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RESEARCH PAPER

Salinity tolerance in chickpea is associated with the ability to 'exclude' Na from leaf mesophyll cells

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Abstract

Salinity tolerance is associated with Na 'exclusion' from, or 'tissue tolerance' in, leaves. We investigated whether two contrasting chickpea genotypes, salt-tolerant Genesis836 and salt-sensitive Rupali, differ in leaf tissue tolerance to NaCl. We used X-ray microanalysis to evaluate cellular Na, Cl, and K concentrations in various cell types within leaflets and also in secretory trichomes of the two chickpea genotypes in relation to photosynthesis in control and saline conditions. TEM was used to assess the effects of salinity on the ultrastructure of chloroplasts. Genesis836 maintained net photosynthetic rates (*A*) for the 21 d of salinity treatment (60 mM NaCl), whereas *A* in Rupali substantially decreased after 11 d. Leaflet tissue [Na] was low in Genesis836 but had increased markedly in Rupali. In Genesis836, Na was accumulated in epidermal cells but was low in mesophyll cells, whereas in Rupali cellular [Na] was high in both cell types. The excessive accumulation of Na in mesophyll cells of Rupali corresponded to structural damage to the chloroplasts. Maintenance of photosynthesis and thus salinity tolerance in Genesis836 was associated with an ability to 'exclude' Na from leaflets and in particular from the photosynthetically active mesophyll cells, and to compartmentalize Na in epidermal cells.

Keywords: Cellular distribution, chickpea (*Cicer arietinum* L.), chloride (Cl), chloroplasts, salinity tolerance, secretory trichomes, sodium (Na), transmission electron microscopy, X-ray microanalysis.

Introduction

Chickpea (*Cicer arietinum* L.) is an important food legume (FAOSTAT, 2016 http://www.fao.org/faostat/en/). Chickpea is grown in regions with soils prone to increasing levels of salt, yet it is sensitive to salinity (Flowers *et al.*, 2010). Salinity adversely affects chickpea germination (Khalid *et al.*, 2001), vegetative growth (Lauter and Munns, 1986*a*; Khan *et al.*, 2015), and especially reproductive processes (Vadez *et al.*, 2007, 2012; Samineni *et al.*, 2011; Turner *et al.*, 2013; Khan *et al.*, 2017). There is, however, variation for salt tolerance within cultivated chickpea genotypes (Vadez *et al.*, 2007; Turner *et al.*,

2013), but the physiological mechanisms conferring these differences in salt tolerance are not fully understood (Khan *et al.*, 2015; Kotula *et al.*, 2015).

Salt tolerance in plants is usually associated with: (i) osmotic adjustment of cells, by increased solute concentrations (Na^+ and Cl^- in vacuoles, organic solutes in cytoplasm), to the more negative osmotic potential of NaCl in the root zone; (ii) regulation of Na^+ and Cl^- accumulation in leaves by 'exclusion' in roots, so as to avoid ion toxicity; and (iii) 'tissue tolerance' which is the ability of tissues to function while containing relatively high internal Na^+ and Cl^- concentrations (Munns and Tester, 2008). Chickpea decreased its leaf osmotic potential by more than the change in osmotic potential around the roots (Sheoran and Garg, 1983; Lauter and Munns, 1987; Khan *et al.*, 2016) and an osmotic treatment of –0.29 MPa (equivalent to that of 60 mM NaCl) did not impair growth of chickpea, whereas 30 mM and 60 mM NaCl substantially reduced growth (Khan *et al.*, 2016). High tissue Na^+ had substantially greater adverse effects than did Cl^- (Khan *et al.*, 2016). Thus, the adverse effects of moderately saline soils on chickpea are predominantly a result of Na^+ toxicity (i.e. inhibition of metabolism, cellular damage, or even cell death resulting from Na^+) in leaf tissues (Khan *et al.*, 2016). Damage to the leaf tissues reduces photosynthesis, and the salt-stressed plants can then be limited by the availability of photosynthates so that both vegetative and reproductive growth declines (Khan *et al.*, 2017).

Indeed, several studies of different chickpea genotypes have found negative correlations between shoot growth and/or yield with leaf Na^+ concentration (Lauter and Munns, 1986*a*; Dua and Sharma, 1997; Turner *et al.*, 2013), whereas in other experiments salt injury correlated with both shoot Na^+ and Cl^- concentrations (Samineni *et al.*, 2011; Khan *et al.*, 2015) or did not find any association with shoot Na^+ concentration (Vadez *et al.*, 2007). Recently, Khan *et al.* (2016) showed that Na^+ salts (without Cl^-) and NaCl equally impaired growth of two chickpea genotypes, whereas Cl^- salts (without Na^+) did not, this being strong evidence that Na^+ toxicity is the predominant adverse effect of salinity in chickpea. Moreover, a tolerant and a sensitive genotype did not differ in leaf Na^+ concentrations after 14 d in 60 mM NaCl, indicating that tissue tolerance of excess Na^+ could be a trait contributing to genotypic differences in salt tolerance in chickpea (Khan *et al.*, 2016).

The present study assessed 'tissue tolerance' to Na^+ in chickpea. A key mechanism determining 'tissue tolerance' is the ability of cells to compartmentalize Na^+ and Cl^- in vacuoles so that Na^+ remains relatively low in the cytoplasm and organelles, such as chloroplasts (Flowers *et al.*, 2015; Munns *et al.*, 2016; Bose *et al.*, 2017). Indeed, damage to chloroplasts can be an important adverse effect of salinity in salt-sensitive species such as rice (Flowers *et al.*, 1985), and may reflect differences in tissue tolerance between genotypes. Additionally, Na^+ and Cl^- could be partitioned between different cell types within leaves; such data are few, but for the salt-tolerant non-halophyte barley, photosynthesis in saline conditions was associated with maintenance of higher K and lower Na in the mesophyll cells, whereas Na and Cl accumulated in epidermal cells (James *et al.*, 2006*b*). In chickpea, photosynthesis was impaired via non-stomatal limitations, and damage to PSII was greater in a salt-sensitive than in a salt-tolerant genotype at similar leaf Na^+ concentrations, but whether genotypes differ in Na^+ accumulation in the leaf mesophyll cells was unknown (Khan *et al.*, 2015, 2016). Moreover, chickpea possesses secretory trichomes on leaves, stems, and pods (Lazzaro and Thomson, 1989), and in saline conditions the secretions have been reported to contain, in addition to organic acids, ~ 231 mM Cl^- and 60 mM Na^+ (Lauter and Munns, 1986*b*). So, chickpea genotypes of contrasting salt tolerance might differ in ion accumulation or secretion via leaf secretory trichomes (speculated on by Flowers *et al.*, 2010; Khan *et al.*, 2015, 2016).

This study investigated concentrations of Na, Cl, and K in various cell types of leaflets, and of secretory trichomes, of two contrasting chickpea genotypes (salt-tolerant Genesis836 and salt-sensitive Rupali), in relation to photosynthetic capacity in saline conditions (controls and two levels of NaCl), via quantitative cryo-SEM X-ray elemental analysis. This is the first investigation of cell-specific element distributions across leaflets of two contrasting chickpea genotypes, in control and saline conditions, and one of only very few studies of cellular element concentrations in plants. In addition, we report on the effects of salinity on the ultrastructure of chloroplasts in the two contrasting genotypes. We addressed the following questions. (i) Do chickpea genotypes with contrasting salt tolerance differ in leaf tissue tolerance to NaCl, assessed as changes in leaflet photosynthesis and chloroplast ultrastructure as related to tissue element concentrations? (ii) Is salt tolerance in chickpea associated with element concentrations in particular leaflet lamina cells or the secretory trichomes?

Materials and methods

Plant material and growth conditions

Two desi-type chickpea (*Cicer arietinum* L.) genotypes that are classified as either salt tolerant (Genesis836) or salt sensitive (Rupali) based on previous experiments with salinized soil (Turner *et al.*, 2013, Kotula *et al.*, 2015) or nutrient solution (Khan *et al.*, 2015, 2016) were used. Seeds were washed with 0.04% (w/v) sodium hypochlorite, the seed coat was pricked, and the seeds were imbibed in aerated 0.5 mM $CaSO_4$ for 3 h and then placed on plastic mesh floating on 10% strength nutrient solution (Khan *et al.*, 2015, 2016; Supplementary Table S1 at *JXB* online). Plants were grown in a phytotron (temperature-controlled glasshouse) between October and December in Perth (Australia) at 20 °C/15 °C day/night. From day 6, plants were exposed to natural sunlight and the nutrient solution was changed to 25% strength. On day 9, four seedlings were transferred to each 4.5 litre pot containing 100% strength nutrient solution. On day 23, the NaCl treatments were imposed in steps of 15 mM (see below). The solution in all pots was renewed weekly.

Treatments and sampling procedure

Three treatments were applied when plants were 23 d old: non-saline control (containing 0.2 mM Na^+ and 0.05 mM Cl^-; Supplementary Table S1), and 30 mM or 60 mM NaCl. The saline treatments were administrated by addition of 15 mM NaCl at 12 h intervals until the final concentrations of 30 mM or 60 mM had been achieved. There were four replicates for each genotype×treatment combination, with each replicate being a pair of two pots with the same treatment so as to provide the number of plants needed for the six sampling times (see below). Each pair of pots was completely randomized with all other pairs, and each pair of pots was re-randomized at the time of each nutrient solution renewal. Immediately before treatments were imposed, an initial sample of one

plant was taken from eight randomly selected pots and one plant was removed from all remaining pots, so that three plants remained in each pot. The subsequent samples were taken at 5, 8, 11, 14, 18, and 21 d after the first addition of 15 mM NaCl. Two pots from the same treatment (acting as one replicate) remained side by side and one plant was taken alternately at each sampling from each pot in the pair. At each sampling, roots and stem bases of plants from the 30 mM and 60 mM NaCl treatments were rinsed three times, for 30 s each time, respectively, with 60 mM and 120 mM mannitol+5 mM $CaSO_4$, and plants from the non-saline controls were rinsed in 5 mM $CaSO_4$. The plant parts were then separated (green leaflets, green petioles and stems, dead leaflets, dead petioles and stems, and roots), the fresh weights recorded, dried at 65 °C for 72 h, and the dry weights recorded.

Leaf gas exchange measurements

The measurements were conducted on the youngest fully expanded leaves after 5, 8, 11, 14, 18, and 21 d of the first 15 mM NaCl application (see above) using a LI-6400 open gas exchange system (LI-COR Biosciences Inc., Lincoln, NE, USA). Net photosynthetic rate (A), intercellular CO_2 concentration (C_i), stomatal conductance (g_s), and transpiration rate (T) were determined at a photosynthetically active radiation of 1500 µmol photons m^{-2} s^{-1} (light saturated; Basu *et al.*, 2007), leaf chamber temperature of 20 °C, relative humidity of 70–80%, and CO_2 concentrations of 400 (ambient) µmol mol^{-1} and 800 (elevated) µmol mol^{-1}. The measurements were taken between 9.30 h and 15.00 h. At the end of measurements, the leaflets within the chamber (middle part of the leaf with 4–6 leaflets) were excised and photographed (for subsequent measurements of surface area using Image J software, National Institutes of Health, Bethesda, MD, USA). The leaflets and petioles were separated, the fresh weights recorded, oven-dried at 65 °C for 72 h and then used to measure ion concentrations (Na^+ and K^+) (described below). The remaining part of the leaf (6–8 leaflets) that was used for gas exchange measurement was flash-frozen in liquid N and stored at –80 °C until freeze drying for subsequent chlorophyll analysis (Supplementary Fig. S9).

Tissue ion analysis

Oven-dried tissues were ground to fine powder in a ball-mill grinder (2010 Geno/Grinder®, SPEX SamplePrep, Metuchen, NJ, USA) and then extracted in 0.5 M HNO_3 (Munns *et al.*, 2010). Extracts were analysed for sodium (Na^+) and potassium (K^+) using a flame photometer (PFP7; Jenway, Dunmow, UK) and chloride (Cl^-) using a chloridometer (model 50CL 1–50; SLAMED, Frankfurt, Germany). The sample size for the leaflets used in the gas exchange measurements was small, so Na^+ and K^+ measurements were prioritized for those samples (extract volumes were also insufficient for Cl^-). The reliability of these analyses was confirmed by taking a reference tissue through the same procedures. Net Na^+, K^+, and Cl^- uptake rates by plants and transport rates from roots to shoots were calculated on a root fresh weight basis as described in James *et al.* (2006*a*).

Light microscopy and transmission electron microscopy (TEM)

Leaflets from the youngest fully expanded leaves were collected at 18 d of treatments, fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer, and stored at 4 °C. Leaflets were then cut into 1 mm segments, washed in phosphate buffer, and post-fixed in 1% OsO_4 in 0.1 M phosphate buffer. After rinsing in deionized (DI) water, samples were dehydrated in a graded series of ethanol (50, 70, 95, 100, and 100%-anhydrous) followed by 100%-anhydrous acetone. Segments were then infiltrated and embedded in Epon-Araldite and left overnight to cure at 60 °C. For light microscopy, sections 1 µm thick were cut using a glass knife, stained with toulidine blue O, and viewed using an AxioScope2 plus equipped with an AxioCam digital camera (Carl Zeiss Microscopy GmbH, Jena, Germany). For TEM, sections 120 nm thick were cut using a diamond knife, collected on copper grids, stained with uranyl acetate and lead citrate, and examined in a transmission electron microscope (JEOL 2100, Tokyo, Japan) at 120 kV. All TEM images were acquired using a digital camera (Gatan Orius 1000, Pleasanton, CA, USA).

Morphological analysis of the leaflet surfaces, especially secretory trichomes, by SEM

Leaflets from the youngest fully expanded leaves were collected 18 d after the treatments had commenced, fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer, and stored at 4 °C. Samples were then briefly washed in DI water, dehydrated in a graded series of ethanol (30, 50, 70, 95, 100, and 100%-anhydrous), and critical point dried by flooding with liquid CO_2 for 1.5 h and then increasing the temperature and pressure to critical point (31 °C, 1200 psi). The samples were sputter-coated with gold and examined with a Zeiss 55 scanning electron microscope at 5 kV. The number and dimensions of secretory trichomes, as well as the number of stomata, on the adaxial and abaxial leaflet surfaces were then analysed using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Cell-specific element analysis by cryo-SEM X-ray microanalysis

Samples for cell-specific elemental analysis were collected from transpiring plants (between 10.00 h and 15.00 h) after 18 d of treatment. Segments of leaflets were excised mid-way along the youngest fully expanded leaf avoiding the central vein, placed on an aluminium grooved pin with OCT, and plunge frozen into liquid N, thereby immediately immobilizing and preserving cellular ions. Samples were stored in liquid N until required, and then prepared and analysed as described in detail in Hayes *et al.* (2018). Briefly, transverse regions of frozen-hydrated leaf segments were prepared by cryoplaning a flat surface using a cryomicrotome, coated with 35 nm chromium without sublimation, and transferred under vacuum to a Zeiss field emission scanning electron microscope fitted with a cryostage, and an Oxford X-Max80 SDD X-ray detector. Samples were analysed at –150 °C, 15 kV, and a 2 nA beam current, in high current mode. Immediately prior to acquisition of each map, the instrument was calibrated and the beam current measured and recorded using a pure copper standard. Elemental maps were acquired at 512 pixel resolution, for >3000 frames with a dwell time of 10 µs per pixel. Drift correction and pulse-pile up correction (empirically determined to correct for O interference of Na quantification; Marshall, 2017) were activated. Analysis and quantification were performed using the Oxford Instruments AZtecEnergy software as described in step by step detail in Hayes *et al.* (2018). Cells analysed were the upper epidermis (UE), palisade mesophyll (PM), spongy mesophyll (SM), and lower epidermis (LE), with 25–73 spectra collected from each cell type from three different leaflets from three different replicate plants (Supplementary Fig. S1). Qualitative elemental analyses were also conducted on head cells of intact secretory trichomes on whole frozen–hydrated leaf segments that were placed on a custom-made stage and coated with 20 nm Cr. Spectra were acquired under the conditions above, for 60 s livetime.

Chlorophyll extraction and analysis

Freeze-dried leaf samples were ground to fine powder using a ball-mill grinder (2010 Geno/Grinder®, SPEX SamplePrep). Chlorophylls were extracted in cold 100% methanol by shaking for 3 h; samples were then centrifuged for 15 min at 1500 rpm (Microfuge® 16 Centrifuge, Beckman Coulter®, Indianapolis, IN, USA) and supernatants were collected, all at 4 °C. Measurements were taken on 200 µl of the sample (or blank) in a 96-well flat-bottom plate (Greiner Bio-One, Frickenhausen, Germany) using a microplate reader (Multiscan® Spectrum Microplate Spectrophotometer, Thermo Electron Corporation, Vantaa, Finland) at wavelengths of 470, 652.4, and 665 nm. Chl *a* and *b* concentrations (Supplementary Fig. S9) were calculated using the equations as described by Wellburn (1994), with modifications to the equations for the pathlength of the microplate reader (with 200 µl) following the method of Warren (2008).

Statistical analysis

Data are presented as means ±SE. Concentrations of Na, Cl, and K in various cell types were analysed using three-way ANOVA with genotype, treatment, and cell type as factors. Growth, tissue ions, and gas exchange parameters were analysed using three-way ANOVA with genotype, treatment, and days of treatment as factors (the last day of treatment was excluded in this analysis due to lack of data for Rupali grown at 60 mM NaCl). Least significant difference (LSD) test at 5% level was conducted to compare the means for the three-way interaction. The effects of genotypes and treatments and their interactions on particular days of treatment were analysed using two-way ANOVA. The effects of days of treatment on genotypes within the treatments were analysed using one-way ANOVA. When significant interactions were detected, a subsequent Tukey test was applied to determine the effects of the treatments. Statistical analysis was performed using MYSTAT 12 (SYSTAT Software Inc., 2007) and GenStat 18th Edition (GenStat VSN International Ltd). Graphs were prepared using GraphPad Prism 7 (Version 7.02, GraphPad Prism Software, Inc.).

Results

The two contrasting chickpea genotypes, salt-tolerant Genesis836 and salt-sensitive Rupali, were chosen for this study based on previous experiments (see the Materials and methods). The growth differences between Genesis836 and Rupali in non-saline control, 30 mM NaCl, and 60 mM NaCl confirmed the previous observations (Khan *et al.*, 2015, 2016) and are presented in Supplementary Results S1; Supplementary Fig. S3. Here, we focus on specific traits of tissue tolerance differentiating the tolerant and sensitive genotypes.

Concentration of Na^+ in leaflets of the youngest fully expanded leaf

Na^+ concentrations in the leaflets of the youngest fully expanded leaf (YFEL) of non-saline plants were low for the duration of the experiment and did not exceed 7.5 mM (on a tissue water basis) for either Genesis836 or Rupali (Fig. 1). Imposition of 30 mM NaCl increased the concentrations of Na^+ in the YFEL leaflets of both genotypes, with average concentrations for the duration of the treatment of 35 mM for Rupali and 46 mM for Genesis836. In the 60 mM NaCl treatment, the concentration of Na^+ in the YFEL leaflets of Genesis836 increased to 94 mM and was steady for the duration of treatment. In contrast, the YFEL leaflet Na^+ concentration in Rupali was similar at 5 d and 8 d after treatment (average of 88 mM), after which it continued to rise and reached 489 mM at 18 d of treatment. Tissue Na^+, Cl^-, and K^+ data for green leaflets of whole shoots, petioles and stems, and roots are presented in Supplementary Fig. S6. Net Na^+, Cl^-, and K^+ uptake rates by roots and transport rates from roots to shoots are presented in Supplementary Results S1; Supplementary Tables S7, S8.

Na, Cl, and K concentrations in various cell types of the lamina of leaflets

Cellular concentrations of Na, Cl, and K were measured in cells of leaflets of the YFELs: upper epidermis (UE), palisade mesophyll (PM), spongy mesophyll (SM), and lower epidermis (LE) (Supplementary Figs S1, S2).

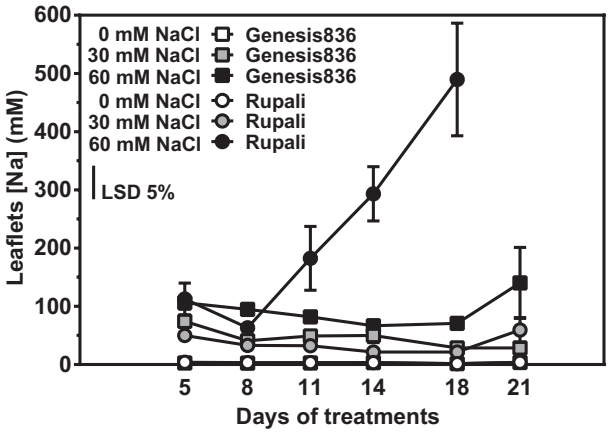

Fig. 1. Concentrations of Na^+ (mM, tissue water basis) in leaflets of the youngest fully expanded leaves that were measured for gas exchange (see Fig. 5). Leaflets which were in the LiCor chamber were excised following the gas exchange measurements and analysed for Na^+ and K^+ (Supplementary Fig. S4). The two chickpea genotypes (Genesis836 and Rupali) were grown in aerated nutrient solution with 0 (non-saline control), 30, or 60 mM NaCl. Treatments were imposed on 23-day-old plants and the measurements were taken at 5, 8, 11, 14, 18, and 21 d of treatments. Na^+ concentration on a tissue water basis was calculated from data on Na^+ measured in extracts of dried tissue samples and the tissue water content from measurements of fresh and dry weights of the leaflets. Data are means ±SE of four replicates. For Rupali at 60 mM NaCl, there were three replicates at 18 d as the other replicate plant had no green leaves, and no green leaves remaining at 21 d. Three-way ANOVA (data up to 18 d of treatments) showed a significant genotype×treatment×days of treatment interaction (P<0.001). Additional statistical analyses are given in Supplementary Table S3.

Sodium

For non-saline control plants, Na concentrations [Na] were low in all cells analysed (Fig. 2) and did not exceed 9.2 mM for either Genesis836 or Rupali (Fig. 3A). The imposition of 30 mM NaCl increased [Na] in Genesis836 to ~95 mM in the UE and LE, but [Na] in PM and SM cells remained relatively low (average 18 mM). In Rupali grown with 30 mM NaCl, [Na] remained low in all cells analysed (average 20 mM). In the 60 mM NaCl treatment, Genesis836 cells did not significantly increase further in [Na], with averages of 105 mM in epidermal cells (UE and LE) and 19 mM in mesophyll cells (PM and SM). In contrast, in Rupali grown with 60 mM NaCl, [Na] dramatically increased in all cell types, ranging from 193 mM in PM to 362 mM in UE (Figs 2, 3A).

Chloride

For non-saline controls, Cl concentrations [Cl] were low in all cells analysed (Fig. 2), ranging from 9 mM in LE in Genesis836 to 18 mM in PM in Rupali (Fig. 3B). The imposition of 30 mM NaCl increased [Cl] in Genesis836 to ~131 mM in epidermal cells (UE and LE) and 230 mM in mesophyll cells (PM and SM). In Rupali with 30 mM NaCl, [Cl] ranged from 62 mM in epidermal cells (UE and LE) to 191 mM in mesophyll cells (PM and SM) (Fig. 3B). At 60 mM, [Cl] did not further increase in epidermal cells (UE and LE) of Genesis836 but it increased to 327 mM in mesophyll cells (PM and SM). In Rupali grown with 60 mM NaCl, [Cl] increased in all cell

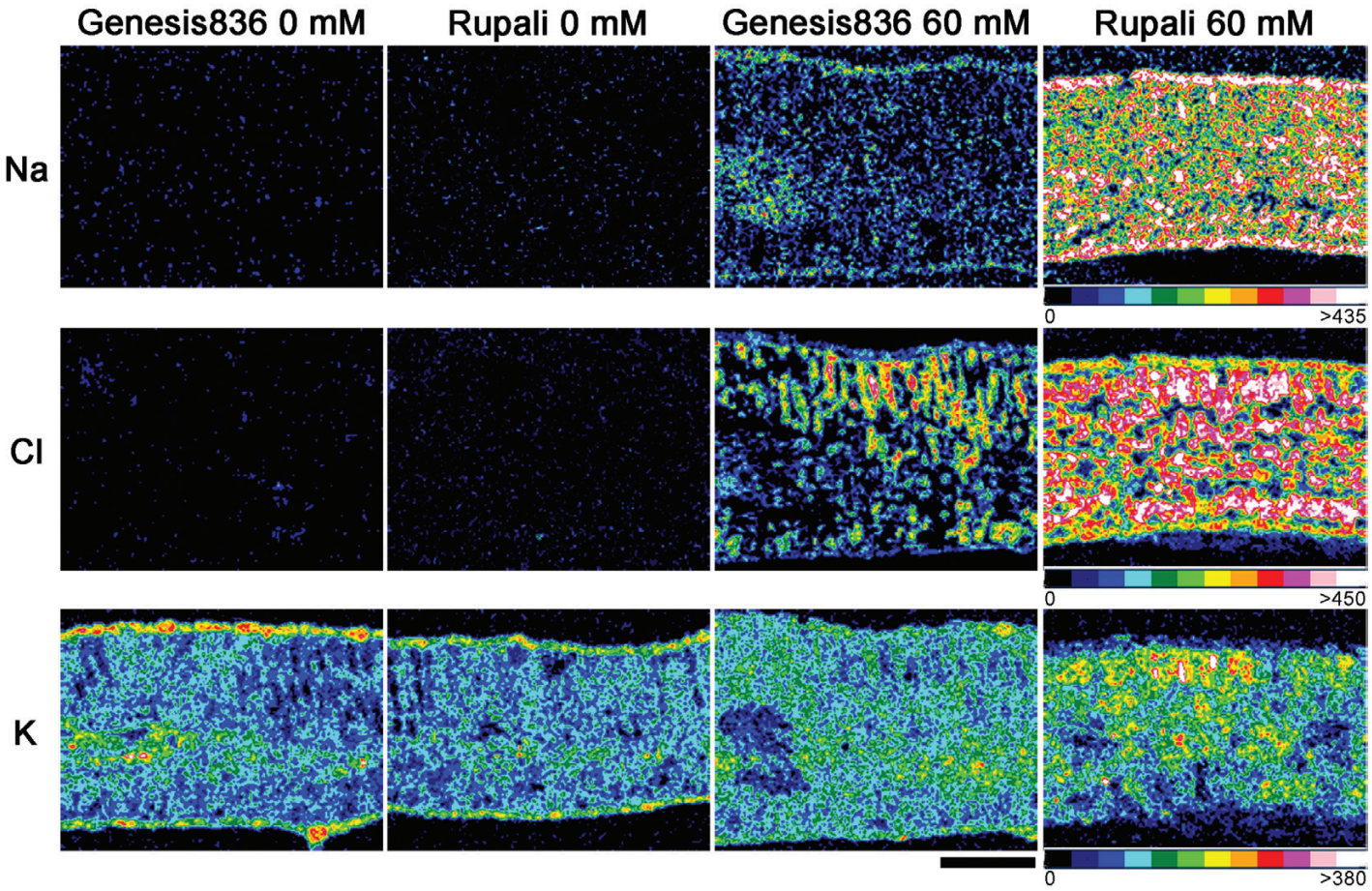

Fig. 2. Typical quantitative element maps of Na, Cl, and K from cryoplaned, frozen–hydrated leaflets of the youngest fully expanded leaves of Genesis836 and Rupali. Plants were grown in aerated nutrient solution with 0 (non-saline control) or 60 mM NaCl for the final 18 d. Treatments were imposed on 23-day-old plants. Elemental concentrations from different cell types are summarized in Fig. 3. For these maps, the concentrations (in mM) are scaled to best reveal element variations across cell layers and treatments, with black=0 (below detection, approximately <5 mM) for all maps, and white ≥435 mM for Na, >450 mM for Cl, and >380 mM for K. The changes in concentration along the colour scale are linear. Scale bar for all images=100 μm.

types, ranging from 301 mM in the UE to ~403 mM in mesophyll cells (Figs 2, 3B).

Potassium

For non-saline controls, K concentrations [K] were similar in Genesis836 and Rupali, ranging from 111 mM in mesophyll cells (PM and SM) to 280 mM in epidermal cells (UE and LE) (Figs 2, 3C). The imposition of 30 mM NaCl treatment reduced [K] in Genesis836 to 70% of control in UE but did not affect [K] in mesophyll cells and LE. In Rupali grown with 30 mM NaCl, [K] declined to on average 75% of control in epidermal cells (UE and LE) and was unaffected in mesophyll cells (PM and SM). The 60 mM NaCl did not cause further changes in [K] in any cells of Genesis836. In Rupali grown with 60 mM NaCl, [K] decreased in epidermal cells to ~85 mM (28% of control), increased in PM to 187 mM (2.2-fold compared to control), and was not affected in SM (Figs 2, 3C).

Potassium/sodium ratio

For non-saline controls, the K/Na ratio was similar in Genesis836 and Rupali, ranging from 93:1 in mesophyll cells (PM and SM) to 220:1 in epidermal cells (UE and LE) (Fig. 3D). The imposition of 30 mM NaCl treatment reduced K/Na in Genesis836 to 4% and 41% of the values in controls for UE and LE, respectively, but did not affect K/Na in mesophyll cells. In Rupali grown with 30 mM NaCl, K/Na declined to on average 53% of the value in control in epidermal cells, 74% in PM, and was unaffected in SM. In 60 mM NaCl treatment, the K/Na ratio in Genesis836 cells was on average 42:1 but in Rupali it declined to on average to 0.7:1 in all cells analysed.

Effects of NaCl on chloroplast ultrastructure

There were no differences in the ultrastructure of chloroplasts in both PM and SM cells in leaflets of the YFEL of Genesis836 and Rupali for the non-saline controls (Fig. 4A, B). In both genotypes, chloroplasts showed organized membrane systems of grana and stroma thylakoids, and with similar numbers of plastoglobuli and large starch granules occupying a major part of the chloroplast interior. Imposition of 30 mM NaCl treatment did not affect the structure of chloroplasts in Genesis836 (Fig. 4C), whereas in Rupali swelling of thylakoids was observed on rare occasions and the number of starch granules appeared to decrease (Fig. 4D). In 60 mM NaCl, for Genesis836, a swelling of thylakoids was observed in a number of chloroplasts, but

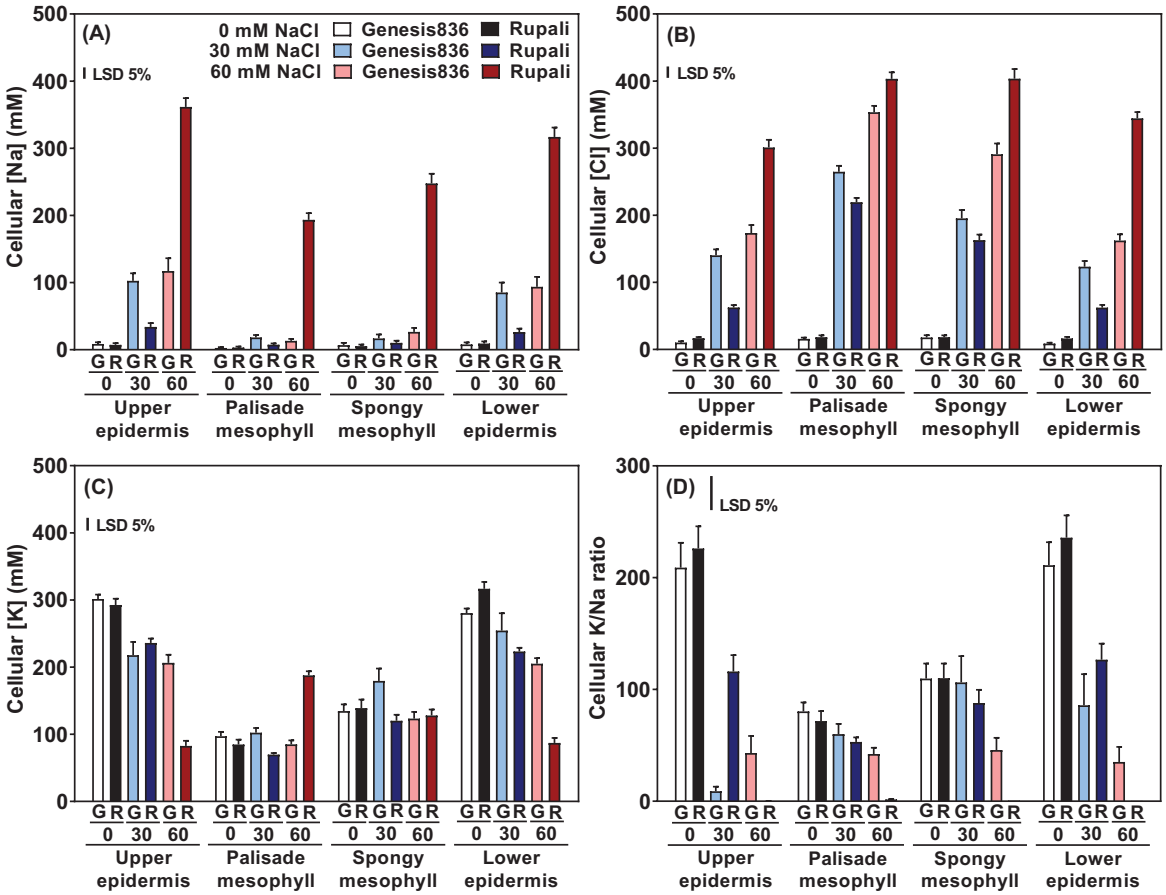

Fig. 3. Concentrations of Na (A), Cl (B), and K (C), and the K/Na ratio (D) in various cell types in the lamina of leaflets of the youngest fully expanded leaves of Genesis836 (G) and Rupali (R) grown in aerated nutrient solution with 0 (non-saline control), 30, or 60 mM NaCl for 18 d. Treatments were imposed on 23-day-old plants. Elemental concentrations were measured by cryo-SEM X-ray microanalysis. The concentrations in mM (mmol kg^{-1} water) are per unit fresh weight from fully hydrated, cryo-fixed cells. Data are means ±SE (n=25–73 cells measured for three different leaflets each from a different replicate plant). There was a significant genotype×treatment×cell type interaction at P<0.001 for cellular concentrations of Na, Cl, and K, and at P<0.05 for K/Na ratio (three-way ANOVA). Bars represent least significant difference (LSD) at P<0.05 for genotype×treatment×cell type interaction. Additional statistical analysis is given in Supplementary Table S10.

thylakoids remained organized into grana and stroma lamellae, and these chloroplasts contained plastoglobuli and large starch granules (Fig. 4E). In contrast, for Rupali in 60 mM NaCl, chloroplasts often had abnormal and spherical shapes with various degrees of damage, such as some chloroplasts only with thylakoids swollen but still arranged into grana and stroma lamellae, but also many severely damaged chloroplasts in which grana and stroma thylakoids were disorganized or collapsed, and the chloroplast envelopes were disrupted (Fig. 4F). These severely damaged chloroplasts did not contain starch granules. In some cells, chloroplasts and the other cellular contents appeared aggregated, which seemed to be associated with breakdown of the tonoplasts.

Leaf gas exchange

The net photosynthetic rate (A) of the YFELs at ambient CO_2 levels were similar for the two genotypes in non-saline and 30 mM NaCl treatments, with an average A of 29.9 µmol CO_2 m^{-2} s^{-1} for the treatment period (Fig. 5A). Imposition of 60 mM NaCl did not affect A in the YFELs of Genesis836 on any day of treatment, whereas A decreased in the YFELs of Rupali at 11 d of the 60 mM treatment onwards and by 14 d was only 6.3 µmol CO_2 m^{-2} s^{-1}. When the concentration of CO_2 during measurements was elevated to 800 µmol mol^{-1}, A did not significantly increase in either genotype, when compared with A at the ambient CO_2 level of 400 µmol mol^{-1} (Fig. 5B).

Similar to A, there were no differences in intercellular CO_2 concentration (C_i) at ambient CO_2 level for the two genotypes when grown in non-saline and 30 mM NaCl treatments and for Genesis836 in 60 mM NaCl, with an average C_i of 319 µmol CO_2 mol^{-1} (Fig. 5C). In Rupali grown with 60 mM NaCl, C_i decreased to 80% of controls at 14 d of treatment. Elevation of the external CO_2 level to 800 µmol mol^{-1} resulted in an ~2-fold increase of C_i in both genotypes, when compared with C_i at the ambient CO_2 level (Fig. 5D).

There were no differences in stomatal conductance (g_s) for the two genotypes in control and 30 mM NaCl treatment, with an average g_s of 1.42 mol H_2O m^{-2} s^{-1} (Fig. 5E). Imposition of 60 mM NaCl caused in Genesis836 a decrease in g_s to ~50% of controls at 5 d and 14 d, and in Rupali g_s declined from 1.14 mol H_2O m^{-2} s^{-1} at 8 d of treatment to 0.07 mol H_2O m^{-2} s^{-1} at 18 d of treatment. Elevation of CO_2 to 800 µmol

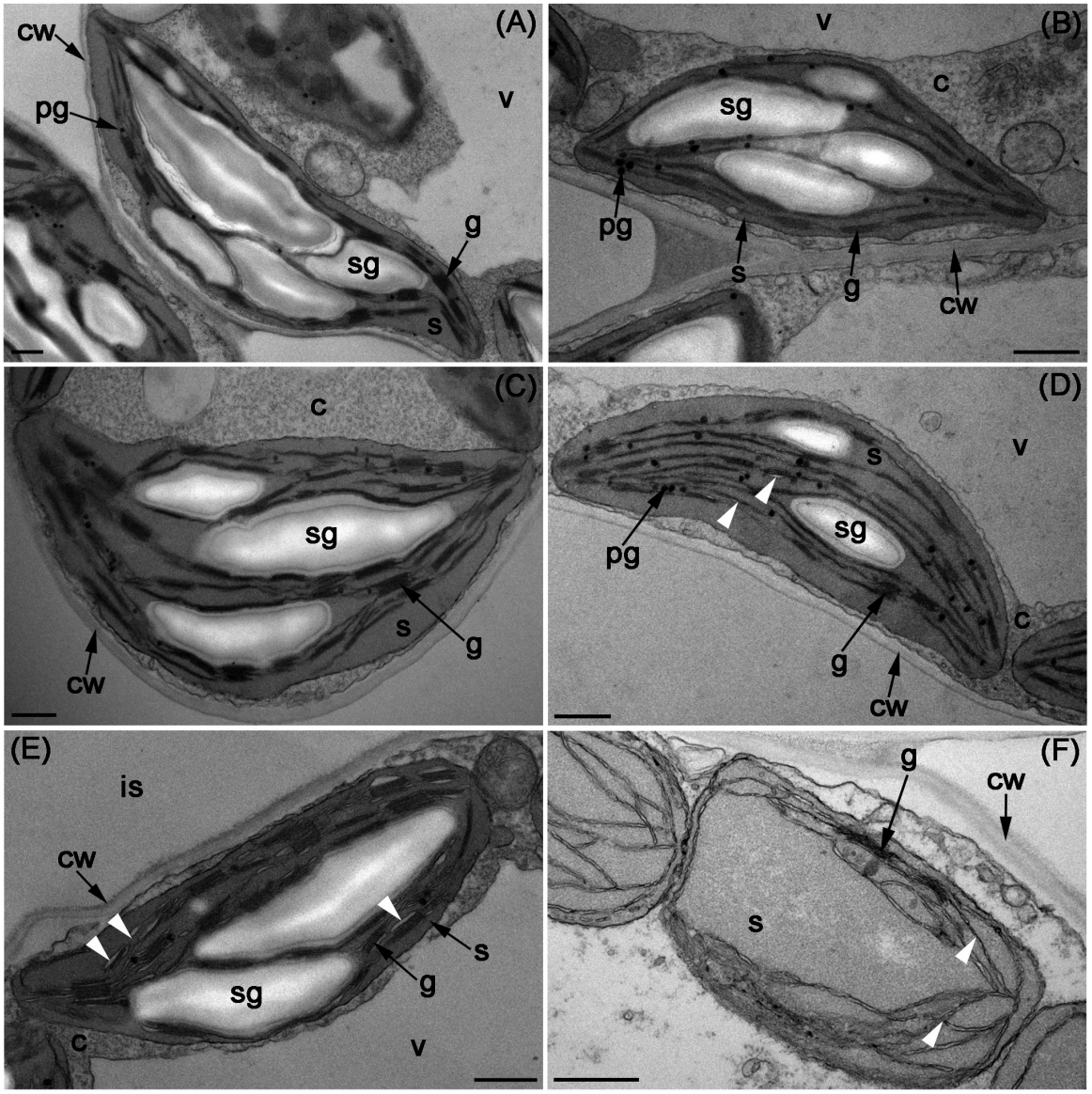

Fig. 4. Transmission electron micrographs of chloroplast ultrastructure in the youngest fully expanded leaves of Genesis836 (A, C, E) and Rupali (B, D, F) grown with 0 (A, B), 30 (C, D), or 60 mM NaCl (E, F) for the final 18 d. White arrowheads indicate examples of swelling of thylakoids (D, E) and damaged granum structure (F). Images are typical of structure seen across two to three replicate leaves. Scale bars=0.5 μm. c, cytoplasm; cw, cell wall; g, granum; is, intercellular space; pg, plastoglobule; s, stroma; sg, starch granule; v, vacuole.

mol^{-1} did not affect the g_s in either genotype when compared with g_s at ambient CO_2 level (Fig. 5F).

Na, Cl, and K concentrations in leaflet secretory trichomes

The secretory trichomes consisted of cluster head cells and three stalk cells (Fig. 6). There were no differences in number and volume of secretory trichomes between genotypes, nor leaflet surfaces (adaxial and abaxial), or among treatments (Supplementary Table S14).

Due to the rarity of obtaining cryoplaned cross-sections through secretory trichomes, only qualitative energy-dispersive X-ray spectra could be obtained from whole head cells of secretory trichomes. These spectra showed K peaks in both Genesis836 and Rupali in the controls and the two salinity treatments (Fig. 7). Neither Na nor Cl peaks were detected in the two genotypes when grown with 0 mM NaCl, and only small peaks of Na and Cl were present for plants from the 30 mM NaCl treatment. At 60 mM NaCl, for Genesis836 the Na and Cl peaks were small, whereas for Rupali the Na peak was comparatively large and a small peak was present for Cl.

Discussion

Chickpea has genotypic variation for salt tolerance (e.g. Vadez *et al.*, 2007; Turner *et al.*, 2013) and amongst various physiological traits, differences in leaf 'tissue tolerance' of Na^+ have been implicated as one factor contributing to tolerance (Khan *et al.*, 2016). Our study demonstrates preferential partitioning of Na within leaflets to epidermis compared with mesophyll

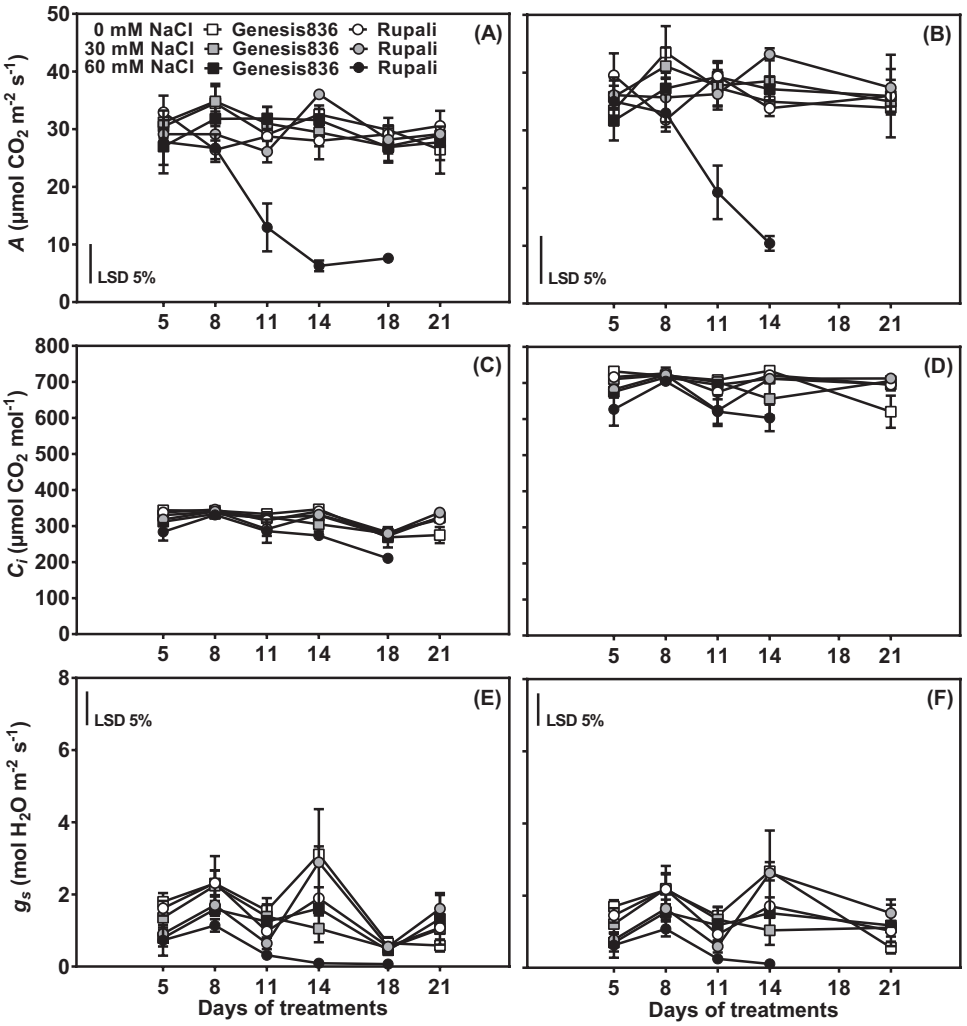

Fig. 5. Gas exchange parameters measured on the youngest fully expanded leaves at a CO_2 concentration of 400 µmol mol^{-1} (A, C, E) and 800 µmol mol^{-1} (B, D, F). Measurements were conducted on two chickpea genotypes (Genesis836 and Rupali) that were grown in aerated nutrient solution with 0 (non-saline control), 30, or 60 mM NaCl. Treatments were imposed on 23-day-old plants and measurements were taken at 5, 8, 11, 14, 18, and 21 d of treatments. Data are means ±SE of four replicates. For Rupali at 60 mM NaCl, only one replicate could be measured at 18 d, and no green leaves were remaining at 21 d. No measurements were conducted at 800 µmol mol^{-1} at 18 d of treatment. There was a significant genotype×treatment×days of treatment interaction for *A* (P<0.001) and g_s (P<0.01), but the interaction was non-significant for C_i (P=0.297) at 400 µmol mol^{-1} CO_2 (three-way ANOVA measured up to 18 d of treatment). Similarly, there was a significant genotype×treatment×days of treatment interaction for *A* (P<0.001) and g_s (P<0.05), but the interaction was non-significant for C_i (P=0.054) at 800 µmol mol^{-1} CO_2 (three-way ANOVA measured up to 14 d of treatment). Bars represent least significant difference (LSD) at P<0.05 for genotype×treatment×days of treatment interaction. Additional statistical analyses are given in Supplementary Tables S11 and S12.

cells in both Genesis836 (salt tolerant) and Rupali (salt sensitive). However, lower Na^+ transport rates from roots to shoots and thus a lower concentration of Na^+ in bulk leaflets accounted for lower Na concentrations in epidermis and mesophyll cells of Genesis836 than Rupali. Low concentrations of Na (<20 mM), and consequently a higher K/Na ratio (44:1) in mesophyll cells, contributed to salt tolerance in Genesis836 as compared with Rupali (salt sensitive). The excessive accumulation of Na (~220 mM) and low K/Na ratio (1.1:1) in mesophyll cells of Rupali corresponded with decreased net photosynthetic rates and structural damage to the chloroplasts.

The preferential accumulation of Na in epidermal cells while maintaining a low concentration of Na in photosynthetically active mesophyll cells has been suggested as an important mechanisms of salinity tolerance in some plants (e.g. barley; Karley *et al.*, 2000). Indeed for chickpea, declines in photosynthetic rates in Rupali grown at 60 mM NaCl were associated with increases in Na concentration in mesophyll cells, whereas photosynthesis was maintained in Genesis836 at 60 mM NaCl and in both genotypes at 30 mM NaCl when Na was partitioned into the epidermis. Fricke *et al.* (1996), using sap extracts from single cells, found that for salt-stressed (up to 150 mM NaCl) barley, Na concentration always increased preferentially in the epidermis but Na also reached high levels in mesophyll cells (302 mM). These increases of Na in mesophyll cells, however, did not affect the photosynthetic performance (Fricke *et al.*, 1996). In a study on salt-treated barley and wheat, James *et al.* (2006*b*) found, using X-ray microanalysis, equal Na distribution between epidermis and mesophyll, but there was a preferential accumulation of K in mesophyll cells. This redistribution of K from epidermis to mesophyll, also observed in other studies on barley (Leigh and Storey, 1993; Cuin *et al.*, 2003; Conn and Gilliham, 2010), presumably enabled a favourable K/Na ratio in the cytoplasm at high leaf Na concentrations (James *et al.*, 2006*b*). In the present

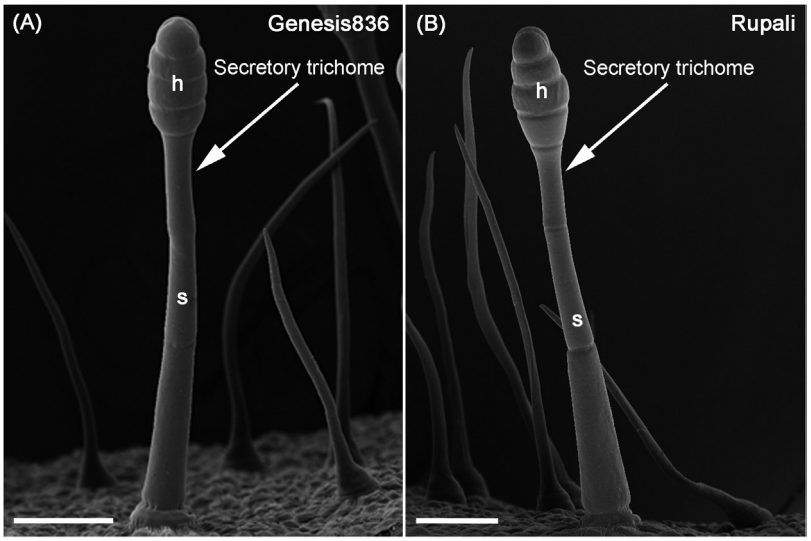

Fig. 6. SEM micrographs of secretory trichomes (with stalk labelled 's' and head 'h') on the youngest fully expanded leaf of Genesis836 (A) and Rupali (B) grown with 60 mM NaCl for 18 d. Several non-secretory trichomes (smaller, hair-like structures) can also be seen in each micrograph. Treatments were imposed on 23-day-old plants. Samples were prepared by critical point drying method, sputter-coated with gold, and examined with a Zeiss 55 at 5 kV accelerating voltage. There were no differences in number and volume of secretory trichomes between genotypes, leaflet surfaces (adaxial and abaxial), or among treatments, with an average number of 4.8 per mm^2 and volume of 15.2×10^{-5} mm^{-3} (Supplementary Table S14). Scale bars=50 μm.

study, maintenance of K in mesophyll cells, but with concurrent decreases in K in the epidermal cells in both genotypes at 30 mM NaCl and Genesis836 at 60 mM NaCl, is indicative of K redistribution also in leaflets of chickpea. Such K redistribution from the epidermal cells, in addition to Na partitioning to epidermal cells, may have contributed to maintenance of an adequate K/Na ratio in mesophyll cells. In Rupali grown at 60 mM NaCl, however, potential redistribution of K from epidermis to mesophyll was not sufficient to compensate for the large increases in mesophyll Na concentration, which consequently resulted in a low K/Na ratio (1.1:1). The high Na concentration and low K/Na presumably contributed to the damage to chloroplasts in the mesophyll of this salt-sensitive genotype.

In contrast to Na, Cl accumulated preferentially in mesophyll compared with epidermal cells of both Genesis836 and Rupali grown with either 30 mM or 60 mM NaCl. The present results are in contrast to previous findings on salt stress in barley where Cl^- was preferentially accumulated in epidermis compared with mesophyll cells (Leigh and Storey, 1993; Fricke *et al.*, 1996; James *et al.*, 2006*b*). Despite the preferential epidermal partitioning, Cl^- reached high levels in mesophyll cells (167 mM) in barley grown in 150 mM NaCl but this did not affect photosynthetic processes (Fricke *et al.*, 1996). Similarly, preferential accumulation of Cl^- in epidermis and relatively low concentration in mesophyll of both salt-tolerant barley and salt-sensitive durum wheat did not explain differences in the photosynthetic capacity between the two species (James *et al.*, 2006*b*). These previous data indicate that cellular compartmentation of Cl^- might not be a factor contributing to salt tolerance (Teakle and Tyerman, 2010). In the present study, we found similar accumulation of Cl at 353–403 mM in palisade mesophyll cells of both salt-tolerant Genesis836 and salt-sensitive Rupali, indicating that lower photosynthetic rates in Rupali grown with 60 mM NaCl were unlikely to be caused by Cl accumulation in these cells. This present finding further supports the conclusion of Khan *et al.* (2016) that salt sensitivity in chickpea is determined by adverse effects of Na^+ in leaflets (i.e. Na^+ toxicity), but not from the relatively high Cl^- concentrations that can co-accumulate in the leaflets.

Reduction of photosynthesis in Rupali grown in 60 mM NaCl was caused by non-stomatal factors rather than by stomatal effects; elevated external CO_2 concentration (800 μmol mol^{-1}) did not lead to recovery of photosynthesis, although the C_i increased 2-fold. This is consistent with a previous study, where no correlation was observed between photosynthesis and C_i but reduced photosynthesis was related to inefficient PSII probably caused by accumulation of Na^+ in leaflets (Khan *et al.*, 2015). A poor PSII activity during salt stress had been suggested to be due to disturbed ionic composition of stroma resulting from entry of Na^+ and Cl^-, which could cause unstacking and distortion of grana, and swelling of thylakoids (rice, Rahman *et al.*, 2000; wheat, Salama *et al.*, 1994). In the present study, swelling of thylakoids, but also disorganization of grana and disruption of the chloroplast envelope, was observed in Rupali grown with 60 mM NaCl. These ultrastructural changes to the chloroplasts occurred at a leaflet Na^+ concentration of ~490 mM and a mesophyll cell Na concentration of ~220 mM, with evidence of breakdown of the tonoplast and aggregation of cellular content in some cells, so that cytoplasmic Na concentrations could have also been high. Breakdown of vacuolar compartmentation occurred in barley at a leaf Na^+ concentration of ~300 mM, which resulted in cytoplasmic Na^+ concentrations of 300 mM and a K/Na ratio of 0.5:1 (James *et al.*, 2006*b*). In the present study, the decline in photosynthetic rates in Rupali began at a leaflet Na^+ concentration of ~180 mM after 11 d of 60 mM NaCl treatment, which may indicate that at this concentration vacuole storage capacity was reached and Na^+ had begun to accumulate in the cytoplasm/chloroplasts, affecting photosynthetic processes. In contrast to Rupali, photosynthetic rates were maintained

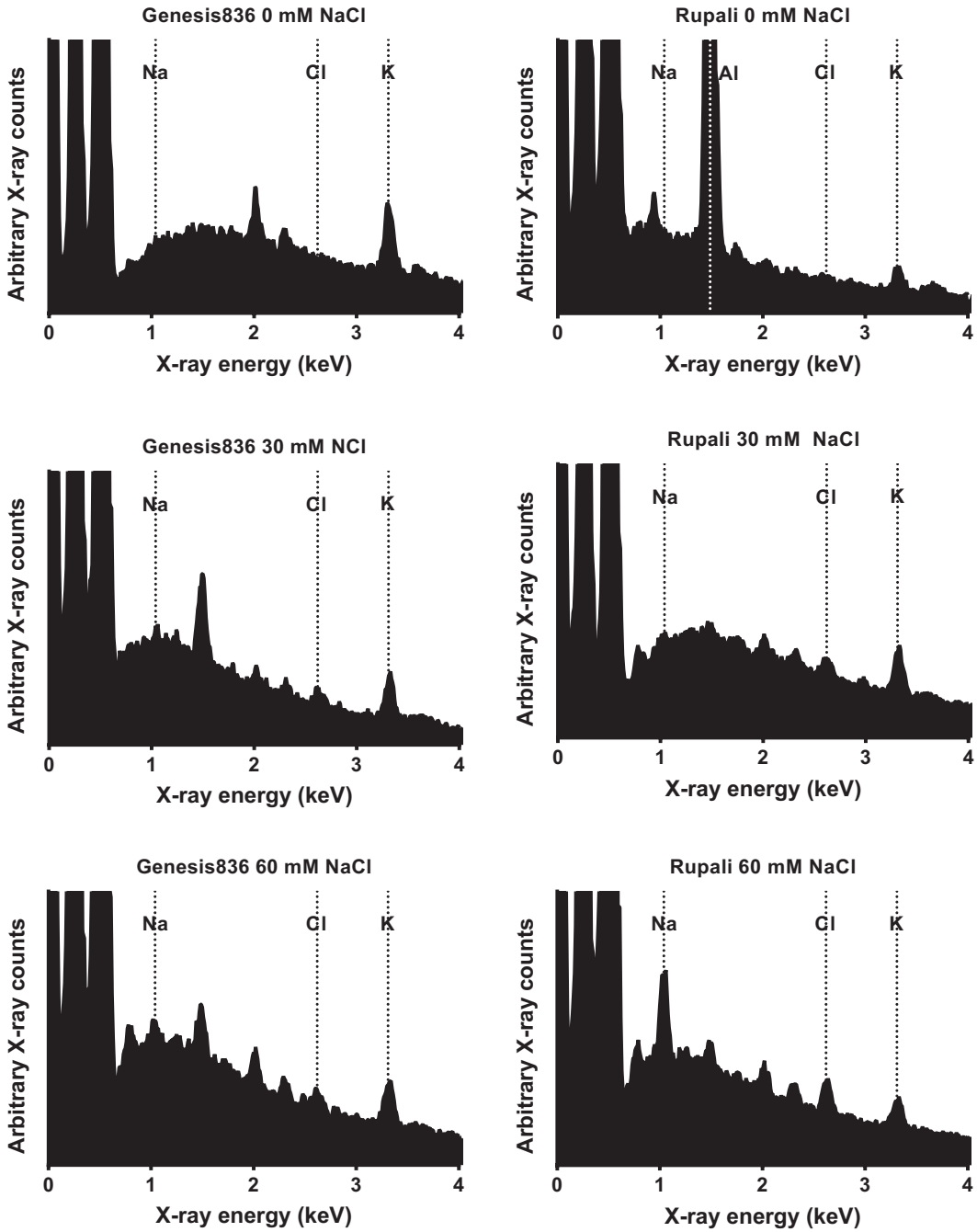

Fig. 7. Qualitative energy dispersive X-ray spectra obtained from frozen–hydrated head cells of intact secretory trichomes from the youngest fully expanded leaves of Genesis836 and Rupali showing relevant Na, Cl, and K peaks. The spectra show clear peaks of K, whereas a large Na peak can only be seen in the spectrum of Rupali from 60 mM NaCl treatment. The large Al peak in the spectrum of Rupali 0 mM NaCl comes from the Al mount on which the leaflet segments were placed during the analysis. Plants were grown in aerated nutrient solution with 0, 30, or 60 mM NaCl for 18 d. Treatments were imposed on 23-day-old plants.

in Genesis836 for the time of treatments, and only minor ultrastructural changes to the chloroplasts (swelling of thylakoids in some cells) were observed after 18 d of treatment, indicating that the majority of Na in mesophyll cells was sequestered into the vacuoles (19 mM). In Genesis836, leaflet Na^+ concentration was ~94 mM for the time of the treatment, with the major proportion of Na^+ partitioned into the epidermis (105 mM after 18 d of 60 mM NaCl treatment).

It has been speculated that tolerance in chickpea to high Na^+ and/or Cl^- concentrations in leaves may be achieved by sequestration of these ions into secretory trichomes and/or excretion from chickpea leaves (Flowers *et al.*, 2010; Khan *et al.*, 2016). We found only small peaks of Cl in X-ray spectra of head cells of secretory trichomes in both Genesis836 and Rupali grown in either 30 mM or 60 mM NaCl, whereas a relatively larger Na peak was seen in these trichomes of Rupali at 60 mM NaCl treatment. Lauter and Munns (1986*b*) found that the Cl^- concentration in secretions on chickpea leaves was ~27% (231 mM) of the total concentrations of anions (Cl^-, malate, and oxalate) and did not differ between salt-tolerant and salt-sensitive genotypes. The Na^+ concentration in leaf surface secretions was only 7% (60 mM) and K^+ <1%

(<8 mM) of the total anions (organic acid anions and Cl^-). Together, these findings indicate that variation in salt tolerance in chickpea (at least for these two genotypes) does not result from Na^+ accumulation in trichomes and/or secretion from leaves (leaf washes were low in Na^+; own unpublished data).

The net Na^+ transport rates from roots to the shoot were 2-fold higher in Rupali as compared with Genesis836. This result indicates that Genesis836, in addition to capacity of cellular compartmentation of Na^+ in leaves, also possesses a greater ability for Na^+ 'exclusion' than Rupali, resulting in a higher Na^+ concentration in leaflets of Rupali. In another study, Khan *et al.* (2016) found a higher shoot Na^+ concentration in Rupali than in Genesis836 after 7 d of 60 mM NaCl treatment, but the shoot Na^+ concentration was then similar after 14 d and 28 d in NaCl. A higher Na^+ concentration in shoots of Rupali during the first several days was related to higher initial uptake of Na^+ in Rupali when compared with Genesis836 (Khan *et al.*, 2016). Results from the present study suggest that the genotypic differences in Na^+ 'exclusion' from the leaflets can persist over a longer period than indicated in Khan *et al.* (2016). This may be due to different time of the year of the experiment (June–August in Khan *et al.*, 2016 versus October–December in the present study) and thus different day length and light intensity, and/or different vapour pressure deficit (e.g. Cl^- content of glandular exudate varied at different humidities; Lauter and Munns, 1986*b*).

In conclusion, preferential compartmentation of Na to epidermis as compared with mesophyll cells was evident in both salt-tolerant Genesis836 and salt-sensitive Rupali. However, better 'exclusion' of Na^+ from leaflets (i.e. reduced rate of Na^+ accumulation) accounted for lower concentrations of Na in both epidermis and mesophyll cells of Genesis836 as compared with Rupali. The capacity to maintain low Na concentration in photosynthetically active mesophyll cells (i.e. better exclusion from leaflets and cellular compartmentation) contributed to salinity tolerance in Genesis836 relative to more sensitive Rupali. High accumulation of Na in mesophyll cells of Rupali resulted in damage to the chloroplasts and reduced net photosynthesis. No genotypic differences were found for accumulation of Cl in mesophyll cells, indicating that differences in salt sensitivity in the two chickpea genotypes studied is not determined by Cl toxicity. Genotypic variation in salt tolerance was also not due to accumulation of Na^+ or Cl^- in secretory trichomes. Future studies are needed to evaluate whether the above findings are consistent for a wider range of contrasting chickpea genotypes.

Supplementary data

Supplementary data are available at *JXB* online.

Fig. S1. Examples of typical cryo-SEM micrographs.

Fig. S2. Cross-section of the lamina of leaflets of Genesis836 and Rupali.

Fig. S3. Dry weight of shoot, relative growth rate of shoot, and dry weight of roots of two chickpea genotypes.

Fig. S4. Concentrations (mM, water basis) of Na^+ in petioles and K^+ in leaflets and petioles of the youngest fully expanded leaves that were measured for gas exchange.

Fig. S5. Concentrations (μmol g^{-1} DW) of Na^+ and K^+ in leaflets and petioles of the youngest fully expanded leaves that were measured for gas exchange.

Fig. S6. Concentrations (mM, tissue water basis) of Na^+, Cl^-, and K^+ in green leaflets of whole shoots, petioles and stems, and roots of two chickpea genotypes.

Fig. S7. Concentrations (μmol g^{-1} DW) of Na^+, Cl^-, and K^+ in green leaflets of whole shoots, petioles and stems, and roots of two chickpea genotypes.

Fig. S8. Transpiration rates (*T*) measured on the youngest fully expanded leaves at a CO_2 concentration of 400 μmol mol^{-1} and 800 μmol mol^{-1}.

Fig. S9. Chl *a* and Chl *b* concentrations in leaflets of the youngest fully expanded leaves of Genesis836 and Rupali that were previously used for gas exchange measurements.

Fig. S10. Relationship of net photosynthetic rates (*A*) and concentration of Na^+ in leaflets of the youngest fully expanded leaves of Genesis836 and Rupali.

Table S1. Composition of nutrient solution used in experiments.

Table S2. *F*-values of two-way ANOVA (factors: 'genotypes' and 'salinity treatments') for shoot DW, shoot RGR, and root DW.

Table S3. *F*-values of two-way ANOVA (factors: 'genotypes' and 'salinity treatments') for concentrations of Na^+ and K^+ in leaflets and petioles of the youngest fully expanded leaves.

Table S4. *F*-values of two-way ANOVA (factors: 'genotypes' and 'salinity treatments') for concentrations of Na^+, Cl^-, and K^+ in leaflets of whole shoots.

Table S5. *F*-values of two-way ANOVA (factors: 'genotypes' and 'salinity treatments') for concentrations of Na^+, Cl^-, and K^+ in petioles and stems.

Table S6. *F*-values of two-way ANOVA (factors: 'genotypes' and 'salinity treatments') for concentrations of Na^+, Cl^-, and K^+ in roots.

Table S7. Na^+, Cl^-, and K^+ net uptake rates by roots of two chickpea genotypes.

Table S8. Na^+, Cl^-, and K^+ net transport rates to shoots of two chickpea genotypes.

Table S9. *F*-values of two-way ANOVA (factors: 'genotypes' and 'salinity treatments') for Na^+, Cl^-, and K^+ net uptake rates and net transport rates.

Table S10. *F*-values of two-way ANOVA (factors: 'genotypes' and 'salinity treatments') for concentratins of Na, Cl, and K and K/Na ratio in various cell types of the youngest fully expended leaves.

Table S11. *F*-values of two-way ANOVA (factors: 'genotypes' and 'salinity treatments') for gas exchange parameters measured at a CO_2 concentration of 400 μmol mol^{-1}.

Table S12. *F*-values of two-way ANOVA (factors: 'genotypes' and 'salinity treatments') for gas exchange parameters measured at a CO_2 concentration of 800 μmol mol^{-1}.

Table S13. *F*-values of two-way ANOVA (factors: 'genotypes' and 'salinity treatments') for concentrations of Chl *a* and *b*.

Table S14. Number of stomata, number of secretory trichomes, and volume of a single secretory trichome on adaxial and abaxial surfaces of two chickpea genotypes.

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