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Investigating inactive, chloroplast localized beta-amylase-9 in *Arabidopsis thaliana*

Nurlybek Mursaliyev
*James Madison University*

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Investigating inactive, chloroplast localized beta-amylase-9 in Arabidopsis thaliana

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by Nurlybek Mursaliyev

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FACULTY COMMITTEE:  HONORS PROGRAM APPROVAL:

Project Advisor:  Jonathan D. Monroe, PhD.,  Barry Falk, Ph.D.,  Professor, Biology  Director, Honors Program

Reader:  Nathan Wright, PhD., Assistant Professor,  Reader:  Christopher Berndsen, PhD., Assistant Professor, Chemistry and Biochemistry  Chemistry and Biochemistry
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Abstract

Plant whole genome sequences started to be available in 1998, and these resources made it possible to identify complete gene families. One of these gene families in *Arabidopsis thaliana* is called the β-amylase gene family, which comprises nine members (BAM1-9). β-amylases play an important role in nighttime starch degradation in chloroplasts producing maltose, which is exported to the cytosol for further metabolism. One of the non-catalytic, plastidic BAMs, β-amylase9 (BAM9), is conserved in flowering plants, suggesting that it plays an important role in plastid function. Arabidopsis plants lacking BAM9 appear to accumulate starch, so we suspect that BAM9 may function to regulate starch metabolism under certain conditions. Amino acid sequence alignments revealed three loops surrounding the active site of BAM9 that are not conserved compared with active BAMs, suggesting that BAM9 may not bind to starch. Starch binding assays supported this hypothesis. The alignments also showed that the active site residues of BAM9 were not all identical to those in the active BAMs suggesting that some other molecule may bind to BAM9 in the active site. Residues in the deeper half of the active site were conserved among the BAM9 orthologs suggesting that these proteins might bind a small carbohydrate, such as maltose. However, experiments with isothermal titration calorimetry showed BAM9 has a low affinity for maltose.
**Introduction**

Starch is one of the major products of photosynthesis in plants and it serves as one of the main components of the human diet. An organism that is used to study starch metabolism in plants is *Arabidopsis thaliana* (Smith et al. 2005). Arabidopsis belongs to the mustard (Brassicaceae) family along with cultivated species such as cabbage and radish. Even though Arabidopsis is not of major agronomic significance, it has been widely used as a model organism in plant research. Arabidopsis has many advantages for basic research, such as its small size, rapid life cycle and prolific seed production. On top of this, the Arabidopsis genome has been sequenced and it can be easily transformed (Rhee et al. 2003).

Starch is a polymer composed of two distinct molecules, amylose and amylopectin, both of which are largely made of $\alpha-1,4$ linked glucose. Amylopectin also contains $\alpha-1,6$ branches (Smith et al. 2005). Helical $\alpha$-linkages and the branched nature of starch makes this molecule an excellent glucose reserve for plants, because this allows plants to store glucose more compactly with many nonreducing ends, and without impacting the osmotic balance of cells.

In flowering plants, starch accumulates in chloroplasts during the day when photosynthesis is active. At night, when all cells require a constant flow of energy and carbon skeletons for building materials, starch is degraded (Smith et al. 2005). Plants that synthesize less starch during the day or have lost the ability to efficiently degrade starch at night, show diminished growth; therefore, starch is important for efficient plant growth (Schulze et al. 1991).

There are many genes that are described in *Arabidopsis thaliana* and other plants that may play roles in starch degradation (Hussain et al. 2003, Duwenig et al. 1997, Matheson et al. 1976, Critchley et al. 2001, Niittylä et al. 2004, Lloyd et al. 2004). The first enzymes that attack starch granules are glucan, water dikinase (GWD) and phosphoglucan, water dikinase (PWD)
These enzymes phosphorylate starch to hydrate the outer chains (Ritte et al. 2002, Bustos et al. 2004; Nakamura, 1996). The phosphorylated starch is linearized by debranching enzymes, limit dextrinase and isoamylase (Nakamura, 1996). The linear soluble glucans are then hydrolyzed by β-amylases releasing maltose as an end product (Niittylä et al., 2004). Maltose is then exported to the cytosol through a maltose transporter on inner membrane of the chloroplast envelope (Niittylä et al., 2004) and converted to other compounds such as sucrose, which is transported to other parts of a plant.

A crystal structure of a soybean β-amylase was obtained showing two maltose molecules bound in the active site, where the terminal four glucose residues of starch would be bound (Mikami et al. 1993). Mikami et al. (1993) identified 14 residues participating in maltose binding (Figure 2). One additional residue was identified by Laederach et al. (1999). Glu186 (soybean β-amylase numbering) and Glu380 are involved in the hydrolysis of maltose from starch. Asp53, His93, Asp101, Arg420, Lys295, Asn381 and Ala382 interact with the terminal maltose in a deeper pocket of the active site; Arg188, Tyr192, Gly298, Phe341 Thr342 and His300 interact with the penultimate maltose in surface pocket of the active site (Mikami et al. 1994; Laederach et al. 1999). Work with the crystal structure of active soybean β-amylase also showed the presence of flexible loop (Mikami et al. 1994). Mikami et al. (1994) showed that movement of the flexible loop depended on the presence of the ligand in the active site. They suggested that the flexible loop blocks the active site when the protein is bound to starch. After hydrolysis of the terminal maltose from the starch, the flexible loop opens the active site and allows freed maltose to exit.
Figure 1. Illustration of the starch degradation pathway in flowering plants. GWD and PWD represent glucan, and phosphoglucan, water dikinase, respectively.
There are nine β-amylase-like genes in *Arabidopsis thaliana*, abbreviated as *BAM1* through *BAM9*. Five out of nine BAM proteins (BAM1 through 4 and -6) have been shown to be located in chloroplast (Fulton et al. 2008, Zeeman, personal communication). BAM1 and BAM3 are the primary active enzymes that hydrolyze starch in vivo (Fulton et al. 2008; Valerio et al. 2011). T-DNA insertion mutants lacking functional proteins in Arabidopsis plants gave more insight into the functions of these genes. *bam1* accumulates starch in guard cells (Valerio et al. 2011) and *bam3* accumulates starch in mesophyll cells in leaves (Fulton et al. 2008). *bam1bam3* double knockouts were shown to accumulate more starch in mesophyll cells than the single *bam3* knockout (Fulton et al. 2008; Kaplan et al. 2005). Moreover, microarray data suggests that BAM3 is expressed at relatively high levels at night suggesting this enzyme is active at night (Smith et al., 2004). Valerio et al. (2011) also showed that BAM1 was induced by osmotic stress in mesophyll cells, suggesting that BAM1 may degrade starch during osmotic stress for...
compatible solute accumulation to counteract water loss from the plant. BAM2 has low enzymatic activity but knockout plants do not have apparent phenotype (Fulton et al. 2008). BAM4 does not seem to degrade starch but the bam4 knockout plant was shown to accumulate starch at the end of the night suggesting that it has a regulatory role (Fulton et al. 2008, Li et al. 2009).

Some BAMs do not have plastid transit peptides and either remain in the cytoplasm or are targeted to the nucleus. BAM5 is localized in cytoplasm of phloem cells. Even though it is located in the cytoplasm where there is no starch, BAM5 can hydrolyze starch (Monroe et al. 1995). The bam5 knockout plant does not seem to differ from wild type plant; therefore the role of BAM5 in Arabidopsis is still unclear (Laby et al. 2001). The function of BAM6 is also unclear. Its gene structure analysis suggests BAM5 and BAM6 are paralogs that resulted from a recent segmental duplication within the Arabidopsis genome (Fulton et al. 2008), but unlike BAM5, BAM6 was shown to be localized to chloroplast (Zeeman, personal communication). BAM7 and -8 are targeted to the nucleus and they possess BRASSINAZOLE RESISTANT1 (BZR1)-type DNA binding domains, which are also found in transcription factors mediating brassinosteroid (BR) responses (Reinhold et al. 2011). They are thought to have a role in controlling plant growth and development (Reinhold et al. 2011). The last member of β-amylase gene family is BAM9, which has not been well described and is the subject of this work.

The only published study on BAM9 thus far indicated that its expression is regulated on a circadian cycle, with the highest peak of expression occurring at the end of the night when most of the starch has been degraded (Chandler et al. 2001). This night/day transition peak of expression has been supported by microarray data presented in other publications as well (Smith et al. 2004; Mockler et al. 2007).
Chandler et al. (2001) cloned and sequenced a BAM9 cDNA, and speculated that BAM9 might have a different catalytic activity because BAM9 did not have one of the two regions that was found to be important for catalytic activity in active soybean β-amylase (Chandler et al. 2001; Mikami et al. 1993). Our lab performed amylase activity assays with purified BAM9 to see whether it had catalytic activity (Fedkenheuer and Monroe, unpublished data). Using starch as a substrate we previously found that BAM9 has no catalytic activity. These findings indicated that BAM9 does not hydrolyze starch.

In order to identify the function of BAM9 in plants, our lab performed studies on Arabidopsis knockouts lacking BAM9. Even though the bam9 knockout is similar to wild type, the bam9bam3 double knockout plants accumulate more starch than either single bam3 or bam9 knockout plants (Steidel and Monroe, unpublished data). These results suggested that BAM9 may have some role in starch metabolism, most likely as a regulator of starch metabolism.

My work has focused on characterizing purified BAM9 protein to identify its ligands, using Isothermal Titration Calorimetry (ITC) and starch binding assays. Identifying BAM9’s ligands may further our understanding of the role of BAM9 in Arabidopsis. I have also analyzed the BAM9 amino acid sequence and created a phylogenetic tree of BAMs from different plant species. In addition, I used a BAM9 3D model to identify conserved sites in the protein that may play roles in association with ligands.
Materials and Methods

Purification of his-tagged BAM9.

BAM9 was expressed with a C-terminal hexahistidine fusion tag in the vector pETDUET using BL-21 strain of E. coli. The plasmid was previously constructed in the Monroe lab. Recombinant E. coli cells were kept in 1 mL 15% glycerol solution at -80°C. Cells were grown in 1 L LB media containing 80 µg/mL carbenicillin. The culture was incubated at 37°C with vigorous shaking (250 rpm) for 4 hours. When the culture reached an OD600 value of 0.6, 1 mM (final concentration) isopropyl β-D-1-thiogalactopyranoside (IPTG) was added and the culture was shaken at 250 rpm at 20°C overnight. The cells were harvested at 10,000 rpm at 4°C for 10 minutes. The cell pellet was re-suspended in 40 mL binding buffer (10 mM imidazole, 50 mM NaH$_2$PO$_4$ pH 8, 0.3 M NaCl) in a 50 mL conical tube. The harvested cells were lysed by sonication with set up program of 60 amp frequency, 5 seconds of sonication (total of 2 minutes) and 20 seconds of the rest on ice.

His-BAM9 was purified by chromatography on Ni$^{2+}$-NTA (Novagen). The soluble fraction after sonication was transferred to new 50 mL conical tube and mixed with 2 mL nickel-resin and was incubated in rotatory-shaker at 4°C at 10 rpm for 1 hour. The nickel-resin was allowed to settle by gravity and the supernatant was discarded. Incubated nickel-resin was resuspended in 40 mL binding buffer and the solution was loaded into column (3x10cm). The nickel-resin was then washed with 20 mL wash buffer (50 mM imidazole, 50 mM NaH$_2$PO$_4$ pH 8, 0.3 M NaCl) to wash off nonspecific bound proteins. The flow through from washing was collected and saved for SDS-PAGE analysis. His-BAM9 protein was eluted with 20 mL elution buffer (250 mM imidazole, 50 mM NaH$_2$PO$_4$ pH 8, 0.3 M NaCl). One milliliter fractions were collected and absorption was analyzed at A280 with spectrophotometer (Nano-vue plus, GE).
The fractions with the highest protein levels were combined and were dialyzed using dialysis tube with MWCO 6-8000 (Spectrum Laboratories) overnight at 4°C against 1 L PBS (25 mM NaH$_2$PO$_4$ pH 7.2, NaCl 100 mM). The dialysis was performed a second time for one hour. Dialyzed proteins were concentrated with a Millipore centrifugal filter (MWCO 10000, Amicon Ultra-15) in a swinging bucket centrifuge at 1800 x g at 4°C for 20 min. The concentration of proteins was determined with the Bio-Rad Protein Assay (Cat# 500-0006) and fractions were analyzed with SDS PAGE. Purified proteins were stored at -80°C. His-tagged BAM3 was purified with the same method.

**3D modeling**

A 3D model of BAM9 was generated using the online program PHYRE v.2.0 (Structural Bioinformatics group, Imperial College, London) and was visualized by Cn3D (NCBI).

**Amino acid sequence alignment and phylogenetic tree analysis**

Sequences of BAM-like proteins were identified using the Basic Local Alignment Search Tool (BLAST) and obtained from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). After removal of the N-terminal chloroplast transit peptides and BRZ domains, sequences were aligned with Clustal Omega (v.1.2.0) and a phylogenetic tree was generated by ClustalW2 phylogeny program from the European Bioinformatics Institute web page (www.ebi.ac.uk). Sequence alignments were visualized using BoxShade program from the Bioinformatics Resource Portal of Swiss Institute of Bioinformatics (www.expasy.org). The phylogenetic tree was visualized by FigTree software v1.4 (Institute of Evolutionary Biology, University of Edinburgh.)
Starch binding assay.

Starch binding assays were performed as described by Steichen et al. (2008) with affinity purified His-BAM9 and His-BAM3 proteins. Corn starch (Sigma) was washed twice with 1 mL of buffer (either 100 mM MES, pH 6, or 100 mM MOPS, pH 7). Into 20 mg of starch in 500 µL of each buffer, 35 µg protein (either His-BAM9 or His-BAM3) were added. The mixture was incubated on a rotory-shaker for 30 min at 4°C. In order to separate starch bound proteins from non-bound proteins, the mixture was centrifuged at 16,100 x g at room temperature for 5 min, and the supernatant was saved. To extract proteins from the starch, the pellet was incubated at 75°C for 10 min in 500 µL of 1% SDS, 100 mM MOPS pH 7 solution. The supernatant and starch extract fractions were analyzed by 4–12% SDS-PAGE and stained with 0.1% Coomassie Brilliant Blue.

Isothermal Titration Calorimetry (ITC) experiments

Isothermal titration calorimetry (ITC) is a method that directly measures the energetics involved in the binding of two components utilizing enthalpy (ΔH) of binding (Velazquez-Campoy et al. 2004). ITC can give the complete thermodynamic profile of a macromolecule binding to its substrate. A thermodynamic profile includes binding affinity (K_a), number of binding sites (n), entropy change (ΔS) and Gibbs energy change (ΔG).

ITC experiments were carried out using a NANO ITC (TA Instruments) with injecting 125 M maltose in 25 mM KH₂PO₄ pH 7.2, 2 mM DTT, 100 mM NaCl into 20 µM protein in the same buffer at 16°C. The initial cell volume was 950 µL. The initial injection volume was 2 µL, and the remaining injections were 4 µL each. Data were analyzed by NanoAnalyze Data Analysis software v.2.4.1 (TA instruments)
Results

*BAM9 purification*

His-BAM9 was purified by chromatography on Ni^{2+}-NTA (Novagen). Figure 3 shows the steps of the purification process. BAM9 has a molecular weight of 52 kD. Cell debris and supernatant are insoluble and soluble fractions, respectively. After centrifugation of sonicated cells, some BAM9 protein was observed in the cell debris, indicating that some of the proteins were denatured during cell growth and/or during the sonication step. Nonetheless, a large amount of relatively pure protein was obtained. The flow through fraction after washing the nickel-resin with wash buffer (methods) does not seem to have a band for BAM9, which indicates the proteins were not lost during washing step before eluting them with elution buffer. The BAM9 concentration determined after the purification was 32 mg/mL.

His-tagged BAM3 was purified with the same method. The molecular weight of BAM3 is 55kD and the final BAM3 concentration was 1.7 mg/mL.
Figure 3. Purification of BAM9 by chromatography on Ni$^{2+}$-NTA (Novagen). Samples from purification steps were run on 4–12% SDS-PAGE and stained with 0.1% Coomassie Brilliant Blue. The black arrow refers to the BAM9 protein at 52kD. Lane 1-Cell Debris after centrifugation of sonicated cells, lane 2-Supernatant after centrifugation of sonicated cells, lane 3-Flow through after the wash step, lane 4- Concentrated Purified BAM9, lane 5-Diluted purified BAM9. MW-Molecular weight ladder with labels to the right in kDa.

Amino acid sequence analysis.

In order to identify differences between BAM9 and active BAMs, we performed an amino acid sequence alignment using BAM3 orthologs and BAM9 orthologs. We chose BAM3 as an active BAM because it is an essential BAM in Arabidopsis starch degradation; we also included soybean BAM5 as a reference in sequence analysis, because it has been crystallized and active site amino acid residues were identified (Mikami et al. 1993).
In order to perform amino acid sequence analysis, 55 BAM-like genes from six completely sequenced and annotated plant genomes were selected using Basic Local Alignment Search Tool (BLAST) by The National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) (Table 1).

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<td>NP_567460, NP_180788, NP_191958, NP_189034, NP_567523, NP_182112, NP_199343, NP_568829, NP_197368</td>
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<tr>
<td><em>Ricinus communis</em></td>
<td>XP_002516865.1, XP_002517513.1, XP_002511858.1, XP_002515712.1, XP_002515712.1, XP_002511857.1, XP_002519919.1</td>
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<td><em>Populus trichocarpa</em></td>
<td>XP_006372990.1, XP_006385589.1, XP_006385389.1, XP_002312750.2, XP_002304400.1, XP_002311706.1, XP_002518196.1</td>
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<td><em>Glycine max</em></td>
<td>NP_001236364.1, XP_003540325.2, XP_003524296.1, XP_003539125.1, NP_001236350.1, XP_006573703.1, XP_003548316.1, XP_003534086.1, XP_003532447.1, XP_003552392.1, XP_003539882.1, XP_003525331.1, XP_003534564.1, NC_016093.1</td>
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<td><em>Solanum lycopersicum</em></td>
<td>NP_001234052.1, XP_004244551.1, XP_004245844.1, NP_001234556.1, XP_004243448.1, XP_004245482.1, XP_004244442.1, XP_004229887.1</td>
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<td><em>Vitis vinifera</em></td>
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In order to identify orthologs of Arabidopsis BAMs among the 55-BAM like genes, a phylogenetic tree was generated (Figure 4). This analysis revealed eight clusters (a – h) that contain closely related genes. Only cluster a does not contain any of the BAMs from Arabidopsis, which indicate these genes do not have orthologs in Arabidopsis (Figure 4). The rest of the seven clusters contain at least one BAM from Arabidopsis. Two clusters contain two Arabidopsis BAMs (Figure 4). Cluster f contains Arabidopsis BAM5 and BAM6 and cluster h contains BAM2 and BAM7.

Clusters c and d contain orthologs of BAM3 and BAM9 respectively, which were used in amino acid sequence alignments in order to identify amino acid sequence differences between BAM9 orthologs and BAM3 orthologs (Figure 4). The fifteen amino acid residues that bind to substrate, identified from active soybean BAM5 (Mikami et al. 1993; Laederach et al. 1999), were perfectly conserved in all BAM3 orthologs (Figure 5), but BAM9 orthologs did not contain all of these residues (Figure 5). Nine amino acid residues were different in Arabidopsis BAM9 compared with soybean BAM5. Asp 54 of soybean BAM5 is Pro in BAM9, Asp101 is Lys, Ala382 is Ser, Glu380 is Gln, Arg188 is Lys, Tyr192 is His, Gly298 is Leu, Phe341 is Ile and Thr 342 is Pro.
Figure 4. Phylogenetic tree of β-amylase like proteins from six different eudicots. Black circles represent eight clusters with closely related genes. Cluster a does not contain any of the Arabidopsis BAMs indicating they are not orthologs of any of Arabidopsis BAMs. Clusters f and h contain two Arabidopsis BAMs and the rest contain one BAMs from Arabidopsis. Cluster d includes BAM9 orthologs and cluster c includes BAM3 orthologs. These two clusters were used in subsequent amino acid sequence analysis.
Figure 5. Alignment of soybean active site residues and their corresponding residues from BAM3 orthologs and BAM9 orthologs. The numbering of amino acids is given only for soybean BAM5 and BAM9. Names of the species and accession numbers of the genes are given on the first and second columns, respectively. Soybean BAM5 amino acid sequences were identified from the paper by Mikami et al. (1993) and Laederach et al. (1999).
Despite the differences from BAM3 orthologs, the deeper pocket amino acids residues in BAM9 orthologs are perfectly conserved among BAM9 orthologs, compared with less conserved outer pocket of the active site (Figure 5). The fact that the deep pocket of the active site is perfectly conserved suggests that BAM9 may bind a small molecule instead of starch. The most reasonable candidate for the BAM9 ligand is a disaccharide such as maltose, because it is the product of starch degradation by the active BAMs. As previously shown in our lab, the absence of hydrolytic activity of BAM9 with starch is supported by the change of glutamic acid (Glu186 in soybean numbering) to glutamine in Arabidopsis BAM9. Mikami et al. (1993) showed that Glu186 and Glu380 act reversibly as proton donors and acceptors during the catalytic activity of soybean BAM5, therefore the uncharged amide group of glutamine probably disrupts the proton exchange that is important for the catalysis.

Comparison of amino acid sequences of BAM3 orthologs and BAM9 orthologs revealed three regions in the BAM9 orthologs that were highly conserved among BAM3 orthologs, but not conserved in BAM9 orthologs (Figure 6). In a 3D model of BAM9 these regions are located around the putative active site (Figure 7), which suggests that the ability of the protein to interact with starch may be disturbed. This observation and the differences in active site residues might suggest that BAM9 does not bind starch as soybean BAM5 does. Moreover, one of the three regions is the flexible loop that is truncated. We speculate that the truncated flexible loop may allow the small carbohydrate ligand to access the active site of BAM9 more easily.
Figure 6. Amino acid sequence alignment of BAM3 orthologs and BAM9 orthologs. Only shown regions are three regions that were significantly different in BAM9 orthologs but were conserved in BAM3 orthologs. Labels on the left are accession numbers of BAM3 orthologs (green) and BAM9 orthologs (blue). Red lines represent three regions that were found to be different in BAM9 orthologs compared with BAM3 orthologs. Truncated flexible loop region is indicated by red box.
Figure 7. Worm representation of the BAM9 model generated using PHYRE and visualized with Cn3D software. α-helices are shown as cylindrical arrows and β-sheets with flat arrows, arrowheads pointing to the C-terminus. Residues of putative active site are shown in blue bubbles. Three regions that were identified being nonconserved in BAM9 orthologs as compared with BAM3 orthologs are shown with red line. The truncated flexible loop is labeled.
Starch binding assay

In order to test the hypothesis that BAM9 does not bind starch, starch binding assay were performed using purified BAM9 and BAM3, which was shown to bind starch (Li et al. 2009). The assays showed that BAM9 does not bind starch at either pH 7 or pH 8, on the other hand BAM3 binds to starch (Figure 8). Since there are nine residues found different out of fifteen in BAM9 active site compared with active BAMs, the ability of BAM9 to interact with starch is probably lost. On top of this, the three regions surrounding active site were also different from active BAMs which may also hinder BAM9’s ability to interact with starch. Interestingly, there was pH dependent affinity observed in BAM3 interaction with starch (Figure 6). BAM3 was able to bind starch more at pH 7 than at pH 8 (Figure 8). This difference in binding correlates with the fact that BAM3 is functional at night, when pH of the stroma is lower than during the day (Werdan et al. 1975).
Figure 8. Starch binding assay with purified BAM9 and BAM3 proteins. After incubating 35 mg of proteins with 20 mg of starch in 500 µL buffer (either 100 mM MES pH 6, or 100 mM MOPS pH 7), starch was separated by centrifugation. The proteins bound to starch were extracted by incubation at 75°C for 10 min in 500 µL of 1% SDS, 100 mM MOPS pH 7 solution. Samples were run in 4–12% SDS-PAGE and stained with 0.1% Coomassie Brilliant Blue. S - Sample from supernatant after centrifugation. P - Sample from extracted pellet. MW-Molecular weight ladder with labels to the left in kDa. The sizes of BAM3 (55 kDa) and BAM9 (52 kDa) are indicated by black arrows.
Since the deep pocket residues of the BAM9 active site were completely conserved in the BAM9 orthologs, we hypothesized that BAM9 might interact with a small carbohydrate instead of starch. The first candidate to test for BAM9 binding was maltose, since it is the product of starch hydrolysis (Smith et al. 2005). In Arabidopsis, products of starch degradation are exported from chloroplast in the form of maltose (Smith et al. 2005). Therefore we hypothesized BAM9 may sense the change in maltose levels in chloroplasts to regulate starch metabolism. In order to test this, we performed isothermal titration calorimetry (ITC). ITC showed that BAM9 does interact with maltose but the apparent affinity to maltose was very weak. Assuming that there is one independent binding site, it was calculated that the dissociation constant ($K_d$) with maltose was $21.5 \pm 3.6$ mM (Figure 9).
Figure 9. Measurement of maltose-BAM9 binding kinetics using isothermal titration calorimetry. A. Titration of 125 mM maltose in 25 mM KH$_2$PO$_4$ pH 7.2, 2 mM DTT, and 100 mM NaCl into 20 μM protein in the same buffer at 16°C. B. Integration plot of the titration peaks and calculations of binding kinetics. Red line indicates fit curve for independent binding. Ka – association constant; dH – enthalpy of binding; n – number of ligand; Kd – dissociation constant; dS – binding entropy.
Discussion

The availability of whole genome sequences of plants made it possible to identify all of the members of gene families, but determining the roles of individual members is challenging. Even though the roles of some β-amylases, such as BAM1 and BAM3, are to function as primary enzymes hydrolyzing starch degradation (Fulton et al. 2008; Valerio et al. 2011), functions of other BAMs are unclear. Even well studied catalytically active BAM5 has no apparent function, because it is localized in cytosol where there is no starch (Monroe et al. 1995, Laby et al. 2001). BAM6 seems to be a paralog of BAM5, but its function is still unclear (Fulton et al. 2008). BAM2 has low enzymatic activity and absence of this gene causes no apparent phenotype (Fulton et al. 2008). Roles of inactive BAMs are even more ambiguous. BAM7 and BAM8 are localized to the nucleus and seem to act as transcriptional regulators of shoot growth and development (Reinhold et al. 2011). The catalytic domains of BAM7 and BAM8 are thought to act as carbohydrate sensors rather than hydrolyzing starch, because they have small or no catalytic activity.

BAM4 was shown to be inactive but the lack of this gene causes Arabidopsis to accumulate starch at the end of the day, which suggests that it may have a regulatory function in starch metabolism (Fulton et al. 2008, Li et al. 2009). Interestingly, BAM4 was shown to bind starch and its function probably involves facilitating starch breakdown in the dark by interacting with starch granules (Li et al. 2009). It was suggested that BAM4 influences starch breakdown as chloroplastic regulator by sensing the concentration of maltose and fine tuning the rate of starch degradation (Fulton et al. 2008). Nevertheless, direct evidence of BAM4 interacting with maltose is lacking.
Similar to BAM4, BAM9 might also be involved in regulating starch metabolism even though it has no hydrolytic activity (Fedkenheuer and Monroe, unpublished data). Since, in this work, we showed that BAM9 does not bind to starch, we exclude direct involvement of BAM9 with starch to regulate starch metabolism as it was proposed with BAM4 (Li et al. 2009). BAM9 has to regulate starch metabolism indirectly by interacting with other proteins. BAM9 might regulate starch metabolism in concert with BAM3, because our lab showed more accumulation of starch in bam3bam9 double knockouts compared with single knockouts (Monroe unpublished), but this requires more investigation in the future.

Comparing the active site amino acids of BAM9 orthologs with BAM3 orthologs, we showed that BAM9 orthologs do not contain all fifteen conserved residues in the active site but residues of the deep pocket of the active site were conserved among all BAM9 orthologs (Figure 5). The conservation of deep pocket of the active site in BAM9 orthologs led us hypothesize that BAM9 may bind a small carbohydrate such as maltose instead of binding starch. However, ITC results showed a very low affinity of BAM9 to maltose with a $K_d$ of 21 mM, which indicates that BAM9 would require high maltose concentration in chloroplast. The exact chloroplast stroma concentrations of maltose has not been reported in literature, but a rough estimation of chloroplast concentration of maltose based on data presented by Weise et al. (2003) and B10NUMB3R5 (2010) indicates that it around 0.5 mM in the daytime and 1.7 mM at night. These values suggest that at normal conditions high maltose concentrations are physiologically not relevant.

Sucrose is synthesized in cytosol of mesophyll cells as one of the primary end products of leaf photosynthesis. Sucrose is used as a means to transport sugar from one part of the plant to another. Mesophyll cells photosynthesizing during the day, on top of storing starch in their
chloroplasts, also export sucrose to non-photosynthesizing cells. Essentially, non-photosynthesizing cells are able to acquire energy and store starch using sucrose from photosynthesizing cells (Smith et al. 2005, Winter et al. 2000). At night, sucrose is also exported when cells degrade the accumulated starch (Smith et al. 2005). Even though sucrose is synthesized in cytosol, Gerrits et al. (2001) showed that sucrose is able to enter chloroplasts where BAM9 is present. Moreover, Lunn et al. (2006) showed that sucrose levels affect trehalose-6-phosphate, which acts as a signal of sugar status in plants (Lunn et al. 2006). Therefore, sucrose and trehalose-6-phosphate are among the main carbohydrate candidates to test for BAM9 binding.

Evidence presented here can further our understanding not only the function of BAM9, but starch metabolism as well. Clearly, amino acid alignments indicated that BAM9 does not possess catalytically important residues and there were differences in sequences surrounding the active site. The results of starch binding assays showed that BAM9 does not bind starch as its active counter parts do. Nonetheless, ITC results showed low affinity to maltose as a carbohydrate ligand so we will pursue other disaccharides as candidate ligands.
References


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