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$\beta$ -amylase 1 (BAM1) degrades transitory starch to sustain proline biosynthesis during drought stress

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5 Diana Pazmino, Alex Costa, Diana Santelia, Paolo Trost, Francesca Sparla  
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1 **β-amylase 1 (BAM1) degrades transitory starch to sustain proline biosynthesis**  
2 **during drought stress**

3

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30

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3

1 **Highlight:**

2 In response to mild and prolonged osmotic stress, BAM1-dependent starch degradation in  
3 mesophyll cells releases carbon skeletons required for the production of osmolytes.

4  
5 **Abstract:**

6 During photosynthesis of higher plants, absorbed light energy is converted into chemical  
7 energy that, in part, is accumulated in the form of transitory starch within chloroplasts. In  
8 the following night, transitory starch is mobilized to sustain the heterotrophic metabolism of  
9 the plant.  $\beta$ -amylases are glucan hydrolases that cleave  $\alpha$ -1,4-glycosidic bonds of starch  
10 and release maltose units from the non-reducing end of the polysaccharide chain. In  
11 Arabidopsis, nocturnal degradation of transitory starch involves mainly  $\beta$ -amylase-3  
12 (BAM3). A second  $\beta$ -amylase isoform,  $\beta$ -amylase-1 (BAM1), is involved in diurnal starch  
13 degradation in guard cells, a process that sustains stomata opening. However, BAM1  
14 does also contribute to diurnal starch turnover in mesophyll cells under osmotic stress.  
15 With the aim of dissecting the role of  $\beta$ -amylases in osmotic stress responses in  
16 Arabidopsis, mutant plants lacking either BAM1 or BAM3 were subject to a mild (150 mM  
17 mannitol) and prolonged (up to one week) osmotic stress. We show here that leaves of  
18 osmotically-stressed *bam1* plants accumulated more starch and less soluble sugars than  
19 both wild-type and *bam3* plants during the day. Moreover, *bam1* mutants were impaired in  
20 proline accumulation and suffered from a stronger lipid peroxidation, again in comparison  
21 with both wild-type and *bam3* plants. Taken together, these data strongly suggest that  
22 carbon skeletons deriving from BAM1 diurnal degradation of transitory starch support the  
23 biosynthesis of proline required to face the osmotic stress. We propose the transitory-  
24 starch/proline interplay as an interesting trait to be tackled by breeding technologies aimed  
25 in improving drought tolerance in relevant crops.

26

27

28

29 **Keywords:** Arabidopsis,  $\beta$ -amylases, drought, proline, transitory starch, osmolytes.

## 1 **Introduction:**

2 Starch is a polymer of D-glucose and represents a convenient way to store carbohydrates  
3 as semi-crystalline and osmotically inert granules composed of about 70-90% by the  
4 highly branched amylopectin polymer and for the remaining 30-10% by the less branched  
5 amylose (Denyer *et al.*, 2001; Zeeman *et al.*, 2002; Streb *et al.*, 2012). As a consequence  
6 of its structure, glucose units embedded in the starch granule may not be immediately  
7 available to satisfy the different demands of the organism in case of urgent request. The  
8 tight regulation of several enzymes involved in starch degradation seems consistent with  
9 the need to speed up the use of starch under particular conditions, i.e. under stress  
10 (Santelia *et al.*, 2015).

11 Two kinds of starch, structurally indistinguishable, are found in plants: secondary and  
12 transitory starch. This physiological distinction is mainly based on different storage organs  
13 and on different rates of synthesis and degradation (Smith *et al.*, 2005). Because of its  
14 commercial relevance, secondary starch has been deeply investigated, also with the aim  
15 of creating new starch structures for industrial applications (Jobling, 2004; Santelia and  
16 Zeeman, 2011; Bahaji *et al.*, 2014). Conversely, the physiology of transitory starch has  
17 become a major topic of research only in last decades (Zeeman *et al.*, 2007; Stitt and  
18 Zeeman, 2012), with increasing evidence of the involvement of transitory starch  
19 metabolism in response to stress (Hummel *et al.*, 2010; Valerio *et al.*, 2011; Santelia *et al.*,  
20 2015; Prasch *et al.*, 2015).

21 Due to their sessile nature, plants have to cope not only with rapid and daily environmental  
22 changes, but they must also balance the energy needed for growth with the energy  
23 required for stress responses. Starch biosynthesis is tightly correlated with photosynthesis,  
24 another process strongly affected by the environment. In the model plant *Arabidopsis*  
25 *thaliana*, half of the photo-assimilates produced by the Calvin-Benson cycle during the day  
26 are typically exported to the cytosol to supply carbon skeletons for anabolic or catabolic  
27 processes, whereas the remaining half is retained in the chloroplast for transitory starch  
28 biosynthesis (Zeeman and ap Rees, 1999). Under normal growth condition, the export of  
29 organic carbon is mediated by two different transport mechanisms, which operate at  
30 different times of the diurnal cycle. During the day, photoassimilates mainly reach the  
31 cytosol via the triose phosphate/phosphate translocator (TPT) (Flügge, 1999), whereas  
32 during the night  $\beta$ -maltose (the major product of starch degradation) and glucose are  
33 exported to the cytoplasm via the maltose (MEX1) (Nittylä *et al.*, 2004) and glucose (GLT  
34 and GT) (Cho *et al.*, 2011; Flügge *et al.*, 2011) transporters, respectively.

1  $\beta$ -Amylases are the only enzymes that produce  $\beta$ -maltose, thereby connecting starch  
2 degradation in chloroplasts with sugar metabolism in the cytoplasm. Several  $\beta$ -amylases  
3 are encoded by the Arabidopsis genome (Lloyd *et al.*, 2005). BAM3 is a major, catalytically  
4 active  $\beta$ -amylase that is necessary for nocturnal starch degradation under physiological  
5 conditions. Conversely, BAM1 is little or not even involved in such process (Fulton *et al.*,  
6 2008; Kötting *et al.*, 2010). However, in response to drought or salt stress, BAM1 becomes  
7 a predominant  $\beta$ -amylase of leaves and is required for starch breakdown in mesophyll  
8 cells (Valerio *et al.*, 2011; Monroe *et al.*, 2014).

9 Water stress has severe negative impacts on plant growth and productivity (Cattivelli *et al.*,  
10 2008; Rockström and Falkenmark, 2010; Osakabe *et al.*, 2014). A common trait of many  
11 plants affected by drought or salinity stress is the accumulation of osmoprotectants such  
12 as proline, glycine betaine, and sugars alcohols (Szabados and Saviourè, 2009; Liang *et al.*  
13 2013). Proline accumulation occurs at very high levels when plants experience  
14 conditions of low water potential. Proline concentration can increase up to 100-fold in  
15 comparison to control conditions (Verbruggen and Hermans, 2008; Szabados and  
16 Saviourè, 2009). However proline does not only function as an osmoprotectant, but it can  
17 also efficiently scavenge reactive oxygen species (ROS), thus protecting the cell from  
18 oxidative damage (Matysik *et al.*, 2002, Bartels and Sunkar, 2005).

19 In plants, proline synthesis occurs both in the cytosol and in the chloroplast, whereas  
20 degradation occurs only in mitochondria. Carbon skeletons for proline biosynthesis are  
21 provided by primary metabolism through the glutamate pool. Whether starch degradation  
22 is involved in this process is currently unknown.

23 To investigate the possible interplay between transitory starch and proline metabolisms  
24 under drought stress, the response to 150 mM mannitol treatments of two single T-DNA  
25 insertion mutants, *bam1* and *bam3*, and wild-type plants was studied and compared. The  
26 findings strongly suggest that in the drought stress response of Arabidopsis, BAM1 and  
27 not BAM3 is the major player of starch degradation in the light, a metabolic pathway that  
28 provides carbon skeletons for the biosynthesis of sucrose and proline to counteract both  
29 osmotic stress and oxidative damage.

30

1 **Material and Methods:**

2 ***Plant materials and growth conditions***

3 Wild-type, T-DNAs and *BAM1* promoter::GUS plants of *Arabidopsis thaliana* (ecotype  
4 Columbia, Col-0) were hydroponically grown at a constant temperature of 22°C, under 12  
5 h light/ 12 h dark cycle with a photosynthetic photon flux density of 110  $\mu\text{mol m}^{-2} \text{sec}^{-1}$ , as  
6 described in Valerio *et al.*, 2011. GUS line and insertion sites of the T-DNA in *bam1*  
7 (SALK\_039895) and *bam3* (CS92461) mutants were already analyzed (Fulton *et al.*, 2008;  
8 Valerio *et al.*, 2011).

9

10 ***Stress conditions***

11 To better analyze the response of *Arabidopsis* plants to drought, previously tested  
12 conditions (300 mM mannitol up to 8 h; Valerio *et al.*, 2011) were changed in order to  
13 obtain a mild (150 mM mannitol) and prolonged (up to 7.5-d) osmotic stress. Mild osmotic  
14 stress was applied to 28/31-d-old plants (excluded 3/4 days of stratification time at 4°C in  
15 darkness), 1 h after switching on the light. Treated plants were transferred to a freshly  
16 prepared hydroponic medium supplemented with 150 mM mannitol. If not differently  
17 specified, plants were harvested either at the end of light (12 h light) or the end of dark (12  
18 h dark), every 12 hours for a maximum of 7.5-d after the beginning of the treatment (DAT).  
19 Samples were immediately frozen in liquid nitrogen and stored at -80°C before analysis.

20

21 ***GUS staining***

22 Histochemical GUS staining was performed as described in Valerio *et al.*, 2011. For each  
23 condition and for each time point, 3 independent transgenic plants were analyzed. Control  
24 and treated (150 mM mannitol) plants were collected every day during the experiment,  
25 always at the end of the 12-h light period. Stained plants were examined by bright-field  
26 microscopy using a Nikon Eclipse 90-I microscope. The images show representative  
27 plants and leaves.

28

29 ***Determination of water loss***

30 The loss of water in leaves was determined as the ratio between the dry weight (DW) and  
31 the fresh weight (FW), measured on single plants collected at 12 h of light and 12 h of  
32 dark, under control or stress conditions, during a 6-d experiment. FW was scored  
33 immediately after excision and DW was determined after incubation at 80°C for 24h. Five  
34 independent biological replicates were analyzed.



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### **Quantification of starch and soluble sugars**

Quantification of starch and soluble sugars were carried out on whole rosette leaves of 3-5 plants for each experimental point. Starch was quantified on bleached leaves as described in Smith and Zeeman, 2006. Quantification of sucrose, glucose and maltose was performed as described in Egli *et al.*, 2010 on freeze-dried supernatants obtained from an extraction of 15 min at 80°C with 80% ethanol. Three independent biological replicas were analyzed.

### **Lipid peroxidation assay**

Oxidative damage was estimated by measuring total lipid peroxidation using the 2-thiobarbituric acid (TBA) assay, as described in Guidi *et al.*, 1999. Briefly, about 200 mg of leaves, powdered in liquid nitrogen, were vigorously mixed with 3 volumes of 0.1% (w/v) trichloroacetic acid (TCA). Samples were centrifuged and 0.5 ml of each supernatant were transferred into a screw cap tube in the presence of 2.0 ml 20% (w/v) TCA and 1.5 µl 0.5% (w/v) TBA. Following a 30 min incubation at 90°C, the reaction was stopped by placing the tubes in an ice-water bath. Samples were centrifuged and the absorbance of the supernatants was monitored at 532 nm, subtracting the non-specific absorption at 600 nm. The amount of MDA-TBA complex was calculated from the extinction coefficient 155 mM<sup>-1</sup> cm<sup>-1</sup>. Three independent biological replicas were analyzed.

### **Proline quantification**

Samples stored at -80°C were ground in liquid nitrogen and free proline content was measured as described by Bates *et al.*, 1973. Briefly, 1.2 ml of 3% 5-sulfosalicylic acid were added to 50 mg of powdered leaves. Samples were centrifuged and appropriate volumes of supernatant were transferred into clean tubes and brought to a final volume of 1 ml with water, mixed with an equal volume of glacial acetic acid and 2.5% ninhydrin reagent (1:1:1). Samples were incubated at 90°C for 1 h, cooled in ice, combined with an equal volume of toluene and mixed vigorously. Following phase partitioning, the absorbance of the upper phase was monitored at 520 nm. The calibration curve was prepared using different proline concentrations as standard. From 3 to 4 independent biological replicas were analyzed.

## 1 **Results:**

### 2 ***Mild osmotic stress induces BAM1 promoter activity***

3 To better understand the activation of *BAM1* in response to mild osmotic stress, the  
4 activity of *GUS* in *Arabidopsis* plants stably transformed with the *BAM1* promoter  
5 controlling the *GUS* reporter gene (*BAM1promoter::GUS plants*) was examined. Adult  
6 plants were exposed to 150 mM mannitol and collected every day for one week.

7 As previously reported in Valerio *et al.*, 2011, in the absence of stress, *GUS* activity of  
8 *BAM1promoter::GUS* plants was mainly confined to guard cells (Figure S1) and almost  
9 absent from mesophyll cells (Fig. 1, right panel). Under mild osmotic stress, a slight  
10 increase in the promoter activity of *BAM1* appeared already at the beginning of the stress,  
11 albeit confined to leaf veins (Fig. 1A-B, left panel). Upon prolonged stress, *GUS* activity  
12 spread to mesophyll cells, first in young leaves and then throughout the whole rosette (Fig.  
13 1C-E, left panel).

14

### 15 ***Water loss in response to stress***

16  $\beta$ -amylase 3 (*BAM3*) is the major isoform responsible for transitory starch degradation at  
17 night (Lao *et al.*, 1999; Fulton *et al.*, 2008). To get insights into the role of *BAM1* in starch  
18 degradation in response to osmotic stress, *bam3* T-DNA mutant plants were also  
19 analyzed. Dehydration rates of *bam1*, *bam3* and wild-type plants in response to 150 mM  
20 mannitol were determined (Figure S2). The obtained data did not show statistically  
21 significant differences among the three genotypes, neither in response to stress nor in  
22 control conditions (Supplementary Table S1). The similar decrease in water content  
23 observed in the three genotypes during the whole experiment, allows a comparison  
24 between genotypes of data expressed on a FW basis.

25

### 26 ***Starch content at the end of the light period***

27 To investigate the involvement of *BAM3*- and *BAM1*-dependent starch degradation  
28 pathways in response to drought stress, starch content was measured in leaves at 12 h  
29 light, before and after the mannitol treatment (Fig. 2 and Figure S3).

30 Consistent with the predominant role of *BAM3* in transitory starch degradation (Fulton *et al.*  
31 *et al.*, 2008), under control growth conditions *bam3* plants showed the well-known starch  
32 excess (*sex*) phenotype, characterized by small plants with high starch content (~3-fold  
33 higher in comparison to wild-type plants) (Fig. 2). Conversely, in comparison to wild-type

1 plants *bam1* mutant did not show any significant change in starch concentration (Fig. 2;  
2 Supplementary Table S2), again in agreement with the literature (Fulton *et al.*, 2008).  
3 In response to osmotic stress, the ratio in starch content between *bam3* and wild-type  
4 samples suddenly decreased from ~3 (in absence of mannitol) to ~2 (in presence of  
5 mannitol), remaining roughly constant throughout the experiment (Fig. 2). On average, the  
6 amount of starch contained in *bam3* plants at the end of the day was reduced by ~50  $\mu\text{mol}$   
7 of glucose equivalents  $\text{g}^{-1}$  FW as a consequence of the stress. Although with a different  
8 timing, an opposite behaviour was observed in *bam1* plants. During the first three days of  
9 experiment starch content in *bam1* plants remained similar to the wild-type, but doubled  
10 wild-type levels from the fourth day on (Fig. 2). An average increase of ~50  $\mu\text{mol}$  glucose  
11 equivalents  $\text{g}^{-1}$  FW was calculated.

12

### 13 **Starch content at the end of the night period**

14 To further analyze the involvement of  $\beta$ -amylases on transitory starch turnover in response  
15 to drought, starch concentration was also measured at the end of the night (12 h dark),  
16 before and after mannitol treatment (Fig. 3). As expected under control condition, wild-type  
17 and *bam1* plants did not differ in their starch content, while *bam3* plants confirmed the sex  
18 phenotype (Fig. 3; Supplementary Table S3)(Fulton *et al.*, 2008).

19 High levels of starch were maintained in *bam3* mutants in the first two days of experiment  
20 (Fig. 3). Conversely, *bam1* plants rapidly responded to 150 mM mannitol with an increase  
21 in starch concentration that, within the first two days of experiment, made them closer to  
22 *bam3* than wild-type plants. Later in the experiment (from 3 to 6 DAT) no significant  
23 differences were observed among the three genotypes in response to 150 mM mannitol  
24 (Fig. 3).

25

### 26 **Lipid peroxidation**

27 A common effect of osmotic stress is the accumulation of free oxygen radicals (Aranjuelo  
28 *et al.*, 2011; Wilhelm and Selmar, 2011) leading to oxidation of unsaturated fatty acids and  
29 membrane damage (Hernandez *et al.*, 1993; Fadzilla *et al.*, 1997). Lipid peroxidation  
30 induced by osmotic stress was evaluated as malondialdehyde (MDA) concentration on  
31 *bam1*, *bam3* and wild-type plants treated with 150 mM mannitol. The exposure to the  
32 osmotic stress increased MDA concentration in all genotypes in a time-dependent manner  
33 (Fig. 4; Supplementary Table S4). However, only *bam1* samples collected at 4.5 DAT  
34 showed a ~2-fold increase of MDA concentration in comparison to wild-type, suggesting

1 that BAM1 is an essential component of Arabidopsis response to the oxidative damage  
2 caused by the osmotic stress.

3

#### 4 **Proline content**

5 Proline is considered a compatible osmolyte and its accumulation in response to different  
6 stresses has been reported in several plant species (Szabados and Savouré, 2009). In  
7 order to test whether proline accumulation in osmotically stressed Arabidopsis plant might  
8 depend on the activity of  $\beta$ -amylases, proline concentration was measured in rosette  
9 leaves of wild-type, *bam1* and *bam3* plants subject to 150 mM mannitol treatments (Fig.  
10 5). In the absence of stress, similar proline concentrations ( $\sim 0.67 \mu\text{mol g}^{-1}$  FW) were  
11 measured in the three genotypes and no significant differences were observed until 2.5  
12 DAT (Fig. 5; Supplementary Table S5). At 3.5 DAT both *bam1* and *bam3* mutants showed  
13 less proline accumulation in respect to the wild-type. However at later time points, only  
14 *bam1* mutant showed a limited accumulation of proline, while *bam3* plants recovered the  
15 same proline concentration of wild-type plants (Fig. 5).

16 Interestingly at 6.5 DAT, the lower proline content of *bam1* mutant in respect to wild-type  
17 (and *bam3* plants) corresponded to  $\sim 37 \mu\text{mol}$  of proline  $\text{g}^{-1}$  FW (Fig. 5). Considering that  
18 the same mutant at the same time point accumulated a surplus of  $\sim 48 \mu\text{mol}$  of glucose  
19 equivalents  $\text{g}^{-1}$  FW (Fig. 2), it seems reasonable that impaired starch degradation was the  
20 reason of the failure in proline accumulation.

21

#### 22 **Soluble sugars**

23 Sucrose, maltose and glucose concentrations were measured in wild-type, *bam1* and  
24 *bam3* plants in response to 150 mM mannitol both at 12 h of light and 12 h of dark (Fig. 6;  
25 Supplementary Table S6). Under control conditions, the concentration of soluble sugars in  
26 all genotypes at the end of the day or at the end of the night, resembled the values already  
27 reported in the literature (Fulton *et al.*, 2008; Hummel *et al.*, 2010). Glucose was higher  
28 than sucrose, that was much higher than maltose, and all three sugars appeared to be  
29 more concentrated at the end of the day than at the end of the night.

30 Alike to what was observed for transitory starch (Fig. 3), during the osmotic stress  
31 experiment, soluble sugars concentrations measured at the end of the night were  
32 essentially similar among the genotypes (Fig. 6, right panels), with the only exception of  
33 maltose in *bam3* mutant at 1 DAT, that was more concentrated than in the wild-type  
34 (Fulton *et al.*, 2008). On the contrary, at the end of the day, *bam1* plants showed a general

1 decrease in sucrose, glucose and maltose concentrations in respect to both wild-type and  
2 *bam3* plants (Fig. 6, left panels). In comparison to wild-type plants at 5.5 DAT, the  
3 absence of BAM1 led to a decrease of  $\sim 2.8 \mu\text{mol}$  of sucrose  $\text{g}^{-1}$  FW,  $\sim 5.9 \mu\text{mol}$  of  
4 glucose  $\text{g}^{-1}$  FW and of  $\sim 55 \text{ nmol}$  of maltose  $\text{g}^{-1}$  FW.

5

## 1 **Discussion:**

2 Plants are sessile organisms with a metabolism that essentially depends on light and  
3 needs to be continuously adapted to environmental changes. A fundamental aspect of this  
4 adaptation consists in the circadian cycles of diurnal synthesis and nocturnal degradation  
5 of transitory starch that allow plants to harmonize with the natural rhythm of light  
6 availability (Stitt and Zeeman, 2012). Nocturnal degradation of transitory starch sustains  
7 basal metabolism and reallocation of organic carbon in the absence of an external input of  
8 energy. On top of that, under stress conditions plants need to redirect transitory carbon  
9 fluxes in order to fuel stress responses, a decision that often implies detrimental effects on  
10 growth. As far as transitory starch is concerned, its degradation and use of the resulting  
11 carbon units for stress responses involve a large set of enzymes, including  $\beta$ -amylases.

12 With the aid of *bam3* and *bam1* knock out mutants (Fulton *et al.*, 2008; Valerio *et al.*,  
13 2011), we have investigated the relative contribution of BAM1 and BAM3 to transitory  
14 starch degradation in response to mild and prolonged osmotic stress. BAM3 is required for  
15 nocturnal starch degradation under physiological conditions (Fulton *et al.*, 2008), while  
16 BAM1 is dispensable for transitory starch degradation in the absence of stress, but is  
17 activated by drought stress at the transcriptional level and post-translationally activated by  
18 reduced thioredoxins (Sparla *et al.*, 2006; Valerio *et al.*, 2011). Under control growth  
19 conditions, rosette leaves of *bam3* mutants contained high levels of starch during the  
20 whole day, always higher than wild-type plants. Under osmotic stress, starch levels of  
21 *bam3* plants suddenly decreased, particularly during the light and became closer to wild-  
22 type levels. Different from *bam3*, under control growth conditions, the levels of leaf starch  
23 in *bam1* mutants were similar to wild-type plants, in agreement with the notion that BAM1  
24 is confined to guard cells until plants start to flower (Valerio *et al.*, 2011; Prasch *et al.*,  
25 2015). However, in response to the osmotic stress, BAM1 shows up also in mesophyll  
26 cells and starch content in *bam1* mutants increased, particularly so at the end of the light  
27 and after several days of stress. In conclusion a mild, prolonged osmotic stress caused a  
28 decrease in daylight starch in plants with no BAM3 and, conversely, an increase in  
29 daylight starch in plants with no BAM1, suggesting that BAM1 is involved in daylight starch  
30 degradation upon stress. This hypothesis fits with both the induction of BAM1 promoter by  
31 the osmotic stress and the redox regulation of BAM1 that favours its activity in the light  
32 (Sparla *et al.*, 2006; Valerio *et al.*, 2011).

33 Plants have evolved several different mechanisms to adequately respond to limited water  
34 availability and proline accumulation has long been reported as a part of the drought

1 stress response (Szabados and Saviouré, 2009). The main pathway of proline biosynthesis  
2 derives from glutamic acid and it can occur both in the cytosol and the chloroplast. Under  
3 stress conditions, however, the plastidial pathway of proline biosynthesis may prevail as a  
4 result of the re-localization of  $\Delta^1$ -pyrroline-5-carboxylate synthetase (P5CS1) into  
5 chloroplasts (Székely *et al.*, 2008). P5CS1 catalyzes the limiting step of proline  
6 biosynthesis and its role in proline accumulation in water stressed plants is recognized  
7 (Székely *et al.*, 2008). Although each of the three genotypes investigated in our study  
8 (*bam1*, *bam3* and Col-0) accumulated proline under osmotic stress, proline concentration  
9 of *bam1* mutants did not reach the same levels reached by wild-type and *bam3* plants.  
10 The lack of adequate proline accumulation in *bam1* mutants correlated with a more severe  
11 oxidative stress in these plants, as judged by the extent of lipid peroxidation. Moreover,  
12 lower proline levels in *bam1* plants went together with lower concentrations of sucrose,  
13 glucose and maltose and, as discussed above, higher levels of starch. Following several  
14 days of stress, starch content in *bam1* plants at the end of the photosynthetic period  
15 exceeded wild-type levels by about 50  $\mu\text{mol}$  of glucose equivalents  $\text{g}^{-1}$  FW. To put this  
16 value into context, proline accumulation in these same plants and under the same  
17 conditions was lower than in wild type plants by 37  $\mu\text{mol}$   $\text{g}^{-1}$  FW, while soluble sugars  
18 (sucrose and glucose) decreased by 12  $\mu\text{moles}$  of hexoses  $\text{g}^{-1}$  FW. Based on these  
19 numbers, the reason why *bam1* plants had less proline and soluble sugars upon stress  
20 may well be that carbon skeletons required to make these osmolytes are stuck into starch  
21 granules and as such not available. Since BAM1 is suggested to play a role in starch  
22 degradation under these conditions, it makes sense that its absence has more dramatic  
23 effects during the day, when BAM1 is redox-activated and P5CS1 is sufficiently  
24 concentrated (Hayashi *et al.*, 2000; Székely *et al.*, 2008) to catalyse the metabolic flux  
25 leading to proline.

26 Although the whole pathway connecting the degradation of transitory starch with the  
27 biosynthesis of proline still remains to be discovered, the presented results strongly  
28 suggest a link between these two metabolic pathways and suggest a role for BAM1 in this  
29 context. Our results suggest that a mild osmotic stress stimulate starch turnover in the light  
30 through the activation of BAM1, both at the transcriptional and post-translational level.  
31 Indeed, BAM1 activity is strictly redox-regulated and since it requires thioredoxin f to be  
32 highly reduced, BAM1 is predicted to be more active under photosynthetic conditions  
33 (Sparla *et al.*, 2006). Based on correlative observations, we propose that maltose derived  
34 from BAM1 degradation of starch upon stress sustains the biosynthesis of proline (and

1 soluble sugars) thereby alleviating the oxidative stress. Since water availability is a major  
2 constraint for modern agriculture, the efforts in selecting crops with better water use  
3 efficiency should take into account this link between starch and proline metabolism.



1 **Supplementary Data:**

2

3 **Supplementary Figure S1:** Activity of BAM1promoter::GUS under control conditions and  
4 in response to 150 mM mannitol.

5

6 **Supplementary Figure S2:** Loss of water in wild-type, *bam1* and *bam3* plants expose to  
7 150 mM mannitol.

8

9 **Supplementary Figure S3:** Starch content in wild-type, *bam1* and *bam3* plants  
10 qualitatively evaluated with Lugol staining.

11

12 **Supplementary Table S1:** p-value from Student's t-tests performed on loss of water.

13

14 **Supplementary Table S2:** p-value from Student's t-tests performed on starch  
15 concentration quantified at 12 h of light.

16

17 **Supplementary Table S3:** p-value from Student's t-tests performed on starch  
18 concentration quantified at 12 h of dark.

19

20 **Supplementary Table S4:** p-value from Student's t-tests performed on degree of lipid  
21 peroxidation.

22

23 **Supplementary Table S5:** p-value from Student's t-tests performed on proline  
24 concentration.

25

26 **Supplementary Table S6:** p-value from Student's t-tests performed on sucrose, glucose  
27 and maltose concentrations quantified at 12 h of light and 12 h of dark.

28

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1 **Figure Legends:**

2

3 **Fig. 1: Activity of BAM1promoter::GUS under control condition and in response to**  
4 **150 mM mannitol treatment.** Plants were grown under 12 h light/ 12 h dark cycle and  
5 osmotic stress was applied 1 h after the beginning of light period. Plants were collected at  
6 the end of light period. GUS activity was measured at 0.5 DAT, panel A; 1.5 DAT, panel B;  
7 3.5 DAT, panel C; 6.5 DAT, panel D and 7.5 DAT, panel E. Scale bar = 1cm. Inset:  
8 magnification of a single leaf. Scale bar = 0.5 cm.

9

10 **Fig. 2: Starch content in wild-type, *bam1* and *bam3* plants measured at 12 h of light**  
11 **in response to drought stress.** Twenty-eight/31-d-old plants hydroponically grown were  
12 expose to 150 mM mannitol 1 h after the switching on the light. Wild-type, *bam1* and  
13 *bam3* plants were collected at 12 h of light before and after mannitol treatment. Values are  
14 the means  $\pm$  SD (n=3 independent biological replicates).

15

16 **Fig. 3: Starch content in wild-type, *bam1* and *bam3* plants at 12 h of dark in**  
17 **response to drought stress.** Twenty-eight/31-d-old plants hydroponically grown were  
18 expose to 150 mM mannitol 1 h after the switching on the light. Wild-type, *bam1* and  
19 *bam3* plants were collected at 12 h of dark before and after mannitol treatment. Values are  
20 the means  $\pm$  SD (n=3 independent biological replicates).

21

22 **Fig. 4: Degree of lipid peroxidation in wild-type, *bam1* and *bam3* plants exposed to**  
23 **osmotic stress.** Lipid peroxidation was measured through TBA assay in wild-type, *bam1*  
24 and *bam3* plants before and after 150 mM mannitol treatment. Plants were collected at 12  
25 h light and different length of treatment. Values are the means  $\pm$  SD (n=3 independent  
26 biological replicates).

27

28 **Fig. 5: Proline content in wild-type, *bam1* and *bam3* plants in response to drought**  
29 **stress.** Proline concentration was measured in whole rosettes of 28/31-d-old wild-type,  
30 *bam1* and *bam3* plants. Plants were collected at 12 h of light before and after 150 mM  
31 mannitol treatment. Values are the means  $\pm$  SD (n=3-4 independent biological replicates).

32

33 **Fig. 6: Sucrose, glucose and maltose content in wild-type, *bam1* and *bam3* plants**  
34 **measured at 12 h of light and at 12 h of dark in response to drought stress.**

1 Arabidopsis plants hydroponically grown were expose to 150 mM mannitol 1 h after the  
2 switching on the light. Whole rosettes of wild-type, *bam1* and *bam3* plants were collected  
3 at 12 h of light (left panels) and 12 h of dark (right panels) before and after 150 mM  
4 mannitol treatment. Values are the means  $\pm$  SD (n=3 independent biological replicates).