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Role of β-amylase1 (BAM1) in the recovery of *Arabidopsis* plants from osmotic stress

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A Project Presented to

The Faculty of the Undergraduate

College of Science and Mathematics

James Madison University

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in Partial Fulfillment of the Requirements

for the Degree of Bachelor of Science

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by Jonathan Michael Schmitz

May 2014

Accepted by the faculty of the Department of Biology, James Madison University, in partial fulfillment of the requirements for the Degree of Bachelor of Science.

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Table of Contents

List of Figures	3
Acknowledgements	4
Abstract	5
Introduction	7
Methods and Materials	12
Results	16
Discussion	20
Appendix	24
Bibliography	31

List of Figures

Figures

1 β-amylase activity in wild-type and *bam5* plants grown under high and low light. 24

2 Diurnal β-amylase activity in WT and mutants of Arabidopsis lacking various BAMs. 25

3 SDS PAGE showing purified BAM1 and -3 enzymes expressed in in *E. coli.* 26

4 Comparison of amylase activity from extracts of Arabidopsis *bam51* and *bam53* leaves and from BAM1 and -3 expressed in *E. coli* at various pHs. 27

5 Impact of osmotic stress on leaf survival. 28

6 Effect of osmotic stress on total water content in wild-type and *bam1* mutants. 29

7 Effect of osmotic stress on β-amylase activity in wild-type, *bam5*, and *bam51* mutants. 30

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Abstract

Starch, a polymer of glucose, is a source of stored energy and carbon for plants. Starch accumulates in chloroplasts during the day and is broken down at night. Many enzymes are involved in this degradation, but the main players are in the β-amylase family of enzymes. In *Arabidopsis*, the β-amylase (BAM) family consists of nine proteins, six of which are plastid targeted. One of these plastidic BAMs is BAM1, which was suggested to be catalytically active during the day in guard cells to aid in the opening of stomata. Also, when exposed to osmotic stress, plants close their stomata, slowing water loss but also preventing photosynthesis. In addition to BAM1's function during the day in guard cells, BAM1 is expressed in mesophyll cells to alleviate the loss of available photosynthate.

BAM5 is an active, cytosolic BAM that can contribute most of the activity in leaves during the day. This high activity masks the activity of the other active BAMs, making it difficult to compare their activities with an amylase assay. We used T-DNA-insertion mutants to genetically eliminate BAM5 in several single and double mutants that also lack BAM1, -2, -3, and/or -6, to remove this high background activity. Amylase assays without this high background activity showed that BAM1 and -3 contribute a majority of the activity in mature leaves while BAM2 and -6 contribute very little activity. This allowed us to compare mutants that lack BAM genes and learn about their activity and expression under various conditions.

During the day, the pH of the stroma is 8.0 and it drops to 7.0 at night. BAM1, due to its location in the stroma, is subject to diurnal pH fluctuations. It was previously observed that assays using a synthetic substrate produced a narrow activity curve in which BAM1 was nearly inactive at pH 8.0, suggesting that BAM1 would not be active during the day. We thought that

the synthetic substrate might be misrepresenting the activity of BAM1 at higher pHs so we used soluble starch as the substrate, which is similar to amylopectin, the natural substrate of BAM1 and -3. Pure BAM1 and -3 expressed in *E. coli* were assayed and compared to assays with extracts made from *bam53* and *bam51.* With starch as the substrate, the pH curves of both BAM1 and BAM3 are broader. Moreover, we observed that at pH 8, BAM3 is less active while BAM1 is more active. The similarities between the pH curves of the crude extracts and corresponding BAMs expressed in *E. coli* confirmed that *bam53* and *bam51* contain primarily BAM1 and BAM3.

Knowing that *bam53* mutants only contained BAM1, we wanted to see how much BAM1 is expressed in mesophyll cells during osmotic stress. To see the effect of osmotic stress on the expression of BAM1, we exposed mutants that lack BAM1 and -5 to osmotic stress and measured their activities with amylase assays. The activity in plants that contained BAM1 was 40% higher under osmotic stress than plants that lacked BAM1. We also monitored the health of these plants over time and observed that, under osmotic stress, *bam1* mutants experienced more chlorosis and wilting than the wild-type.

We reasoned that due to the activity and location of BAM1 in mesophyll cells, BAM1 may hydrolyze starch, providing carbon skeletons for the production of osmolytes. These osmolytes help plants take up water through osmosis. The increased activity of plants containing BAM1 combined with *bam1* plants experiencing less chlorosis, anthocyanin production, and wilting as the wild-type suggests that BAM1 plays a crucial role in the survival of osmotic-stress.

Introduction

Starch, a polymer of glucose, is a source of stored energy and carbon for plants. During the day, triose phosphate is generated via photosynthesis, half of which is either utilized or converted to sucrose for export to roots and developing leaves where it provides carbon skeletons that aid cellular respiration and growth. The remaining half of triose phosphate remains in the chloroplast where it is used for starch synthesis (Nittylä et al., 2004). Starch accumulates in chloroplasts during the day and is broken down during the night to provide sugars and carbon skeletons that aid in respiration and growth during the night when photosynthesis is inactive and no longer providing sucrose (Zeeman et al., 2007). This regular diurnal cycle and the rates of starch synthesis and degradation are flexible and are readily adjusted by the plant in response to changes in day/night length, temperature, and availability of water (Smith and Stitt, 2007; Graf et al., 2010). There are many enzymes involved in starch degradation, but it is recognized that enzymes in the β-amylase family are the main players in starch degradation at night (Smith et al., 2003).

In *Arabidopsis thaliana*, the β-amylase (BAM) family consists of nine proteins, six of which, BAM1-4, -6, and -9, are plastid targeted, where starch is accumulated (Lao et al., 1999; Fulton et al., 2008; S. Zeeman, personal communication). BAMs function by catalyzing the removal of maltose from the nonreducing ends of starch (Mikami et al., 1994). The BAM family of proteins are 50-60 kD in size and are similar in structure (Adachi et al., 1998).

Based on the crystal structure of soybean BAM5, catalytically active BAMs have a "flexible loop" consisting of seven residues that extend into the solvent (Mikami et al., 1994). This flexible loop opens to allow starch to enter the active site, closes to induce hydrolysis, and

then opens again to allow maltose to leave the active site while starch remains bound to the protein. This mechanism allows for a processive hydrolysis of starch instead of requiring the BAMs to reassociate with starch each time a maltose is cleaved. *Arabidopsis* has been a model organism for understanding starch metabolism and it is useful for the understanding of the specific roles of the 9 BAM proteins.

BAM1 is a plastid-targeted catalytically active β-amylase (Lao et al., 1999). Mutants lacking BAM1 contained more starch in the guard cells during the day than the wild-type (Valerio, et al., 2011). When extracts from wild-type and *bam1* mutants were assayed, extracts of *bam1* mutants were slightly less active compared to the wild-type (Fulton et al., 2008). Using the BAM1 promoter fused to the reporter gene encoding β-glucuronidase (GUS). Valerio et al. (2011) showed that BAM1 is limited to guard cells, where it is thought to aid their opening and closing under normal conditions. The presence of BAM1 in the guard cells allows it to break down starch during the day to provide carbon skeletons for malate synthesis (Outlaw and Manchester, 1979). Under normal conditions in a plant, potassium is accumulated in guard cells, allowing them to open to let CO_2 enter leaves. As this potassium accumulates, its positive charge also accumulates. Malate is then accumulated as a counter ion to the positively-charged potassium to prevent an undesirable accumulation of charge.

Using the same transgenic plants containing the BAM1 promoter driving expression of GUS, BAM1 was also shown to be highly expressed in mesophyll cells during osmotic stress (Valerio et al., 2011). Assays showed an increase in amylase activity under osmotic stress in wild-type plants compared to *bam1* plants (Valerio et al., 2011). Compatible solute synthesis from the breakdown of starch could be used by the plant to counteract osmotic stress during

drought situations (Valerio et al., 2011). During drought it is difficult for a plant to obtain the little water that remains in the soil. When the water content inside the plant is higher than the water content in the soil, water has a tendency to flow to the soil, causing the plant to become osmotically stressed. When a plant is exposed to osmotic stress, the plant produces abscisic acid (ABA), which causes stomata to close, slowing water loss (Kempa et al., 2008). While this may prevent water loss, the closing of guard cells also prevents photosynthesis. To alleviate the loss of available photosynthate by the closing of stomata, BAM1 is upregulated in mesophyll cells (Valerio et al., 2011). This suggests that BAM1 may hydrolyze starch in order to generate the necessary carbon skeletons for the production of osmolytes. These osmolytes could help mesophyll cells maintain a low water potential, allowing roots to take up water through osmosis.

BAM3 is a plastid-targeted, catalytically active β-amylase (Kaplan and Guy, 2005; Sparla et al., 2006). Plants lacking *bam3* accumulated more starch at the end of the night than the wild-type plants (Fulton et al., 2008). Our unpublished results showed that *bam1*, *bam2*, and *bam6* mutants do not accumulate starch. This suggests that BAM3 may be the predominant functional BAM during the night. BAM3 is also induced after 2 hours of cold stress at 4°C (Kaplan and Guy, 2005). As temperatures are typically cooler at night, induction by the cold suggests that BAM3 might be a cold-tolerant enzyme. Interestingly, while osmotic stress initiates an up-regulation of BAM1, it also causes down-regulation of BAM3 (Kaplan and Guy, 2005; Sparla et al., 2006). The close relationship between the inverse expression of BAM1 and BAM3 is interesting as BAM1 and BAM3 seem to be the main players in starch degradation during the day and night, respectively.

Assays suggested that BAM4 has no activity, but BAM4 is thought to be a regulator of starch metabolism by interacting with other proteins, such as BAM1 and -3 to stimulate starch degradation (Fulton et al., 2008; Li et al., 2009). Using GFP, a marker protein fused to the BAM4 coding sequence, BAM4 was localized to the choloroplasts (Fulton et al., 2008). Mutants that lack BAM4 accumulate more starch and less maltose than the wild-type, and *bam4* mutants that also lack either BAM1 or -3 have more starch and less maltose than mutants that solely lack BAM4 (Fulton et al. 2008). This suggests that BAM4 does not contribute to the degradation of starch entirely, but it may combine with BAM1 and -3 in order to facilitate starch degradation. *bam4* mutants accumulate starch, suggesting that BAM4's presence and expression in the cell might be due to its role as a regulatory protein (Fulton et al., 2008). BAM2 has catalytic activity, but its activity is 25 to 50 times lower than BAM1 or -3 (Fulton et al., 2008). Nothing has been published about BAM6, but it is apparently active and localized to the chloroplasts (S. Zeeman, personal communication).

BAM9 is the last protein in the BAM family which is plastid-targeted protein. Unlike the catalytically active BAM proteins, comparisons between orthologs of BAM9 show a deletion in the section of the gene that codes for the flexible loop. Amylase assays also showed that BAM9 is catalytically inactive (Fedkenhauer and Monroe, unpublished). BAM9 was also experimentally shown to be expressed at high levels during the night/day transition (Chandler et al., 2001). Microarray data from six different experiments shows that, in all cases, expression of BAM9 peaks just as night transitions into day (Smith et al., 2004; Mockler et al., 2007). Due to this peak just as the day begins, and the data showing that BAM9 is catalytically inactive, BAM9, like BAM4, may have a regulatory function.

BAM5 is catalytically active but is not localized to plastids and can make up a relatively substantial portion of the β-amylase activity in extracted leaves (Lin et al., 1988; Monroe & Preiss, 1990; Wang, et al., 1995; Laby et al., 2001). With a high background of BAM5 activity, it is difficult to measure the activities of the plastidic BAM enzymes. For this research, we constructed a series of double mutants, each lacking BAM5 and one of the active, plastidic BAMs: *bam51*, *bam52*, *bam53*, and *bam56*. These mutants allowed us to see the activity that they contribute without the interference of BAM5.

The focus of this thesis is on the catalytically active plastidic enzyme, BAM1. I focused on BAM1's function during the day, its expression during osmotic stress, and how BAM1 may aid a plant's recovery after drought. I also investigated BAM1's function by comparing it to another catalytically active plastidic enzyme, BAM3. The pH of the stroma during the day is around 8.0, and at night it drops to 7.0 (Kramer et al., 1999). BAM1, due to its location in the stroma, is subject to diurnal pH fluctuations. Amylase assays comparing BAM1 and -3 over a pH scale using a synthetic substrate, p-nitrophenylmaltopentoaside (PNPG5), produced a narrow activity curve that showed no difference in activity between the two enzymes, moreover, BAM1 had little activity at pH of 8 (Fulton et al., 2008; Li et al., 2009). This suggested that BAM1 could not be active during the day if the stromal pH is around 8 (Kramer et al., 1999). We performed amylase assays utilizing starch as the substrate to show how BAM1 functions differently from BAM3. Using starch, the activity curve of BAM1 is wider than curves previously published, exposing conditions in which it might be a functional starch-degrading enzyme.

Methods and Materials

Growth of *Arabidopsis thaliana* plants

Seeds were scattered on 5” pots containing Sunshine Mix #3 Soil (Sun Gro Horticulture Distribution Inc. Bellvue, WA, USA) moistened with water in order to create a lawn of young plants. After one to two weeks of growth, seedlings were transplanted five plants to a pot and watered with 5L nutrient solution [consisting of tap water with 5 mM KNO_3, 2.5 mM KPO_4 (pH 5.5), 2 mM $MgSO_4$, 2 mM $Ca(NO_3)_2$, 0.05 mM FeNaEDTA, 70 mM H_3BO_3, 14 mM $MnCl_2$, 0.5 mM $CuSO_4$, 1 mM $ZnSO_4$, 0.2 mM $NaMoO_4$, 10 mM NaCl, and 0.01 mM $CoCl_2$], per tray, containing eight pots. Plants were grown on growth carts (Growers Supply Co. Ann Arbor, MI, USA) under fluorescent lights (300-350 µmol m^{-2} s^{-1}) at 22°C under a diurnal cycle of 12 hours with the lights on and 12 hours with the lights off. Plants were watered as needed by sub irrigation with tap water. For osmotic stress experiments, pots were watered with 200 mL of 200 mM mannitol in nutrient solution, and monitored over the course of one week.

Quantification of water loss in osmotically-stressed plants

Mature rosettes from plants under osmotic stress and normal conditions were dried in pre-tared paper bags at 80ºC for 48 hours. The percent water content was calculated from the fresh weight and the dry weight of the plants.

Preparation of enzyme extracts and amylase assays

Mature leaves were ground by mortar and pestle with sand and extraction buffer (50 mM MES pH 6.0, 2 mM DTT, 5 mM EDTA). Extracts were centrifuged at 13,000 rpm for 10 minutes and the supernatant was used as the enzyme for amylase assays. Amylase activity was determined in 0.5 mL of 50 mM MES (pH 6.0) with 10 mg/mL Lintner soluble starch as

(Pfanstiehl Laboratories, Waukegan, IL) substrate. Duplicate reactions were started with the addition of enzyme and were stopped by immersion in a boiling water bath for 3 min. Reducing sugars were measured by the Somogyi-Nelson assay (Nelson 1944).

Expression of BAM1 and -3 in *E. coli*

Plasmids containing BAM1 and -3 were a gift from H. Reinhold (ETH Zurich, Switzerland). Constructs of BAM1 and -3 were created by removing the transit peptide sequences, as described by (Fulton et al., 2008). These cDNA constructs containing BAM1 and BAM3 were cloned into the pJET1.2 vector and then transferred into the pET29a vector by cutting pET29a with the EcoRI and NotI restriction enzymes. DH5-α *E. coli* cells containing the BAM1 and BAM3 plasmids in pET29a were streaked onto LB agar plates containing 50 µg/mL kanamycin (kanamycin^{50}) in order to confirm purity. To express the proteins, DNA was isolated by standard mini prep and then transformed into competent BL-21+ *E. coli* cells. We used 2 ng/µL DNA per transformation, selecting transformants on a LB agar containing kanamycin^{50}. The culture tubes were confirmed by restriction digest to contain the pET29a plasmid containing either BAM1 or BAM3.

E. coli cultures with the pET29a plasmid containing either BAM1 or BAM3 were streaked onto kanamycin^{50} plates in order to confirm their purity. Culture tubes containing 3 mL of LB broth and kanamycin^{50} were inoculated with colonies that contained the pET29a plasmid and were shaken at 250 rpm for overnight at 37°C. Flasks with 250 mL of media were inoculated with the 3mL cultures grown overnight and the flasks were shaken at 250 rpm at 37°C until the optical density (A_{600}) was at 0.6. At this point, 0.5 M IPTG was added and shaken at 250 rpm at 20°C overnight.

Purification of BAM1 and -3

Cultures were centrifuged at 10,000 G for 20 minutes at 4ºC and the supernatant was removed. The pellet was transferred to a conical tube and frozen for ten minutes at -80°C. The frozen pellet was then resuspended in binding buffer (0.1 M $NaH_2PO_4^{3-}$, 10 mM imidazol, and 0.1 M NaCl, pH 8.0). Resuspended cells were sonicated on ice for five seconds and then allowed to cool for 20 seconds, repeating on and off, for a total of two minutes at 50 amplitude. Sonicated cells were centrifuged at 10,000 G at 4°C for 30 minutes and the supernatant was transferred into a clean conical tube.

Washed nickel-nitrilotriacetic acid agarose (Ni-NTA) His-Bind Resin (QIAGEN) was added to the supernatant in the conical tube at a ratio of 4 mL resin:30 mL supernatant and the solution was mixed gently, making sure no bubbles formed in the solution. This resin-supernatant solution was then mixed by rotation at 5 RPM for one hour at 4°C. This solution was then transferred to a 3 cm diameter column, allowing the nickel beads to settle at the bottom and the binding buffer to drain. The resin was washed with 30 mL of additional binding buffer and allowed to drain. The resin was then washed with 30 mL of washing buffer (0.1 M $NaH_2PO_4^{3-}$, 40 mM imidazol, and 0.1 M NaCl, pH 8.0) and allowed to drain. Once washed five times, the bound protein was eluted with elution buffer (0.1 M $NaH_2PO_4^{3-}$, 250 mM imidazol, and 0.1 M NaCl, pH 8.0) and was collected in 20 1.5 mL microcentrifuge tubes. Fractions were analyzed with the Bio-Rad Protein Assay (Bio-Rad Laboratories Inc. Hercules, CA, USA), in which protein concentration was measured using a Synergy H1 Hybrid Multi-Mode Microplate Reader (Bio-Tek) set at 595 nm. The microcentrifuge tubes that had the highest concentration of protein were pooled and the contents were transferred to a Spectra/Por dialysis tube, (6000-8000

molecular weight cutoff; Spectrum, Rancho Dominguez, CA) and dialyzed against 1L of dialysis buffer (20 mM MOPS, 0.1 M NaCl, and 0.2 mM tris(2-carboxyethyl) phosphine (TCEP), pH 8.0) at 4°C overnight. Dialyzed proteins were confirmed to be the correct size via SDS PAGE, in which proteins were boiled for 5 minutes with SDS buffer (5.5% β-mercaptoethanol, 5 mM Tris, 38.4 mM glycine, and 0.1% SDS, pH 8.3) and then run on a Mini-PROTEAN TGX precast gel (Bio-Rad Laboratories Inc. Hercules, CA, USA) for 1hr. Dialyzed proteins were then transferred into Amicon Ultra-4 Centrifugal Filters and centrifuged at 3000 RPM for 30 minutes to reduce the total volume. Concentrated proteins were then aliquoted into microcentrifuge tubes and frozen at -80°C for storage.

Results

In *Arabidopsis* there are four plastidic BAMs that could be active, but they cannot be assayed in the presence of BAM5 due to its high level of activity. Due to this background activity, it would have been hard to investigate the functions of BAM1 without first understanding how and when BAM5 contributes this background activity.

BAM5 activity under high-light and low-light

In some conditions, BAM5 makes up most of the total BAM activity during the day (Caspar et al., 1989), making it difficult to measure the activity of the other active BAMs. We wondered if light intensity was a factor in this increased amylase activity during the day. To answer this, plants were grown in the green house and the growth room in the lab to test the effect of full sun (high-light) and fluorescent bulbs (low-light) on amylase activity (Figure 1). Under high-light, wild-type plants had over four times the activity compared to the wild-type plants grown under low light. Under high-light, wild-type activity was three fold higher than *bam5* mutants indicating that 70% of the wild-type activity in high-light grown plants was due to BAM5. Under low light, wild-type plants and *bam5* mutants differed by an insignificant amount. The lower activity in *bam5* gave us confidence that they lack BAM5 and will allow us to measure the activity of the other active BAMs. All subsequent experiments were performed with plants that were grown under low-light.

Activity of wild-type, *bam5*, *bam51*, *bam52*, *bam53*, and *bam56*

To determine which of the plastid-targeted BAMs contribute to leaf plastid activity, mutant plants grown under a diurnal cycle were assayed and their activities varied greatly from the wild-type plants. *bam5* plants were 20% less active than the wild type (Figure 2). Compared

to the *bam5* mutant, *bam52* and *bam56* mutants had no loss of activity. *bam51* plants were 50% less active, and *bam53* plants were 60% less active compared to the *bam5* mutant. This suggests that BAM2 and -6 may not contribute any amylase activity in mature leaves. The combined activity in *bam53* and *bam51* equals the total activity of *bam5* suggesting that BAM1 and -3 contribute most of the plastidic amylase activity in mature leaves. There were no major differences in activity between the extracts made from 4 hr into the day or night.

Confirmation of purity of BAM1 and -3 proteins

In order to confirm that BAM1 and -3 contribute most of the plastidic amylase activity in mature leaves, we needed to express them in *E. coli* and compare pure enzyme activity to crude leaf extract activity. Before pure enzyme activity could be determined, we needed to ensure that our BAM1 and -3 expressed in *E. coli* were pure. Proteins were run on a SDS page gel compared to a Precision Plus 'All Blue' protein standard to confirm if their size was correct. High and low concentrations were used to see if bands of an incorrect size were visible, to confirm if the proteins were pure. Although the lanes were somewhat overloaded, BAM1 was between 75kD and 50kD, close to the expected size of 64.6 kD. BAM3 was slightly above 50 kD, close to the expected size of 55.5 kD (Figure 3). Less intense bands are found in all the samples, indicating minor contamination, but compared to how strong the bands of the correct size were, BAM1 and -3 constituted a majority of the total protein.

Fulton et al. (2008) measured the activities of BAM1 and -3 grown in *E. coli* on a pH scale and found little difference between BAM1 and -3. The synthetic substrate, p-nitrophenlymaltopentoaside (PNPG5), was used for these pH curves, which produces narrow activity curves that are widened when starch is used as the substrate (Sparla et al., 2006). We

wondered if using starch as the substrate would show differences that were not observed in Fulton et al. (2008).

The pH curve produced by *bam53* resembles the pH curve produced by the corresponding *E. coli* grown BAM1 (Figure 4). Similarly, pH curve produced by *bam51* resembles the pH curve produced by the corresponding *E. coli* grown BAM3. The similarities in the curves, combined with the purity of the corresponding *E. coli* grown BAMs (Figure 3), give us confidence that *bam53* only contains BAM1 and *bam51* only contains BAM3. The pH optima of BAM1/*bam53* are 6.5 and BAM3/*bam51* are 6.0. BAM1/*bam53*'s activity gradually decreases from pH 7 to pH 10. BAM3/*bam51*'s activity rapidly decreases from pH 7 and is at half its activity at pH 8 relative to its maximum activity at pH 6 (Figure 4). This suggests that BAM1 could be functional during the day while the pH of the stroma is 8.0, and BAM3 would be less functional.

Leaf survival under osmotic stress

Following the protocol of Valerio et al. (2011), wild-type plants and *bam1* mutants were exposed to 450 mM mannitol in order to induce osmotic stress, but they died within a few days (data not shown). We thought that this death might have been a result of the concentration of mannitol being too high for plants to survive, so we used a lower concentration of mannitol and observed the plants over time. Wild-type plants exposed to 200 mM mannitol for 5 days looked much like the control wild-type plants, albeit with a slightly darker pigmentation compared to the control plants (Figure 5). Under osmotic stress, *bam1* mutants experienced chlorosis and wilting. To quantify the degree to which leaves wilted, water content was measured in rosettes under osmotic stress and normal conditions. Under osmotic stress, *bam1* mutants lost three times as

much water compared to wild-type plants under similar conditions (Figure 6). Chlorosis and wilting in mutants lacking BAM1 under osmotic stress suggests that BAM1 plays a crucial role in surviving osmotic stress.

BAM1 activity under osmotic stress

Valerio et al. (2011) measured the β-amylase activities of plants under osmotic stress and found that treated wild-type plants contained more activity compared to the untreated wild-type plants and *bam1* mutants were less active compared to the untreated *bam1* mutants (Valerio et al., 2011). BAM5 is induced by sugar (Mita et al., 1995) which accumulates under osmotic stress (Valerio et al., 2011). Wild-type plants and *bam1* mutants both contain BAM5, which may make the data in Valerio et al. (2011) difficult to interpret. With mutants that lack both BAM1 and BAM5, we can now determine if osmotic stress affects BAM1 activity in leaf extracts.

Wild-type *Arabidopsis thaliana* plants exposed to osmotic stress from 200 mM mannitol nutrient solution had 40% higher activity than the control plants watered with nutrient solution, similar to results previously published (Valerio et al., 2011; Figure 7). *bam5* mutants experienced a similar increase, but importantly, there was no difference between the control and the osmotically-stressed *bam51 mutants*. Osmotic stress had an similar effect in both wild-type and *bam5* mutants, while osmotic stress had no effect on *bam51* mutants. This increase in the plants that have BAM1 indicates that BAM1 is responsible for the increase in β-amylase activity under osmotic stress.

Discussion

In *Arabidopsis*, the BAM family consists of nine genes, which encode proteins that play an important role in starch degradation. Four of the active BAM proteins are localized to the plastids, where starch degradation occurs, but the high background activity of BAM5, a cytosolic BAM, has prevented much understanding about these active, plastidic BAMs (BAM1, -2, -3, and -6) because its activity masks the activity of the other BAMs. Without BAM5, the differences between the BAMs could be easily seen. A plant without BAM5 with neither -2 or -6 has the same loss in activity as a plant without BAM5 (Figure 2). This suggests that even though BAM2 and -6 are active plastid-targeted BAMs, they do not play a substantial part in mature leaf starch degradation. Also, the combination of activity in *bam51* and *bam53* is nearly equivalent to the activity of *bam5,* suggesting that BAM1 is the only active BAM in *bam53* and BAM3 is the only active BAM in *bam51*.

There are two pieces of evidence that suggest that BAM1 is active in guard cells: the BAM1 promoter drives GUS expression in the guard cells of wild-type plants and *bam1* mutants accumulate starch in guard cells during the day (Outlaw and Manchester, 1979; Valerio, et al., 2011). Lower activity in *bam1* mutants compared to wild-type plants suggest that BAM1 is functional and plays a role in starch degradation during the day (Fulton et al., 2008). During the day, the pH of the stroma of chloroplasts is around 8.0 and drops to around 7.0 during the night (Kramer et al., 1999). Since BAM1 is plastid targeted, it is likely that BAM1 would be exposed to the fluctuating pH of the stroma. Assays measuring the total amylase activity of corresponding BAM1 and -3 expressed in *E. coli* over a pH scale using a synthetic substrate, p-nitrophenlymaltopentoaside (PNPG5), produced a narrow activity curve that indicated there was

no difference between the activities of BAM1 and -3 and showed that BAM1 was completely inactive at a pH of 8 (Fulton et al., 2008; Li et al., 2009). This contradicted the idea that BAM1 is active during the day, when the stromal pH is 8.0, as BAM1 is inactive at a pH of 8.0 (Sparla et al., 2006).

We repeated the pH curves performed by Fulton et al. (2008) using soluble starch as the substrate, which is similar to the natural substrate of BAM1 and -3, amylopectin. Pure BAM1 and -3 (Figure 3) were assayed and compared to assays with extracts made from *bam53* and *bam51* (A and B of Figure 4). Using starch as the substrate widens the pH curve of both BAM1 and BAM3, showing that at pH 8, BAM3 is less active while BAM1 is more active. The similarities between the crude extracts and corresponding BAMs expressed in *E. coli* pH curves further suggests that *bam53* and *bam51* contain primarily BAM1 and BAM3, respectively, because if the crude extracts of *bam53* or *bam51* contained anything other than BAM1 or -3, they would differ from the corresponding *E. coli* BAMs. This not only shows that BAM1 can function during the day when the pH of the stroma is 8.0, but it also shows that BAM1 is likely to be more active during the day than BAM3.

Valerio et al. (2011) measured amylase activity in plants under osmotic stress and found that activity was increased in wild-type plants but not in *bam1* mutants. They concluded that BAM1 was upregulated under stress in plants that contain BAM1. However, their *bam1* plants contained BAM5, which is known to be induced by sugars (Mita et al., 1995). Due to the accumulation of sugars under osmotic stress, it is uncertain whether the increase in activity is due to BAM1 or -5. In order to remove that uncertainty, BAM5 was knocked out to see the effect of osmotic stress induced by a 200 mM mannitol nutrient solution on BAM1 activity. 200 mM

mannitol was chosen because it gives results of desiccation in leaves without killing the wild-type plants (Figure 5). Wild-type and *bam5* plants had increased amylase activity due to the treatment, while *bam51* plants had the same activity between the control and treatment, suggesting that BAM1 is indeed induced by osmotic stress (Figure 7).

bam1 plants experienced signs of wilting under osmotic stress imposed by treatment with 200 mM mannitol, whereas the wild-type plants experience little to no signs of wilting and chlorosis (Figure 5). This provides a phenotype for the increased activity that was observed (Figure 7). These two experiments indicate that the same level of osmotic stress that induces BAM1 activity also leads to a stress-sensitive phenotype in the bam1 mutant.

Our data suggests reasons for how BAM1 functions using different methods that both agree with and expand on previously published data. BAM1 is capable of functioning during the day, as stated by Valerio et al. (2011) because of its wide pH profile compared to BAM3. BAM1 is upregulated during osmotic stress, suggested by the increased activity in wild-type plants and *bam5* mutants under osmotic stress but no increase in activity in *bam51* mutants under osmotic stress. This is further evidence for the hypothesis that BAM1 may function in the response of plants to osmotic stress. Under osmotic stress, lower water availability in the soil creates a water potential in the cells that causes the plant to lose water. BAM1 may help provide carbon skeletons and osmolytes, through the breakdown of starch, reversing the water potential in the cells, allowing the plant to take up water from the soil.

Removing BAM5 allows one to characterize for the first time the individual properties of plasticic BAMs and how they are influenced by stress. Using knock-out or knock-down mutants lacking BAM5 and the active BAMs, clear differences can be observed without the background

activity of BAM5 (Figure 2; Figure 5). Without the masking power of BAM5, further assays may uncover more functions of BAM1 and the rest of the β-amylase family.

Appendix

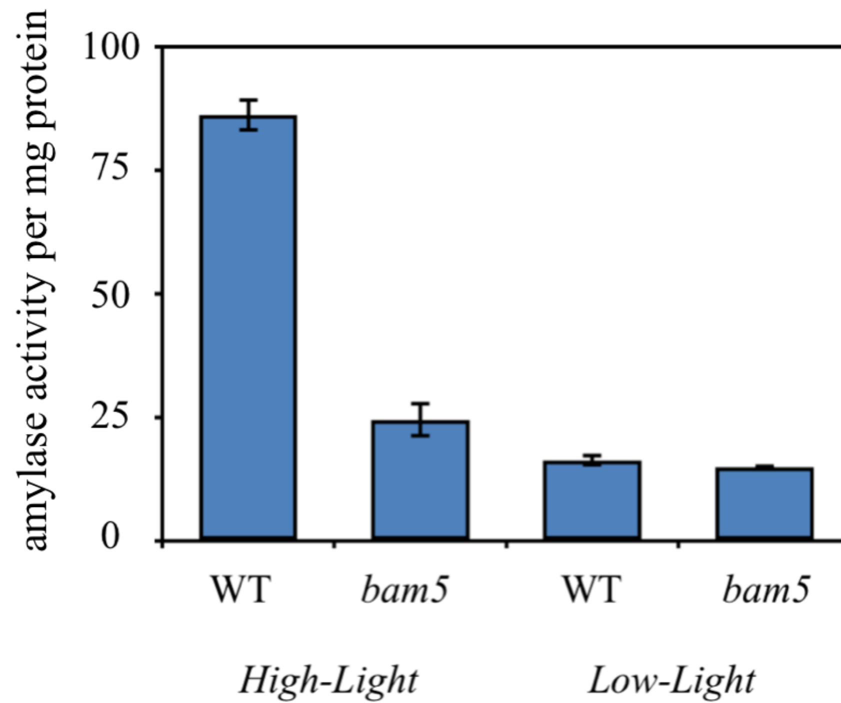

Figure 1. β-amylase activity in wild-type and *bam5* plants grown under high and low light.

Total β-amylase activity was determined in crude leaf extracts using soluble starch as the substrate. Plants were grown in the greenhouse under full sun (high-light) and in our grow room in the lab (low-light) for two weeks. Each value is the mean ± SD of three replicate extracts.

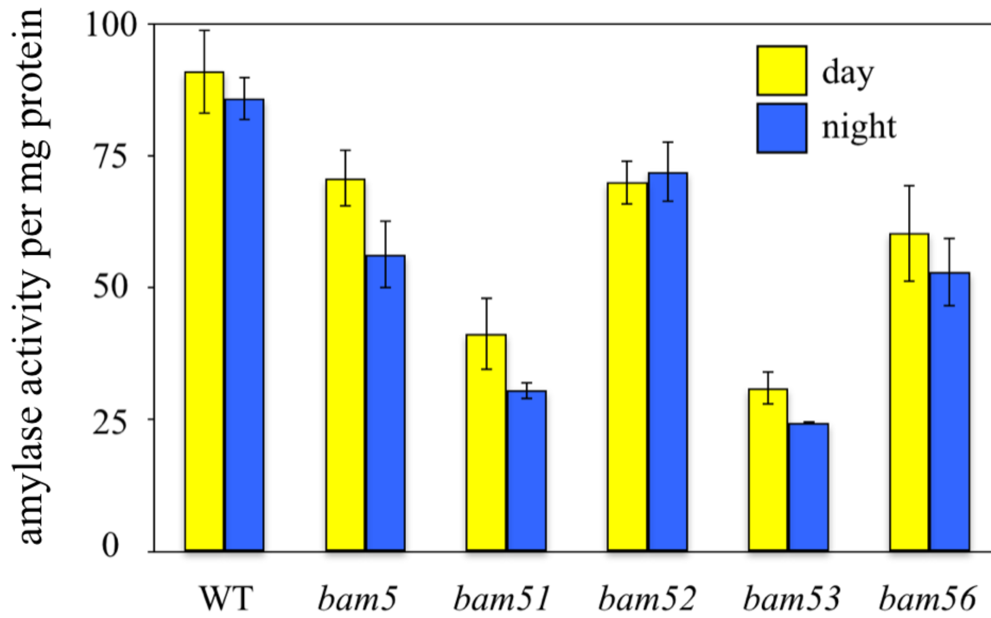

Figure 2. Diurnal β-amylase activity in WT and mutants of Arabidopsis lacking various BAMs.

Total β-amylase activity from Arabidopsis crude leaf extracts was determined using starch as the substrate. Plants were grown under low light and harvested 4 h into the day (12 PM) and 4 h into the night (12 AM). Each value is the mean ± SD of three replicate crude leaf extracts.

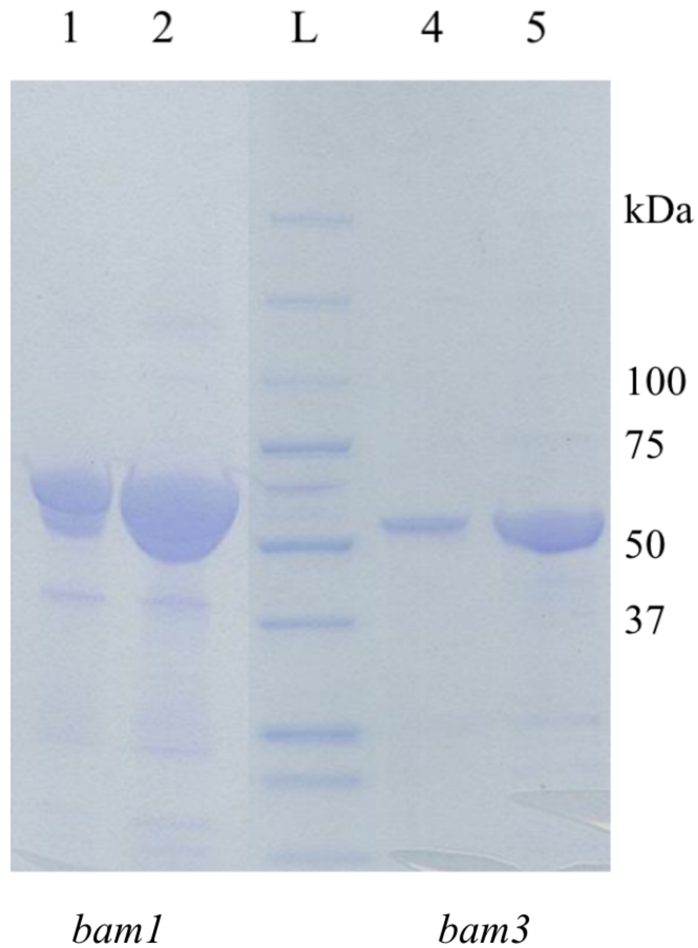

Figure 3. SDS PAGE showing purified BAM1 and -3 enzymes expressed in in *E. coli*.

Lanes “1” and “2” contain BAM1 at low (10% protein in water) and high (50% protein in water) concentrations. The “L” lane was the Precision Plus ‘All Blue” protein standards. Lanes “4” and “5” were BAM3 at low (10% protein in water) and high (50% protein in water) concentrations.

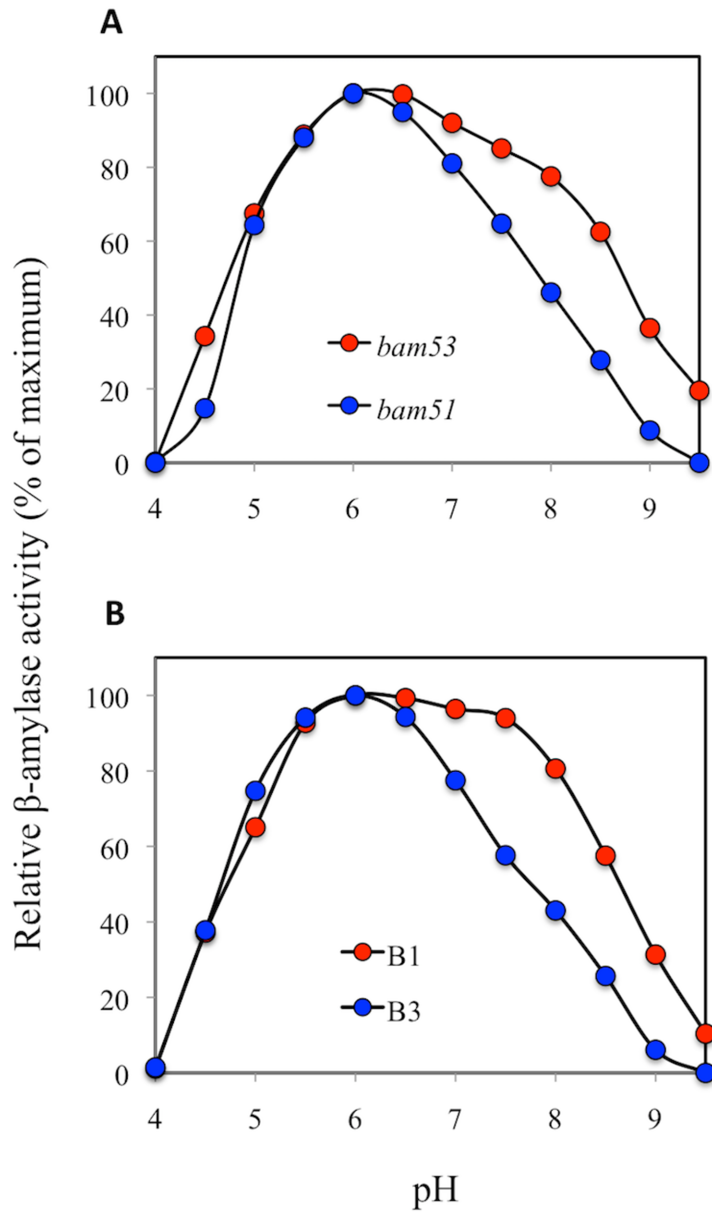

Figure 4. Comparison of amylase activity from extracts of Arabidopsis *bam51* and *bam53* leaves and from BAM1 and -3 expressed in *E. coli* at various pHs.

(A) pH curves for total β-amylase activity in *bam53* and *bam51* crude leaf extracts.
(B) pH curves for β-amylase activity of BAM1 and -3 expressed in *E. coli*. Each value is the mean of two replicate assays.

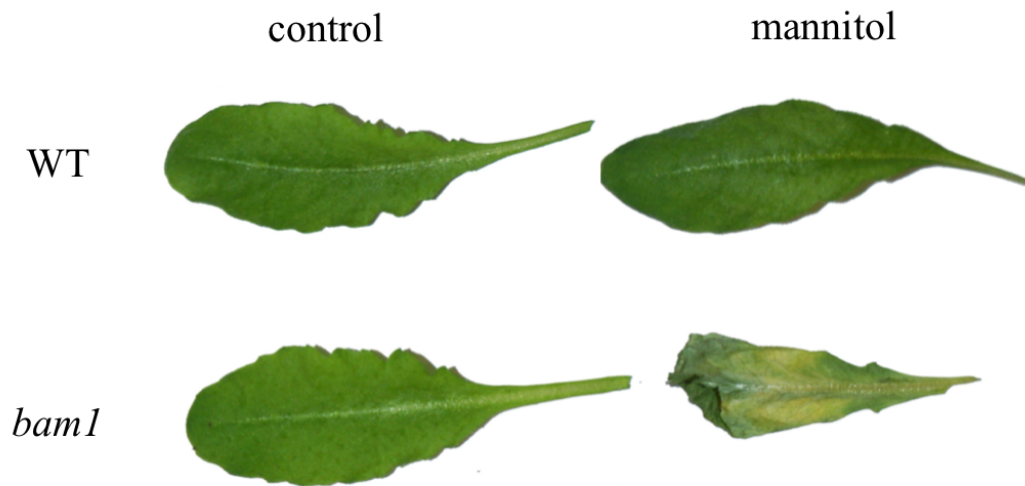

Figure 5. Impact of osmotic stress on leaf survival.

Arabidopsis plants were watered with either 200 mL of 200 mM mannitol in nutrient solution (mannitol) or watered with 200 mL of nutrient solution (control). After 5 days a typical leaf was excised for photography.

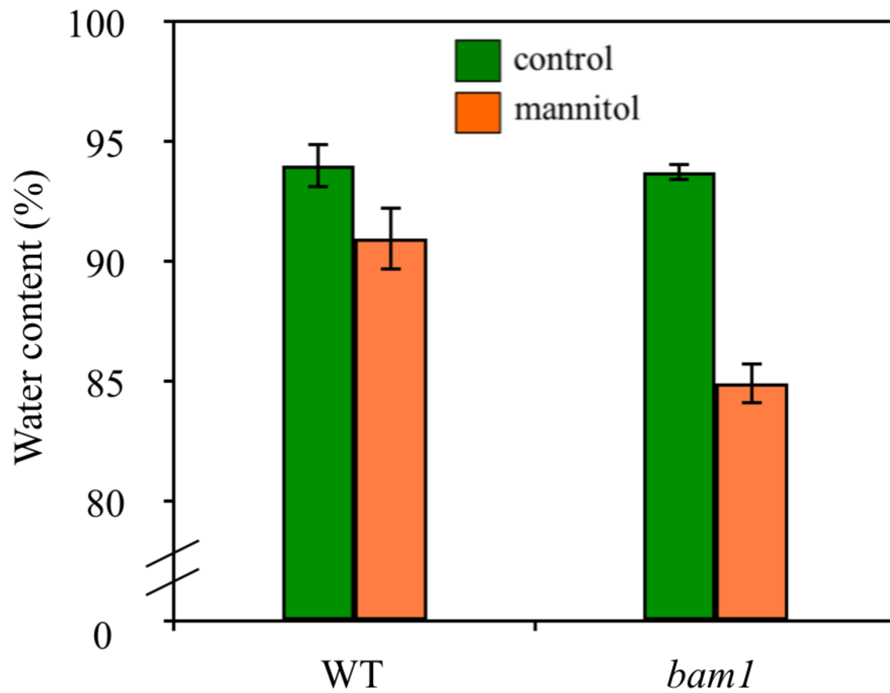

Figure 6. Effect of osmotic stress on total water content in wild-type and *bam1* mutants. Arabidopsis plants were grown under low-light and watered with either 200 mL of 200 mM mannitol in nutrient solution (mannitol) or watered with 200 mL of nutrient solution (control). Each value is the mean ± SD of 10 pairs of *Arabidopsis* rosettes.

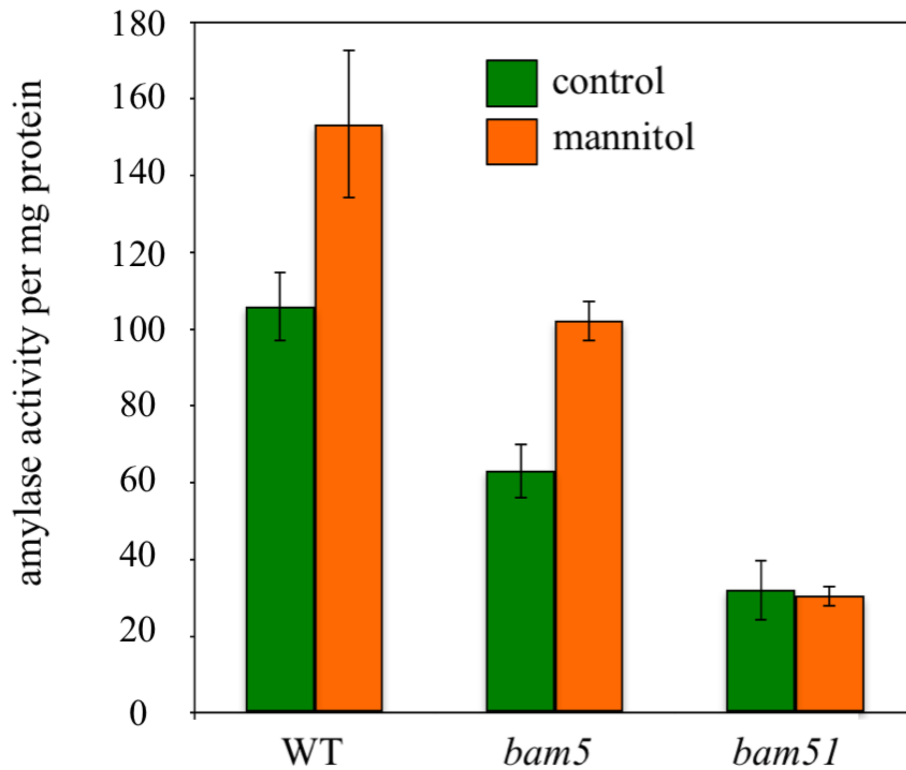

Figure 7. Effect of osmotic stress on β-amylase activity in wild-type, *bam5*, and *bam51* mutants.

Arabidopsis plants were were grown under low-light and watered with either 200 mL of 200 mM mannitol nutrient solution (mannitol) or watered with 200 mL of nutrient solution (control). Each value is the mean ± SD of three replicate crude leaf extracts.

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