# 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 to cope with saline environment. Not much direct supporting evidence, however, was given. *Chenopodium quinoa* plants were grown under saline conditions for 5 weeks. One day prior to commencement of salinity stress EBC from all leaves and petioles were gently removed using soft cosmetic brush. Physiological, ionic and metabolic changes in brushed and non-brushed leaves were compared. Gentle removal of EBC did neither initiate wound metabolism nor affected physiology and biochemistry of control-grown plants but had a pronounced effect on salt-grown plants resulting in a salt-sensitive phenotype. Of 91 detected metabolites, more than half (50) were significantly affected by salinity. Removal of EBC has dramatically modified these metabolic changes, with the biggest differences reported for gamma-aminobutyric acid (GABA), proline, sucrose and inositol, affecting ion transport across cellular membranes (as shown in electrophysiological experiments). This work provides the first direct evidence for the role of EBC in salt tolerance in halophytes and attributes this to (1) key role of EBC as a salt dumper to externally sequester salt load; (2) improved $K^+$ retention in leaf mesophyll and (3) EBC being 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 (i.e. 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). Because of this, in lay terms they are often referred to as 'salt loving plants'. Halophytes flourish under conditions that kill 99% of crop species and thus are considered as 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 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 that was found in a large number of species from different families (Flowers *et al.* 2015; Yuan *et al.* 2016). One type of salt glands are 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 the 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 postulated 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 reserve 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 &

65 Vera-Estrella, 2015; Oh *et al.* 2015). However, most of these roles were simply postulated
66 based on circumstantial evidence and not proven in direct experiments. Therefore, the
67 question of whether EBC are essential for salinity stress tolerance remains to be answered in
68 direct experiments.

69 Until now, most studies on salt bladders were conducted using the inducible CAM plant
70 *Mesembryanthemum crystallinum*. Starting from classical CAM physiological studies by
71 Winter and co-authors in 70ies (e.g. Winter, 1973), following research has provided a
72 significant conceptual advance in our understanding of various aspect of cell-specific
73 regulation under saline conditions (Barkla *et al.* 2012; Barkla & Vera-Estrella, 2015; Oh *et al.*
74 2015). However, from both physiological and biochemical point of view, *M. crystallinum*
75 plants are rather unique and have some features (e.g. a transition from C3 to CAM
76 metabolism under stress conditions and a pronounced succulency, Adams et al 1992, 1998)
77 that are hardly found in any crop species. When wild type and *M. crystallinum* mutant
78 lacking EBCs were confronted with salinity stress, the EBC mutant showed a significant
79 impairment in seed yield. However, this impairment was not due to inability of the
80 bladderless mutant to sequester salt in EBC as, contrary to expectations, shoots of wild-type
81 plants had approximately 1.5-fold higher $Na^+$ and $Cl^-$ content than the mutant under saline
82 conditions (Agarie *et al*., 2007). *De facto*, these findings questioned the role of EBC as
83 external storage space for the salt load. Thus, being extremely interesting, these observations
84 still do not prove a direct role for salt bladders as a component of the salt tolerance
85 mechanism. Moreover, given the above unique physiological and anatomical features of *M.*
86 *crystallinum*, it is rather difficult to translate these findings into breeding concepts for salt
87 tolerance in traditional crops.

88 Over the last decades our research focus was on *Chenopodium quinoa*. This
89 recretohalophyte C3 species advanced to a pseudo-cereal plant of a high economic value that

possesses a combination of highly orchestrated physiological traits conferring 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). The simple anatomy of *C. quinoa* EBC complex makes it also an ideal model to study 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 mechanism, while old leaves retain $Na^+$ in leaf cell vacuoles (Bonales-Allatorre *et al.* 2013a). In this study, we used *C. quinoa* plants to provide direct evidence for the role of EBC in salinity stress tolerance. Our underlying working hypothesis was two-fold. First, we assumed that EBC operates as external $Na^+$ storage and their removal should increase the salt load in the leaf lamina, affecting plant performance under saline conditions. Second, we hypothesised that EBC may preferentially accumulate some metabolites known for their ability to modulate plant ionic relations. Indeed, it was shown earlier that some of so-called "compatible solutes" may in fact possess a strong ability to block ion channels mediating plant ionic homeostasis (e.g. the role of choline in vacuolar $Na^+$ sequestration originating 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). Some compatible solutes are also known to possess an ability to scavenge reactive oxygen species (ROS; Peshev *et al*., 2013; Smirnoff and Cumbes, 1989) and thus, if accumulated in high concentration, may potentially prevent ROS-induced changes  activation of the broad range of $Na^+$, $K^+$ and $Ca^{2+}$ permeable ion channels (see Demidchik and Maathuis, 2007 for a review).

Here we show that removing EBC results in a salt-sensitive growth phenotype. We also show that when exposed to salinity stress, *C. quinoa* undergoes a significant shift in its

115 metabolite profile, and that removal of EBC impacts metabolite homeostasis in the leaf
116 lamina. Comparing salt induced metabolic and ionic changes, we discuss transporters likely
117 involved in ion sequestration in EBC.
118
119 **Materials and Methods**
120 ***Plant materials and growth conditions***

121 Three plant species, quinoa (*Chenopodium quinoa* Willd), its close relative *Chenopodium*
122 *album* L., and *Atriplex lentiformis* L. were used in this study. Quinoa seeds were a gift from
123 Prof SE Jacobsen (University of Copenhagen, Denmark). Atriplex seeds were obtained from
124 Wildseed Tasmania (Sorrel, Australia), and *C. album* seeds were obtained from Rühlemann's
125 Kräuter & Duftpflanzen (Horstedt, Germany). Plants were grown from seeds in 20 cm
126 diameter pots filled with standard potting mix (Chen *et al.* 2007) under temperature-
127 controlled glasshouse conditions (mean day/night temperatures 26/20 °C; humidity 65%; day
128 length 15 h) at the University of Tasmania in Hobart, Australia, between November 2015 and
129 March 2016. Ten seeds were sown in each pot and then thinned to leave 4 uniform plants per
130 pot. Experiments were organised in a completely randomised design, with each treatment
131 including at least four pots (with four plants in each). Of these 6 to 8 uniform plants were
132 later selected for sampling. All experiments were replicated three times, with consistent
133 results.
134
135 ***Experiments with intact plants (Experiment 1)***

136 Quinoa plants were grown for 5 weeks under control conditions. One day prior to
137 commencement of salinity stress EBC of all leaves were gently removed from both sides of
138 the leaves using soft cosmetic brush (Fig. 1A, B). Also removed were all EBC on the stem

and petioles. Plants were then irrigated with 400 mM NaCl for 5 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 each 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 non-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 the treatments (~ 5 weeks for *C. quinoa* and *C. album* and ~8 weeks for *A. lentiformis*). At a seven-leaf stage, the shoot apex was excised (chopped off), leaving six leaves remained on the plant's shoot (Fig. 1C). Then EBC of all remained leaves were gently brushed from both upper and lower surfaces of leaves using a soft cosmetic brush (Fig. 1B). Salinity stress was imposed then as described in the section above, and all the emerging lateral buds were removed on a regular basis keeping the constant number of leaves during the entire experiment.

***Physiological assessment***

Fresh weights of the shoot biomass were determined immediately after harvest. Dry weights of the plants were measured after drying plants in the 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 the

163 measurements were carried out on the mid-portion part of the topmost leaves in chopped
164 plants and on the young fully expanded leaves in non-chopped plants. For plant nutrient
165 analysis, two types of measurements were conducted. In the first one, approximately 0.1 g of
166 dry matter was added to 7 ml of nitric acid and microwave-digested for 15 min. The digested
167 material was diluted to a final volume of 15ml, and leaf $Na^+$ and $K^+$ content was measured
168 using Flame Photometer (PFP7, Jenway, UK). In the second set of measurements, a freeze-
169 thaw method (Cuin *et al.* 2009) was used. Appropriate leaves were collected, placed in
170 Eppendorf tubes and immediately placed in the freezer (-18$^o$C). Before measurements, the
171 samples were thawed, and samples were hand squeezed to extract all the sap. The collected
172 sample was thoroughly mix and measured for its $K^+$and $Na^+$ concentration (in mM per water
173 basis) using flame photometry as described above. Chloride concentration in the squeezed
174 samples was measured using Cl-selective microelectrodes using the MIFE system (see details
175 below).

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

180 ***Metabolite extraction***

181 For each sample, approximately, 100 mg of leaf was harvested and immediately frozen in
182 liquid nitrogen and stored at -80$^o$C until freeze-drying that was carried out using Alpha 1-2
183 LDplus (Martin Christ, Osterode, Germany). Aliquots (10mg) of homogenized, freeze-dried
184 leaf material were transferred to Eppendorf tubes and accurate weights recorded. Methanol
185 (MeOH, 500 µL) containing the internal standards (D-Sorbitol-$^{13}C_6$ (0.02 mg/mL) and L-
186 Valine-$^{13}C_5$,$^{15}N$ (0.02 mg/mL), Sigma Aldrich (Australia), was added to the sample tubes.
187 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 folds with 50% MeOH, and aliquots of 100µL from the 10 fold diluted supernatant were transferred to clean Eppendorf tubes and dried in 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 (Student 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

238 previous publications (Shabala *et al*., 2006; Chen *et al*., 2007). Commercially available liquid
239 ion exchangers $K^+$, $Na^+$ and $Cl^-$ cocktails were used (catalogue number 60031, 71747 and
240 24902, respectively; both from Sigma-Aldrich, St Louis, MO, USA). Youngest fully mature
241 quinoa leaves were harvested from 5 weeks old plants that were grown under ambient light in
242 a temperature-controlled glasshouse (between 19°C and 26°C and average humidity of
243 approximately 65%) at the University of Tasmania. Seeds were sown in 2-L plastic pots filled
244 with standard potting mix and irrigated with either water (for mesophyll measurements) or
245 100 mM NaCl (for stalk cell measurements).

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

256 For $Cl^-$ and $Na^+$ flux measurements in stalk cells, the petioles of youngest fully mature
257 quinoa leaves were excised and immobilised in the measuring chamber containing 4 mL of
258 BSM (0.5 mM KCl, 0.1mM CaCl2, pH 5.5) for 1.5 h prior to the measurements. Ion fluxes
259 were then measured under control conditions for 5 min and 5 mM GABA was applied by
260 pipetting and mixing the required volume of stock solutions into the bathing solution. For
261 mock controls, the same amount of BSM solution was added to the chamber.
262

## Results

### *Gentle removal of EBC does not affect 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. The mechanical disturbance to plant tissues may activate a broad array of mechano-sensing channels (Monshausen & Haswell, 2013) and thus could result in significant changes in the growth patterns of plant organs, in a process termed thigmomorphogenesis (Coutand, 2010).

In our hands and in the absence of salt stress, however, 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 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 ($P > 0.05$) were net $CO_2$ assimilation rates (Pn; Fig 2F) and stomatal conductance (Gs; Fig 2 G) between brushed and non-brushed leaves. Thus, the above visual difference is most likely explained by different light reflection properties from the leaf surface (albedo effect). We also compared leaf $K^+$ content between treatments. With the major cationic osmolyte $K^+$, no statistically significant difference in leaf content was found between brushed and non-brushed leaves (Fig. 2E). To study brushing effects on the leaves' metabolism, we monitored the metabolic profiles using GC-MS (Table S1) but did not find significant ($P < 0.05$) altered levels of amino acids, sugars and 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 bulk of leaf being disturbed. Therefore, under control conditions, removal of salt bladders by a gentle brushing of the leaf surface seem not induce any damage or caused 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 way” by rubbing the leaf surface with fingers. Such removal has resulted 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 the gentle brushing became visible, however, for plants grown under saline (400 mM NaCl for 5 weeks) conditions (Fig. 3) showing significantly ($P < 0.05$) smaller biomass (Fig. 3C).

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

We then tested how does a plant respond, or - with bladders removed from developing leaves, or - that were not allowed growing young leaves. The rationale behind this study was that in intact plants removal of EBC might potentially impact formation and development of

new leaves, while in decapitated plants this developmental aspect was eliminated. To answer this question, the shoot apex from 4-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 with 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 dumpers (e.g. 30% increase; Fig. 4E).

To confirm the role of bladders, we performed brushing experiments with another halophyte species having rather dense EBC (Fig. S3), *Atriplex lentiformis*. As with *C. quinoa* observations, removal of EBC has 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 have used *C. album*, a close relative of *C. quinoa*. The ecotype we selected had only very few EBC on the leaf surface (and only in very young leaves; Fig S3) and, therefore, cannot rely on $Na^+$ sequestration in EBC as a dominant tolerance mechanism. As expected, brushing of the leaf surface of bladderless *C. album* has not resulted in 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 in addition, they will also need to synthesize metabolites that serve as compatible solutes to compensate in the cytoplasm the difference towards the increase in vacuole $Na^+$ 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 present grown in control and salt treatments (Table 1). Of those, more than half metabolites (50) were statistically significantly affected upon salt treatment (based on Student 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, including threitol (2.36 fold), rhamnose (1.52 fold), inositol (1.61 fold). Three sugars acids and one sugar alcohol decreased, including erythronate (-1.67 fold), threonate (-4.13 fold), galactonate (-2.54 fold) and arabitol (-1.65 fold). Two sugar phosphates decreased, including 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 leaves were compared to their bladder baring counterparts pronounced changes were observed (Tables S2, Figs 7 A and B). Using GC-MS, we have identified 11 known and 5 unknown metabolites which were significantly

362 different in salt-treated leaves with EBC removed (Table S2) when compared to salt treated
363 intact leaves. Interestingly, all significantly changed metabolites decreased in leaves with
364 EBC removed, which we confirmed with quantitative GC-MS and LC-MS assays: GABA
365 content dropped from 1.5 ± 0.19 to 0.5 ± 0.17 mM (-3 fold), proline and inositol from 2.75 ±
366 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),
367 respectively. At the same time, sucrose content increased from 0.27 ± 0.01 mM in samples
368 with EBC to 0.86 ± 0.27 mM (3.19 fold). Besides the latter metabolites GC-MS analysis
369 revealed relative changes for another amino acids; aspartate (-2.82 fold), four organic acids;
370 citrate (-2.59 fold), glycolate (-1.72 fold), oxalate (-6.25 fold) and threonate (-3.68 fold).
371 Only two sugar phosphates decreased; glycerol-3-phosphate (-2.28 fold) and inositol-1-
372 phosphate (-2.17 fold). Threitol was the only sugar which increased in salt-treated leaves with
373 EBC removed compared to intact salt-treated leaves (1.77 fold). There was also an in
374 decrease of kampferol (0.33 fold) and uracil (0.29 fold).

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

386

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

We have next tried to establish a causal relationship between observed changes in a leaf metabolic profile and plant ionic relations. Accordingly, we have hypothesised that changes in some of above metabolite concentrations caused by brushing may be essential to control transport of ions across cellular membranes in leaf mesophyll and maintain cytosolic $K^+/Na^+$homeostasis. We have 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)). Addition of the hydroxyl radical-generating Cu/ascorbate mix to leaf mesophyll has resulted in a massive $K^+$ efflux across the plasma membrane (Fig 8A; open symbols). Pre-treating leaf with exogenously applied 8 mM sucrose (mimicking increase in sucrose level in brushed cells) has completely mitigated this ROS-induced $K^+$ efflux (Fig 8 A, B) thus improving cytosolic $K^+/Na^+$ ratio.

We then studied effect of GABA on ion loading into EBC by looking at its impact on $Na^+$ and $Cl^-$ transport from 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 has 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 was

mostly circumstantial. We show here that a gentle removal of EBC that did not cause thigmomorphogenic responses (Kamano *et al.* 2015; Moulia *et al.* 2015) results in a salt-sensitive phenotype, providing the first direct evidence for the 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 resulted 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 plant responses to salinity stress (Fig. S4). This indicates that the presence of EBC increase chenopods 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 young leaves used in our study was ~80 μm, and cell density was about 85 cell per $mm^2$ (Fig. S5), 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. At the same time, leaf lamina thickness was ~ 120 μm, making a corresponding volume of the leaf lamina 0.12 $\mu L^3$. This makes the ratio between the volume of EBC and the volume of the leaf lamina in quinoa leaves to reach 1/3, with EBC representing about 25% of the total aerial

437 volume. At the same time, the difference in $Na^+$ sap concentration in the leaf lamina between
438 brushed and non-brushed leaves was about 150 mM (Fig. 4E). This allows to estimate the
439 $Na^+$ concentration of quinoa EBC being around 850 mM. A similar calculation made for
440 chloride results in the estimated $Cl^-$ concentration in EBC being around 1M (Fig 3E).
441
442 ***Salt dumping in EBC may cost plants less compared with intracellular sequestration***
443 In mechanistic terms, EBC may be considered as "inverted vacuoles" (Shabala *et al.* 2014a).
444 However, the carbon cost of internal and external sequestration mechanisms may be different
445 and should be considered. As cell volume is proportional to the 3rd power of diameter while
446 the surface area – to the 2nd power, the volume to area ratio is increasing with increased cell
447 diameter (Table S3). This implies that the carbon cost related to the formation of the cell wall
448 decreases as the cells become bigger (a 10-fold increase in efficiency for a 10-fold increase in
449 diameter). Thus, assuming both epidermal and mesophyll cells possess the same set of
450 transporters for $Na^+$ sequestration, the carbon cost will be an order of magnitude lower in
451 EBC. Given that plants need to allocate a substantial amount of carbon for *de novo* synthesis
452 of compatible solutes for osmotic adjustment under saline conditions (Flowers *et al.* 2015;
453 Flowers & Colmer, 2015), the ability to reduce the amount of carbon for cell wall deposits
454 may be a critical factor conferring salinity stress tolerance at the whole-plant level. This
455 suggestion is fully consistent the generalised energy balanced model proposed by Munns and
456 Gilliham (2015) showing that stress tolerance mechanisms represent additional costs to the
457 plant required to deal with the salt load in the soil, and at high salinity there will be zero
458 growth, as the total costs to the plant equal energy gain.
459
460 ***Removing EBC compromises leaf $K^+$ retention ability***

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

473 The loss of mesophyll $K^+$ retention ability may be also causally related to changes in
474 the leaf metabolic profile (discussed below) and, specifically, the difference in oxalate
475 content (6-fold lower in brushed leaves compared with intact counterparts; Table 1). Earlier
476 Jou et al. (2007) have suggested that in *M. crystallinum* plants calcium oxalate crystals
477 present in EBC can serve as a regulatory site for intracellular $K^+$. According to this
478 suggestion, $K^+$ is remobilised from the crystals to increase cytosolic $K^+$ concentration in
479 nearby leaf mesophyll cells under conditions of reduced $K^+$ uptake and compromised leaf $K^+$
480 retention (under saline conditions).

481

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

483 Salt treatment of intact leaves induced a strong shift in the metabolite profile when compared
484 to untreated intact leaves (Table 1, Fig. 6) which aligns well with reported metabolite changes
485 upon salinity in halophytes (Kumari et al 2015). As described before, the 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). Opposite to reported metabolite effects we found a decrease in sucrose and glucose in salt treated intact quinoa leaves compared untreated leaves. However, reported changes did not encounter any reference to the involvement of bladders towards the metabolite changes. Only one study reported metabolic changes upon salinity in EBC's specifically prepared from *Mesembryanthemum crystallinum* which under salt also showed a decrease in most organic acids with 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 observed before 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 such as 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 as seen in our experiment (Gong *et al.* 2005). Reduction of organic acids, and in particular TCA cycle intermediates have been correlated with both decreased TCA cycle activity and increase draw on carbon structures for synthesis of compounds required for coping with the 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 been associated with osmotic stress, such as salinity, providing an increase of cellualar osmolarity and providing energy and building blocks for

511 osmoprotectants, such as inositol, and to scavenge ROS (Kumari et al 2015, Widod et al 2009,
512 Sanchez et al 2008). In most reports, sucrose, fructose and glucose have been increased after
513 salt stress in plants. However, in quinoa glucose, as well as glucose-6-phosphate, decreased
514 massively under salt treatment down to 7% of the levels in control leaves, which may indicate
515 increased consumption of glucose through glycolysis or as a building block of salt-response
516 carbohydrates or glycoproteins. For instance, ribose and inositol, which derive from glucose-
517 6-phosphate, which also decreased significantly, were strongly increased. Similarly, glycine,
518 serine and ethanolamine, which derive from 3-phosphoglyceric acid, and tyrosine and
519 phenylalanine, which derive from phosphoenolpyruvate, all significantly increase (Fig. 6).
520 Ethanolamine is a precursor for the synthesis of glycine betaine, a well-known osmolyte
521 shown to increase under salinity stress in plants (Suzuki *et al.* 2003). Tyrosine and
522 phenylalanine are phenolic amino acids and are precursors of alkaloids and other secondary
523 metabolites which have been shown to accumulate in plants under salinity stress.
524
525 ***Salt metabolism in leaves that lost the ability for external salt sequestration in EBC***
526 Our analysis (Table S1) revealed that metabolite composition of intact leaves and leaves with
527 EBC removed were very similar indicating that removal of EBC has no effect on the
528 metabolite profiles of leaves when grown in control conditions. However, when plants were
529 grown in salt conditions, a number of metabolites were significantly altered when intact
530 leaves were compared to those with EBC removed (Tables S2). Supervised and unsupervised
531 clustering analysis (Figs 7A and 7B) clearly shows separations between the two treatments
532 which reflects the differences in metabolite levels. GC-MS analysis detected 16 metabolites
533 of which interestingly 15 were significantly decreased and only one was increased in the salt
534 treated leaves with EBC removed (Table S2). Here, we focus on the effect of brushing on

535 GABA- , inositol- and sucrose biology; the metabolites with known ability to regulate ionic
536 relations in plants.

537 GABA is a non-protein amino acid shown to modulate anion fluxes across the plasma
538 membrane (Ramesh *et al.* 2015). In the current work, we show that brushing EBC reduces
539 leaf GABA content by ~ 3 fold, from 1.5 to 0.5 mM, in quinoa. Given the fact that EBC
540 represent about a quarter of the total leaf volume (see above), the estimated concentration of
541 GABA in EBC should be therefore at least 10 fold higher than in brushed leaf blade, e.g.
542 about 5-6 mM. This is clearly in the physiological range for reported effects of GABA on ion
543 channels activity in plants (Shabala *et al.* 2014a, b; Ramesh *et al.* 2015; Gilliham and
544 Tyerman, 2016) and can therefore modulate salt loading into EBC, as shown in Fig 9. Indeed,
545 application of 5 mM GABA has significantly reduced the magnitude of net $Cl^-$ and $Na^+$ efflux
546 from the stalk cell (Fig 9) suggesting that increase accumulation of GABA in EBC may
547 feedback on the rate of salt loading in salt bladders. Future studies should reveal the
548 molecular nature and intracellular targets of such potential GABA targets in stalk and EBC.

549 The cyclic polyol myo-inositol is used in all organisms in many different metabolic
550 pathways. Additionally, inositol plays an important role in plant osmotic adjustment (Adams
551 *et al.* 2005). Importantly, both animal and plant studies have suggested that inositol transport
552 may be tightly coupled with transport of $Na^+$. Myo-inositol concentrations increased in salt-
553 stressed plants (Zhai *et al.* 2016), and expression of IMT (myo-inositol phosphate synthase)
554 is enhanced in response to salt stress. Nelson *et al.* (1999) hypothesized that the loading of
555 $Na^+$ into the xylem is coupled to myo-inositol transport, and that myo-inositol acts as a
556 facilitator of the $Na^+$ uptake and long-distance transport in halophytes. It remains to be
557 answered if such a mechanism may operate in EBC.

558 The last aspect that warrants the discussion is a significant (3-fold) increase in leaf
559 sucrose levels upon removal of EBC (from 0.27 to 0.86 mM). We believe that this
560 phenomenon may be explained by an increased demand for non-enzymatic ROS scavenging
561 in bladder-less leaves; this notion is strongly supported by our observations that leaf
562 mesophyll cells treated with exogenously supplied sucrose had better ability to tolerate
563 oxidative stress and retain $K^+$ in the cytosol (Fig 8A, B). Salinity stress is known to result in a
564 significant accumulation of various forms of ROS (Mittler *et al.* 2011; Bose *et al.* 2014), with
565 hydrogen peroxide, superoxide radicals, and hydroxyl radicals being the dominant ones. Of
566 these, only the first two can be handled (kept under control) by means of enzymatic
567 antioxidants. At the same time, hydroxyl radicals represent the most aggressive forms of ROS
568 (Demidchik, 2014), causing damage to key cellular structures and significantly disturbing
569 intracellular ion homeostasis (Demidchik *et al.* 2010), compromising leaf photosynthetic
570 performance (Shabala *et al.* 2016). Sugars have been proposed to play a direct role in non-
571 enzymatic antioxidant scavenging (Uemura & Steponkus, 2003; Van den Ende & Valluru,
572 2009; Foyer & Shigeoka, 2011; Stoyanova *et al.* 2011; Peshev *et al.* 2013); this is fully
573 supported by our electrophysiological data (Fig 8). When sugars are compared at the same
574 molar concentration, their greatest antioxidant capability is strongly correlated with their total
575 number of hydroxyl groups, explaining why sucrose (with eight OH groups) is better
576 compared with other sugars such as glucose and fructose (with five OH groups) (Smirnoff &
577 Cumbes, 1989). Therefore, sucrose is well-suited to protect the leaf mesophyll, when
578 hydroxyl radical production is expected to increase due to the failure of plants to load
579 excessive salt into EBC.

580 In conclusion, this work provides the explicit evidence for the important role of EBC as
581 component of salinity tolerance mechanisms in halophytes species and attribute this role to
582 several mechanisms such as EBC role as external NaCl storage space; improved $K^+$ retention

583 in leaf mesophyll; and a storage space for several metabolites known to modulate plant ionic
584 relations.

585 **Acknowledgments**

586 This work was supported by the ARC Discovery grant DP150101663 to Sergey Shabala and
587 Rainer Hedrich and by funding provided by King Saud University. Nadia Bazihizina is a
588 recipient of the Marie Curie Fellowship (grant 700001). Metabolite analyses were conducted
589 at Metabolomics Australia (School of BioSciences, The University of Melbourne, Australia),
590 a NCRIS initiative under Bioplatforms Australia Pty Ltd. The authors thank Mrs Himasha
591 Mendis of Metabolomics Australia for sample extraction, GC-MS untargeted data acquisition
592 and analysis.

593

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## 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 the 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 being removed from one half of the leaf. D – a summary of treatment and sampling protocols.

**Figure 2**. The gentle removal of EBC does not alter plant phenotype (A, B) or has 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).

**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 $P < 0.05$.

**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 $P < 0.05$.

839 **Figure 5**. Effect of EBC removal on growth and physiological characteristics of *Atriplex*
840 *lentiformis* plants. A-C – typical images of brushed and non-brushed plants grown under
841 control and salt conditions. D – shoot fresh weight; E – leaf $Na^+$ content. Br – brushed
842 leaves; NBr – non-brushed leaves; NBr-Br - leaves that were non-brushed during the salt
843 exposure but from which EBC were removed prior to elemental analysis. Data is mean ±
844 SE (n = 5 to 8). Data labelled with different low case letters is significantly different at $P <$
845 0.05.
846 **Figure 6**. Pathway map of metabolite differences between control and salt treated intact
847 quinoa leaves (with EBC present). Metabolic pathway and graphs were created using
848 VANTED (Junker et al 2006). Bars represent control (green) and salt treated (blue) (n = 5).
849 **Figure 7**. Partial Least Square Discriminant Analysis (A) and Hierarchical Cluster Analysis
850 combined with heatmap analysis (B) of untargeted GC-MS of salt treated quinoa leaves
851 with EBC present (non-brushed) and EBC removed (brushed). The shady circles in Panel
852 A indicate a 95% confidence level.
853 **Figure 8**. Effect of sucrose on $K^+$ retention in quinoa leaf mesophyll exposed to ROS. A –
854 hydroxyl radical-induced transient net $K^+$ flux kinetics measured from mesophyll cells
855 pre-treated with 8 mM of sucrose for 1.5 h prior to onset of oxidative stress. Hydroxyl
856 radicals were generated by applying 0.1/0.3 mM Cu/ascorbate mix (see Demidchik *et al*.,
857 2003 for details). The sign convention is "efflux negative". Values are means ± SE (n =
858 10). B -steady state $K^+$ fluxes in mesophyll tissues of quinoa leaves before and after (30
859 min) the addition of Cu/ascorbate mix. Values are means ± SE (n = 10). *Significant at P
860 < 0.001.
861 **Figure 9**. Effect of GABA on $Na^+$ and $Cl^-$ efflux from the stalk cells in quinoa. A – a
862 representative transient net $Na^+$ flux from the stalk cells from plants germinated and grown
863 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 were 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 is 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 the gentle brushing, EBC is dis-attached 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 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.

912 **Table 1**. Comparison of GC-MS untargeted metabolite profiles of non-brushed leaves with
913 and without salinity treatment. CNB = Control Non-Brushed; TNB = Treated Non-Brushed.
914 Data are presented as x-fold with CNB set to 1 (n=5). Blue cells indicate statistical
915 significance determined with Students t-test (P > 0.05) and green cells indicate statistical
916 significance following Benjamin-Hochberg False Discovery Rate correction.
917

| | 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** |

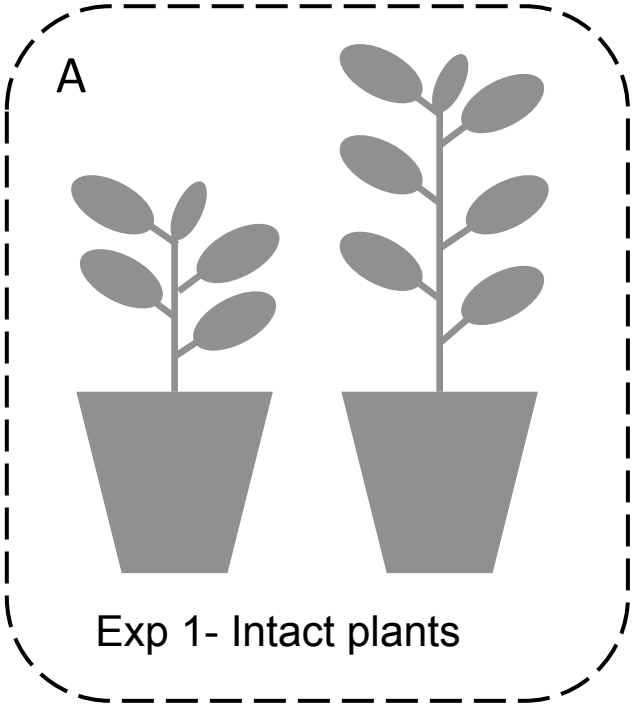
| | | | | | | |
|---|---|---|---|---|---|---|
| **Ribonate** | 1.000 | ± | 0.371 | -1.456 | ± | 0.168 |
| **Ribose** | 1.000 | ± | 0.072 | 2.000 | ± | 0.431 |
| **Sucrose** | 1.000 | ± | 0.035 | **-1.927** | ± | **0.147** |
| **Threitol** | 1.000 | ± | 0.184 | **2.366** | ± | **0.082** |
| **Threonate** | 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 |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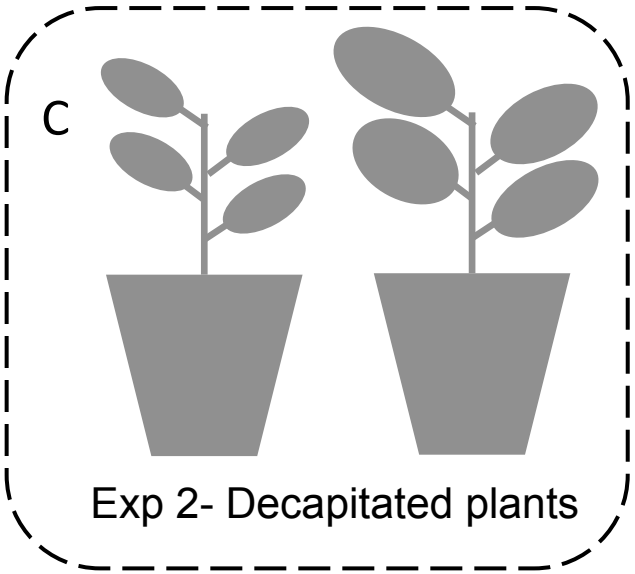
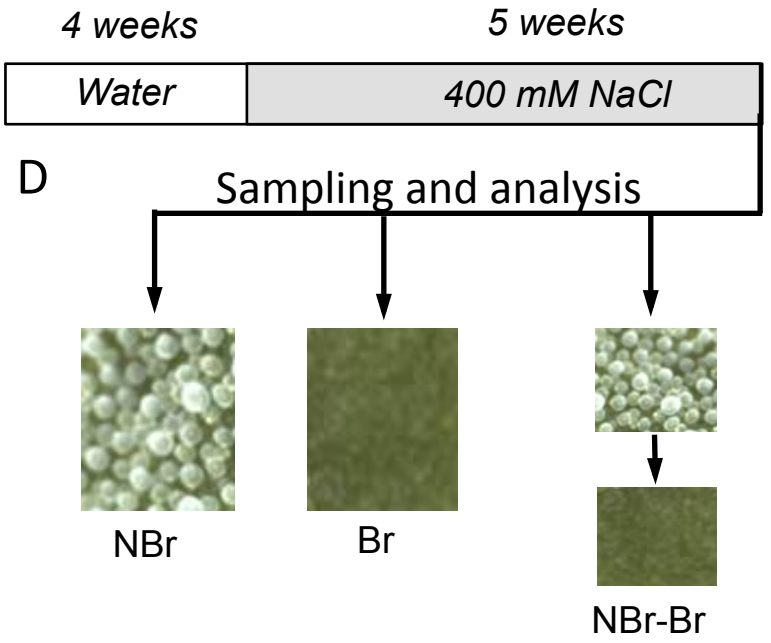
Exp 1- Intact plants


B


C
Exp 2- Decapitated plants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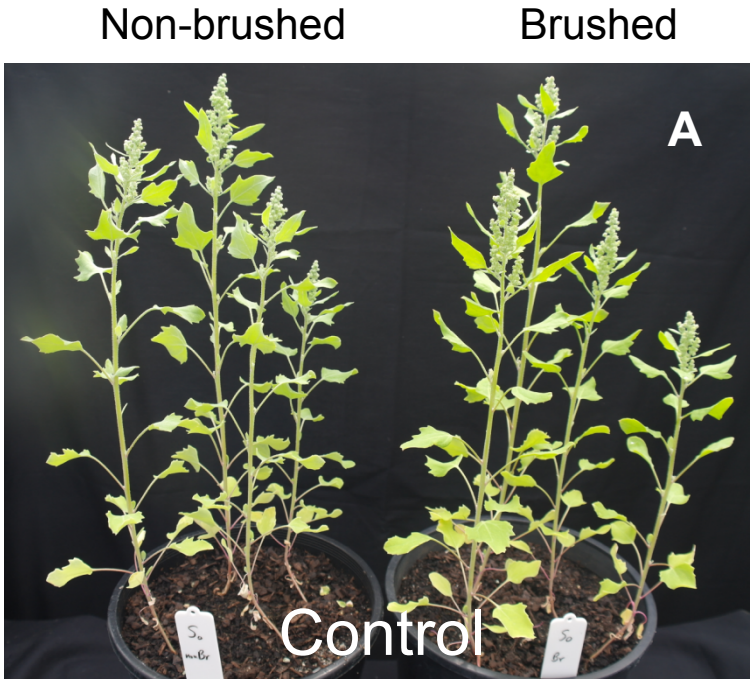
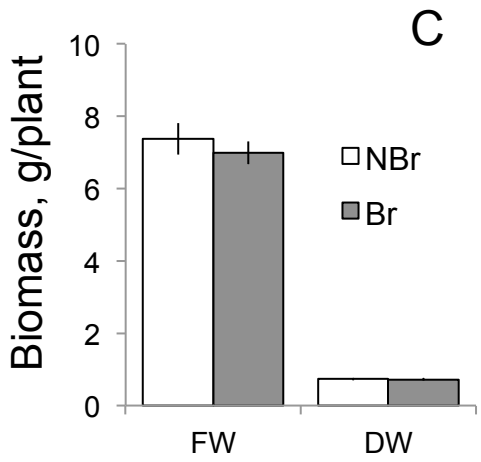
5 weeks
Water
400 mM NaCl
D
Sampling and analysis
NBr
Br
NBr-Br


Non-brushed
Brushed
A
Control


Non-brushed
Brushed
B
Control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Biomass, g/plant
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6
4
2
0
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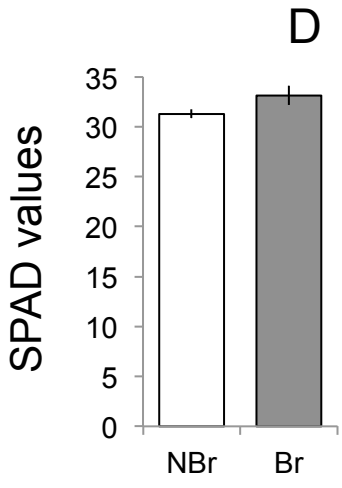
Br
FW
DW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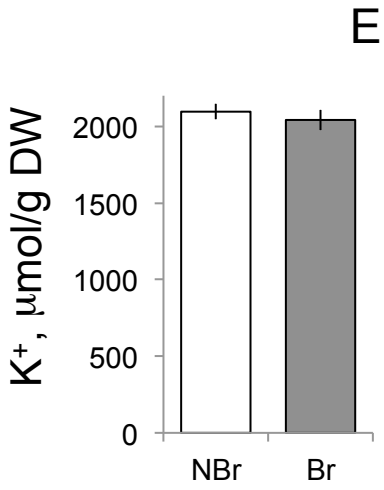
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K+, µmol/g DW
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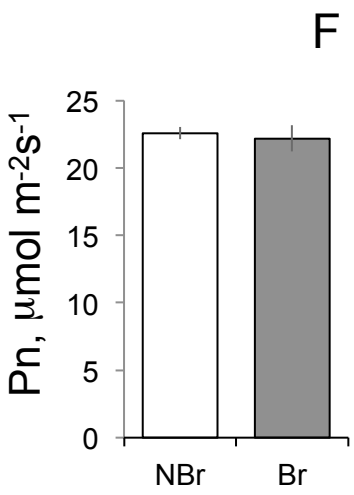
1500
1000
500
0
NBr
Br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Pn, µmol m-2s-1
25
20
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NBr
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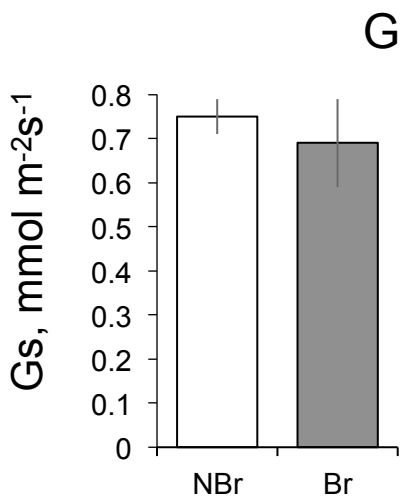
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Gs, mmol m-2s-1
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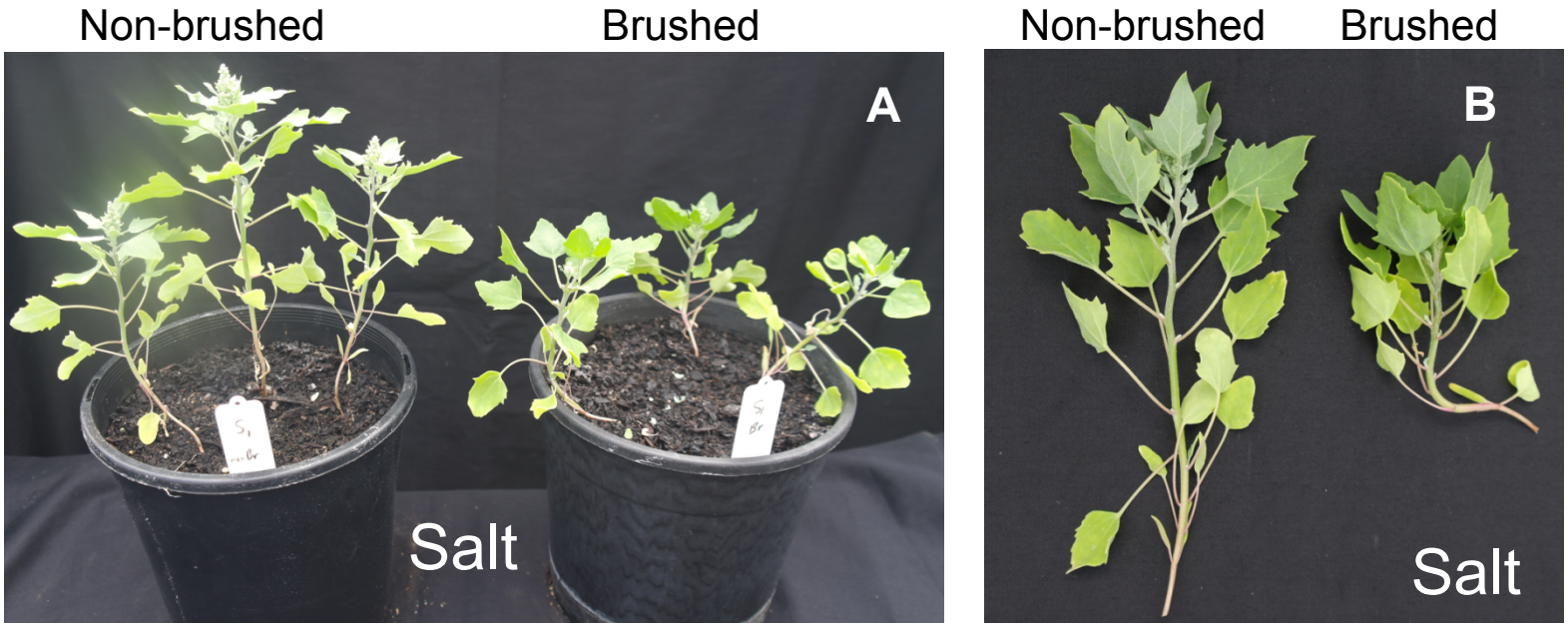
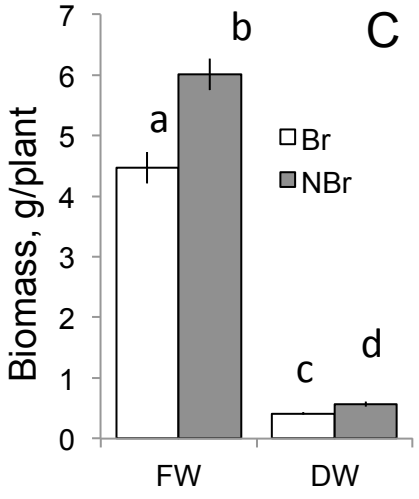
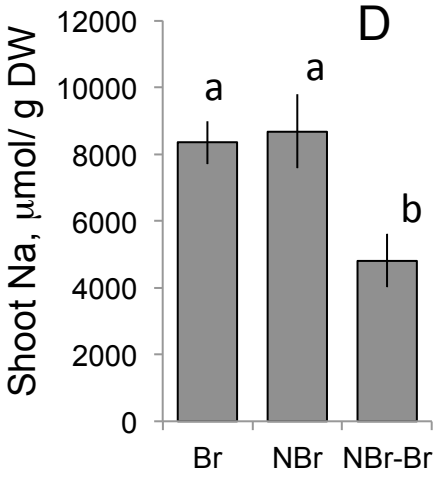
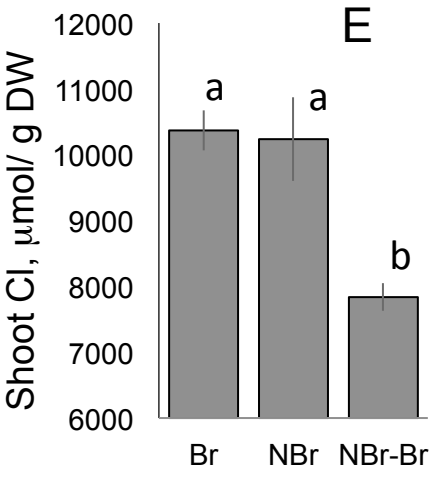
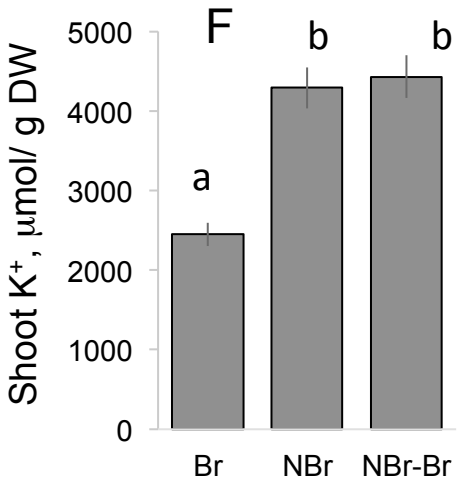
0.3
0.2
0.1
0
NBr
Br

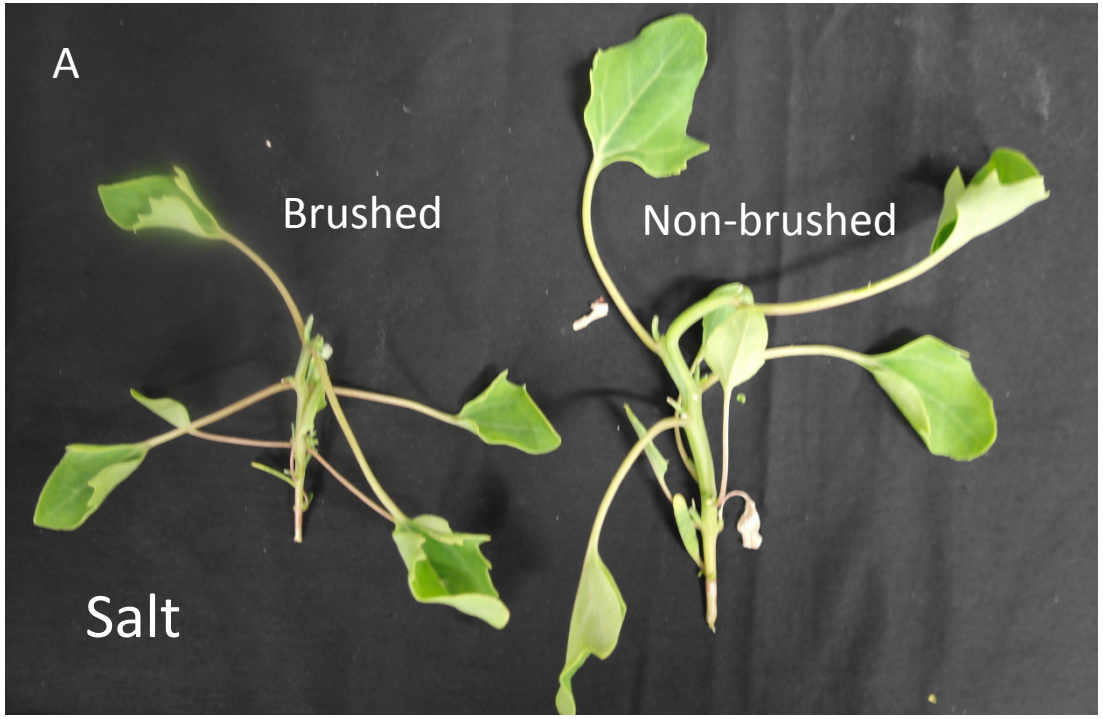

Non-brushed
Brushed
A
Salt
Non-brushed
Brushed
B
Salt

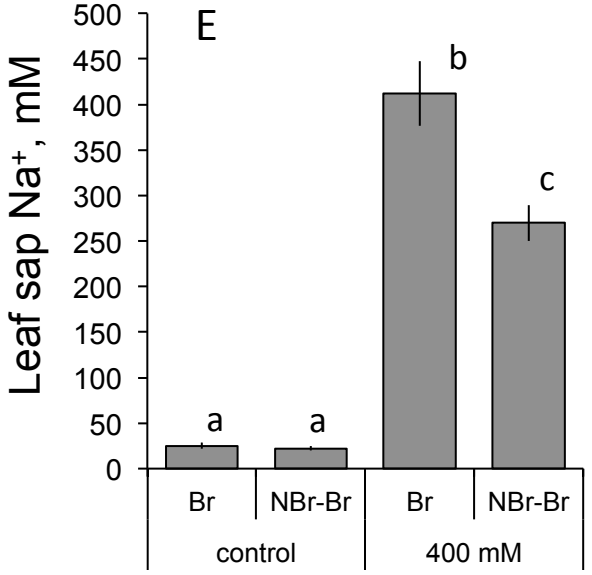

C
Biomass, g/plant
0
1
2
3
4
5
6
7
a
b
c
d
Br
NBr
FW
DW

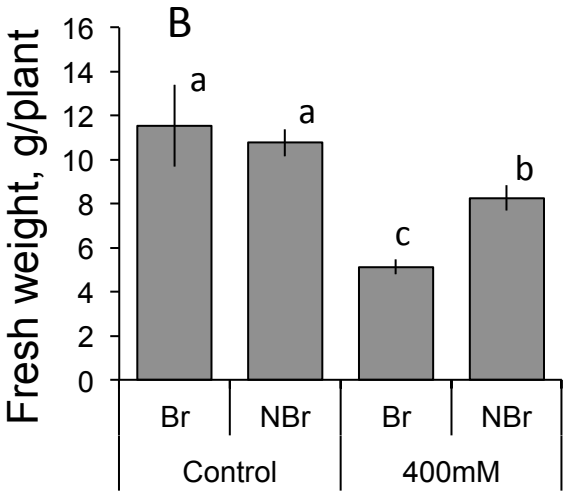

D
Shoot Na, µmol/ g DW
0
2000
4000
6000
8000
10000
12000
a
a
b
Br
NBr
NBr-Br

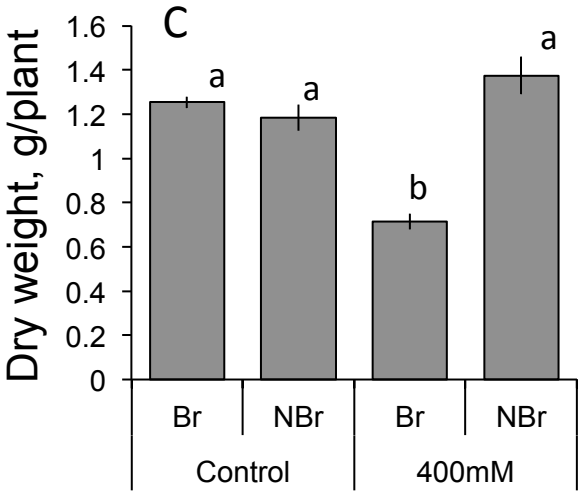

E
Shoot Cl, µmol/ g DW
6000
7000
8000
9000
10000
11000
12000
a
a
b
Br
NBr
NBr-Br

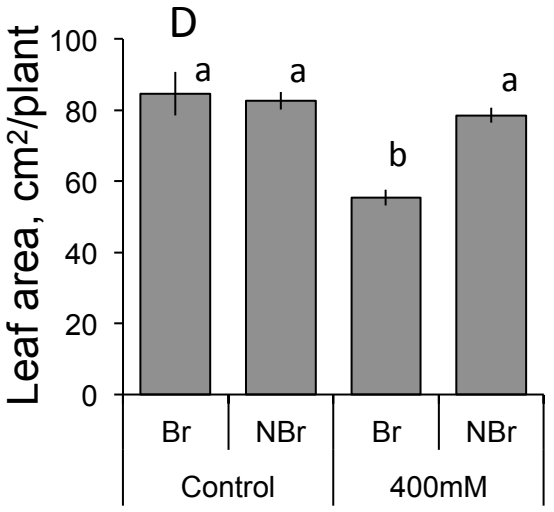

F
Shoot K+, µmol/ g DW
0
1000
2000
3000
4000
5000
a
b
b
Br
NBr
NBr-Br

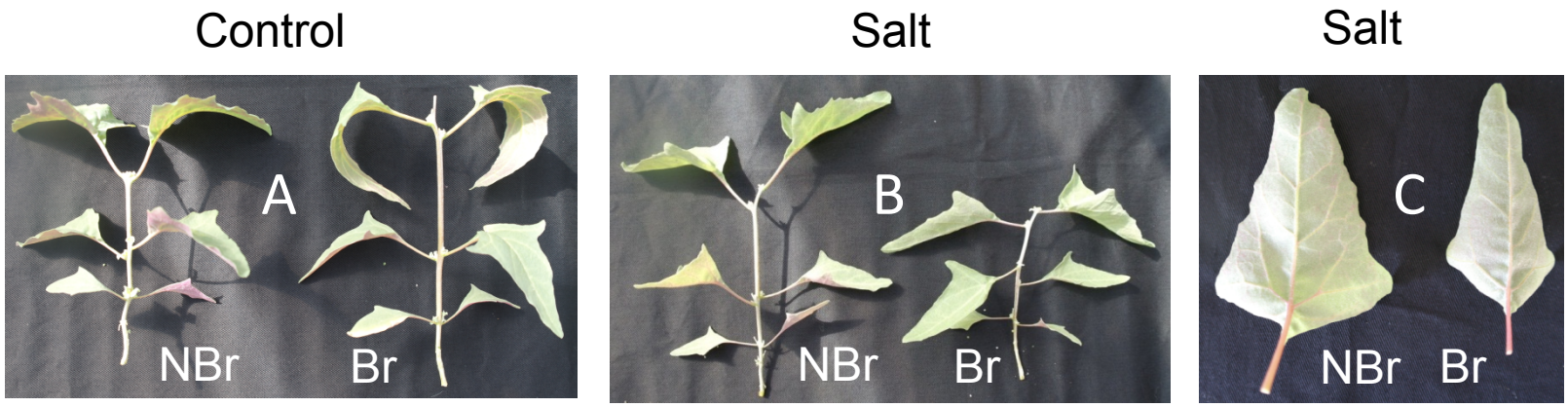

A
Brushed
Non-brushed
Salt

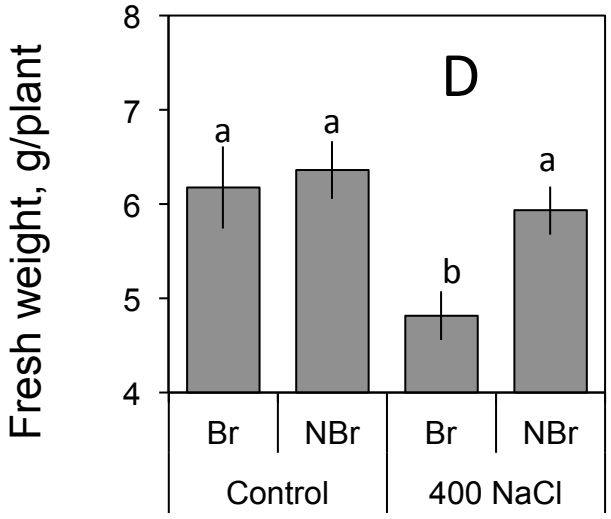

E
Leaf sap Na+, mM
500
450
400
350
300
250
200
150
100
50
0
a
a
b
c
Br
NBr-Br
Br
NBr-Br
control
400 mM


B
Fresh weight, g/plant
16
14
12
10
8
6
4
2
0
a
a
c
b
Br
NBr
Br
NBr
Control
400mM


C
Dry weight, g/plant
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
a
a
b
a
Br
NBr
Br
NBr
Control
400mM


D
Leaf area, cm2/plant
100
80
60
40
20
0
a
a
b
a
Br
NBr
Br
NBr
Control
400mM


Control
Salt
Salt
A
B
C
NBr
Br
NBr
Br
NBr
Br


Fresh weight, g/plant
D
8
7
6
5
4
a
a
b
a
Br
NBr
Br
NBr
Control
400 NaCl

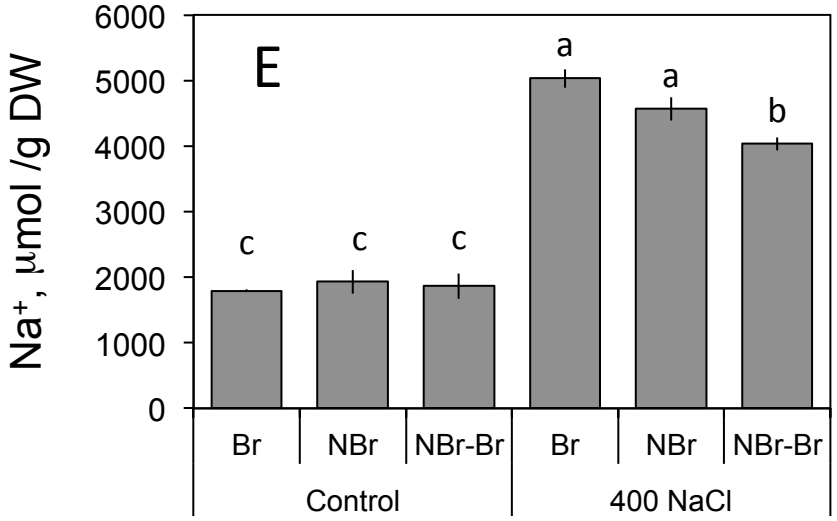

Na+, µmol /g DW
E
6000
5000
4000
3000
2000
1000
0
c
c
c
a
a
b
Br
NBr
NBr-Br
Br
NBr
NBr-Br
Control
400 NaCl

Fig 6

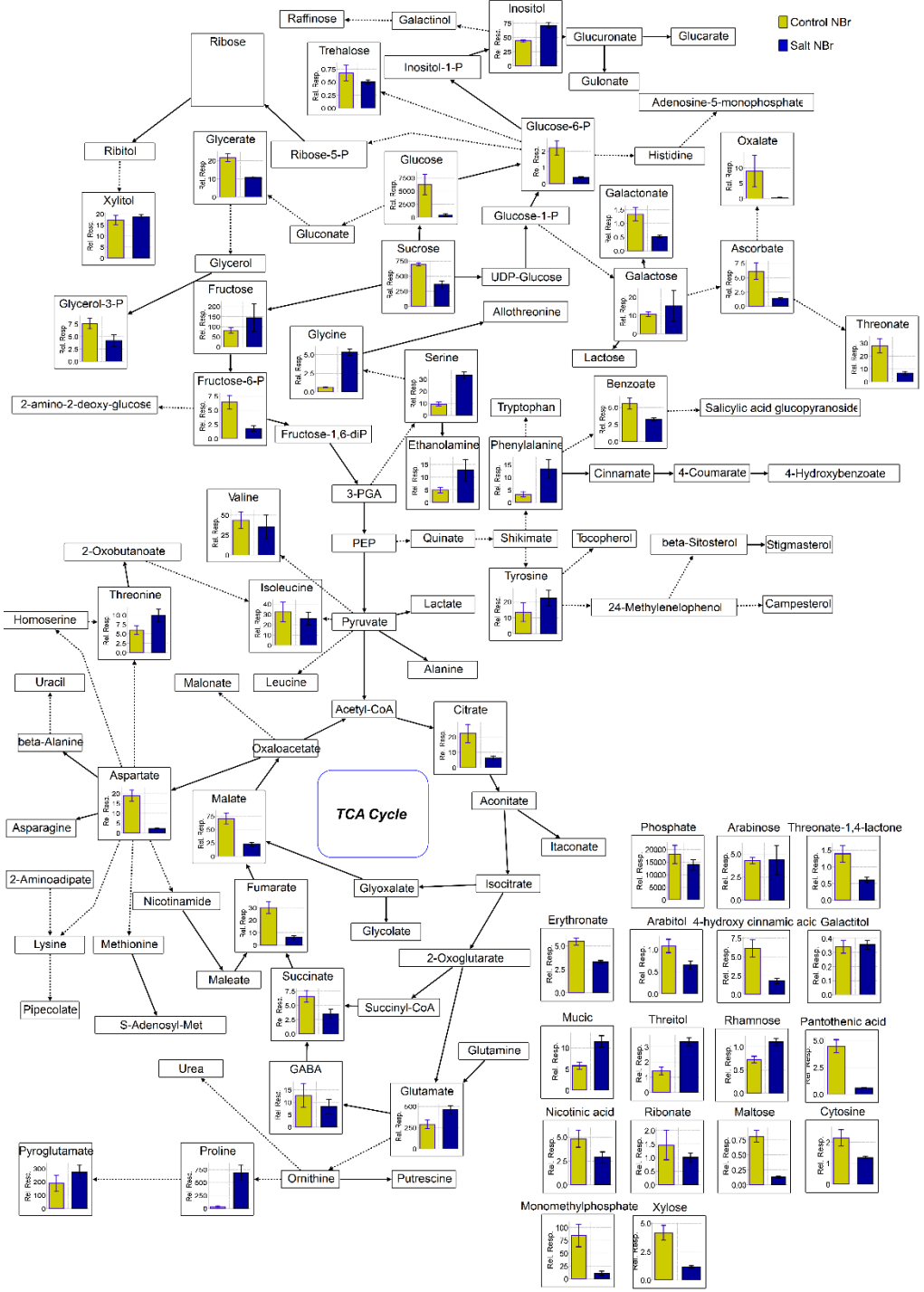
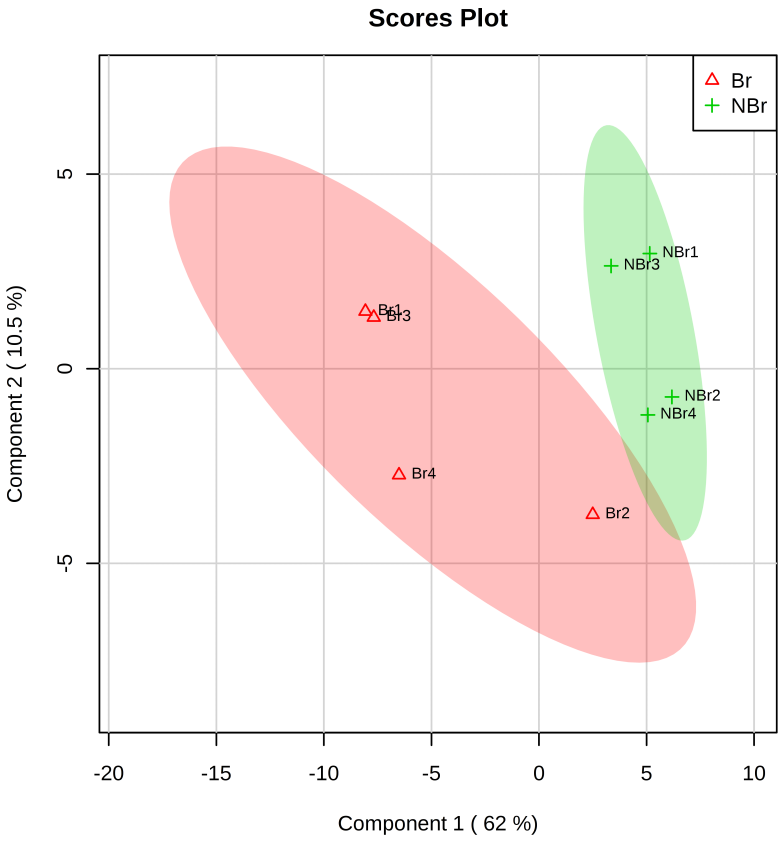
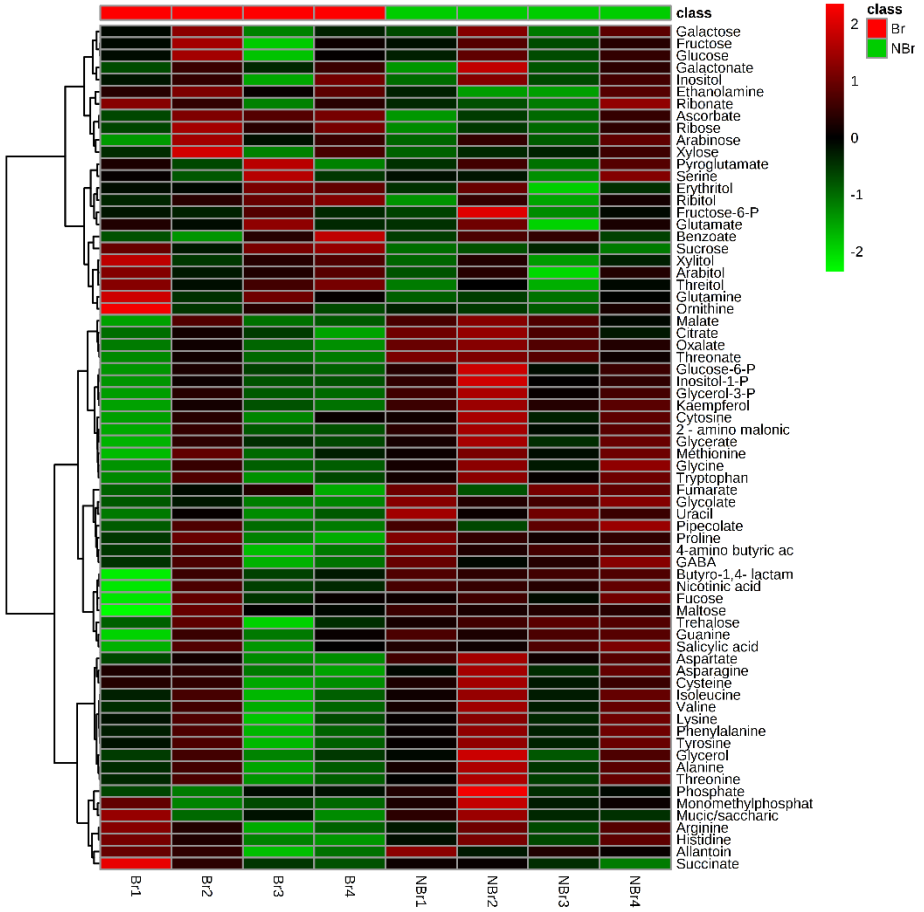
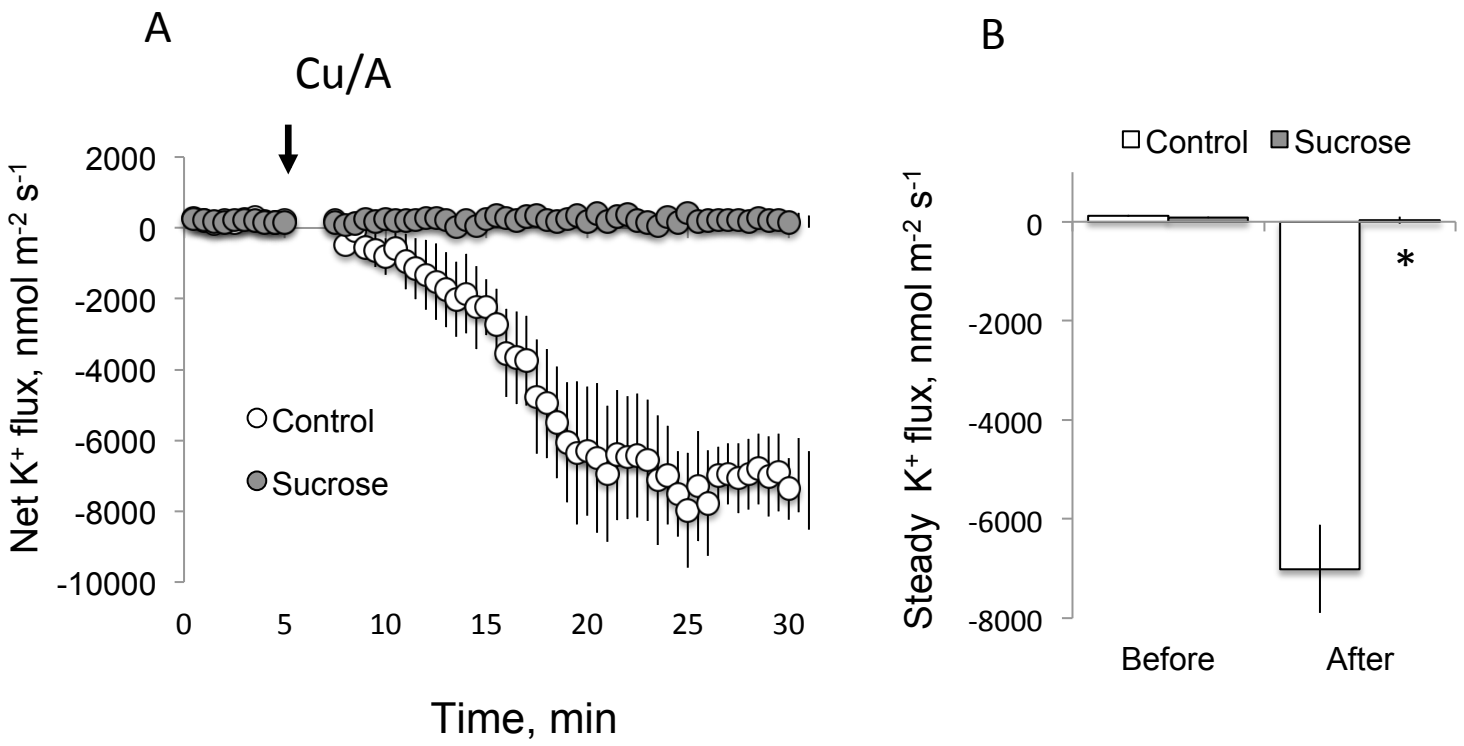
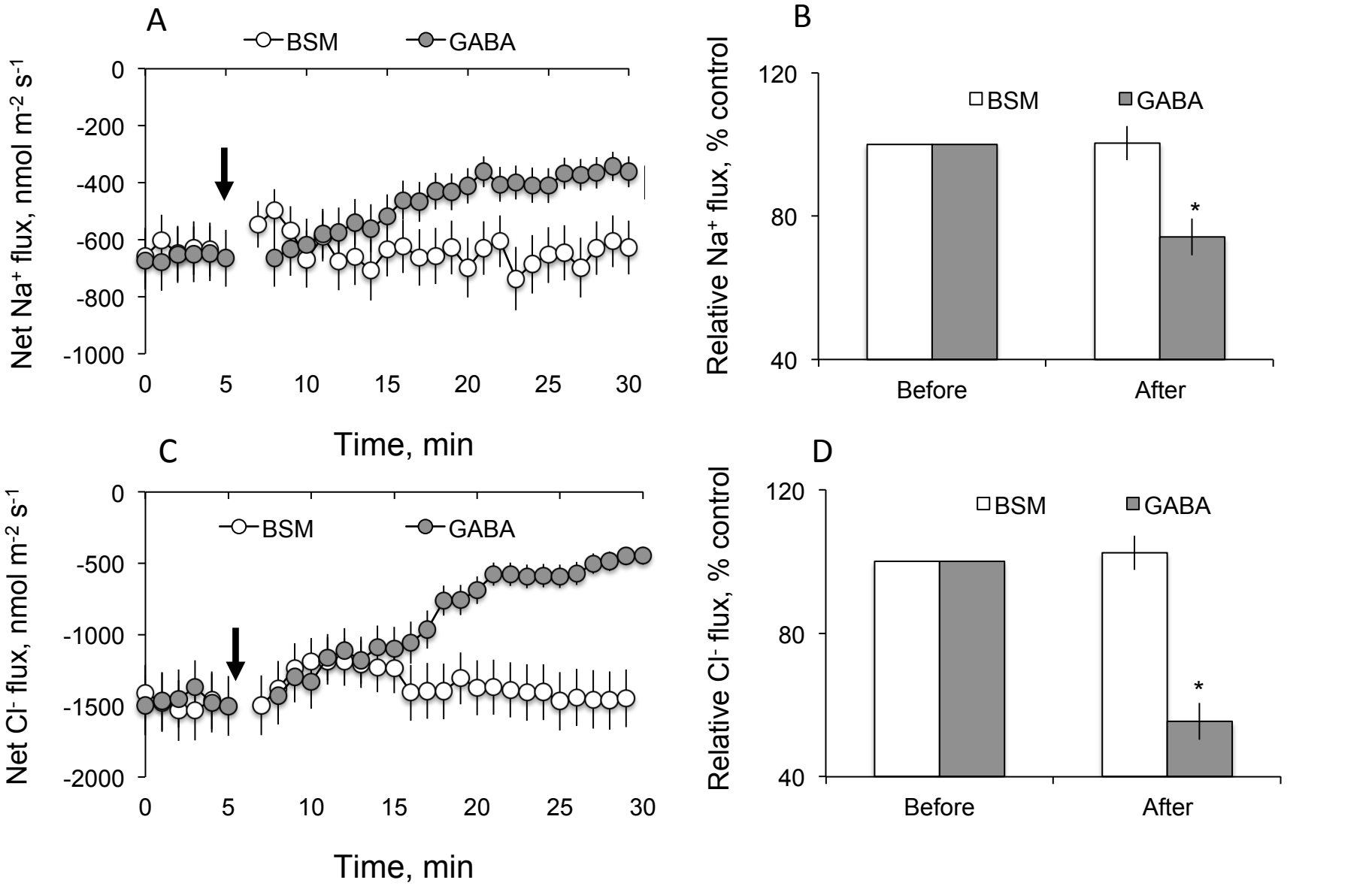

Control NBr
Salt NBr
Raffinose
Galactinol
Inositol
Glucuronate
Glucarate
Gulonate
Inositol-1-P
Trehalose
Ribose
Ribitol
Xylitol
Glycerate
Ribose-5-P
Gluconate
Glucose
Glucose-6-P
Adenosine-5-monophosphate
Histidine
Oxalate
Glucose-1-P
Galactonate
Ascorbate
Sucrose
UDP-Glucose
Galactose
Lactose
Glycerol
Glycerol-3-P
Fructose
Allothreonine
Glycine
Serine
Threonate
Fructose-6-P
2-amino-2-deoxy-glucose
Fructose-1,6-diP
Tryptophan
Benzoate
Salicylic acid glucopyranoside
Ethanolamine
Phenylalanine
Cinnamate
4-Coumarate
4-Hydroxybenzoate
3-PGA
Valine
PEP
Quinate
Shikimate
Tocopherol
beta-Sitosterol
Stigmasterol
2-Oxobutanoate
Tyrosine
24-Methylenelophenol
Campesterol
Threonine
Isoleucine
Lactate
Homoserine
Pyruvate
Alanine
Uracil
Malonate
Leucine
Acetyl-CoA
Citrate
beta-Alanine
Oxaloacetate
Aspartate
Malate
TCA Cycle
Aconitate
Asparagine
Itaconate
Phosphate
Arabinose
Threonate-1,4-lactone
2-Aminoadipate
Fumarate
Glyoxalate
Isocitrate
Nicotinamide
Glycolate
Erythronate
Arabitol
4-hydroxy cinnamic acic
Galactitol
Lysine
Methionine
2-Oxoglutarate
Maleate
Succinate
Succinyl-CoA
Mucic
Threitol
Rhamnose
Pantothenic acid
Pipecolate
S-Adenosyl-Met
Glutamine
Urea
GABA
Glutamate
Nicotinic acid
Ribonate
Maltose
Cytosine
Pyroglutamate
Proline
Ornithine
Putrescine
Monomethylphosphate
Xylose

Fig 7


Scores Plot
Br
NBr
Br1
Br3
Br4
Br2
NBr1
NBr3
NBr2
NBr4
Component 2 ( 10.5 %)
Component 1 ( 62 %)
-20
-15
-10
-5
0
5
10
5
0
-5


class
Br
NBr
2
1
0
-1
-2
Galactose
Fructose
Glucose
Galactonate
Inositol
Ethanolamine
Ribonate
Ascorbate
Ribose
Arabinose
Xylose
Pyroglutamate
Serine
Erythritol
Ribitol
Fructose-6-P
Glutamate
Benzoate
Sucrose
Xylitol
Arabitol
Threitol
Glutamine
Ornithine
Malate
Citrate
Oxalate
Threonate
Glucose-6-P
Inositol-1-P
Glycerol-3-P
Kaempferol
Cytosine
2 - amino malonic
Glycerate
Methionine
Glycine
Tryptophan
Fumarate
Glycolate
Uracil
Pipecolate
Proline
4-amino butyric ac
GABA
Butyro-1,4- lactam
Nicotinic acid
Fucose
Maltose
Trehalose
Guanine
Salicylic acid
Aspartate
Asparagine
Cysteine
Isoleucine
Valine
Lysine
Phenylalanine
Tyrosine
Glycerol
Alanine
Threonine
Phosphate
Monomethylphosphat
Mucic/saccharic
Arginine
Histidine
Allantoin
Succinate
Br1
Br2
Br3
Br4
NBr1
NBr2
NBr3
NBr4


A
Cu/A
2000
0
-2000
-4000
-6000
-8000
-10000
Net K+ flux, nmol m-2 s-1
Control
Sucrose
0
5
10
15
20
25
30
Time, min
B
Control
Sucrose
0
-2000
-4000
-6000
-8000
Steady K+ flux, nmol m-2 s-1
*
Before
After


A
BSM
GABA
Net Na+ flux, nmol m-2 s-1
0
-200
-400
-600
-800
-1000
0
5
10
15
20
25
30
Time, min
B
Relative Na+ flux, % control
120
80
40
BSM
GABA
*
Before
After
C
Net Cl- flux, nmol m-2 s-1
0
-500
-1000
-1500
-2000
BSM
GABA
0
5
10
15
20
25
30
Time, min
D
Relative Cl- flux, % control
120
80
40
BSM
GABA
*
Before
After