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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. ß-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 ß-amylase-3 (BAM3). A second ß-amylase isoform, ß-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 ß-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.

Introduction:

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

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

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

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

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

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

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

Material and Methods:

Plant materials and growth conditions

Wild-type, T-DNAs and *BAM1* promoter::GUS plants of *Arabidopsis thaliana* (ecotype Columbia, Col-0) were hydroponically grown at a constant temperature of 22°C, under 12 h light/ 12 h dark cycle with a photosynthetic photon flux density of 110 µmol m^{-2} sec^{-1}, as described in Valerio *et al.*, 2011. GUS line and insertion sites of the T-DNA in *bam1* (SALK_039895) and *bam3* (CS92461) mutants were already analyzed (Fulton *et al.*, 2008; Valerio *et al.*, 2011).

Stress conditions

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

GUS staining

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

Determination of water loss

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

ß-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 ß-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

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

Proline content

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

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

Soluble sugars

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

Alike to what was observed for transitory starch (Fig. 3), during the osmotic stress experiment, soluble sugars concentrations measured at the end of the night were essentially similar among the genotypes (Fig. 6, right panels), with the only exception of maltose in *bam3* mutant at 1 DAT, that was more concentrated than in the wild-type (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 ~ 2.8 µmol of sucrose g^{-1} FW, ~ 5.9 µmol of
4 glucose g^{-1} FW and of ~55 nmol of maltose g^{-1} FW.
5

Discussion:

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

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

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

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

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

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

Supplementary Data:

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

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

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

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

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

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

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

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

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

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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 ± 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 ± 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 ± 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 ± 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.

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