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Relative Expression of a Salinity Stress-Responsive Na^+/H^+ Exchanger (NHX) in Root and Leaf Tissues of the African Leafy Vegetable, *Amaranthus dubius*

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ABSTRACT

Amaranthus dubius, an African leafy vegetable (ALV), is an easy-to-grow, annual shrub and a highly nutritious food source, containing elevated levels of essential nutrients in the leaves. Many ALVs, including *A. dubius,* can tolerate salinity stress, enabling their cultivation on marginal land. However, the widespread propagation of *A. dubius* as a stable food source has thus far not been realised due partially to the high frequency at which hybridisation occurs, resulting in high genotypic and phenotypic variability. Therefore, to increase the agricultural output capacity of this species on salt-affected marginal lands, it is important to screen, select and then clonally propagate the identified salinity-tolerant genotypes to ensure true-to-type fidelity in the regenerated population. It is also important, thereafter, to elucidate their underlying gene expression of the stress response. The present study exposed 4-week-old *A. dubius* seedlings to 100, 200 and 400 mM NaCl to determine their degree of salt tolerance. Genotypes were then screened, selected and clonally propagated through cuttings, based on high growth rates and biomass, and salt tolerance. Generally, growth and physiological parameters decreased as substrate salinity increased. However, individual salt-stressed genotypes demonstrated similar vigour to nonstressed plants and were able to maintain total protein and chlorophyll concentrations despite increasing salinity. The relative expression of an NHX1-like transcript was quantified in 15 genotypes using degenerately primed real-time qPCR. The relative expression of the putative NHX1 gene was 6.7 times greater in root tissues of seedlings treated with 400 mM NaCl (10.7 ± 1.8) compared to the roots of untreated seedlings (1.6 ± 1.3), and 2.8-fold more than leaf tissues harvested from seedlings treated with 400 mM NaCl. Furthermore, the relative electrical conductivity (EC) of root tissues was 10 times greater than the EC of leaf tissues from the same 400 mM NaCl treatment. Numerous genotypes yielded similar chlorophyll content between 200 and 400 mM NaCl treatments, with genotypes salinity-1 (S1) ($3.5 \pm 0.2\,\mu g/cm^2$) and S34 ($4.0 \pm 0.4\,\mu g/cm^2$) having the highest concentrations of chlorophyll in the 400 mM group, which was positively correlated with total protein content. Following micropropagation through direct organogenesis, selected clones maintained true-to-type traits such as similar chlorophyll, protein and NHX1-like expression as their parent plants when exposed to 400 mM NaCl. This study revealed that some genotypes demonstrated salt stress tolerance capabilities rivalling established halophytes by regulating the constitutive or inducible expression of an NHX1-like protein in roots and leaves. The correlation between protein content and NHX1-like expression was nonlinear and nonproportional, demonstrating the complexity of this response and necessitating further exploration of specific protein families or functional groups conferring salinity tolerance in this species.

1 | Introduction

Soil salinisation is an indirect but undeniable consequence of the global climate crisis (Bannari and Al-Ali 2020). Rising temperatures increase evaporation in arid and semi-arid regions, concentrating salts in the soil. Unpredictable precipitation patterns compound this issue by restricting salt leaching or displacing and depositing salts from deeper layers into the surface soil. Additionally, rising ocean levels facilitate saltwater incursion into coastal and low-lying areas, contaminating freshwater and marginal land. Salinity stress is characterised by excess Na^+ ions, which disrupt the fragile ionic and osmotic equilibria required for many vital biochemical pathways (Mahajan and Tuteja 2005; Tuteja, Gill, and Tuteja 2011). Elevated soil salinity (> 40 mM NaCl) severely decreases the agricultural output capacity of many conventionally cultivated staple crops, including maize (*Zea mays*) and rice (*Oryza sativa*), by impeding water and nutrient uptake, hindering germination, respiration and photosynthesis, culminating in wilted, stunted and diseased crops (Machado and Serralheiro 2017; Huang et al. 2019; Ben-Asher et al. 2021). Furthermore, salinity stress generates an electropositive potential across cellular membranes, manifesting as chlorosis and necrosis (Tuteja, Gill, and Tuteja 2011).

Some plants trigger a detoxification process in response to salinity stress, including upregulating the production of transmembrane antiporters such as Na^+/H^+ exchangers (NHX) (Yokoi et al. 2002). The NHX family of proteins utilise the inductive potential of protons to actively transport toxic Na^+ against the electrochemical gradient and away from sensitive intracellular components (Yokoi et al. 2002). Certain crucifers (*Brassica* spp.), cucurbits (*Cucumis* spp.) and mallows (*Gossypium* spp.) demonstrate moderate (40–200 mM NaCl) salinity stress tolerance by regulating ion homeostasis, particularly maintaining favourable intracellular K^+ and Na^+ levels, achieved through the selective uptake and active transport of these ions, as well as compartmentalisation of Na^+ in vacuoles (Kumar et al. 2009; Akrami and Arzani 2019; Sharif et al. 2019). Extremely salt-tolerant (200–2000 mM NaCl) halophytes, such as *Kochia sieversiana* and *K. prostrata*, utilise similar mechanisms of ion sequestration but to a greater extent than their salt-sensitive glycophytic relatives (Karimi et al. 2005; Yang et al. 2007; Rahman et al. 2021). Cytosolic enzymes isolated from halophytes are similarly intolerant to salinity compared to their glycophytic counterparts, underscoring the importance of Na^+ compartmentalisation across species (Greenway and Osmond 1972; Glenn, Brown, and Blumwald 1999; Flowers and Colmer 2008).

Salinity stress has been shown to significantly affect the chlorophyll content in many plants, including tomato (*Solanum lycopersicum*), pepper (*Capsicum annuum*), peanut (*Arachis hypogaea*), radish (*Raphanus sativus*) and beet (*Beta vulgaris*), culminating in reduced photosynthetic efficiency and impeding overall growth (Jamil et al. 2007; Cao et al. 2018; Xie et al. 2019; Abdelaal, Mazrou, and Hafez 2020). Excess soil salinity induces oxidative stress which disrupts chlorophyll synthesis and degrades chloroplast membranes, leading to a decrease in total chlorophyll concentration (Idder et al. 2019; Abdelaal, Mazrou, and Hafez 2020; Liu, Xu, et al. 2023). Subsequently, excessive light energy is absorbed, further damaging the photosynthetic apparatus and decreasing carbon assimilation (Wang et al. 2021). The reduction in chlorophyll content under salinity stress has also been linked to changes in photosynthetic and metabolic pathways, modulating gene expression related to photosynthetic capacity and carbon metabolism (Kamyab et al. 2016; Lima-Melo et al. 2016; Singh et al. 2020; Li, Chu, et al. 2022). Salinity stress therefore also significantly alters the expression of proteins, affecting total protein concentrations in plant tissues, which represents another indicator of adaptation to salinity stress (Suárez 2005; Hossain, Nouri, and Komatsu 2011; Cheng et al. 2015; Attia et al. 2021).

Reduced chlorophyll and protein content under salinity stress have been correlated with increased electrolyte leakage, indicating cellular membrane damage (Klemm et al. 2002; Shahid et al. 2015; Liu, Xu, et al. 2023). Therefore, electrical conductivity (EC) represents yet another parameter relevant to assessing the impact of salinity stress on plants, serving as a measure of ion leakage and compartmentalisation (Niu et al. 2017; Rao et al. 2019; Ehosioke et al. 2020). The quantification of these parameters in response to increasing levels of salinity stress provides valuable insights into plant physiology and biochemistry and has been used to identify and evaluate salinity-resistant wheat (*Triticum aestivum*) (Jabeen et al. 2022), sorghum (*Sorghum bicolor*) (Bavei et al. 2011), barley (*Hordeum vulgare*) (Mohammed, Morrison, and Baldwin 2021), cotton (*Gossypium hirsutum*) (Ramani et al. 2018) and thale cress (*Arabidopsis thaliana*) (Kalaji et al. 2016) varieties.

Some *Amaranthus* species, locally in South Africa and around the world, grow on marginal land and have demonstrated a high degree of resistance to salinity stress (Russell, Rathinasabapathi, and Hanson 1998; Wang et al. 1999; Omamt, Hammes, and Robbertse 2005; Delano-Frier et al. 2011). However, stress responses have not been genetically quantified in many indigenous amaranths, including the hardy annual shrub, *A. dubius*, which has demonstrated remarkable stress tolerance abilities and contains elevated levels of essential nutrients (Leung 1968; Ferrarotto 2003; Odhav et al. 2007; Muriuki, Sila, and Onyango 2014; Dhanya et al. 2017; Hoang et al. 2020; Akter et al. 2022; Moyo, Amoo, and Staden 2022). Identifying mechanisms underlying stress tolerance can facilitate selective breeding and crop improvement programmes, enabling the cultivation of crops resilient to climate change–induced stresses, fortifying food security. Furthermore, elucidating the unique adaptive strategies of naturalised species encourages their conservation for future transformative and sustainable agricultural strategies, aiding both scientific understanding and practical applications in agriculture and food systems.

The only known allotetraploid in the genus, *A. dubius*, emerged via interspecific hybridisation (Sauer 1967; Waselkov, Boleda, and Olsen 2018). Increased ploidy is correlated with significantly greater genotypic variability, facilitating hybridisation and contributing to a multitude of morphological and physiological adaptations that influence the capacity of this species to thrive in harsh environments (Omamt, Hammes, and Robbertse 2005; Khaing et al. 2013). Resilience in stressful environments combined with elevated nutritional contents reinforces the practicality of *A. dubius* as a crop that can alleviate malnutrition, improve resource-use efficiency and offer economic potential in impoverished and marginalised regions, directly addressing the

global climate and food security crises. Nevertheless, indigenous foods are underutilised due to the popularisation and commercialisation of excessively processed and more conveniently supplied 'Westernised' diets, which often rely on resource-intensive agricultural practices (Dweba and Mearns 2011). In *A. dubius*, intraspecific variability and interspecific hybridisability present obstacles to extensive cultivation due to inherently unpredictable field growth characteristics (Wulff 1988; Sindhu 2002; Stetter and Schmid 2017; Viljoen et al. 2018; Areington, Neto, and Watt 2022).

Identification and clonal propagation of superior *A. dubius* genotypes merges traditional knowledge with modern agricultural practices, promoting sustainable, consistent and economically viable food systems. This targeted approach minimises undesirable genetic variation, thereby enhancing consistency and uniformity for efficient scaling of genotypes with superior traits, such as high biomass and stress tolerance. Therefore, developing biotechnological techniques to quantify stress responses and screen for these superior genotypes is needed. A better understanding of the salinity tolerance strategies employed by *A. dubius* is necessary to improve and promote the widespread cultivation of this species in salt-affected regions. It is hypothesised that *A. dubius* specimens growing on marginal land express orthologous NHX proteins to aid salinity detoxification and Na^+ compartmentalisation. Additionally, quantifying the expression of NHX transcripts provides a genotypic basis for identifying salinity-tolerant cultivars.

Quantifying the relative expression of the gene of interest (GOI) in response to stress necessitates extracting RNA from tissues and performing subsequent quantitative and qualitative assessments on the extracts. Quantification of the extracted RNA is needed to ensure that equal amounts of RNA from each sample are used in downstream analyses, as variations can affect the accuracy of gene expression measurements (Kashima et al. 2020). Moreover, quality control is essential to assess the integrity and purity of the RNA extracts because degraded or contaminated RNA can also lead to inaccurate gene expression data (Carvalhais et al. 2013). These analyses provide a foundation for understanding the molecular basis of stress tolerance, identifying stress-responsive genes and developing strategies for enhancing stress resilience within the species of interest.

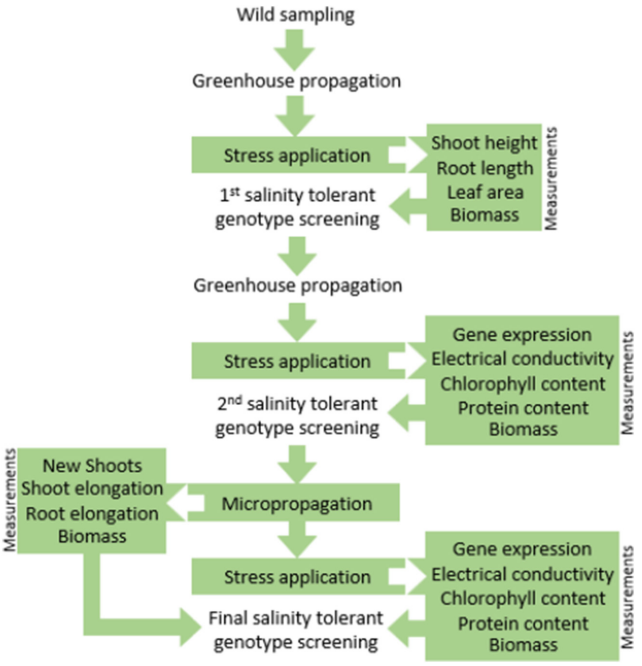
The genome of *A. dubius* has not been sequenced, posing an obstacle to quantifying putative NHX expression levels because there are no known sequences from which to create polymerase chain reaction (PCR) primers. Therefore, a method for developing degenerate primers by aligning multiple known amino acid sequences of highly conserved NHX proteins from other well-studied members of the same class (eudicots) as *A. dubius* is required. This study investigated the ability of *A. dubius* to tolerate salinity stress by quantifying the underlying genetic expression and regulation of this stress response in root and leaf tissues to screen for salinity-tolerant genotypes. The aim was to develop RNA extraction and real-time quantitative polymerase chain reaction (RT-qPCR) protocols to quantify the expression of potential NHX antiporters synthesised by *A. dubius* in response to saline environmental conditions. Furthermore, micropropagation of tolerant genotypes through direct organogenesis was explored because this approach allows for year-round, rapid multiplication of contaminant-free, true-to-type clones.

2 | Materials and Methods

Scheme 1 summarises the experimental design for this study, outlining the sequence of growth, physiology and gene expression analyses and measurements used to screen and select salinity-tolerant genotypes of *A. dubius* from the wild population. There were three screening steps following salinity stress treatments wherein the best-performing genotypes were selected at each step for further experiments. The first two screening steps were carried out in a greenhouse, while the final screening occurred following micropropagation and subsequent greenhouse acclimatisation of the identified salinity-tolerant clonal genotypes.

2.1 | Plant Material

Over 200 seeds obtained from wild *A. dubius* populations in Desainagar, South Africa (29°36′47.1″ S 31°09′08.8″ E), were individually sown into seedling tray inserts (37 mm width × 60 mm height) containing a 1:1 mixture of coco peat (Grovida, South Africa) and seedling mix (Grovida, South Africa). For species verification, a specimen was deposited to the Bews Herbarium (Pietermaritzburg, University of KwaZulu-Natal, accession: NU0094621). The trays were placed inside mist tents within the greenhouse at the School of Life Sciences (Westville campus, University of KwaZulu-Natal) and watered daily at 4 pm for 5 min with municipal water using an automatic system. Each insert of the seedling tray received an average of 15 mL of water per day. After 1 week, following cotyledon emergence, the seedlings were individually transferred to plastic flowerpots (90 mm diameter × 100 mm depth) with the same substrate as the seedling trays but with added vermiculite (10% v/v) (Grovida, South



SCHEME 1 | Flow diagram of the experimental design for this study.

Africa) to improve aeration, water retention and nutrient exchange (Rajkumar et al. 2017; Xu et al. 2021). Dr. Fisher's Classic Multifeed (AECI, South Africa) solution (1.3 g/L) was applied to this substrate once every 7 days at a volume of 10 mL per seedling.

2.2 | Salinity Stress Application

There were 50 individual seedlings (representing 50 genotypes from 50 seeds) arranged randomly for each of the four treatments: 0 (control), 100, 200 and 400 mM NaCl (Saarchem, South Africa). To mitigate salt shock, salinity stress was gradually applied in increments of 50 mM NaCl/day (50 mL) up to the required concentrations following seedling establishment, indicated by the emergence of the fifth or sixth true leaves, approximately 4 weeks post-germination. Thereafter, the pots were watered with 50 mL of each treatment solution at 9 am inside a mist tent for 5 days. Additionally, the seedlings were misted with municipal water for 5 min in the evening at a rate of ~50 mL per pot to prevent NaCl hyperaccumulation within the substrate. Salinity stress was induced only in the soil to mimic the real-world scenario of cultivation on marginal land afflicted by soil salinisation due to climate change and unsustainable irrigation. Applying salt to the substrate simulated the response of *A. dubius* under conditions reflective of the intended cultivation environment.

2.3 | Seedling Growth

Before starting the salinity stress treatments, baseline data were obtained for seedling height, measured using a 1000 mm stainless steel ruler (Tork Craft, South Africa). One week after continuous salinity stress application, seedling height, root length as well as foliar and root fresh masses (FM) were measured ($n = 50$). A 1-week experimental duration enabled efficient observations of the acute response of *A. dubius* seedlings to salt stress to assess the impact of salinity on growth, physiology and genetic expression. These responses may occur rapidly and reach a plateau after a certain period, justifying a week-long experimental duration to capture the critical timeframe during which significant changes in physiology and molecular regulation are expected to occur (Sottosanto, Saranga, and Blumwald 2007; Kollist et al. 2019). Moreover, a shorter experimental period might not capture the full spectrum of responses, and prolonged exposure to high levels of salinity can lead to seedling death, even in tolerant genotypes. Limiting the experimental duration to 1 week allows evaluation of the sublethal effects of salt stress, providing insights into the early stages of plant responses without compromising the viability of tolerant seedlings.

Total leaf area was determined by Version 4.0 of the generative pretrained transformer (GPT-4) architecture developed by OpenAI (USA), which was prompted to identify and calculate the area of leaves in an image against an internal reference scale, adapted from Minervini, Abdelsamea, and Tsaftaris (2014) and Hariadi et al. (2018). Sample images were converted into hue, saturation and value colour space components and then segmented to create a binary representation, highlighting green-coloured areas. Morphological operations such as erosion and dilation were applied to clean up the binary image and remove noise, facilitating the identification of contours to outline the boundaries of the green area. Finally, the area of all contours was summed in GPT-4 using the OpenCV function cv2.contourArea (Open Source Computer Vision, Russia) to obtain the total leaf area ($n = 50$).

2.4 | Genotype Tracking of Salinity-Tolerant Clones

A seedling vigour (V) index rating system (Equation 1) was developed to calculate the stress tolerance ability of salt-treated seedlings as a function of the untreated control.

Equation 1: Seedling vigour index (V):

$$V = \frac{a}{\overline{a}^c} + \frac{b}{\overline{b}^c} + \frac{c}{\overline{c}^c} + \frac{d}{\overline{d}^c} + \frac{e}{\overline{e}^c} \quad (1)$$

where a = height, b = root length, c = leaf area, d = foliar FM, e = root FM and $\overline{a}^c, \overline{b}^c, \overline{c}^c, \overline{d}^c, \overline{e}^c$ = mean values of corresponding measurements for untreated (control) seedlings.

This novel seedling growth index equation was necessary to combine multiple stress-affected growth parameters into a single rating (V) to comprehensively compare and identify the best-performing genotypes at the optimal harvestable age and condition for consumption—young and unblemished leaves and stems. Moreover, most stress tolerance and vigour rating indices incorporate a single parameter, typically foliar FM, and assume linearity between stressors and growth, inadequately quantifying and oversimplifying potentially nonlinear and complex stress responses (Ranal 2006; Castan, Gomes-Junior, and Marcos-Filho 2018).

Shoot cuttings (5–10 cm in length, diagonally cut below the node) from the 10 best-performing genotypes from each salinity stress treatment, identified by Equation (1), were individually maintained in 100-mL beakers filled with 80-mL distilled water supplemented with 1% indole-3-butyric acid (IBA) (PBR Trading International, South Africa) for approximately 8 days under ambient greenhouse conditions until adventitious root emergence and growth to at least 1 cm. Thereafter, whole plants (roots and shoots) were transferred to plastic flowerpots (120 mm diameter × 150 mm depth) containing seedling mix with added vermiculite (10% v/v) (Grovida, South Africa). These selected genotypes were then returned to the mist tents and watered daily with municipal water for 3 min in the evenings at a rate of ~80 mL per pot. Dr. Fisher's Classic Multifeed solution (1.3 g/L) was applied to the substrate once every 7 days at a volume of 15 mL per genotype. Clonal cuttings from the selected genotypes were allowed to regrow in mist tents for 4 weeks before 0 (control), 100, 200 and 400 mM NaCl treatments were reapplied over 1 week ($n = 3$).

2.5 | Electrical Conductivity

Following salinity treatments, soil EC values were generated by homogenising one part of the soil sample with five parts distilled water. The solution was then mechanically shaken (SPO-8 Platform Shaker, Labcon, South Africa) for 8 h at 100 rpm. To

obtain EC values, the leachate was collected via vacuum filtration and measured with a PocketPro+ conductivity meter (Hach Lange, Germany) (Rayment and Lyons 2010). The EC of leaf and root samples was similarly assessed—one part of the dried sample (apical leaf or root tissue) was homogenised in five parts of distilled water using a mortar and pestle, shaken for 8 h at 100 rpm and vacuum filtered before measuring. There were 17 genotypes ($n = 17$) represented in three technical replicates ($n = 3$).

2.6 | Chlorophyll Content

The chlorophyll content of a 1 cm^2 leaf disc excised from newly developed, unblemished leaves of each genotype was spectrophotometrically quantified at the conclusion of the second and third screening stages (Scheme 1) ($n = 3$). The leaf discs were briefly rinsed in distilled water, individually transferred into 2-mL microcentrifuge tubes containing 1.5-mL 99.9% chilled ethanol (Protea Lab Services, South Africa) and stored on ice in the dark for 24 h. The samples were then centrifuged (Eppendorf 5710R, Germany) for 5 min at 10,000 rpm and 4°C. The resultant supernatant was analysed by a UV-1800 spectrophotometer (Shimadzu, Japan) at wavelengths of 660 nm (chlorophyll-a) and 640 nm (chlorophyll-b). Thereafter, the total chlorophyll content was calculated (adapted from Ritchie 2008).

2.7 | Protein Content

The Bradford method of total protein quantification was used to assess the protein concentrations in roots and leaves of selected cutting and in vitro propagated genotypes (Bradford 1976). Homogenisation was achieved by grinding ~100 mg of tissue in liquid nitrogen (LN_2) (Afrox, South Africa), and proteins were extracted using TRIzol Reagent (Life Technologies, Netherlands) according to the manufacturer's specifications. Coomassie Brilliant Blue dye (Merck, Germany) was added to each sample ($n = 3$), resulting in a shift in the dye's absorption spectra, measured at 595 nm using a spectrophotometer (Shimadzu, Japan). A calibration curve was established using serial dilutions (12.5, 25, 50, 75, 100 and 150 μg/mL) of the protein standard, bovine serum albumin (BSA) (Merck, Germany). The unknown protein concentration of the samples was then determined using linear regression analysis of the measured absorbance values plotted against the standard curve.

2.8 | RNA Extraction, Quantification and Qualitative Control

Foliar tissue, amounting to three unblemished young leaves, and root tip tissue samples were randomly excised from three of the tallest and most vigorously elongating biological replicates from each stress treatment. The tissue samples were rinsed with distilled water for 10 s and blotted dry with autoclaved paper towel. The samples were sectioned, weighed and frozen in LN_2 and then immediately prepared for RNA extraction or stored at −80°C in an ultra-freezer (NuAire, USA).

Total RNA was isolated from foliar tissue for each salinity stress treatment using TRIzol Reagent (Life Technologies, Netherlands) according to the manufacturer's specifications. The RNA concentration was quantified using the RiboGreen protocol for the NanoDrop 3300 Fluorospectrometer (Thermo Fisher Scientific, USA). RNA quality was evaluated by running 5 μg of the extract through electrophoresis on 1% tris-acetate-ethylenediaminetetraacetic acid (TAE) agarose gel (Thermo Fisher Scientific, USA) supplemented with 1% NaOCl (Reckitt Benckiser, South Africa) for 30 min at 110 V (Aranda, LaJoie, and Jorcyk 2012). All RNA samples were aliquoted and stored at −80°C in an ultra-freezer until needed for RT-qPCR analysis.

2.9 | RT-qPCR Parameters

RT-qPCR analysis was completed using the GoTaq 2-Step RT-qPCR System (Promega, USA). The cDNA template was synthesised from 1 μg of total RNA as per the manufacturer's instructions. The qPCR final reaction volume of 20 μL was composed of 1 μL cDNA template (equivalent to 1 ng starting quantity of RNA), 10 μL of GoTaq qPCR Master Mix (2X), 1 μL of both primers (500 nM) (see Section 2.10) and 7 μL of nuclease-free water. No template controls (NTCs) were performed with 8 μL of nuclease-free water to account for the lack of cDNA, thus ensuring a constant final reaction volume.

The RT-qPCR thermocycling reactions were performed with three biological and two technical replicates of each salt stress treatment using the Eco Real-Time PCR System (Illumina, USA). All assays were carried out under the following conditions: one cycle of 95°C for 2 min, 40 cycles of 95°C for 15 s and 68°C for 1 min for the degenerate NHX1 primer pair (see Section 2.10) and 60°C for 1 min for the reference gene primer pairs. A melting curve was generated from 95°C to 65°C to verify amplicon specificity and identify erroneous hairpin and primer dimer formation. The size of the amplicon was estimated using gel electrophoresis (Armstrong and Schulz 2015).

2.10 | Primer Design

Degenerate NHX1 forward and reverse primers were developed using Base-By-Base with j-CODEHOP (Brodie et al. 2004; Tu et al. 2018) which identified degeneracies within aligned sequences and accounted for the inherent redundancy of amino acid and codon pairing. Multiple known NHX1 amino acid sequences expressed in salinity-tolerant eudicots obtained from the National Center for Biotechnology Information protein databases were aligned using a constraint-based multiple alignment tool (COBALT) (Papadopoulos and Agarwala 2007). DNA sequence outputs which adhered to all other generic primer design parameters, such as melting temperature, length, guanine–cytosine content and secondary structure formation, were selected for further analysis. The degenerate primers used for NHX1 quantification were as follows:

5′-ATGTCCCACTACACCTGGCAYAA-3′ (forward)

5′-GTCGGTCACGAACCTCCATTTC-3′ (reverse)

The reference gene used for RT-qPCR normalisation was malate dehydrogenase (MDH). Primers for this reference gene of an ancestral species were developed by González-Rodríguez et al. (2019) with the following sequences:

5′-TGCTCCCAACTGCAAGGTTC-3′ (forward)

5′-ACCAAGTGCCCTGTTGTGAT-3′ (reverse)

2.11 | RT-qPCR Analysis

LinRegPCR (Ruijter et al. 2009) was used to determine the fluorescence threshold and the mean RT-qPCR efficiency per amplicon. The generated quantitation cycle (Cq) values and RT-qPCR efficiencies (E) of the NHX GOI and reference gene (MDH) were used to calculate the relative quantification (RQ) for each salt stress treatment as described by Equation (2) (Pfaffl et al. 2004). These data were then expressed as relative fold changes per salt stress treatment. BestKeeper was used to assess the stability of the reference gene by calculating the standard deviation (SD) and coefficient of variation (CV%) (Pfaffl et al. 2004).

Equation 2: Pfaffl method of relative quantification:

$$\mathrm{RQ} = \frac{\left(E_{\mathrm{unknown}}\right)^{\Delta \mathrm{Cq}_{\mathrm{unknown}}}}{\left(E_{\mathrm{control}}\right)^{\Delta \mathrm{Cq}_{\mathrm{control}}}} \quad (2)$$

where $\Delta \mathrm{Cq}_{\mathrm{unknown}} = \mathrm{Cq}$ of NHX1 in control treatment—Cq of NHX1 in unknown treatment and $\Delta \mathrm{Cq}_{\mathrm{control}} = \mathrm{Cq}$ of MDH in control treatment—Cq of MDH in unknown treatment.

The relative quantification (RQ) of the GOI was calculated by determining the RT-qPCR efficiencies (E) and differences in quantitation cycles (ΔCq) between test samples (100, 200 and 400 mM NaCl) and the control (0 mM NaCl).

2.12 | In Vitro Propagation of Selected Genotypes

Nodal explants ($n = 6$) harvested from the cuttings of the selected genotypes of *A. dubius* were clonally propagated in vitro, using the protocol established for this species by Shaik, Dladla, and Watt (2022). The explants were decontaminated in 1% (v/v) sodium hypochlorite (NaOCl) with two drops of Tween 20 (Bayer, South Africa) for 10 min and then transferred to semi-solid shoot multiplication medium (half-strength Murashige and Skoog (MS) basal medium (Murashige and Skoog 1962), 30 g/L sucrose, 2 mg/L 6-benzylaminopurine (BAP) and 0.5 mg/L indole-3-acetic acid (IAA), pH 5.7) for 3 weeks and then subcultured onto shoot elongation medium (half-strength MS, 30 g/L sucrose, 0.1 mg/L BAP and 0.1 mg/L IAA, pH 5.7) for 2 weeks, before finally being transferred to rooting medium (half-strength MS, 30 g/L sucrose and 0.1 mg/L IAA, pH 5.7) for a further 2 weeks. All media were steam sterilised in an HL-341 autoclave (Gemmy Industrial Corporation, Taiwan) at 121°C and 15 psi for 20 min.

The number of emergent shoots from individual explants was recorded weekly throughout the shoot multiplication period. Thereafter, the shoot elongation and root proliferation rates were measured during growth on the respective medium. Acclimatisation of rooted plantlets was performed by potting in soil (Section 2.1) and covering with clear plastic bags for 2 weeks in a grow room. Thereafter, the plastic bags were removed and the pots were transferred to a greenhouse mist tent for 4 weeks (Shaik, Dladla, and Watt 2022), following which the yield of individual explants was recorded. Wild-type specimens which were not exposed to stress treatments were used as a control. Once the acclimatised clones grew to the equivalent vegetative stage (5–6 leaves) of their parents in the initial stress treatment assay, they were subjected to the same salinity stress treatments and subsequent processing, extraction and transcript quantification (Section 2.2).

2.13 | Data Analyses

Physiological growth, EC, protein concentration, chlorophyll content and gene expression data were analysed using the Statistical Package for the Social Sciences (SPSS) version 29.0 (IBM, USA). The datasets were assessed for normality (Shapiro–Wilk test) and metavariance (Levene's test) to satisfy the assumptions of the post hoc test. Significant interactions among and between treatments were identified by univariate or multivariate ANOVAs followed by Tukey post hoc testing. A probability of $p < 0.05$ was used as the threshold for statistical significance. A paired samples t-test was used to determine differences between pre- and post-micropropagation parameters.

Hierarchical cluster analysis using Ward's method with Euclidean distances as the metric for NHX1 activity and chlorophyll and protein content in root tissues of selected clones enabled grouping of genotypes with similar salinity stress responses. Principal component analysis (PCA) of these same parameters was used to reduce dimensionality and identify components that accounted for the most variance within each dataset (Mehraj and Shimasaki 2017).

FIGURE 1 | Representative 1-month-old genotypically unique *Amaranthus dubius* seedlings after undergoing salinity stress treatments for 1 week. (A) 0 (control), (B) 100, (C) 200 and (D) 400 mM NaCl.

3 | Results and Discussion

3.1 | Growth Characteristics Under Salinity Stress

The median initial height of all seedlings at the start of the salinity stress treatments was 8.5 ± 0.8 cm. Figure 1 depicts the representative range of genotypically unique seedling growth forms following exposure to salinity stress for each of the tested treatments for 1 week. Some seedlings from the 100, 200 and 400 mM NaCl treatment groups exhibited stunted growth with leaf and stem chlorosis (Figure 1B2,C2,D2). However, other specimens undergoing the same treatments showed vigorous growth (Figure 1B1,C1,D1), indicating genotype-specific variation. Seedlings from the untreated control group demonstrated typical *A. dubius* growth characteristics and appearance, that is, vibrantly green, simple, alternate and elliptic-ovate–shaped leaves (Figure 1A).

Table 1 lists the median ± SD growth characteristics of *A. dubius* seedlings following each salinity stress application. Generally, growth parameters decreased as NaCl concentrations rose, except for height in the 100 mM NaCl group (26.7 ± 4.6 cm) which was not significantly different compared to the untreated control (26.5 ± 4.45 cm). Additionally, in the 100 mM NaCl group, the number of leaves (17.5 ± 3.9) and foliar FM (52.9 ± 6.2 g) did not differ from the control (15.7 ± 3.5, 56.9 ± 4.4 g, respectively). Interestingly, the number of leaves on specimens from the 400 mM NaCl group (15.3 ± 3.9) was also similar to the untreated control and shared similarity with the 200 mM NaCl group (14.8 ± 3.8), demonstrating genotypic stability of this parameter. However, the leaves of many specimens treated with NaCl were smaller, exhibiting irregular, furled and rounded morphology (Figure 1B2,C2,D2). These observations were quantitatively reflected in the leaf area measurements of samples in the 100 (184.6 ± 10.8 cm^2), 200 (150.9 ± 11.4 cm^2) and 400 (119.8 ± 11.6 cm^2) mM NaCl groups in comparison to the untreated control (202.8 ± 8.5 cm^2), demonstrating significant decline with each increasing NaCl treatment.

Additional parameters sharing similarity among the salinity treatment groups included root length and root FM, which were similar between the 100 (50.7 ± 7.7 cm, 21.3 ± 5.7 g) and 200 (46.9 ± 6.5 cm, 19.1 ± 5.7 g), and 200 and 400 (45.1 ± 8.9 cm, 16.3 ± 7.9 g) mM NaCl treatments. Furthermore, the SDs of root-related parameters were relatively more stable than the SDs of other parameters, demonstrating limited variability across treatments and potentially indicating a degree of robustness and resilience, especially in conjunction with the observed similarities in root length and FM across salinity treatments. Moreover, foliar FM was similar between the 100 and 200 (48.2 ± 7.9 g) mm NaCl groups but demonstrated increasing SD as salinity increased. Similarly, the SD of other growth parameters increased as salinity increased (with the exception of the number of leaves), indicating variability of plant height, foliar FM and leaf area within the species in response to salinity stress, and further demonstrating the high degree of intraspecific variability reported in previous studies on *A. dubius* (Potluri and Persad 1998; Sindhu 2002; Celine et al. 2007; Arti, Kurrey, and Suranjna 2018; Nyonje et al. 2022; Areington, Neto, and Watt 2022; Shaik, Dladla, and Watt 2022; Amma and Rajalakshmi 2023).

TABLE 1 | Growth parameters of 1-month old *Amaranthus dubius* seedlings undergoing salinity stress treatments for 1 week.

Treatment (mM NaCl)	Survival (%)	Height (cm)	Root length (cm)	Number of leaves	Foliar FM (g)	Root FM (g)	Leaf area (cm^2)	Vigour (*V*)
0 (control)	90	$26.5^{a} \pm 4.4$	$58.7^{a} \pm 6.4$	$15.7^{ab} \pm 3.5$	$56.9^{a} \pm 4.4$	$28.4^{a} \pm 4.1$	$202.8^{a} \pm 8.5$	$5.0^{a} \pm 0.4$
100	82	$26.7^{a} \pm 4.6$	$50.7^{b} \pm 7.7$	$17.5^{a} \pm 3.9$	$52.9^{ab} \pm 6.2$	$21.3^{b} \pm 5.7$	$184.6^{b} \pm 10.8$	$4.5^{b} \pm 0.5$
200	72	$23.0^{b} \pm 5.7$	$46.9^{bc} \pm 6.5$	$14.8^{b} \pm 3.8$	$48.2^{b} \pm 7.9$	$19.1^{bc} \pm 5.7$	$150.9^{c} \pm 11.4$	$3.9^{c} \pm 0.6$
400	50	$19.4^{c} \pm 6.8$	$45.1^{c} \pm 8.9$	$15.3^{ab} \pm 3.9$	$39.0^{c} \pm 16.6$	$16.3^{c} \pm 7.9$	$119.8^{d} \pm 11.6$	$3.4^{d} \pm 0.9$

Note: Different letters indicate statistically significant differences for each parameter (ANOVA with Tukey post hoc test; $p < 0.05$), median ±SD, $n = 50$.
Abbreviation: FM, fresh mass.

The 10 most vigorously growing genotypes from each salinity treatment were determined by applying Equation (1) (summarised in Table 1) and selected for further assessment. The condition in salinity-stressed genotypes where $V \geq 5$ was observed in nine genotypes treated with 100 mM NaCl and two genotypes treated with 200 mM NaCl, signifying cumulatively greater than or equal performance to the untreated control and indicating the absence of stress-induced growth constraints. However, achieving such equivalence was rare due to the overall detrimental impact of salinity stress on growth, evidenced by the significant decreases in vigour as salinity concentrations increased. Therefore, the top-performing genotypes ($n = 10$) from each salinity stress treatment were selected for further analyses, allowing for a more focused evaluation of the most promising specimens, while also maintaining sample size and reducing ambiguity in the selection process.

Comparatively, the number of leaves, height and biomass of another amaranth, *A. tricolor*, significantly decreased when treated with 10,000 ppm (approximately 171 mM NaCl), demonstrating decreased protein production, cell size and division (Siswanti and Riesty 2021). Furthermore, water spinach (*Ipomoea aquatica*) genotypes undergoing 75 mM NaCl in vitro, exhibited stunted growth and decreased seedling height, yielding inconsistent biomass (Ibrahim, Abas, and Zahra 2019), emphasising the varied responses of different genotypes to salinity stress, which was also observed in this study. Studies have reported salinity tolerance and adaptability of spinach (*Spinacia oleracea*) (Ferreira et al. 2018). However, those results were obtained under low-to-moderate levels of salinity stress (< 120 mM NaCl). Moreover, the harmful effects on plant growth and yield from excess salinity are mainly caused by osmotic stress and ion toxicity, resulting in cellular dehydration and growth inhibition (Hauser and Horie 2010; Zhu et al. 2010; Mahdy and Fathi 2012; Ramadoss et al. 2013). Additionally, the overexpression of certain genes has been found to enhance salt tolerance, suggesting the potential for genetic engineering to improve growth parameters under salinity stress (Singla-Pareek, Reddy, and Sopory 2003).

The findings of the present study demonstrated that multiple growth parameters of different *A. dubius* genotypes are significantly affected by varying levels of salinity stress and can be used to preliminarily evaluate the salinity tolerance capabilities of this species to identify tolerant genotypes. However, repeated exposure to salinity stress and further investigations are needed to elucidate the physiological and molecular responses of the identified genotypes, supplementing these initial findings and further screening for salinity tolerance. Therefore, clonal cuttings from the initially identified genotypes were allowed to regrow in mist tents for 4 weeks before 0 (control), 100, 200 and 400 mM NaCl treatments were reapplied. Only 17 clonal genotypes survived re-exposure to the 400 mM NaCl treatment and were therefore labelled as salinity (S) tolerant (S1, S4, S6, S10, S11, S12, S13, S16, S17, S22, S23, S25, S28, S32, S33, S34 and S40). These genotypes were selected for further gene expression, EC, protein, chlorophyll and micropropagation analyses.

3.2 | Relative Gene Expression

The electrophoresis gels for RNA extracted from *A. dubius* roots (Figure 2) and leaves (Figure 3) depict two distinct bands per lane, representing 28S and 18S ribosomal RNA, which are indicative of pure and high-quality RNA samples with minimal degradation. The regulation of a putative Na^+/H^+ transmembrane exchanger (NHX) expressed in 15 of the 17 selected genotypes of *A. dubius* was quantified in response to increasing levels of soil salinity stress (0 (control), 100, 200 and 400 mM NaCl) and plotted in Figure 5. Furthermore, Figure 4 shows the electrophoresis gel of the resultant RT-qPCR amplicons, demonstrating a single band across all samples between 100 and 200 bp. This result is congruent with the degenerate primer locations (823–975 bp) on the aligned NHX1 sequences. The relative expression of the NHX1-like gene was normalised against the expression of the housekeeping gene-encoding MDH. BestKeeper inspection determined acceptable expression of this reference gene throughout all treatments (SD < 0.2, CV% < 7.0) (Pfaffl et al. 2004), and LinRegPCR analysis determined that all samples were

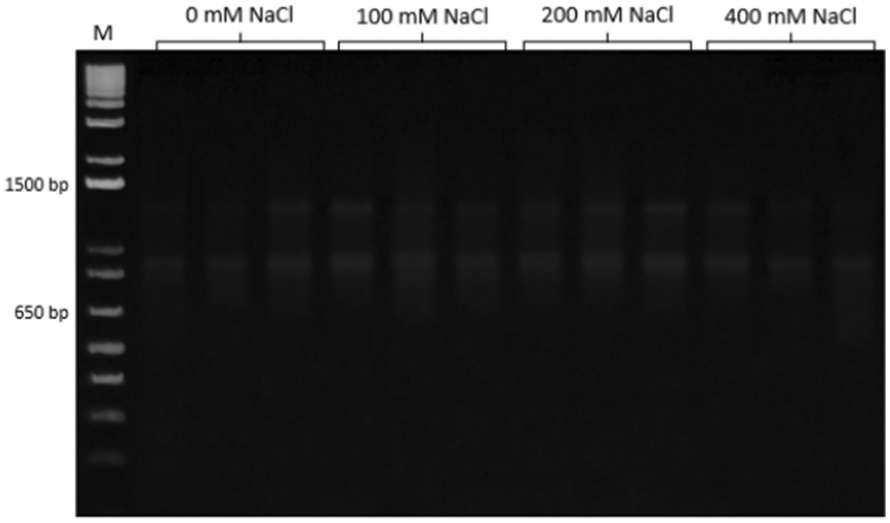


FIGURE 2 | Electrophoresis of 5 μg total RNA extracts from *Amaranthus dubius* roots exposed to salinity stress (0 (control), 100, 200 and 400 mM NaCl). *M* = 1 Kbp marker (Invitrogen, USA).

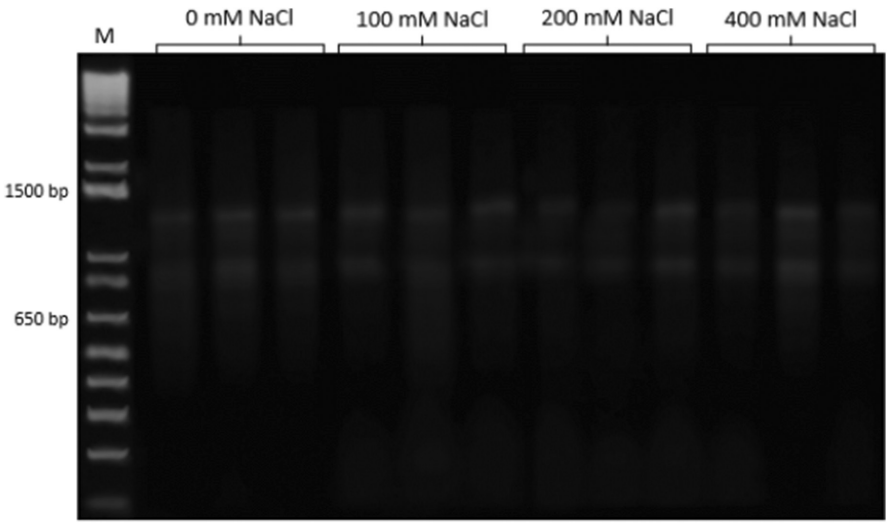


FIGURE 3 | Electrophoresis of 5 μg total RNA extracts from *Amaranthus dubius* leaves exposed to salinity stress (0 (control), 100, 200 and 400 mM NaCl). *M* = 1 Kbp marker (Invitrogen, USA).

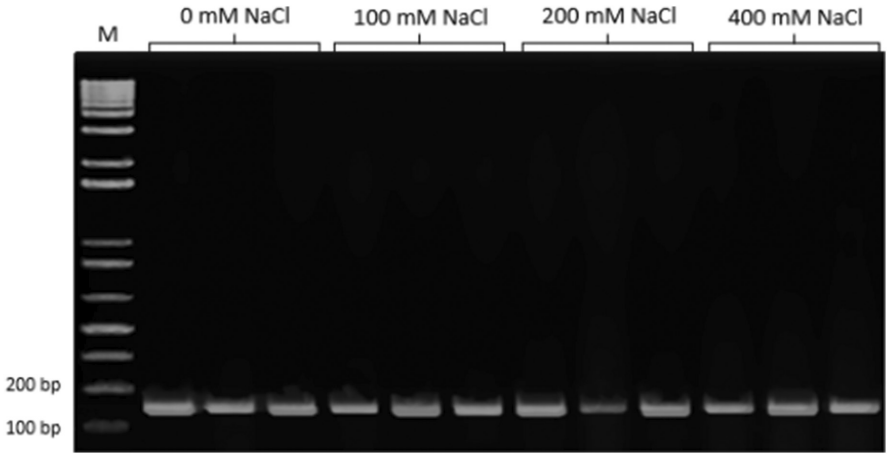


FIGURE 4 | Electrophoresis of 5 μL RT-qPCR product of salinity-stressed *Amaranthus dubius* specimens. *M* = 1 Kbp marker (Invitrogen, USA).

adequately efficient ($1.8 < E < 2.2$) (Ruijter et al. 2009). Finally, melt curve analysis confirmed no detectable hairpin or primer dimer constructions.

The general trend depicted in Figure 5 shows that putative NHX1 expression in leaf and root tissues increased as more NaCl was added to the substrate. However, leaf tissue from Genotypes S1, S11, S23, S25, S33 and S34 and root tissue from S16 exhibited increased GOI expression when treated with 100 or 200 mM NaCl but demonstrated significant decline when treated with 400 mM NaCl. Furthermore, Genotype S4 exhibited stable leaf and root expression of the GOI throughout the control and 100 mM NaCl groups, then, during the 200 and 400 mM NaCl treatments, demonstrated downregulated leaf expression while root expression was simultaneously upregulated. Untreated leaf tissue from Genotypes S1, S12, S17, S25, S32 and S34 yielded undetectable NHX1-like transcripts for RT-qPCR quantification. Similarly, untreated root tissue from Genotypes S6, S10, S17 and S33 did not exhibit sufficiently detectable expression. The observed lack of transcript expression in untreated samples followed by quantifiable expression in treated samples of the same genotypes was indicative of tissue-specific inducible expression of the NHX1-like gene in response to salinity stress (Mohamed 2019; Wan et al. 2019; Yang et al. 2020). Conversely, S4, S11, S16, S22, S23 and S28 demonstrated constitutive expression of the GOI in leaf and root tissues.

Overall, in leaf tissues, the median ± SD fold change of putative NHX1 transcripts compared to the untreated control (0.5 ± 0.1) was 4.5 ± 1.7, 9.7 ± 2.2 and 8.2 ± 2.3 in the 100, 200 and 400 mM NaCl treatments, respectively. In root tissues, GOI expression was 2.5 ± 1.6, 4.5 ± 2.7 and 6.5 ± 1.8 times more than the untreated control (1.6 ± 1.3) in the presence of 100, 200 and 400 mM NaCl, respectively. Notably, the relative expression of GOI transcripts in root tissues was significantly greater than in leaf tissues of genotypes exposed to 400 mM NaCl, with S11 demonstrating the highest (31.4-fold) and S32 yielding the lowest (1.3-fold) differences in expression between the tissue types among all samples. Genotypes S13 and S40 did not yield quantifiable NHX1-like transcripts in any treatment group and were omitted from Figure 5. However, these genotypes survived the highest tested level of salinity stress (400 mM NaCl), indicating that

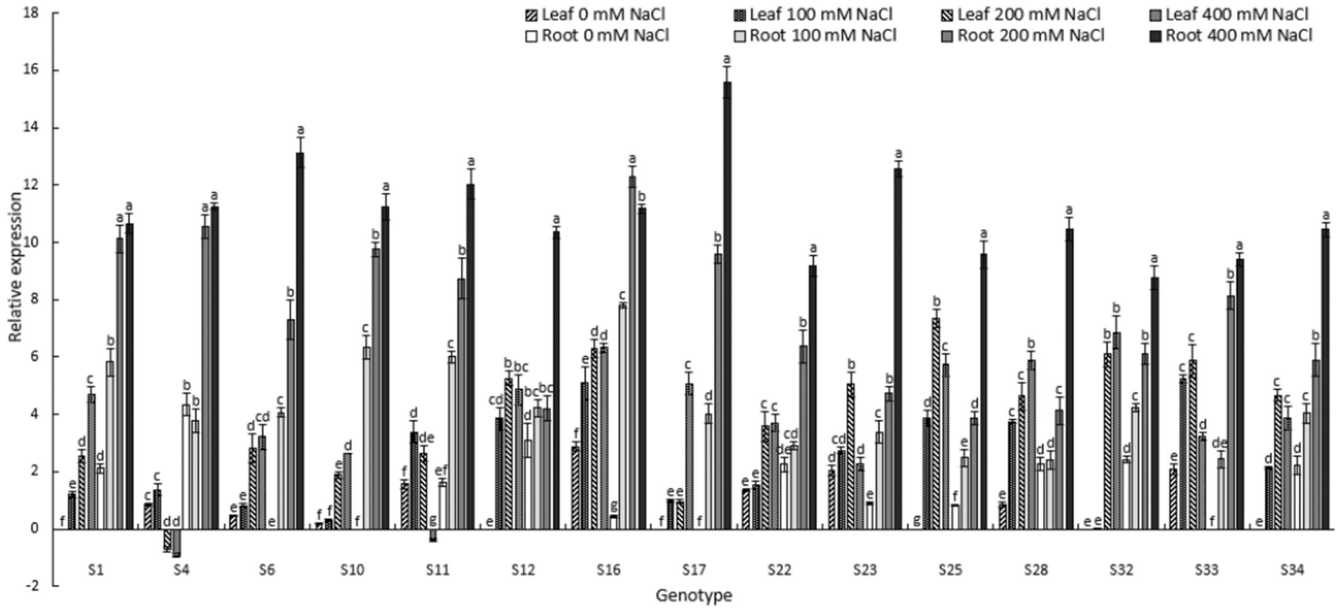


FIGURE 5 | The relative expression of an NHX1-like gene in leaf and root tissues of salinity (S)-tolerant *Amaranthus dubius* genotypes propagated through cuttings and exposed to 0 (control), 100, 200 and 400 mM NaCl. Different letters indicate statistically significant differences for a genotype (ANOVA with Tukey post hoc test; $p < 0.05$), mean ± SD, $n = 3$.

there are other, unknown mechanisms involved in the observed salinity tolerance of these genotypes, necessitating further research. Genotypes S13 and S40 may harbour a diverse set of genes or alleles that collectively contribute to salinity tolerance. Multifunctionality and redundancy in genetic pathways could compensate for the observed lack of NHX1-like transcripts. These genotypes may have alternative ion transporters or antiporters other than NHX1 to regulate ion homeostasis under high salinity conditions. Genotypes S13 and S40 might possess osmotic adjustment mechanisms, synthesising and accumulating compatible solutes, such as proline or glycine betaine, which are osmoprotectants, helping cells maintain turgor pressure and cellular integrity under saline conditions (Yang et al. 2017; Maqsood et al. 2020). Salinity stress often leads to the production of reactive oxygen species (Hasanuzzaman et al. 2021). Genotypes S13 and S40 may possess robust antioxidant defence mechanisms, including enzymes like superoxide dismutase or catalase, which protect against oxidative damage associated with salinity stress (Hasanuzzaman et al. 2021). Additionally, the root systems of these genotypes may exhibit specific morphological or architectural adaptations that enhance nutrient and water uptake efficiency under saline conditions, including changes in root length, branching patterns or the development of specialised root structures (Shelden and Munns 2023).

For the majority of tested *A. dubius* genotypes, the increased function of NHX1 in roots, sequestering excess Na^+ ions, was critical for salt tolerance. Under saline conditions, plants accumulate high levels of Na^+ ions, which can be toxic to plant cells. NHX1 helps to prevent the accumulation of excess Na^+ ions in the cytosol by transporting them into vacuoles. This not only helps to maintain a low cytosolic Na^+ concentration but also creates an osmotic gradient that helps to prevent water loss from the plant's cells. The biochemistry of salinity tolerance in *A. dubius* involves various mechanisms, including osmotic adjustment and antioxidant defence pathways (Hoang et al. 2020). The present study demonstrated the ability of *A. dubius* to regulate ion homeostasis by sequestering excess Na^+ ions through the action of the Na^+/H^+ antiporter NHX1.

Table 2 lists the relative expression of NHX1 transcripts in leaf and root tissues of a variety of plants exposed to salinity stress. Similar to the overall findings presented in Figure 5, osmotolerant rice (*O. sativa*) on basal salt medium supplemented with NaCl demonstrated greater NHX1 expression in roots than in leaves. Furthermore, Zhang et al. (2008) investigated the halophytic Indian walnut (*Aeluropus littoralis*) growing on perlite aerated with a culture solution containing NaCl (root application) and Moshaei et al. (2014) examined *A. littoralis* growing in sand irrigated with 500 mM NaCl (foliar application), both showing greater NHX1 expression in roots than in leaves—albeit to a lesser extent in the latter study. Contrastingly, rose gum (*Eucalyptus grandis*), silver grass (*Miscanthus sinesis*) and palisade grass (*Urochloa brizantha*) in hydroponic culture augmented with NaCl (root application), wheatgrass (*Agropyron elongatum*) in soil irrigated with a NaCl solution (foliar application) and transgenic *A. thaliana* in soil supplemented with NaCl (root application) showed greater NHX1 expression in leaves than in roots. These results demonstrated that the mode of application of NaCl does not necessarily influence tissue-specific NHX1 regulation.

The relative expression of NHX1-like transcripts in *A. dubius* leaves (3.9 ± 2.3) was comparable to the levels of NHX1 reported in leaves of wheat (*T. aestivum*) (3.5), *M. sinensis* (5.5) and transgenic tobacco (*Nicotiana tabacum*) (4.1), and the halophytes, goatgrass (*Aegilops tauschii*) (5.7) and nitre bush (*Nitraria sibirica*) (5.4). Additionally, relative root NHX1 expression in *A. littoralis* (8) and sprangletop (*Leptochloa fusca*) (8.5) were similar to the expression of putative NHX1 transcripts in *A. dubius* roots (10.7 ± 1.8). The selected *A. dubius* genotypes endured similar levels of salinity stress (400 mM

TABLE 2 | Relative expression of NHX1 transcripts in leaf and root tissues of various plant species compared to *Amaranthus dubius*.

Species	Common name	Age (week)	NaCl application (mM)	Tissue	Relative NHX1 expression	Reference
Amaranthus dubius	Sugarcane herb	6	400 (root)	Leaf	3.9 ± 2.3	Present study
				Root	10.7 ± 1.8	
Aegilops tauschii[b]	Goatgrass	6	150 (ND)	Leaf	5.7	Abbas et al. (2021)
Aeluropus littoralis[b]	Indian walnut	5	400 (root)	Leaf	2	Zhang et al. (2008)
				Root	8	
		7	400 (foliar)	Leaf	1.9	Moshaei et al. (2014)
				Root	2.3	
Agropyron elongatum	Wheatgrass	16	120 (foliar)	Leaf	16	Sheikh-Mohamadi et al. (2022)
				Root	5	
Arabidopsis thaliana[a]	Thale cress	5	200 (root)	Leaf	1.3	Huyen et al. (2022)
		2	50 (root)	Root	1.1	Liu, Wei, et al. (2023)
Carthamus tinctorius	Safflower	4	200 (foliar)	Leaf	12	Shaki, Maboud, and Niknam (2018)
Eucalyptus grandis	Rose gum	16	150 (root)	Leaf	9	García, Gamboa, and Krauskopf (2019)
				Root	5	
Glycine max	Soybean	2	170 (root)	ND	9	Sun et al. (2019)
Leptochloa fusca[b]	Sprangletop	6	500 (root)	Root	8.5	Panahi et al. (2013)
Miscanthus sinesis	Silver grass	4	300 (root)	Leaf	5.5	Sun et al. (2021)
				Root	1.6	
Nicotiana tabacum	Tobacco	8	300 (foliar)	Leaf	1.8	Li, Mu, et al. (2022)
Nicotiana tabacum[a]					4.1	
Nitraria sibirica[b]	Nitre bush	10	400 (root)	Leaf	5.4	Tang et al. (2021)
Oryza sativa IR28	Rice	2	150 (root)	Leaf	16	Nguyen et al. (2023)
				Root	16	
Oryza sativa DP				Leaf	19	
				Root	102	

(Continues)

TABLE 2 | (Continued)

Species	Common name	Age (week)	NaCl application (mM)	Tissue	Relative NHX1 expression	Reference
Triticum aestivum	Wheat	8	150 (foliar)	Leaf	3.5	Al-Ashkar et al. (2021)
Urochloa brizantha	Palisade grass	4	200 (root)	Leaf	17	Silva et al. (2021)
				Root	-5	

Abbreviation: ND, no data.
[a]Denotes transgenic species.
[b]Denotes halophytic species.

NaCl) as the typical halophytes *N. sibirica* (400 mM NaCl) and *A. littoralis* (400 mM NaCl). Moreover, salinity-tolerant *A. dubius* genotypes survived higher concentrations of NaCl than transgenic varieties of *A. thaliana* (200 mM NaCl), eggplant (*Solanum melongena*) (200 mM NaCl) and *N. tabacum* (300 mM NaCl). In comparison to the other food crops listed in Table 2, the selected *A. dubius* genotypes in the present study were tolerant to higher levels of salinity stress than the osmotolerant *O. sativa* (150 mM NaCl), *T. aestivum* (150 mM NaCl) and soybean (*Glycine max*) (170 mM NaCl).

The expression levels of NHX1 transcripts are compared in Table 2 between *A. dubius* and halophytes such as *N. sibirica* (Tang et al. 2021), *A. littoralis* (Zhang et al. 2008) and *L. fusca* (Panahi et al. 2013) undergoing salinity stress at concentrations of NaCl similar to those presently tested. While some halophytes can survive higher concentrations of NaCl, they offer limited nutritional value for human consumption compared to *A. dubius* (Petropoulos et al. 2018). Therefore, halophytes have primarily been investigated to produce transgenic varieties of popular crop species such as *Z. mays* and *N. tabacum*. However, transgenic constitutive overexpression of NHX1 has failed to yield crops that can tolerate similar levels of salinity stress to halophytic species or that demonstrated by *A. dubius* in the present study. Therefore, *A. dubius* presents an opportunity to diversify food sources, especially in salt-affected regions facing increasing environmental challenges due to climate change.

3.3 | Electrical Conductivity

The EC measurements for the selected clonal genotypes of *A. dubius* propagated via cuttings and exposed to 0, 100, 200 and 400 mM NaCl are depicted in Figure 6 ($n = 3$). At higher concentrations of salinity (> 200 mM NaCl), all leaf ECs were significantly lower than the EC values of the corresponding treatment in roots. Leaf and root ECs generally increased with each increasing salinity stress treatment. However, there was no significant increase in leaf EC between the 0 and 100 mM NaCl treatments of Genotypes S1, S4, S6, S10, S13, S16, S17, S22, S23, S32, S33, S34 and S40. Between 100 and 200 mM NaCl treatments, leaf ECs were similar in Genotypes S1, S4, S6, S10, S11, S12, S16, S17, S22, S23, S25, S32 and S40. Additionally, leaf EC did not significantly increase between 200 and 400 mM NaCl treatments for Genotypes S4, S6, S10, S11, S13, S22, S23, S28, S33 and S34. Notably, Genotypes S4 and S10 did not show a significant EC increase in leaf tissues throughout the 0, 100, 200 and 400 mM NaCl treatments, indicating adaptive mechanisms for ionic compartmentalisation away from leaf tissues to cope with increasing levels of salinity stress. Contrastingly, the root EC in Genotype S12 did not increase when treated with higher NaCl concentrations (> 200 mM NaCl), demonstrating a diminished ability to compartmentalise ions in root tissues, resulting in elevated leaf EC.

The measured EC values showed correlation with higher expression of NHX1 in roots, and thus, accumulation of Na^+. Practically, root accumulation of Na^+ is beneficial because only the leaves and young stems of *A. dubius* are consumed. This

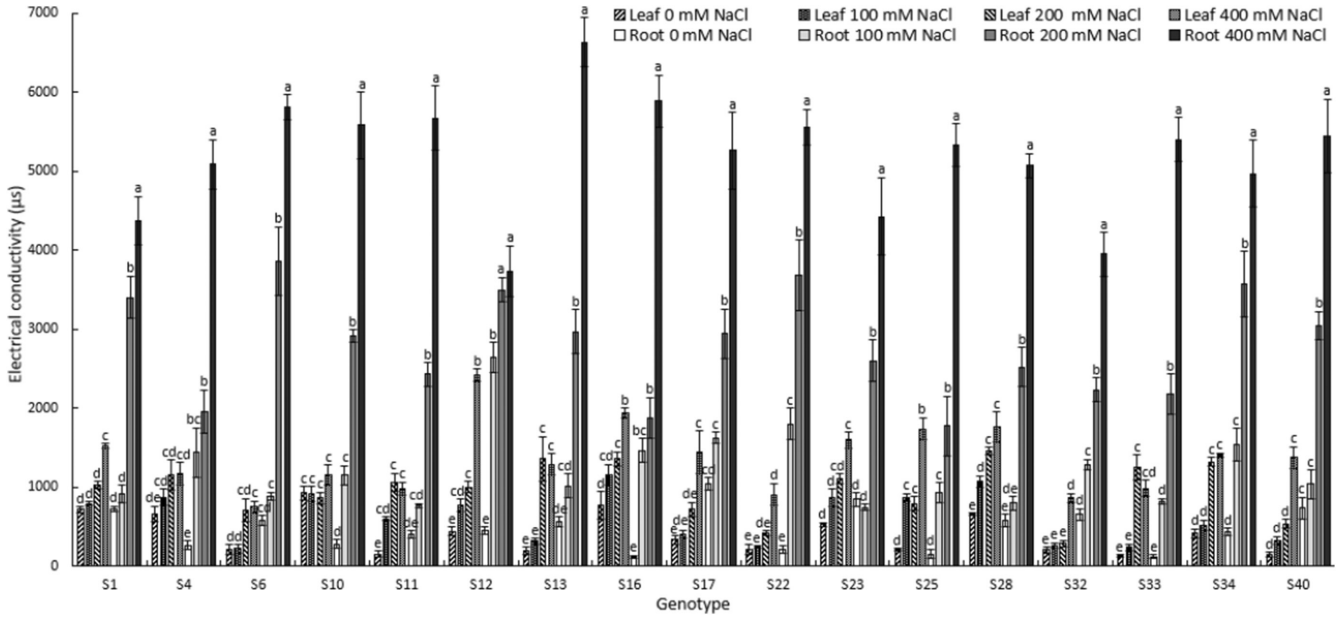


FIGURE 6 | The effect of salinity treatments on the electrical conductivity (EC) of soil, roots and leaves of *Amaranthus dubius* propagated through cuttings. Error bars denote ± SD from the mean ($n = 3$). Different letters indicate significant differences in EC values of leaf, root or soil samples across salinity stress treatments (0, 100, 200 or 400 mM NaCl). *Denotes similarity between 100 and 200 mM NaCl treatments and leaf tissue samples (ANOVA with Tukey post hoc test; $p < 0.05$).

physiological response helps maintain the quality and safety of the edible plant portions, as high salt concentrations in leaves can adversely affect taste, texture and nutritional content, rendering them less palatable and potentially harmful for consumption. Vacuolar transmembrane NHX1 antiporters actively transport Na^+ cations away from the cytosol using the electrochemical gradient generated by proton movement across the membrane (Apse et al. 1999). The compartmentalisation of toxic Na^+ from the sensitive cellular machinery in the cytosol ultimately confers salinity stress tolerance. Previous studies further support the present findings of NHX1 expression generally being higher in roots than in leaves, which is thought to be due to the greater need for salt tolerance in the roots (Yokoi et al. 2002; Mahajan and Tuteja 2005; Munns, James, and Läuchli 2012; Tuteja, Gill, and Tuteja 2011).

Because only Na^+ was added to the substrate, EC was considered to be a reliable proxy for Na^+ concentration in specific tissues (Ramani et al. 2018; Mohammed, Morrison, and Baldwin 2021; Jabeen et al. 2022). Higher EC values correlate with higher Na^+ concentrations and coupled with the NHX1 expression data, offer a comprehensive view of the physiological response to salinity. Na-CoroNa green labelling and alternative approaches such as the use of compartment-specific markers in conjunction with other fluorescent dyes or advanced imaging techniques like ion-specific probes coupled with confocal microscopy could be explored in future studies. Na-CoroNa green is a useful tool for visualising Na^+ within cells, but it does not provide absolute specificity for vacuolar Na^+. The fluorescence can sometimes be observed in other compartments, leading to potential misinterpretation of results (Park et al. 2009; Wu et al. 2015; Wu et al. 2018). Therefore, ensuring the accuracy and reliability of fluorescence labelling requires rigorous optimisation and validation.

3.4 | Chlorophyll Content

The total leaf chlorophyll content in *A. dubius* clonal genotypes propagated via cuttings under different NaCl treatments is visually represented in Figure 7. Generally, chlorophyll content decreased as salinity increased, with the exception of Genotype S32, which demonstrated similar chlorophyll content throughout the 0, 100 and 200 mM NaCl groups, and S34, which maintained similar chlorophyll throughout the 100, 200 and 400 mM NaCl treatments. Furthermore, Genotypes S4, S6, S10, S11, S13, S16, S28 and S33 yielded similar chlorophyll between the untreated control and 100 mM NaCl treatment groups. Moreover, Genotypes S1, S4, S6, S10, S12, S16, S23, S25, S33 and S40 yielded similar chlorophyll content between 200 and 400 mM NaCl treatments. In the 400 mM NaCl group, Genotypes S1 ($3.5 \pm 0.2\,\mu g/cm^2$) and S34 ($4.0 \pm 0.4\,\mu g/cm^2$) had the highest concentrations of chlorophyll.

In other studies, salinity-tolerant genotypes of various plants exhibited better growth performance and higher chlorophyll content than salinity-sensitive genotypes, indicating the importance of the NHX1 gene in quantifying salinity tolerance (Abbas et al. 2015; Su et al. 2019; Irshad et al. 2022). NHX1 expression has been shown to increase in many plants exposed to salinity stress (Table 2) and is associated with the maintenance of ion homeostasis to mitigate salinity stress (Barragán et al. 2012). Additionally, a reduction in chlorophyll content under salinity stress has been consistently observed, leading to a decline in photosynthetic activity (Gengmao et al. 2015; Khatar, Mohammadi, and Shekari 2017; Tufail et al. 2017; Ghorbani et al. 2018; Krishna 2018; Shaki, Maboud, and Niknam 2018; Sarkar et al. 2019; Uddin et al. 2019; Jabeen et al. 2022; Lu et al. 2022; Navyashree and Ashvathama 2022). Furthermore, the reduction in chlorophyll content has been linked to the

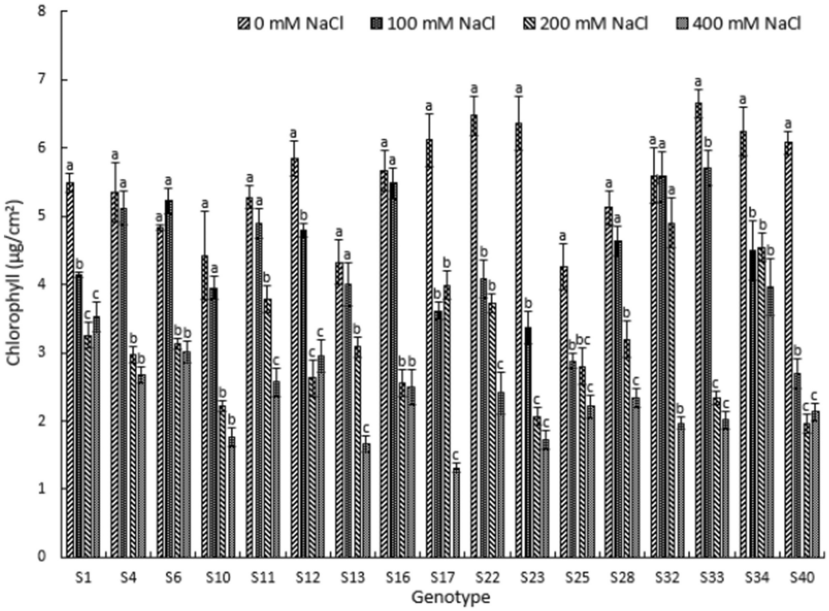


FIGURE 7 | Chlorophyll content in the leaves of selected genotypes of *Amaranthus dubius* propagated through cuttings and undergoing 0 (control), 100, 200 and 400 mM NaCl soil treatments. Different letters indicate statistically significant differences for a genotype (ANOVA with Tukey post hoc test; $p < 0.05$), mean ± SD, $n = 3$.

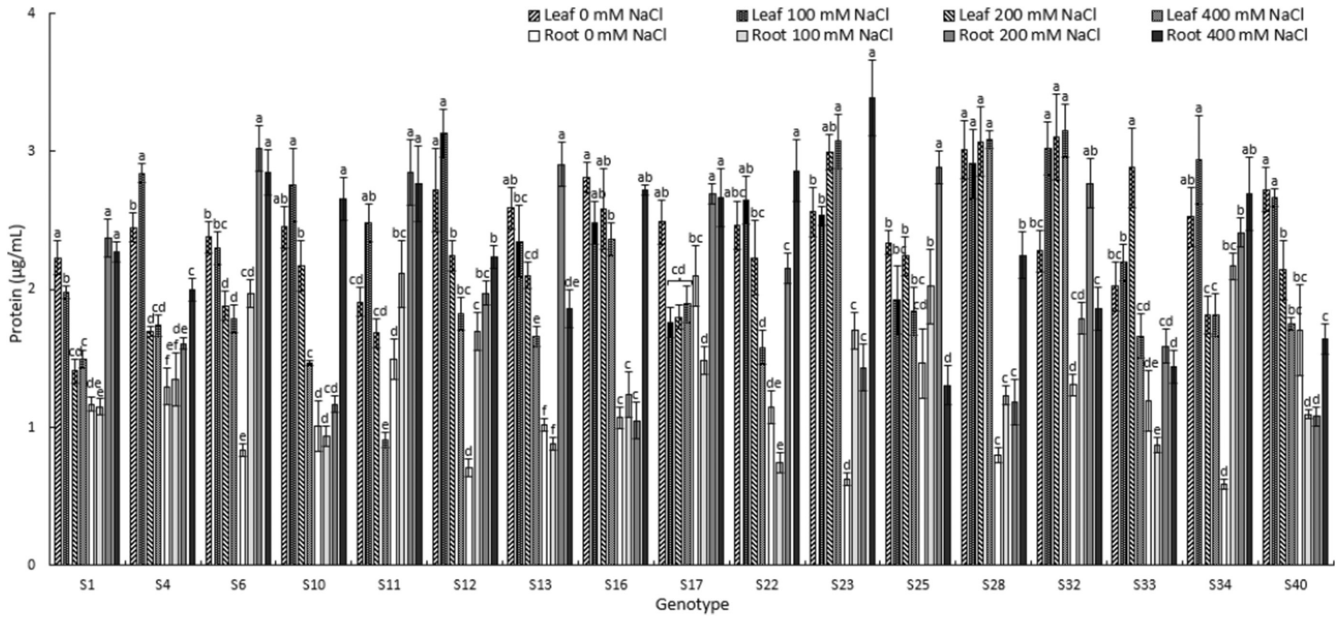


FIGURE 8 | Protein concentration of leaves and roots of salinity (S)-tolerant *Amaranthus dubius* genotypes propagated through cuttings and exposed to salinity stress (0 [control], 100, 200 and 400 mM NaCl). Different letters indicate statistically significant differences for a genotype (ANOVA with Tukey post hoc test; $p < 0.05$), mean ± SD, $n = 3$.

destabilisation of chlorophyll pigments and the pigment–protein complex under salinity stress (Uddin et al. 2019). These findings collectively suggest an inverse relationship between NHX1 expression and chlorophyll content under salinity stress, indicating that NHX1 expression increases while chlorophyll content decreases in response to salinity stress. This relationship can be considered a biological index to evaluate plant tolerance to stress for genotype screening and future breeding programmes (Ghorbani et al. 2018).

3.5 | Protein Content

The total protein content of selected *A. dubius* clonal genotypes propagated via cuttings is represented in Figure 8. Under the highest applied salinity treatment (400 mM NaCl), leaves from Genotypes S23, S28 and S32 contained the most proteins (3.1 ± 0.2, 3.1 ± 0.1 and 3.2 ± 0.2 µg/mL, respectively). Additionally, the roots from Genotype S23 contained the highest protein concentration (3.4 ± 0.3 µg/mL) when treated with 400 mM NaCl. The general

trend observed among most genotypes was a decrease in leaf proteins and an increase in root proteins as higher concentrations of NaCl were applied to the substrate, with the exception of S23, S25, S28, S32 and S33, wherein leaf protein concentrations were maintained or even surpassed root proteins at higher salinity stress treatments. Leaf protein of Genotypes S28 and S32 showed a positive correlation with increased leaf NHX1 expression. This result signified possible adaptive resource allocation mechanisms, prioritising preservation of photosynthetic capacity within the leaves of these genotypes.

These findings could be the result of accumulation of stress-responsive proteins, such as NHX1, transcription factors, antioxidants and osmoprotectants, mediating osmotic and ionic homeostasis. In genotypes demonstrating lower leaf protein compared to roots, the observed increase in root proteins was correlated with greater NHX1 expression in S4 and S11 only. For the remaining genotypes, there was no correlation between protein content and NHX1 expression. Genotypes S1, S4, S6, S10, S11, S12, S16, S17, S22, S23, S28 and S34 demonstrated a positive correlation between root protein and root NHX1 expression between 0 and 400 mM NaCl treatments. However, the only linear correlation between root protein and root NHX1 expression throughout all treatments was observed in S10, which was not proportional.

These results suggest that the relationship between total protein content and NHX1 expression is nonlinear and non-proportional, involving changes in specific protein families or functional groups which is not reflected in a proportional change in total protein content. These findings are congruent with similar studies examining NHX1 expression (Yokoi et al. 2002; Leidi et al. 2010; Ahmad et al. 2020; Cui et al. 2020; Long et al. 2020), wherein the correlation between NHX1 expression and protein content varied depending on the specific genotypes and cultivars, the severity and duration of the stress and the influence of various signalling pathways regulating gene expression and protein synthesis under salinity stress.

All tested genotypes demonstrated similar or increased root protein content as salinity stress was increased, indicating that these plants can adapt to salinity stress by enhancing or maintaining protein synthesis. In the EC and chlorophyll assays, Genotypes S1, S4, S6, S10, S11, S12, S13, S16, S23, S25, S28, S32, S33, S34 and S40 maintained each parameter throughout some, or all of the salinity stress treatments. The stability of EC levels in the leaves of tested genotypes suggests effective osmoregulation and ion homeostasis. Consistent chlorophyll levels suggest that these genotypes can withstand salinity-induced disruptions to the photosynthetic apparatus, maintaining the energy-capturing process necessary for plant growth. The ability to maintain essential physiological parameters under salinity stress indicates that these genotypes possess efficient stress response systems. Genotypes demonstrating robust salinity tolerance physiology have potential applications in agriculture, especially in areas affected by soil salinity.

3.6 | Cluster and Principal Component Analyses (PCA)

The dendrogram shown in Figure 9 obtained from the hierarchical cluster analysis revealed two major clusters. One cluster contained genotypes (S1, S4, S12, S22, S25, S28, S32, S33 and S34) that exhibited higher Na^+/H^+ exchanger (NHX) activity, leaf chlorophyll and root protein accumulation under salinity stress, indicating more robust salinity tolerance. The other cluster comprised genotypes (S6, S10, S11, S16, S17 and S23) with relatively lower NHX activity, chlorophyll and protein, denoting lesser salinity tolerance. PCA demonstrated a clear separation of selected genotypes along Components 1 and 2, which accounted for 53% and 34% of the total variance, respectively, indicating that these two components effectively summarised the data contained in the original variables. The variables contributing most significantly to this separation were chlorophyll and protein contents.

3.7 | Micropropagation

Multiple parameters and the mean agricultural output (foliar FM ± SD) of salinity-tolerant *A. dubius* clonal genotypes following micropropagation and acclimatisation are listed in Table 3 ($n = 10$). Overall, there was an average of 3.4 ± 0.6 new shoots per explant during the 3-week multiplication period, and the subsequent average shoot and root elongation rates were 2.8 ± 0.6 and 1.7 ± 0.3 cm/week, respectively. The number of new shoots per explant was higher in comparison to the micropropagation of *A. dubius* by Shaik, Dladla, and Watt (2022), wherein each explant yielded an average of two new shoots. This outcome is likely due to genotype-specific variability unintentionally selected for in earlier stages of the present study which identified stress-tolerant genotypes, indicating a correlation between stress tolerance and shoot production in vitro. Genotypes that experienced stress during the earlier screening stages may retain a 'stress memory', enabling a more efficient response during subsequent micropropagation, manifesting in increased shoot production (Ding, Fromm, and Avramova 2012; Sun et al. 2021).

Micropropagated clonal Genotypes S1, S4, S11, S13, S22, S25, S32 and S40 yielded the highest foliar FM (55.5 ± 5.2 g), which was similar to the foliar FM of untreated seedlings (56.9 ± 4.4 g) from the initial salinity stress assay and significantly higher than the control (39.8 ± 4.4 g) over a similar culture duration. Genotypes S6, S12, S13, S16, S22, S25, S33 and S40 produced the most shoots on the shoot multiplication medium. Genotypes S4, S10, S11, S12, S16, S22, S23, S32 and S34 demonstrated the fastest shoot elongation rates on the elongation medium. Genotypes S4, S11, S16, S22, S23, S28, S33 and S40 showed the fastest root elongation rates when transferred to the rooting medium. Overall, Genotypes S4, S11, S22 and S40 performed the best throughout micropropagation and subsequent acclimatisation, with S4 and S11 having faster shoot elongation than S40 and S40 generating more shoots than S4 and S11. Genotype S22 demonstrated the highest values for all measured parameters throughout micropropagation.

3.8 | Salinity Stress Tolerance Post-micropropagation

Compared in Table 4 are pre- and post-micropropagation physiological and genetic parameters (protein, chlorophyll and

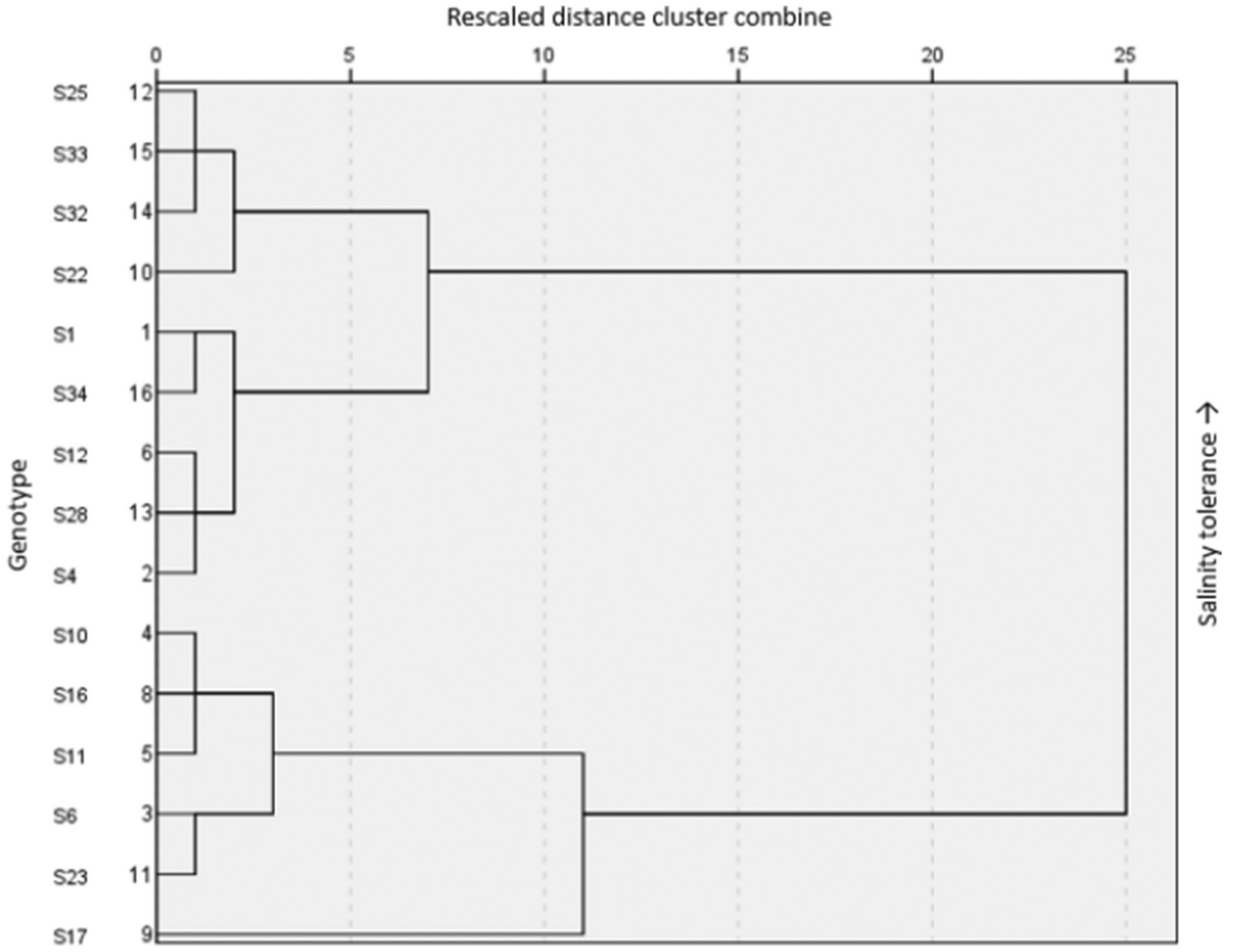


FIGURE 9 | Hierarchical cluster analysis dendrogram (Ward's method with Euclidean distance) of selected genotypes responses to NHX activity and chlorophyll and protein content.

NHX1 expression) of the identified salinity tolerant genotypes which also demonstrated superior growth throughout micropropagation (S4, S11, S22 and S40). Following acclimatisation, when 400 mM NaCl was reapplied to the substrate, RT-qPCR analysis revealed that the micropropagated clonal genotypes displayed putative NHX1 gene expression profiles similar to their parent genotypes, with the exception of S4, showing a marginal decrease in expression. However, none of the other compared parameters (protein and chlorophyll content) yielded significant differences ($p < 0.05$), indicating that micropropagation and subsequent acclimatisation did not have a negative effect on the ability of the selected genotypes to tolerate salinity stress. This result is indicative of true-to-type cloning which is a consequence of the direct organogenesis pathway of morphogenesis used by Shaik, Dladla, and Watt (2022).

The similarity in putative NHX1 gene expression profiles between micropropagated clonal genotypes and their parent genotypes suggests that the process of micropropagation has maintained genetic stability in terms of NHX1 gene expression. The decrease in NHX1 expression in S4 may be a specific response to cellular stress induced during micropropagation. The genotype might employ alternative stress response mechanisms that compensate for the reduced NHX1 expression. The regulatory networks governing NHX1 expression are complex and interconnected. Changes in other components of these networks during micropropagation could indirectly influence NHX1 expression in S4. Epigenetic modifications, such as DNA methylation or histone modifications, can influence gene expression without altering the underlying DNA sequence (Singroha et al. 2022), resulting in decreased NHX1 expression. Altered transcriptional regulation, possibly through changes in transcription factor activity or accessibility of regulatory elements, could also affect NHX1 expression by influencing the expression of transcriptional regulators (Lu et al. 2022). Additionally, post-transcriptional modifications, such as alternative splicing, could contribute to the observed decrease in NHX1 expression in Genotype S4. Furthermore, microRNAs (miRNAs) can post-transcriptionally regulate gene expression (Bartel 2009). Changes in miRNA levels during micropropagation could potentially target NHX1 transcripts, leading to reduced expression. The slight deviation in relative expression demonstrated by Genotype S4 indicates a subtle variation in this response to micropropagation or acclimatisation compared to the other genotypes, but ultimately did not impact the measured physiological parameters.

TABLE 3 | Growth measurements from the micropropagation of selected salinity (S)-tolerant *Amaranthus dubius* genotypes.

Genotype	Number of new shoots	Shoot elongation (cm/week)	Root elongation (cm/week)	Foliar FM (g)
S1	$3.3^{bcdef} \pm 0.4$	$2.1^{def} \pm 0.6$	$1.1^{h} \pm 0.2$	$50.3^{abc} \pm 3.4$
S4	$2.1^{fg} \pm 0.6$	$3.7^{ab} \pm 0.3$	$2.2^{ab} \pm 0.2$	$55.6^{a} \pm 4.9$
S6	$4.0^{abcd} \pm 0.3$	$1.9^{ef} \pm 0.8$	$1.7^{cdef} \pm 0.4$	$40.7^{d} \pm 3.2$
S10	$3.1^{cdefg} \pm 1.2$	$3.9^{a} \pm 0.4$	$1.1^{h} \pm 0.2$	$44.2^{bcd} \pm 5.3$
S11	$1.9^{g} \pm 0.6$	$3.6^{ab} \pm 0.6$	$2.3^{ab} \pm 0.3$	$56.2^{a} \pm 3.3$
S12	$4.2^{abc} \pm 0.6$	$3.6^{ab} \pm 0.6$	$1.4^{efgh} \pm 0.2$	$44.3^{bcd} \pm 2.0$
S13	$3.9^{abcde} \pm 0.6$	$2.8^{bcde} \pm 0.5$	$1.8^{bcdef} \pm 0.2$	$57.9^{a} \pm 7.2$
S16	$4.4^{ab} \pm 0.8$	$3.1^{abc} \pm 1.0$	$2.1^{abcd} \pm 0.3$	$43.5^{bcd} \pm 1.3$
S17	$2.8^{defg} \pm 0.4$	$1.5^{f} \pm 0.5$	$1.2^{gh} \pm 0.3$	$45.1^{bcd} \pm 13.3$
S22	$3.7^{abcde} \pm 0.5$	$3.0^{abcd} \pm 0.6$	$1.9^{abcde} \pm 0.1$	$57.7^{a} \pm 5.5$
S23	$2.9^{defg} \pm 0.5$	$3.5^{ab} \pm 0.4$	$2.3^{a} \pm 0.5$	$39.7^{d} \pm 5.8$
S25	$4.0^{abcd} \pm 0.5$	$1.8^{f} \pm 0.6$	$1.3^{fgh} \pm 0.5$	$55.5^{a} \pm 6.9$
S28	$3.3^{bcdef} \pm 0.7$	$2.2^{cdef} \pm 0.5$	$2.0^{abcd} \pm 0.3$	$45.4^{bcd} \pm 3.1$
S32	$2.7^{efg} \pm 0.7$	$3.3^{ab} \pm 0.5$	$1.1^{gh} \pm 0.4$	$52.1^{ab} \pm 3.6$
S33	$4.7^{a} \pm 0.9$	$1.8^{f} \pm 0.7$	$1.9^{abcd} \pm 0.4$	$41.9^{cd} \pm 5.6$
S34	$2.0^{g} \pm 0.7$	$3.3^{ab} \pm 0.7$	$1.6^{defg} \pm 0.4$	$38.0^{d} \pm 5.1$
S40	$4.5^{ab} \pm 0.7$	$1.9^{ef} \pm 0.8$	$2.2^{abc} \pm 0.3$	$58.9^{a} \pm 6.9$
Control	$2.5^{efg} \pm 0.9$	$1.4^{f} \pm 0.4$	$0.8^{h} \pm 0.3$	$39.8^{d} \pm 4.4$
Mean	3.4 ± 0.6	2.8 ± 0.6	1.7 ± 0.3	48.6 ± 5.1

Note: Different letters indicate statistically significant differences among genotypes (ANOVA with Tukey post hoc test; $p < 0.05$), mean ± SD, $n = 6$.
Abbreviation: FM, fresh mass.

TABLE 4 | Comparisons between pre- and post-micropropagation genetic (root NHX1-like relative expression [RE]) and physiological parameters (root protein and leaf chlorophyll content) of identified salinity-tolerant *Amaranthus dubius* genotypes re-exposed to 400 mM NaCl, mean ± SD (paired samples *t*-test, $n = 3$).

	Root NHX1-like (RE)		Root protein (μg/mL)		Chlorophyll (μg/cm²)	
Genotype	Before	After	Before	After	Before	After
S4	$11.3^{*} \pm 0.1$	$10.9^{*} \pm 0.2$	1.9 ± 0.1	2.0 ± 0.1	2.7 ± 0.1	2.5 ± 0.3
S11	12.0 ± 0.5	12.6 ± 0.5	2.8 ± 0.3	2.5 ± 0.3	2.6 ± 0.2	3.2 ± 0.5
S22	9.2 ± 0.4	9.1 ± 0.3	2.9 ± 0.2	2.4 ± 0.6	2.4 ± 0.3	2.0 ± 0.4
S40	10.4 ± 0.3	10.1 ± 0.1	1.6 ± 0.1	1.4 ± 0.3	2.1 ± 0.1	2.1 ± 0.1

$^{*}p < 0.05$.

The consistent levels of protein and chlorophyll content further underscore the robustness of the chosen genotypes in maintaining crucial biochemical components necessary for cellular functions and photosynthetic processes. This stability observed in the measured genetic and physiological parameters may be attributed to the inherent adaptability and stress tolerance mechanisms within the selected genotypes. In the context of screening for superior genotypes, these findings suggest that the selected genotypes maintain desirable traits conferring salinity tolerance and stress resilience during micropropagation. This stability could serve as a valuable criterion for identifying superior genotypes with consistent physiological performance, laying the foundation for their potential utilisation in breeding programmes or crop improvement strategies.

4 | Conclusion

The observed trends in NHX1 expression, ionic compartmentalisation, chlorophyll maintenance and protein fluctuations

collectively contribute to a more holistic understanding of how different genotypes of *A. dubius* respond to salinity stress. The quantification of putative NHX1 expression in leaf and root tissues under varying NaCl concentrations revealed distinct patterns among different genotypes. Notably, tissue-specific constitutive or inducible NHX1 expression, which decreased in leaves and increased in root tissues, indicated adaptive mechanisms for the compartmentalisation of toxic ions away from sensitive leaf tissues. Additionally, the quantification of NHX1-like gene expression in leaf and root tissues served as a molecular marker, highlighting the differential regulation of this salinity-responsive gene among roots and leaves of different genotypes.

Interestingly, the observed nonlinear and nonproportional changes in protein content underscore the complexity of the molecular response to salinity stress, emphasising the need for a more detailed exploration of specific protein families or functional groups involved in salinity tolerance mechanisms of this species. Cumulatively, this study provided a robust foundation to screen for salinity-tolerant genotypes through many salinity-affected parameters. This integrated approach enhanced the understanding of the intricate interplay between genetic factors and adaptive mechanisms, facilitating the development of resilient crop varieties tailored to thrive in saline environments.

The selection and screening of 17 salinity-tolerant clonal *A. dubius* genotypes, with S4, S11, S22 and S40 emerging as promising candidates, demonstrating exceptional performance in the ionic, chlorophyll and protein content assays. These genotypes also demonstrated superior performance throughout micropropagation and subsequent acclimatisation. The selected genotypes could be suitable for cultivation on marginal lands affected by salinity, increasing crop productivity on otherwise underutilised lands, promoting sustainable agricultural practices.

These findings call for a better understanding of the physiology and genetics of *A. dubius* in response to salinity stress, establishing best practices to ensure that people benefit from this crop and providing a better understanding of the suitability and broader impact on food security of this species. Further investigations are needed to quantify the elemental composition in the leaves of the presently identified salinity-tolerant clonal genotypes to determine if the demonstrated salt tolerance ability is maintained while remaining palatable and nutritious. In roots, Na^+ vacuole-specific localisation can be confirmed using compartment-specific markers in conjunction with fluorescent dyes or advanced imaging techniques like ion-specific probes coupled with confocal microscopy. The sequence of the NHX1-like transcript is of interest to molecular biologists, providing a molecular basis for taxonomic classification, aiding in future phylogenetic studies, elucidating evolutionary relationships by identifying sequence variations among species or broader taxa and resulting in more robust and accurate reconstructions of evolutionary trees. Additionally, the maintenance of superior *A. dubius* clones in vitro represents an invaluable resource of disease-free stock for use in future practical and scientific explorations of this species. However, the scope of micropropagation extends beyond the laboratory, necessitating field trials to validate the performance of clones under natural environmental conditions, providing additional insights into their adaptability, productivity and interaction with biotic and abiotic factors in agricultural settings.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are openly available in Yabalena (https://yabelana.ukzn.ac.za/) at https://doi.org/10.25410/ukzn.25163633.v1.

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