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RESEARCH PAPER

Metabolic responses to salt stress of barley (*Hordeum vulgare* L.) cultivars, Sahara and Clipper, which differ in salinity tolerance

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Abstract

Plants show varied cellular responses to salinity that are partly associated with maintaining low cytosolic Na^+ levels and a high K^+/Na^+ ratio. Plant metabolites change with elevated Na^+, some changes are likely to help restore osmotic balance while others protect Na^+-sensitive proteins. Metabolic responses to salt stress are described for two barley (*Hordeum vulgare* L.) cultivars, Sahara and Clipper, which differed in salinity tolerance under the experimental conditions used. After 3 weeks of salt treatment, Clipper ceased growing whereas Sahara resumed growth similar to the control plants. Compared with Clipper, Sahara had significantly higher leaf Na^+ levels and less leaf necrosis, suggesting they are more tolerant to accumulated Na^+. Metabolite changes in response to the salt treatment also differed between the two cultivars. Clipper plants had elevated levels of amino acids, including proline and GABA, and the polyamine putrescine, consistent with earlier suggestions that such accumulation may be correlated with slower growth and/or leaf necrosis rather than being an adaptive response to salinity. It is suggested that these metabolites may be an indicator of general cellular damage in plants. By contrast, in the more tolerant Sahara plants, the levels of the hexose phosphates, TCA cycle intermediates, and metabolites involved in cellular protection increased in response to salt. These solutes remain unchanged in the more sensitive Clipper plants. It is proposed that these responses in the more tolerant Sahara are involved in cellular protection in the leaves and are involved in the tolerance of Sahara leaves to high Na^+.

Key words: Barley, GC-MS, metabolomics, salt stress, tissue tolerance.

Introduction

Plants respond in many ways to salinity and at a number of levels (Munns and Tester, 2008). For example, extensive investigations of salt stress responses using transcriptomics approaches have been described for rice (Walia *et al.*, 2005, 2007; Kumari *et al.*, 2009) and *Lotus japonicus* (Sanchez *et al.*, 2008*b*). Complementing these studies are detailed analyses of changes of the proteome following salt stress in *Arabidopsis* (Jiang *et al.*, 2007), rice (Nohzadeh Malakshah *et al.*, 2007; Zang and Komatsu, 2007), potato (Aghaei *et al.*, 2008), and *Physcomitrella patens* (Wang *et al.*, 2008). It is expected that exposure of plants to salinity will also result in a wide range of metabolic responses. While changes in levels of several metabolites following salt stress are well documented, less well documented are the changes that occur in the metabolome overall (Zuther *et al.*, 2007; Sanchez *et al.*, 2008*a*, *b*). One of the best described metabolic responses is the increase in intracellular concentration of a range of soluble, neutral organic compounds

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Abbreviations: TCA, tricarboxylic acid; GABA, γ-aminobutyric acid; GC-MS, gas chromatography-mass spectrometry; HCA, hierarchical cluster analysis; PCA, principal component analysis.

that are collectively termed 'compatible solutes' (Bohnert *et al.*, 1995; Bohnert and Shen, 1998).

Compatible solutes are small hydrophilic molecules that are non-toxic (thus 'compatible') and do not perturb cellular functions even when present in high concentrations (Yancey, 2005). Increased concentrations of compatible solutes in the cytoplasm can contribute to reducing the water potential in the cytoplasm by balancing the decreased water potential associated with Na^+ accumulation in the vacuoles and the extracellular volume. These neutral organic compounds can also alleviate the inhibitory effects of high ion concentrations on enzymatic activity without interfering with protein structure and function. For example, they may act as antioxidants in scavenging free radicals (Smirnoff and Cumbes, 1989), and can help stabilize membranes (Hare *et al.*, 1998). The most common compatible solutes in plants are polyhydroxy compounds (sucrose, oligosaccharides, and polyhydric alcohols) and nitrogen-containing compounds (proline, other amino acids, quaternary ammonium compounds, and polyamines) (Bohnert *et al.*, 1995; Hare *et al.*, 1998). However, it is noteworthy that increases in amino acid concentrations have been attributed simply to arising from the decrease in growth rate that results from stress (Munns, 2002). Such accumulation may not, therefore, be an adaptive response to salinity but simply a secondary consequence of reduced growth.

Other effects of salinity on metabolism and metabolite levels include changes in photosynthesis and respiration (Chaves *et al.*, 2009; Lawlor and Tezara, 2009). Photosynthetic responses to salinity stress are highly complex and affect processes at a range of levels in the leaf. The intensity, duration, and speed of onset of the stress will all influence the way the plant responds to reduced water availability due to salinity. A reduction of photosynthetic activity will limit the availability of energy and photoassimilate for metabolism, resulting in increased catabolism of stored carbohydrates via respiration in order to provide cells with energy. Under conditions of long-term salt stress, when damage may have been caused and cells show symptoms of necrosis, other effects of salinity may also influence metabolism (Flexas *et al.*, 2004; Chaves *et al.*, 2009). Comparisons of data from transcript analyses following drought or salt stress show that plants respond by changing gene expression as well as physiological and biochemical processes (Chaves *et al.*, 2009). Most apparent is that drought and, to a greater extent, salinity stress both result in reduced expression of photosynthetic genes (for a review, see Chaves *et al.*, 2009).

When grown under conditions of environmental stress, the metabolic homeostasis of plants is disrupted and altered. For example, the response to increased salt involves dramatic changes in the activity of a number of genes and proteins which ultimately lead to changes in plant metabolism. The application of metabolomics technologies for a more comprehensive cellular metabolite analysis offers a rational approach to investigate the implications of these metabolic alterations on a broad scale. Metabolomics employs a range of analytical technologies (mass spectrometry (MS)- or NMR-based techniques), the most common of which is GC-MS which allows rapid, non-biased, and simultaneous analysis of a large number of small molecules (Kopka *et al.*, 2004; Roessner, 2007; Roessner and Beckles, 2009).

Studies of plant responses to salinity stress and the differences between salt-sensitive and salt-tolerant species using metabolomics approaches have revealed an interesting diversity of patterns (for a review, see Sanchez *et al.*, 2008*a*). First, there are differences in constitutive levels of metabolites between tolerant and sensitive varieties/species; and, second, different species show conserved as well as divergent metabolite responses in response to salinity. These patterns have been demonstrated through the comparison of *Arabidopsis thaliana* with its salt-tolerant relative *Thellungiella halophila* (Gong *et al.*, 2005) as well as when a range of different rice (*Oryza sativa*) cultivars with different levels of salinity tolerance were compared (Zuther *et al.*, 2007). Under control conditions, the salt-tolerant *T. halophila* had higher levels of sucrose, fructose, glucose, proline, citrate, malate, and succinate compared with *Arabidopsis*. However, following salt stress, *T. halophila* showed greater increases in inositol, galactinol, and raffinose and reductions in fumarate, malate, phosphate, and aspartate than did *Arabidopsis* (Gong *et al.*, 2005).

Zuther *et al.* (2007) demonstrated a clear separation of the metabolic phenotypes of sensitive and tolerant rice cultivars which was more obvious in the root than the leaf. The roots of the more tolerant varieties had lower levels of the TCA cycle intermediates and other organic acids, and higher amino acid levels. Increases in certain amino acids (including proline), sugars (including sucrose, fructose, and glucose) and polyols (including inositols) have been reported that are common for a number of species under investigation, for example, grapevine (Cramer *et al.*, 2007), the halophyte *Limonium latifolium* (Gagneul *et al.*, 2007), *Arabidopsis* (Gong *et al.*, 2005; Sanchez *et al.*, 2008*a*), *Lotus japonicus* (Sanchez *et al.*, 2008*b*), and rice (Zuther *et al.*, 2007). In addition, a clear reduction in a range of organic acids involved in and associated with the TCA cycle has been demonstrated in all these species. High Na^+ stress often correlates with a strong decrease of K^+. A recent study on the effects of K^+ deficiency on the metabolism of leaves and roots of *Arabidopsis* has demonstrated an interesting pattern of strong increases of sugars and high nitrogen-containing amino acids in both roots and leaves whereas certain organic acids and other amino acids decrease only in roots (Armengaud *et al.*, 2009).

Barley (*Hordeum vulgare* L.) is an important cereal, grown primarily for animal feed and as a raw material for alcohol production. Barley production in Australia is affected by increasing dryland salinity which severely limits growth and reduces yields (Rengasamy *et al.*, 2003). Barley is notable in that it can maintain growth whilst accumulating high concentrations of Na^+ in its leaves (Munns *et al.*, 1988). The high tissue tolerance of barley is likely to involve sequestration of Na^+ into intracellular vacuoles and the synthesis of compatible solutes that accumulate in the cytoplasm to balance the osmotic potential of the vacuolar Na^+. Thus, barley is a plant of particular interest for metabolomic

studies and with the longer term aim of transferring the tissue tolerance trait to other commercial species such as wheat and rice which have much lower Na^+ tissue tolerance.

In the current study, a metabolomics analysis was conducted on the parents of a mapping population, Clipper and Sahara (the former is a commercial Australian cultivar and the latter a North African landrace) following salinity stress. The two cultivars were found to differ in their growth and metabolite responses to the imposed salinity stress. This differential response is discussed in terms of the potential involvement of particular metabolites in either a specific, adaptive response to salt stress, or to general cellular damage/ageing as a result of the stress treatment.

Materials and methods

Plant material and growth conditions

Seeds of two cultivars of barley (*Hordeum vulgare* L.) cv. Clipper and cv. Sahara were obtained from Dr Tim Sutton from the Australian Centre for Plant Functional Genomics (ACPFG), University of Adelaide, Australia. Seeds were surface-sterilized in 70% ethanol for 1 min and then soaked in 10% sodium hypochlorite for 15 min. Seeds were washed thoroughly with water for 15 min, germinated in aerated water overnight, and then placed on moist filter paper for 2–3 d until the first leaf of the seedlings emerged. Individual seedlings were then suspended in a hydroponic nutrient solution composed of 2 mM $Ca(NO_3)_2$, 5 mM KNO_3, 5 mM NH_4NO_3, 2 mM $MgSO_4$, 0.1 mM KH_2PO_4, 0.5 mM Na_2SiO_3, 0.05 mM NaFe(III)EDTA, 5 μM $MnCl_2$, 5 μM $ZnSO_4$, 0.5 μM $CuSO_4$, 0.1 μM $NaMoO_3$, and 5 μM H_3BO_3. The solution was gently aerated and replaced every 3–4 d. Plants were grown in controlled conditions at 21/18 °C day/night, with a photoperiod regime of 16/8 h day/night at 200 μmol m^{-2} s^{-1} photon irradiance at plant height. NaCl treatment was started 7 d after germination. When salt stress was applied, the NaCl concentration was initially 50 mM and then increased the following day to the final concentration of 100 mM NaCl. $CaCl_2$ was added to a final concentration of 3.3 mM to maintain levels of available Ca^{2+}. Plants were grown for varying periods before being harvested for fresh and dry weight determinations. The second youngest leaf of five individual plants were separately harvested and immediately shock frozen in liquid nitrogen 4 h into the light regime and separately homogenized into a powder using a mortar and pestle under liquid nitrogen. Aliquots of the powder from the 3 week and 5 week (w) time points were used for elemental analysis and from the 24 h, 3 w, and 5 w for metabolomic analyses. Root tissue from each individual plant was harvested and immediately shock frozen in liquid nitrogen from 3-week-old and 5-week-old plants for metabolomic analysis.

Growth measurements

Shoot height, root length, and fresh and dry weights were recorded. For each measurement, four individual plants were used. Differences between control and NaCl-treated samples were tested by one-way and two-way ANOVAs (analysis of variance) using the statistical software package MINITAB (Minitab® Statistical Software, Minitab Inc., Pennsylvania, USA). Significance differences are compared using Tukey's pair-wise comparisons, with differences of $P < 0.05$ being considered as significant.

Elemental analysis in leaf tissue

An aliquot of the frozen ground leaf (second youngest) samples was dried in an oven at 80 °C for 24 h, and sent to Dr Yuri Shavrukov (ACPFG, University of Adelaide) for elemental analysis as described in Jeffries *et al.* (1999).

Extraction, derivatization and analysis of barley leaf and root metabolites

Metabolite analysis by GC-MS was carried out as described by Roessner *et al.* (2006). Separate aliquots of the frozen and ground root and leaf tissue of five individual plants per cultivar and treatment were used for metabolite extraction. A polar metabolite fraction, obtained from approximately 60 mg homogenized leaf and 120 mg homogenized root tissue, was extracted in 350 μl of 100% methanol and 20 μl ribitol and norleucine (0.2 mg ml^{-1} ribitol and norleucine in water) added as internal standards for quantification. Samples were then processed, derivatized (both TMS and TBS), and analysed by GC-MS as described in Jacobs *et al.* (2007).

Statistical analysis

The data were analysed as described in Roessner *et al.* (2001) and are presented as fold changes compared with the reference, which is set to 1. Fold changes less than 1 were inverted and multiplied by −1 to aid interpretation. Differences between two treatments were considered significant when the P value (calculated using a Student's t test) was <0.05. Principal component analysis (PCA) was carried out using Pirouette 3.11 software (Infometrix Inc, Woodinville, US) on the response data per gram FW for each individual metabolite and measurement following $\log_{10}$ transformation. Heatmap analysis combined with hierarchical cluster analysis of the same data set as used in the PCA analyses, but combined for roots and leaves, was carried out using the R program called made4 as described in Culhane *et al.* (2005).

Results

General growth performance of hydroponically grown Sahara and Clipper plants

When grown hydroponically, Sahara and Clipper showed differential responses to salt. Sahara was less affected by the presence of 100 mM NaCl, there being an absence of leaf symptoms even after 5 w exposure (data not shown). Furthermore, an obvious reduction in biomass was seen in Clipper (Fig. 1). This reduction coincided with the onset of

leaf senescence that started at the tip of the older leaves at around 2 w and became more severe after 4 w. By week 6, most leaves displayed extensive regions of necrotic tissue (data not shown). Fresh and dry weights were used to monitor the physiological response of each cultivar to salinity (Fig. 1). After 3 w, a significant decrease in growth in the presence of salt was apparent in both cultivars (Fig. 1A). When the growth of salt-treated plants is plotted as a percentage of the control, differences in the responses of the cultivars can be seen after 4 w (Fig. 1B). Growth of Sahara recovered relative to the control after 4 w in salt, whereas Clipper plants stopped growing after 2 w and did not resume growth.

Roots are in direct contact with the growth solution and can be profoundly affected by salt stress. Roessner *et al.* (2006) have shown previously that Clipper roots are longer than Sahara roots. After 6 w of salt treatment, roots of both cultivars were reduced in length to a similar extent, i.e. a 46% reduction in Clipper and 43% in Sahara (Fig. 1C). Roots exposed to 100 mM NaCl were essentially the same length throughout the experiment. Exposure to 100 mM NaCl for 6 w also resulted in similar decreases in shoot height for both: i.e. a reduction of 19% in Clipper and 14% in Sahara (Fig. 1D). It is notable, however, that both the salt-treated and control Clipper plants reached their maximum height after 2 w, whereas both treated and control Sahara plants grew throughout the experiment. Examination of plants indicated that the recovery in biomass production observed in Sahara was due to an increase in leaf number following Na^+ treatment (data not shown).

Sodium and potassium levels in the second youngest leaf

Plant responses to 100 mM NaCl were determined by measuring Na^+ concentrations in the second youngest leaf by flame photometry following exposure to salt for 3 w and 5 w. Table 1 shows that the concentration of Na^+ in leaves was much higher in plants grown under salt stress, regardless of variety. In control plants, the K^+/Na^+ ratio varied between 390 and 1050, highlighting the high concentration of K^+ relative to Na^+. After salt treatment, there was a 30–40-fold increase in Na^+ levels in Clipper, at both 3 w and 5 w, thus causing the K^+/Na^+ ratio to fall to 6 after 3 w and to recover slightly to 31 after 5 w. This increase in the K^+/Na^+ ratio was brought about by a 63% reduction in leaf Na^+ between 3 w and 5 w and a 52% increase in leaf K^+. There was a similar 30-fold increase in Na^+ levels after 3 w in Sahara following exposure to elevated salt and a more modest 13-fold increase after 5 w. Despite this smaller increase after 5 w, the K^+/Na^+ ratio was almost identical to that of Clipper. For comparison, the K^+/Na^+ ratios in floral apices of barley cv. Clipper plants grown in gravel beds and watered with solutions containing 100 or 175 mol m^{-3} NaCl were 3 and 8, respectively (Munns *et al.*, 1988). The floral apex K^+/Na^+ ratio in control plants was 28.

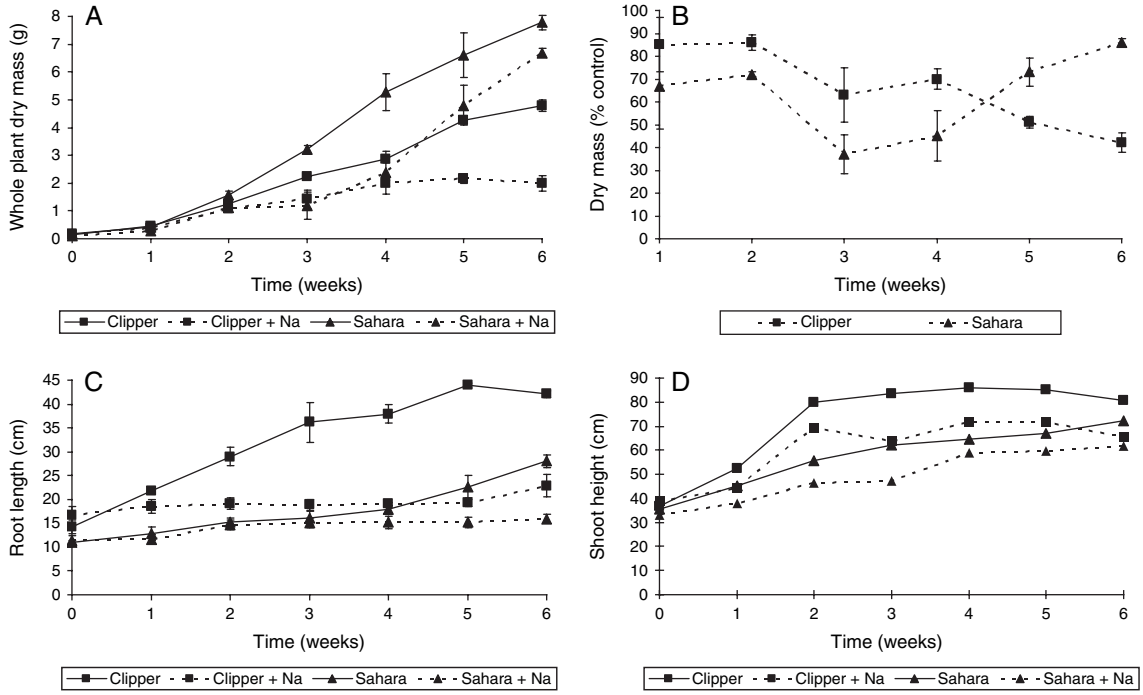

Fig. 1. Dry weight, root length, and shoot height measurements of Clipper and Sahara plants grown hydroponically in control (1 mM) and salt-treated (100 mM NaCl) conditions. Barley plants were 1 week old when 50 mM NaCl was added to the growth solution of half the plants. The following day the salt concentration was raised to 100 mM. A continuous line indicates plants grown in control solution and a dotted indicates plants with the salt treatment. Squares refer to Clipper and triangles to Sahara. A) Whole plant dry weight: B) Dry weight expressed as % of the relevant control: C) Root length: D) Shoot height. Error bars are S.E. of the mean of 5 independent samples in all cases.

Table 1. K^+ and Na^+ concentrations (μg g^{-1} DW) in leaves of Clipper and Sahara after 3 w and 5 w of growth

Barley plants were grown hydroponically in control or elevated salt (100 mM NaCl) for 3 w and 5 w. The second youngest leaves were harvested and analysed for Na^+ and K^+ levels using flame photometry. The concentration of Na^+ and K^+, the K^+/Na^+ ratio, and the Na^+ fold increase in the leaves of both cultivars are presented. Values are the means ±SE (n=5).

	Time	K		Na		K^+/Na^+ ratio		Na^+ fold
		Control	Treated	Control	Treated	Control	Treated	
C	3	3020±720	1670±160	7.8±2.1	299±27	390	6	38
	5	2590±160	3470±170	3.4±0.2	112±30	760	31	33
S	3	32 600±4930	16 996±5770	31±4.2	1037±256	1050	16	33
	5	30 860±3708	15 910±1480	40±3.7	514±39	770	31	13

In an initial study, analysis of root Na^+ and K^+ at 6 h, 24 h, 1 w, and 2 w in both cultivars showed the same pattern as that in shoots, with no significant differences detected between cultivars in the presence or absence of salt (data not shown). Analyses of root Na^+ and K^+ at 3 w and 5 w were not subsequently performed.

Metabolic responses in leaves after salt stress

Clipper and Sahara showed no differences in their K^+/Na^+ ratios after 5 w of salt stress, despite obvious physiological differences in responses to salinity (Table 1). This suggested that the two cultivars may operate different cellular mechanisms in response to high levels of Na^+ in their tissues. Here, a GC-MS-based strategy was used to investigate metabolite changes in the Clipper and Sahara cultivars following treatment with 100 mM NaCl for 24 h, 3 w, and 5 w. The objective was to compare metabolic responses between the cultivars and to monitor how short- and long-term salt imposition affects the metabolite profiles. Given that Na^+ levels in leaves are important determinants in salinity tolerance, leaf metabolite changes were examined initially.

A total of 72 compounds of known structure, comprising 23 amino acids, 20 organic acids and 29 sugars, were identified in leaf extracts of barley. A number of metabolites (30) were also found that could neither be identified nor classified using either commercial libraries such as NIST or the MSRI library (Kopka *et al.*, 2004). Only those metabolites that could be chemically identified are described here and are summarized in Table 2 and in Supplementary Tables S1 to S4 at *JXB* online).

Figure 2 shows a principal component analysis of barley leaf metabolite data. Except for the Clipper 3 w control data set, there is a clear separation between short-term (24 h) and long-term (3 w and 5 w) responses in the first principal component, which accounts for 27.5% of the total variance. For both Clipper and Sahara, the short-term metabolite cluster is to the left of the long-term cluster. Short-term salt stress also caused a smaller metabolite separation than long-term stress, with the Clipper 24 h data set forming a single cluster and the Sahara 24 h salt-treated cluster being slightly above the control cluster, that is, these two data sets are separated by the second principal component. For long-term (3 w and 5 wk) salt stress, the salt-treated cluster is to the right of the respective control (i.e. separated by the first principal component), except for 5 w salt-treated Sahara which is directly above the control cluster. The metabolome of Clipper leaves showed fewer developmental changes initially, as the 3 w control cluster is close to the 24 h control cluster. Investigation of the loadings for the first principal component revealed contributions from a number of metabolites with sugars and organic acids mainly responsible for the separation. Examination of loadings for the second principal component identified proline and citramalate as being the main metabolites contributing to the separation. Citramalate was found in Sahara leaves after short-term and long-term salt stress, but was not found in Clipper leaves after 5 w in salt stress.

After 24 h in 100 mM NaCl, only a few metabolites changed their concentrations in Clipper and Sahara leaves (Table 2; see Supplementary Tables S1 and S2 at *JXB* online). In Sahara leaves, all significant changes were as a result of a decrease in metabolite concentration of between −1.2-fold and −10-fold. Metabolites at lower concentrations than the respective control plants were lysine, quinate, shikimate, succinate, threonate, threonate 1,4-lactone, 1-monohexadecanoglycerol, 1-monooctodecanoglycerol, fructose, galacturonate, glucose, maltose, sorbitol, and xylose (Table 2; see Supplementary Tables S1 and S2 at *JXB* online). The greatest decrease (−10-fold) was observed in sorbitol. In Clipper leaves, five metabolites increased and five others decreased in concentration (Table 2; see Supplementary Tables S1 and S2 at *JXB* online). Metabolites that increased 2–3-fold were valine and the sugars galactinol, gluconate, raffinose, and trehalose. Metabolites that decreased −2 to −14 fold were dehydroascorbate and the sugars fructose, galacturonate, glucose, and xylose.

A greater number of changes in metabolite concentrations were noted in Clipper and Sahara leaves after 3 w of salt exposure (Table 2; see Supplementary Tables S1 and S2 at *JXB* online). Not only was the number of changes greater, but the magnitude of these changes was also generally larger. In Sahara leaves, the levels of several amino acids and their derivatives were −2 to −6-fold lower in salt-treated plants (Table 2; see Supplementary Tables S1 and S2 at *JXB* online). These were alanine, aspartate,

Table 2. Metabolite ratios in 100 mM salt-treated Sahara and Clipper leaves compared to controls for different lengths of time (normalized values for the controls are not shown; see Supplementary Tables 3 and 4 at *JXB* online)

Data obtained from TBS and TMS analysis of salt-treated Clipper (C) and Sahara (S) leaves were normalized to the mean response calculated for the respective untreated samples at the same stage of growth. Time periods are 24 h, 3 w, and 5 w. Values are presented as the ratios ±% SE of five independent determinations. Normalized ratios less than 1.0 were inverted and multiplied by −1. Values that are significantly (P <0.05) higher were indicated in bold type and blue (strength of colour correlated with strength of change) and those that are significantly lower are in italics, underlined, and yellow (strength of colour correlated with strength of change). Also indicated in colour are metabolite changes more than 2-fold (blue increase and yellow decrease) although not statistically significant.

Amino acids	**24 h C ± SE**	**24 h S ± SE**	**3 w C ± SE**	**3 w S ± SE**	**5 w C ± SE**	**5 w S ± SE**
5-Oxoproline	1.03 ± 0.09	1.17 ± 0.11	1.24 ± 0.38	−1.07 ± 0.13	−1.04 ± 0.36	−1.04 ± 0.23
β-alanine	1.06 ± 0.23	1.22 ± 0.11	**3.26 ± 0.08**	1.32 ± 0.16	1.36 ± 0.47	1.71 ± 0.25
Alanine	−1.41 ± 0.17	−1.78 ± 0.16	−1.29 ± 0.30	*−5.56 ± 0.25*	*−5.00 ± 0.15*	−1.14 ± 0.24
Asparagine	1.25 ± 0.20	1.28 ± 0.13	**2.80 ± 0.21**	−1.72 ± 0.22	−1.56 ± 0.18	2.16 ± 0.43
Aspartate	1.37 ± 0.20	1.67 ± 0.14	**3.36 ± 0.06**	*−1.96 ± 0.17*	−1.49 ± 0.26	1.60 ± 0.17
GABA	1.15 ± 0.22	−1.15 ± 0.26	**12.37 ± 0.14**	−1.47 ± 0.18	**43.32 ± 0.46**	2.47 ± 0.45
Glutamate	1.92 ± 0.68	1.10 ± 0.57	**7.98 ± 0.28**	**3.43 ± 0.17**	1.81 ± 0.43	1.80 ± 0.29
Glutamine	1.49 ± 0.27	−1.82 ± 0.54	**65.05 ± 0.41**	−1.41 ± 0.52	5.95 ± 0.56	1.08 ± 0.52
Glycine	1.31 ± 0.15	−1.25 ± 0.10	**2.42 ± 0.09**	*−1.70 ± 0.11*	1.49 ± 0.14	1.34 ± 0.14
Homoserine	−1.14 ± 0.04	1.15 ± 0.15	**15.43 ± 0.37**	**2.04 ± 0.11**	*−4.76 ± 0.41*	1.70 ± 0.76
Isoleucine	1.31 ± 0.21	1.18 ± 0.16	1.36 ± 0.36	−1.04 ± 0.65	3.04 ± 0.29	**3.00 ± 0.05**
Leucine	1.50 ± 0.22	1.23 ± 0.18	1.25 ± 0.38	1.36 ± 0.72	**4.17 ± 0.27**	**4.71 ± 0.11**
Lysine	1.48 ± 0.23	*−3.85 ± 0.09*	−1.26 ± 0.21	2.85 ± 0.59	6.08 ± 0.49	**10.12 ± 0.12**
N-acetylglutamate	1.05 ± 0.56	1.33 ± 0.49	**94.00 ± 0.35**	2.55 ± 0.30	1.73 ± 0.36	1.92 ± 0.43
Phenylalanine	1.20 ± 0.04	−1.19 ± 0.09	1.12 ± 0.23	1.11 ± 0.40	6.16 ± 0.60	**3.72 ± 0.25**
Proline	2.04 ± 0.70	1.75 ± 0.30	**40.71 ± 0.35**	−1.51 ± 0.60	**44.52 ± 0.40**	**10.14 ± 0.28**
Putrescine	−1.31 ± 0.13	−1.15 ± 0.11	**3.46 ± 0.11**	*−2.56 ± 0.13*	**4.18 ± 0.34**	**1.83 ± 0.07**
Serine	1.97 ± 0.48	1.74 ± 0.23	**5.31 ± 0.34**	*−4.00 ± 0.18*	*−2.17 ± 0.38*	3.07 ± 0.37
Threonine	1.06 ± 0.34	1.00 ± 0.07	**10.99 ± 0.53**	**11.57 ± 0.13**	1.54 ± 0.55	1.51 ± 0.19
Tryptophan	1.69 ± 0.23	−1.51 ± 0.18	1.40 ± 0.21	1.18 ± 0.24	20.75 ± 0.68	5.47 ± 0.55
Tyramine	1.31 ± 0.06	−1.07 ± 0.11	1.67 ± 0.21	*−2.22 ± 0.05*	7.48 ± 0.49	2.16 ± 0.30
Tyrosine	1.61 ± 0.07	1.00 ± 0.08	−1.89 ± 0.36	3.39 ± 0.35	2.84 ± 0.52	**4.63 ± 0.20**
Valine	**1.90 ± 0.09**	1.32 ± 0.21	**2.07 ± 0.24**	1.23 ± 0.38	2.14 ± 0.29	**2.41 ± 0.15**
Organic acids	**24 h N ± SE**	**24 h S/N ± SE**	**3 w N ± SE**	**3 w S/N ± SE**	**5 w N ± SE**	**5 w S/N ± SE**
α-ketoglutarate	1.14 ± 0.08	1.02 ± 0.04	−1.49 ± 0.20	**2.63 ± 0.22**	*−7.69 ± 0.09*	−1.29 ± 0.28
Aconitate	1.81 ± 0.40	1.18 ± 0.06	1.81 ± 0.40	**4.96 ± 0.13**	1.06 ± 0.36	1.29 ± 0.31
Ascorbate	1.14 ± 0.05	1.61 ± 0.18	−1.15 ± 0.13	**2.49 ± 0.17**	*−4.12 ± 0.02*	5.46 ± 0.58
Citramalate	1.20 ± 0.11	−1.22 ± 0.11	1.84 ± 0.47	1.69 ± 0.20	n.d.	2.27 ± 0.60
Citrate	1.16 ± 0.10	−1.03 ± 0.07	*−3.22 ± 0.44*	**9.72 ± 0.18**	*−12.50 ± 0.24*	*−2.63 ± 0.71*
Dehydroascobate	*−2.32 ± 0.20*	−1.30 ± 0.12	1.21 ± 0.15	**5.32 ± 0.05**	−1.79 ± 0.32	−1.08 ± 0.55
Erythronate	1.36 ± 0.05	−1.07 ± 0.03	1.58 ± 0.16	1.11 ± 0.11	*−2.85 ± 0.11*	−1.29 ± 0.14
Fumarate	1.18 ± 0.08	−1.18 ± 0.04	2.61 ± 0.40	−1.07 ± 0.10	*−2.44 ± 0.07*	−1.16 ± 0.43
Glycerate	1.35 ± 0.11	−1.11 ± 0.02	1.34 ± 0.18	1.27 ± 0.17	*−2.27 ± 0.15*	−1.45 ± 0.12
Isocitrate	1.20 ± 0.06	1.28 ± 0.06	1.37 ± 0.36	**14.64 ± 0.22**	*−6.25 ± 0.48*	−1.15 ± 0.90
Malate	1.25 ± 0.09	−1.11 ± 0.06	1.15 ± 0.27	**1.49 ± 0.09**	*−4.54 ± 0.17*	−1.85 ± 0.55
Maleate	1.77 ± 0.32	−1.41 ± 0.11	**4.18 ± 0.56**	−1.25 ± 0.05	2.12 ± 0.76	*−2.00 ± 0.25*
Monomethylphosphate	−1.14 ± 0.31	1.12 ± 0.16	**2.25 ± 0.43**	**7.57 ± 0.12**	*−2.44 ± 0.18*	1.24 ± 0.41
Oxalate	1.82 ± 0.47	1.43 ± 0.23	3.62 ± 0.48	−1.03 ± 0.11	2.53 ± 0.19	1.00 ± 0.23
Quinate	1.01 ± 0.09	*−1.31 ± 0.03*	1.17 ± 0.34	1.61 ± 0.31	*−2.17 ± 0.30*	2.08 ± 0.10
Ribonate	1.42 ± 0.06	1.00 ± 0.04	−1.09 ± 0.19	**2.27 ± 0.11**	*−2.56 ± 0.04*	−1.78 ± 0.26
Shikimate	1.11 ± 0.09	*−1.49 ± 0.02*	−1.19 ± 0.13	1.14 ± 0.14	*−2.32 ± 0.12*	*−2.22 ± 0.35*
Succinate	1.30 ± 0.10	*−1.20 ± 0.02*	1.19 ± 0.26	**2.50 ± 0.18**	*−2.04 ± 0.15*	−1.69 ± 0.11
Threonate	1.44 ± 0.06	*−1.18 ± 0.04*	1.55 ± 0.23	**1.73 ± 0.09**	*−2.04 ± 0.10*	2.08 ± 0.17
Threonate−1,4−lactone	−1.23 ± 0.06	*−1.40 ± 0.07*	**1.70 ± 0.10**	**2.75 ± 0.15**	*−1.78 ± 0.13*	−1.61 ± 0.04
Sugars	**24 h N ± SE**	**24 h S/N ± SE**	**3 w N ± SE**	**3 w S/N ± SE**	**5 w N ± SE**	**5 w S/N ± SE**
1,6-Anhydroglucose	−1.89 ± 0.15	−1.51 ± 0.34	5.26 ± 0.35	1.06 ± 0.14	1.02 ± 0.19	2.13 ± 0.38
1-Monohexadecanoglycerol	−1.51 ± 0.25	*−2.78 ± 0.63*	1.11 ± 0.25	4.45 ± 0.51	*−2.32 ± 0.08*	1.16 ± 0.21
1-Monooctodecanoglycerol	−1.06 ± 0.24	*−2.70 ± 0.58*	*−2.44 ± 0.29*	2.83 ± 0.54	*−2.50 ± 0.21*	1.58 ± 0.19
2−Keto-gluconate	1.52 ± 0.11	−1.08 ± 0.07	1.35 ± 0.14	**2.62 ± 0.04**	−1.39 ± 0.28	−1.43 ± 0.16
2-*O*-glycerol-β-D-galactose	1.55 ± 0.16	1.04 ± 0.06	1.30 ± 0.14	**7.87 ± 0.14**	*−2.27 ± 0.08*	**3.86 ± 0.17**
3PGA	−1.41 ± 0.10	−1.04 ± 0.02	−1.36 ± 0.12	**7.20 ± 0.16**	−1.96 ± 0.31	1.38 ± 0.28
Diethylglycerol	1.76 ± 0.08	1.01 ± 0.05	1.39 ± 0.38	**2.26 ± 0.18**	*−1.51 ± 0.19*	−1.47 ± 0.18

Table 2. *Continued*

Sugars	24 h N ± SE	24 h S/N ± SE	3 w N ± SE	3 w S/N ± SE	5 w N ± SE	5 w S/N ± SE
Digalactosylglycerol	1.57 ± 0.17	1.08 ± 0.05	**1.98 ± 0.13**	**2.73 ± 0.20**	−1.07 ± 0.23	**3.40 ± 0.20**
Fructose	*−4.00 ± 0.14*	*−3.33 ± 0.10*	2.37 ± 0.29	−1.04 ± 0.06	−1.11 ± 0.32	*−2.50 ± 0.22*
Fructose-6-P	−1.16 ± 0.19	−1.56 ± 0.12	1.23 ± 0.16	**4.94 ± 0.26**	1.68 ± 0.29	1.39 ± 0.24
Galactinol	**2.34 ± 0.15**	1.26 ± 0.06	−1.56 ± 0.41	**2.82 ± 0.10**	1.29 ± 0.18	**2.52 ± 0.18**
Galactonate	1.39 ± 0.07	−1.20 ± 0.38	1.17 ± 0.12	**3.28 ± 0.10**	−1.39 ± 0.17	1.15 ± 0.10
Galacturonate	*−3.85 ± 0.07*	*−2.70 ± 0.10*	1.56 ± 0.19	**2.70 ± 0.09**	1.75 ± 0.26	3.31 ± 0.45
Glucarate	−1.02 ± 0.31	1.02 ± 0.06	1.41 ± 0.12	**2.04 ± 0.13**	−1.25 ± 0.12	1.35 ± 0.13
Gluconate	**3.33 ± 0.38**	−1.20 ± 0.10	1.15 ± 0.28	**14.88 ± 0.35**	−1.56 ± 0.10	*−3.20 ± 0.32*
Glucose	*−2.38 ± 0.27*	*−1.96 ± 0.16*	**5.63 ± 0.45**	1.20 ± 0.11	−1.01 ± 0.37	−1.85 ± 0.36
Glucose-6-P	−1.07 ± 0.18	−1.78 ± 0.12	1.46 ± 0.26	**4.92 ± 0.24**	−1.18 ± 0.40	1.65 ± 0.34
Glycerol	−1.12 ± 0.12	−1.23 ± 0.13	1.80 ± 0.29	1.13 ± 0.06	3.07 ± 0.37	**2.10 ± 0.05**
Glycerol-3-P	1.64 ± 0.17	1.19 ± 0.17	−1.23 ± 0.14	**5.06 ± 0.18**	−1.41 ± 0.51	**4.27 ± 0.11**
Inositol	1.22 ± 0.09	1.05 ± 0.02	−1.02 ± 0.11	**1.88 ± 0.09**	*−3.13 ± 0.12*	1.10 ± 0.10
Maltose	1.38 ± 0.19	*−1.40 ± 0.07*	*−2.00 ± 0.50*	**3.66 ± 0.11**	*−2.22 ± 0.23*	1.35 ± 0.11
Melezitose	1.16 ± 0.18	−1.05 ± 0.07	1.39 ± 0.24	1.06 ± 0.32	−2.04 ± 0.39	1.96 ± 0.27
Melibiose	1.62 ± 0.12	1.11 ± 0.09	−1.15 ± 0.12	**8.63 ± 0.12**	−1.19 ± 0.18	1.06 ± 0.18
Raffinose	**2.35 ± 0.14**	1.18 ± 0.04	1.15 ± 0.52	**5.59 ± 0.27**	−1.11 ± 0.20	**4.24 ± 0.23**
Sorbitol	−1.50 ± 0.20	*−10.00 ± 0.11*	−1.10 ± 0.10	*−2.00 ± 0.30*	*−5.00 ± 0.19*	**6.54 ± 0.35**
Sucrose	1.18 ± 0.12	−1.14 ± 0.08	−1.07 ± 0.18	**2.25 ± 0.04**	−1.69 ± 0.11	**2.03 ± 0.22**
Trehalose	**2.06 ± 0.10**	1.11 ± 0.08	*−2.13 ± 0.19*	1.40 ± 0.13	*−2.70 ± 0.09*	**2.68 ± 0.16**
Xylitol	1.04 ± 0.02	1.03 ± 0.02	1.22 ± 0.14	1.06 ± 0.09	−1.06 ± 0.03	−1.19 ± 0.08
Xylose	*−14.28 ± 0.08*	*−1.69 ± 0.11*	**1.93 ± 0.17**	**2.27 ± 0.14**	−1.88 ± 0.23	1.19 ± 0.21

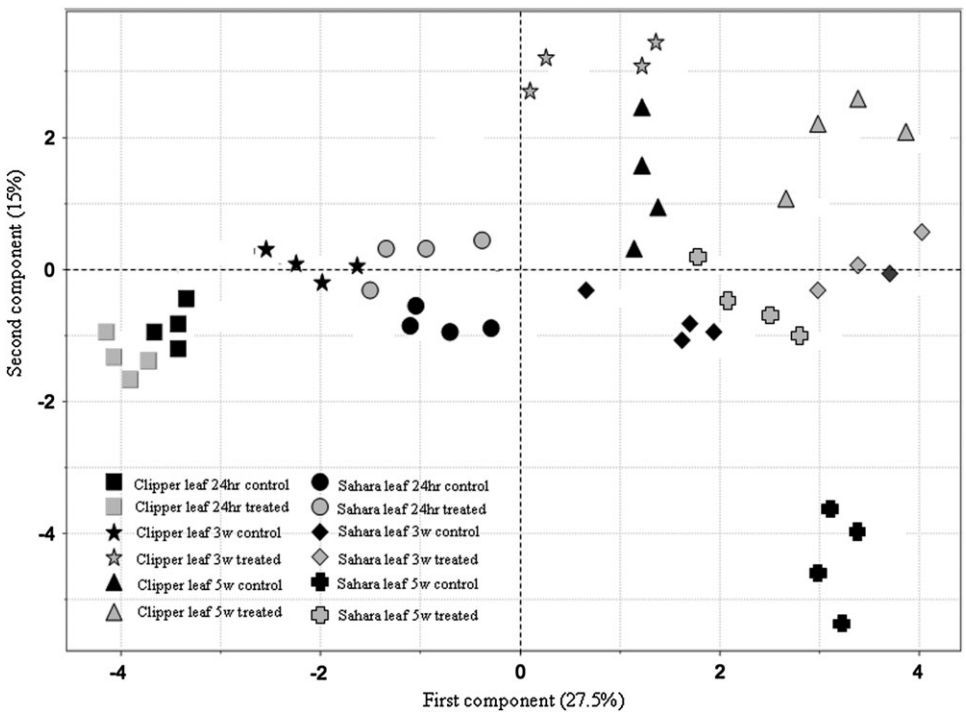

Fig. 2. Principal component analysis (PCA) of leaf metabolite profiles of Clipper and Sahara grown in control (1 mM Na^+) or salt-treated (100 mM NaCl) conditions for varying lengths of time. Time periods are 24 h, 3 w, and 5 w. Each data point represents the metabolite profile of a single sample. The distance between samples was calculated as described in the Materials and methods using the log-transformed, normalized single measurements from which the means presented in Supplementary Tables S1 and S2 at *JXB* online were calculated. Principal components 1 and 2 account for 42.5% of the variance in the data.

glycine, putrescine, serine, and tyramine. All other changes were due to increases in metabolite concentration in salt-treated Sahara leaves. The levels of amino acids that were 2–12-fold higher were glutamate, homoserine, and threonine. Organic acids and sugars present at higher levels included intermediate metabolites of the hexose phosphate

pool and tricarboxylic acid (TCA) cycle, polyols, sugars, and ascorbate. The hexose phosphate pool metabolites at 5–7-fold higher levels were 3-PGA, glucose-6-P, and fructose-6-P; the TCA cycle intermediates at 2–15-fold higher levels were α-ketoglutarate, aconitate, citrate, isocitrate, malate, and succinate; the polyols at 2–5-fold higher levels were inositol and glycerol-3-P; and the sugars at 2–6-fold higher levels were 2-ketogluconate, galactinol, galactonate, galacturonate, glucarate, maltose, raffinose, and sucrose. Ascorbate and dehydroascorbate levels were 2–5-fold higher in salt-treated Sahara leaves. The biggest changes, 14-fold increases, were in gluconate and isocitrate.

A different set of metabolic responses was observed in Clipper leaves after 3 w of salt stress (Table 2; see Supplementary Tables S1 and S2 at *JXB* online). Only three metabolites were at lower concentrations as a result of the salt treatment: citrate, 1-mono-octodecanoglycerol, and trehalose. The fold reductions were all −3-fold or less. All other changes were increases. Amino acids at 2–65-fold higher concentrations were β-alanine, asparagine, aspartate, GABA, glutamte, glutamine, glycine, homoserine, proline, putrescine, serine, threonine, and valine. GABA, homoserine, and threonine all showed a greater than 10-fold increase in concentration, and glutamine and proline both showed a greater than 40-fold increase in concentration. The greatest increase at 94-fold was observed in N-acetylglutamate. Organic acids and sugars showing a 2–6-fold increase were monomethylphosphate, threonate-1,4-lactone, digalactosylglycerol, glucose, and xylose.

Further differences in the metabolite profiles of Sahara and Clipper leaves were found following 5 w exposure to salt. No metabolites were present in lower amounts in Sahara leaves, but a few amino acids and sugars were present in higher amounts (Table 2; see Supplementary Tables S1 and S2 at *JXB* online). Of the amino acids and their derivatives, the levels of isoleucine, leucine, phenylalanine, putrescine, tyrosine, and valine were 5-fold higher or less, and levels of lysine and proline were 10-fold higher after salt stress. Sugars and sugar alcohols present at 2–6-fold higher levels were galactinol, raffinose, sorbitol, sucrose, trehalose, 2-*O*-glycerol-β-D-galactose, digalactosyglycerol, glycerol, and glycerol-3-P. No significant fold changes were noted in levels of any organic acids. A greater range of changes (both higher and lower) was found in Clipper leaves after 5 w of salt stress. The majority of organic acids, polyols, and trehalose, and a few amino acids, were present in reduced amounts (see Supplementary Table S1 at *JXB* online). Organic acids at −2 to −12-fold levels than those in control plants were TCA cycle intermediates and the antioxidant ascorbate. Levels of polyols, such as inositol, and other sugar alcohols, such as 2-*O*-glycerol-β-D-galactose, 1-monohexadecanoglycerol, 1-mono-octodecanoglycerol, diethylglycerol, and sorbitol, were −1.5 to −5-fold lower. A few amino acids and derivatives were present at higher levels, including GABA (43-fold), leucine (4-fold), proline (45-fold), and putrescine (4-fold). The only amino acid present at lower levels after 5 w of salt stress was alanine (−5-fold).

Metabolic responses in roots after salt stress

In Clipper and Sahara roots, a total of 62 compounds were identified (see Supplementary Tables S3 and S4 at *JXB* online). The same compounds were present in both cultivars. Several metabolites were only identified in Clipper and Sahara leaves but not in roots: glutamine, tryptophan, α-ketoglutarate, ascorbate, galactonate, isocitrate, threonate-1,4-lactone, 1,6-anhydroglucose, glycerol, and melibiose. Levels of other metabolites that were detected but could not be identified will not be described here.

Figure 3 shows a PCA of root metabolite data. Clusters of metabolite data from roots subjected to a short-term salt stress were separated from those subjected to a long-term salt stress. The clusters of Clipper and Sahara short-term data sets were to the right of the clusters of long-term data sets. The short-term salt stress data sets for Clipper and Sahara roots form a single cluster with their control data sets. These data sets are not resolved by principal components 1 and 2. For the long-term (3 w and 5 w) data sets, the salt-treated clusters are to the left of the respective control data sets, except 3 w salt-treated Clipper which is below the control. Examination of the loadings for the first and second principal components showed a number of metabolites that contribute to cluster separation. For the first principal component, aconitate, malate, and 3PGA are the main metabolites responsible for separation. For the second principal component, monomethylphosphate and citramalate are the main metabolites responsible for separation. Citramalate was not detected in Clipper or Sahara roots after 3 w and 5 w of salt stress which therefore caused a large variation for this metabolite reflected in its impact onto the loadings.

Supplementary Table S3 at *JXB* online shows the range of metabolites identified in Sahara roots and how their levels changed after 24 h, 3 w, and 5 w of salt stress. After a short-term exposure to salt, levels of polyols and trehalose rose 2–3-fold (see Supplementary Table S3 at *JXB* online). Polyols at a higher concentration were glycerol-3-P and inositol. Levels of many more metabolites changed in Sahara roots after 3 w exposure to salt. In all cases, the respective metabolite was present at a lower concentration in salt-treated roots. Metabolites found to decrease −3 to −7-fold were homoserine, fumarate, glycerate, quinate, fructose, gluconate, glucose, and sorbitol. Levels of monomethylphosphate and threonate were found to decrease −16-fold and −9-fold, respectively. After 5 w of salt stress, only one metabolite, erythronate, was present at levels higher (6-fold) than those in control roots. In all other cases the metabolite was present at a level below that in the control (see Supplementary Table S3 at *JXB* online). Levels of two amino acids, lysine (−50-fold) and tyramine (−100-fold), were substantially lower. Decreases of −2 to −20-fold were also noted for many organic acids: dehydroascorbate, malate, maleate, quinate, monomethylphosphate, shikimate, succinate, and threonate. Levels of the sugar alcohol, inositol, were −2-fold lower. Larger reductions were noted in levels of the sugars fructose (−33-fold), glucose (−13-fold), and the sugar alcohol, diethylglycerol (−50-fold).

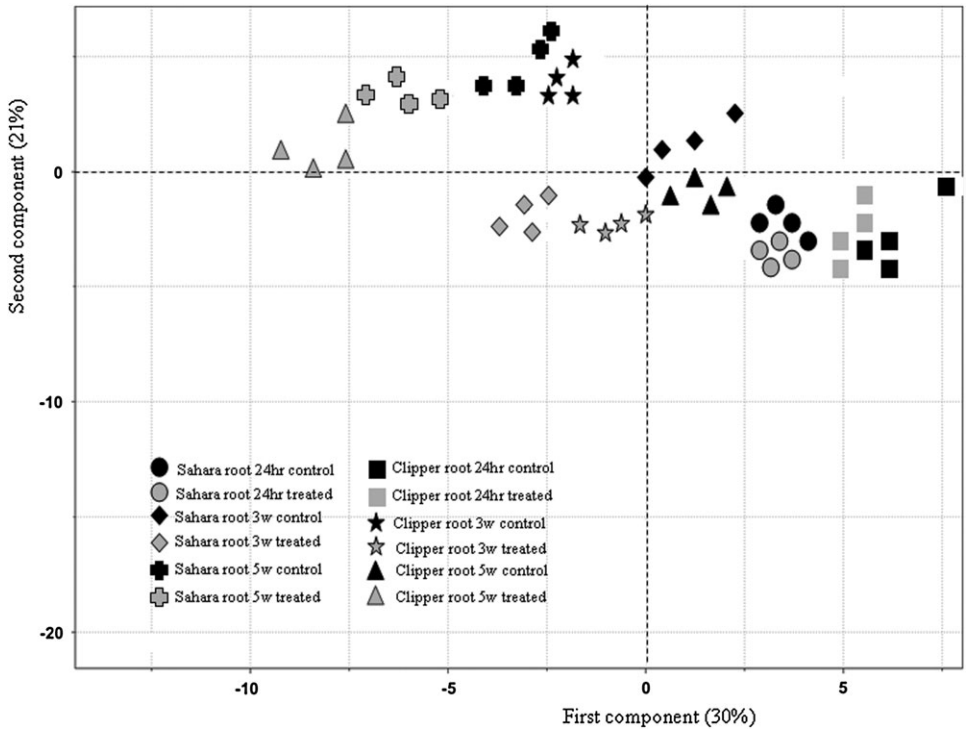

Fig. 3. Principal component analysis (PCA) of root metabolite profiles of Clipper and Sahara grown in control (1 mM Na^+) or salt-treated (100 mM NaCl) conditions for varying lengths of time. Time periods are 24 h, 3 w, and 5 w. Each data point represents the metabolite profile of a single sample. The distance between samples were calculated as described in the Materials and methods using the log-transformed, normalized single measurements from which the means presented in Supplementary Tables S3 and S4 at *JXB* online were calculated. Principal components 1 and 2 account for 51% of the variance in the data.

Metabolic responses in Clipper roots after 24 h salt stress included decreases in the levels of most amino acids and three sugars; the levels of inositol and trehalose were 2-fold higher (see SupplementaryTable S4 at *JXB* online). Amino acids and their derivatives present at −2 to −10-fold lower levels were alanine, asparagine, homoserine, isoleucine, leucine, putrescine, serine, threonine, tyrosine, and valine. The biggest reduction was in β-alanine, which was present at −14-fold lower levels. Levels of three sugars, fructose, glucose, and raffinose, fell by −4 to −7-fold. By 3 w of salt exposure, levels of β-alanine, alanine, GABA, glycine, proline, and putrescine were all above those of control plants. β-alanine (20-fold) and proline (117-fold) showed the biggest increases. Levels of several organic acids, erythronate, malate, ribonate, succinate, threonate, were all −2 to −5-fold lower in salt-exposed roots. Fructose-6-P (−6-fold), 2-ketogluconate (−3-fold), galacturonate (−3-fold), and glucarate (−3-fold) were sugars at lower levels, whereas sorbitol (18-fold) and trehalose (6-fold) were sugars at higher levels. Many organic acids were at lower levels in Clipper roots after 5 w of salt stress and included decreases of −4 to −50-fold in TCA cycle metabolites, such as citrate, fumarate, malate, and succinate. Sugars, such as 2-ketogluconate, fructose, glucose, and xylose, were −3 to −11-fold lower than in control plants. Levels of proline (8-fold), sorbitol (7-fold), and trehalose (9-fold) were higher after 5 w of salt stress, whereas levels of asparagine (−9-fold) and homoserine (−25-fold) were lower.

A global perspective of metabolite changes in roots and leaves

All data from roots and leaves of both cultivars are also summarized in the form of a heatmap in Fig. 4 to provide a global perspective of metabolite changes in response to salt treatment. Noteworthy are the cluster formations of metabolites representing similar patterns of metabolites throughout the data set. A clear difference in metabolite levels between root and leaf tissue can be observed with most amino acids being at much higher levels in the roots compared to the leaves and all other analysed classes of compounds showing the opposite pattern. Most sugars are grouped together with a few exceptions, for example, glucose- and fructose-6-phosphate formed a distinct cluster representing their tight metabolic correlation which has been previously observed (Roessner *et al.*, 2001). Also apparent is that the root and leaf samples were clustered in distinct groups after 5 w of treatment (Fig. 4). In general, the more distinct clusters relating to treatment were found for the leaf samples particularly after a longer salt treatment, demonstrating that the amplitude of the changes was greater in leaf rather than in root tissue due to salt treatment. In summary, this type of heatmap analysis provides a powerful global visualization of how metabolite levels alter due to a treatment. In addition, an overview of similarities not only between the samples under analysis but also the metabolite pattern throughout the data set can also

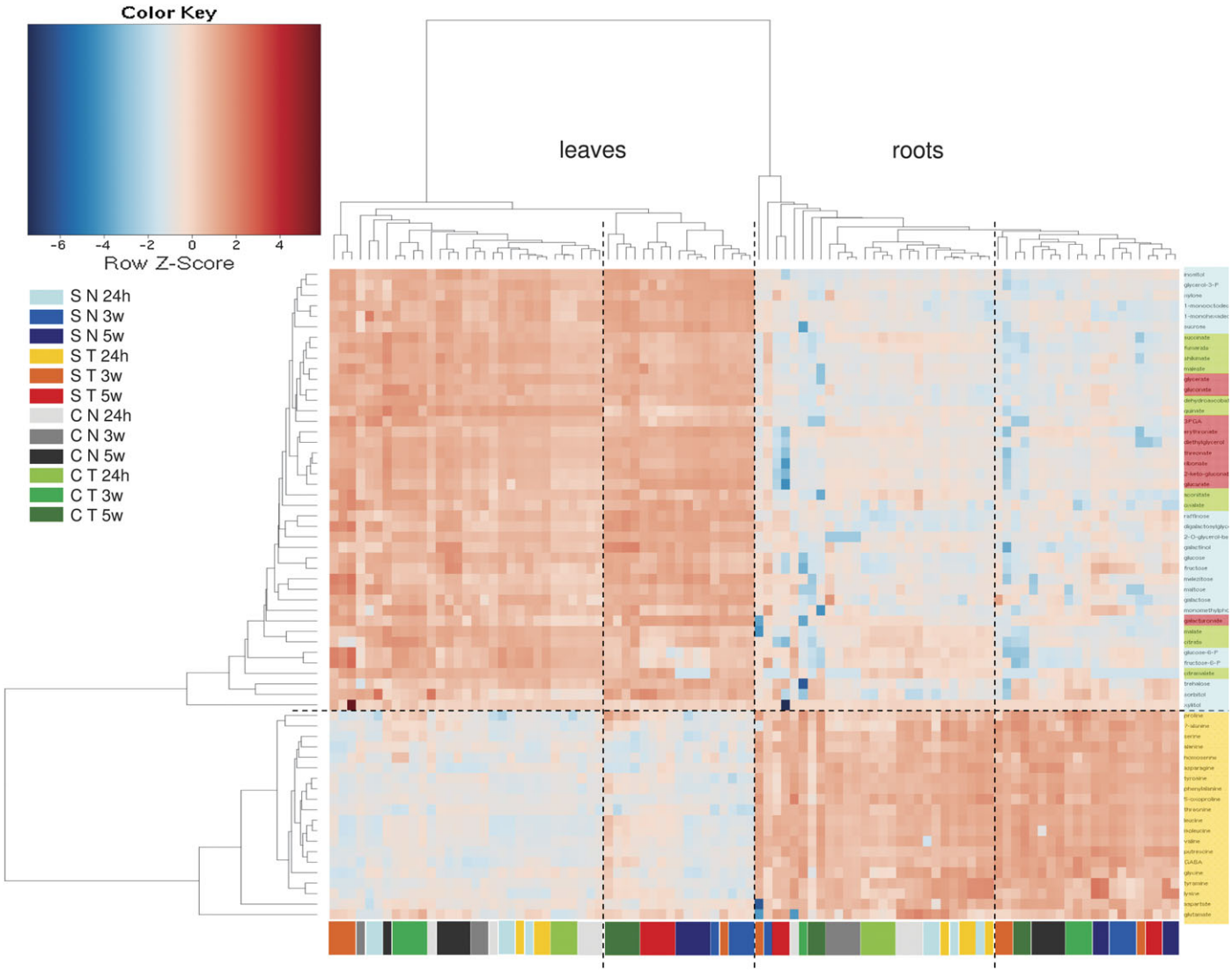

Fig. 4. Heatmap analysis combined with hierarchical cluster analysis of the same data set as used in Figs 2 and 3 using the R program called made4 as described in Culhane *et al.* (2005). Metabolites are coloured as follows: amino acids in orange, organic acids in green, sugars in light blue, and sugar acids in red. Samples are colour coded as the legend on the figure. S, Sahara; C, Clipper; N, non-treated; S, salt-treated. Picture courtesy of Tim Erwin.

be gleaned thereby suggesting possible relationships between particular metabolites or within a metabolite class.

Discussion

Sahara grows better in high salt despite accumulating higher leaf Na^+ levels

When grown hydroponically under conditions of high salinity the two barley cultivars, Clipper and Sahara, performed very differently over the 6 w period. Clipper was severely affected, having a growth reduction of up to 58% of dry weight (compared with 14% in Sahara). In addition, Clipper leaves had developed extensive areas of necrosis by the end of the experimental period. Different responses were seen in Clipper and Sahara after 3 w of salt treatment: Clipper essentially ceased growing, whereas Sahara resumed growing at rates similar to those of untreated plants (Fig. 1). By this time, Sahara also had significantly higher leaf Na^+ concentrations but had less leaf necrosis than Clipper, suggesting that Sahara leaves are more tolerant to salt than those of Clipper. This may be, at least partly, as a result of a higher tissue K^+/Na^+ ratio (16 in Sahara and 6 in Clipper; Table 1). Previous studies have demonstrated that the ability of barley to maintain photosynthetic capacity, and hence maintain better salinity tolerance, is associated with higher K^+, lower Na^+, and a higher cytosolic K^+/Na^+ ratio (James *et al.*, 2006).

Only a few metabolites change after short-term (24 h) exposure to NaCl

Following 24 h salt treatment only a small number of metabolites responded with more changes being observed in the roots of Clipper than in the roots of Sahara and the

leaves of both plants. A number of amino acids and sugars (e.g. fructose, glucose, and raffinose) decreased in Clipper roots. Interestingly, in roots of both cultivars an increase in inositol and trehalose (see Supplementary Tables S3 and S4 at *JXB* online) was observed following 24 h salt treatment indicating a role of these two known osmoprotectants (Yancey, 2005) in the short-term osmotic response. While this has not previously been noted in barley, a recent study of *Arabidopsis* cell cultures following 24 h of salt stress also revealed an increase in the levels of inositol (Kim *et al.*, 2007). Fricke *et al.* (2006) reported that solute levels in the elongation zone of the leaf of the barley cultivar Golf rose 8–20 h after the addition of 100 mM NaCl to the hydroponic solution. This assumption was supported by the change in external osmolarity caused by the addition of NaCl. Unfortunately, they did not analyse organic solutes in this experiment. It was proposed that the change in osmolarity was caused by the increase in inorganic solutes, such as K^+ and nitrate (Fricke *et al.*, 1994), however, organic solutes such as inositol and trehalose could also have contributed to the observed change. The fact that only a few metabolites responded after 24 h of treatment may suggest that a change of metabolite levels may not be possible within such a short time frame (Gibon *et al.*, 2006). It would also have been interesting to determine whether transcript and protein levels as well as metabolic enzyme activities respond within 24 h of treatment.

Increases in amino acids in Clipper leaves after salt stress may be related to tissue damage

More than half of all amino acids analysed were statistically significantly increased in Clipper leaves after 3 w and 5 w of salt stress with some quite dramatic accumulations, for example, up to 94-fold in N-acetylglutamate levels (Table 2). In 3 w treated Sahara leaves a completely different response was observed with only a few amino acids being slightly increased and a similar number of them being slightly decreased (Table 2). After 5 w of treatment the amino acid profiles in Sahara showed more similarities to those of Clipper at 3 w suggesting that Sahara leaves are also beginning to sustain cell damage. Increased amino acid levels have previously been observed following different stress treatments (Kaplan *et al.*, 2004; Nikiforova *et al.*, 2006; Zuther *et al.*, 2007; Sanchez *et al.*, 2008*a*, *b*; Armengaud *et al.*, 2009) and in some cases have been related to senescence and cell damage (Roessner *et al.*, 2006; van Doorn and Woltering, 2008). Our data suggest that the onset of senescence may have been earlier in Clipper leaves since there were increased levels of amino acids as early as 3 w after salt treatment, whereas this pattern was only seen in Sahara at the 5 w time point. Increased levels of amino acids have been observed in rice cultivars (Zuther *et al.*, 2007) and *Lotus japonicus* (Sanchez *et al.*, 2008*b*) following salt treatment; both plants being considered to be salt sensitive. On the other hand, such increases in amino acid profiles were not observed in the halophyte *Limonium latifolium* (Plumbaginaceae; Gagneul *et al.*, 2007), the salt-tolerant tree *Populus euphratica* (Brosche *et al.*, 2005), nor the salt-cress *Thellungiella halophila*. In these plants only proline and a few other amino acids (e.g. aspartate) were increased. It is concluded, therefore, that with the exception of proline, most increases in amino acid levels in Clipper leaves after salt stress may not be part of an adaptive response to salt, but rather an indicator of general stress and cell damage.

A similar scenario was evident in the root data with increased levels of amino acids in Clipper roots after 3 w of treatment (see Supplementary Table S3 at *JXB* online) whereas there were no changes in Sahara roots. It has already been shown, using a proteomics approach, that high salt treatment initiated programmed cell death (PCD) in rice root tips and this was correlated with reduced ATP production and an alteration in the activity of a number of metabolic enzymes (Tsunezuka *et al.*, 2005; Chen *et al.*, 2009). This again may indicate that increased levels of amino acids following a stress treatment may be correlated with senescence and cell damage or death.

Levels of certain amino acid have also been observed to increase in *Arabidopsis* leaves and roots when grown under K^+-deficient conditions (Armengaud *et al.*, 2009) with stronger increases evident in the leaves. The authors proposed that the increased levels of amino acids may act to balance the loss of charge due to the strongly reduced K^+ levels in those plants (Armengaud *et al.*, 2009). In our study both Clipper and Sahara leaves showed reduced K^+ levels to about half of the control levels following salt treatment. It is therefore important to consider if the Na^+ toxicity symptoms observed in Clipper leaves also partly reflects a response to decreased K^+ levels. Sahara as the more tolerant variety may be better adapted to cope with decreased K^+ levels in its leaves. Further investigations are required to decipher the interdependence, if any, between the metabolic responses to Na^+ toxicity and K^+ deficiency and their role in salinity tolerance mechanisms.

GABA and putrescine as potential senescence markers

In concurrence with the hypothesis that increased amino acids are related to senescence and cell damage, it has been shown that glutamate and glutamine typically become more available during leaf senescence (Ansari *et al.*, 2005). These molecules, both of which increased statistically in 3 w salt-treated Clipper leaves, can be converted to GABA (which also increased) by glutamate decarboxylase (GAD). GABA has been shown to be a stress marker (Bouche and Frommer, 2004) and an important molecule in changing nitrogen and carbohydrate metabolism (Fait *et al.*, 2008). Another metabolite which has previously been shown to respond to drought and salt stress is putrescine, a member of the polyamine family (Alcazar *et al.*, 2006; Groppa and Benavides, 2008); this was increased in Clipper leaves after 3 w and 5 w of salt stress treatment, whereas in Sahara leaves it only increased at 5 w. Polyamines are aliphatic nitrogen compounds which are positively charged at physiological pH allowing interaction with negatively

charged macromolecules such as DNA, RNA, proteins or phospholipids. It has previously been shown that putrescine and spermidine levels, as well as the activities of polyamine biosynthetic enzymes, increased once rice leaves entered an advanced stage of senescence (Chien and Kao, 1991). Roessner *et al.* (2006) showed that boron-stressed tips of Clipper leaves, where necrosis was already visible, had strongly increased levels of putrescine (6-fold) compared with the leaf tips of untreated plants. By contrast, there were no changes in putrescine levels in the leaf tips of Sahara, the boron-stress-tolerant variety, following treatment with very high levels of boron (1000 μM) for 3 w (Sutton *et al.*, 2007). A similar picture was observed for GABA with 7-fold increases in boron-treated Clipper leaf tips compared with untreated tips and no changes in Sahara. It is concluded that the increased putrescine and GABA levels observed here in salt-treated Clipper leaves and roots may also represent an indicator of the onset of senescence and cell damage in the leaf tissue.

Are reduced levels of organic acids a marker of reduced growth following salt stress?

There was a marked difference in the levels of organic acids in Clipper and Sahara leaves following salt-stress. In Sahara leaves after 3 w of salt stress, the levels of several metabolites from the tricarboxylic (TCA) cycle, including citrate (10-fold), aconitate (5-fold), isocitrate (15-fold), α-ketoglutarate (3-fold), succinate (2.5-fold), and malate (1.5-fold) all increased (Table 2). This possibly suggests an increase in the flow of carbon from glycolysis through this pathway leading to an increased production of NADH, $FADH_2$, and ATP. Because the increased TCA cycle activity occurred at a time when growth of Sahara plants was resuming after the salt treatment (Fig. 2), it is possible that the additional energy and reducing equivalents being generated were used to support growth. Direct measurement of dark respiration rates in salt-stressed and control conditions would be needed to confirm that the resumption of growth in Sahara was concomitant with, or followed by, an increased flux of carbon through glycolysis and the TCA cycle. Interestingly, levels of almost all OAs determined in this study were significantly decreased in 5 w treated Clipper leaves (Table 2). Again, it is proposed that levels of organic acids, especially those that are part of the TCA cycle, may represent the metabolic activity of the tissue under investigation; that is, higher levels of these organic acids compared with the control are correlated with the ability of the plant to improve its growth when stressed (as for Sahara). Lower levels may be correlated with reduced metabolic activity and, therefore, reduced growth (as in Clipper). Increases in respiration in order to cater for the increasing demand on energy have been proposed as a mechanism of short-term adaption to salinity stress (Bloom and Epstein, 1984; for a review, see Lambers *et al.*, 1998). A long-term response in many cases is a decrease in respiration and this has been correlated with a decrease in the demand for energy as growth is slowed following a decrease of leaf water potential due either to salt or drought stress (for a review, see Lambers *et al.*, 1998). This is consistent with our data where a decrease in organic acids is seen, proposed to be correlated with a decrease in respiration, which then is reflected in the reduced growth rate in the Clipper plants following salt treatment. By contrast, an increase was observed, especially of the TCA intermediates in Sahara which resumes growth following salt stress. To test this hypothesis, the photosynthetic, photorespiration, and dark respiration rates in both Clipper and Sahara leaves would need to be determined.

An increase in the levels of ascorbate and its degradation products, dehydroascorbate and threonate, possibly also including threonate-1,4-lactone (Green and Fry, 2005) was found in 3 w treated Sahara leaves (Table 2). Ascorbate is a major antioxidant in plants protecting them, via the ascorbate–glutathione and xanthophyll cycles, against oxidative damage resulting from aerobic metabolism and photosynthesis (Smirnoff, 1996). Higher ascorbate levels may have supported the better performance of Sahara when grown under conditions of high salinity. It is also noteworthy that after 5 w of salt treatment none of the organic acids had increased (Table 2) suggesting that any increase in respiration and antioxidant activity was transient.

Increased sugar levels in leaves are correlated with better growth performance of Sahara after salt stress

A large number of sugar and sugar acid levels were significantly increased in 3 w treated Sahara leaves, whereas increases only in the levels of fructose and glucose were observed in 3 w treated Clipper leaves. Interestingly, these sugars did not change in Sahara leaves. It has been shown that accumulation of sugars is a common response to drought, salinity, and low-temperature stress (for reviews, see Gupta and Kaur, 2005; Wingler and Roitsch, 2008). Sugars act not only as osmoprotectants in helping to maintain osmotic balance and to stabilize macromolecules under stress conditions, but can also provide an immediate energy source to plants restarting growth after a period of stress-induced dormancy (Yancey, 2005). Increased levels of sugars following salt stress have also been described in *Lotus japonicus* (Sanchez *et al.*, 2008*b*), *Arabidopsis* and *Thellungiella* with greater increases in sugars in the salt-acclimated *Thellungiella* (Gong *et al.*, 2005), the halophyte species *L. latifolium* (Gagneul *et al.*, 2007), and the salt-tolerant tree *P. euphratica* (Brosche *et al.*, 2005). It is worth noting that no increases in sugars were observed in different rice cultivars following salt stress but rather there were decreases in fructose and sucrose levels (Zuther *et al.*, 2007).

There were also increases in the levels of certain metabolites involved in the biosynthesis of these sugars. For example, the trisaccharide raffinose (galactosyl-sucrose) is produced by the condensation of galactinol (α-galactosyl-*myo*-inositol) and sucrose to produce raffinose and inositol (Tapernoux-Lüthi *et al.*, 2004). Levels of galactinol and inositol were both 2–3-fold higher in salt-treated Sahara leaves compared with controls. It has been proposed that inositol is an

effective free radical scavenger (Smirnoff and Cumbes, 1989) since it has previously been shown to accumulate to high levels in salt-stressed plants (Ishitani *et al.*, 1996; Gong *et al.*, 2005; Gagneul *et al.*, 2007; Brosche *et al.*, 2005).

Conclusions

The data presented here provide a new perspective on the salt responses of two barley cultivars. When grown hydroponically, different responses to salt stress were observed in the two cultivars, possibly due to the large difference in the leaf K^+/Na^+ ratios. Sahara, the better-adapted cultivar, displayed tolerance to high internal salt concentrations without apparent cell damage suggesting that this cultivar may have mechanisms either to maintain a higher K^+/Na^+ ratio in the cytoplasm through compartmentation of Na^+ into the vacuole or by increasing the metabolite levels to cope with the increased osmotic potential. Associated with the higher tissue tolerance in Sahara leaves was an increase in sugars, polyols, and a large number of organic acids which may either act as osmoprotectants or provide more readily available carbon sources for better growth performance compared with Clipper. By contrast, Clipper leaves, which showed symptoms of necrosis following salt stress, showed higher levels of amino acids which may be correlated with cell damage. A large decrease in organic acid levels in Clipper may reflect a reduced rate of metabolism as a response to salinity. Therefore, it is concluded that the metabolic differences in the responses of Clipper and Sahara following long-term salt exposure may be correlated with their differential abilities to sequester Na^+ into the vacuole to avoid cellular damage.

Supplementary data

Supplementary data are available at *JXB* online.

Supplementary Table S1. Metabolite ratios in 100 mM salt-treated Sahara leaves compared to controls for different lengths of time

Supplementary Table S2. Metabolite ratios in 100 mM salt treated Clipper leaves compared to controls for different lengths of time

Supplementary Table S3. Metabolite ratios in 100 mM salt treated Sahara roots compared to controls for different lengths of time

Supplementary Table S4. Metabolite ratios in 100 mM salt-treated Clipper roots compared to controls for different lengths of time

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