

Alma Mater Studiorum Università di Bologna
Archivio istituzionale della ricerca

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

This is the final peer-reviewed author's accepted manuscript (postprint) of the following publication:

Published Version:

Zanella, M., Borghi, G.L., Pirone, C., Thalmann, M., Pazmino, D., Costa, A., et al. (2016). β -amylase 1 (BAM1) degrades transitory starch to sustain proline biosynthesis during drought stress. JOURNAL OF EXPERIMENTAL BOTANY, 67(6), 1819-1826 [10.1093/jxb/erv572].

Availability:

This version is available at: <https://hdl.handle.net/11585/559691> since: 2022-02-14

Published:

DOI: <http://doi.org/10.1093/jxb/erv572>

Terms of use:

Some rights reserved. The terms and conditions for the reuse of this version of the manuscript are specified in the publishing policy. For all terms of use and more information see the publisher's website.

This item was downloaded from IRIS Università di Bologna (<https://cris.unibo.it/>).
When citing, please refer to the published version.

(Article begins on next page)

1 This is the final peer-reviewed accepted manuscript of:

2 β -amylase 1 (BAM1) degrades transitory starch to sustain proline
3 biosynthesis during drought stress

4 Martina Zanella, Gian Luca Borghi, Claudia Pirone, Matthias Thalmann,
5 Diana Pazmino, Alex Costa, Diana Santelia, Paolo Trost, Francesca Sparla
6 Author Notes

7 Journal of Experimental Botany, Volume 67, Issue 6, March 2016, Pages
8 1819–1826, <https://doi.org/10.1093/jxb/erv572>

9
10 The final published version is available online at:
11 <https://doi.org/10.1093/jxb/erv572>

12
13 Rights / License:

14 The terms and conditions for the reuse of this version of the manuscript are specified in
15 the publishing policy. For all terms of use and more information see the publisher's
16 website.

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

[†]Martina Zanella^{1,2}, [†]Gian Luca Borghi¹, Claudia Pirone¹, Matthias Thalmann², Diana Pazmino², Alex Costa³, Diana Santelia², Paolo Trost¹, Francesca Sparla¹

¹Department of Pharmacy and Biotechnology FaBiT, University of Bologna, Via Irnerio 42, 40126 Bologna, Italy

²Institute of Plant Biology, University of Zürich, Zollikerstrasse 107, CH-8008 Zurich, Switzerland

³Department of Bioscience, University of Milan, Via Celoria 26, 20133 Milano, Italy

[†]MZ and GLB equally contributed to this work

e-mail addresses: Martina Zanella (martina.zanella@biol.ethz.ch); Gian Luca Borghi (borghi.gianluca@yahoo.com); Claudia Pirone (claudia.pirone@studio.unibo.it); Matthias Thalmann (m.thalmann@botinst.uzh.ch); Diana Pazmino (diana.pazmino@botinst.uzh.ch); Alex Costa (alex.costa@unimi.it); Diana Santelia (dsantelia@botinst.uzh.ch).

Corresponding author:

Francesca Sparla

e-mail: francesca.sparla@unibo.it

phone: +39 0512091281; fax: +39 051 051242576

Co-corresponding author:

Paolo Trost

e-mail: paolo.trost@unibo.it

phone: +39 0512091329; fax: +39 051 051242576

Running title: The interplay between starch and proline under osmotic stress

Date of submission: 12 November 2015

Number of figures: 6 (all figures coloured online-only)

Number of supplementary figures: 3

Number of supplementary tables: 6

1

2 ***Total word count: 5870***

3

Highlight:

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

Abstract:

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

Keywords: *Arabidopsis*, β -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 β -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 β -Amylases are the only enzymes that produce β -maltose, thereby connecting starch
2 degradation in chloroplasts with sugar metabolism in the cytoplasm. Several β -amylases
3 are encoded by the Arabidopsis genome (Lloyd *et al.*, 2005). BAM3 is a major, catalytically
4 active β -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 β -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).

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.

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.

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.

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⁻¹ cm⁻¹. 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.

Results:

Mild osmotic stress induces BAM1 promoter activity

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

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

Water loss in response to stress

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

Starch content at the end of the light period

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

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

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

Starch content at the end of the night period

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

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

Lipid peroxidation

A common effect of osmotic stress is the accumulation of free oxygen radicals (Aranjuelo *et al.*, 2011; Wilhelm and Selmar, 2011) leading to oxidation of unsaturated fatty acids and membrane damage (Hernandez *et al.*, 1993; Fadzilla *et al.*, 1997). Lipid peroxidation induced by osmotic stress was evaluated as malondialdehyde (MDA) concentration on *bam1*, *bam3* and wild-type plants treated with 150 mM mannitol. The exposure to the osmotic stress increased MDA concentration in all genotypes in a time-dependent manner (Fig. 4; Supplementary Table S4). However, only *bam1* samples collected at 4.5 DAT 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 Saviouré, 2009). In
7 order to test whether proline accumulation in osmotically stressed Arabidopsis plant might
8 depend on the activity of β -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 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 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 g^{-1} FW, $\sim 5.9 \mu\text{mol}$ of
4 glucose g^{-1} FW and of $\sim 55 \text{ nmol}$ of maltose 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 β -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 Δ^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 phototosynthetic period
15 exceeded wild-type levels by about 50 μmol of glucose equivalents 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 μmol g^{-1} FW, while soluble sugars
18 (sucrose and glucose) decreased by 12 μmoles of hexoses 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

29 **Acknowledgements:**

30 This work was funded by the Progetto Strategico STARCHitecture-University of Bologna
31 (to FS, PT) and by the Swiss National Science Foundation SNSF-Grant 31003A_147074
32 (to DS). MZ was supported by a short-term fellowship of the Faculty of Mathematics,
33 Physics and Natural Science (University of Bologna, Italy). We thank Samuel C. Zeeman
34 for providing *bam3* T-DNA insertion mutant plants.

Literature:

- Aranjuelo I, Molero G, Erice G, Avice JC, Nogués S.** 2011. Plant physiology and proteomics reveals the leaf response to drought in alfalfa (*Medicago sativa* L.). *Journal of Experimental Botany* 62, 111-123.
- Bahaji A, Li J, Sánchez-López ÁM, Baroja-Fernández E, Muñoz FJ, Ovecka M, Almagro G, Montero M, Ezquer I, Etxeberria E, Pozueta-Romero J.** 2014. Starch biosynthesis, its regulation and biotechnological approaches to improve crop yields. *Biotechnology Advances* 32, 87-106.
- Bartels D and Sunkar R.** 2005. Drought and salt tolerance in plants. *Critical Reviews in Plant Sciences* 24, 23-58.
- Bates IS, Waldren RP, Teare ID.** 1973. Rapid determination of free proline for water stress studies. *Plant Soil* 39, 205-207.
- Cattivelli L, Rizza F, Badeck F-W, Mazzucotelli E, Mastrangelo AM, Francia E, Marè C, Tondelli A, Stanca AM.** 2008. Drought tolerance improvement in crop plants: An integrated view from breeding to genomics. *Field Crops Research* 105, 1-14.
- Cho MH, Lim H, Shin DH, Jeon JS, Bhoo SH, Park YI, Hahn TR.** 2011. Role of the plastidic glucose translocator in the export of starch degradation products from the chloroplasts in *Arabidopsis thaliana*. *New Phytologist* 190, 101-112.
- Denyer K, Johnson P, Zeeman SC, Smith AM.** 2001. The control of amylose synthesis. *Journal of Plant Physiology* 158, 479-487.
- Egli B, Kölling K, Köhler C, Zeeman SC, Streb S.** 2010. Loss of cytosolic phosphoglucomutase compromises gametophyte development in *Arabidopsis*. *Plant Physiology* 154, 1659-1671.
- Fadzilla NM, Finch RP, Burdon RH.** 1997. Salinity, oxidative stress and antioxidant responses in shoot cultures of rice. *Journal of Experimental Botany* 48, 325-331.
- Flügge U-I.** 1999. Phosphate translocators in plastids. *Annual Review of Plant Physiology and Plant Molecular Biology* 50, 27-45.
- Flügge U-I, Häusler RE, Ludewig F, Gierth M.** 2011. The role of transporters in supplying energy to plant plastids. *Journal of Experimental Botany* 62, 2381-2392.
- Fulton DC, Stettler M, Mettler T, Vaughan CK, Li J, Francisco P, Gil M, Reinhold H, Eicke S, Messerli G, Dorken G, Halliday K, Smith AM, Smith SM, Zeeman SC.** 2008. Beta-AMYLASE4, a noncatalytic protein required for starch breakdown, acts upstream of three active beta-amylases in *Arabidopsis* chloroplasts. *Plant Cell* 20, 1040-1058.

- Guidi L, Bongi G, Ciompi S, Soldatini GF.** 1999. In *Vicia faba* leaves photoinhibition from ozone fumigation in light precedes a decrease in quantum yield of functional PSII centres. *Journal of Plant Physiology* 154, 167-172.
- Hayashi F, Ichino T, Osanai M, Wada K.** 2000. Oscillation and regulation of proline content by P5CS and ProDH gene expressions in the Light/Dark cycles in *Arabidopsis thaliana* L. *Plant Cell Physiology* 41, 1096-1101.
- Hernandez JA, Corpas FJ, Gomez M, del Rio LA, Sevilla F.** 1993. Salt-induced oxidative stress mediated by active oxygen species in pea leaf mitochondria. *Physiologia Plantarum* 89, 103-110.
- Hummel I, Pantin F, Sulpice R, Piques M, Rolland G, Dauzat M, Christophe A, Pervent M, Bouteillé M, Stitt M, Gibon Y, Muller B.** 2010. *Arabidopsis* plants acclimate to water deficit at low cost through changes of carbon usage: an integrated perspective using growth, metabolite, enzyme, and gene expression analysis. *Plant Physiology* 154, 357-372.
- Jobling S.** 2004. Improving starch for food and industrial applications. *Current Opinion in Plant Biology* 7, 210-218.
- Kötting O, Kossmann J, Zeeman SC, Lloyd JR.** 2010. Regulation of starch metabolism: the age of enlightenment? *Current Opinion in Plant Biology* 13, 321-328.
- Lao NT, Schoneveld O, Mould RM, Hibberd JM, Gray JC, Kavanaugh TA.** 1999. An *Arabidopsis* gene encoding a chloroplast targeted β -amylase. *Plant Journal* 20, 519-525.
- Liang X, Zhang L, Natarajan SK, Becker DF.** 2013. Proline mechanisms of stress survival. *Antioxidant Redox Signalling* 19, 998-1011.
- Lloyd JR, Kossmann J, Ritte G.** 2005. Leaf starch degradation comes out of the shadows. *Trends in Plant Science* 10, 130-137.
- Matysik J, Alia, Bhalu B, Mohanty P.** 2002. Molecular mechanisms of quenching of reactive oxygen species by proline under stress in plants. *Current Science* 82, 525-532.
- Monroe JD, Storm AR, Badley EM, Lehman MD, Platt SM, Saunders LK, Schmitz JM, Torres CE.** 2014. β -Amylase1 and β -amylase3 are plastidic starch hydrolases in *Arabidopsis* that seem to be adapted for different thermal, pH, and stress conditions. *Plant Physiology* 166, 1748-1763.
- Nittylä T, Messerli G, Trevisan M, Chen J, Smith AM, Zeeman SC.** 2004. A previously unknown maltose transporter essential for starch degradation in leaves. *Science* 303, 87-89.
- Osakabe Y, Osakabe K, Shinozaki K, Tran LS.** 2014. Response of plants to water

stress. *Frontiers in Plant Science*, doi: 10.3389/fpls.2014.00086.

Prasch CM, Ott KV, Bauer H, Ache P, Hedrich R, Sonnewald U. 2015. β -amylase1 mutant *Arabidopsis* plants show improved drought tolerance due to reduced starch breakdown in guard cells. *Journal of Experimental Botany* 66, 6059-6067.

Rockström J and Falkenmark M. 2010. Semiarid crop production from a hydrological perspective: gap between potential and actual yields. *Critical Reviews in Plant Sciences* 19, 319-346.

Santelia D and Zeeman SC. 2011. Progress in *Arabidopsis* starch research and potential biotechnological applications. *Current Opinion in Biotechnology* 22, 271-280.

Santelia D, Trost P, Sparla F. 2015. New insights into redox control of starch degradation. *Current Opinion in Plant Biology* 25, 1-9.

Smith AM and Zeeman SC. 2006. Quantification of starch in plant tissues. *Nature Protocols* 1, 1342-1345.

Smith AM, Zeeman SC, Smith SM. 2005. Starch degradation. *Annual Review of Plant Biology* 56, 73-98.

Sparla F, Costa A, Lo Schiavo F, Pupillo P, Trost P. 2006. Redox regulation of a novel plastid-targeted beta-amylase of *Arabidopsis*. *Plant Physiology* 141, 840-850.

Stitt M and Zeeman SC. 2012. Starch turnover: pathways, regulation and role in growth. *Current Opinion in Plant Biology* 15, 282-292.

Streb S, Eicke S, Zeeman SC. 2012. The simultaneous abolition of three starch hydrolases blocks transient starch breakdown in *Arabidopsis*. *Journal of Biological Chemistry* 287, 41745-41756.

Szabados L and Savouré A. 2009. Proline: a multifunctional amino acid. *Trends in Plant Science* 15, 89-97.

Székely G, Abrahám E, Cséplő A, Rigó G, Zsigmond L, Csiszár J, Ayaydin F, Strizhov N, Jásik J, Schmelzer E, Koncz C, Szabados L. 2008. Duplicated P5CS genes of *Arabidopsis* play distinct roles in stress regulation and developmental control of proline biosynthesis. *Plant Journal* 53, 11-28.

Valerio C, Costa A, Marri L, Issakidis-Bourguet E, Pupillo P, Trost P, Sparla F. 2011. Thioredoxin-regulated beta-amylase (BAM1) triggers diurnal starch degradation in guard cells, and in mesophyll cells under osmotic stress. *Journal of Experimental Botany* 62, 545-555.

Verbruggen N and Hermans C. 2008. Proline accumulation in plants: a review. *Amino Acids* 35, 753-759.

Wilhelm C and Selmar D. 2011. Energy dissipation is an essential mechanism to sustain the viability of plants: the physiological limits of improved photosynthesis. *Journal of Plant Physiology* 168, 79-87.

Zeeman SC, Tiessen A, Pilling E. 2002. Starch synthesis in *Arabidopsis*. Granule synthesis, composition, and structure. *Plant Physiology* 129, 516-529.

Zeeman SC and ap Rees T. 1999. Changes in carbohydrate metabolism and assimilate partitioning in starch-excess mutants of *Arabidopsis*. *Plant Cell Environment* 22, 1445-1453.

Zeeman SC, Smith SM, Smith AM. 2007. The diurnal metabolism of leaf starch. *Biochemical Journal* 401, 13-28.

Figure Legends:

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

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

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

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

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

Fig. 6: Sucrose, glucose and maltose content in wild-type, *bam1* and *bam3* plants 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).