# Epidermal bladder cells confer salinity stress tolerance in the halophyte quinoa and *Atriplex* species

**Running title**: EBC in salinity stress responses

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**Abstract**

Epidermal bladder cells (EBC) have been postulated to assist halophytes in coping with saline environments. However, little direct supporting evidence is available. Here, *Chenopodium quinoa* plants were grown under saline conditions for five weeks. One day prior to salinity treatment, EBC from all leaves and petioles were gently removed using a soft cosmetic brush and physiological, ionic and metabolic changes in brushed and non-brushed leaves were compared. Gentle removal of EBC neither initiated wound metabolism nor affected the physiology and biochemistry of control-grown plants but did have a pronounced effect on salt-grown plants, resulting in a salt-sensitive phenotype. Of 91 detected metabolites, more than half were significantly affected by salinity. Removal of EBC dramatically modified these metabolic changes, with the biggest differences reported for GABA, proline, sucrose and inositol, affecting ion transport across cellular membranes (as shown in electrophysiological experiments). This work provides the first direct evidence for a role of EBC in salt tolerance in halophytes and attributes this to (1) a key role of EBC as a salt dump for external sequestration of sodium; (2) improved $K^+$ retention in leaf mesophyll and (3) EBC as a storage space for several metabolites known to modulate plant ionic relations.

**Key words**: epidermal bladder cells, halophyte, metabolic profile, sodium sequestration.

## Introduction

Halophytes constitute less than 0.4% of all land plants (Yuan *et al.* 2016). While the precise definition of the term halophyte is still a matter of debate (e.g. Flowers & Colmer, 2008), in a broad sense, it defines plant species that naturally inhabit saline environments and benefit from having substantial amounts of salt in the growth media (Shabala, 2013). Consequently, in lay terms they are often referred to as 'salt-loving plants'. Halophytes flourish under conditions that would kill 99% of crop species, so are considered a viable alternative to conventional agriculture in saline areas (Glenn *et al.* 1999; Ruan *et al.* 2010; Panta *et al.* 2014; Ventura *et al.* 2015).

The superior salinity tolerance in halophytes is achieved via an orchestrated performance of a large number of physiological mechanisms and anatomical and morphological features (Bohnert *et al.* 1995; Barkla & Pantoja, 1996; Bressan *et al.* 2001; Flowers & Colmer, 2008; Shabala & Mackay, 2011; Barkla *et al.* 2012; Adolf *et al.* 2013; Ozgur *et al.* 2013; Bose *et al.* 2014; Shabala *et al.* 2014a; Yuan *et al.* 2016). Amongst the latter, the ability to secrete salt through specialised leaf structures termed salt glands, is arguably one of the most remarkable features of halophytes, which is found in a large number of species from different families (Flowers *et al.* 2015; Yuan *et al.* 2016). One type of salt gland is the epidermal bladder cells (EBC), which are modified trichomes (Shabala *et al.* 2014a) of a spherical shape, typically with an average diameter of 1 mm and a cell volume of about 500 nL (Adams *et al.* 1998).

EBC have long been suggested to play an important role in plant performance under saline conditions. Some suggested roles include: (i) sequestration sites for excessive salt load, (ii) storage of metabolites (iii) a secondary epidermis for protection against UV radiation, (iv)

external water reservoirs and (v) a reservoir for ROS-scavenging metabolites and organic osmoprotectants (Steudle *et al.* 1975; Jeschke & Stelter, 1983; Rygol *et al.* 1989; Adams *et al.* 1992, 1998; Vogt *et al.* 1999; Ibdah *et al.* 2002; Agarie *et al.* 2007; Jou *et al.* 2007; Barkla & Vera-Estrella, 2015; Oh *et al.* 2015). However, most of these roles are postulations based solely on circumstantial evidence; they lack direct experimental evidence. Hence, the question as to whether EBC are essential for salinity stress tolerance is not yet unequivocally answered.

To date, most studies on salt bladders were conducted on the inducible CAM plant *Mesembryanthemum crystallinum*. The classical CAM physiological studies by Winter and co-authors in 1970s (e.g. Winter, 1973) and the subsequent research have provided a significant conceptual advance in our understanding of various aspect of cell-specific regulation under saline conditions (Barkla *et al.* 2012; Barkla & Vera-Estrella, 2015; Oh *et al.* 2015). However, from both a physiological and biochemical point of view, *M. crystallinum* plants are unique and have some features (e.g. a transition from C3 to CAM metabolism under stress conditions and a pronounced succulency, Adams et al 1992, 1998) that is rarely found in any crop species. When wild type and *M. crystallinum* mutants lacking EBCs were confronted with salinity stress, the EBC mutant showed a significant impairment in seed yield. However, this impairment was not due to an inability of the bladderless mutant to sequester salt in EBC as, contrary to expectations, shoots of wild-type plants had approximately 1.5-fold higher $Na^+$ and $Cl^-$ content than the mutant under saline conditions (Agarie *et al*., 2007). *De facto,* these findings questioned the role of EBC as an external storage space for the excess salt load. Thus, although extremely interesting, these observations do not prove a

direct role for salt bladders as a component of the plant's salt tolerance mechanism. Moreover, given the above unique physiological and anatomical features of *M. crystallinum*, it is rather difficult to translate these findings into breeding concepts for salt tolerance in traditional crops.

Over the last decade, our research has focused on *Chenopodium quinoa*. This recretohalophyte C3 species has become a pseudo-cereal plant of a high economic value. It possesses a combination of highly orchestrated physiological traits that confer its superior salinity stress tolerance (Jacobsen, 2003; Hariadi *et al.* 2011; Adolf *et al.* 2013; Bonales-Allatorre *et al.* 2013a, b; Shabala *et al.* 2013, 2014a, b). Moreover, the simple anatomy of the *C. quinoa* EBC complex makes it an ideal model for studying mechanism of salt sequestration in salt bladders (Shabala *et al.* 2014a). Early studies have shown that quinoa plants rely on both external (salt bladders) and internal (mesophyll cell vacuoles) $Na^+$ sequestration (Bonales-Allatorre *et al.* 2013a, b). Younger plants with higher EBC density and underdeveloped vacuoles in mesophyll cells rely predominantly on bladder-based sequestration mechanisms, while old leaves retain $Na^+$ in the leaf cell vacuoles (Bonales-Allatorre *et al.* 2013a). In this study, we used *C. quinoa* plants to provide direct evidence for a role of EBC in salinity stress tolerance. Our underlying working hypothesis was two-fold. First, we assumed that EBC operate as external $Na^+$ storage and their removal should increase the salt load in the leaf lamina, so affecting plant performance under saline conditions. Second, we hypothesised that EBC may preferentially accumulate certain metabolites that are known for their ability to modulate plant ionic relations. Indeed, it was shown earlier that some of so-called "compatible solutes" could possess a strong ability to block ion channels

mediating plant ionic homeostasis (e.g. the role of choline in vacuolar $Na^+$ sequestration, which originates from its ability to block slow vacuolar channels, Pottosin *et al.,* 2014; or improved $K^+$ retention in plant tissues treated with exogenous glycine betaine, Cuin and Shabala, 2005). In addition, some compatible solutes also act as scavengers of reactive oxygen species (ROS; Peshev *et al*., 2013; Smirnoff and Cumbes, 1989), so if accumulated in high concentrations, may potentially prevent ROS-induced changes in the activation of a broad range of $Na^+$, $K^+$ and $Ca^{2+}$ permeable ion channels (see Demidchik and Maathuis, 2007 for a review).

Here we show that the removal of EBC results in a salt-sensitive growth phenotype. We also demonstrate that when exposed to salinity stress, *C. quinoa* undergoes a significant shift in its metabolite profile, and that EBC removal impacts upon metabolite homeostasis in the leaf lamina. Comparing salt-induced metabolic and ionic changes, we discuss the transporters likely involved in ion sequestration in the EBC.

## Materials and Methods

### *Plant materials and growth conditions*

Three plant species, quinoa (*Chenopodium quinoa* Willd), its close relative *Chenopodium album* L., and *Atriplex lentiformis* L. were used in this study. The quinoa seeds were a gift from Prof SE Jacobsen (University of Copenhagen, Denmark). Atriplex seeds were obtained from Wildseed Tasmania (Sorrel, Australia), and *C. album* seeds were obtained from Rühlemann's Kräuter & Duftpflanzen (Horstedt, Germany). Plants were grown from seed in 20 cm diameter pots filled with standard potting mix (Chen *et al.* 2007), under temperature-

controlled glasshouse conditions (mean day/night temperatures 26/20 °C; humidity 65%; day length 15 h) at the University of Tasmania in Hobart, Australia, between November 2015 and March 2016. Ten seeds were sown in each pot and later thinned to leave four uniform plants per pot. Experiments were organised in a completely randomised design, with each treatment including at least four pots (with four plants in each). Of these six to eight uniform plants were later selected for sampling. All experiments were replicated three times, and showed consistent results.

***Experiments with intact plants (Experiment 1)***

Quinoa plants were grown for five weeks under control conditions. One day prior to the commencement of salinity stress, EBC of all leaves were gently removed from both sides of the leaf using a soft cosmetic brush (Fig. 1A, B). Also removed were all the EBC on the stem and petioles. Plants were then irrigated with 400 mM NaCl for five weeks (Fig. 1D). As new leaves emerged, EBC were removed from the leaf surface and petioles on a regular basis (3-4 times per week) until the experiment was stopped and plants harvested for analysis (Fig. 1D). All lateral buds were also removed on a regular basis. Three types of measurements were conducted for both control and salt-treated plants: (1) Non-brushed (intact) leaves (abbreviated here as NBr); (2) leaves that were brushed shortly before commencing the salt stress (abbreviated as Br); and (3) leaves that were not brushed during the salt exposure but from which EBC were removed prior to elemental analysis (abbreviated as NBr-Br) (Fig. 1D).

***Experiment with decapitated plants (Experiment 2)***

Plants were grown until 7-leaf stage under control conditions prior to commencement of treatments (~ five weeks for *C. quinoa* and *C. album* and ~8 weeks for *A. lentiformis*). At the seven-leaf stage, the shoot apex was excised (chopped off), leaving six leaves remaining on the plant's shoot (Fig. 1C). Then the EBC of all the remaining leaves were gently brushed from both the upper and lower surfaces of leaves using a soft cosmetic brush (Fig. 1B). Salinity stress was then imposed as described in the above section, and all the emerging lateral buds were removed on a regular basis, keeping the number of leaves constant during the entire experiment.

***Physiological assessment***

The fresh weight of the shoot biomass was determined immediately after harvest. Dry weights of the plants were measured after drying the plants in an oven at 65 °C for 72 h. Leaf chlorophyll content was measured (in arbitrary units) by the Minolta SPAD-502 meter (Konica Minolta Sensing, Tokyo, Japan). Net $CO_2$ assimilation (Pn) and stomatal conductance (Gs) were measured using the LiCor 6400 gas exchange system (Lincoln, NE, USA) under full sunlight (around mid-day) under glasshouse conditions. All measurements were carried out on the mid-portion part of the topmost leaves in chopped plants and on the young fully expanded leaves in non-chopped plants. For plant nutrient analysis, two types of measurements were conducted. In the first, approximately 0.1 g of dry matter was added to 7 ml of nitric acid and microwave-digested for 15 min. The digested material was diluted to a final volume of 15 ml, and leaf $Na^+$ and $K^+$ content was measured using Flame Photometer (PFP7, Jenway, UK). In the second set of measurements, a freeze-thaw method (Cuin *et al.*

2009) was used. Appropriate leaves were collected, placed in Eppendorf tubes and immediately placed in a -18 $^{o}$C freezer . Before measurement, the samples were thawed and hand squeezed to extract all the sap. The collected sample was thoroughly mix and measured for its $K^+$ and $Na^+$ concentration (in mM per water basis) using flame photometry as described above. Chloride concentration in the squeezed samples was measured using Cl-selective microelectrodes using the MIFE system (see details below).

To determine the variability of physiological measurements, the experimental data were subjected to analysis of variance (SAS Institute, Cary, NC, USA). The Least Significance Difference (LSD) at P=0.05 probability level was used to compare means between the treatments.

***Metabolite extraction***

For each sample, approximately 100 mg of leaf was harvested and immediately frozen in liquid nitrogen and stored at -80$^{o}$C until freeze-drying, which was carried out using Alpha 1-2 LDplus (Martin Christ, Osterode, Germany). Aliquots (10 mg) of homogenized, freeze-dried leaf material were transferred to Eppendorf tubes and accurate weights were recorded. Methanol (MeOH, 500 µL) containing the internal standards (D-Sorbitol-$^{13}C_6$ (0.02 mg/mL) and L-Valine-$^{13}C_5$,$^{15}N$ (0.02 mg/mL), Sigma Aldrich (Australia), was added to the sample tubes. The samples were vortexed, then incubated in a Thermomixer at 70 $^{o}$C with a mixing speed of 850 rpm for 15 minutes, followed by a 15 minutes of centrifugation at 13,000 rpm (15, 900 x *g*). The MeOH supernatant was transferred into a 1.5 mL Eppendorf tube and set aside. Water (500 µL, Milli Q grade) was added to the remaining sample pellet and vortexed before being centrifuged for 15 minutes at 13,000 rpm (15, 900 x *g*). The supernatant was

removed and combined with the MeOH supernatant (supernatant "A"). This supernatant was used for GC-MS untargeted and targeted analysis.

***Derivatisation for GC-MS analysis***

Derivatisation for GC-MS analysis was carried out as described in Dias *et al.* (2015). The derivatised sample was then left for 1 h before 1 µL was injected onto the GC column using a hot needle technique. Splitless and split (1:10) injections were done for each sample.

***Untargeted GC-MS analysis***

Untargeted GC-MS analysis and data analysis were carried out as described in Hill *et al.* (2013).

***Quantification of sucrose and inositol using GC-MS***

An aliquot of supernatant "A" was further diluted 10-fold with 50% MeOH, and aliquots of 100 µL from the 10-fold diluted supernatant were transferred to clean Eppendorf tubes and dried under vacuum using a Rotational Vacuum Concentrator (RVC 2-33 CD plus, John Morris Scientific, Pty Ltd, Melbourne, Australia). Sucrose and inositol were quantified as described in Dias *et al.* (2015). Calculated concentrations (concentrations based on response of standards and their expected concentrations) were exported and the final concentrations were expressed in mM on a fresh weight basis.

***Quantification of GABA and proline using LC-MS***

Quantification of gamma-aminobutyric acid (GABA) and proline was done as described in Boughton *et al.* (2011). Calculated concentrations (concentrations based on response of standards and their expected concentrations) were exported and the final concentrations were expressed in mM on a fresh weight basis.

***Statistical and further data analysis***

Statistical analysis (Students t-test including Benjamini-Hochberg False Discovery Rate correction, Partial Least Square Discriminant Analysis (PLSDA) and heat map in combination with hierarchical cluster analysis) of untargeted GC -MS was generated through the web-based, open-source metabolomics data analysis tool MetaboAnalyst version 3.0. To generate PLSDA scores plots, area responses for all features detected are normalised to the fresh weight and internal standard before uploading into MetaboAnalyst. Normalised responses were $\log_{10}$ transformed to achieve normal distribution. 2-D PLSDA scores plot (Fig. 7A) were performed based on the sample group information provided and selected PCs component 1 and component 2. Heat maps were generated from GC-MS data (Fig 7B).

GC-MS untargeted data was mapped on an author-created metabolite network of the primary metabolism via the built-in graph editor in VANTED (http://vanted.ipk-gatersleben.de/) (Junker *et al.* 2006). Bar charts indicate relative response per metabolite from control non-brushed and salt treated non-brushed leaves (Fig. 6).

***MIFE electrophysiology***

Net ion fluxes were measured from quinoa leaf mesophyll and stalk cells using the

Microelectrode Ion Flux Estimation (MIFE, University of Tasmania, Hobart, Australia) technique. The full details on the principles and methods of this technique are available in our previous publications (Shabala *et al*., 2006; Chen *et al*., 2007). Commercially available liquid ion exchangers $K^+$, $Na^+$ and $Cl^-$ cocktails were used (catalogue number 60031, 71747 and 24902, respectively; all from Sigma-Aldrich, St Louis, MO, USA). Youngest fully mature quinoa leaves were harvested from five week old plants, grown under ambient light in a temperature-controlled glasshouse (between 19°C and 26°C and average humidity of approximately 65%) at the University of Tasmania. Seeds were sown in 2-L plastic pots filled with standard potting mix and irrigated with either water (for mesophyll measurements) or 100 mM NaCl (for stalk cell measurements).

For $K^+$ flux measurements in the mesophyll, the abaxial epidermis of youngest fully mature quinoa leaves was removed using fine tweezers, and leaf segments of ~5-8 mm were cut and left floating (peeled side down) overnight in buffered Tris/MES basal salt medium (BSM: 0.5 mM KCl, 0.1mM $CaCl_2$, pH 5.5) solution to eliminate possible confounding wounding effects. The following day, leaf segments were immobilised in the measuring chamber containing either 4 mL of buffered Tris/MES BSM or 4 mL of buffered Tris/MES BSM with the addition of 8 mM sucrose for 1.5 h prior to the measurements. Ion fluxes were measured under control conditions for 5 min and then the hydroxyl-radical generating (Demidchik *et al*., 2003) Cu/ascorbate mixture (0.1/0.3 mM) was applied by pipetting and mixing the required volume of stock solutions into the bathing solution.

For $Cl^-$ and $Na^+$ flux measurements in stalk cells, the petioles of youngest fully mature quinoa leaves were excised and immobilised in the measuring chamber containing 4 mL of

BSM (0.5 mM KCl, 0.1mM CaCl2, pH 5.5) for 1.5 h prior to measurement. Ion fluxes were then measured under control conditions for 5 min, then 5 mM GABA was applied by pipetting and mixing the required volume of stock solutions into the bathing solution. For mock controls, the same amount of BSM solution was added to the chamber.

## Results

### *Gentle removal of EBC does not affect the physiology and biochemistry quinoa plants*

To study the role of epidermal bladder cells in salt tolerance of *C. quinoa* plants, these trichome-like structures were removed with a soft cosmetic brush. Such a mechanical disturbance to plant tissues could activate a broad array of mechano-sensing channels (Monshausen & Haswell, 2013), thus potentially result in significant changes in the growth patterns of plant organs by the process termed thigmomorphogenesis (Coutand, 2010).

Nonetheless, in our hands and in the absence of salt stress, removal of EBC by a gentle brushing did not result in any obvious growth phenotype (Fig. 2A, B). Both brushed and non-brushed plants had the same fresh and dry weights (Fig. 2C). While non-brushed plants had slightly a greener appearance to the naked eye (Fig. 2B), there was no significant (at $P < 0.05$) difference in the leaf chlorophyll content between treatments (measured as SPAD value; Fig. 2D). Also, similar between brushed and non-brushed leaves ($P > 0.05$) were net $CO_2$ assimilation rates (Pn; Fig 2F) and stomatal conductance (Gs; Fig 2 G). Thus, the above visual difference is most likely explained by different light reflective properties of the leaf surface (albedo effect) due solely to the presence or absence of bladder cells. We also compared leaf $K^+$ content between treatments. No statistically significant difference in leaf

content was found between brushed and non-brushed leaves (Fig. 2E) for this major cationic osmolyte. To study brushing effects on the leaf metabolism, we monitored the metabolic profiles using GC-MS (Table S1), but did not find significantly ($P < 0.05$) altered levels of amino acids, sugars or sugar alcohols. Thus, the unique anatomy of the epidermis-stalk cell-EBC complex (Fig. S1) of predetermined breaking zone/junction allows EBC to be removed without the bulk of leaf being disturbed. Therefore, under control conditions, the removal of salt bladders by gentle brushing of the leaf surface seems not to induce any damage or cause thigmo-morphogenetic effects.

As a "negative control" and in order to ensure sensitivity of the metabolite profiling method, we also removed EBC in more 'cruel ways" by rubbing the leaf surface with fingers. Such removal does result in an altered plant phenotype (Fig. S2), with brushed plants being more stunted and having (Fig. S2A) smaller leaves (Fig. S2B). None of these plants were used in further studies.

***Removal of EBC results in a salt-sensitive phenotype***

The effect of gentle brushing became visible, however, for plants grown under saline (400 mM NaCl for five weeks) conditions (Fig. 3), showing significantly ($P < 0.05$) smaller biomass (Fig. 3C).

We then looked at how the presence of EBC affects the accumulation of $Na^+$, $K^+$ and $Cl^-$ in the leaf lamina. To do this, we brushed EBC (containing accumulated salt) off salt-grown plants immediately prior to analysis (abbreviated as NBr-Br in Fig 3) and compared $Na^+$, $K^+$ and $Cl^-$ contents in the leaf lamina with those where EBC were removed prior to the

imposition of salinity stress (Br treatment). Our working hypothesis was that NBr-Br leaves should have less $Na^+$ and $Cl^-$ (the two components of salt) accumulated in leaf lamina as they possessed the capability to sequester a large part of the salt load into EBC during plant growth. Indeed, this appeared to be true; plants that had EBC during exposure to salinity accumulated only ~60% of both $Na^+$ and $Cl^-$ in their leaf lamina compared with plants from which EBC were removed before NaCl treatment (Fig 3D, E). Brushed plants also had nearly 50% lower potassium content in leaves compared to non-brushed counterparts (Fig 3F). This suggests that about 50% of the total $K^+$ and 40% $Na^+$ and $Cl^-$ taken up by leaves is stored in EBC.

Next, we tested how does the plant responds when either the bladders were removed from developing leaves, or when we prevented the generation of new, growing young leaves. The rationale behind this study was that in intact plants, the removal of EBC might potentially impact upon formation and development of new leaves; in decapitated plants, this developmental aspect was eliminated. To answer this question, the shoot apex from four-week old plants was removed to have just a fixed number of leaves (Fig. 1C). Brushing of EBC under saline conditions again resulted in a salt-sensitive phenotype (Fig. 4A), with both fresh (FW) and dry (DW) weight and leaf surface area being significantly bigger in non-brushed plants (Fig. 4B-D). Similar to the trend with plants possessing an apex, we found that bladder-free individuals accumulated ca. 410 mM $Na^+$ in the leaf lamina compared with only 270 mM in those allowed to have EBC operating as salt dumps (e.g. 30% increase; Fig. 4E).

To confirm the role of bladders, we performed brushing experiments with another halophyte species that has rather dense EBC (Fig. S3), *Atriplex lentiformis.* As with *C.*

*quinoa* observations, removal of EBC resulted in a salt-sensitive phenotype in *A. lentiformis* (Fig. 5A-C), with brushed plants accumulating more $Na^+$ (Fig. 5E) and having reduced biomass under saline conditions (Fig. 5D). As a negative bladder control, we used *C. album*, a close relative of *C. quinoa*. The ecotype we selected had very few EBC on the leaf surface (and only in very young leaves; Fig S3), thus cannot rely on $Na^+$ sequestration in EBC as a dominant tolerance mechanism. As expected, brushing of the leaf surface of bladderless *C. album* did not result in a salt-sensitive phenotype (Fig S4AB), and no significant (at $P < 0.05$) difference in leaf $Na^+$ content was observed between brushed and non-brushed plants (Fig S4C).

***Salinity induces pronounced changes in leaf metabolic profile***

Salt-grown plants need to adjust osmotically to hyperosmotic conditions. Under such a scenario, salt tolerant plants take up salt and store it in the vacuole. Thus, we hypothesised that they will also need to synthesize metabolites to serve as compatible solutes that would compensate in the cytoplasm for the rise in vacuole osmolality due to the increased $Na^+$ and $Cl^-$. Using GC-MS, we analysed the salt and bladder dependent change in *C. quinoa* leaves. We detected 91 metabolites in leaves with EBC, grown in control and salt treatments (Table 1). Of these, more than half of the metabolites (50) were statistically significantly affected upon salt treatment (based on Students t-test $P < 0.05$), while 46 remained significant following False Discovery Rate correction using the Benjamin-Hochberg method (Chong *et al.* 2015). Among them, six amino acids, 11 organic acids, 13 sugars and sugar alcohols, and 17 unidentified metabolites were significantly changed. Most amino acids such as proline

(16.79 fold), glycine (8.38 fold), phenylalanine (4.09 fold), serine (3.51 fold) and glutamate (1.59 fold) were significantly increased. Aspartate was the only amino acid that decreased (-8.26 fold). Interestingly, apart from succinate and nicotinate, which did not significantly change, 10 organic acids significantly decreased (between -32.78 and -1.71 fold) and with only one (mucic / saccharate) being increased (1.99 fold). Three sugars increased, threitol (2.36 fold), rhamnose (1.52 fold), inositol (1.61 fold). Three sugars acids and one sugar alcohol decreased, erythronate (-1.67 fold), threonate (-4.13 fold), galactonate (-2.54 fold) and arabitol (-1.65 fold). Two sugar phosphates decreased, glucose-6-phosphate (-5.34 fold) and fructose-6-phosphate (-3.59 fold). There were also significant decreases in primary sugars including xylose (-3.60 fold), maltose (-6.37 fold), glucose (-13.80 fold) and sucrose (-1.93 fold). In addition, monomethylphosphate (-7.76 fold) and cytosine (-1.74 fold) significantly decreased (Table 1).

***Removal of EBC affects plant metabolic adaptation to salinity***

Removal of EBC of plants grown in control conditions did not alter the metabolite profile (Table S1). When the metabolic profile of salt-grown bladderless leaves was compared with their bladder-bearing counterparts, pronounced differences were observed (Tables S2, Figs 7 A and B). Using GC-MS, we identified 11 known and 5 unknown metabolites, which were significantly different in salt-treated leaves with EBC removed (Table S2) compared to salt-treated intact leaves. Interestingly, all significantly changed metabolites decreased in leaves with EBC removed, which we confirmed with quantitative GC-MS and LC-MS assays: GABA content dropped from 1.5 ± 0.19 to 0.5 ± 0.17 mM (-3 fold), proline and inositol from

2.75 ± 0.13 to 1.3 ± 0.26 mM (-2.12 fold) and from 0.31 ± 0.3 to 0.25 ± 0.05 mM (-1.24 fold), respectively. At the same time, the sucrose content increased from 0.27 ± 0.01 mM in samples with EBC to 0.86 ± 0.27 mM (3.19 fold). Besides the latter metabolites, GC-MS analysis revealed relative changes for another amino acid; aspartate (-2.82 fold), four organic acids; citrate (-2.59 fold), glycolate (-1.72 fold), oxalate (-6.25 fold) and threonate (-3.68 fold). Only two sugar phosphates decreased; glycerol-3-phosphate (-2.28 fold) and inositol-1-phosphate (-2.17 fold). Threitol was the only sugar which increased in salt-treated leaves with EBC removed compared to intact salt-treated leaves (1.77 fold). There was also a decrease of kampferol (0.33 fold) and uracil (0.29 fold).

PLSDA and unsupervised Hierarchical Cluster Analysis (HCA) combined with Heat Map Analysis are routinely used methods for visualisation of metabolite profiling data and was applied to our samples to ascertain the overall patterns of metabolite profiles as determined with untargeted GC-MS (Figs 7A, 7B). Analysis of GC-MS data revealed a clear separation between intact salt-treated leaves and salt-treated leaves with EBC removed. The score plots (Fig. 7A) also demonstrate that following the removal of EBC, the biological variation of metabolite levels is much higher, indicated by a larger distribution of samples within the PLSDA plot, while the biological variation of metabolite levels of intact leaves is relatively smaller, as demonstrated by a more stringent clustering. HCA combined with Heat Map Analysis also revealed a clear separation between salt-treated intact leaves and salt-treated leaves with EBC removed (Fig. 7A).

***GABA and sucrose modulate ion transport across mesophyll and stalk cell plasma membrane***

We next attempted to establish a causal relationship between the observed changes in a leaf metabolic profile and the plant ionic relations. Accordingly, we hypothesised that changes in some of above metabolite concentrations caused by brushing may be essential for controlling transport of ions across the cellular membranes of the leaf mesophyll and thus in the maintenance of cytosolic $K^+/Na^+$ homeostasis. We tested this hypothesis by measuring the ability of mesophyll cells to retain $K^+$ upon exposure to oxidative stress (associated with salinity – both in glycophytes (Mittler *et al*., 2011) and halophytes (Bose *et al*., 2014)). The addition of the hydroxyl radical-generating Cu/ascorbate mix to the leaf mesophyll resulted in a massive $K^+$ efflux across the plasma membrane (Fig 8A; open symbols). Pre-treating leaves with exogenously-applied 8 mM sucrose (mimicking the increase in sucrose levels in brushed cells) completely mitigated this ROS-induced $K^+$ efflux (Fig 8 A, B) so potentially improving the cytosolic $K^+/Na^+$ ratio.

We then studied the effect of GABA on ion loading into EBC by looking at its impact on $Na^+$ and $Cl^-$ transport from the stalk cells (Fig 9). In salt grown plants, a substantial $Na^+$ and $Cl^-$ efflux (of about -700 and 1500 nmol $m^{-2}$ $s^{-1}$, respectively) was measured from the stalk cell under steady conditions before GABA treatment (Fig 9A,C). Application of 5 mM GABA significantly reduced this efflux by about 25% for $Na^+$ and 50% for $Cl^-$ (Fig 9B,D; both significant at $P < 0.05$). No such changes were measured in mock controls when the equivalent amount of BSM solution was added to the bath instead of GABA (Fig 9).

## Discussion

The physiological role of EBC in plant adaptive responses to salinity has been a matter of numerous experimental and review papers (see Introduction), but the reported evidence is mostly circumstantial. Here we show that the gentle removal of EBC, which did not cause any thigmomorphogenic response (Kamano *et al.* 2015; Moulia *et al.* 2015), results in a salt-sensitive phenotype. This provides the first direct evidence for a role of EBC in salt tolerance in halophytes.

### *EBC act as major $Na^+$ and $Cl^-$ store, rescuing growth under salinity stress*

Removal of EBC and preventing the ability of halophyte plants to sequester $Na^+$ and $Cl^-$ in external structures results in a salt-sensitive phenotype in both *C. quinoa* (Figs 3, 4) and *A. lentiformis* (Fig. 5) plants. At the same time, brushing *C. album* leaves with very few or no bladder cells present (Fig. S3) did not alter the plant's responses to salinity stress (Fig. S4). This indicates that the presence of EBC increases chenopod salinity stress tolerance.

The CAM plant *M. crystallinum* accumulates up to 0.4-1.2 M $Na^+$ in EBC when grown under saline conditions (Adams *et al.* 1998, Barkla *et al.* 2002, Oh *et al.* 2015). Similar numbers were reported for other halophyte species. For example, in *Atriplex gmelini* 80% of all $Na^+$ accumulated in young leaves were located in EBC (Tsutsumi *et al.* 2015), reaching concentrations close to 500 mM.

In *M. crystallinum*, EBC remain compressed to the epidermal surface in unstressed plants but expand to comprise up to 25% of the total aerial volume once the plants have responded to stress (Steudle *et al.* 1975; Barkla *et al.* 2002). The same is true for quinoa (Fig. S5). The

typical cell diameter of EBC in the young leaves used in our study was ~80 μm, the density was about 85 cells per $mm^2$ (Fig. S5), and the overall volume of all EBC on one side of the leaf was ~0.02 $\mu L^3$, or 0.04 $\mu L^3$, assuming EBC are distributed uniformly on both sides. The leaf lamina thickness is ~ 120 μm, making the corresponding volume of the leaf lamina 0.12 $\mu L^3$. Thus the ratio between the volume of EBC and the volume of the leaf lamina in quinoa leaves reaches 1/3, with EBC representing about 25% of the total aerial volume. At the same time, the difference in $Na^+$ sap concentration in the leaf lamina between brushed and non-brushed leaves is about 150 mM (Fig. 4E). Thus, we estimate the $Na^+$ concentration of quinoa EBC to be around 850 mM. A similar calculation for chloride results in an estimated $Cl^-$ concentration in EBC of about 1 M (Fig 3E).

***Salt dumping in EBC may cost plants less compared to intracellular sequestration***

In mechanistic terms, EBC may be considered as "inverted vacuoles" (Shabala *et al.* 2014a). Nonetheless, the carbon cost of internal and external sequestration mechanisms may be different and should be considered. As the cell volume is proportional to the 3rd power of the diameter while the surface area is to the 2nd power, the volume to area ratio increases with increased cell diameter (Table S3). This implies that the carbon cost related to the formation of the cell wall decreases as the cells become bigger (a 10-fold increase in efficiency for a 10-fold increase in diameter). Thus, assuming both epidermal and mesophyll cells possess the same set of transporters for $Na^+$ sequestration, the carbon cost will be an order of magnitude lower in EBC. Given that plants need to allocate a substantial amount of carbon for *de novo* synthesis of compatible solutes for osmotic adjustment under saline conditions (Flowers *et al.*

2015; Flowers & Colmer, 2015), the ability to reduce the amount of carbon for cell wall deposits may be a critical factor conferring salinity stress tolerance at the whole-plant level. This suggestion is fully consistent the generalised energy balanced model proposed by Munns and Gilliham (2015) showing that stress tolerance mechanisms represent additional costs to a plant required to deal with the salt load in the soil, so at high salinity there may be zero growth, the total costs to the plant will equal any energy gain.

***Removing EBC compromises leaf $K^+$ retention ability***

Over the last decade, the ability of cells to maintain cytosolic $K^+$ homeostasis and retain $K^+$ under saline conditions has emerged as one of the critical mechanisms conferring salinity tissue tolerance in both root and shoot tissues (Anschutz *et al.,* 2014; Shabala and Pottosin, 2014; Wu *et al*., 2015; Shabala *et al*., 2016). In this work, we have shown that brushed quinoa plants accumulated much less $K^+$ in leaf lamina, with leaf sap $K^+$ concentration being nearly 2-fold lower in plants with EBC removed prior to salinity exposure (Fig 3E). It remains to be answered as to whether such better $K^+$ retention is associated with better control of membrane potential in mesophyll cells of non-brushed plants, or is related to the prevention of ROS accumulation in these cells under saline conditions. Both voltage and ROS- inducible pathways of $K^+$ leak operate in plant cells under saline conditions (reviewed in Shabala and Pottosin, 2014; Shabala *et al*., 2016), and follow-up experiments are required to reveal which of these pathways was affected by brushing.

The loss of the mesophyll $K^+$ retention ability may be also causally related to changes in the leaf metabolic profile (discussed below) and specifically, the difference in oxalate

content (6-fold lower in brushed leaves compared with intact counterparts; Table 1). Earlier, Jou et al. (2007) suggested that in *M. crystallinum* plants, calcium oxalate crystals present in EBC can serve as a regulatory site for intracellular $K^+$. According to this suggestion, $K^+$ is remobilised from the crystals to increase the cytosolic $K^+$ concentration in nearby leaf mesophyll cells under conditions of reduced $K^+$ uptake and compromised leaf $K^+$ retention (such as under saline conditions).

***Effect of salinity on metabolic profile in quinoa leaves***

Salt treatment of intact leaves induced a strong shift in the metabolite profile when compared to untreated intact leaves (Table 1, Fig. 6), which aligns well with reported metabolite changes upon salinity in halophytes (Kumari et al 2015). As described before, major changes also found in quinoa leaves were an increase of proline and inositol (and other polyols) accompanied by a decrease in organic acids, including TCA cycle intermediates (Kumari et al 2015). Contrary to reported metabolite effects, we found a decrease in sucrose and glucose in salt treated intact quinoa leaves compared to untreated leaves. However, the reported changes did not include any reference to the involvement of bladders towards the metabolite changes. Only one study reported metabolic changes upon salinity in EBC specifically prepared from *Mesembryanthemum crystallinum*, which under salt stress, also showed a decrease in most organic acids and an increase in proline and fructose (Barkla and Vera-Estrella 2015).

Proline, a known osmolyte involved in salt responses in plants (Szabados & Savoure, 2010) increased more than 16-fold, which has been previously observed in salt treated quinoa cotelydons (Ruffino *et al.* 2010). Proline accumulates in several plant species under stressful

environmental conditions including salt, drought, heat and cold, where it mitigates the adverse effects of stress in multiple ways including protecting cell structures, protein integrity and enhancing enzyme activities (Szabados & Savoure, 2010). Most of the organic acids decreased, a metabolic phenotype previously observed in salt-treated barley, rice, Arabidopsis and grapevine (Gong *et al.* 2005; Cramer *et al.* 2007; Zuther *et al.* 2007; Widodo *et al.* 2009). The halophyte *Thellungiella* showed a similar reduction in organic acids to that seen in our experiments (Gong *et al.* 2005). Reduction of organic acids, and in particular TCA cycle intermediates have been correlated with both decreased TCA cycle activity and an increased drawing on carbon structures for the synthesis of compounds required for coping with stress (Sanchez et al 2008, Widodo et al 2009). For instance, the precursor for proline synthesis is glutamate, which also decreased significantly. Glutamate is derived from 2-oxoglutarate, a TCA cycle intermediate.

Increased sugars have also been associated with osmotic stresses such as salinity, providing an increase in cellular osmolarity and providing energy and building blocks for osmoprotectants, such as inositol, and for scavenging ROS (Kumari et al 2015, Widod et al 2009, Sanchez et al 2008). In most reports, sucrose, fructose and glucose have all increased after salt stress in plants. However, in quinoa, glucose, as well as glucose-6-phosphate, decreased massively under salt treatment, down to 7% of the levels in control leaves. This may indicate an increased consumption of glucose through glycolysis or as a building block of salt-response carbohydrates or glycoproteins. For instance, ribose and inositol, which derive from glucose-6-phosphate (which also decreased significantly), were strongly increased. Similarly, glycine, serine and ethanolamine, which derive from 3-phosphoglyceric

acid, and tyrosine and phenylalanine, which derive from phosphoenolpyruvate, all significantly increased (Fig. 6). Ethanolamine is a precursor for the synthesis of glycine betaine, a well-known osmolyte known to increase under salinity stress in plants (Suzuki *et al.* 2003). Tyrosine and phenylalanine are phenolic amino acids and are precursors of alkaloids and other secondary metabolites that have been shown to accumulate in plants under salinity stress.

***Salt metabolism in leaves that have lost the ability for external salt sequestration in EBC***

Our analysis (Table S1) revealed that the metabolite composition of intact leaves and leaves with EBC removed were very similar, indicating that removal of EBC has no effect on the metabolite profiles of leaves when grown in control conditions. However, when plants were grown in salt conditions, a number of metabolites were significantly altered when intact leaves were compared to those with EBC removed (Tables S2). Supervised and unsupervised clustering analysis (Figs 7A and 7B) clearly shows separations between the two treatments, which reflects the differences in metabolite levels. GC-MS analysis detected 16 metabolites of which interestingly, 15 were significantly decreased and only one was increased in the salt treated leaves with EBC removed (Table S2). Here, we focus on the effect of brushing on GABA , inositol and sucrose biology; the metabolites with a known ability to regulate ionic relations in plants.

GABA is a non-protein amino acid known to modulate anion fluxes across the plasma membrane (Ramesh *et al.* 2015). In the current work, we show that brushing EBC reduces leaf GABA content by ~ 3-fold, from 1.5 to 0.5 mM, in quinoa. Given that EBC represent

about a quarter of the total leaf volume (see above), the estimated concentration of GABA in EBC should be therefore at least 10 fold higher than in brushed leaf blade, i.e. about 5-6 mM. This is clearly within the physiological range for reported effects of GABA on ion channels activity in plants (Shabala *et al.* 2014a, b; Ramesh *et al.* 2015; Gilliham and Tyerman, 2016) and can therefore modulate salt loading into EBC, as shown in Fig 9. Indeed, application of 5 mM GABA significantly reduced the magnitude of net $Cl^-$ and $Na^+$ efflux from the stalk cell (Fig 9), suggesting that increase accumulation of GABA in EBC may feed back on the rate of salt loading in salt bladders. Future studies should reveal the molecular nature and intracellular targets of such potential GABA targets in stalk and EBC.

The cyclic polyol myo-inositol is used in all organisms in many different metabolic pathways. Additionally, inositol plays an important role in plant osmotic adjustment (Adams *et al.* 2005). Importantly, both animal and plant studies have suggested that inositol transport may be tightly coupled with transport of $Na^+$. Myo-inositol concentrations increased in salt-stressed plants (Zhai *et al.* 2016), and expression of IMT (myo-inositol phosphate synthase) is enhanced in response to salt stress. Nelson *et al.* (1999) hypothesised that the loading of $Na^+$ into the xylem is coupled to myo-inositol transport, and that myo-inositol acts as a facilitator of the $Na^+$ uptake and long-distance transport in halophytes. It remains to be answered whether such a mechanism operates in EBC.

The last aspect that warrants the discussion is a significant (3-fold) increase in leaf sucrose levels upon removal of EBC (from 0.27 to 0.86 mM). We believe that this phenomenon may be explained by an increased demand for non-enzymatic ROS scavenging

in bladder-less leaves; a notion strongly supported by our observations that leaf mesophyll cells treated with exogenously supplied sucrose have a better ability to tolerate oxidative stress and retain $K^+$ in the cytosol (Fig 8A, B). Salinity stress is known to result in a significant accumulation of various forms of ROS (Mittler *et al.* 2011; Bose *et al.* 2014), with hydrogen peroxide, superoxide radicals, and hydroxyl radicals being the dominant ones. Of these, only the first two can be handled (kept under control) by means of enzymatic antioxidants. At the same time, hydroxyl radicals represent the most aggressive forms of ROS (Demidchik, 2014), causing damage to key cellular structures and significantly disturbing intracellular ion homeostasis (Demidchik *et al.* 2010), compromising leaf photosynthetic performance (Shabala *et al.* 2016). Sugars have been proposed to play a direct role in non-enzymatic antioxidant scavenging (Uemura & Steponkus, 2003; Van den Ende & Valluru, 2009; Foyer & Shigeoka, 2011; Stoyanova *et al.* 2011; Peshev *et al.* 2013) as fully supported by our electrophysiological data (Fig 8). When sugars are compared at the same molar concentration, their greatest antioxidant capability is strongly correlated with their total number of hydroxyl groups, explaining why sucrose (with eight OH groups) is better compared with other sugars such as glucose and fructose (with five OH groups) (Smirnoff & Cumbes, 1989). Therefore, sucrose is well-suited to protect the leaf mesophyll when hydroxyl radical production is expected to increase due to the failure of plants to load excessive salt into EBC.

In conclusion, this work provides the explicit evidence for the important role of EBC as a component of salinity tolerance mechanisms in halophytes species. This role can be attributed to several mechanisms including EBC role as external NaCl storage space, improved $K^+$

retention in leaf mesophyll and as a storage space for several metabolites known to modulate plant ionic relations.

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**References:**

Adams M.A., Richter A., Hill A.K. & Colmer T.D. (2005) Salt tolerance in Eucalyptus spp.: identity and response of putative osmolytes. *Plant, Cell and Environment* 28, 772-787.

Adams P., Thomas J.C., Vernon D.M., Bohnert H.J. & Jensen R.G. (1992) Distinct cellular and organismic responses to salt stress. *Plant and Cell Physiology* 33, 1215–1223.

Adams P., Nelson D.E., Yamada S., Chmara W., Jensen R.G., Bohnert H.J. & Griffiths H. (1998) Growth and development of *Mesembryanthemum crystallinum* (Aizoaceae). *New Phytologist* 138, 171-190.

Adolf V.I., Jacobsen S.E. & Shabala S. (2013) Salt tolerance mechanisms in quinoa (Chenopodium quinoa Willd.). *Environmental and Experimental Botany* 92, 43–54.

Agarie S., Shimoda T., Shimizu Y., Baumann K., Sunagawa H., Kondo A., …, Cushman J.C.

(2007) Salt tolerance, salt accumulation, and ionic homeostasis in an epidermal bladder-cell-less mutant of the common ice plant *Mesembryanthemum crystallinum*. *Journal of Experimental Botany* 58, 1957-1967.

Barkla B.J. & Pantoja O. (1996) Physiology of ion transport across the tonoplast of higher plants. *Annual Review of Plant Biology* 47, 159-184.

Barkla B.J., Vera-Estrella R., Camacho-Emiterio J. & Pantoja O. (2002) $Na^+/H^+$ exchange in the halophyte *Mesembryanthemum crystallinum* is associated with cellular sites of $Na^+$ storage. *Functional Plant Biology* 29, 1017-1024.

Barkla B.J., Rosario V. & Pantoja O. (2012) Protein profiling of epidermal bladder cells from the halophyte *Mesembryanthemum crystallinum*. *Proteomics* 12, 2862-2865.

Barkla B.J. & Vera-Estrella R. (2015) Single cell-type comparative metabolomics of epidermal bladder cells from the halophyte *Mesembryanthemum crystallinum*. *Frontiers in Plant Science 6*.

Bohnert H.J., Nelson D.E. & Jensen R.G. (1995) Adaptations to environmental stresses. *The Plant Cell* 7, 1099–1111.

Bonales-Alatorre E., Pottosin I., Shabala L., Chen Z.H., Zeng F., Jacobsen S.E. & Shabala S. (2013a) Differential activity of plasma and vacuolar membrane transporters contributes to genotypic differences in salinity tolerance in a halophyte species, *Chenopodium quinoa*. *International Journal of Molecular Sciences* 14, 9267-9285.

Bonales-Alatorre E., Shabala S., Chen Z.H. & Pottosin I. (2013b) Reduced tonoplast fast-activating and slow-activating channel activity is essential for conferring salinity tolerance in a facultative halophyte, quinoa. *Plant Physiology* 162, 940-952.

Bose J., Shabala L., Pottosin I., Zeng F., Velarde-Buendía A.M., Hariadi Y. & Shabala S. (2014) Kinetics of xylem loading, membrane potential maintenance, and sensitivity of $K^+$-permeable channels to ROS: physiological traits that differentiate salinity tolerance between pea and barley. *Plant, Cell and Environment* 37, 589–600.

Boughton B.A., Callahan D.L., Silva C., Bowne J., Nahid A., Rupasinghe T., …, Roessner U. (2011) Comprehensive profiling and quantitation of amine group containing metabolites. *Analytical Chemistry* 83, 7523-7530

Bressan R.A., Zhang C., Zhang H., Hasegawa P.M., Bohnert H.J., and Zhu J.K. (2001) Learning from the Arabidopsis experience. The next gene search paradigm. *Plant Physiology* 127, 1354-1360.

Chen Z., Cuin T.A., Zhou M., Twomey A., Naidu B.P. & Shabala S. (2007) Compatible solute accumulation and stress-mitigating effects in barley genotypes contrasting in their salt tolerance. *Journal of Experimental Botany* 58, 4245-4255.

Chong E.Y., Huang Y., Wu H., Ghasemzadeh N., Uppal K., Quyyumi A.A., Jones D.P. & Yu T. (2015) Local false discovery rate estimation using feature reliability in LC/MS metabolomics data. *Scientific Reports* 5.

Coutand C. (2010) Mechanosensing and thigmomorphogenesis, a physiological and biomechanical point of view. *Plant Science* 179, 168-182.

Cramer G.R., Ergül A., Grimplet J., Tillett R.L., Tattersall E.A., Bohlman M.C., …, Quilici D. (2007) Water and salinity stress in grapevines: early and late changes in transcript and metabolite profiles. *Functional and Integrative Genomics* 7, 111-134.

Cuin T.A. & Shabala S. (2005) Exogenously supplied compatible solutes rapidly ameliorate

NaCl-induced potassium efflux from barley roots. *Plant and Cell Physiology* 46, 1924-1933

Cuin T.A., Tian Y., Betts S.A., Chalmandrier R. & Shabala S. (2009) Ionic relations and osmotic adjustment in durum and bread wheat under saline conditions. *Functional Plant Biology* 36, 1110-1119.

Davenport R. (2002) Glutamate receptors in plants. *Annals of Botany* 90, 549-557.

Demidchik V. & Maathuis F.J.M. (2007) Physiological roles of nonselective cation channels in plants: from salt stress to signalling and development. *New Phytologist* 175, 387-404.

Demidchik V., Shabala S.N., Coutts K.B., Tester M.A. & Davies J.M. (2003) Free oxygen radicals regulate plasma membrane $Ca2^{+}$-and $K^{+}$-permeable channels in plant root cells. *Journal of Cell Science* 116, 81-88.

Demidchik V., Cuin T.A., Svistunenko D., Smith S.J., Miller A.J., Shabala S., Sokolik A. & Yurin V. (2010) Arabidopsis root $K^{+}$-efflux conductance activated by hydroxyl radicals: single-channel properties, genetic basis and involvement in stress-induced cell death. *Journal of Cell Science* 123, 1468-1479.

Demidchik V. (2014) Mechanisms and physiological roles of $K^{+}$ efflux from root cells. *Journal of Plant Physiology* 171, 696-707.

Dias D.A., Hill C.B., Jayasinghe N.S., Atieno J., Sutton T. & Roessner U. (2015) Quantitative profiling of polar primary metabolites of two chickpea cultivars with contrasting responses to salinity. *Journal of Chromatography B* 1000, 1-13.

Flowers T.J. & Colmer T.D. (2008) Salinity tolerance in halophytes. *New Phytologist* 179, 945–963.

Flowers T.J. & Colmer T.D. (2015) Plant salt tolerance: adaptations in halophytes. *Annals of Botany* 115, 327-331.

Flowers T.J., Munns R. & Colmer T.D. (2015) Sodium chloride toxicity and the cellular basis of salt tolerance in halophytes. *Annals of Botany* 115, 419-431.

Foyer C.H. & Shigeoka S. (2011) Understanding oxidative stress and antioxidant functions to enhance photosynthesis. *Plant Physiology* 155, 93-100.

Gilliham M. & Tyerman S.D. (2016) Linking metabolism to membrane signaling: the GABA–malate connection. *Trends in Plant Science* 21, 295-301.

Glenn E.P., Brown J.J. & Blumwald E. (1999) Salt tolerance and crop potential of halophytes. *Critical Reviews in Plant Sciences* 18, 227–255.

Gong Q., Li P., Ma S., Indu Rupassara S. & Bohnert H.J. (2005) Salinity stress adaptation competence in the extremophile *Thellungiella halophila* in comparison with its relative *Arabidopsis thaliana*. *The Plant Journal* 44, 826-839.

Hariadi Y., Marandon K., Tian Y., Jacobsen S.E. & Shabala S. (2011) Ionic and osmotic relations in quinoa (*Chenopodium quinoa* Willd.) plants grown at various salinity levels. *Journal of Experimental Botany* 62, 185-193.

Hill C.B., Dias D.A. & Roessner U. (2015) Current and emerging applications of metabolomics in the field of agricultural biotechnology. In *Advances in Food Biotechnology* (ed R. V. Ravishankar), pp. 13-26. John Wiley & Sons Ltd.

Ibdah M., Krins A., Seidlitz H.K., Heller W., Strack D. & Vogt T. (2002) Spectral dependence of flavonol and betacyanin accumulation in *Mesembryanthemum crystallinum* under enhanced ultraviolet radiation. *Plant, Cell and Environment* 25,

1145–1154.

Jacobsen S.E. (2003) The worldwide potential for quinoa (*Chenopodium quinoa* Willd.). *Food Reviews International* 19, 167-177.

Jeschke W.D. & Stelter W. (1983) Ionic relations of garden orache, *Atriplex hortensis* L.: growth and ion distribution at moderate salinity and the function of bladder hairs. *Journal of Experimental Botany* 34, 795-810.

Jou Y., Wang Y. & Yen H.E. (2007) Vacuolar acidity, protein profile, and crystal composition of epidermal bladder cells of the halophyte *Mesembryanthemum crystallinum*. *Functional Plant Biology* 34, 353–359.

Junker B.H., Klukas C. & Schreiber F. (2006) VANTED: a system for advanced data analysis and visualization in the context of biological networks. *BMC Bioinformatics* 7, 109.

Kamano S., Kume S., Iida K., Lei K.J., Nakano M., Nakayama Y. & Iida H. (2015) Transmembrane topologies of $Ca^{2+}$-permeable mechanosensitive channels MCA1 and MCA2 in *Arabidopsis thaliana*. *Journal of Biological Chemistry* 290, 30901-30909.

Kumari A., Das P., Parida, A.K. & Agarwal P.K. (2015) Proteomics, metabolomics, and ionomics perspectives of salinity tolerance in halophytes. *Frontiers in Plant Science* 6, 537.

Mittler R., Vanderauwera S., Suzuki N., Miller G., Tognetti V.B., Vandepoele K., …, Van Breusegem F. (2011) ROS signaling: the new wave?. *Trends in Plant Science* 16, 300-309.

Monshausen G.B. & Haswell E.S. (2013) A force of nature: molecular mechanisms of mechanoperception in plants. *Journal of Experimental Botany* 64, 4663-4680.

Moulia B., Coutand C. & Julien J.L. (2015) Mechanosensitive control of plant growth: bearing the load, sensing, transducing, and responding. *Frontiers in Plant Science* 6, 52.

Munns R. & Gilliham M. (2015) Salinity tolerance of crops–what is the cost? *New Phytologist* 208, 668-673.

Nelson D.E., Koukoumanos M., Bohnert H.J. (1999) Myo-inositol-dependent sodium uptake in ice plant. *Plant Physiology* 119, 165-172.

Oh D.H., Barkla B.J., Vera-Estrella R., Pantoja O., Lee S.Y., Bohnert H.J. & Dassanayake M. (2015) Cell type-specific responses to salinity–the epidermal bladder cell transcriptome of *Mesembryanthemum crystallinum*. *New Phytologist* 207, 627-644.

Ozgur R., Uzilday B., Sekmen A.H. & Turkan I. (2013) Reactive oxygen species regulation and antioxidant defence in halophytes. *Functional Plant Biology* 40, 832–847.

Panta S., Flowers T., Lane P., Doyle R., Haros G. & Shabala S. (2014) Halophyte agriculture: Success stories. *Environmental and Experimental Botany* 107, 71–83.

Peshev D., Vergauwen R., Moglia A., Hideg É. & Van den Ende W. (2013) Towards understanding vacuolar antioxidant mechanisms: a role for fructans? *Journal of Experimental Botany* 64, 1025-1038.

Pottosin I., Bonales-Alatorre E. & Shabala S. (2014) Choline but not its derivative betaine blocks slow vacuolar channels in the halophyte *Chenopodium quinoa*: Implications for salinity stress responses. *FEBS Letters* 588, 3918-3923.

Ramesh S.A., Tyerman S.D., Xu B., Bose J., Kaur S., Conn V., …, Feijó J.A. (2015) GABA signalling modulates plant growth by directly regulating the activity of plant-specific anion transporters. *Nature Communications* 6, 7879

Ruan C.J., da Silva J.A.T., Mopper S., Qin P. & Lutts S. (2010) Halophyte improvement for a salinized world. *Critical Reviews in Plant Sciences* 29, 329-359.

Ruffino A.M.C., Rosa M., Hilal M., González J.A. & Prado F.E. (2010) The role of cotyledon metabolism in the establishment of quinoa (*Chenopodium quinoa*) seedlings growing under salinity. *Plant and Soil* 326, 213-224.

Rygol J., Zimmermann U. & Balling A. (1989) Water relations of individual leaf cells of *Mesembryanthemum crystallinum* plants grown at low and high salinity. *The Journal of Membrane Biology* 107, 203-212.

Sanchez D.H., Siahpoosh M.R., Roessner U., Udvardi M. & Kopka J. (2008) Plant metabolomics reveals conserved and divergent metabolic responses to salinity. *Physiologia Plantarum* 132, 209-219.

SAS Institute Inc. (2003) *SAS/STAT User's Guide, version 9.1.3,* Cary, N.C., SAS Institute Inc.

Shabala L., Ross T., McMeekin T. & Shabala S. (2006) Non-invasive microelectrode ion flux measurements to study adaptive responses of microorganisms to the environment. *FEMS Microbiology Reviews* 30, 472-486.

Shabala S.N. & Mackay A.S. (2011) Ion transport in halophytes. *Advances in Botanical Research* 57, 51-187.

Shabala S. (2013) Learning from halophytes: physiological basis and strategies to improve abiotic stress tolerance in crops. *Annals of Botany* 112, 1209–1221.

Shabala S., Hariadi Y. & Jacobsen S.E. (2013) Genotypic difference in salinity tolerance in quinoa is determined by differential control of xylem $Na^+$ loading and stomatal density.

*Journal of Plant Physiology* 170, 906-914.

Shabala S., Bose J. & Hedrich R. (2014a) Salt bladders: do they matter? *Trends in Plant Science* 19, 687–691.

Shabala S., Shabala L., Barcelo J. & Poschenrieder C. (2014b) Membrane transporters mediating root signalling and adaptive responses to oxygen deprivation and soil flooding. *Plant, Cell and Environment* 37, 2216-2233.

Shabala L., Zhang J., Pottosin I., Bose J., Zhu M., Fuglsang A.T., …, Bacic A. (2016) Cell-type-specific $H^+$-ATPase activity in root tissues enables $K^+$ retention and mediates acclimation of barley (*Hordeum vulgare*) to salinity stress. *Plant Physiology* 172, 2445-2458.

Shabala S, Bose J, Fuglsang AT, Pottosin I (2016) On a quest for stress tolerance genes: membrane transporters in sensing and adapting to hostile soils. *Journal of Experimental Botany* 67, 1015-1031.

Smirnoff N. & Cumbes Q.J. (1989) Hydroxyl radical scavenging activity of compatible solutes. *Phytochemistry* 28, 1057-1060.

Steudle E., Lüttge U. & Zimmermann U. (1975) Water relations of the epidermal bladder cells of the halophytic species *Mesembryanthemum crystallinum*: direct measurements of hydrostatic pressure and hydraulic conductivity. *Planta* 126, 229-246.

Stoyanova S., Geuns J., Hideg É. & Van Den Ende W. (2011) The food additives inulin and stevioside counteract oxidative stress. *International Journal of Food Sciences and Nutrition* 6, 207-214.

Suzuki M., Yasumoto E., Baba S. & Ashihara H. (2003) Effect of salt stress on the

metabolism of ethanolamine and choline in leaves of the betaine-producing mangrove species *Avicennia marina*. *Phytochemistry* 64, 941-948.

Szabados L. & Savoure A. (2010) Proline: a multifunctional amino acid. *Trends in Plant Science* 15, 89-97.

Tsutsumi K., Yamada N., Cha-um S., Tanaka Y. & Takabe T. (2015) Differential accumulation of glycinebetaine and choline monooxygenase in bladder hairs and lamina leaves of *Atriplex gmelini* under high salinity. *Journal of Plant Physiology* 176, 101-107.

Uemura M. & Steponkus P.L. (2003) Modification of the intracellular sugar content alters the incidence of freeze-induced membrane lesions of protoplasts isolated from *Arabidopsis thaliana* leaves. *Plant, Cell and Environment* 26, 1083-1096.

Van den Ende W. & Valluru R. (2009) Sucrose, sucrosyl oligosaccharides, and oxidative stress: scavenging and salvaging? *Journal of Experimental Botany* 60, 9-18.

Ventura Y., Eshel A., Pasternak D. & Sagi M. (2015) The development of halophyte-based agriculture: past and present. *Annals of Botany* 115, 529-540.

Vogt T., Ibdah M., Schmidt J., Wray V., Nimtz M. & Strack D. (1999) Light-induced betacyanin and flavonol accumulation in bladder cells of *Mesembryanthemum crystallinum*. *Phytochemistry* 52, 583-592.

Widodo J.H., Patterson E.N.E., Tester M., Bacic A. & Roessner U. (2009) Metabolic responses to salt stress of barley (*Hordeum vulgare* L.) cultivars, Sahara and Clipper, which differ in salinity tolerance. *Journal of Experimental Botany* 60, 4089-4103.

Winter K. (1973) Studies on NaCl-induced crassulacean acid metabolism in *Mesembryanthemum crystallinum*. *Planta* 109, 135-145.

Yuan Z., Druzhinina I.S., Labbé J., Redman R., Qin Y., Rodriguez R., …, Lin F. (2016) Specialized microbiome of a halophyte and its role in helping non-host plants to withstand salinity. *Scientific Reports 6*.

Zhai H., Wang F., Si Z., Huo J., Xing L., An Y., He S. & Liu Q. (2016) A myo-inositol-1-phosphate synthase gene, IbMIPS1, enhances salt and drought tolerance and stem nematode resistance in transgenic sweet potato. *Plant Biotechnology Journal* 14, 592-602.

Zuther E., Koehl K. & Kopka J. (2007) Comparative metabolome analysis of the salt response in breeding cultivars of rice. In *Advances in Molecular Breeding Toward Drought and Salt Tolerant Crops* (eds M.A. Jenks, P.M. Hasegawa & S.M. Jain), pp. 285-315. Springer Netherlands.

**Figure legends**

**Figure 1**. Details of experimental design used in this study. Br – brushed leaves; NBr – non-brushed leaves; NBr-Br - leaves that were non-brushed during salt exposure but from which EBC were removed prior to elemental analysis. A, C – cartoons illustrating two types of experiments conducted (on intact and decapitated plants, accordingly). B - *Chenopodium quinoa* leaf with EBC removed from one half of the leaf. D – a summary of treatment and sampling protocols. The scale bar in is 1 mm.

**Figure 2**. The gentle removal of EBC does not alter plant phenotype (A, B) or have any significant impact on its agronomical or physiological characteristics in *Chenopodium quinoa* plants grown under control conditions. C – shoot fresh (FW) and dry (DW) weigh; D - chlorophyll content (SPAD readings); E - leaf $K^+$ content; F – net $CO_2$ assimilation, Pn; G – stomatal conductance, Gs. Data are mean ± SE (n = 5 to 8). The scale bar is 5 cm.

**Figure 3**. Removal of EBC from salt-grown *Chenopodium quinoa* plants results in a salt-sensitive phenotype (A, B, C) and has a major impact on ionic relations in leaf lamina. A, B – typical images of brushed (Br; with EBC removed prior to salt stress onset) and intact (non-brushed; NBr) quinoa plants grown for 5 weeks at 400 mM NaCl. C – shoot fresh (FW) and dry (DW) weight; D – leaf $Na^+$ content; E – leaf sap $K^+$ concentration. Data are mean ± SE (n = 5 to 8). Data labelled with different low case letters is significantly different at $P < 0.05$. The scale bar is 5 cm.

**Figure 4**. Salt-sensitive phenotype resulting from the gentle removal of EBC from leaves of decapitated *Chenopodium quinoa* plants (in which shoot apex was removed to have just a fixed number of leaves). A – typical images of brushed and non-brushed plants; B, C –

shoot fresh (B) and dry (C) weight of control and salt-grown plants; D – leaf surface area; E – leaf sap $Na^+$ content. Data are mean ± SE (n = 5 to 8). Data labelled with different low case letters is significantly different at $P < 0.05$. The scale bar is 5 cm.

**Figure 5**. Effect of EBC removal on growth and physiological characteristics of *Atriplex lentiformis* plants. A-C – typical images of brushed and non-brushed plants grown under control and salt conditions. D – shoot fresh weight; E – leaf $Na^+$ content. Br – brushed leaves; NBr – non-brushed leaves; NBr-Br - leaves that were non-brushed during the salt exposure but from which EBC were removed prior to elemental analysis. Data is mean ± SE (n = 5 to 8). Data labelled with different low case letters is significantly different at $P < 0.05$.

**Figure 6**. Pathway map of metabolite differences between control and salt treated intact quinoa leaves (with EBC present). Metabolic pathway and graphs were created using VANTED (Junker et al 2006). Bars represent control (green) and salt treated (blue) (n = 5).

**Figure 7**. Partial Least Square Discriminant Analysis (A) and Hierarchical Cluster Analysis combined with heatmap analysis (B) of untargeted GC-MS of salt treated quinoa leaves with EBC present (non-brushed) and EBC removed (brushed). The shaded circles in Panel A indicate a 95% confidence level.

**Figure 8**. Effect of sucrose on $K^+$ retention in quinoa leaf mesophyll exposed to ROS. A – hydroxyl radical-induced transient net $K^+$ flux kinetics measured from mesophyll cells pre-treated with 8 mM of sucrose for 1.5 h prior to onset of oxidative stress. Hydroxyl radicals were generated by applying 0.1/0.3 mM Cu/ascorbate mix (see Demidchik *et al*., 2003 for details). The sign convention is "efflux negative". Values are means ± SE (n =

10). B -steady state $K^+$ fluxes in mesophyll tissues of quinoa leaves before and after (30 min) the addition of Cu/ascorbate mix. Values are means ± SE (n = 10). *Significant at P < 0.001.

**Figure 9**. Effect of GABA on $Na^+$ and $Cl^-$ efflux from the stalk cells in quinoa. A – a representative transient net $Na^+$ flux from the stalk cells from plants germinated and grown in the presence of 100 mM NaCl. B – relative $Na^+$ fluxes from stalk cells (% of initial values) after the addition of 5 mM GABA to the bath. Values are means ± SE (n = 5). C – a representative transient net $Cl^-$ flux from the stalk cells from plants germinated and grown in the presence of 100 mM NaCl. D – relative $Cl^-$ fluxes from stalk cells (% of initial values) after the addition of 5 mM GABA to the bath. Values are means ± SE (n = 5). In controls, the appropriate amounts of BSM solution was added instead of GABA. For all MIFE data, the sign convention is "efflux negative". *Significant at P < 0.05.

**Supplementary Information**

**Table S1.** Untargeted GC-MS metabolite profile comparisons of brushed versus non-brushed grown under control conditions. CNB = Control Non-Brushed; CB = Control Brushed. Data are presented as x-fold with CNB set to 1 (n = 5). Blue cells indicate statistical significance determined with Students t-test (P > 0.05).

**Table S2.** Untargeted GC-MS metabolite profile comparison of brushed versus non-brushed quinoa leaves grown under saline conditions. TNB = Treated Non-Brushed; TB = Treated Brushed. Data are presented as x-fold with TNB set to 1 (n = 5). Blue cells indicate statistical significance determined with Students t-test (P > 0.05).

**Table S3.** Geometrical consideration and cell volume to surface ratio for cells of different size in the context of carbon cost efficiency associated with cell wall formation. The bigger the cell diameter, the less carbon is required per volume unit.

**Figure S1**. Anatomical structure of epidermal bladder cell (EBC) –stalk cell (SC) complex in *Chenopodium quinoa* leaves (A). Upon gentle brushing, EBC is detached from the SC, causing no damage to leaf lamella (B).

**Figure S2**. Severe leaf brushing results in altered plant phenotype. Quinoa plants were grown under control conditions and EBC were mechanically removed from each newly developed leaf by severe brushing causing thigmomorphogenetic responses. As a result, brushed plants were more stunted (A) and had smaller leaves (B).

**Figure S3**. Differences in EBC density between young (top panels) and old (lower panels) leaves of three species used in this study. *Atriplex lentiformis* (another halophyte species) had EBC density even higher than *Chenopodium quinoa*, while in closely related

*Chenopodium album*, only a few EBC could be observed in one field of view in young (but not old) leaves. Hence, adaptive strategy of *C. album* to saline stress is different from two other species and do not rely on salt sequestration in EBC.

**Figure S4**. Effect of leaf brushing on physiological and agronomical characteristics of *Chenopodium album* plants. A – shoot fresh weigh; B - shoot dry weight; C – shoot $Na^+$ content. Mean ± SE (n = 5 to 7). Plants were treated with 400 mM NaCl for 5 weeks. Br – brushed; NBr – non-brushed. Different low-case letters indicate significant difference at $P < 0.05$. As seen from the figure, contrary to *Chenopodium quinoa* plants, brushing the leaf surface of *C. album* did not result in a salt-sensitive phenotype and did not led to higher $Na^+$ accumulation in the leaf lamina.

**Figure S5**. Cross-section of salt-grown *Chenopodium quinoa* leaf showing the relative size of EBC compared to the thickness of the leaf lamina.

**Table 1**. Comparison of GC-MS untargeted metabolite profiles of non-brushed leaves with and without salinity treatment. CNB = Control Non-Brushed; TNB = Treated Non-Brushed. Data are presented as x-fold with CNB set to 1 (n=5). Blue cells indicate statistical significance determined with Students t-test (P > 0.05) and green cells indicate statistical significance following Benjamin-Hochberg False Discovery Rate correction.



| | Control NBr | | | Salt NBr | | |
|---|---|---|---|---|---|---|
| **ORGANIC ACIDS** | **x-fold** | | **sem** | **x-fold** | | **sem** |
| **4_hydroxy cinnamic acid** | 1.000 | ± | 0.183 | **-3.404** | ± | **0.201** |
| **Ascorbic acid/ Iso ascorbic** | 1.000 | ± | 0.240 | **-4.637** | ± | **0.172** |
| **Benzoate** | 1.000 | ± | 0.141 | **-1.708** | ± | **0.061** |
| **Citrate** | 1.000 | ± | 0.266 | **-3.626** | ± | **0.235** |
| **Glycerate** | 1.000 | ± | 0.097 | **-2.010** | ± | **0.022** |
| **Fumarate** | 1.000 | ± | 0.152 | **-4.915** | ± | **0.248** |
| **Malate** | 1.000 | ± | 0.142 | **-3.147** | ± | **0.176** |
| **Mucic/saccharic** | 1.000 | ± | 0.142 | **1.998** | ± | **0.118** |
| **Nicotinic acid** | 1.000 | ± | 0.185 | -1.675 | ± | 0.195 |
| **Oxalate** | 1.000 | ± | 0.567 | **-32.783** | ± | **0.271** |
| **Pantothenic acid** | 1.000 | ± | 0.134 | **-7.667** | ± | **0.044** |
| **Pyroglutamate** | 1.000 | ± | 0.317 | 1.459 | ± | 0.178 |
| **Succinate** | 1.000 | ± | 0.152 | -1.895 | ± | 0.245 |
| **Threonate-1,4-lactone** | 1.000 | ± | 0.178 | **-2.299** | ± | **0.144** |
| **SUGARS AND SUGAR ALCOHOLS** | **x-fold** | | **sem** | **x-fold** | | **sem** |
| **Arabinose** | 1.000 | ± | 0.078 | 1.015 | ± | 0.361 |
| **Arabitol** | 1.000 | ± | 0.140 | **-1.650** | ± | **0.127** |
| **Erythronate** | 1.000 | ± | 0.066 | **-1.665** | ± | **0.048** |
| **Fructose** | 1.000 | ± | 0.165 | 1.772 | ± | 0.482 |
| **Fructose-6-P** | 1.000 | ± | 0.190 | **-3.587** | ± | **0.258** |
| **Galactitol** | 1.000 | ± | 0.138 | 1.042 | ± | 0.084 |
| **Galactonate** | 1.000 | ± | 0.177 | **-2.539** | ± | **0.070** |
| **Galactose** | 1.000 | ± | 0.122 | 1.409 | ± | 0.548 |
| **Glucose** | 1.000 | ± | 0.313 | **-13.796** | ± | **0.442** |
| **Glycerol-3-P** | 1.000 | ± | 0.139 | -1.819 | ± | 0.277 |

| | | | | | | |
|---|---|---|---|---|---|---|
| **Glucose-6-P** | 1.000 | ± | 0.197 | **-5.336** | ± | **0.100** |
| **Inositol** | 1.000 | ± | 0.043 | **1.605** | ± | **0.066** |
| **Inositol** | 1.000 | ± | 0.036 | **1.611** | ± | **0.064** |
| **Maltose** | 1.000 | ± | 0.124 | **-6.374** | ± | **0.169** |
| **Rhamnose** | 1.000 | ± | 0.097 | **1.519** | ± | **0.067** |
| **Ribonate** | 1.000 | ± | 0.371 | -1.456 | ± | 0.168 |
| **Ribose** | 1.000 | ± | 0.072 | 2.000 | ± | 0.431 |
| **Sucrose** | 1.000 | ± | 0.035 | **-1.927** | ± | **0.147** |
| **Threitol** | 1.000 | ± | 0.184 | **2.366** | ± | **0.082** |
| **Threonate** | 1.000 | ± | 0.193 | **-4.137** | ± | **0.180** |
| **Trehalose** | 1.000 | ± | 0.220 | -1.347 | ± | 0.079 |
| **Xylose** | 1.000 | ± | 0.156 | **-3.602** | ± | **0.093** |

| **OTHERS** | **x-fold** | | **sem** | **x-fold** | | **sem** |
|---|---|---|---|---|---|---|
| **Monomethylphosphate** | 1.000 | ± | 0.259 | **-7.757** | ± | **0.350** |
| **Cytosine** | 1.000 | ± | 0.177 | **-1.742** | ± | **0.047** |
| **Diethylene_glycol** | 1.000 | ± | 0.313 | -1.559 | ± | 0.165 |
| **Phosphate** | 1.000 | ± | 0.199 | -1.297 | ± | 0.144 |

| | **Control NBr** | | | **Salt NBr** | | |
|---|---|---|---|---|---|---|
| ***AMINO ACIDS AND AMINES*** | **x-fold** | | **sem** | **x-fold** | | **sem** |
| **Aspartate** | 1.000 | ± | 0.154 | **-8.260** | ± | **0.158** |
| **Ethanolamine** | 1.000 | ± | 0.212 | 2.567 | ± | 0.329 |
| **GABA** | 1.000 | ± | 0.378 | -1.523 | ± | 0.335 |
| **Glutamate** | 1.000 | ± | 0.226 | **1.588** | ± | **0.105** |
| **Glycine** | 1.000 | ± | 0.117 | **8.283** | ± | **0.085** |
| **Isoleucine** | 1.000 | ± | 0.295 | -1.254 | ± | 0.243 |
| **Phenylalanine** | 1.000 | ± | 0.329 | **4.098** | ± | **0.276** |
| **Proline** | 1.000 | ± | 0.261 | **16.792** | ± | **0.185** |
| **Serine** | 1.000 | ± | 0.160 | **3.511** | ± | **0.081** |
| **Threonine** | 1.000 | ± | 0.322 | 2.002 | ± | 0.168 |
| **Tyrosine** | 1.000 | ± | 0.433 | 1.643 | ± | 0.220 |
| **Valine** | 1.000 | ± | 0.239 | -1.249 | ± | 0.425 |

| **UNKNOWNS** | **x-fold** | | **sem** | **x-fold** | | **sem** |
|---|---|---|---|---|---|---|
| **UN_2_276_13.279** | 1.000 | ± | 0.229 | -1.657 | ± | 0.102 |
| **UN_3_205_13.816** | 1.000 | ± | 0.382 | -2.702 | ± | 0.158 |
| **UN_4_262_14.466** | 1.000 | ± | 0.050 | -1.026 | ± | 0.353 |

| | | | | | | |
|---|---|---|---|---|---|---|
| **UN_6_306_16.105** | 1.000 | ± | 0.095 | **1.728** | ± | **0.055** |
| **UN_7_306_16.255** | 1.000 | ± | 0.092 | 1.113 | ± | 0.038 |
| **UN_8_292_16.499?** | 1.000 | ± | 0.151 | **-9.271** | ± | **0.101** |
| **UN_9_204_18.259** | 1.000 | ± | 0.136 | **-2.080** | ± | **0.230** |
| **UN_10_217_18.860** | 1.000 | ± | 0.403 | **-3.342** | ± | **0.196** |
| **UN_11_292_19.232** | 1.000 | ± | 0.125 | **-1.767** | ± | **0.144** |
| **UN_12_275_20.337** | 1.000 | ± | 0.086 | **-1.898** | ± | **0.023** |
| **UN_13_285_20.524** | 1.000 | ± | 0.367 | -1.635 | ± | 0.211 |
| **UN_14_275_20.752** | 1.000 | ± | 0.161 | 1.287 | ± | 0.091 |
| **UN_15_273_21.216** | 1.000 | ± | 0.095 | **-14.250** | ± | **0.331** |
| **UN_16_361_21.963** | 1.000 | ± | 0.433 | **-15.054** | ± | **0.404** |
| **UN_17_174_23.147** | 1.000 | ± | 0.181 | **-2.802** | ± | **0.109** |
| **UN_18_319_23.921** | 1.000 | ± | 0.074 | **-9.420** | ± | **0.041** |
| **UN_19_445_25.068** | 1.000 | ± | 0.166 | **-3.338** | ± | **0.114** |
| **UN_20_204_25.590** | 1.000 | ± | 0.214 | **-4.366** | ± | **0.148** |
| **UN_21_290_26.044** | 1.000 | ± | 0.088 | **-4.665** | ± | **0.064** |
| **UN_22_318_27.667** | 1.000 | ± | 0.174 | 1.389 | ± | 0.297 |
| **UN_23_321_28.613** | 1.000 | ± | 0.073 | **-1.508** | ± | **0.023** |
| **UN_24_191_29.266** | 1.000 | ± | 0.211 | 1.157 | ± | 0.078 |
| **UN_25_204_30.286** | 1.000 | ± | 0.056 | -1.048 | ± | 0.048 |
| **UN_26_328_31.254** | 1.000 | ± | 0.122 | **-6.257** | ± | **0.126** |
| **UN_27_318_31.709** | 1.000 | ± | 0.159 | **-2.158** | ± | **0.057** |
| **UN_31_297_33.138** | 1.000 | ± | 0.158 | 1.387 | ± | 0.123 |
| **UN_32_327_33.739** | 1.000 | ± | 0.197 | -1.613 | ± | 0.180 |
| **UN_33_647_35.483** | 1.000 | ± | 0.284 | **-4.125** | ± | **0.307** |
| **UN_154_7.967** | 1.000 | ± | 0.418 | -1.548 | ± | 0.255 |
| **UN_14_275_20.752** | 1.000 | ± | 0.134 | 1.418 | ± | 0.086 |
| **UN_18.033** | 1.000 | ± | 0.118 | 1.083 | ± | 0.047 |

Fig 1

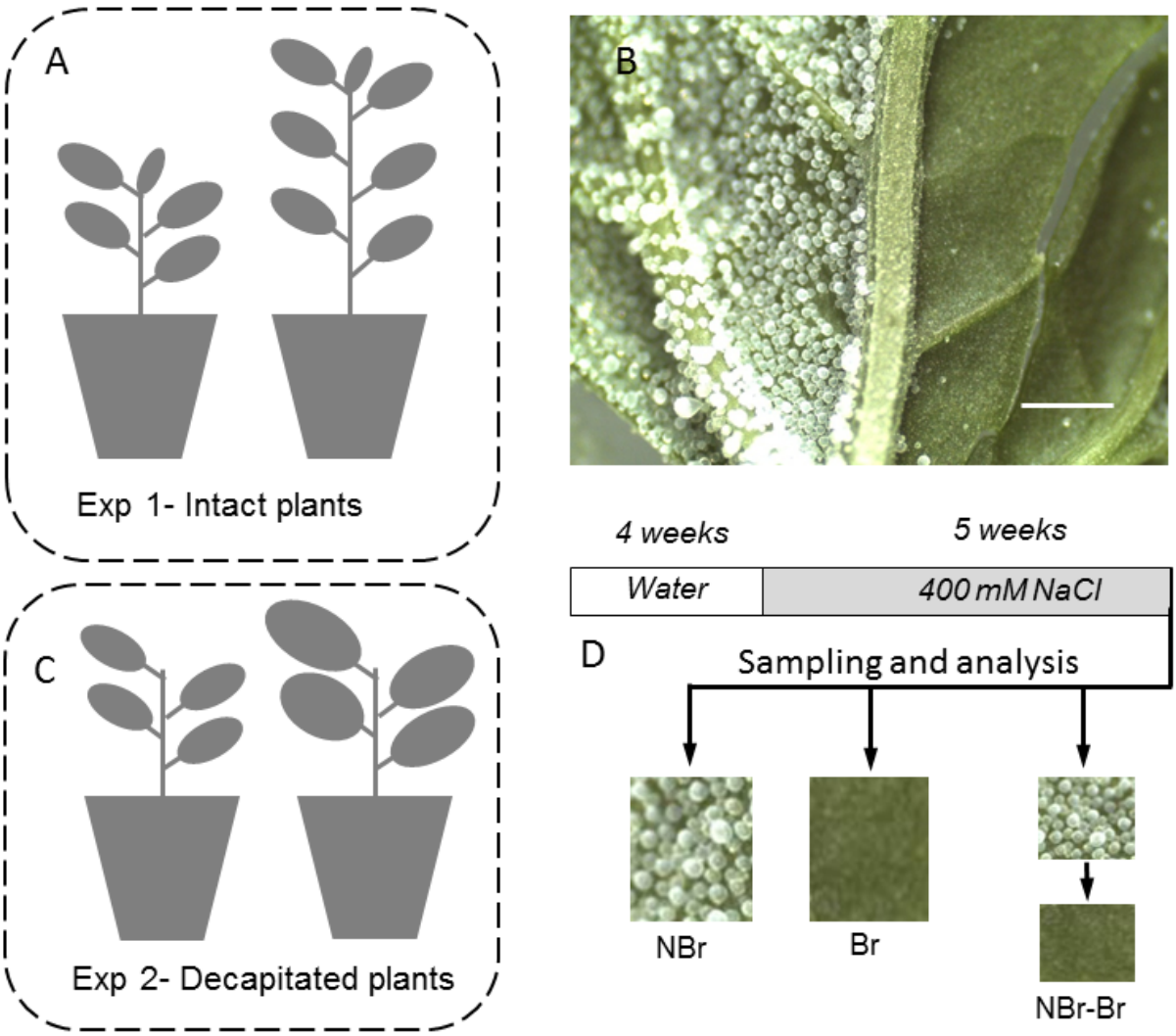


Fig 2

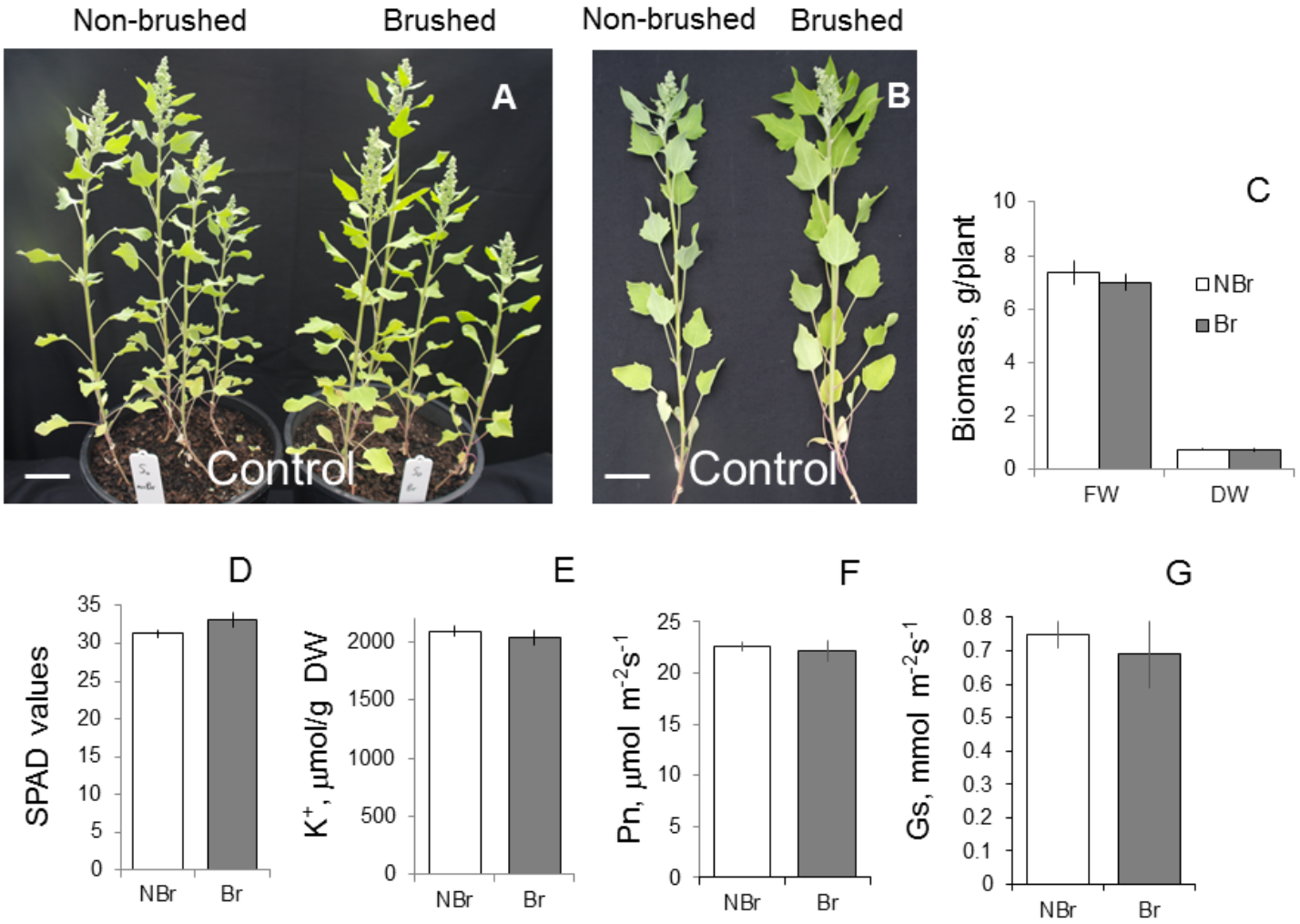


Fig 3

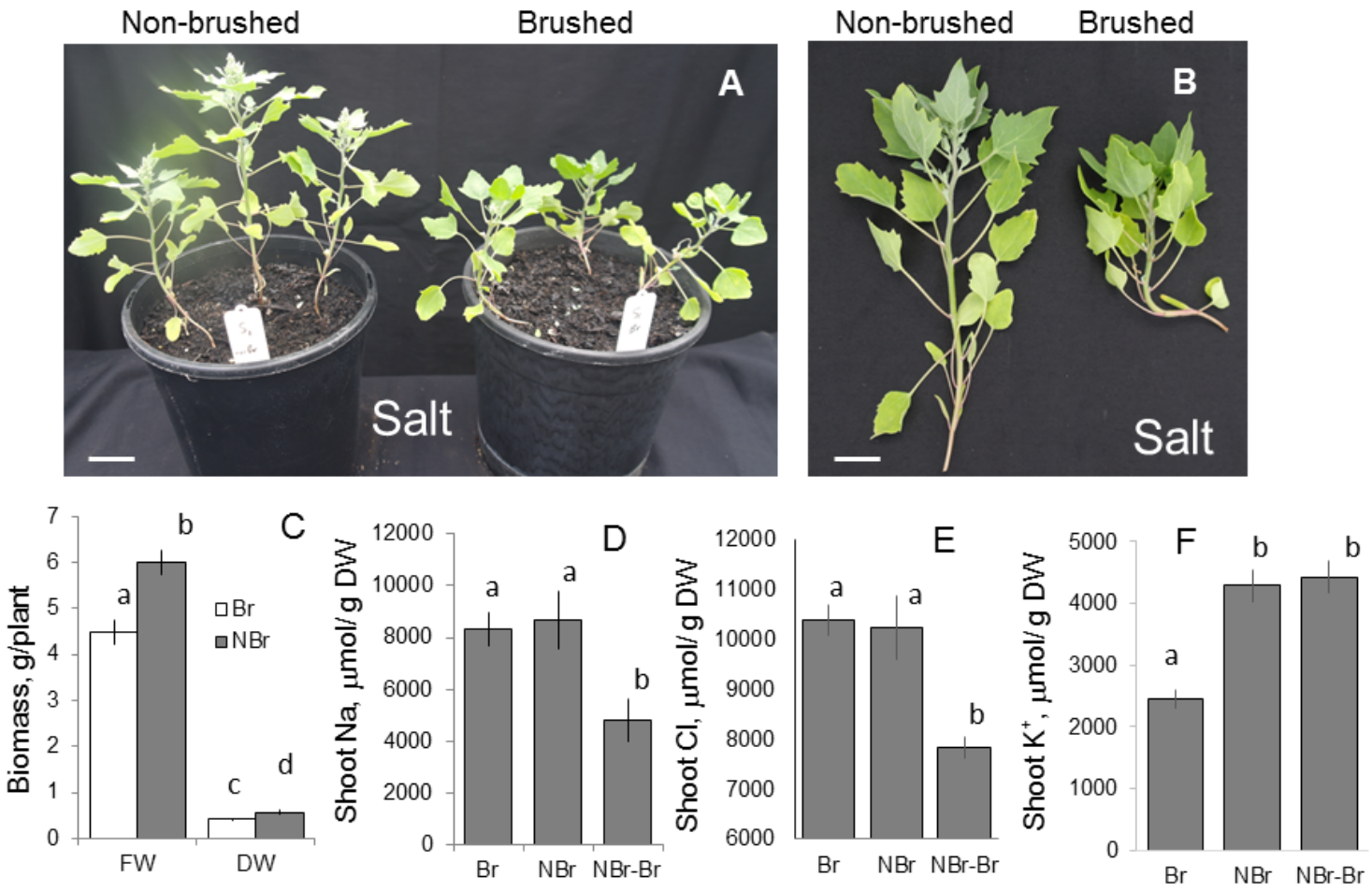
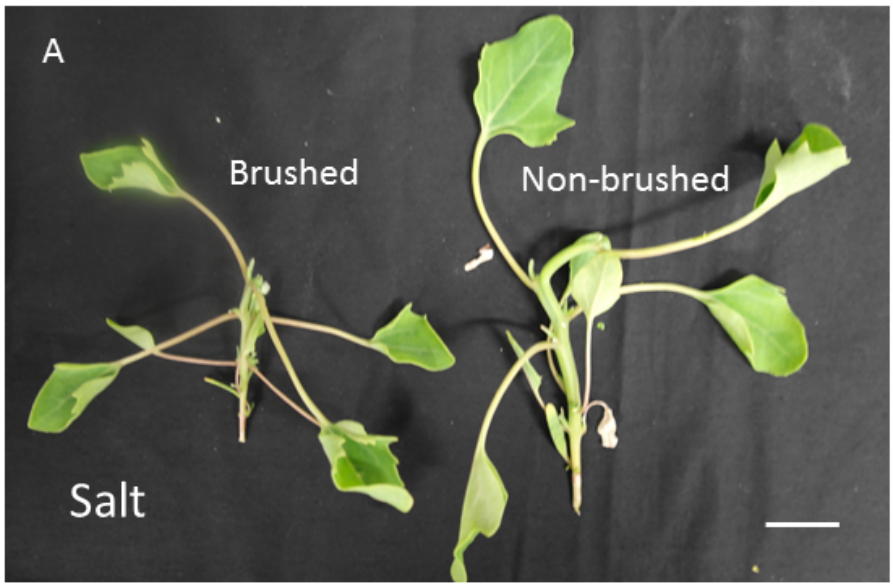
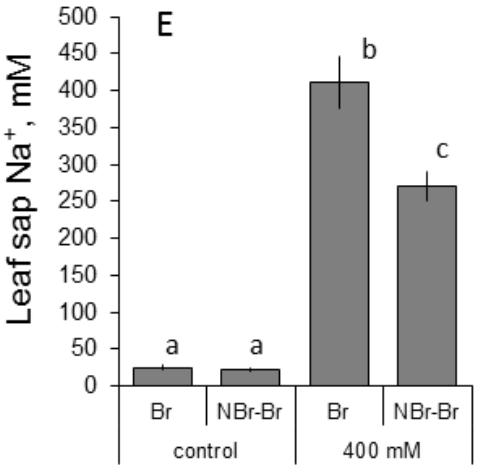
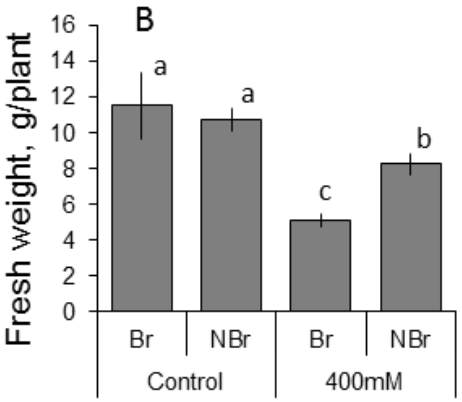
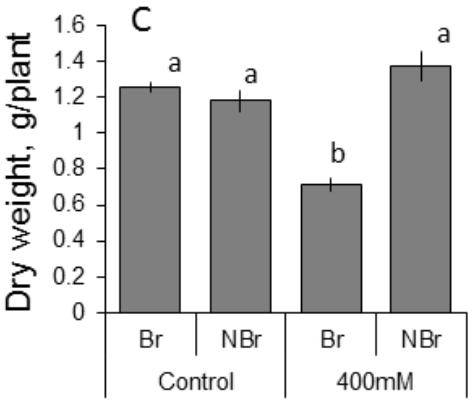


Fig 4



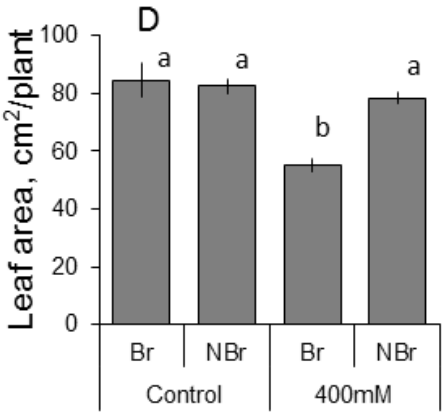
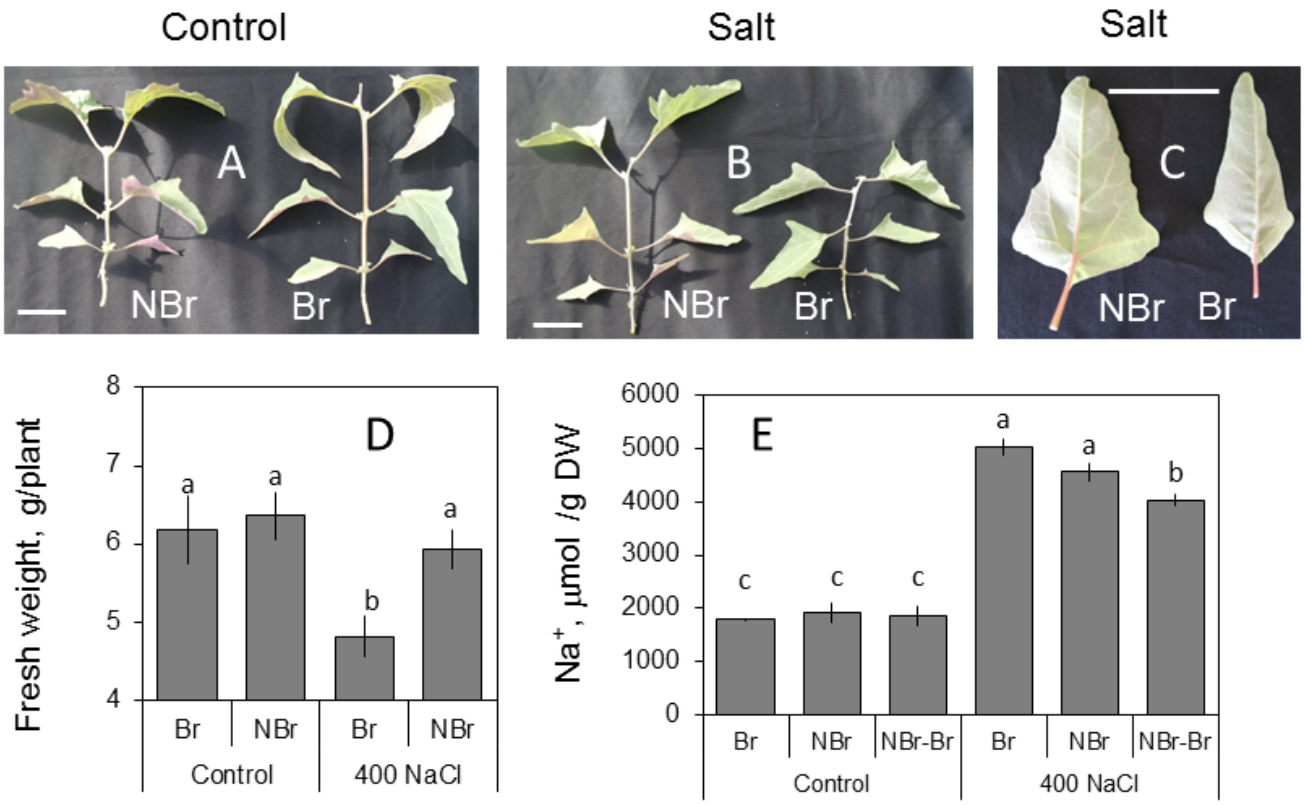



Fig 5



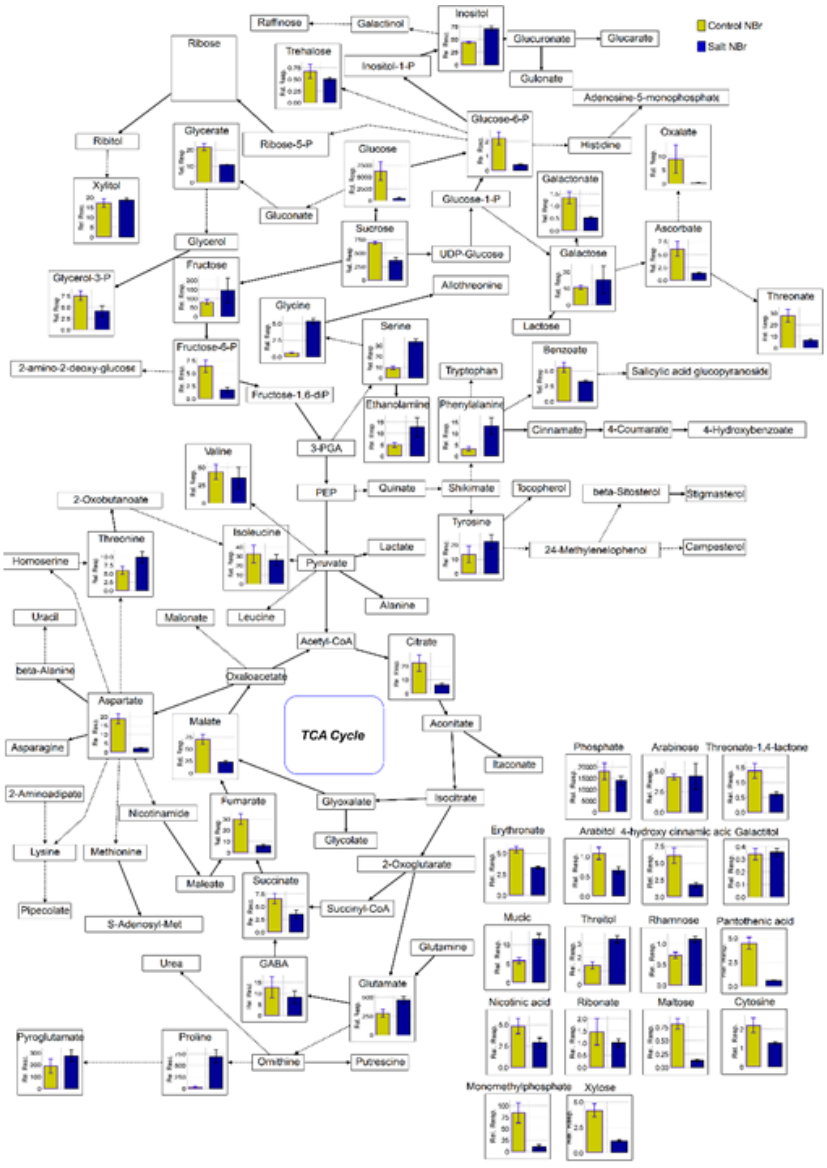
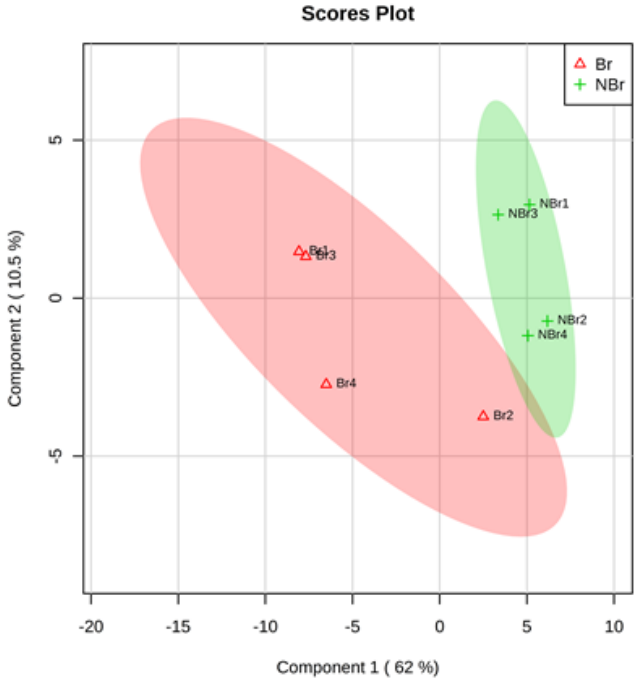



Fig 6

Fig 7



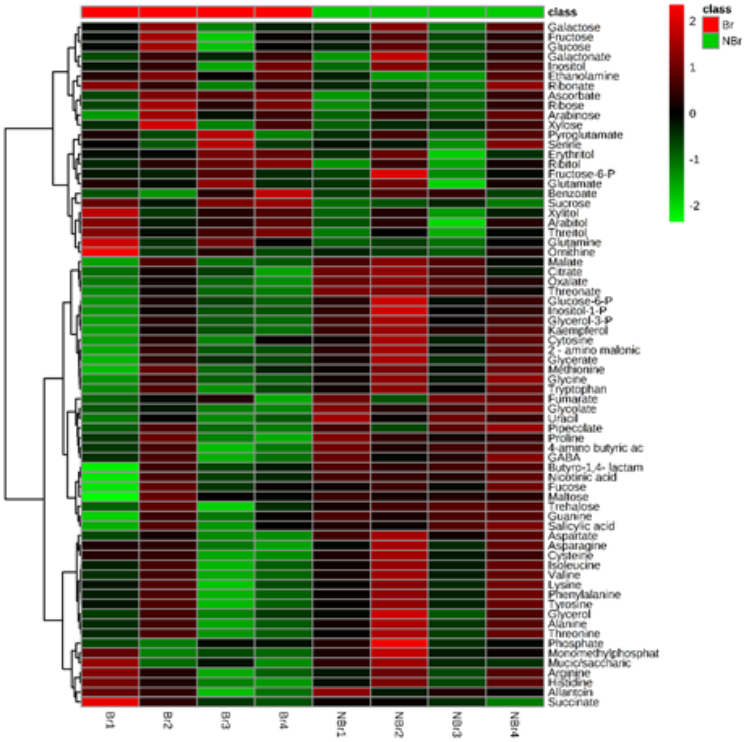



Fig 8

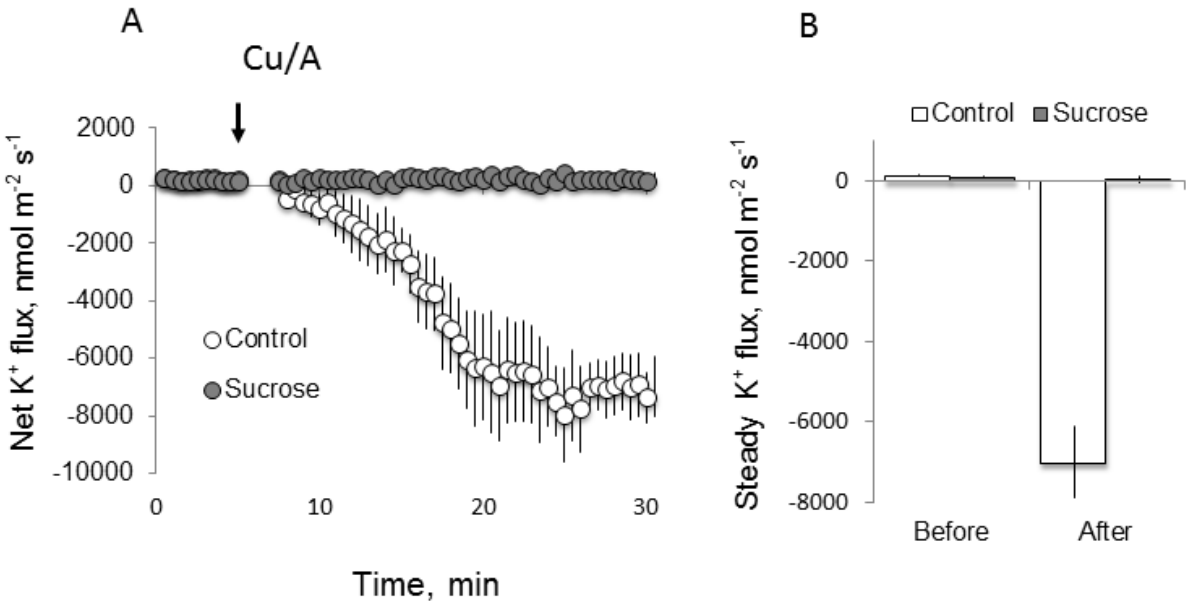
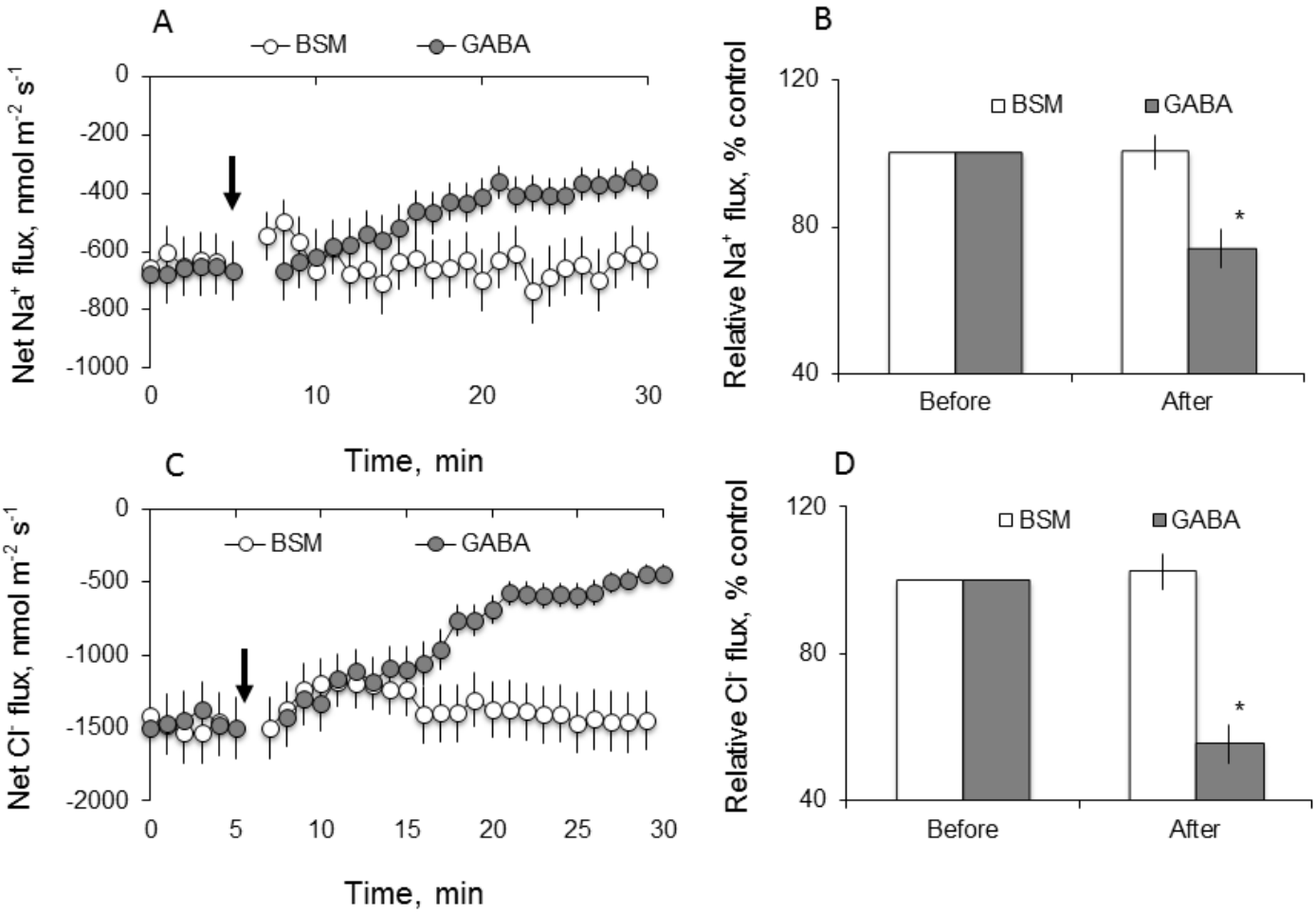


Fig 9



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