nonspecifically, and therefore was identified as a false positive result.

Retransformations for 40aM1 were unsuccessful; none of the transformations resulted in

colonies. The other colonies from the master stocks from the first screen were unable to be tested because the yeast in the master stocks died in the fridge before their prey plasmids could be isolated.

From the second cDNA library screen, no potential positive interaction partners have been tested in this manner due to time restraints but they are currently being tested and will be investigated by other members in the lab.

Discussion

β -amylase 2 is one of nine BAM proteins in *Arabidopsis thaliana*. Phenotypic analysis indicates that BAM2 serves a role in starch degradation, but details about its function are still unknown. BAM2 was previously shown to have very low β -amylase activity in vitro, and *bam2* knockouts showed no phenotype in young leaves (Fulton et al., 2008). However, our work found that BAM2 appears to play a role in starch degradation in older plants (Monroe et al., 2014) and, more recently, we found that BAM2's activity increases significantly in the presence of salt, especially KCl. Because *BAM2* is persistent in the genome of most land plants and we were able to observe a role for BAM2 in older plants, even though at the time there was little reported catalytic activity, we used yeast two-hybridization to determine if BAM2 binds another protein, potentially as part of a regulatory function. After constructing an appropriate BAM2 bait vector, we first validated that the BAM2-pAS1 vector was suitable for use in the cDNA library screen by testing for self-activation by the bait vector and basal or leaky expression of the reporter genes. Yeast transformed with BAM2-pAS1 were plated on media lacking tryptophan and histidine to select for yeast that were successfully transformed with the BAM2-pAS1 vector and to check for expression of the *HIS3* reporter gene. Because *HIS3* has leaky expression, the media also contained increasing concentrations of 3-AT, a competitive inhibitor of the *HIS3* enzyme. Because no colonies grew on media with 25 mM 3-AT or higher, it was determined that the BAM2 bait vector was not significantly activating expression of *HIS3* and that 25 mM of the inhibitor was appropriate to counteract the leaky expression of *HIS3*. BAM2-pAS1 yeast were also tested with an X-gal assay to confirm that the presence of *BAM2* in pAS1 did not activate expression of the *lacZ* reporter gene. No colonies turned blue, indicating that BAM2 did not cause the yeast to express the *lacZ* reporter gene. Together, these two tests validated that BAM2

was suitable for use in the cDNA library screen using a concentration of 25 mM 3-AT in the media.

After validating the BAM2-pAS1 vector, the yeast two-hybrid screen was conducted with the *Arabidopsis* leaf and root cDNA library of prey plasmids. Colonies growing on CM-Trp,-Leu,-His + 25 mM 3-AT indicated that there was interaction between the bait and prey proteins because *HIS3* was expressed, allowing histidine synthesis to occur. The *HIS3*-positive colonies were also tested with an X-gal assay to determine *lacZ* expression, which would also be seen if there was interaction between the bait and prey proteins. The first screen had a transformation efficiency of 1×10^3 CFU/mL and resulted in 42 *HIS3*-positive colonies, of which 23 were also *lacZ*-positive (Table 1). Midway through the process of identifying potential interaction partners through miniprepping and sequencing prey vectors, the master stocks of yeast died; thus, the screen was repeated. In the second screen, there was a transformation efficiency of 3- to 4×10^4 CFU/mL and resulted in 82 *HIS3*-positive colonies with 55 colonies also being *lacZ*-positive (Table 1). The higher transformation efficiency in the second screen meant that there was a higher chance that BAM2 had an opportunity to interact with each prey plasmid represented in the cDNA library.

Before the master stocks for screen 1 died, prey plasmids from six colonies were miniprepped and sequenced. Of these six, false positive testing was attempted for three prey vectors by transforming the vector into yeast alone, BAM2-pAS1 yeast, pSEIII2-pAS1 yeast, and empty-pAS1 yeast to test for false positive interactions. For the prey plasmid 7aM1, only the transformation of pSEIII2-pAS1 yeast with 7aM1 was successful, but this resulted in only one colony. Combined with the failed transformations into BAM2-pAS1 yeast, empty-pAS1 yeast, and yeast alone, it is possible that the DNA concentration in the 7aM1 miniprep was insufficient

or of too poor quality for the transformation or that the yeast strains were not sufficiently competent. All of the 40aM1 transformations, which were done at the same time as the 7aM1 transformations, failed, providing further evidence that the yeast strains were not sufficiently competent, though it is possible that the 40aM1 miniprep was also insufficient or of too poor quality.

The retransformation of 15aL1 into BAM2-pAS1 yeast and resultant growth on media lacking histidine indicated that the interaction was repeatable; however, transformation of 15aL1 into pSEIII2-pAS1 yeast and empty-pAS1 yeast also resulted in growth on media lacking histidine, suggesting that *HIS3* was being expressed in these transformations as well. Growth on these control plates indicated interaction between the protein encoded in 15aL1 and the products of both the unrelated bait vector and the empty bait vector. The X-gal assays on the colonies indicated that *lacZ* was also expressed in all the colonies. Expression of the *HIS3* and *lacZ* reporter genes in the transformation of 15aL1 with BAM2-pAS1 yeast indicated that the interaction between BAM2 and the protein encoded by the 15aL1 vector was true; however, expression of the reporter genes when 15aL1 was transformed with the unrelated bait and with the empty bait indicated that the protein encoded in 15aL1 may interact with the pAS1 vector or that 15aL1 is a sticky protein that interacts nonspecifically. The results from the transformations with 15aL1 led to its identification as a false positive and its elimination as a potential interactor with BAM2.

Most of the sequenced prey plasmids from the two screens have not yet been tested to identify false positives due to time restraints. However, we were able to identify the sequenced genes using BLAST-n and found information about the encoded proteins through UniProt to assess the potential validity of their interactions by other means. BLAST searches of the six prey

sequences from the first screen revealed six unique genes, while searches of 38 successful prey sequences from the second screen revealed 19 individual genes (Table 2). While most of the genes were inserted into the prey vector in frame with the GAL4 activation domain, two prey vectors had cDNA genes inserted backwards into the vector. In these cases, the proteins expressed would not have been the protein from the true gene; therefore, they were eliminated as false positive results since whatever protein was expressed likely does not exist in *Arabidopsis*. Two genes were identified from both screens: LHCb1.1 and At2g24020 (Table 2). Also of note, several sequences from the second screen were identified independently multiple times. Eight prey sequences from the second screen were identified as At2g24020, eight as LEA14, five as At5g07340, and two sequences were identified as At5g26710 (Table 2).

In analyzing the genes, putative cellular localization was the primary criterion used for assessing the potential validity of the prey protein's interaction with BAM2. Because BAM2 is plastid-localized in *Arabidopsis*, the analysis focused on genes that were likely to be chloroplast localized as well. There were six genes identified that encode proteins that are predicted to be targeted to the chloroplast. Many of the identified chloroplast-targeted proteins are involved in photosynthesis. Chlorophyll a-b binding proteins 1, 2, and 3 (LHCb1 genes) were identified from the screens (Table 2). These three proteins are very similar and have highly conserved C-terminal regions that were represented in the recovered prey plasmids (Silva et al., 2015). Chlorophyll a-b binding proteins 1, 2, and 3 are all involved in harvesting light for the photosystem complexes in photosynthesis. Also targeted to the chloroplast and involved in photosynthesis is the photosystem I reaction center subunit V protein, also known as PSI-G. PSI-G is part of photosystem I, an integral component of the electron transport chain in photosynthesis (Jensen et al., 2002). The ribulose biphosphate carboxylase (RuBisCO) small

chain 1A protein was also identified in the screen (Table 2). RuBisCO small chain 1A combines with other subunits to comprise RuBisCO (Izumi et al., 2012), an enzyme involved in carbon fixation in photosynthesis. Contrary to the other chloroplast-targeted proteins, the At2g24020 gene encodes a chloroplast protein not thought to be involved in photosynthesis. At2g24020 was isolated from prey vectors from both the first and second screen and encodes an uncharacterized protein.

The Arabidopsis Information Resource, TAIR (<http://www.arabidopsis.org/>), was used to analyze expression of these proteins using expressed sequence tags (ESTs). ESTs are short sequences (200-800 nucleotides) derived from cDNA libraries that can give an indication of the relative expression of genes (Nagaraj et al., 2006). Because photosynthesis is such an important process for the plant, proteins involved with photosynthesis pathways tend to have high expression rates. EST analysis revealed that the chlorophyll a-b binding proteins 1, 2, and 3 are represented in the database by 17332, 1911, and 1814 ESTs, respectively, indicating that they are relatively highly expressed genes, with chlorophyll a-b binding protein 1 expressed the highest. PSI-G has 2486 EST hits, and RuBisCO small chain 1A has 29560 ESTs, which makes sense given that RuBisCO is one of the most highly expressed proteins in plants. The large number of ESTs for these proteins suggests that they are highly expressed and thus may comprise a disproportionate number of cDNAs in the prey library. Additionally, these proteins involved in photosynthesis are generally well-characterized and no involvement with starch degradation has yet been reported. For these reasons, the interaction observed between these proteins and BAM2 may have been due to their high numbers in the cDNA library of prey plasmids, although false positive testing is needed before any can be eliminated.

As opposed to the proteins involved in photosynthesis, At2g24020 is represented by only 48 ESTs, indicating that it has a much lower relative expression and thus was likely represented in the cDNA library of prey plasmids fewer times. The fact that At2g24020 was isolated nine separate times across the two screens, combined with the lower number of ESTs, makes At2g24020 an interesting potential interaction partner for BAM2. Zybaïlov et al. (2008) identified the At2g24020 protein in the chloroplast proteome and reported that the protein had 182 amino acids including the chloroplast transit peptide, but the protein is uncharacterized so its structure and function are unknown.

A potential structure for the At2g24020 protein was obtained by submitting the amino acid sequence to Phyre2 (<http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index>) to generate a homology model (Figure 5). Phyre2 predicted the structure of the At2g24020 protein to be similar to that of the uncharacterized protein hp0035 from *Helicobacter pylori*, which is a small protein composed of two alpha helices. The model generated by Phyre2 covered 50%, or 91 residues, of the At2g24020 protein, with 100% confidence. Though false positive testing has not yet been done for the At2g24020 vector, it is possible that the At2g24020 protein represents a novel protein involved in starch metabolism. Therefore, the At2g24020 protein is of interest.

From the cDNA library screens, sequenced prey vectors also revealed several genes for proteins that were not localized to the chloroplast (Table 2). While most have not been tested for being false positives, it is possible that these proteins are still pertinent. The prey proteins not predicted to be plastid-localized may interact with BAM2 before BAM2 is translocated to the chloroplast in the cell. Additionally, because targeting-peptide prediction is only a prediction, it is possible that some of the identified proteins do not have a detected transit peptide but are still targeted to the chloroplast and thus may still have a true interaction with BAM2. However, it

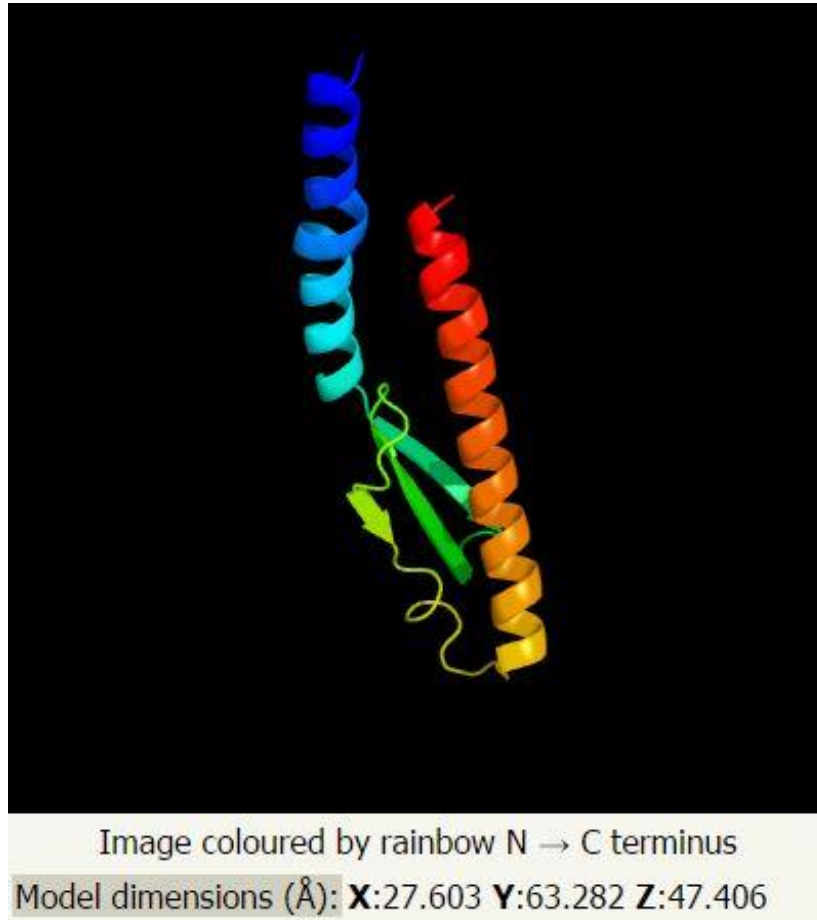


Figure 5. Phyre2-generated model of the At2g24020 protein.

would not be surprising if many of these proteins are shown to be false positives as about 50% of prey plasmids are typically eliminated as false positives via retransformation (Tian et al., 2012).

BAM2 is a conserved gene encoding a protein whose function is unknown. In this yeast two-hybrid experiment, we screened a cDNA library of prey plasmids for proteins that *BAM2* might interact with, potentially as part of *BAM2*'s hypothesized role in regulating some aspect of starch metabolism. We identified several proteins encoded by potentially-interacting prey vectors, including several targeted to the chloroplast. Of the prey proteins that were plastid-

localized, most were involved in photosynthesis; however, one was an uncharacterized protein and thus may represent a new protein involved in starch metabolism. Additionally, there are still a few remaining prey plasmids that need to be sequenced. In addition to the merits of contributing to elucidating the function of BAM2, this project involved significant protocol development. Through this project, the protocol for yeast two-hybridization has been optimized and can now be used to study the other BAM proteins that are potentially regulatory, BAM4 and BAM9. Further work in this project will consist of conducting further false positive testing. Once false positives have been eliminated, remaining interaction(s) will be further verified by overexpressing the prey protein(s) and conducting additional biochemical analysis to validate their role in regulating starch metabolism.

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