Wheat mitochondrial respiration shifts from the TCA cycle to the GABA shunt under salt stress

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Summary

- Mitochondrial respiration and tricarboxylic acid (TCA) cycle activity are required during salt stress in plants to provide ATP and reductants for adaptive processes such as ion exclusion, compatible solute synthesis and reactive oxygen species (ROS) detoxification. However, there is a poor mechanistic understanding of how salinity affects mitochondrial metabolism, particularly respiratory substrate source.

- To determine the mechanism of respiratory changes under salt stress in wheat leaves, we conducted an integrated analysis of metabolite content, respiratory rate, and targeted protein abundance measurements. Also, we investigate the direct effect of salt on mitochondrial enzyme activities.

- Salt treated wheat leaves exhibit higher respiration rate and extensive metabolite changes. The activity of the TCA cycle enzymes pyruvate dehydrogenase complex and the 2-oxoglutarate dehydrogenase complex were shown to be directly salt sensitive. Multiple lines of evidence showed that the γ-aminobutyric acid (GABA) shunt was activated under salt treatment.

- During salt exposure, key metabolic enzymes required for the cyclic operation of the TCA cycle are physiochemically inhibited by salt. This inhibition is overcome by increased GABA shunt activity, which provides an alternative...
carbon source for mitochondria that bypasses salt-sensitive enzymes to facilitate the increased respiration of wheat leaves.

**Keywords:** Pyruvate Dehydrogenase, γ-aminobutyric acid (GABA) Shunt, Mitochondria, Salinity, Sodium Chloride, SRM Mass Spectrometry, Wheat.

**Introduction**

Soil salinity is a major agricultural issue that affects crop yield around the world (Munns & Tester, 2008). Soil salinity leads to salinity stress in plants which is characterised by reductions to photosynthesis and growth (Sudhir & Murthy, 2004; Chaves *et al.*, 2009; Kafi, 2009). The impairment of photosynthesis is partly due to the toxic effects of high salt concentrations on the photosynthetic machinery, which limits carbon assimilation and therefore curtails plant growth (Chen *et al.*, 2015). Salinity stressed plants are also characterized by a reallocated carbon balance, with a higher portion of assimilated carbon diverted away from growth and towards respiration and maintenance (Jacoby *et al.*, 2011; Flowers *et al.*, 2015). Previous studies have shown that mitochondrial respiration is required during salt stress in plants, because it provides adenosine triphosphate (ATP) and reductants that fuel adaptive processes including ion exclusion, compatible solute synthesis, and reactive oxygen species (ROS) detoxification (Munns & Tester, 2008). An extensive literature has studied the metabolite contents of salt treated leaves and roots in both dicot and monocot species, and the consensus results are characterised by an accumulation of sucrose and amino acids, most notably proline, and a marked depletion of organic acids, especially intermediates of the tricarboxylic acid (TCA) cycle (Johnson *et al.*, 2003; Cramer *et al.*, 2007; Kim *et al.*, 2007; Zuther *et al.*, 2007). The mechanism for these changes is not clear, but this metabolic reprogramming has a wide-ranging potential impact on physiological processes such as charge balance, ammonium detoxification and compatible solute accumulation (Sanchez *et al.*, 2008).

Plants can deploy different physiological strategies to cope with salinity stress, such as ion exclusion, osmotic tolerance and tissue tolerance (Tyerman *et al.*, 2019). These tolerance strategies are mediated by diverse respiratory mechanisms, as evidenced by the wide range of respiratory responses observed across different plant species via respiratory rate measurements of salt treated plants (Jacoby *et al.*, 2011). Multiple studies have reported that salt-stressed plants exhibit changes to the ratio of CO₂ released over O₂ consumption (respiration quotient), potentially indicating that mitochondria oxidise a differential set of substrates under salinity (Cramer *et al.*, 2007;
Lambers et al., 2008; Jacoby et al., 2013). Ultimately, the nature of the substrates respired will exert a large influence on the plant’s overall metabolic homeostasis, especially in modulating the balance between growth, compatible solute biosynthesis, and energy production.

Mitochondrial respiration is often positioned at the centre of plant metabolic networks, because the TCA cycle links energy metabolism with both carbon and nitrogen metabolism (Jacoby et al., 2013; Nunes-Nesi et al., 2013). Chemical inhibition of TCA cycle components can activate alternative metabolic routes through the TCA cycle, which are usually dependent on protein activity, substrate availability and energy demand (Foyer et al., 2011; Krasensky & Jonak, 2012; Nunes-Nesi et al., 2013). There are multiple alternative routes of TCA cycle metabolism that have been reported in plants, including the malate to pyruvate pathway and the γ-aminobutyric acid (GABA) shunt pathway. In cases where the TCA cycle becomes deficient in oxaloacetate due to its utilisation as a carbon skeleton for amino acid synthesis, the malate to pyruvate pathway bypasses oxaloacetate, enabled via malate production from glucose-derived phosphoenolpyruvate and the conversion of malate into pyruvate by malic enzyme (ME) (Kazachkova et al., 2013). Similarly the GABA shunt is a pathway that bypasses the 2-oxoglutarate dehydrogenase complex (OGDC) catalysed step in the TCA cycle (Studart-Guimaraes et al., 2007; Che-Othman et al., 2017). In the GABA shunt pathway, glutamate that can be produced from 2-oxoglutarate is decarboxylated into GABA by glutamate decarboxylase (GAD). GABA then enters the mitochondria and is catabolized by γ-aminobutyrate transaminase (GABA-T) forming succinyl semialdehyde (SSA). The final step is the conversion of SSA into succinate by succinyl semialdehyde dehydrogenase (SSADH) (Michaeli et al., 2011).

It has been proposed that the GABA shunt activity is important in stress adaptation in plants by regulating cytosolic pH, limiting ROS production, regulating nitrogen metabolism and bypassing steps in the TCA cycle (Renault et al., 2010; Akcay et al., 2012; Che-Othman et al., 2017; Carillo, 2018). However, it is currently unclear how environmental stress inhibits the TCA cycle and why the GABA shunt represents a suitable alternative. Furthermore, there is an incomplete knowledge of how mitochondrial substrate carriers respond to stress in plants (Lee & Millar, 2016).
Here we examined how salt stress affects the respiratory metabolism of wheat plants, measuring the abundance of metabolites and proteins involved in the TCA cycle, GABA shunt and mitochondrial membrane transport. We characterise specific steps of mitochondrial metabolism that are particularly sensitive to salt, and postulate that the induction of salt-tolerant respiratory pathways provide a potential mechanism to explain the metabolite changes that are often observed in salt-treated plants.

Materials and Methods

Plant growth

Wheat plants (*Triticum aestivum* L. var Wetsonia and Wyalkatchem were grown in a supported hydroponic system according to Munns and James (2003) with some modifications. The seeds were germinated in Petri dishes containing filter paper dampened with dimethylcarbamothioylsulfanyl N,N-dimethylcarbamodithioate (Thiram) solution (1.4 g/L) for four days then transferred into hydroponic solution (half strength modified Hoagland solution containing 1 mM NH$_4$NO$_3$; 0.5 mM KH$_2$PO$_4$; 0.25 mM CaCl$_2$; 50 μM KCl; 0.5 mM MgSO$_4$; 25 μM H$_3$BO$_3$; 2 μM MnSO$_4$; 2 μM ZnSO$_4$; 0.5 μM CuSO$_4$; 0.5 μM Na$_2$MoO$_4$ and 0.5 mM Fe-EDTA) in pots filled with gravel to support the roots. The hydroponic pumps were turned off for 15 out of every 30 minutes, for aeration. Seedlings were grown in a laboratory growth chamber with 16/8 hours light/dark cycle, light intensity 500 μmol m$^{-2}$ s$^{-1}$, 28/22°C day/night temperature and constant 65% humidity. Salt treatments were commenced at day eight after emergence. NaCl was added to the nutrient solution via 25 mM increments at 9 am and 4 pm every day for three days to reach a final NaCl concentration of 150 mM. NaCl concentration was maintained at 150 mM until harvest. Nutrient solutions were replaced weekly.

Measurements of photosynthesis and respiration

CO$_2$ fluxes of the third leaf were measured at day 11 of salt treatment using a portable infrared gas analyser (IRGA) systems equipped with a 600 mm$^2$ leaf chamber. The measurement was commenced at least two hours after the start of the illumination period in the growth cabinet. To measure photosynthesis, transpiration and stomatal conductance, the middle part of the third leaf was clamped in the leaf chamber. The flow rate through the leaf chamber was set to 300 μmol s$^{-1}$ and the light-emitting diode (LED) light source in the leaf chamber was set to photosynthetically active radiation (PAR) of 500 μmol m$^{-2}$ s$^{-1}$. For respiration measurements, a whole leaf with a fresh weight from 400 to 600 mg was folded and inserted into the darkened leaf chamber to
obtain a measurable CO₂ flux. Flow rate through the leaf chamber was 150 μmol s⁻¹.

The chamber block temperature (T_{Blc}) was set to match the ambient air temperature (T_{Air}) which was around 23°C and relative humidity was around 55 to 65%. CO₂ efflux was logged every minute for 30 minutes. The CO₂ efflux rate was calculated as the average rate over the last ten minutes of the measurement period, after CO₂ efflux reached its steady state.

Leaf O₂ consumption was measured using a liquid-phase oxygraph system with a Clark-type oxygen electrode. Wheat leaves of fresh weight 90 mg to 100 mg were cut into pieces of approximately 10 mm in length, and equilibrated in respiration buffer containing 10 mM HEPES, 10 mM MES and 2 mM CaCl₂, pH 7.2 in the dark for 30 minutes. The O₂ consumption rate of the leaf was measured by immersing the leaf into 2 mL of respiration buffer in a darkened electrode chamber for at least 15 minutes at 25°C while monitoring the depletion of dissolved O₂ over time using Oxygraph Plus v1.02 software.

**Biomass and elemental sodium content**

Root, shoots and leaf 1, 2 and 3 (the first, second and third emerging leaves) were separated upon harvested. The fresh weight of all harvested tissue was recorded. Tissue was dried at 65°C for 3 days, and dry weight recorded. Sodium content of dried tissue was then quantified via flame photometry. First, dried leaf tissue was ground to a fine powder in liquid nitrogen, and the leaf powder was transferred into 10 mL of 0.5 M HCl and shaken for 2 days using a rotating shaker. The extracts were then filtered through 0.45 µM syringe-driven filter into 20 mL glass vials. Sodium content of the filtrates was measured using a flame photometer.

**Mitochondrial isolation, protein extraction and digestion**

Mitochondria were isolated from whole shoots by differential centrifugation followed by a 0-4.4% (w/v) PVP and self-forming Percoll gradient based on the methods of Jacoby et al. (2013) and Kerbler and Taylor (2017). Mitochondrial proteins were precipitated via acetone treatment, and the digested peptide samples were extracted using solid phase C-18 macrospin columns.

**MRM optimisation and targeted proteins quantification**
Selected reaction monitoring (SRM) mass spectrometry of peptide transitions was optimized using trypsin digested isolated mitochondrial extracts run on an Agilent 6430 triple quadrupole (QqQ) mass spectrometer with an HPLC Chip Cube source by the method described in Taylor et al. (2014).

**MRM data analysis**

Raw MS data obtained from an Agilent Technologies 6430 QqQ mass spectrometer were imported into Skyline, and peptides with fewer than three reliable transitions were removed. Differences in protein abundance between mitochondria isolated from control versus salt treated plants were analysed using MSstats version 2.1.6 operated on R version 3.1.0 (Broudy et al. 2014; Chang et al. 2012; Choi et al. 2014; Surinova et al. 2013). The MSstats input file was generated from Skyline software (MacLean et al., 2010). The peak intensity of the transitions was log2 transformed and normalized across runs using the equalized median method to remove biases between MS runs. Three transitions were used for each peptide, and unique peptides were used for each protein.

**Gas Chromatography – Mass Spectrometry (GC-MS)**

Metabolites were extracted according to the modified procedure of Shingaki-Wells et al. (2011). Analysis of samples via GC-MS followed the procedure described previously (Howell et al., 2009). Metabolite abundance values in each sample were measured relative to the abundance of a spiked internal standard (ribitol). Raw GC-MS data pre-processing and statistical analysis were performed using MetabolomeExpress software (version 1.0; http://www.metabolome-express.org). Detailed methods have been previously reported (Carroll et al., 2010).

**Protein extraction for measuring the abundance of GABA shunt enzymes**

GAD abundance was measured in whole leaf protein extracts, while both GABA-T and SSADH were measured in high-speed pellets. Cytosolic extracts and high speed pellets were both obtained from leaf 3 harvested from control and salt-treated plants.

**Total RNA extraction for qRT-PCR analysis**

Total RNA was extracted from the third leaf using Spectrum Plant Total RNA Kit following the manufacturer’s instructions. The DNA in the sample was digested using On-Column Deoxyribonuclease 1(DNase 1) Digestion Set following the manufacturer’s
instructions. The RNA concentration and quality were determined using NanoDrop 1000 Spectrophotometer and agarose gel electrophoresis.

Real-time PCR of GABA shunt genes

cDNA synthesis from the RNA sample was carried out using the iScript cDNA synthesis kit following the manufacturer’s instructions. Synthesised cDNA was purified from the samples using QIAquick PCR Purification Kit following the manufacturer’s instructions. An equal concentration of cDNA was used for quantitative RT-PCR in all samples. Quantitative RT-PCR analysis was carried out using a LightCycler 480 instrument, with LightCycler 480 SYBR Green I Master reaction mix. The gene encoding Regulatory Particle Triple-A ATPase 5A (RPT5A) was used as the reference transcript for the qRT-PCR experiment, and was selected by examining an RNASeq dataset assembled for wheat (www.wheatproteome.org). A number of transcripts were assessed for stability across wheat development and RPT5A was found to be the most stable reference. To calculate transcript expression, each cDNA sample was analysed in duplicate, and relative expression was calculated by normalizing versus the expression level measured at the first day in control plants. RPT5A primer sequences were RPT5A-fwd (5’- CCTGCTTTACTCCGTTCGG-3’) and RPT5A-rev (5’-TCCACACAAACAGCTTCG-3’). GAD, GABA-T, and SSADH transcripts’ primer sequences were as follows: GAD-fwd, 5’-TGCCGGAGAACTCGATCCCCAAG-3’, GAD-rev, 5’-CGGTTCTGGAGCTCGGTGGTGAC-3’, GABAT-fwd, 5’-TATGATGTCCACCGCTTTGA-3’, GABAT-rev, 5’-CTGACAGCGAGGGTTATTCC-3’, SSADH-fwd, 5’-CTACGACGGGAAGACCATCGAG-3’, and SSADH-rev, 5’-ATGCTTGCTCAACCTTCTCGG-3’.

GAD activity assay

200 mg of ground leaf 3 tissue was added to 1 mL of protein extraction buffer (150 mM potassium phosphate, 5 mM EDTA, 1 mM MgCl₂, 0.5 % (w/v) PVP, 3 mM β-mercaptoethanol, 0.2 mM pyridoxal phosphate (PLP), 10 % (w/v) glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF) pH 5.8) at 4°C and vortexed thoroughly. The mixture was centrifuged at 500 x g for 30 minutes at 4°C. The supernatant was used for the GAD activity assay and protein concentration in the supernatant was determined via Bradford assay. GAD activity was measured by quantifying the amount of GABA produced in the carboxylation reaction through enzymatic assay using GABase following the manufacturer’s instructions. For experiments under varying salt conditions, the GABase assay of the control GAD assay (0 mM NaCl) was supplemented.

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with 48.75 mM NaCl. The increase of OD340 nm was recorded plate reading spectrophotometer. The amount of GABA was calculated according to an external calibration curve of GABA. GABA standards with concentrations of 0, 12.5, 25, 50, 100 µM were prepared by dissolving GABA in GAD assay buffer.

GABA shunt inhibition

3-mercaptopropionic acid (3-MP) was used to inhibit GAD activity while Vigabatrin (gamma-vinyl-GABA) was used to inhibit GABA-T activity. 3-MP and Vigabatrin were both dissolved in water and applied at a final concentration of 10 mM and 0.5 mM, respectively. To determine the degree of respiratory inhibition, leaf O$_2$ consumption was measured using an O$_2$ electrode as described above, and after a 15 minute measurement one of the inhibitors was added into the respiratory buffer. The measurement proceeded for another 15 minutes and the percentage of respiratory reduction was calculated by comparing O$_2$ consumption rates before and after the addition of the inhibitors.

mtPDC, mtOGDC and MDH activity assays

Activity assays of mtPDC, mtOGDC and malate dehydrogenase (MDH) were carried out according to the method of Huang et al. (2015). For E3 supplemented assays, 2 U mL$^{-1}$ commercial porcine lipoamide dehydrogenase was included in the assay reaction buffer.

mtPDC E1, E2 and E3 activity assays

To assay E1 activity, the reaction was initiated by adding 100 µg of mitochondrial protein into the assay medium consisting of 50 mM TES-NaOH pH 7.5, 5 mM pyruvate, 0.2 mM TPP, 1.5 mM Fe(CN)$_6^{3-}$ and 10 mM MgCl$_2$. E1 activity was spectrophotometrically assayed based on the reduction rate of FeCN at 420 nm. The backward activity of E2 was initiated by adding 100 µg mitochondrial protein into the assay medium containing 50 mM TES-KOH pH 7.2, 10 mM acetyl phosphate, 1.5 mM dihydrolipoamide, 75 µM Coenzyme A, and 2 U ml$^{-1}$ phosphoacetyltransferase. E2 activity was measured based on the ester formed in acetyl-lipoamide at 232 nm. The E3 forward activity was monitored by the rate of NADH formation at 340 nm in 1 mL of assay medium consisting in 50 mM TES-KOH (pH 8.5), 2 mM NADH, and 1 mM lipoamide.

mtPDC partial purification
A wheat seedling lawn was grown in the dark on soil for ten days in a growth chamber with 65% humidity and 23°C temperature. Five hundred grams of dark grown wheat shoots were harvested for mitochondrial isolation to obtain at least 20 mg of mitochondrial protein for each replicate. mtPDC was partially purified using the method of Millar et al. (1998). To examine the effect of 150 mM NaCl on the binding of E1 and E3 subunits to the E2 core of mtPDC, 150 mM NaCl was supplemented into the mtPDC buffer immediately after the freeze/thaw step, before beginning the 200,000 x g centrifugation step. The activity of mtPDC and its E1, E2 and E3 subunits were measured as described above.

Results

Sodium content, growth, photosynthesis and respiration

To evaluate the extent of sodium accumulation in wheat leaves, sodium content in the third leaf of control and salt-treated plants was measured over 11 days (Figure 1a). Leaf sodium content of salt treated plants accumulated approximately 4 fold from day 2 to day 11, with a significant difference compared to control first occurring at day 3 (Figure 1a). The impact of this sodium accumulation on biomass accumulation, photosynthesis and respiration was then determined (Figure S1). At day 11 after salt treatment commenced, sodium content was around 10 fold higher in salt treated plants compared to matched control plants. At this time point, both photosynthesis rate and biomass of the third leaf were significantly decreased under salt treatment (Figure 1b and c), while respiration rate measured both by CO₂ production and O₂ consumption was significantly higher compared to control plants (Figure 1d and e).

Metabolite changes following salt exposure

To examine changes in metabolite abundance in wheat leaves under salt stress, leaf 3 at day 11 of salt treatment was harvested from control and salt-stressed plants and metabolite profiling was undertaken by GC-MS. This enabled the quantification of metabolites involved in carbon metabolism including sugars, TCA cycle intermediates and amino acids. Investigating the data, it is evident that salt treatment is characterised by marked increases in the abundance of many amino acids, for example proline accumulated more than 180 fold and lysine was 85 fold higher in abundance compared to the control (Figure 2). There were also increases in the abundance of alanine, glutamate and GABA. All sugars analysed in this study showed significant increases in abundance except glucose, which was significantly decreased by approximately 4 fold.
Analysis of organic acid abundance revealed that there was a large depletion in aconitate abundance in salt-stressed plants (more than 155 fold) compared to control plants. Citrate, malate and fumarate also showed significant decreases in abundance under salt treatment. In contrast two organic acids exhibited a different pattern, with significant increases in the abundance of both succinate and 2-oxoglutarate being seen in the salt treated plants.

Changes in the abundance of proteins involved in the TCA cycle, mitochondrial membrane transport and the GABA shunt pathway

To examine mitochondrial protein abundance changes in wheat leaves under salt stress, leaf 3 at day 11 of salt treatment was harvested from control and salt-stressed plants and SRM mass spectrometry was applied to quantify a targeted set of proteins involved in the TCA cycle, mitochondrial membrane transport and the GABA shunt. For the 38 TCA cycle proteins examined, 6 showed statistically significant differential abundance (P<0.01, log2 fold > ± 0.26) in response to salt treatment (Table S1). These 6 proteins are displayed in Figure 3a. There were significant increases in the abundance of a particular isoform of succinate dehydrogenase subunit 2 following salt exposure (Figure 3a). Interestingly another isoform of succinate dehydrogenase subunit 2 along with aconitase, mtPDC E2 subunits and mtOGDC E2 subunits all decreased in abundance following salt exposure (Figure 3a). Of the 22 mitochondrial membrane transport proteins examined, 8 showed statistically significant changes in abundance in response to salt treatment (Table S2). The 8 proteins are displayed in Figure 3b. This analysis revealed that there were significant increases in the abundance of a branched chain amino acid carrier, a phosphate carrier, and VDAC 1 while there was a decrease in abundance of two pyruvate carrier isoforms, VDAC and TIM9 (Figure 3b). Examining the abundance of GABA shunt components, the results showed that 3 proteins exhibited statistically significant changes in abundance in response to salt treatment (Table S3). These 3 proteins are displayed in Figure 3c. This revealed that there were significant increases in abundance in two isoforms of SSADH and a single isoform of glutamate dehydrogenase (GDH).

GABA shunt induction and it effect on respiration

To determine whether changes in GABA level and GABA shunt protein abundance (Figures 2 & 3) indicated a transcriptional activation of the GABA shunt pathway, the temporal expression of GAD, GABA-T and SSADH genes was examined (Figure 4a).
Over a time course from 2-11 days after commencing the salt treatment, the relative transcript abundance of each gene was compared between salt treated versus control plants, using primers designed to the conserved regions of GAD, GABA-T and SSADH genes (Mazzucotelli *et al.*, 2006; Al-Quraan *et al.*, 2013). All genes show higher expression in salt-treated plants compared to control plants in at least one timepoint. For both GABA-T and GAD this elevated gene expression was transient, with salt treated plants only exhibiting significantly higher transcript abundance after day 2 or day 3 of salt stress respectively, whereas there were no significant differences at later stages in the time course. For SSADH there was a clear induction of gene expression under sustained salt treatment, with a statistically significant difference between control and salt treated plants emerging at day 7, and increasing toward day 11 where SSADH transcript level was 8 fold higher in salt treated plants compared to control. To examine the abundance of other proteins related to the GABA shunt, further SRM assays were developed for 7 GABA shunt proteins and isoforms (Table S4). The abundance of these proteins was measured in whole leaf extracts and organelle enriched high speed pellets, and for the 7 proteins examined, 4 showed statistically significant changes (P=<0.01, log2 fold > ± 0.26) in abundance in response to salt treatment (Table S4). These 4 proteins are displayed in Figure 4b. There were statistically significant increases in the abundance of SSADH, GDH, GABA-T and GLT in salt-treated plants compared to control, however, there was no significant difference in GAD abundance.

As GAD transcript and protein abundance did not increase significantly under salt treatment, a time course of GAD activity in the third leaves in control and salt treated was carried out to determine whether GABA shunt activation involved a post-translational induction of GAD activity (Figure 4c). GAD activity in both control and salt-treated plants increased as the plants developed from day 2 to day 11, but GAD activity in salt-treated plants was significantly higher compared to control plants from day 5 after salt treatment commenced. To determine if GAD itself is a salt sensitive enzyme, GAD activity was measured following the direct addition of 150 mM NaCl to the assay medium (Figure 4d). This showed that GAD activity was significantly lower in the presence of NaCl, indicating that GAD is directly, albeit mildly, inhibited by salt.

To analyse whether the timing of GABA shunt induction was synchronous with changed GABA abundance over time, GABA content in the third leaves of salt treated plants was measured over a time course from day 2 to day 11 (Figure 4e). This showed that GABA content was maintained at a significantly higher level in salt-treated plants compared to
control plants from day 3 to day 11 after salt treatment commenced. To investigate whether wheat respiration was reliant on GABA shunt activity following exposure to salt, leaves were treated using either Vigabatrin or 3-mercaptoproprionic acid (3-MP) that inhibit GABA-T and GAD activity, respectively. The respiration rate of control and salt-stressed wheat leaves was then compared before and after the addition of GABA shunt inhibitors (Figure 4f). The application of 0.5 mM Vigabatrin and 10 mM 3-MP reduced the respiration rate in salt-stressed wheat leaves by approximately 20%, whereas there was no inhibition of respiratory rate in control plants following the addition of either inhibitor.

The direct effect of salt on TCA cycle enzyme activity

To investigate whether the measured reduction in the protein abundance of mtPDC and mtOGDC components in vivo indicated a susceptibility of these protein complexes to salt, the activities of mtPDC and mtOGDC in mitochondria isolated from untreated leaves were measured in assay media supplemented with different concentrations of NaCl. For comparison, the activity of malate dehydrogenase (MDH) was also measured. This showed that the activity of both the mtPDC and mtOGDC from isolated mitochondria decreased in a manner proportional to the NaCl concentration in the assay media (Figure 5a). In contrast the activity of a MDH was not affected by up to 300 mM NaCl (Figure 5a). To investigate whether the reduced mtOGDC and mtPDC activity in the presence of NaCl was due to the reported dissociation of the labile E3 subunit from the mtOGDC and mtPDC complex, excess exogenous porcine lipoamide dehydrogenase were added to the mtPDC and mtOGDC assay media at 2 U mL$^{-1}$. This approach has previously been adopted to circumvent the negative effect of E3 detachment on the activity of these enzymes (Poulsen & Wedding, 1970; Karam & Bishop, 1989; Millar et al., 1998). The addition of exogenous E3 reduced the inhibition of mtOGDC in 150 mM NaCl but did not affect the inhibition of mtPDC (Figure 5b). The percent inhibition was then calculated for both mtPDC and mtOGDC in 150 mM NaCl with and without the additional E3 (Figure 5c). This showed that excess E3 increased the relative mtOGDC activity in 150 mM NaCl from 15% to 33% representing a two-fold higher relative activity of mtOGDC in the presence of 150 mM NaCl compared to the control.

Direct effect of NaCl on mtPDC components and their activity

To determine if the reduced mtPDC activity observed in vitro following salt addition was due to the reduced activity of individual enzymes of the complex, activity assays

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for each subunit were conducted in mitochondrial extracts in assay media with or
without 150 mM NaCl (Figure 6a). This showed that contrary to the activity of mtPDC
as a whole, the activities of both the E1 and E3 subunits increased in the presence of 150
mM NaCl. The activity of the E2 subunit using standard methods was too slow to
measure in these extracts. To obtain an E2 activity we therefore partially purified wheat
mtPDC samples based on the method described in Millar et al. (1998) which allowed us
to measure E2 directly. This showed that E2 also increased in activity when the assay
medium was supplemented with 150 mM NaCl (Figure 6a).

Despite the lack of PDC stimulation by exogenous E3 (Figure 5c), we wanted to more
directly determine whether salt exposure induces the dissociation of mtPDC subunits
and loss of mtPDC activity. To do this, we measured the activity of mtPDC and its E1,
E2 and E3 subunits in isolated wheat mtPDC complexes, which were purified with or
without 150 mM NaCl in the isolation medium (Millar et al., 1998). If E1 and E3
subunits dissociated upon NaCl treatment, then they would remain in the supernatant
during the purification procedure, whereas the large E2 core will sediment during
centrifugation. Following these isolations, we observed no difference in mtPDC, E1 or
E2 specific activity between control purification versus salt supplemented purification
(Figure 6b). However, E3 activity showed a significant decrease in activity when
comparing mtPDC complexes purified in the presence of salt versus those purified
under low salt conditions (Figure 6b). This indicated that whilst some E3 subunit
dissociated from mtPDC under NaCl treatment, it did not limit the overall activity of the
enzyme. When purified wheat mtPDC was directly assayed in medium containing 150
mM NaCl its activity was again significantly lowered compared to the control (Figure
6c).

**Discussion**

Under salt treatment, wheat seedlings exhibit a dramatic accumulation of sodium
coupled with reductions in photosynthesis and biomass, indicating that salinity exerts a
major impact on plant growth and physiology. Measurements of respiration in the dark
showed that in contrast to these inhibited processes, respiration rate increases in wheat
under salt stress. However, these observations in isolation do not fully explain the
mechanistic changes to the respiratory apparatus that occur during the onset of salinity
stress. In this study, we provide multiple lines of evidence to show that wheat
mitochondrial respiration alters its carbon source under salt stress. This conclusion is
derived from an integrated analysis of metabolite levels, respiratory substrate utilisation
assays, targeted quantification of protein abundance values, and enzymological
measurements that investigate the direct physiochemical effect of salt on enzyme
function. We conclude that salt treatment induces a coordinated set of metabolic
changes in wheat cells, involving the bypass of salt-sensitive steps in primary
metabolism (Figure 7). We postulate that this reconfiguration of respiratory metabolism
provides a mechanistic explanation accounting for the altered metabolic fingerprint of
salt affected plants.

Salinity stress depletes the capacity and activity of the early steps in pyruvate utilization
by mitochondrial respiration

Changes in protein abundance showed that both pyruvate transporters and subunits of
the mtPDC and mtOGDC in the mitochondrial matrix were lower in leaves of salt
stressed wheat plants compared to control plants (Figure 3). At the same time leaves had
increased alanine abundance, which can indicate pyruvate accumulation (Rocha et al.,
2010) as well as decreased citrate and aconitate content, potentially indicating a
decreased capacity for acetyl-CoA-dependent synthesis of these organic acids. These
data strongly suggested that mitochondrial pyruvate oxidation is impaired in wheat
leaves under salt stress.

Activity assays of mtPDC and mtOGDC showed these enzymes were inhibited by the
direct addition of salt to the assay medium, occurring at concentrations as low as 50 mM
NaCl. This was consistent with our previous report of a greater sensitivity of pyruvate +
malate compared to glutamate + malate-dependent respiration in isolated wheat
mitochondria exposed to increasing salt concentrations (Jacoby et al., 2015). We were
particularly interested in dissecting the mechanism of salt-induced enzyme inhibition.

Both mtPDC and mtOGDC are multi-enzyme complexes consisting of three subunits;
E1 (2-oxo acid dehydrogenase) and E2 (acetyltransferase) specific to each complex, and
the same E3 (lipoamide dehydrogenase (Guan et al., 1995; Luethy et al., 1995; Chen et
al., 2014). The activity of each individual mtPDC subunit was measured in media
containing salt, and all three enzymes showed increased activity when NaCl
concentrations were raised from 0 mM to 150 mM. A similar finding was reported in
Bacillus stearothermophilus where E3 activity reached its highest level at 200 mM NaCl
(Hiromasa et al., 2003). Therefore, we concluded that the salt-induced reduction of
mtPDC and mtOGDC activity is likely caused by altered quaternary structure of these protein complexes.

The decreased mtPDC and mtOGDC activities on addition of salt in vitro may be due to the instability of the protein complexes under high salt conditions, which induces a dissociation of the constituent subunits (Behal et al., 1994; De Marcucci et al., 1995; Millar et al., 1999; Katz et al., 2007). This instability might lead to protein degradation and explain the reduced protein abundance in vivo that was observed in this study after a period of salt treatment (Figure 3a). The hypothesis that protein complex instability causes salt sensitivity is supported by our observation that addition of excess E3 into the assay solution partially restored mtOGDC activity under high-salt conditions (Figure 5c). Furthermore, we report that E3 dissociates from the mtPDC complex during purification in the presence of 150 mM NaCl (Figure 6b). In mammals, the E2 subunit of mtPDC has been shown to contain a subunit binding domain, where E1 and E3 are attached through a weak noncovalent bond involving salt bridge interaction (Mande et al., 1996; Jung et al., 2003; Patel et al., 2014). While there is still a lack of detailed information about the mtOGDC E2-E3 interaction, it has been proposed that the E3 subunit is linked to the mtOGDC E2 core by physically binding to a single E1 subunit (Rice et al., 1992; McCartney et al., 1998). Alternatively, salt-induced inhibition might be underpinned by disruptions to the active-site coupling and substrate channelling mechanisms in these protein complexes, which are highly dependent on the specific conformation of the lipoate-bound lipoyl domain in the E2 subunit (Perham et al., 2002; Taylor et al., 2004). The swinging action of the E2 lipoyl domain enables active site coupling between the E2-bound E1 and E3 components of the mtPDC (Thelen et al., 1998; Perham et al. 2002). This conformationally flexible domain could be particularly sensitive to salt-induced deformation, and its dysfunction would perturb the coordinated transfer of substrates and products between the individual subunits.

**GABA shunt activation provides an alternative pathway for mitochondrial respiration due to PDC and OGDC inhibition**

Succinyl semialdehyde dehydrogenase (SSADH) and glutamate dehydrogenase (GDH) both exhibited increased abundance under salt exposure in mitochondrial samples (Figure 3c). Both GDH and SSADH are part of GABA shunt pathway that can provide an alternative carbon source for the TCA cycle (Bouché & Fromm, 2004). Induction of the GABA shunt under salinity is supported by increased levels of succinate and 2-
oxoglutarate (Figure 2), and increased GABA shunt activity provides a potential
explanation accounting for the increased respiration rate observed in salt-treated wheat
leaves, despite the reduced potential for pyruvate oxidation by TCA cycle. To date we
have not identified any GABA permease peptides in isolated wheat mitochondrial
membrane extracts, which meant its abundance could not be directly assessed. The
directionality of the GDH reaction is contested in the literature, with multiple studies
concluding that stress-induced GDH accumulation mediates the deamination of
 glutamate into 2-oxoglutarate to maintain the 2-oxoglutarate abundance in the cell,
while the production of glutamate occurs primarily through the GS/GLT pathway (Lea
& Miflin, 2003; Foyer et al., 2011; Terce-Laforgue et al., 2015). However, this function
of GDH has been questioned by previous studies of salinity stress, suggesting that
increased GDH activity performs an aminating role to synthesise glutamate from 2-
oxoglutarate (Kumar et al., 2000; Wang et al., 2007; Wang et al., 2012). In this study,
we observed a lower abundance of mtOGDC subunits and higher abundance of a 2-
oxoglutarate/malate translocator (Figure 2), which indicated that 2-oxoglutarate may be
transported across the mitochondrial membrane and utilised outside of the TCA cycle.
SSADH is responsible for providing succinate into TCA cycle via the oxidation of
succinate semialdehyde (SSA). In-vitro assays have shown that SSADH specifically
oxidises SSA and uses nicotinamide adenine dinucleotide (NAD\(^+\)) to produce NADH
(Bouché & Fromm, 2004). The resulting NADH can then be utilised by the
mitochondrial electron transport chain. SSADH is negatively regulated by NADH and
ATP, which suggests that GABA shunt activity is regulated via feedback control,
enabling the modulation of GABA shunt activity relative to the redox and energy status
in the cell (Shelp et al., 1999; Bouché & Fromm, 2004).

A number of previous studies have linked the responses of GABA shunt components to
salinity stress. For example, a microarray study of the temporal response of gene
expression in young seedlings of Arabidopsis to various stresses by Kilian et al. (2007)
revealed that GAD3/4 and GABA-T were upregulated under salt stress in Arabidopsis
seedlings, and Renault et al. (2010) found that the expression of genes encoding GAD,
GABA-T and SSADH are up-regulated under salt stress in cultured Arabidopsis cells.
Similarly by analysing GAD gene expression and GAD enzyme activity under salt stress
in Nicotiana sylvestris, Akcay et al. (2012) found that GAD activity was increased, and
the activity profile was correlated with GAD gene expression. In this study we collected
multiple lines of evidence for enhanced GABA shunt activity under salinity stress in

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wheat, such as significant increases in GAD activity, GABA abundance, expression of GAD, GABA-T and SSADH genes, together with the increased protein abundances of NADP-GDH isoform 1, NADH-GLT, GABA-T and SSADH isoform 2 (Figure 4). Increases in the abundance of NADP-GDH and NADH-GLT are likely indicators that nitrogen assimilation into glutamate was also up-regulated under salinity stress in wheat, although this increase in glutamate could also come from protein degradation. As glutamate is the precursor of many stress-related metabolites including GABA (Forde & Lea, 2007), up-regulation of NADP-GDH and NADH-GLT might be important in maintaining glutamate abundance during the activation of GABA shunt under salinity stress. This is particularly important because it has been shown that glutamate level can be drastically affected when GAD is overexpressed or compromised (Baum et al., 1996; Takayama et al., 2015). GAD is the main enzyme responsible for GABA production in plants (Baum et al., 1993; Fait et al., 2008). In this study, GAD activity increased even though there were no significant differences in transcript and protein abundance between control and salt-treated plants, suggesting that increased GAD activity was mediated via post-translational regulation.

To determine if there was a link between GABA shunt induction and respiratory rate, 3-mercaptopropionic acid (3-MP) and Vigabatrin were used to inhibit the activity of GAD and GABA-T in this study. Vigabatrin, a GABA analogue, is an effective competitive inhibitor of GABA-T in plants (Clark et al., 2009; Trobacher et al., 2013). Figure 4 showed that only salt-stressed wheat leaves exhibited a significant reduction in respiration rate when the GABA shunt pathway was inhibited at either the GAD or the GABA-T step, indicating that wheat respiration becomes more dependent on GABA shunt activity under salt stress. These observations are also consistent with a study by Bao et al. (2015), which investigated how each individual GABA shunt enzyme contributes to salt tolerance in tomato via gene silencing. In that study, the silencing of SlGADs and SlGABA-Ts led to increased ROS accumulation and salt sensitivity. In another study, Yin et al. (2014) found that respiratory rate increased proportionally to higher GAD activity under salt stress in soybean. These findings bolster the proposal that GABA shunt provides an alternative carbon source for the TCA cycle and supports mitochondrial respiration during salinity stress in wheat.

TCA cycle inhibition and GABA shunt induction may explain salinity induced changes in metabolite profiles
As reported in other species (Sanchez et al., 2008), we observed a large accumulation of amino acids under salt stress in wheat, epitomised by proline which showed the largest increase in abundance following salt exposure among all metabolites analysed in this study. Proline is an osmoprotectant known to accumulate during various stresses and confers tolerance to salt stress (Szekely et al., 2008; Mattioli et al., 2009). Besides reducing cytosolic osmotic potential, proline is also important in stabilizing protein structures (Rajendrakumar et al., 1994; Russo et al., 2003), scavenging free radicals (Alia et al., 2001; Signorelli et al., 2015) and influencing intracellular ion homeostasis (Chin & Shabala, 2005). Alongside proline, there was an accumulation of arginine and ornithine that are also derived from glutamate. Their accumulation is consistent with an induction of the GABA shunt. There were also significant increases in the abundance of TCA cycle intermediates 2-oxoglutarate and succinate. In the TCA cycle, 2-oxoglutarate is produced from oxidative decarboxylation of isocitrate via IDH (Foyer et al., 2011) and it can also be produced from deamination of glutamate via glutamate dehydrogenase (Masclaux-Daubresse et al., 2006). The increased 2-oxoglutarate abundance might be the result of increased activity of one or both of these enzymes. In this study, we observed a lower abundance of several organic acids, most notably citrate and aconitate. These metabolites are immediately downstream from pyruvate in the TCA cycle, and we hypothesise that their lower abundance is caused by inhibited pyruvate oxidation. Lower citrate abundance was also observed in a wide range of plants under salinity stress (Sanchez et al., 2008), whereas the decrease in aconitate was only observed in this study. In our data, lysine exhibited an 80-fold abundance increase, and methionine and asparagine increased by 15-20 fold. These amino acids may come from multiple sources including protein degradation and de novo synthesis through nitrogen assimilation into carbon skeletons derived from glycolysis and TCA cycle intermediates (Gilbert et al., 1998). But their accumulation would also be consistent with reduction of oxaloacetate utilisation by the TCA cycle due to inhibition of mtPDC.

Conclusion

From this study, we show that wheat mitochondrial respiration appears to alter its substrate source under salt stress (Figure 7). Salinity induces a reduction in the abundance of the pyruvate carrier and pyruvate dehydrogenase subunits, which lowers the capacity for cyclic operation of the TCA cycle to provide reductant for respiration. The decrease in pyruvate utilisation is accompanied by reductions in aconitate and
citrate abundance. We propose a potential mechanism underpinning reduced mtPDC and mtOGDC activities and abundance under salinity, whereby these large multi-enzyme complexes undergo subunit dissociation due to direct physiochemical effects of NaCl. Our data provide multiple lines of evidence for the activation of the GABA shunt pathway during salt exposure, such as increases in the abundance of the 2-oxoglutarate/malate carrier, GDH, GABA-T, SSADH, and GAD activity, accompanied by the reduction of mtOGDC abundance. The increases in GABA shunt activity would provide an alternative carbon supply into the TCA cycle through succinate production, which would bypass both mtPDC and mtOGDC steps of the TCA cycle. Application of chemical inhibitors confirmed that the GABA shunt makes a higher contribution to respiratory metabolism under salt stress. Synthesising our results, we propose a mechanistic explanation for salt induced changes to the metabolome, involving a metabolic reorganisation to bypass salt-sensitive mitochondrial enzymes such as mtPDC and mtOGDC, with the decrease in cyclic TCA cycle operation compensated via an increased contribution of the GABA shunt to respiratory substrate supply.

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Author Contributions
N.L.T. and A.H.M. conceived the research plans; N.L.T. and A.H.M. supervised the experiments; M.H.C-O. and R.P.J. performed the experiments; M.H.C-O., R.P.J., A.H.M. and N.L.T. wrote the article.

Fig. 1 Physiology responses of wheat (Triticum aestivum) to salinity stress. (a) Sodium content in leaf 3 of control (blue line) and salt treated (red line) plants. Black line represents salt concentration in the growth medium of salt treated plants. Error bars represent ± SEM. Stars indicates significant difference between control and salt treated plant with $P < 0.05$, $n = 4$. (b) Dry weight, (c) photosynthesis rate, (d) $O_2$ consumption rate and (e) $CO_2$ production rate of wheat leaf 3 in control and 11-d salt-treated plants. Error bars represent ± SEM. Stars indicate significant difference with $P \leq 0.05$, $n = 3$. This article is protected by copyright. All rights reserved
Fig. 2 Changes in abundance of metabolites following salt exposure. Changes in metabolite abundances in wheat (*Triticum aestivum*) leaf 3 at day 11 after salt treatment. Stars indicate significant difference with $P \leq 0.05, n = 3$.

Fig. 3 Significant changes in the abundance of targeted proteins in response to wheat (*Triticum aestivum*) leaf salt exposure. Significant changes in the abundance of the targeted proteins that are involved in the (a) tricarboxylic acid (TCA) cycle, (b) mitochondrial membrane transport and (c) proteins of the γ-aminobutyric acid (GABA) shunt. Comparisons were made between control vs 150 mM NaCl treatment in leaf 3 at day 11 after salt treatment. $P \leq 0.01$, log$_2$ fold $> \pm 0.26$, error bars represent ± SEM ($n = 4$).

Fig. 4 GABA shunt induction in wheat (*Triticum aestivum*) and its effect of respiration. (a) Transcript abundance of glutamate decarboxylase (GAD), γ-aminobutyrate transaminase (GABA-T) and succinate semialdehyde dehydrogenase (SSADH) in leaf 3 of control (blue line) and salt treated (red line) plants, relative to control plants at day 2 after salt treatment commenced. Error bars represent ± SEM, stars indicate significant difference between control and salt treated treated plants with $P \leq 0.05, n = 4$. (b) Significant changes of γ-aminobutyric acid (GABA) shunt related proteins in the leaf 3 of salt treated plants compared to control. Error bars represent ± SEM, $n = 4$. (c) GAD activity in total protein samples extracted from leaf 3 of control (blue line) and salt treated (red line) plants. Error bars represent ± SEM, stars indicate significant difference between control and salt treated treated plants with $P \leq 0.05, n = 4$. (d) GAD activity in the total protein extracts from leaf 3 of control plants assayed in the absence or salt or in 150 mM NaCl. Error bars represent ± SEM, stars indicate significant difference between control and salt treated treated plants with $P \leq 0.05, n = 4$. (e) Relative GABA content in leaf 3 of control (blue line) and salt treated (red line) plants. Error bars represent ± SEM, stars indicate significant difference between control and salt treated treated plants with $P \leq 0.05, n = 4$. (f) Percentage of respiration rate of leaves harvested from control and salt-stressed plants after the addition of the GABA shunt inhibitors. Error bars represent ± SEM, stars indicate significant difference between control and salt treated supplemented assay with $P \leq 0.05, n = 6$.

Fig. 5 Effect of NaCl on mtPDC, mtOGDC and MDH activity in wheat (*Triticum aestivum*). (a) The activity of pyruvate dehydrogenase (mtPDC), 2-oxoglutarate

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dehydrogenase (mtOGDC) and malate dehydrogenase (MDH) under different salt concentrations. Error bars represent ± SEM. Different letters indicate significant difference in the activity of the enzymes between different salt concentrations with \( P \leq 0.05, n = 3 \). (b) Activity of mtOGDC and mtPDC without and with the supplement of external E3 in assay buffers with and without 150 mM NaCl. Error bars represent ± SEM, stars indicate significant difference with \( P \leq 0.05, n = 3 \). (c) The relative activity of mtPDC and mtOGDC in the presence of 150 mM NaCl with and without the supplement of external E3 compared to control (0 mM NaCl). Error bars represent ± SEM, stars indicate significant difference with \( P \leq 0.05, n = 3 \).

**Fig. 6** Effect of NaCl on the subunits of PDC in wheat (*Triticum aestivum*). (a) The activity of the E1, E2 and E3 subunits of wheat pyruvate dehydrogenase (mtPDC) under control (0 mM NaCl) and 150 mM NaCl. Error bars represent ± SEM, stars indicate significant difference with \( P \leq 0.05, n = 3 \). (b) Relative activity of mtPDC and its E1, E2 and E3 subunits in isolated mtPDC samples from control (0 mM NaCl) salt supplemented purified (SSP) mtPDC samples and 150 mM NaCl SSP mtPDC samples. Error bars represent ± SEM. Stars indicate significant difference of the activity of the enzymes between different salt concentration with \( P < 0.05, n = 3 \). (c) Activity of purified wheat mtPDC in assay medium with or without 150mM NaCl. Error bars represent ± SE of the mean. Stars indicate significant difference of the activity of the enzymes between different salt concentration with \( P < 0.05, n = 3 \).

**Fig. 7** A proposed model for the changes in tricarboxylic acid (TCA) cycle activity including a reduction in pyruvate uptake and the increase γ-aminobutyric acid (GABA) shunt activity following salt treatment of in wheat (*Triticum aestivum*). Reduced pyruvate uptake led to the depletion of citrate and aconitate and reduced NADH production in the TCA cycle up to 2-oxoglutarate. A decrease in 2-oxoglutarate dehydrogenase (mtOGDC) abundance also slows the conversion of 2-oxoglutarate into succinate and reduces the production of NADH. Increased GABA shunt activity and an increase in abundance of the malate/2-oxoglutarate carrier, Succinate semialdehyde dehydrogenase (SSADH) and GABA provide succinate from an alternative carbon source into the TCA cycle. The noncyclic form of the TCA cycle is also consistent with increased amino acids biosynthesis that use 2-oxoglutarate and oxaloacetate as carbon skeletons. Highlighted enzymes in circles; mtPPC, pyruvate carrier; mtPDC, pyruvate dehydrogenase; mtOGDC, 2-oxoglutarate dehydrogenase; OMT, 2-oxoglutarate/malate
translocator; GDH, glutamate dehydrogenase; MDH, malate dehydrogenase; SSADH, succinate semialdehyde dehydrogenase; BAC, Branched-chain amino acid carrier. Red boxes indicate a decrease in abundance of an enzyme, while green boxes indicate an increase in abundance following salt treatment in this study. Red arrows indicate a decrease in abundance of a metabolite, while green arrows indicate an increase in abundance of a metabolite following salt treatment in this study.

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Supporting Information

Figure S1. Physiology responses of wheat (Triticum aestivum) to salinity stress.

Table S1. Change in abundance of targeted TCA cycle proteins following salt exposure.

Table S2. Change in abundance of targeted mitochondrial membrane transport proteins following salt exposure.

Table S3. Change in abundance of targeted GABA Shunt proteins following salt exposure.

Table S4. Change in abundance of targeted GABA Shunt proteins following salt exposure.