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ORIGINAL ARTICLE

Understanding the role of root-related traits in salinity tolerance of quinoa accessions with contrasting epidermal bladder cell patterning

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Abstract

Main conclusion **To compensate for the lack of capacity for external salt storage in the epidermal bladder cells, quinoa plants employ tissue-tolerance traits, to confer salinity stress tolerance.**

Abstract Our previous studies indicated that sequestration of toxic Na^+ and Cl^- ions into epidermal bladder cells (EBCs) is an efficient mechanism conferring salinity tolerance in quinoa. However, some halophytes do not develop EBCs but still possess superior salinity tolerance. To elucidate the possible compensation mechanism(s) underlying superior salinity tolerance in the absence of the external salt storage capacity, we have selected four quinoa accessions with contrasting patterns of EBC development. Whole-plant physiological and electrophysiological characteristics were assessed after 2 days and 3 weeks of 400 mM NaCl stress. Both accessions with low EBC volume utilised Na^+ exclusion at the root level and could maintain low Na^+ concentration in leaves to compensate for the inability to sequester Na^+ load in EBC. These conclusions were further confirmed by electrophysiological experiments showing higher Na^+ efflux from roots of these varieties (measured by a non-invasive microelectrode MIFE technique) as compared to accessions with high EBC volume. Furthermore, accessions with low EBC volume had significantly higher K^+ concentration in their leaves upon long-term salinity exposures compared to plants with high EBC sequestration ability, suggesting that the ability to maintain high K^+ content in the leaf mesophyll was as another important compensation mechanism.

Keywords Quinoa · Salinity tolerance · Epidermal bladder cells · Compensation mechanism · Sodium · Potassium

Abbreviations

BSM	Basic salt media
EBCs	Epidermal bladder cells
GORK	Guard cell outward-rectifying K^+ channel
MIFE	Microelectrode ion flux estimation
NHX	$Na^+(K^+)/H^+$ exchanger
SKOR	Stelar K^+ outward rectifier
S(HBV)	Salt-sensitive accession with high bladder volume
S(LBV)	Salt-sensitive accession with low bladder volume
SOS1	Salt overly sensitive 1
T(HBV)	Salt-tolerant accession with high bladder volume
T(LBV)	Salt-tolerant accession with low bladder volume

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Introduction

Halophytes are considered as plants with a superior ability to use specialized mechanisms to survive under high-saline conditions (Shao et al. 2014). Many of them are able to compartmentalise toxic ions such as Na^+ and Cl^- in the

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specialized epidermal bladder cell (EBC). The latter trait enables halophytes to effectively take away excessive salt from the metabolically active tissues and transfer it into EBCs operating as salt dumps, therefore, contributing to overall salinity tolerance (Shabala et al. 2014). Our previous study showed that sequestration of toxic Na^+ ions into EBCs is an efficient mechanism contributing to salinity tolerance in quinoa, as compromising this ability by the mechanical removal of EBC resulted in a salt-sensitive phenotype (Kiani-Pouya et al. 2017). Also, our large-scale quinoa germplasm screening revealed a strong correlation between the bladder's storage capacity and salinity tolerance indicating that the larger bladders with higher densities had a positive role in salinity tolerance (Kiani-Pouya et al. 2019). The molecular identity of key transporters involved in accumulation of Na^+ and Cl^- in EBCs in quinoa has been recently revealed (Bohm et al. 2018) and characterized at the functional level.

However, at least 50% of all halophytes do not utilize glands or EBCs to optimize their tissue ion concentrations (Shabala 2013). For instance, *Suaeda* is representative of a group of very tolerant halophyte plants that are able to accommodate salt in the shoots without the need for salt compartmentalisation into external bladders (Flowers and Colmer 2008). Given that these plants still possess superior salinity tolerance capability suggests the existence of multiple mechanisms for salinity tolerance, allowing plants to compensate for the lack of EBC sequestration ability. These mechanisms are numerous (Bressan et al. 2001; Shabala and Mackay 2011; Barkla et al. 2012; Ozgur et al. 2013; Shabala et al. 2014), and it remains to be answered which of them plays a major role to compensate quinoa plants for the absence of external salt storage in EBCs, to deal with the salt stress.

In most cases, the lack of EBC is compensated by the pronounced succulency in plant shoots, allowing large amounts of salt to be stored in enlarged vacuoles in specialised storage tissues (Flowers and Colmer 2008; Zeng et al. 2018). In lay terms, these plants simply switched from external to internal salt storage. However, the succulency is typically found in halophytic dicots and often not observed in grass species (Flowers and Colmer 2008); it is also not pronounced in quinoa. This suggests that other compensation mechanisms (such as root traits) may also confer salinity tolerance in halophytes. What are their roles in quinoa?

In this work, we hypothesised that EBC sequestration is not the only mechanism behind the salinity tolerance in quinoa, and plants with low EBC volume (and, hence, compromised ability for the external Na^+ storage) should have compensation mechanism(s) to deal with excessive salt. Amongst possible candidates are efficient osmotic adjustment and osmoprotection, traits that maintain optimal ion homeostasis, and developmental and physiological control of stomatal operation (Flowers and Colmer 2008; Munns and Tester 2008; Shabala and Mackay 2011; Shabala et al. 2014). Of specific interest are mechanisms regulating Na^+ and K^+ transport and homeostasis (Schroeder et al. 2013).

Plants reduce the amount of accumulated Na^+ by its efficient exclusion at the root level. This exclusion is mediated by the plasma membrane-localized salt overly sensitive 1 (SOS1) transporter (Qiu et al. 2002). *SOS1* homologues *CqSOS1A* and *CqSOS1B* are present in quinoa (Maughan et al. 2009) and have a high level of similarity to *SOS1* homologs of other plant species. Upon salinity exposure, *CqSOS1* were up-regulated in both root and shoot tissues in quinoa (Ruiz-Carrasco et al. 2011). Another important trait conferring salinity tolerance is vacuolar Na^+ sequestration that allows plants to avoid cytosolic Na^+ toxicity (Munns et al. 2019). The current consensus is that this process is mediated by Na^+/H^+ antiporters encoded by *NHX* genes. Eight *NHX* isoforms are known to be present in Arabidopsis genome (Bassil and Blumwald 2014). Four of these (*AtNHX1–AtNHX4*) are located at tonoplast; two (*AtNHX7* and *AtNHX8*) at the plasma membrane; and two (*AtNHX5* and *AtNHX6*) are localised in endosomes. Initially described as Na^+/H^+ antiporters involved in vacuolar Na^+ sequestration (Apse et al. 1999), NHX exchangers were later shown to also have high affinity to K^+ (Jiang et al. 2010; Bassil et al. 2011). As a result, depending on their intracellular localisation NHX exchangers are capable to transport either K^+ or Na^+ into the vacuole or endosome in exchange for H^+ efflux to the cytosol (NHX1–6) and Na^+ efflux out of the cell in exchange for H^+ influx into the cell (plasma membrane-bound NHX7–8) (Bassil et al. 2012).

The other suitable traits may be related to regulation of K^+ transport and homeostasis. Halophytes like quinoa also rely heavily on the use of inorganic ions to maintain cell turgor (Shabala 2013). Potassium is the major cation present in the cytosol (with concentrations 100 mM and above) and thus is critical for this purpose. However, a massive K^+ leakage from the cytosol of root and leaf tissues occurs under salinity stress in all plants (Shabala et al. 2006) which leads to K^+ pool depletion and may trigger programmed cell death in plant tissues through activation of enzymes associated with protein catabolism (Demidchik et al. 2010). This leak is mediated by the GORK outward-rectifying K^+ efflux channel. Discovered first in the guard cells (hence, the name GORK for Guard Cell Outward-Rectifying K^+) (Hosy et al. 2003), this channel is also widely expressed in the root epidermis (Véry et al. 2014; Rubio et al. 2019) and is a main pathway of salt-stress-induced K^+ loss from both root and leaf cells (Shabala et al. 2016a, b; Wu et al. 2018). Salinity stress significantly depolarizes the plasma membrane making channel (e.g. AKT or KAT)-mediated K^+ uptake thermodynamically impossible (Shabala et al. 2016a, b). Thus, an ability to control expression levels and

activity of GORK channels may be critical for salinity tolerance and operate as a part of the compensation mechanism. Also important may be an ability to control long-distant K^+ transport from the root to the shoot. Xylem K^+ loading is mediated by SKOR (Stellar K^+ Outward-Rectifying) channels (Ishikawa et al. 2018). SKOR channels are present in the plasma membrane of stelar parenchyma and activate in a time-dependent manner at membrane potentials slightly positive of the Nernst potential of K^+ (Wegner and Raschke 1994; Gaymard et al. 1998). Thus, in this work, we hypothesised that in the absence of high external salt storage capacity for Na^+can be then compensated by a better K^+ retention in plant tissues and/or more efficient K^+ transport from roots to shoots.

In this study, four quinoa accessions were selected from our previous experiment that possessed different EBC patterning and contrasting salt tolerance. These accessions were used in a series of glasshouse and laboratory experiments to understand the mechanistic basis of compensatory mechanisms conferring salinity stress tolerance in genotypes lacking EBC sequestration capacity.

Materials and methods

Plant material and growth conditions

Two relatively salt-sensitive quinoa accessions (195 and Q30) and two salt-tolerant accessions (Q68 and Q21) were used in this study (Fig. 1). The origin and related information of employed accessions in this study are provided in Table 1. Throughout the text, these are abbreviated as

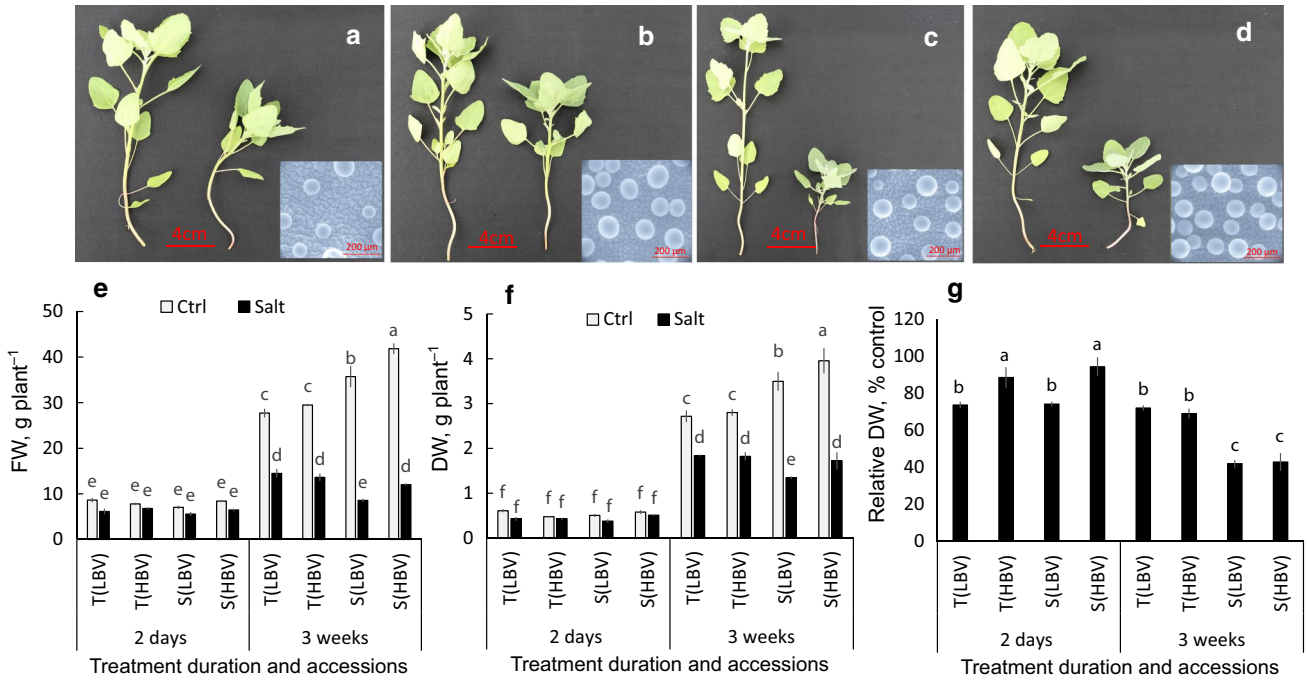

Fig. 1 Biomass and epidermal bladder cells (EBCs) patterning of selected accessions. Accessions used are: **a** accession Q21, salt-tolerant with low EBC volume; abbreviated as T(LBV); **b** accession Q68, salt-tolerant with high EBC volume, T(HBV); **c** accession Q30, salt-sensitive with low EBC volume, S(LBV); **d** accession 195, salt-sensitive with low EBC volume, S(HBV). The insets are scanning electron microscope images of leaf surface showing bladder density in each accession. **e–g** Fresh and dry weights of four quinoa accessions grown under control and saline (400 mM NaCl) conditions. Mean ± SE ($n = 5$). **e** Fresh weight (g plant^{-1}). **f** Dry weight (g plant^{-1}). **g** Relative weight (% control). Data labelled with different lower case letters are significantly different at $P < 0.05$. *FW* fresh weight, *DW* dry weight; *Ctrl* control

Table 1 The origin and related information of employed quinoa accessions

Accession code	Accession name	Country of origin	Donor/expedition
Q21	CICA	Peru-Puno	Universidad Nacional del Altiplano, Puno
Q30	Amarillo	Peru-Puno	Universidad Nacional del Altiplano, Puno
Q68	Negra pampa	Bolivia	Universidad Mayor de San Andres
195	CHEN 195	Peru	Braunschweig Genetic Resources Centre: 32277

S (for sensitive) and T (tolerant) and referred as HBV and LBV for high- and low-EBC bladder volume, respectively. Plants were grown from seeds in 20 cm diameter pots filled with a mixture of 70% sand and 30% perlite under temperature-controlled glasshouse conditions at the University of Tasmania in Hobart, Australia. Seeds were irrigated with a half-strength modified Hoagland's nutrient solution. The nutrient solution composition was as follows: KNO_3 (3 mM), Ca $(NO_3)_2$ $4H_2O$ (2.5 mM), KH_2PO_4 (0.17 mM), $MgSO_4.7H_2O$ (1.5 mM), Fe as sodium ferric diethylenetriamine pentaacetate (NaFeDTPA) (50 µM), H_3BO_3 (23 µM), $MnSO_4$ H_2O (5 µM), $ZnSO_4$ $7H_2O$ (0.4 µM), $CuSO_4$ $7H_2O$ (0.2 µM), and H_2MoO_4 (0.1 µM). After germination, the nutrient solutions were replaced with a full-strength modified Hoagland's solution. Day/night temperatures were 22 °C and 16 °C; the mean humidity 74% and a day length 16 h (incandescent lights were set at 6.00–9.00 and 16.00–22.00 h to give the day length h). Fifteen seeds were sown in each pot. Germinated seedlings were then thinned to leave four uniform plants per pot a few days before salinity treatment commenced. Experiment was organised in a completely randomised design, with each treatment including four replications. Seedlings were watered for 14 days with nutrient solution and the salt stress was then commenced at 15th day after sowing, with NaCl salt added to the irrigation water. Plants were watered twice daily, and salinity concentration was increased by 50 mM increments over 4 days to reach a final concentration of 400 mM NaCl. Plants then were maintained under salt stress for 3 weeks. Different physiological and electrophysiological parameters were assessed after short (2 days) and long term (3 weeks) of 400 mM NaCl stress.

Leaf sap Na^+ and K^+ concentration

To measure Na^+ and K^+ concentrations, the youngest fully expanded leaf was harvested at 2 days and 3 weeks after imposing to 400 mM NaCl. The harvested leaves were placed into Eppendorf tubes and immediately frozen. For ion content and osmolality determinations, the leaves were thawed and the sap then was extracted through squeezing the leaves. To remove debris, the extracted sap was centrifuged at 5000 g for 5 min. About 25–50 µL of the collected leaf sap was diluted with an appropriate volume of distilled water to measure K^+ and Na^+ contents using a flame photometer (Corning 410C, Essex, UK). Five replicates were used for each treatment.

MIFE non-invasive ion flux measurements

Net Na^+ and K^+ fluxes were measured using non-invasive ion-selective vibrating microelectrodes (the MIFE technique; University of Tasmania, Hobart, Australia). The principles of MIFE ion flux measurements and details of microelectrodes fabrication and calibration are fully described elsewhere (Shabala et al. 1997, 2006). In brief, borosilicate glass capillaries (GC150-10; Clark Electrochemical instruments, Pangbourne, Berks, UK) were pulled out using a vertical puller, then dried overnight at 225 °C, and silanized with tributilchlorosilane (Cat. no. 90796; Fluka, Busch, Switzerland). Electrodes were then back-filled using backfilling solutions (200 mM KCl for K^+ and 500 mM NaCl for Na^+) and tips of respective electrodes were front-filled with commercially available ionophore cocktails (Na^+ catalogue No. 71176 and K^+, catalogue No. 71176; both from Sigma-Aldrich, Castle Hill, NSW, Australia) and finally calibrated with respective standards. The electrodes that had a slope above 50 mV per decade with a correlation above 0.999 were used for measurements.

MIFE experimental protocols for Na^+ and K^+ flux measurements

In this work, we were interested in comparing SOS1-mediated root extrusion ability. The GUS staining experiments have suggested that *SOS1* transcripts are highly expressed in the root apex but not detected in the mature zone (Shi et al. 2002); this explains our choice of the elongation zone for Na^+ flux measurements. At the same time, correlation between root K^+ retention ability and salinity tolerance was reported for the mature (Chen et al 2005, 2007) but not elongation (Wu et al. 2018) zones. In a light of this, our K^+ flux measurements were conducted in the mature root zone.

For K^+ flux measurement, seeds of quinoa accessions were surface sterilized with 10% of commercial bleach (White King, Vic, Australia) for 10 min and then rinsed thoroughly with deionized water for 30 min. Seeds were sown on the filter paper in 90-mm Petri dish and grown for 3 days in an incubator at 24 °C. The germinated seedlings were then immobilized in a Petri dish and pre-conditioned in a Basic Salt Media solution (BSM: 0.1 mmol/L $CaCl_2$ and 0.5 mmol/L KCl) for 30 min. Steady-state K^+ fluxes were recorded for 5 min from the mature zone of the root (5 mm from the root tip). Then, treatment of 200 mM NaCl was administered and net fluxes of K^+ were measured for further 30 min.

To measure Na^+ efflux, plants were grown in a mixture of 70% sand and 30% perlite for 2 weeks with tap water and then continue growing under non-saline and 400 mM NaCl conditions for additional three weeks as described above. A so-called 'recovery protocol' (Cuin et al. 2011) was then used to measure the magnitude of Na^+/H^+ exchanger-mediated Na^+ efflux from the epidermal root tissue. An apical root segment was cut and thoroughly rinsed with 10 mM $CaCl_2$ solution, to remove apoplastic NaCl. The root segment was then transferred into a clean chamber containing

Na^+-free BSM solution (with/without 0.1 mM amiloride). Na^+ flux measurements were conducted in the elongation zone, between 250 and 500 µm from the root cap.

RNA extraction and RT-qPCR experiments

Harvesting plant samples for real-time qPCR was carried out at 3 weeks after imposing 400 NaCl salt stress. About 100 mg of fresh roots were harvested and used immediately for extraction. The total RNA was extracted from roots by grinding in a liquid nitrogen using Isolate II RNA Mini Kit (Bioline, NSW, Australia) according to the manufacturer's instructions. The first-strand cDNA was synthetised using QuantiTect Reverse Transcription Kit (Qiagen). Relative transcript levels of *CqSOS1*, *CqNHX1*, *CqSKOR*, *CqGORK* and *CqEF-1a* as a reference gene were determined using a real-time qPCR analysis by Qiagen Rotor-gene PCR system. The sequences and primers information of these genes were previously reported in studies with quinoa (Zou et al. 2017; Bohm et al. 2018). RT-qPCR experiments were as follows: 95 °C for 2 min, 95 °C for 10 s, 55 °C for 15 s and 72 °C for 20 s. Amplified gene products were detected using QuantiNova SYBR Green PCR Kit (Qiagen). The $2^{-\Delta\Delta Ct}$ method as described by Livak and Schmittgen (2001) was used to analyse the relative expression levels of the studied genes. The control gene (*CqEF-1a*) was used for normalization of the test gene transcript. Each data point consisted of three biological and two technical replicates and is shown as mean ± SE. Details on primers are presented in Suppl Table S1. The reported data are presented as relative values (e.g. transcript levels under saline conditions divided by the corresponding values under control conditions).

Statistical analysis

Data were analysed using IBM SPSS Statistics 24 software (IBM corp. Armonk, NY, USA). Unless stated otherwise, the presented data represent a mean of five replicates and are accompanied by the standard errors. A one-way analysis of variance and treatment mean separations was performed using Duncan's multiple range test at 5% level of significance.

Results

Biomass and EBCs characteristics of selected accessions

Four contrasting quinoa accessions (Fig. 1) were chosen for this study from a large-scale screening experiment (Kiani-Pouya et al. 2019). Accessions Q68 and Q21 with 68.9% and 71.9% relative DW under saline conditions were classified as salt-tolerant plants (T), representing HBV and LBV accessions, respectively. Accessions Q195 and Q30 with a relative DW of 41.9% and 42.8% respectively, were deemed as salt sensitive. They also had contrasting EBC volume (HBV and LBV, respectively; Fig. 1). The specific information on the bladder density and EBC diameters of studied accessions grown in the presence of 400 mM NaCl is given in Table 2. As one can see, the difference in the EBC volume between HBV and LBV varieties for salt-tolerant and salt-sensitive plants were 2.2- and 1.8-fold, respectively (Table 2). Based on calculations shown in Table 2, in HBV accessions, EBC comprised about one-third of the total aerial volume, representing a major potential sink for external Na^+ storage.

Leaf and root sap ionic analysis

Two days after imposing 400 mM salt stress, Na^+ contents in leaves and roots of both salt-sensitive and tolerant plants significantly increased compared to control plants; the numbers were even higher after 3 weeks of salt stress (Fig. 2a, b). In leaves, the highest Na^+ content was recorded in accession S(HBV) and there was not significant difference among other accessions (Fig. 2a). Consistent with this result, S(LBV) had Na^+ concentration as low as salt-tolerant plants (Fig. 2a). In roots, Na^+ concentration in T(LBV) was the

Table 2 Bladder-related information of four quinoa accessions grown under 400 mM NaCl conditions

Accession	Bladder density (cell mm^{-2})	Bladder diameter (µm)	Bladder volume (µl mm^{-2})	% of total aerial volume
Salt-tolerant				
T(LBV)	17.5 ± 1.3	111.5 ± 5.5	0.025 ± 0.001	17.5 ± 1.23
T(HBV)	29.8 ± 2.2	120.3 ± 2.7	0.055 ± 0.002	31.5 ± 2.45
Salt-sensitive				
S(HBV)	82.4 ± 8.6	84.6 ± 2.3	0.056 ± 0.002	31.8 ± 2.3
S(LBV)	58 ± 3.5	80.2 ± 3.3	0.031 ± 0.001	20 ± 1.91

For the sake of simplicity, it is assumed that EBCs are of a spherical shape and their density on the both leaf surfaces was equal. The thickness of leaf lamina was assumed being ~ 120 µm. The total aerial volume is the sum of leaf lamina and EBCs volumes. Mean ± SE ($n = 5$)

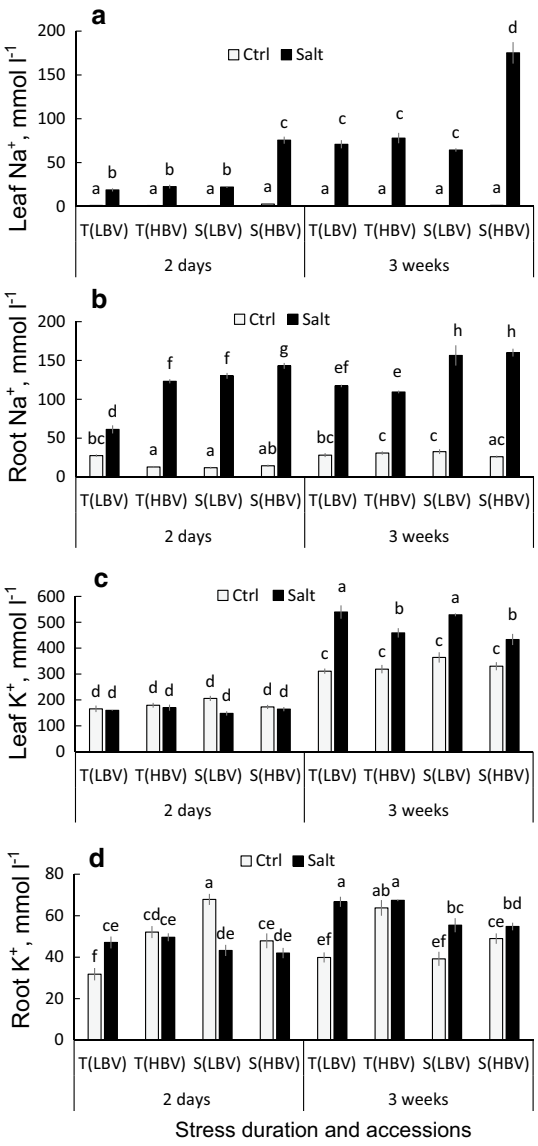

Fig. 2 Na^+ and K^+ concentrations (mmol/L) of leaf and root sap of four quinoa accessions grown under control and saline (400 mM NaCl) conditions. Mean ± SE ($n = 5$). **a** Leaf sap Na^+. **b** Root sap Na^+. **c** Leaf sap K^+. **d** Root sap K^+. Data labelled with different lower case letters are significantly different at $P < 0.05$

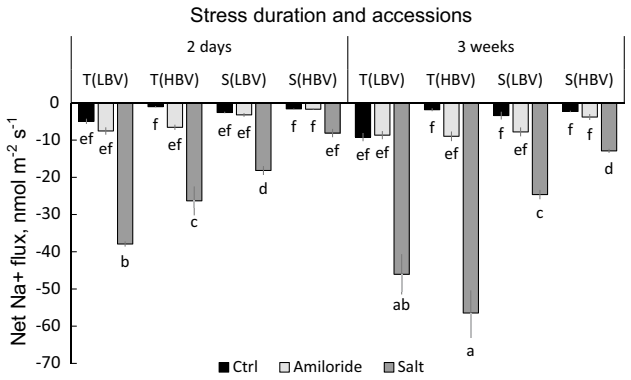

Fig. 3 Net Na^+ fluxes measured in 'recovery protocols' from the elongation zone (250–500 µm from root tip) of four quinoa accessions after pre-conditioning in Basic Salt Media solution (BSM: 0.1 mmol/L $CaCl_2$ and 0.5 mmol/L KCl) containing 100 µM amiloride for 20 min. Before measurements, plants were grown in a mixture of 70% sand and 30% perlite for 2 weeks with tap water and then continue growing under non-saline and 400 mM NaCl conditions for additional 3 weeks. Mean ± SE ($n = 6$–8). Prior to measurements, plant roots were gently removed from the pot media. An apical root segment was cut and thoroughly rinsed with 10 mM $CaCl_2$ solution, to remove apoplastic NaCl. The root segment was then quickly transferred into a clean chamber containing Na^+-free BSM solution (with/without 0.1 mM amiloride) and immobilized for MIFE measurements. Net Na^+ flux (a proxy for SOS1 activity) was measured as described in "Materials and methods" section for 5–10 min. The first 20 min after root immobilization were discarded to account for the Donnan exchange in the cell wall and removal of the apoplastic Na^+. Inserts in each panel denote the steady-state Na^+ efflux 5 min after the removal of the pre-conditioning solution. Data labelled with different lower-case letters are significantly different at $P < 0.05$. The flux convention is "efflux negative"

lowest after 2 days of salt exposure, whereas T(HBV) had high Na^+ concentration that was similar to S(LBV) (Fig. 2b). However, after 3 weeks of salinity stress, both salt-tolerant accessions had the same Na^+ concentration in their roots which were significantly lower compared with those in salt-sensitive plants (Fig. 2b).

There was not significant difference in the leaf K^+ content amongst accessions either before stress or after 2 days of salt exposure. After 3 weeks of salinity stress, leaf K^+ concentration was significantly increased in all accessions (Fig. 2c). T(LBV) and S(LBV) had significantly higher K^+ concentration compared to their counterparts (Fig. 2c). K^+ concentration in roots of all accessions increased significantly upon long-term salinity exposure, with salt-tolerant accessions having higher K^+ concentration than salt-sensitive plants; however, there was not significant difference between plants with high and low EBC volume (Fig. 2d).

Na^+ flux from the root

Salt-treated and control roots of quinoa accessions grown for 2 days and 3 weeks under 400 mM NaCl were compared for their ability to exclude Na^+. When transferred to Na^+-free media, net Na^+ efflux was recorded from the root epidermis in all accessions. This efflux was strongest in the root elongation zone (where SOS1 Na^+/H^+ exchanger is predominantly located; Shi et al. 2002) and observed only in salt-grown plants (Fig. 3). Net Na^+ efflux was higher in plants exposed to longer salinity treatments (Fig. 3). At short-term salt stress, the highest net Na^+ efflux (-37.9 nmol m^{-2} s^{-1}) was measured from salt-tolerant accession T(LBV) and the lowest (-8.1 nmol m^{-2} s^{-1}) signal was from salt-sensitive accession S (HBV) (Fig. 3). Under long-term salinity stress,

accession T(HBV) with − 66.5 nmol m^{-2} s^{-1} and S(HBV) with − 12.9 nmol m^{-2} s^{-1} had the highest and lowest Na^+ efflux, respectively (Fig. 3). Root pre-treatment with 0.5 mM amiloride (a known inhibitor of Na^+/H^+ SOS1 exchanger; Wu et al. 2019) reduced root Na^+ extruding ability by ca 90% (Fig. 3).

NaCl-induced K^+ flux from root

Adding 200 mM NaCl to the bath solution resulted in a rapid and massive net K^+ efflux from mature root zone in all accessions (Fig. 4a). The peak K^+ efflux was highest in salt-sensitive accessions compared with their salt-tolerant counterparts. The lowest response was measured from T(LBV) (Fig. 4b). In both sensitive and tolerant groups, varieties with LBV had lower net K^+ efflux compared with varieties with HBV (Fig. 4b), indicative of a compensation mechanism. Both salt-tolerant accessions showed a lower steady-state K^+ efflux compared to sensitive plants after exposure to salt stress where K^+ efflux for T(LBV) and T(HBV) were − 37.5 and − 33.2 nmol m^{-2} s^{-1}, respectively, and these values for S(LBV) and S(HBV) were − 63.3 and − 66.2 nmol m^{-2} s^{-1} (Fig. 4c).

Transcript levels of salt transporters under saline conditions

The transcript levels of *CqSOS1*, *CqNHX1*, *CqGORK*, and *CqSKOR* were measured in root tissues of all quinoa accessions grown for 3 weeks under 400 mM salinity stress compared to their corresponding plants grown under non-saline conditions, and then normalized to the expression level of housekeeping gene *CqEF-1a*.

A comparative analysis of *CqSOS1* indicated that this gene was up-regulated under saline conditions in all accessions (Fig. 5). However, the expression level of salt-tolerant genotypes was significantly higher compared with their sensitive counterparts. The highest increase in *CqSOS1* expression level (more than five-fold) was observed in T(HBV) (Fig. 5). Similar to *CqSOS1*, the transcript level of *CqNHX1* was higher in salt-tolerant plants compared to sensitive ones, with the strongest response in T(LBV) (Fig. 5). On the

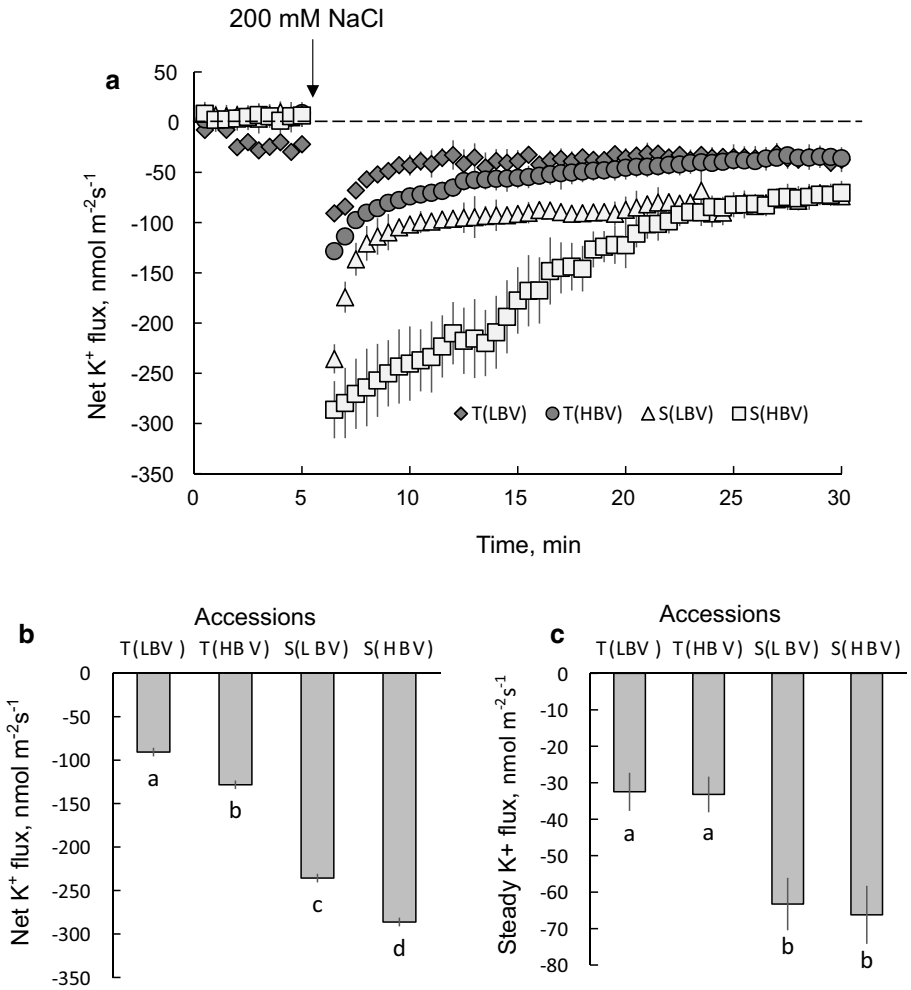

Fig. 4 **a** Kinetics of NaCl-induced K^+ efflux from roots of four quinoa accessions. Net K^+ fluxes were measured from the mature zone (5 mm from root tip) of 3-day-old seedlings pre-conditioned in Basic Salt Media solution. **b** Peak K^+ efflux values from root samples exposed to 200 mM NaCl. **c** Steady-state K^+ efflux measured from root samples 40 min after exposure to salt stress. Mean ± SE ($n = 6$–8). Data labelled with different lower case letters are significantly different at $P < 0.05$

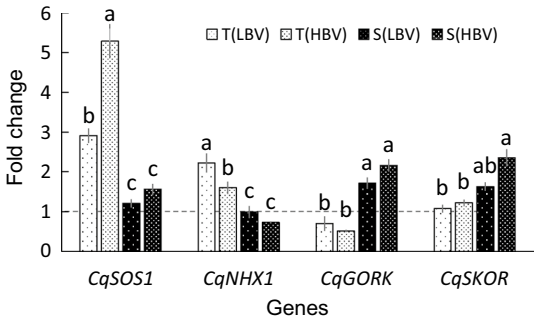

Fig. 5 The relative transcript level of *CqSOS1, CqNHX1*, *CqSKOR*, *CqGORK* in roots of four quinoa accessions (from plants exposed to 400 mM NaCl for 3 weeks). Each data point is mean ± SE of three technical replicates each representing six biological samples

contrary to the above mentioned trends, the transcript level of *CqGORK* and *CqSKOR* genes in salt-tolerant accessions either remained unchanged or reduced while the activity of these genes increased in salt-sensitive accessions under saline conditions (Fig. 5).

Discussion

Na^+ exclusion ability from the root compensates for the lack of EBC

Na^+ exclusion from the shoot and its restriction from entry to the plant at the root level are among the most important salinity tolerance mechanisms in plant species. However, as the amount of Na^+ that can be retrieved from the shoot and moved back to the root is very small (Lei et al. 2014), Na^+ exclusion from roots has long been recognised as a key physiological characteristic contributing to salt tolerance (Munns and Tester 2008). In the presence of EBCs, quinoa plants have the ability to sequester toxic Na^+ away from metabolically active cellular compartments in the leaf. In the absence of a possibility for large external Na^+ storage in low EBC volume (LBV) accessions, Na^+ exclusion at the root level and maintaining low Na^+ concentration in leaves could be considered as primary compensation mechanism (Fig. 2a, b). Three major lines of evidence support this notion. First, both T(LBV) and S(LBV) accessions had lower Na^+ content at the root level after 2 days of salt stress in comparison to their counterparts with high EBC volume (Fig. 2b). Second, for the same conditions, within each group (e.g. either sensitive or tolerant) low bladder-bearing genotypes showed significantly higher rate of net Na^+ efflux (Fig. 3) compared with their HBV counterparts. Third, both LBV accessions had lower leaf Na^+ concentration after 3 weeks of salinity stress showing that they were able to limit Na^+ entry to the shoot in the absence of high external Na^+ storage (Fig. 2a). Thus, these accessions employed Na^+ exclusion from the root uptake as a compensation mechanism for the lack of a high EBC volume. This finding suggests that an active Na^+ efflux system is present to remove the Na^+ out of the root cells of plants with low EBC volume. The most suitable candidates for this role are Na^+/H^+ exchangers at the plasma membrane encoded by *SOS1* gene. Earlier pharmacological and genetic experiments showed operation of such exchangers in roots of wheat (Cuin et al. 2011; Feki et al. 2014), Arabidopsis (Ullah et al. 2016) and barley (Wu et al. 2019). Gene expression results are consistent with the notion that the plasma membrane Na^+ efflux transporter *CqSOS1* operates in the removal of Na^+ out of the root cells (Fig. 5), with higher *CqSOS1* transcript levels found in salt-tolerant accessions. Consistent with previous findings (Ruiz-Carrasco et al. 2011), salinity exposure resulted in a five-fold upregulation of *CqSOS1* transcript levels in T(HBV). This is in agreement with the observed changes in root Na^+ concentration of studied plants in physiological experiments (Fig. 2b).

Vacuolar NHX1 exchangers play a critical role in the intracellular Na^+ sequestration in glycophytes (Apse et al. 1999; Zhang et al. 2001; Xu et al. 2008). Although some recent evidence suggest that these transporters may also play a significant role in regulating vacuolar K^+ loading and cytosolic pH (Bassil and Blumwald 2014), their (original) role in vacuolar Na^+ sequestration was not disapproved and, to the best of our knowledge, they remain the only known transporting proteins fulfilling this role. In the current study, the significantly higher transcript level of *CqNHX1* at the root level in T(LBV) compared to T(HBV) may indicate that this antiporter has an active role in compartmentalisation of Na^+ into the vacuole in roots thus conferring a compensation strategy when plants lack high EBC volume as an external Na^+ storage in the shoot (Fig. 5).

K^+ retention as a component of the compensation mechanism

K^+ plays a vital role in many cell functions. A strong correlation between salinity tolerance and root K^+ retention ability has been found in glycophytes such as wheat (Cuin et al. 2011), rice (Feng et al. 2019), maize (Cao et al. 2019) and barley (Chen et al. 2007). Quinoa as a halophytic plant has a high ability to retain K^+ under unfavorite conditions (Bonales-Alatorre et al. 2013). In a line with the role of K^+ in salinity tolerance, T(LBV) and S(LBV) had significantly higher K^+ concentration in their leaves at long-term (3 weeks) salinity stress compared to plants with high EBC sequestration ability (Fig. 2c), suggesting that the ability to maintain high K^+ content under saline condition may play an important role as a component of the tissue-tolerance mechanism, to compensate for the lack of ability to sequester salt lead in EBCs. Indeed, higher K^+ content in LBV genotypes

(presumably stored in the vacuoles) will allow for longer buffering of cytosolic K^+, thus giving plants more time to sequester excessive Na^+ in the vacuoles, in the absence of the external storage space. Based on the thermodynamical calculation and using reported numbers for salt-stress induced K^+ flux values from plant tissue, Wu et al. (2018) showed that a typical vacuolar K^+ pool may provide efficient cytosolic K^+ buffering between 100 and 400 min. In this context, higher shoot K^+ content in LBV may give them a competitive advantage and "buy more time" to induce transcriptional changes for some key genes involved in adaptation to salinity stress. Also, given the fact that plants with low EBC sequestration may have higher Na^+ concentration in the mesophyll cells, higher levels of K^+ will make K^+/Na^+ ratio in this tissue more optimal.

Potassium is known to activate over 50 enzymes (Marschner et al. 1995) including Rubisco and enzymes that play a role in the chlorophyll biosynthesis. Thus, reduced K^+/Na^+ ratio in the cytosol as a result of accumulation of higher concentration of Na^+ in leaf may compromise plant's CO_2 assimilation capacity. Consistent with this notion, Wu et al. (2015) showed that K^+ retention ability in photosynthetically active mesophyll tissue was an important characteristic contributing to the overall salt tolerance in barley plants. Under this circumstance, the higher ability of quinoa to avoid reduction of K^+ content and to keep optimal K^+/Na^+ ratio in their cytosol, as observed in T(LBV) and S(LBV), may be considered as an important compensation strategy for the lack of EBC sequestration ability. NaCl-induced K^+ leakage of studied accessions showed that accessions with low EBC volume [T(LBV) and S(LBV)] had smaller K^+ loss when exposed to 200 mM NaCl (Fig. 4a–c) and also had significantly higher leaf K^+ content than their counterparts with HBV [T(HBV) and S(HBV)] (Fig. 2c). As the loss of K^+ from leaves may trigger programmed cell death (Shabala 2009) and accelerate leaf senescence, such K^+ retention in the mesophyll may be a critical component of the tissue-tolerance mechanism. For example, Arabidopsis *gork1-1* mutant plants which lacking functional outward-rectifying K^+ channels had ten-fold lower number of cells undergoing programmed cell death compared with wild type (Demidchik et al. 2010). The mechanism behind is that reactive oxygen species activated K^+ efflux through GORK channels in wild-type plant that resulted in significantly higher K^+ loss from plant cells that stimulates programmed cell death (Demidchik et al. 2014).

NHX genes may preferentially transport K^+ compared to Na^+ (Venema et al. 2003). It has been shown that vacuolar K^+ contents in double mutants of Arabidopsis plant lacking *nhx1nhx2* genes was one-third that of wild-type root (Bassil et al. 2011) and leaf cells (Barragan et al. 2012) demonstrating the significant importance of *NHXs* in the regulation of K^+ homeostasis. Thus, the lowest NaCl-induced K^+ leakage in T(LBV) (Fig. 4a, b) may be potentially explained by the higher expression level of *CqNHX1* gene compared to other accessions (Fig. 5). This is also consistent with the finding of the biggest increase in root K^+ content in T(LBV) and in accord with the above notion of the vacuolar K^+ pool being a buffer for cytosolic K^+ homeostasis.

The highest *CqSKOR* transcript level was found in S(HBV) accession (Fig. 5). However, the shoot K^+ content in this accession was lower or equal to other genotypes (Fig. 2) suggesting that transcriptional changes alone were not sufficient to provide adequate supply of K^+ to the shoot and pointing out at the importance of post-translational regulation.

Salinity stress up-regulated transcript levels of CqGORK; this upregulation was highest in salt-sensitive accessions (Fig. 5). While the physiological rationale behind these changes remains to be explicitly demonstrated, a plausible explanation could be in K^+ efflux playing a charge balancing role. Sodium influx into cytosol results in substantial (by 60–80 mV; Shabala et al. 2016a, b) depolarization of plasma membrane potential (MP); the extent of this depolarization is higher in salt-sensitive varieties. Plants can restore MP values by either increasing the rate of H^+ pumping by H^+-ATPase (Bonales-Alatorre et al. 2013) or by increased K^+ efflux. As the first option comes with substantial energy cost, GORK-mediated K^+ efflux might be considered by plants as a more advantageous strategy.

In conclusion, quinoa species employ diverse strategies to deal with salinity stress. While their main mechanism involves external salt storage in epidermal bladder cells, varieties lacking this capacity may compensate for it by having better K^+ retention in the cell (a tissue-tolerance trait) and enabling higher Na^+ extrusion from the root. The latter trait is typical of glycophytes and is consistent with the notion of quinoa being a facultative halophyte (Adolf et al. 2013).

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Compliance with ethical standards

Conflict of interest The authors have no conflict of interest to declare.

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