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A Comparative Salt Shock Stress Response in the Wild *Brassica oleracea* and Doubled Haploid Genotypes

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ABSTRACT

The study was conducted to compare the early response between the wild (S1), founder (DHLS150), and doubled haploid (DH) lines to salt shock. A single dose of 250 mM NaCl was used to expose the plants to salt shock. Data analyzed at a two-point time; 24 hr post-treatment (24 hr pt), and 14 days post-treatment (2 wk pt) showed a significant increase 24 hr pt in leaf Na^+ content ($P<0.05$) in the parent and DH lines, however, K^+ and Ca^{2+} levels remained significantly unchanged ($P>0.05$), and K^+/Na^+ ratio reduced, which indicates osmotic shock. Similarly, the K^+/Na^+ ratio showed an improvement of 2 wk pt in some of the S1 parent and DH lines, recuperating phase. The qRT-PCR analysis showed significant variation in gene expression, that show an mRNA level of sodium/proton exchanger (*NHX1*), potassium transporter 9 (*KT9*) and potassium uptake permease (*KUP11*), vacuolar H^+ adenosine triphosphatase (*V- ATPase - G*), and chloride gated channel (*V-CLC*) in both the parent and DH lines. The results further revealed that the S1 and DH lines maintained higher levels of K^+, Ca^{2+}, and K^+/Na^+ ratio in leaves through ion-selective capacity. Therefore, our results conclude that the adaptability of *Brassica oleracea* to salt shock and prolonged salt stress could be associated with ion selectivity, and Na^+ exclusion and the response between the parent and DH lines are similar hence the DH lines can be used for the brassica breeding program.

INTRODUCTION

The two distinct phenomena; salt stress and salt shock are both triggered by the application of salt. However, the terminologies 'salt shock' and 'salt stress' are used interchangeably, the salt stress is the exposure of plants to salinity, the main component has been the NaCl in two ways either by gradual exposure to increasing levels of NaCl or by exposure to low levels of salinity, and could be both ways (Yuri, 2013). On the other hand, a salt shock is an extreme form of salt stress, where the plants are exposed suddenly to a high level of salinity. The two main components of salt stress/shock are mechanisms responsible for salinity tolerance in plants involving both tolerances to osmotic and ionic stresses (Munns and Tester, 2008). Induction of salt shock leads to the immediate response of osmotic shock due to large differences in osmolarity (osmotic pressure) between external solutes with a high concentration of NaCl and internal in the cell cytoplasm. The osmotic phase causes inhibition of water uptakes as a result of increased salt content in the soil around the roots (Munns and Tester, 2008). The mechanism to reduce the effect of osmotic stress has been attributed to osmotic adjustment, in which the plants accumulate more both inorganic and organic solutes (Munns et al., 2020). Other important mechanisms to confront osmotic stress have been directed to reduce transpiration leading to water loss, which depends on stomatal closure and stomatal density (Flowers et al., 2010; Albaladejo, 2017). In addition, ionic stress is the specific component of salt stress that the plants experience when growing in saline soils. Halophytes in this regard are considered as Na^+ - includers that is, their tolerance is associated with high Na^+ accumulation in the leaves (Flowers et al., 2010). The mechanisms also involved either removing the added Na^+ from cells through membrane-bound Na^+/H^+ antiporters or via sequestration of excess Na^+ and K^+ into the leaf vacuoles through the antiporters in the membrane such as proton exchangers Na^+/H^+ (Blumwald and Pool, 1985; Zhu, 2000; Bassil et al., 2012). Additionally, ion homeostasis is ubiquitous to membrane proteins that catalyzed the electroneutral exchange of Na^+ and or H^+ across the membrane, thereby playing an essential role in cellular Na^+/K^+ and pH homeostasis (Rodriguez-Rosales et al., 2009; Leidi et al., 2010; Barragán et al., 2012). The calcium-permeable non-selective cation channels (NSCC) contribute both directly and indirectly to the Na^+ entry into the cell. The influx of Na^+ through these channels stimulates depolarization of the plasma membrane, activating K^+ outward-rectifying channels (KOR) thereby reducing the net passive K^+ uptake through the inward-rectifying K^+ channels (KIR) (Demidchik et al., 2003; Demidchik et al., 2014; Albaladejo, 2017). Accumulation of cGMP suppressed the influx of Na^+ by deactivation process via NSCC and allow the apoplastic Ca^{2+} into the cell cytoplasm through cyclic nucleotide-gated channels (CNGC). Increase cytoplasmic Ca^{2+} indicates abiotic stress, and consequently, triggers cascade reactions via the activation of cytosolic calcium induces calmodulin (CaM)-dependent kinases leading to the activation of other plasma membranes H^+-ATPases (Bose et al., 2014a). Restoring membrane voltage and inhibiting depolarisation-activated NSCC and processes that lead to the reduction of Na^+ influx into the cell and efflux of K^+, improve plant tolerance to salt stress have been considered to be important response mechanisms (Yadav et al., 2012; Shabala, 2003; Bose et al., 2014a; Mostofa et al., 2015).

Investigation of these membrane transporters like SOS1 (Salt Overly Sensitive 1), which excludes Na^+ out of the root and facilitates its loading into the xylem, High-Affinity Potassium Transporters 1 (HKT1s), which involved in Na^+ retrieval from the xylem under salt stress, as well as NHX1 (Na^+/H^+ exchanger 1) to develop salt-tolerant plants has been reported (Munns et al., 2012; Nieves-Cordones et al., 2016; Jaime-Pérez et al., 2017). For example, High-Affinity Potassium Transporters (HKTs), which perform functions of Na^+/K^+ symporter and Na^+ uniporter present in the plasma membrane of different plant crops including wheat, rice, and *Arabidopsis* have been shown to improve cytoplasmic K^+ and salt-tolerant (Waters et al., 2013). Research by Berthomieu et al. (2003) reported the critical role of HKTs in sodium recirculation in *Arabidopsis* from the shoots to the roots by carrying Na^+ from the shoot into the phloem and subsequently releasing it back into the roots. Different isoforms of HKT genes, HKT1;4 isolated from salt-tolerant durum wheat cultivars expressed in Xenopus oocytes have been shown to exhibit higher Na^+ selectivity (Daldoul et al., 2014). Also, Sunarpi et al. (Sunarpi et al., 2005) have reported the localization of HKTs proteins to the membrane of xylems' parenchyma cells and linked it to salt tolerance in *Arabidopsis*. They also reported that the overexpression of HKT in Arabidopsis HKT1; 1 in the root stele causes improvement by 37-64% in resilient phenotype. In addition, Salt Overly Sensitive transporters such as (SOS1) have been reported to involve in the transportation of Na^+ as indicated by studies carried out on mutant yeast that lacks Na^+-ATPases and Na^+/H^+ antiporter activity (Kinclova-Zimmermannova et al., 2004). Further studies indicated that AtSOS1 transporters work as anti-porters in Na^+ transport in *Arabidopsis* plants (Shi et al., 2003; Kinclova-Zimmermannova et al., 2004). Overexpression of SOS1 genes isolated from a halophyte Salicornia brachiate has been shown to improve salt tolerance in tobacco (Yadav et al., 2011). An excess Na^+ accumulation distorts by altering the K^+ homeostasis, in such a manner that the Na^+/K^+ ratio has been considered as an indicator of salt tolerance index not only in glycophytes but also in halophytes (Cai and Gao, 2020; Kiani-Pouya et al., 2020). Therefore, K^+ transporters could be key determinants of salt tolerance, which include KT, and KUP (KT/HAK/KUP family).

Different forms of *B. oleracea* have been selected by farmers for domestication to produce crops with distinct uses and characteristics such as broccoli, Brussels sprouts, cabbage, cauliflower, and kale,

among others. The diversification of *B. oleracea*, caused by selection processes, leads to a differentiation of several botanical varieties or groups, such as Italica, gemmifera, capitata, botrytis, and acephala, corresponding, respectively, to the above-mentioned crops (Branca et al., 2011). The wild *B. oleracea* are adaptive to many environmental conditions and show a level of tolerance against abiotic factors, including temperature, salinity, and water stresses (Ashraf et al., 1999; Ashraf et al., 2008; Cuartero et al., 2006). The cultivated *B. oleracea* and its wild species are included in the primary gene pool (Thampson et al., 2010; Peter et al., 2012). Higher allelic diversity found in the wild species is rich in variability than in the cultivated one (Thampson et al., 2010; Peter et al., 2012). Characterization and exploitation of such variability in the wild *B. oleracea* C – genome would pave a way for improving brassica oleracea vegetables and facilitate crop breeding and conservation strategies (Peter et al., 2012). The use of doubled haploid lines in hybrid production helps to reduce the time for plant breeding (Thampson et al., 2010; Peter et al., 2012). The advantages of using DH lines include the elimination of residual heterozygosity and heterogeneity and also help in the assessment of quantitative traits (Peter et al., 2012). Accurate assessment of the levels and patterns of genetic diversity can be useful in crop breeding for diverse applications which include; analysis of genetic variability in cultivars (Smith and Dinnen-Zopfy, 1984; Cox et al., 1988), identifying diverse parental combinations to create segregating progenies with maximum genetic variability for further selection (Barrett and Kidwell, 1998), and introgression of desirable genes from diverse germplasm into the available genetic base (Thompson et al., 2010). In this research work, homozygous DH lines “Diversity Fixed Foundation Sets” (DFFS) developed from the F1 materials derived from crosses between the wild parent *Brassica oleracea* S1 lines and the founder lines (DHSL150) of the core collections using microspore culture was utilized (Pink et al., 2008).

Therefore, in the present study, we aimed to examine the effects of sudden salt shock, exposure to 250 mM NaCl and monitor the early response of the parent lines; the wild *Brassica oleracea*, and cultivated rapid-cycle DHLS150 and DH lines. At the same time, we have taken physiological and gene expression approaches to get an insight into the initial salt response mechanisms by assessing parameters like mineral homeostasis and qRT-PCR analysis of the expressed genes, and by comparing the early response between the parent and DH lines would establish and unravel the mechanism employed by B. oleracea, and show the effect of traits introgression between the parent and DH lines.

MATERIALS AND METHODS

Seed Collections

The seeds were from Gene Bank, Warwick Crop Centre, Wellesbourne Campus, the University of Warwick, UK. The six accession lines of wild S1 parent lines, rapid-cycle founder line (DHLS150), and seven accession lines of doubled haploids (DHs) were collected and sowed in 4 x 10 tray filled with M2 Compost soil and kept under a controlled environment Glasshouse, Phytobiology Unit, University of Warwick.

Growth Chamber Condition, Soil Compositions, and Seeds Sowing

The growth chamber was a controlled glasshouse with an average temperature between 20^0 C day and 18^0 C night. It was supplemented with available light (400 W SONT lamps), photoperiod, which was set at 03:00 – 19:00 hrs to ensure photosynthesis. The growth chamber was also maintained under a reliable controlled internal environment, containing air handling and drainage for the enhancement of natural light penetration, and pest control combined with alternative energy solutions to reduce the carbon footprint. An M2 soil compost used was designed to suit the growth of a wide variety of bedding plants. It provides a good quality nutrient supply, and its physicochemical constituents include; pH = 5.3 – 6.0, nitrogen (N) = 192 mg/L, phosphorus (P) = 98 mg/L, and potassium (K) = 319 mg/L and finally, the size of the soil particle is between 0 – 10 mm respectively. The seeds were sown using two seeds per hole in a 20 x 10 plastic tray filled with M2 compost soil. Germination was monitored between 6 – 7 days after seeds were sown and then daily for a period of three weeks. As described, fully germinated seeds were when radicles had fully emerged from the seed coat and out of the soil compost. They were regularly watered twice per week for a period of four weeks (28 days) and re-transplanted into the bigger pots (7 cm x 10 cm) at week six before set-out into a completely randomized experimental design.

Salt Shock Recruitment

Experiment 1

In the first screening experiment for salt shock stress, two (2) accessions of the wild *B. oleracea*: *B. oleracea* (C07079A-S1) and *B. bourgaei* (C07007-S1), and a founder rapid-cycle line DHSL150 (C04099) and four accessions of doubled haploid (DH) lines: C10025-DH, C13001-DH, C10121-DH, and C10128-DH were recruited.

Experiment 2

In the second salt shock experiment, related accession lines, not in experiment one (1) were further selected and the DH lines derived from the F1 materials of those wild accessions based on the outcome of the first screening experiment were selected to further study their response mechanisms to salt shock stress. These include the main founder line (DHLS150), *B. bourgaei* (C07007-S1), *B. oleracea* (C07060-S1), and *B. oleracea* (C07079A-S1), and the DH lines: C10025-DH, C13013-DH, C13001-DH, C10128-DH, and C10121-DH, respectively.

Salt Shock Stress Inducement and Sample Collections

The salt shock was induced when the plants were at 42 days old (6 weeks) using a single dose of freshly prepared 250 mM NaCl on the treated plants while untreated plants (control) were administered only tap water. Salt leakages through an opening beneath the plants were blocked using a plastic cover throughout the experimental period to ensured maximum treatment. Twenty-four hours post-treatment (24 hr pt), samples from leaf-four (#4) were collected in an Eppendorf, quickly flash-frozen in liquid nitrogen, and 50 mL test tubes of both treated and non-treated plants for both RNA and mineral analyses. The plants were watered 24 hr pt after samples were collected with non-salty water and on day 14 post-treatment (2 weeks pt), another set of samples from leaf five (#5) as described, and all samples were kept at -80^0 C for RNA analysis until used.

ICP-MS Analysis of Mineral Content

A 0.5g of the harvested leaves material (oven-dried at 80 ^{0}C for 12 hr) was finely grounded using pestle and mortar, to expose more surface area that ensures complete digestion efficiency was placed into a standard 50 mL PTFE digestion tubes. 2 mL of 69% ICP-MS grade nitric acid was added, and loaded into the microwave digestion system (*MARSX* 5 CEM Corporation, USA), for 34 minutes. The concentrations of Na^+, K^+, and Ca^{2+} were determined using ICP – MS (Agilent 7500 series) in the Department of Chemistry, University of Warwick.

Gene Expression Analysis

RNA Isolation and Reverse transcription

RNA isolation was carried out strictly as according to the manufacturer's guidelines using an RNeasy Plant mini kit (QIAGEN) and the quality and quantity of the RNA were checked using a Nanodrop spectrophotometer (Thermo Fisher Scientific; Waltham, USA) and the RNA integrity was verified on a 1.5% agarose gel. The cDNA, briefly, was synthesized by using an equivalent volume representing 2.5µg of the extracted RNA. Two masters mixed were prepared; the first one was made in a 1.5 ml Eppendorf tube which contained an equivalent of 2.5µg total RNA, 1µl oligo (dT) primer (20 mM) and DEPC-treated used to make up the volume to 20µl. The content was mixed and centrifuged briefly and incubated at 65^0 C for 5 min. The second master-mixed was prepared using 10.0µl 5xRT buffer, 5.0µl DTT (20 mM), 1.0µl of premixed dNTPs, and 0.5µl RT Superscript II respectively. The tubes were placed on ice for 2 min before being placed in the water bath at 65^0 C. A 16.5µl of the second prepared master-mixed was pipetted into each tube, and the final volume was brought up to 50µl with molecular water and mixed. The tubes were then incubated at 42^0 C for 1 hr after which the synthesized cDNA was diluted with 200µl of molecular water and the quality of each synthesized cDNA was tested using a Nanodrop spectrophotometer (Thermo Fisher Scientific; Waltham, USA) and kept at -20 ^{0}C until required.

qRT-PCR Assay of Stress Responsive Genes

Changes in the expression of ten stress-responsive genes involved were quantified using quantitative real-time PCR analysis. These are selected membrane ion transporter transcripts that showed significant variation in both tolerant and susceptible lines obtained from RNA-Seq (data not shown) exposed to salt shock were chosen for qPCR validation (Table 1.0). Transcript-specific primers were designed to amplify a specific cDNA sequence of the transcripts in our samples. The sequences of mRNAs of the transcripts of interest were obtained by using their individual transcripts IDs and downloaded from (https://plants.ensembl.org/Brassica_oleracea/) were blasted at (https://www.ncbi.nlm.nih.gov/blast/). The primers were designed using DNASTAR (Lasergene 14) software. The parameters used are as follows: *Tm*, 55^{0}C to 62^{0}C, differences not >2^{0}C between the primers in a pair was insured; primer length, 19- 24 bp; GC content, 45-55%; amplicon length, 100-150 bp. Whenever possible, primers were designed to span introns and caution was made to insured that only primer (forward and reverse) yielding a single product in conventional PCR and qPCR was used in the validation. Quantitative PCR (qPCR) was performed using an Mx3005P multiplex quantitative PCR system (Agilent Stratagene). 5.0µl cDNA samples prepared from the total RNA extracted from three biological replicates of the experimental plants were used. A master-mixed was prepared using 10.0µl SYBGREEN as a detection probe and 2.5µl each of primer pair (Forward and Reverse). Plate set-up was prepared using a randomized design using a color code that represents treated and the control samples in three replicates randomly, each sample was included three times per pair primer and with the housekeeping primer genes (b-Tubulin). The qPCR thermocycler was set using the parameters; denatured temperature, 95 ^{0}C for 5 min; annealing temperatures, 62 – 55 ^{0}C for 1.0 min, and 72^0 C for 30 sec and 45 cycles.

Table 1.0: List of Genes and their respective forward and reverse primers sequence used

Gene ID	Gene name	Forward Sequence	Reverse Sequence
Bo1g022080	*KT*	TGGCGGAAAGGGTAGAAACAT	TGGATGAAGAAGCTACTAAG
Bo1g158860	*V-type G*	CCAGCAGAGGAGGAGGAGGTG	TTCGATTTTGGCATCAGTCTCTTG
Bo2g024320	*V-type a*	AATGGCTCCACTGCAACTTCTC	GTTTTCATTTTCACTTTTATCGCT
Bo4g012670	*KT 9*	TGGTGTTCTGTCTTTCGTTTTCTG	TGATAACGGAGAAGGTGGGACT
Bo4g039050	*KUP11*	ACAATGGGTGGTGGTGGTGA	TATGGATGAAGAAGCTGGTCGG
Bo4g145930	*V-type a*	GGTGGTGGTGGTGGTGGTT	TGCTCATCTCACCGTCTCTTACCT
Bo5g131740	*CAX3*	AGATGTCCAAACCCGCCGTCAG	GATTCTCCTCGGCGCAGCGTT
Bo8g030800	*V-CLC*	TCTTGCTACGAGCTCTCCAGTCCT	AACGGCGAGGTTGTTGGTGAAA
Bo9g003910	*ECA2*	GAAGTCTTTATCTCCGTGGTCGTG	GATGTCCAAACCCGCCGTCAG
Bo9g010200	*NHX1*	TCGTTTTTGGATTCTTTCGTAT	GTATTGTCATTGGCCATCTCTTGG
LI61-qRNA-Btub	B-tubulin	TCATGGATCTGGAGCCTGGAAC	GGAATGGCAAACCTGAAACCC

Data Analysis and Statistics

Statistical analysis

A qPCR analysis was conducted and data was analyzed using an Mx3005P multiplex quantitative PCR system (Agilent Stratagene, Germany). The housekeeping gene (β-Tubulin) was used for normalization. Genotype-specific Ct values for the unknown samples and control were calculated using baseline-corrected, ROX-normalised parameters. Three technical replicates were included in each plate, and average Ct values for each gene were normalized within the plate housekeeping gene by a method of Livak and Schmittgen (2001). The average *Ct* values from the three biological replicates were further analyzed using Microsoft Excel (2016) to calculate the mean, standard deviation (SD), % Coefficient of variance (%CV), SEM, and Log2 Fold was used to determine the relative gene expression of salt responsive genes. Analysis of variance (ANOVA) was carried out using F-test to test for significant variation between different time points. Differences were considered significant at $P < 0.05$. To minimize bias in comparisons of the varieties, which differed in salinity tolerance, all data were normalized to the control. Data presented in the text, figures, and tables are values of mean ± standard error of the mean of three replicates ($n = 3$).

RESULTS

Initial Salt Shock Experiment: Variation in Leaf Mineral Content

The initial salt shock study, involving one of parent founder line DHLS150 and some DH lines; C10025, C10121, C10128, C13001 was investigated in the presence of high salt concentration (250 mM NaCl). The plant's response to NaCl treatment was manifested by an increase in Na^+ leaf content and reduced Na^+/K^+ ratio 24 hr post-treatment in the leaves (6wk) **Figure 1**. The overall result indicates no significant variation in the traits that control the physiological response between the parent line DHLS150 and the DH lines regarding the excess Na^+ which indicate osmotic shock among the lines with Na^+ concentration between 1.5 – 1.9 mg/L. The levels of K+ and Ca2+ significantly remained higher, especially the Ca2+ in all the lines. However, two-week (14 ds) post-treatment, the ICP-MS analysis of the younger leaves (8wk) revealed that the trend was reversed and the plants were able to exclude Na^+ from the younger leaves (8wk) and improved K^+ retention whereby increasing the Na^+/K^+ ratio (osmotic tolerance) as compared to the 24 hr pt. In comparison between the two-point time data, showed significant variation between the 'older' (6wk) and the 'younger' (8wk) leaves. Improve K^+ retention and Na^+ exclusion observed may indicate genetic similarities and the effect of introgression of quality traits that control the physiological response into the DH lines.

The Ca^{2+} level remained relatively unchanged above 5.0 mg/L in all the lines despite salt treatment except C10128-DH line 14 ds post-treatment (**Figure 1**). This suggests that the effect of Na^+-Ca^{2+} interaction does not affect the transportation of calcium into the xylem tissues in *B. oleracea* genotypes under salt shock stress. The C10121-DH line showed a higher retention capacity for both Na^+ and K^+ ions in the leaves without affecting the Ca^{2+} level in both younger and old leaves. This could suggest that in this DH line that the physiological response employ to handle the osmotic phase of salt shock stress may differ from its first parent DHSL150, and may be due to the homozygous effect.

The computed Na^+/K^+ ratio was reduced 24 hr post-treatment as a result of higher Na^+ concentrations in the cytoplasm of the cell. This indicates susceptibility to the osmotic phase of salt shock stress, which could be due to a compromised transport system across the xylem and parenchyma tissues.

However, the transport within and across the cells have shown to be adjusted 14 ds post-treatment whereby the *B. oleracea* lines showed an improved Na^+/K^+ as presented in **Figure 2**. Additionally, an improved Na^+/K^+ ratio was higher in the DH lines as compared to the parent line (DHLS150), especially for C13001-DH and C10121-DH, respectively (**Figure 2**). This could be important for osmotic adjustment and homozygous effect (inherited double traits from both parents) and can be a useful indicator of introgression and for plant breeding purposes.

It further suggests that the *B. oleracea* lines may have evolved a mechanism to reduce the Na^+ influx (excluders) and improve the level of K^+, which is important in ameliorating the effect of excess salt that ensures growth. A correlation analysis conducted between the physiological parameters at two time-points showed a weak correlation between Na^+ and K^+ 24 hr post-treatment in older leaves (R^2 = 0.0978), while Ca^{2+} and Na^+ showed a strong positive correlation (R^2 = 0.6808), which suggest possible role played by calcium in signaling pathway in salt stress response. The situation was reversed 14 ds post-treatment when younger leaves were analyzed. The improved K^+ level observed correlates well with a reduced level of Na^+ which showed tolerance and good for *B. oleracea* lines used (R^2 = 0.9732) (**Figure 3**).

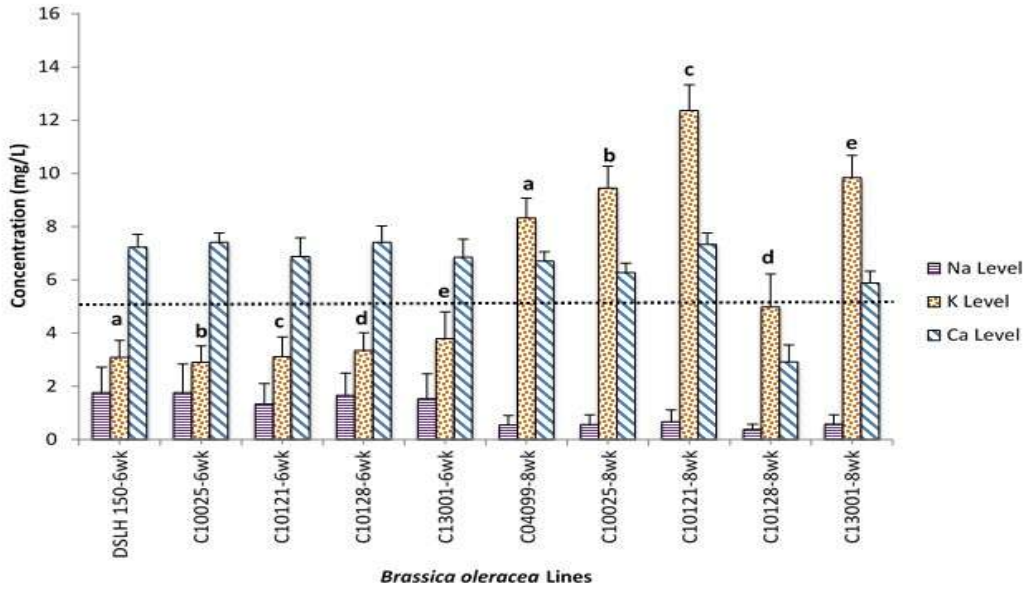

Figure 1: The effect of salt on the levels of leaf mineral content in *B. oleracea* genotypes.
Data represent mean ± SEM (*n* = 3). A significant difference (P<0.05) was determined using **paired one-tailed** Student's T-test by comparing two-time points, and a similar letter indicates a comparison between the two-time points and significant difference. **Note:** DHLS150 (accession number: C04099) is the founder parent line and DH lines with accession numbers; C10025, C10121, C10128, and C13001.

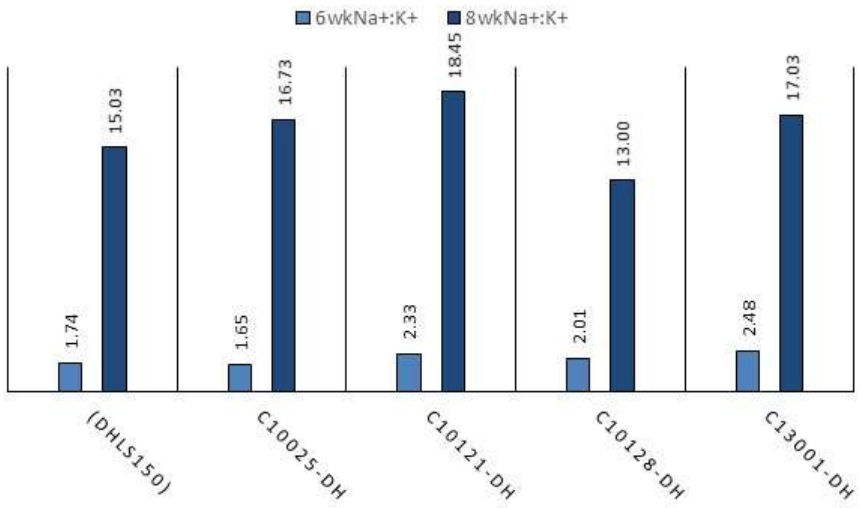

Figure 2: A comparison between K^+/Na^+ ratio using 250 mM NaCl in *B. oleracea* genotypes.
Note: DHLS150 (accession number: C04099) is the founder parent line and DH lines with accession numbers; C10025, C10121, C10128, and C13001.

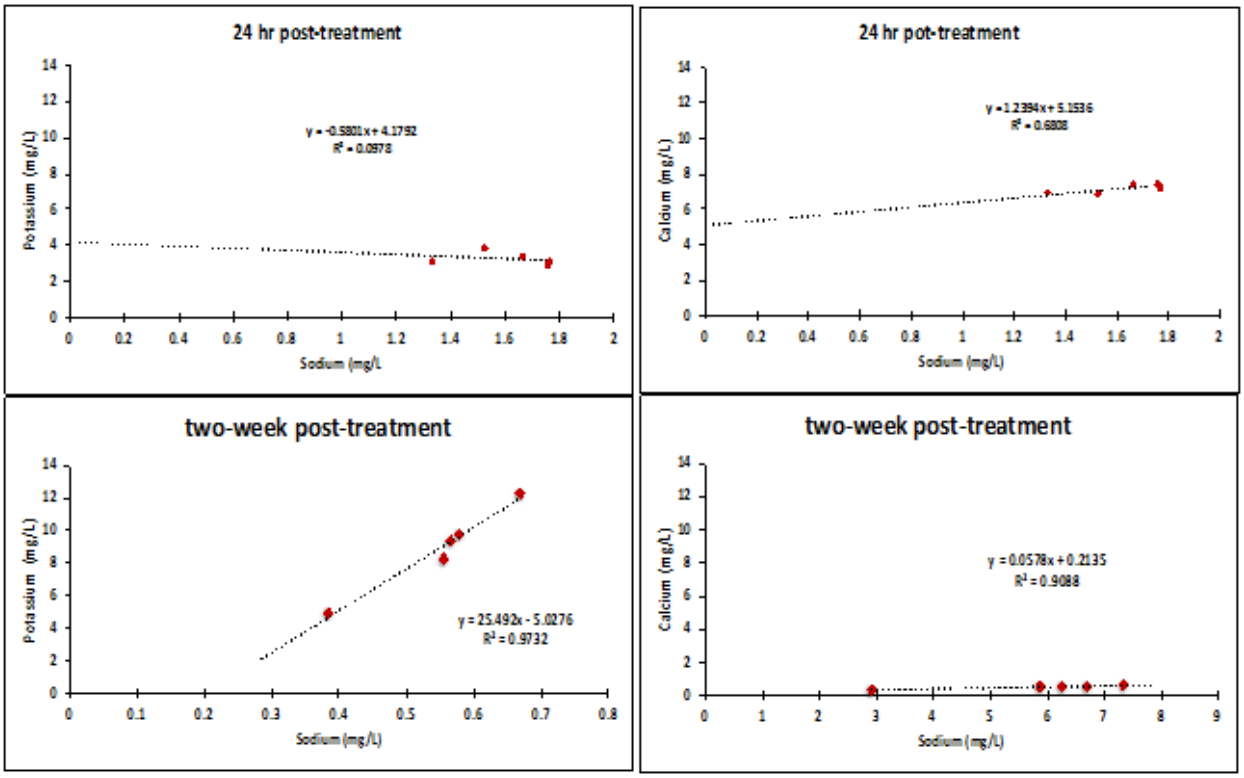

Figure 3: Correlation analysis of leaf mineral content in *B. oleracea* genotypes exposed to high salt concentrations.

Second Salt Shock Experiment: Leaf Mineral Content

In the second salt shock experiment involving more parent lines; the wild S1; *B. bourgaei* (C07007-S1), B. oleracea-S1 (C07060); *B. oleracea-S1* (C07079A) and the founder line (DHLS150), and fixed DH lines; C10025-DH; C13013-DH; C13001-DH, C10128-DH, and C10121-DH were analyzed and the ICP-MS result obtained showed that Na^+ level increased in all the lines as compared to the control group (**Figure 4A**) and the level of K^+ remained relatively higher 24 hr post-treatment. A significant increase in Na^+ level above 4.0 mg/L was observed in the founder line DHLS150, the DH lines; (C10025-DH, C13001-DH, C10121-DH), and C13013-DH ($P < 0.05$ and 0.001), respectively. This corroborated with our initial salt shock screening, indicating the same physiological responses. The newly introduced wild S1 parent lines (second parent lines) have shown variation in their response to salt shock treatment, however, no significant differences were observed between the treated and the control plants as presented in **Figure 4A**. Two-week post-treatment, all the lines have shown features of accumulating more Na^+ contrary to the initial salt shock screen. Generally, Na^+ accumulation is accompanied by K^+ reduction, as it was found in the salt-treated B. oleracea lines two-week post-treatment (**Figure 4B**). Thus, a K^+ reduction of about 50% was observed in salt-treated B. oleracea lines (**Figure 4D**). An inverse relation seems also to exist in one of the wild S1 parent lines; B. oleracea-S1 (C07060), where the K^+ level was higher against the control (**Figure 4D**).

The K^+/Na^+ ratio obtained using the wild S1 parent lines and DH genotypes has shown a reduced K^+/Na^+ ratio due to an increased Na^+ influx. This was observed more in the older leaves 24 hr post-treatment (**Figure 4**), as compared to the younger leaves 14 ds post-treatment. The highest K^+/Na^+ observed was in the wild *B. oleracea*-S1 (C07060-S1), and (C07079A-S1) followed by a doubled haploid line (C10128-DH). This observation corroborates well with the observed Na^+/K^+ ratio in the initial salt shock screening, whereby *B. oleracea* genotypes show susceptibility to salt treatments. In addition, the treated *B. oleracea* were able to cushion the effects associated with excess salt by improving their selectivity of K^+ over Na^+ thereby reducing the Na^+ intake, which resulted in a high cellular K^+/Na^+ ratio 2 weeks post-treatment. Furthermore, some DH lines have shown higher cellular Na^+/K^+ as compared with the founder parent line DHLS150 (**Figure 5**). Due to the effects of salt shock stress on ion homeostasis, the K^+/Na^+ ratio was affected by increasing Na^+ influx against K^+ thereby reducing the Na^+/K^+ ratio 24 hr post-treatment (**Figure 5**), thus indicating susceptibility to osmotic shock as a result of high salt concentrations.

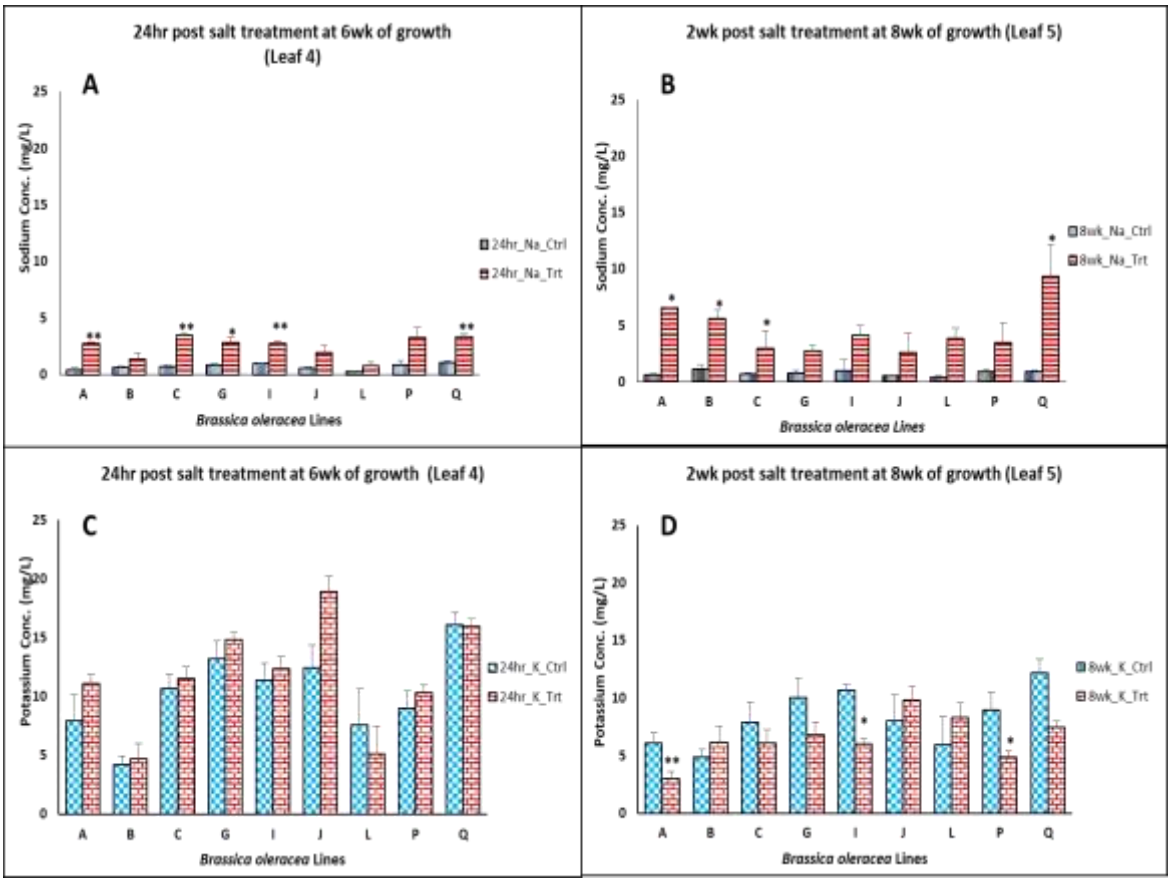

Figure 4: Changes induced by salt shock stress and physiological traits related to osmotic homeostasis in B. oleracea lines twenty-four hours and 2 week post-treatment.

Note: A = the main founder line (DHLS150), **B** = *B. bourgaei* (C07007-S1), **C** = C10025-DH; **G** = C13013-DH; **I** = C13001-DH; **J** = B. oleracea-S1 (C07060); **L** = C10128-DH; **P** = *B. oleracea-S1* (C07079A); and **Q** = C10121-DH. Data represents mean (n = 3) and error bars show the standard error of the mean (SEM). Significant different by comparing the mean of untreated control vs treated plants determine using **paired one-tailed** Students t-test as indicated by an asterisk (* = $p < 0.05$; ** = 0.001).

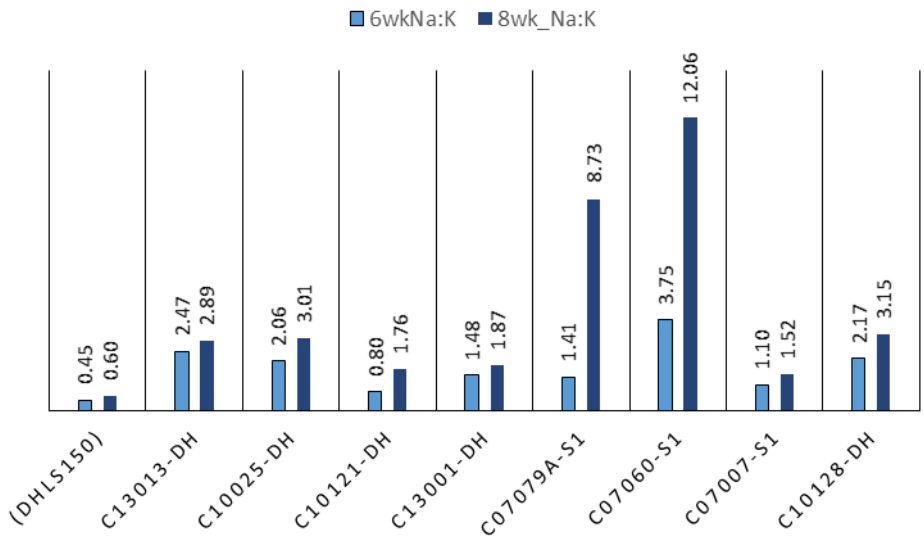

Figure 5: A Comparison of Na^+/K^+ between the wild *B. oleracea*, the founder DHLS150 and derived DH lines at six weeks (6wk) 24 hr post-treatment and eight weeks (8wk) 14 days post-treatment, respectively.

Expression of Salt Shock Stress Response Genes Involve in Na^+ and K^+ Homeostasis

To analyze the transcripts expressed in response to the sudden salt shock, the genes involved in the K^+, Na^+, and Ca^{2+} homeostasis were analyzed. A relative gene expression analysis was carried out on some selected genes: *V-CLC*, *ECA2* and *CAX3,* potassium uptake (*KUP11*), *KT9*, *NHX1* and *V-type-a1,* and *V-type-G* using leaves from the *B. oleracea* lines, 24 hr post-treatment and14 days (2 wk) post-treatment and the two relative gene expression were compared between the parent lines and DH lines. The gene for voltage-gated chloride channels (V-*CLC*) plays a significant role that actively regulates the movement of anions and thus preventing excess chloride from reaching toxic levels in the cytoplasm of the cell. The result shows that V-*CLC* was highly expressed 24 hr pt in the founder line *B. oleracea* (DHLS150), wild S1 *B. oleracea* lines; (C07060-S1), (C07079A-S1), so also in the two of the DH lines; C13013-DH and C13001-DH, respectively ($P < 0.05$) as presented in (**Figure 6 A-F)**. This may indicate the involvement of the *V-CLC* gene in salt shock stress response, especially the osmotic phase. Interestingly, the expression of the *V-CLC* gene by the DH lines may unveil similarities in the pathways involved in both parent and DH lines and show introgression of traits that respond physiologically the same as the parent lines. However, a significant reduction was observed in the relative expression of the *V-CLC* 14 days post-treatment in all the lines except for the C10121-DH genotype (**Figure 6 A-F**), which may suggest variation due to genetic differences between the lines.

The Ca^{2+}/H^+ exchanger (*CAX3*), which has also been shown to be involved in Na^+/H^+ shuttling between the cytoplasm was significantly expressed in the founder line DHLS150, wild *B. oleracea;* (C07060-S1) and (C07079A-S1) and C13013-DH line ($P<0.05$) which may indicate its involvement in Na^+ homeostasis in early-stage (osmotic phase) and may suggest its involvement in the early response mechanism in these accessions of B. oleracea to salt shock. It ensured the optimal cytoplasmic pH for normal enzymatic reactions in salt shock stress response (**Figure 6: A, B, E&F)**. The other two DH lines; (C10121-DH) and (C13001-DH) have shown lower expression of *CAX3*. This data corroborates with their physiological response in terms of Na^+ and Ca^{2+} homeostasis which indicates a higher Ca^{2+}/Na+ ratio 24 hr post-treatment. Furthermore, variation in *CAX3* expression between the parent lines and the DH lines especially the C10121-DH, and C13001-DH could be due to biological and physiological variations, which can further be linked to variations in recombinant alleles. The result also indicates an increase in the relative expression of *ECA2* (Ca^{2+}-ATPases) an endoplasmic reticulum-bound transporter as against the untreated control 24 hr post-treatment (24 hr pt) in all the *B. oleracea* genotypes with exception of C10121-DH, which showed lower expression of both *ECA2* and *CAX3* genes.

The relative expression of potassium transporter family (K^+/Na^+ *HKT*); *KT9, KT*, and potassium uptake (*KUP11*) have shown that the *KT9* was highly expressed in the wild S1 parent lines; (C07060-S1) and (C07079A-S1) 24 hr post-treatment in respect to the untreated control lines (**Figures 6**). Furthermore, *KUP11* also showed a similar expressional pattern in one of the DH lines C13001-DH, which suggests its involvement in the early salt shock stress response (osmotic phase) in these lines. The relative expression of *KT9 & KUP11* in the founder line DHLS150 and some DH lines was lower at both time-points, however, it signifies the involvement of these genes in osmotic adjustment and ensuring cytoplasmic K^+ is maintained, which corroborated well with the level of K^+ ion 2 weeks post-treatment. This result read well with physiological data where (C07060-S1) and (C07079A-S1) have shown an appreciable level of K^+/Na^+ ratio 14 ds post-treatment (**Figure 5)**.

The relative expression of cation exchanger of (*NHX1*) has shown a significant variation between the wild S1 parent lines; *B. oleracea*; (C07060-S1), (C07079A-S1) and the founder second parent line, DHLS150. The *NHX1* showed a lower relative expression 24 hr pt in the founder line DHLS150 genotype while the wild S1 lines, especially *B. oleracea* (C07060- S1) and *B. oleracea* (C07079A-S1) have shown a higher relative expression when compared to the untreated line (**Figures 6**). The doubled haploid genotypes; C13013-DH and C10121-DH both have similar expressions of *NHX1* similar to the parental lines. Fourteen days (14 ds) post-treatment (2 wk pt), the level of *NHX1* was reduced in all the *B. oleracea* genotypes. This may indicate similarity in the pathways involved in physiological under salt shock stress conditions. However, the C13001-DH genotype shows a unique expression different from the other DH genotypes of *B. oleracea* wild S1 extraction 24 hr post-treatment. This variation could be a result of differences in allelic recombination that may affect physiological responses. The variation due to relative expression of *NHX1* in some *B. oleracea* wild S1; (C07060-S1) & (C07079A-S1) and founder DHLS150 24 hr pt could suggest higher sodium in the growing medium and reduction in NHX1 14 ds post-treatment could suggest the plant's ability to exclude the excess Na^+ or compartmentalization in the vacuole of the cells (**Figures 6**).

The result showed non-significant expression of both *V-type-a1* and *V-type-G* in all the *B. oleracea* lines 24 hr post-treatment. But there is significant variation in the expressional pattern between the wild S1 lines and the doubled haploid genotypes (**Figures 6**).

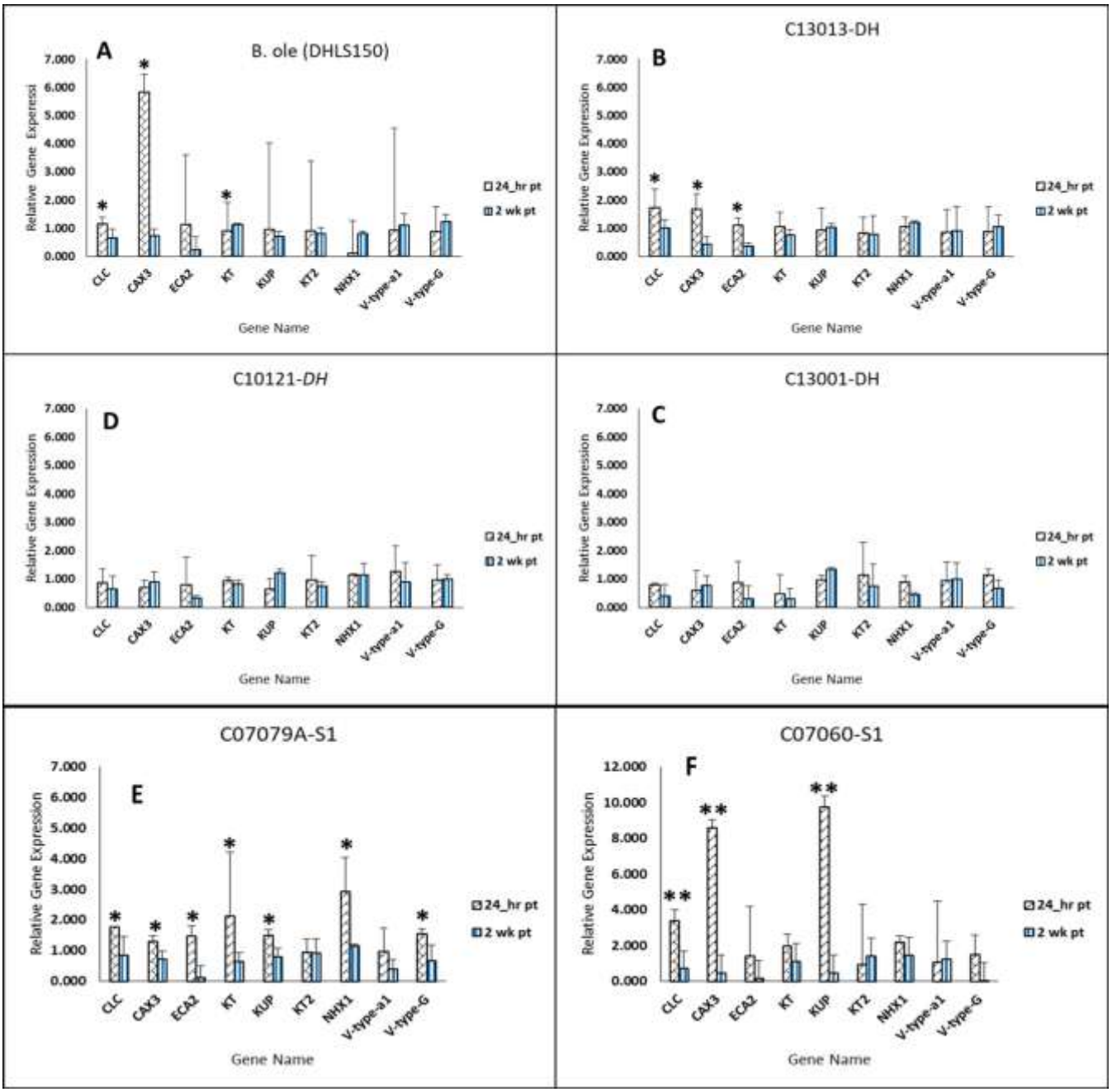

Figure 6: Comparison of relative Gene Expression level of K^+, Na^+ and Ca^{2+} transporter genes in *B. oleracea* under salt treatment.

Values represent means±SEM of normalized data with housekeeping gene β-Tubulin (n = 9). Twenty-four-hour post-treatment (24_hr pt) and 14 days post-treatment (2 wk pt). Asterisks indicate a significant difference between the two time points ($p < 0.05$ & 0.01).

DISCUSSION

Plants exposed to higher salt experience stress due to the accumulation of Na^+ and Cl^- ions, which cause membrane damage, nutrient imbalance, enzymatic inhibition, and metabolic dysfunction (Munns and Tester, 2008). The basic response of plants exposed to higher salt has been K^+ efflux from the cells caused by excess Na^+ (Nedjimi and Daoud, 2009). The huge Na^+ influx in the plant growth medium would create a plasma membrane depolarisation which further activates membrane-bound cation channels, the guard cell outward rectifying potassium channels (GORK), which stimulate Na^+ diffusion into the cell, and K^+ efflux thereby increasing Na^+ content (Blumwald et al., 2000; Demidchik et al., 2002). Salt-induced stress disrupts the K^+/Na^+ ratio and interferes with K^+ homeostasis (Siaei et al., 2012; Tuncturk et al., 2008).

From the results, it was observed that salt stress-induced causes an increase in leaf Na^+ content twenty-four hours post-treatment in all the *B. oleracea* lines. An increase in Na^+ leaf content following salt stress has been reported in many studies (Essah, 2002; Yasar et al., 2002; Kusvuran et al., 2007) and that of canola genotypes (Tuncturk et al., 2011). The level of potassium was affected two weeks post-treatment. Reduction in potassium level due to salt stress was widely reported e.g., studies by (Ashraf et al., 2008; Essah, 2002; Yasar et al., 2006; Li et al., 2006; Badeh-Hagh et al., 2008). Potassium plays a critical role in the neutralization reactions of anion and regulates cell membrane polarisation, osmoregulation, likewise being an important factor in the activity of some enzymes that are involved in many metabolic pathways (Very et al., 2014). The reduction of potassium level was attributed to the entry of the higher amount of Na^+ into plant roots cell by non-selective cation channels (NSCC) that cause K^+ efflux or leakage through guard cell outward rectifying potassium channel (GORK) and stellar K^+ outward rectifying channel (SKOR) (Rahman et al., 2017). Other potassium transporters that might be implicated are membrane-bound protein channels actively involved in the transportation of potassium like Shaker K^+ channel, High-affinity potassium (HAK), potassium uptake (KUP), potassium transporter (KT), and high-affinity potassium transporter (HKT) amongst others (Very et al., 2014; Wang and Wu, 2013; Shabala and Pottosin, 2014).

Higher Na^+ accumulation was observed two-week post-treatment leading to a reduction of K^+/Na^+ ratio, which might be a result of disruption of ion homeostasis. The Na^+ influx and K^+ efflux have been associated with increased ROS production that could lead to the activation of NSCC (Maathuis, 2009). A similar report has shown that higher Na^+ could lead to disruption of ion homeostasis under salt stress conditions (Tuncturk et al., 2008; Wu and Wang, 2012). From the results, the Ca^{2+} level has been relatively unaffected in all the *B. oleracea* genotypes despite higher Na^+ in the growth medium. Research has shown that the addition of exogenous calcium in salt medium promotes membrane stability, thus ameliorating salt toxicity by decreasing Na^+ influx through NSCC and indirectly inhibiting K^+ efflux through the GORK channel in plants (Nedjimi and Daoud, 2009; Shabala and Posttosin, 2014; Essah et al., 2003; Shabala et al., 2006). More so, exogenous calcium has been shown to cause a reduction in the uptake and transport of Na^+ and further prevent it from binding to the cell wall (Kurth et al., 1986; Rubio et al., 2003). Other functions of cellular and vacuolar calcium include blockage of the fast vacuole (FV) channel in a voltage-dependent and independent reaction preventing Na^+ from being leaked back into the vacuole and ultimately their transport into the cell (Albaladejo, 2017; Tikhonova et al., 1997). Improved Ca^{2+} level observed in *B. oleracea* genotypes might be attributed to the improved K^+ level observed thus, leading to a higher K^+/Na^+ ratio. Although an increase in Na^+ leaf content was observed, the *B. oleracea* lines were able to improve and retain their Ca^{2+} level. This could be a plus to the wild S1 and DH lines because optimum concentrations of Ca^{2+} have been shown to play a crucial role in both physiological processes of plants and increase plant resistance to abiotic stresses and a higher extracellular Ca^{2+}/Na^+ ratio causes a reduction in Na^+ influx (Rengel, 2006). In addition, Ca^{2+} participates in the regulatory mechanism thereby enabling the plants to adjust to adverse conditions; high temperature, cold injury, drought stress, and salt stress (Arora et al., 2000; Bowler and Fluhr, 2000; Mozafari et al., 2008; Joshi et al., 2012).

The voltage-gated dependant chloride channels were reported to be located in the thylakoids membrane in *Arabidopsis* which function in fine-tuning the proton motive force (PMF) and enable plants to adjust the variability of light during photosynthesis (Andrei et al., 2015). Under salt stress conditions, excess Cl^- anions tend to affect membrane polarisation and the electrochemical gradient thus, affecting the cytoplasmic pH. Most of the critical enzymes present in thylakoids are of photosynthetic importance, for example, ribulose -1, 5- bisphosphate carboxylase/oxygenase (Rubisco), an important regulatory enzyme in Calvin Cycle, which was reported to be affected by salinity stress (Bose et al., 2017). Our result shows that the relative expression of *V-CLC* was higher in some *B. oleracea* genotypes 24 hr pt and showed reduced expression 14 ds post-treatment. This could suggest its significance in safeguarding the chloroplasts from deleterious effects of excess Cl^- ion under salt stress but not necessarily reducing the excess cytoplasmic sodium, because these *B.*

oleracea genotypes have shown higher sodium leaf content 24 hr post-treatment (**Figure 4**).

High-affinity potassium transporter family are proteins that act both as Na^+/K^+ symporter and or uniporter. Studies have proven their presence in the plasma membrane in the different plant's cell membranes, *viz.* include; wheat, rice, and *Arabidopsis,* and their role in salinity tolerance (Waters et al., 2013). Analysis of our result has shown that *KT9* and *KUP11* were both expressed 24 hr post-treatment in some *B. oleracea* genotypes. Potassium transporter genes are of a three-member family in plants, namely; Shakers K^+ channel, *HAK* (High-Affinity K)*/KUP* (K Uptake)/*KT* (K Transporter), and *HKT* (High- affinity K Transporter) and are active at the plasma membrane (Wang and Wu, 2013; Shabala and Pottosin, 2013; Very et al., 2014). Under salt stress conditions, plants struggle to maintain a certain level of potassium concentrations to counterbalance the effects of excess sodium to ensure their osmotic potential (Shabala and Pottosin, 2014; Su et al., 2002). Potassium plays a critical role in enzymes activities as a cofactor and in the regulation of water movement into the cell to maintain cell turgor. Positive expression of *KT* and *KUP11* in some *B. oleracea* genotypes can be a counter mechanism to reduce the effects of excess sodium, particularly in K^+-Na^+ homeostasis. Studies indicate that *AKT1* in *P. tenuiflora* (*PutAKT1*) was up-regulated under both excess/deficient potassium conditions and under salt conditions, which signifies its role in potassium homeostasis (Ardie et al., 2010). Also, its overexpression has been shown to improve salt tolerance in *Arabidopsis* through an increased K^+ uptake (Ardie et al., 2010). The expression of these genes by *B. oleracea* genotypes could be the reason for their improved Na^+/K^+ ratio two-week post-treatment (**Figure 5**).

Plants Ca^{2+} - ATPases are of two classes; IIA and IIB: i.e., Type IIA endoplasmic reticulum bound (ECA as ER-type Ca^{2+}-ATPase) and type IIB, which auto-inhibit Ca^{2+}- ATPase (ACA) (Baxter et al., 2010). The endoplasmic reticulum-bound *ECA2* isoform has shown lower relative expression following salt stress in all *B. oleracea* lines. Although no report was available to suggest the key role of *ECA2* in salt tolerance, some studies have suggested its possible involvement in potassium and calcium transport (Edelist et al., 2009). For example, using constitutive expression studies, genes related to *KT1, KT2,* and *ECA1* have shown to be associated with *SOS1* genes that regulate both potassium and calcium transport in the halophyte *H. paradoxus* (Edelist et al., 2009).

From our results, the level of relative expression of NHX1 a membrane ion transporter, and tonoplast-bound Na^+/H^+ exchangers has shown to be highly expressed in some B. oleracea genotypes that were shown to have high K^+/Na^+ ratios (**Figures 2 & 5**) and less expressed in others that showed a reduction in K^+/Na^+ ratio. Many studies have described the vacuolar NHX1 proteins as integral membrane antiporters that catalyze the exchange of cations across tonoplast membrane under the influence of electrochemical gradient generated by the activities of other vacuolar H^+-ATPases and other proton pumps (Blumwald et al., 2000; Yao et al., 2010; Xu et al., 2013). In Arabidopsis, different isoforms of Na^+/H^+ exchanger (NHX) i.e., NHX1-4 have been identified to involve in K^+/Na^+ exchange, for H^+ in the vacuole (Bassil et al., 2012; Barragán et al., 2012; McCubbin et al., 2014). Regulation of cytoplasmic pH is critical to plant cells under salinity stress, several studies involving E. coli, yeast, plants, and animal have suggested that NHX-type antiporters act mechanistically to leak protons out to fine-tune the luminal pH of specific intracellular compartments (Reguera et al., 2014). High expression of NHX1 and V-type-ATPases in B. oleracea genotypes 24 hr pt may be as a result of excess Na+ and other protons. This could lead to significant membrane depolarization thus causing transport of excess ions to the vacuole under an electrochemical gradient established by V-type-ATPases, i.e., V-type-a and V-type-G genes whose were shown to be highly expressed in some B. oleracea genotypes in response to salt stress. Another important role played by NHX1 genes, in addition to sequestration, have been associated with cytosolic K^+ uptake into the cell vacuole, as reported by studies of nhx1/nhx2 knockout (Barragán et al., 2012; Liu et al., 2013). These observations were further elaborated were it shown that overexpression of NXH1 genes in transgenic soybean led to a reduction of Na+ in the shoots and more of K^+ in both roots and shoots, suggesting its role in K+ homeostasis (Liu et al., 2013). Under salt stress conditions, an appreciable K^+/Na^+ ratio is critical to the plants' survival as established by studies using two amaranth species (Estrada et al., 2021). It was reported that transgenic rice expressed higher NHX1genes exhibits higher K^+ content in shoots under salt environment (Estrada et al., 2021). Importantly, some of our B. oleracea genotypes have shown high expression of NHX1 genes and improved K^+/Na^+ ratio two-week post-treatment (**Figure 4**).

CONCLUSION

In conclusion, the main mechanism used by *B. oleracea* to maintain osmotic homeostasis could be based on its ability to reduce excess Na^+ and improve K^+ uptake under salt shock stress. Furthermore, the high constitutive expression levels of genes involved in Na^+ and K^+ homeostasis could be a key point in the osmotic tolerance displayed by the wild S1 parent as against the founder DHLS150 cultivated line. The study constituted an important turning point and advances our knowledge on the doubled haploid (DH) lines that were first exposed to salt shock, the response indicates a level of introgression of traits and can be a milestone in the study of brassica vegetables. Finally, the DH lines could also be used further on research related to full salinity stress to unravel more efficient responses by the fact that they grow quickly and within a short time, not like the wild S1 parent lines.

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