Role of *Arabidopsis* RAB5 GEF *vps9a* in maintaining potassium levels under sodium chloride stress

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Abstract
Salt stress is one of the major factors impacting crop productivity worldwide. Through a variety of effector and signaling pathways, plants achieve survival under salinity stress by maintaining high cytosolic potassium/sodium ion (K⁺/Na⁺) ratios, preventing Na⁺ cytotoxicity, and retaining osmotic balance. Ras-related protein 5 (Rab5) members are involved in the trafficking of endosomes to the vacuole or plasma membrane (PM). The vacuolar protein sorting-associated protein 9 (*vps9a*) encodes the single guanine nucleotide exchange factor (GEF) that activates all three known Rab5 proteins in *Arabidopsis thaliana*. Previous work from our group has reported the critical function of *vps9a* for the operation of salt-induced endocytic pathway, as well as the expansion of endomembrane compartments under saline stress conditions. Here we show an additional role of *vps9a* in plant response to salt stress via maintenance of K⁺ status of the cell rather than Na⁺ homeostasis. Our results show that roots from *vps9a-2* mutant, subjected to 100 mM NaCl, display alterations in transcript levels of genes involved in the K⁺ homeostasis pathway. Concurrent with the observed sensitivity of *vps9a-2* mutant under NaCl stress, exposure to low K⁺ environments resulted in growth retardation, and reduced rate of endocytosis. Furthermore, *vps9a-2* mutant displays reduced expression of auxin reporter, Direct Repeat-5 (DR5), and alterations in polarity and abundance of auxin efflux carrier PIN-FORMED2 (PIN2). Imposition of NaCl stress was found to be restrictive to the elongation capacity of cells in the root elongation zone of *vps9a-2* mutant. Together our results indicate that alterations in K⁺ homeostasis and associated cellular changes causing increased cell wall pH, contribute to diminished root growth and compromised survival of *vps9a-2* mutant under salt stress conditions.

**KEYWORDS**

*Arabidopsis*, DR5, K⁺ homeostasis, PIN2, RAB5, salt stress response

1 | INTRODUCTION

Soil salinity is the major abiotic stress-causing decline in crop productivity globally. Several pathways contribute toward the ability of plants to respond and cope with salinity stress. At the cellular level, the ability to perceive stress and prevent cytotoxicity is the key to establish salt tolerance. Therefore, proteins transported to the PM and vacuoles form one of the crucial targets for the salinity response.
pathway in plants. At the physiological level, the ability to retain K+ in photosynthetically active zones such as the shoot contributes toward resistant phenotype. Thus, strategies involving efficient uptake, prevention of efflux, and loading of K+ to the stele under salt stress become important parameters for survival.

Activity of PM ATPases leads to the generation of the electrochemical gradient necessary for maintaining a cytosolic K+ concentration of around 100 mM and cytosolic pH at 7.3 (Shen et al., 2013). Saline soil conditions lead to PM depolarization and perturbation of cytosolic K+ concentrations due to increased Na+ uptake and concomitant K+ efflux from the root and mesophyll tissue (Carden et al., 2003; Shabala et al., 2005, 2006; Shabala, 2000).

Transport vesicles or endosomes enable intracellular movement of cargo (including proteins, lipids, nutrients) by endocytic uptake of nutrients and recycling of PM proteins (Faini et al., 2013; Fan et al., 2015; Murphy et al., 2005). Earlier studies have reported that alterations in abundance and distribution of PM proteins such as PIN are mediated by the repression of endocytosis that in turn impact growth rates (Kitakura et al., 2011). Rab-GTPases are members of the ras superfamily of regulatory GTPases that are involved in intracellular membrane trafficking pathways and have a significant role in a plethora of cellular processes including growth and development, cell division, plant defense and immune responses, self-incompatibility (Boutté et al., 2009; Brandizzi, 2018; Chow et al., 2008; Dhonukshe et al., 2006; Geldner et al., 2003; Inada et al., 2016, 2017; Ivanov & Gaude, 2009; Kwon et al., 2013; Liu et al., 2017; Pereira-Leal & Seabra, 2001; Zhang et al., 2007). Rab-GTPases transition from active guanosine triphosphate (GTP: membrane bound) to inactive guanosine diphosphate (GDP: cytosolic) forms, via a reaction catalyzed by specific guanidine exchange factors (GEFs) (Zhen & Stemmark, 2015). Three Rab5 members are described in Arabidopsis namely Rha1 (Rab5F2a) and Ara7 (Rab5F2b), that regulate endosome-vacuole transit pathway and plant-specific Ara6 (Rab5F1) that regulates endosome to PM trafficking pathway (Ebine et al., 2012; Lee et al., 2004; Ueda, 2001; Ueda et al., 2004).

While the activation of mammalian Rab5 involves several distinct GEFs, all three Arabidopsis Rab5 members are specifically activated by vps9a expressed ubiquitously in contrast to vps9b that is localized to pollen and embryo sac (Carney et al., 2006; Goh et al., 2007). Homozygous mutant lines of Arabidopsis with defects in vps9a are shown to have distinct phenotypes. Complete disruption of the vps9a domain as for vps9a-1T-DNA insertion line, leads to embryonic lethality, while partial vps9a domain disruption as in the vps9a-2 line display defects in the root system (Goh et al., 2007). Earlier work from our group has reported the salt-sensitive phenotype of Arabidopsis vps9a-2 mutant (Baral et al., 2015).

The present work aims at understanding the molecular basis of the observed NaCl sensitivity of vps9a-2 mutant. Our results identify an important correlation between salt-sensitive phenotype of vps9a-2 mutant and defects in maintaining K+ levels rather than due to Na+ exclusion/sequestration pathways. We show that vps9a-2 mutant display root growth retardation under high Na+ as well as low K+ environments indicating the relevance of K+ homeostasis mechanisms during imposed salinity stress. Analysis of root transcript levels in vps9a-2 mutant subjected to NaCl stress revealed the modulation of genes such as guard cell outward-rectifying K+ (Gork), Arabidopsis H+-ATPase 4 (Aha4), steral K+ outward rectifying (Skor), cation/H+ exchanger 17 (Chx17), and high-affinity K+ transporter 5 (Hak5), known to have a prominent role in various aspects related to K+ homeostasis. Notably, while vps9a-2 mutant shows comparable levels of FM4-64 uptake under control K+ conditions (10 mM), exposure to low K+ conditions (1 µM) resulted in a marked reduction in endocytic capacity, in addition to compromised growth ability.

Vacular sequestration is driven by transmembrane H+ gradients. We show that vps9a-2 mutant display aberrations in capacity for vacular acidification, display increased root membrane depolarization, and cell wall alkalization upon salt stress exposure. Anatomically, we observe a limited capacity of cells in the root elongation zone to undergo elongation, leading to greater damage in vps9a-2 mutant, following exposure to salt stress. Interestingly, we a observed reduction in the expression of yellow fluorescence protein (YFP) tagged, auxin response reporter, direct repeat 5 (DR5), in addition to alterations in polarity and abundance of the auxin efflux protein, PIN2 in vps9a-2 mutant. These changes were more acute upon exposure to saline stress. Based on these results, we propose that vps9a-mediated modulation via DR5 and auxin response protein such as PIN2 could be one of the factors leading to interference in the establishment/maintenance of H+ proton gradient and influence K+ uptake. A combinatorial outcome of these processes reflects as lower shoot K+ levels contributing to the salt-sensitive, phenotype in vps9a-2 mutant.

2 | MATERIALS AND METHODS

2.1 | Plant growth and treatment

Arabidopsis seeds were sterilized and vernalized for 2 days at 4°C prior to plating. Seeds were plated on complete medium (1/2 MS with 1% sucrose, 1% agar and buffered with MES at pH = 5.8) and grown vertically under standard conditions with 16 hr light and 8 hr dark cycle. For treatment, 4-day-old seedlings were transferred to plates with different treatment conditions/durations as mentioned for each experiment. Exposure to NaCl stress was performed in plates supplemented with 100 mM NaCl for indicated duration and then grown as mentioned above.

For experimental membrane depolarization conditions, intracellular K+ concentrations (100 mM) were matched with high external K+ conditions (by external addition of 90 mM KCl, to achieve a final 100 mM KCI/K+ concentration), thereby equilibrating the
concentration gradient. For high pH, the medium was buffered with 20 mM HEPES at (pH = 7.2) or 50 mM bicine (pH = 7.6) instead of MES. Low K+ media was used as detailed in (Cheong et al., 2007) and indicated concentrations of K+ were achieved following supplementation with KCl.

2.2 Lines used

Arabidopsis thaliana ecotype Columbia-0 (WT), vps9a-2 mutant (Goh et al., 2007), PIN2:PIN2-GFP (Xu & Scheres, 2005), vps9a:vps9a-GFP (gift from Prof. Takashi Ueda), DR5-YFP (gift from Prof. Kalika Prasad), ara6-2 (SAIL_98-E08), ara7 (WiscDsLox355 B06), rha1 (SAIL_596-A03), syp21 (CS68714), syp22 (CS68717) mutant Arabidopsis lines were used for various experiments. vps9a-2 mutant line expressing DR5-YFP, was generated by crossing vps9a-2 mutant and DR5-YFP Arabidopsis lines. vps9a-2 mutant was phenotypically identified by the growth pattern from F1 seeds and the presence of YFP cassette was confirmed by PCR using YFP-specific primers. YFP expression was assessed by confocal imaging. Positive lines from the F4 generation were used for the experiments. vps9a-2 mutant line expressing PIN2:PIN2-GFP was generated by crossing vps9a-2 mutant and PIN2:PIN2-GFP Arabidopsis lines. vps9a-2 mutant was phenotypically identified by the growth pattern from F1 seeds and the presence of PIN2:PIN2-GFP cassette was confirmed by PCR using GFP-specific primers. GFP expression was assessed by confocal imaging. Positive lines from the F3 or F4 generation were used for the experimental analysis. For comparing differences in the growth pattern of vps9a-2 mutant in comparison to WT plants and to account for them during the analysis of the effect of various treatment/conditions, phenotypic assessment of root growth retardation has been represented following normalization to respective control conditions including 0 mM NaCl, 10 mM K+ or pH = 5.8, as mentioned individually for each figure/experiment. Percentage root growth inhibition (I) in response to salt stress/treatment was calculated with respect to respective growth under control conditions (0 mM NaCl) as follows (Baral et al., 2015):

$$ I = \left[ 1 - \frac{(\Delta L/L)_{\text{stress}}}{(\Delta L/L)_{\text{control}}} \right] \times 100 $$

where $\Delta L/L = (\text{Final length following treatment}) - \text{Initial length}$ (length at day 0)/Initial length (length at day 0).

In experiments to assess effects of low (50 or 1 μM) and high (100 mM KCl) external K+; values are normalized to 10 mM external K+ (0 mM KCl) as the control condition. Similarly, for experiments to study response to alkaline stress, root length measurements are represented following normalization to control condition, pH = 5.8.

2.4 Determination of ion content by flame photometry

Four-day-old seedlings were transferred to plates supplemented with either (0 mM NaCl) or (100 mM NaCl) and grown for 10 days. Following incubation, plants were removed gently and washed quickly 3-times with milli-Q water. Root and shoot tissues were separated and stored in glass vials. The harvested material was dried completely at 55°C for 2 days and tissue dry weight (DW) was determined. Six hundred μl of 70% HNO3 per 5 mg DW was added and the mixture heated for 10 min at 70°C to facilitate digestion. Perchloric acid (1.2 ml) was added and the sample mix heated at 120°C in a silicone bath till the volume was reduced to 500 μl. Samples were diluted and quantitated using Systronics flame photometer 128. Instrument calibration was carried out using standards with known quantities of Na+ and K+ (10, 20, 50, and 100 ppm of NaCl and KCl). Ion content in samples was calculated using the following formula (Choi et al., 2014):

$$ \text{Ion Content (mg/g DW)} = \frac{\text{Amount of ion (ppm) × Total Sample Volume (L)}}{\text{Sample DW (g)}} \text{ (Choi et al., 2014).} $$

Ion content determination was performed using equivalent DW tissue to ascertain the use of identical tissue amounts for experimental analysis. For comparing differences in vps9a-2 mutant background, data are represented following normalization to values obtained for WT plants grown under control conditions (0 mM NaCl). In order to quantify the effects of NaCl treatment in WT or vps9a-2 mutant background, data are depicted as normalized to values obtained for the respective WT or vps9a-2 mutant plants grown under control conditions (0 mM NaCl), denoted as 1.

2.5 Real-time PCR

Gene primers were designed using QuantPrime (Arvidsson et al., 2008). The list of primers used is detailed in Figure S1. WT or vps9a-2 mutant were grown for 4 days and then transferred to plates under control (0 mM NaCl) or subjected to salt stress (100 mM NaCl) for 10 days. RNA from root tissue was extracted using Trizol reagent. Total RNA was subjected to DNase I treatment and used for cDNA synthesis with Superscript III followed by RNase H (Thermo Fisher) treatment according to the manufacturer’s protocol. cDNA was used along with SYBR green mix for carrying out real-time PCR using Applied Biosystems instrument 7500. Fold changes were calculated using the ΔΔCT method with WT control and Ubc21 gene for normalization (Livak & Schmittgen, 2001). At least three independent biological replicates were performed for the experiment.
2.6 | Confocal imaging

Confocal imaging was performed on 5-day-old seedlings in most experiments and details of treatment are mentioned for each individual experiment. Probes were purchased from Thermo Scientific. FM4-64 stocks (1.25 mM) were prepared in DMSO and used at a final concentration of 2 μM for staining. FM4-64 fluorescence was imaged following excitation at 561 nm and emission was collected at 670–770 nm. FM4-64 arbitrary fluorescence unit values are represented following normalization to values obtained for WT plants grown under control conditions (10 mM K⁺), denoted as 1. For GFP imaging, 488 nm laser was used with an excitation peak at 510 nm and emission was collected at 500–600 nm. For YFP imaging, 488 nm laser was used with an excitation peak at 514 nm and emission was collected at 500–545 nm. Imaging was performed on Olympus FV 3000 confocal microscope and quantification was performed using ImageJ software. For the determination of DR5-YFP intensity, a region of interest (ROI) of 40 μm was used. All fluorescence intensities were quantified using FIJI software ImageJ and represented as corrected total cell fluorescence (CTCF) values. In order to quantify the relative expression of DR5 or PIN2, data are plotted following normalization to values obtained for WT plants grown under control conditions (0 mM NaCl) denoted as 1.0. The effect of NaCl treatment obtained intensity values were normalized to the respective WT or mutant plants under control (0 mM NaCl) conditions, denoted as 100%.

2.7 | Vacuolar pH measurements

Staining protocol as described by (Bassil et al., 2013) was adopted. pH determinations were based on a calibration curve generated with buffers prepared for pH 5.8–7.4 with increments of 0.4 pH units. pH calibration solutions were made in 50 mM ammonium acetate and 50 mM 2-morpholinoethanesulfonic acid (MES) (for pH standards 5.8–6.2) or 50 mM (4-[2-(hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES) (for pH standards 6.6–7.4). Required pH values were obtained by adjustment using 50 mM Bis-tris propane (BTP) (Sigma, MO, USA). 2′,7′-Bis-(2-carboxyethyl)-5-(and-6) carboxyfluorescein, acetoxyethyl ester (BCECF-AM) (1.6 mM stock) was used with a working concentration of 10 μM along with 0.02% pluronic acid (Sigma). BCECF-AM preloaded 5-day-old wild-type plants were incubated in respective pH standard solutions containing 10 μM nigericin and valinomycin (Sigma, MO, USA) for 15 min and then imaged at 20x magnification, following sequential excitation at 445 and 488 nm and emission recorded at 510–550 nm. Fluorescence intensity ratios were determined, and pH was plotted as log regression to generate an in-situ calibration curve, used to determine pH values for experimental plants.

2.8 | DiBAC₄(3) staining

Plasma membrane potential was determined using the voltage-sensitive fluorescent dye bis-(1,3-dibarbituric acid)-trimethine oxanol, DiBAC₄(3) (Dejonghe et al., 2016). The dye exhibits an enhanced fluorescence when bound to depolarized membranes and conversely hyperpolarization is indicated by a decrease in fluorescence. Probe was used at a working concentration of 10 μM and staining was carried out for 30 min. Seedlings were imaged with excitation/emission of 488/520 nm. Fluorescence intensities were quantified using FIJI software ImageJ and represented as corrected total cell fluorescence (CTCF) values. Obtained fluorescence values were normalized with those for WT control plants (0 mM NaCl) denoted as 1 to obtain relative DiBAC₄(3) intensity. Error bars represent SEM of at least 25 cells from 4 to 7 different plants.

2.9 | Determination of apoplast/cell wall pH

Cell wall pH was determined using the method described by (Barbez et al., 2017), with indicated modifications. Four-day-old seedlings were subjected to 100 mM NaCl stress and stained using 1 mM 8-Hydroxypyrene-1,3,6-trisulfonic acid trisodium salt (HPTS) for 30 min. Seedlings were imaged sequentially first with excitation at 445 nm and emission at 505–550 nm; followed by excitation at 405 nm and emission at 440–470 nm. Background subtraction was performed and fluorescence intensities were quantified using the FIJI software of ImageJ. Ratio of intensities at 445/405 nm was determined and plotted. Since in situ calibration curves can vary with each experimental set, ratios of intensities have been normalized to values obtained for WT plants treated with 100 mM NaCl as described in an earlier study (Barbez et al., 2017). The experiment was repeated three times with similar results.

2.10 | Statistical analysis

All statistical analysis was performed using unpaired, non-parametric Student’s t test, and significance values determined for individual experiments are detailed in the figure legend and represented as “p < .05,” “p < .01,” “p < .001,” and “**p < .001.” Values were considered comparable and differences statistically not significant with p ≥ .05. Data analysis was performed, and graphs were plotted using GraphPad Prism software version 8.

3 | RESULTS

3.1 | vps9a-2 mutant display root growth inhibition and lower K⁺ levels upon exposure to salt stress

Maintenance of high cytosolic K⁺/Na⁺ ratios is important for cell survival and exposure to high saline environment disrupts these ratios (Munns & Tester, 2008). Salt-sensitive plants, therefore, end up having increased intracellular Na⁺ concentrations and a limited ability to accumulate K⁺ in their cells (Mahmood ur et al., 2019). Due to the importance of K⁺ as a macronutrient and its involvement in several
metabolic processes, alteration of intracellular K⁺ levels, either by changes in uptake or translocation to the shoot, result in compromised plant growth thereby affecting yield and productivity (Yeo et al., 1990). Our results show that vps9a-2 mutant display retarded root growth phenotype in the presence of 100 mM NaCl (Figure 1a, left and middle panel; Baral et al., 2015). To confirm the phenotype, we additionally assessed the effects of salt stress on vps9a-complemented lines. Following salt stress treatment, vps9a-2 mutant displayed higher percentage of root growth inhibition (60.02 ± 4.75%) compared to WT (37.5 ± 2.45%) and vps9a-complementation rescued the root growth retardation phenotype (43.0 ± 1.45%) to values comparable to WT (Figure 1a,b). Having confirmed the comparability
in phenotypes of vps9a-complemented lines to WT (p > .05) both in the absence and presence of NaCl; we performed all further experiments with WT and vps9a-2 mutant lines.

In order to assess for ionic imbalances, we determined Na$^+$ and K$^+$ contents in root and shoot tissues. Four-day-old WT or vps9a-2 mutant seedlings were subjected to either control (0 mM NaCl) or stress (100 mM NaCl) conditions for 10 days and Na$^+$ and K$^+$ ion contents were analyzed using flame photometry and normalized to WT plants grown under control conditions (0 mM NaCl). Root tissue from WT and vps9a-2 mutant plants showed comparable levels of Na$^+$ (1.0 ± 1.08 ± 0.01; Figure 1c), under control conditions. In contrast, exposure to salt stress, resulted in increased root Na$^+$ content in both WT (5.95 ± 0.63) and vps9a-2 mutant plants (4.88 ± 0.92; Figure 1c). Shoot Na$^+$ levels under control conditions were (1.0 vs. 1.27 ± 0.12) in WT and vps9a-2 mutant, respectively (Figure 1d). Robust increase in shoot Na$^+$ levels following exposure to salt stress was observed in both WT and vps9a-2 mutant (32.83 ± 3.25 vs. 32.01 ± 2.54; Figure 1d), in comparison to WT control conditions. Although an increase in Na$^+$ contents following salt stress was higher in the shoot as compared to root tissue, the extent of increase in both root and shoot tissue were comparable between the genotypes (p > .05; Figure 1c,d). In order to determine the specific effect of NaCl treatment, we also represent ionic content values following normalization to respective WT and vps9a-2 mutant control plants to 1 (Figure S2). No significant difference was observed in root Na$^+$ level increase in WT and vps9a-2 mutant, 5.93 ± 0.632 and 4.480 ± 0.691, respectively (Figure S2a). Similar analysis on shoot Na$^+$ levels showed no statistical significance, (30.10 ± 4.81 vs. 25.87 ± 2.37) for WT and vps9a-2 mutant, respectively (Figure S2b).

Assessment of K$^+$ levels in root and shoot tissues from WT and vps9a-2 mutant plants subjected to salt stress, revealed a converse pattern to that observed for Na$^+$ levels, as K$^+$ levels reduced following NaCl treatment. In comparison to WT plants under control conditions, root K$^+$ levels were found to be lower in vps9a-2 mutant plants, (1.0 vs. 0.78 ± 0.08) (*p < .05, Figure 1e). Following salt stress, root K$^+$ levels in both WT and vps9a-2 mutant undergo a decrease (0.83 ± 0.04 vs. 0.65 ± 0.05) and the difference between both the genotypes subjected to salt stress achieved a similar level of statistical significance (*p < .05, Figure 1e). While shoot K$^+$ levels under control (0 mM NaCl) conditions are comparable for both genotypes (1.0 vs. 0.97 ± 0.23, p > .05, Figure 1f), K$^+$ levels following exposure to salt stress revealed a greater reduction (0.51 ± 0.03 vs. 0.33 ± 0.008) in vps9a-2 mutant (***p < .001, Figure 1f). In order to further identify the effect of NaCl treatment alone, ionic content values were normalized to those obtained for respective plants under control conditions. Root K$^+$ levels in WT and vps9a-2 mutant were found to be comparable at 0.83 ± 0.036 and 0.84 ± 0.062, respectively (Figure S2c). In contrast, shoot K$^+$ content, following NaCl stress exposure in WT and vps9a-2 mutant, was significantly different, 0.51 ± 0.033 and 0.345 ± 0.014, respectively (**p < .0001, Figure S2d). These results indicate that both WT and vps9a-2 mutant plants accumulate Na$^+$ under saline stress and do so in a comparable manner. In both genotypes, accumulation of Na$^+$ is accompanied by a simultaneous decline in tissue K$^+$; however, this change is much more pronounced in shoot and root tissue from vps9a-2 mutant plants (Figure 1e,f). These results indicate that the major defect in vps9a-2 mutant is linked to the inability to sustain K$^+$ homeostasis.

In order to understand the contribution of individual Rab5 mutants namely ara6, ara7, rha1, and SNARE protein mutant syp21 and syp22 under control and 100 mM NaCl-stress conditions, we performed photometric ion estimation experiments. In contrast to the reduced K$^+$ levels in vps9a-2 mutant, individual Rab5 mutants, ara6, ara7, and rha1 display higher root K$^+$ levels. This could be due to the compensatory function of the other two members in the absence of one of the Rab5 members downstream of vps9a (Figure S3). Additionally, ara6, ara7, and rha mutants display a growth phenotype comparable to WT. While these observations are of interest, for the purpose of the current study, we focussed on the role of vps9a since it is the single GEF activating all individual Rab5 members.

### 3.2 | vps9a-2 mutant plants are hypersensitive to low external K$^+$ conditions

Ion estimation revealed the compromised ability of vps9a-2 mutant to maintain K$^+$ homeostasis. Since there is a close link in the maintenance of Na$^+$ and K$^+$ balance under high saline environments, we next tested whether defects in the maintenance of low K$^+$ levels resulted in the observed salt sensitivity of vps9a-2 mutant. Low-affinity uptake systems mediate uptake at higher K$^+$ concentrations, while high-affinity uptake system takes over in low K$^+$ regimes. AtAkt1 functions at concentrations between 10
and 50 µM and AtHak5 are the sole high-affinity uptake system functioning at concentrations below 10 µM external K⁺ (Nieves-Cordones et al., 2010; Rubio et al., 2010). In order to ensure activation of high-affinity uptake systems in our experimental setup, we individually assessed the performance of WT and vps9a-2 mutant under exclusively low K⁺ conditions (in the absence of NaCl stress) containing either 50 or 1 µM external K⁺. Four-day-old plants grown under control conditions (10 mM K⁺) were transferred to
either similar (10 mM K\(^+\)) or low K\(^+\) conditions containing either 50 or 1 µM external K\(^+\) and grown for a further 7 days (Figure 2a). In order to evaluate the effect of stress treatment in each genotype, root growth measurements were calculated following normalization to values obtained for respective WT or vps9a-2 mutant plants grown under control (10 mM K\(^+\)) conditions. Compared to WT, percentage root growth inhibition under 50 µM low K\(^+\) was significantly higher in vps9a-2 mutant (39.52 ± 4.69% vs. 78.97 ± 4.12%) and a similar increase in root growth inhibition was observed at 1 µM low K\(^+\) conditions (30.74 ± 5.2% vs. 79.17 ± 4.36%) in vps9a-2 mutant (\(****p < .0001\), Figure 2a,c). Whilst two individual K\(^+\) concentrations (50 or 1 µM), were experimentally tested to represent a low K\(^+\) environment, both concentrations yielded a comparable extent of root growth inhibition (\(p > .05\), Figure 2c). These results demonstrate that hypersensitivity of vps9a-2 mutant to low K\(^+\) external conditions, is independent of NaCl stress, indicating the critical role of vps9a in moderating K\(^+\) levels in plant cells.

Endocytic pathways allow for uptake of extracellular material and experimental evidence demonstrates the important role of Rab5 proteins in the endocytic pathway (Šmaj et al., 2004). We used FM4-64 internalization as a probe to investigate endocytosis in the vps9a-2 mutant line. Our results show comparable FM4-64 uptake under control (10 mM) external K\(^+\), in both WT and vps9a-2 mutant, 1 versus 1.045 ± 0.052 (Figure 2b, upper panel). Exposure to low K\(^+\) (1 µM) resulted in greater FM4-64 fluorescence intensity in WT as compared to vps9a-2 mutant, 1.848 ± 0.086 versus 1.293 ± 0.087, indicating a compromise in the low K\(^+\)-induced endocytic capacity in vps9a-2 mutant (\(****p < .0001\), Figure 2b, lower panel and D). To further confirm our observations, we tested FM4-64 uptake in vps9a-2 mutant functionally complemented with vps9a: vps9a-GFP, subjected to low K\(^+\) conditions. We observed an increase in GFP spots (corresponding to vps9a expression) and FM4-64 marked red spots (corresponding to low K\(^+\)-induced endocytosis) under low (1 µM) external K\(^+\) conditions (Figure S4) and a degree of co-localization was observed. These results confirm the role of vps9a in plant response to low K\(^+\) conditions.

### 3.3 Altered expression K\(^+\) homeostasis genes in vps9a-2 mutant under salt stress

Defects in components of the K\(^+\) homeostasis pathway could occur due to aberrations in processes such as the inability to establish or maintain pH gradients, enhanced K\(^+\) efflux, and/or inability to turn on high-affinity K\(^+\) uptake mechanisms. In order to gain an insight into the possible mechanism(s) affected in vps9a-2 mutant plants, we analyzed changes in the expression pattern of genes associated with K\(^+\) homeostasis. Representative genes for each category such as Gork (K\(^+\) efflux), Aha4 and Skor (pH gradient/membrane potential/loading of K\(^+\) to shoots), Chx17 (pH-induced low K\(^+\)), and Hk5 (high-affinity uptake system) were analyzed in root tissue from WT or vps9a-2 mutant subjected to 100 mM NaCl stress for 10 days (Figure 3).

Voltage-gated channel, guard cell outward-rectifying K\(^+\) (Gork), is also expressed in root outer cell layers (epidermal, root hairs, and cortex) in Arabidopsis and forms a major pathway for salt stress-induced K\(^+\) leakage from root cells (Demidchik et al., 2010, 2014). RT-PCR analysis indicates significantly higher Gork transcript in vps9a-2 mutant in comparison to WT, under control conditions (1.0 vs. 1.86 ± 0.264) (\(p < .05\), Figure 3a). In addition, while a 2.5-fold increase in transcript levels was observed in salt-stressed WT roots, no such induction was observed in roots from vps9a-2 mutant (2.46 ± 0.22 vs. 1.85 ± 0.40) (\(p < .05\), Figure 3a). Although Gork transcripts in vps9a-2 mutant appear to be unchanged upon salt stress exposure, interestingly the inherent 1.9-fold higher Gork expression in vps9a-2 mutant under control conditions also correlates with low root K\(^+\) content (Figure 1e).

Arabidopsis Aha4 gene encodes a plasma membrane H\(^+\) ATPase isoform, expressed in the stele region of root and homoygous Aha4 disruption shows NaCl sensitivity with increased Na\(^+\) to K\(^+\) ratio in leaf tissues (Vitart et al., 2001). RT-PCR results show that Aha4 expression in vps9a-2 mutant was reduced to 50% of WT under both salt-stress and control conditions (\(****p < .001\), Figure 3b).

Stelar K\(^+\) outward rectifying (Skor) channel is involved in K\(^+\) loading in the xylem (Isayenkov & Maathuis, 2019; Nieves-Cordones et al., 2016; Véry & Sentenac, 2003). Loss of function skor mutants display low K\(^+\) content in the shoot tissue (Gaymard et al., 1998) and Skor over-expression leads to increased stress tolerance (Long-Tang et al., 2018). Our transcript analysis results show comparable levels of Skor transcript in both WT and vps9a-2 mutant (1.0 vs. 1.16 ± 0.392) under control conditions and no change in the transcript levels was observed in WT plants following salt stress. However, vps9a-2 mutant shows dramatically decreased Skor transcript levels under salt conditions (0.944 ± 0.235 vs. 0.049 ± 0.018) compared to WT under stress (\(p < .1\), Figure 3c). In view of the critical role for xylem loading in root to shoot solute transmission, the observed dramatic
decrease in the expression of a major xylem-loading K\(^+\) transporter suggests a possible role for Skor as a cause for poor K\(^+\) accumulation in vps9a-2 mutant shoots under salt stress.

Chx17 belongs to members of the CPA gene family in Arabidopsis and is localized in pre-vacuolar compartments (PVC) and PM of root tissue, where it aids K\(^+\) retention under salt stress (Chanroj et al., 2012; Jia et al., 2018; Sun et al., 2015). Transcript analysis showed that WT and vps9a-2 mutant maintain similar levels of expression under control conditions (1.0 vs. 1.07 ± 0.15), whereas upon NaCl stress vps9a-2 mutant showed high induction (0.246 ± 0.03 vs. 1.29 ± 0.33) compared to similarly treated WT roots (\(*p < .05,\) Figure 3d). These results establish that vps9a-2 mutant has defects linked to both salt stress and K\(^+\) homeostasis and consequently common pathways mediating responses to these specific stresses such as H\(^+\) maintenance are affected in vps9a-2 mutant.

AtHak5 is another important gene, specifically induced upon low K\(^+\) conditions, and has been considered as a marker for low K\(^+\) levels (Gierth et al., 2005). Transcript analysis indicate that in comparison to WT, vps9a-2 mutant show a 32-fold increase in transcript under control conditions (1.0 vs. 32.43 ± 8.25) (\(**p < .01,\) Figure 3e) and ~45-fold induction under salt stress conditions (4.0 ± 1.81 vs. 179.7 ± 55.16) compared to WT plants exposed to NaCl stress.

**Figure 3** Real-time transcript analysis showing expression of genes involved in potassium homeostasis under salt stress conditions. Four-day-old seedlings of wild-type (WT) or vps9a-2 mutant (vps9a-2) grown in the absence (control) or presence (NaCl) of 100 mM NaCl for 10 days. Root tissue was harvested, and real-time PCR analysis was performed as detailed in the Materials and Methods section. Expression levels for each indicated gene (a–e) were determined by normalizing to Ubc21 used as internal control. Fold change was calculated using ∆∆Ct method by normalizing the obtained values to WT control roots. Error bars represent mean ± SEM (n = 3) of at least three independent biological replicates, for each gene analyzed. Asterisk represents statistical significance as calculated by Student's \(t\) test and indicated as \(*p < .05, **p < .01, ***p < .001\).


2

+ mutant roots inherently experience higher degree of $K^+$ deficiency under control conditions that are further exacerbated upon saline stress exposure.

**3.4** Enhanced disruption of membrane potential in vps9a-2 mutant exposed to salt stress

It has been described that changes in ionic composition cause an alteration of plasma membrane potential and $aha2$ mutant are shown to display sensitivity to membrane depolarizing conditions such as high external $K^+$ (Haruta et al., 2010). Similar association of plasma membrane depolarization with stress and hormone response has also been reported (Bates & Goldsmith, 1983; Shabala et al., 2015). Since our results show alterations in tissue ionic composition, we next investigated the phenotypes displayed under membrane depolarizing-induced conditions. Membrane depolarization conditions were generated experimentally, as detailed in the Materials and Methods section, and root growth inhibition evaluated for both WT and vps9a-2 mutant lines. In comparison to WT, percentage root growth inhibition in response to 100 mM external KCl was significantly higher in vps9a-2 mutant, $(64.64 \pm 1.79\% \text{ vs. } 90.42 \pm 3.64\%)$ (**$p < .0001$, Figure 4a,c). In order to investigate the status of PM potential upon NaCl stress in vps9a-2 mutant, we used DiBAC$_4$(3), a small negatively charged fluorescent probe that binds PM. Membrane depolarization leads to lower internal negative charge and enhanced probe binding causing an increase in fluorescence intensity. Under control conditions DiBAC$_4$(3) fluorescence was comparable for both genotypes, 1 versus $1.045 \pm 0.06$ (Figure 4d). Exposure to NaCl stress for 30 min leads to an increase in observed DiBAC$_4$(3) intensity in both genotypes (Figure 4b, lower panel); however, in comparison to WT, enhancement was significantly greater for vps9a-2 mutant, $1.715 \pm 0.031 \text{ versus } 3.044 \pm 0.09$ (****$p < .0001$, Figure 4d). These results show that loss of vps9a results in increased membrane depolarization observed by incremented DiBAC$_4$(3) fluorescence intensity) under salt stress conditions and further support the regulatory role of vps9a in pH regulation, maintenance of K$^+$ levels, and membrane potential gradient under these conditions.

**3.5** Anatomical changes in vps9a-2 mutant displaying hypersensitivity to NaCl stress

In order to identify initial events that could trigger hypersensitivity and growth retardation phenotype upon extended exposure to saline stress, we investigated the effects of short-term NaCl stress. WT and vps9a-2 mutant plants were exposed to 6 and 24 hr of saline stress condition and confocal imaging was carried out to visualize anatomical alterations. Roots from vps9a-2 mutant were found to display drastically reduced elongation zone and essentially lacked distinct separation between the differentiation zone bearing lateral roots/root hairs and the meristematic zone (Figure 5a,b and Figure S5a,b). Cells from vps9a-2 mutant display increased swelling at the 24 hr time point; a phenotype that is markedly different in comparison to WT plants. We conclude that vps9a-2 mutant subjected to saline stress conditions undergo early cell deformation and mis-arrangement and appear to be twisted (Figure 5a,b) and these early anatomical changes lead to enhanced hypersensitivity observed upon prolonged exposure to saline stress.

**3.6** vps9a-2 mutant display lower vacuolar acidification under salt stress

The observed inability of vps9a-2 mutant to undergo elongation under NaCl-stress conditions, could either be due to a compromise in the inherent vacuolar expansion capacity or due to changes in the localization of proteins that facilitate maintenance of acidic cell wall pH necessary for cell elongation (Dünser & Kleine-Vehn, 2015). We, therefore, investigated the vacuolar pH of vps9a-2 mutant in comparison to WT plants subjected to 100 mM NaCl stress for 24 hr (Figure 5c). An in-situ calibration curve was generated for WT seedlings subjected to known pH values (Bassil et al., 2013). Excitation ratios at 488/445 nm wavelengths were calculated using the indicated pH standards and a log regression curve was generated (Figure S7a,b). Vacuolar pH determinations were made based on the calibration curve. We observe that in comparison to WT, vps9a-2 mutant epidermal vacuolar pH, under control conditions, was 0.3 pH units lower ($6.57 \pm 0.024 \text{ vs. } 5.77 \pm 0.02$). Upon exposure of WT and vps9a-2 mutant to 24 hr NaCl stress, pH values corresponded to ($5.80 \pm 0.014 \text{ vs. } 5.74 \pm 0.020$) (Figure 5d). Thus, while the WT vacuoles undergo acidification under saline stress, observed change in vacuolar pH ($\Delta$ pH) in vps9a-2 mutant was minimal ($0.257 \pm 0.028$ vs. $0.099 \pm 0.052$) (**$p < .01$, Figure 5e).

**3.7** Apoplast pH determination and alteration of auxin response in vps9a-2 mutant

Thus, far our results demonstrate that vps9a-2 mutant display sensitive phenotype following perturbations in the maintenance of pH gradient. PM H$^+$-ATPases are one of the major players associated with pH maintenance and studies have implicated their important role in the root adaptation to alkaline stress by mediating proton secretion (Haruta et al., 2010).

To determine the influence on the regulation of H$^+$ gradient formation/maintenance, we evaluated the phenotype of vps9a-2 mutant under high pH. Results from these experiments show that vps9a-2 mutant display greater sensitivity to alkalization (pH = 7.2 and 7.6)
In comparison to WT, root growth inhibition was found to be more pronounced in vps9a-2 mutant (34.71 ± 4.10% vs. 60.00 ± 4.07%) and (69.71 ± 2.31% vs. 93.0 ± 4.03%), at pH = 7.2 and 7.6, respectively. These results reveal the alkaline pH sensitivity of vps9a-2 mutant and indicate the role of vps9a on H⁺ gradient maintenance.
PM H⁺-ATPase is regulated by numerous factors under abiotic stress conditions (Palmgren, 2001) and auxins are one of the key factors involved in the regulation of the pump, which in turn leads to cell elongation (Rober-Kleber et al., 2003). Dynamics of auxin response and visualization of spatial auxin response pattern is often monitored using the synthetic auxin-responsive promoter.
increased activity of PM H^+ modulating proton secretion in the root tip to maintain primary root efflux carrier, PIN2, in the adaptation of roots to alkaline stress, by Arabidopsis thaliana mechanisms, requiring the combined action of auxin-efflux carrier vps9a-2 (Figure S6b). In order to identify whether this observed decline in salt stress, we analyzed results following normalization to respective plants under control conditions. We observed that when compared to WT-PIN2 lines, for PIN2-GFP expression following stress conditions, respectively, for WT and vps9a-2 mutant lines. Values represented are mean ± SEM from 4 to 6 plants with **p ≤ .01, ***p < .0001 indicating statistical significance.

As observed, DR5-YFP was maximally expressed in the quiescent center of the root and adjacent columella cells (Figure S6a). Under control conditions, DR5-YFP expression was found to be (100 vs. 86.47 ± 4.70%) in WT and vps9a-2 mutant plants, respectively. Application of 100 mM NaCl stress for 24 hr resulted in severely reduced YFP expression corresponding to (79.81 ± 3.85% vs. 36.61 ± 6.87%), respectively (**p < .001, Figure S6b) in WT and vps9a-2 mutant lines, respectively. These results indicate that despite inherently lower DR5-YFP expression in vps9a-2 mutant, under control conditions, auxin response is additionally attenuated in vps9a-2 mutant plants subjected to salt stress (86.47 ± 4.70% vs. 36.61 ± 6.87%, ***p < .0001, Figure S6b). In order to identify whether this observed decline in DR5-YFP expression in vps9a-2 mutant is, indeed, a response to salt stress, we analyzed results following normalization to respective plants under control conditions. We observed that when compared to WT, there was an enhanced decrease in DR5-YFP levels in salt-stressed vps9a-2 mutant (79.81 ± 3.848% vs. 41.99 ± 9.569%), respectively (**p < .01, Figure S6c). Based on these results, we conclude that the observed reduction in DR5-YFP expression in vps9a-2 mutant has a salt-specific component.

Establishment of auxin gradients is supported by cellular efflux mechanisms, requiring the combined action of auxin-efflux carrier family of (PIN) proteins, comprising of eight members in Arabidopsis thaliana. Earlier evidence has demonstrated the importance of auxin efflux carrier, PIN2, in the adaptation of roots to alkaline stress, by modulating proton secretion in the root tip to maintain primary root elongation. Arabidopsis, pin2 mutants are reported to exhibit the decreased activity of PM H^+-ATPase and depressed root elongation under alkaline conditions (Xu et al., 2012). In view of the reported role of PIN2 in various environmental stress conditions, for the present study, we focussed our analysis on the role of PIN2 in salt-induced root inhibition observed in vps9a-2 mutants.

We tested the effects of vps9a in PIN2 trafficking, by generating PIN2-GFP lines in vps9a-2 mutant background (vps9a-2-PIN2) as described in Materials and Methods and comparing them to corresponding WT lines (WT-PIN2), for PIN2-GFP expression following exposure to 100 mM NaCl stress. We observed that WT-PIN2 lines show pronounced GFP expression in the apical PM region and no lateral expression (Figure 6, left panel). In contrast, vps9a-2-PIN2 lines showed reduced apical PM expression and laterally diffused GFP localization under control conditions (Figure 6b,c) and the fluorescent intensity was (100 vs. 77.43 ± 4.17%) in WT and vps9a-2 mutant (**p < .0001, Figure 6e)). Even though, vps9a-2 mutant line shows an inherently lower PIN2-GFP expression under control conditions, subsequent addition of NaCl stress results in further reduction in PIN2-GFP expression (77.43 ± 4.17 vs. 45.31 ± 1.8%, ***p < .0001, Figure 6e)). We confirmed salt-specific decrease by normalizing obtained values with respective control conditions. As shown, in comparison to WT, exposure to salt stress results in greater reduction in levels of PIN2-GFP expression in vps9a-2 mutant, 80.75 ± 1.25% versus 65.48 ± 2.84%, respectively (**p < .0001, Figure S6d), showing salt-specific part of the response. On the basis of these results, we propose that observed alteration in abundance and mis-localization of PIN2, in vps9a-2 mutant exposed to NaCl stress and consequent failure to establish polarity, necessary for cell expansion in the elongation zone, could be one of the factors that impact PM-ATPase-mediated cell wall acidification.

Since cell wall forms the first point of response to external stress stimulus and further cellular events that mediate subsequent growth, apoplast pH was evaluated under NaCl stress conditions. We used HPTS for determining the extent of cell wall alkalization. HPTS has a true isosbestic point at 415 nm in the excitation spectrum, flanked by two excitation peaks whose respective emission intensities vary based on pH. Consequently, fluorescence intensity at excitation of 445 nm corresponds to the deprotonated form, while excitation at 405 nm detects the protonated form of HPTS. Thus, higher 445/405 ratio can be correlated to alkalization (Overy et al., 1995). In comparison to WT, exposure to 6 hr-NaCl stress resulted in higher cell wall pH in vps9a-2 mutant (Figure 6f,G). The observed increased alkalization could in turn result in growth retardation and susceptibility of vps9a-2 mutant to salt stress.

4 | DISCUSSION

Our results reveal that salt stress exposure causes enhanced root growth inhibition in vps9a-2 mutant in comparison to WT, and this
**FIGURE 6** Phenotype of vps9a-2 mutant under alkaline stress conditions, expression of PIN2:PIN2-GFP in WT or vps9a-2 mutant lines and apoplastic pH measurements following exposure to salt stress conditions. Four-day-old WT and vps9a-2 mutant were subjected to control pH = 5.8 or alkaline stress (pH = 7.2 or 7.6) conditions. (a) Representative plate showing sensitivity of vps9a-2 mutant to alkaline stress (pH = 7.2 or pH = 7.6) in comparison to control (pH = 5.8). (d) Root lengths were measured after 7 days of treatment and root length inhibition (%) under alkaline pH stress was plotted after normalization to respective WT or vps9a-2 mutant plants grown under control (pH = 5.8) conditions. Error bars represent SEM of values obtained for at least 10 plants and the experiment was independently repeated 4-5 times. (b and c) Four-day-old WT expressing PIN2:PIN2-GFP (WT-PIN2) or vps9a-2 mutant expressing PIN2:PIN2-GFP (vps9a-2-PIN2), were subjected to 0 mM NaCl (control) or 100 mM NaCl stress for 24 hr (as indicated). Fluorescence pattern of PIN2-GFP in root tissue from WT (b, c: left panel) and vps9a-2 mutant lines (b, c: right panel). (b) Representative image of a region of cells displaying altered PIN2-GFP polarity in root tissue from WT and vps9a-2 mutant (as indicated). (c) Representative image showing PIN2-GFP signal intensity for the entire root (in pseudo-color). Signal intensity is indicated by the bar on the extreme right. (e) Apical fluorescence intensity was determined and normalized to WT control (taken as 100%), and statistical significance determined by non-parametric t test, is denoted with asterisk. Error bars depict SEM (from 3 to 4 plants). (f) Apoplastic pH was measured using HPTS following 6 hr treatment with 100 mM NaCl in 4-day-old seedlings. Representative confocal microscopy images showing the fluorescence at excitation wavelengths of 445, 405 nm, and merged are shown (left, middle, and right, respectively, panel f). Quantification of fluorescence intensities (445/405) after normalization with WT plants subjected to 100 mM NaCl is shown (g). Statistical significance is denoted by ***p < .001, ****p < .0001
phenotype is rescued in vps9a-complemented plants (Figure 1a,b). It has been reported that the estimated combinatorial effect of high salinity and low K+ could cause Na+/K+ ratios over 1,000-fold, a value that exceeds the K+/Na+ selectivity of K+ channels (Isayenkov & Maathuis, 2019; Maathuis, 1999). Our evaluation of K+ and Na+ status in WT and vps9a-2 mutant lines showed an interesting response to salt stress in vps9a-2 mutant that manifests itself by a decrease in growth rates primarily because of low K+ retention capabilities rather than the conventional avoidance response involving Na+ exclusion or sequestration. 

Ion content determination revealed that Na+ content was comparable under control conditions (0 mM NaCl), in root and shoot tissue from either genotype and similarly the extent of Na+ increase in response to salt stress is similar for both WT and vps9a-2 mutant (Figure 1c,e). However, K+ contents differ substantially between the genotypes and while under control non-stress conditions, vps9a-2 mutant display reduced (~12%) root K+ levels; application of NaCl stress resulted in a significant decrease in K+ content to 18% and 36% in WT and mutant lines, respectively (Figure 1f). Statistical analysis and calculation based on normalization with respective control conditions was performed to evaluate the salt-specificity of these changes. We find that extent of reduction in root K+ levels for both WT and vps9a-2 mutant upon exposure to NaCl stress was similar, (12% and 14%, respectively; Figure S2c). Interestingly, while shoot K+ levels in both WT and vps9a-2 mutant line are similar under control conditions, exposure to salt stress results in greater decrease in shoot K+ levels, 49% versus 67%, respectively (**p < .001, Figure S2d). Based on these results, we conclude that salt sensitivity of vps9a-2 mutant is independent of Na+ cytotoxicity and could instead be due to the exaggerated effects of salt-imposed K+ deficiency. Our results are in concordance with reports demonstrating the correlation of Arabidopsis salt-tolerant accessions with ability to maintain K+ (Sun et al., 2015). Defects in K+ homeostasis of vps9a-2 mutant could be due to differential K+ accumulation capacity (primarily driven by H+-ATPases energized membrane potential gradients and efflux) or distribution mechanisms (Nieves-Cordones et al., 2014). Significantly higher root growth inhibition under exclusively low K+ concentrations, (Figure 2c) and specific cellular perturbations in K+ homeostasis under NaCl stress, confirm the compromised K+ uptake ability of vps9a-2 mutant. Precise maintenance of K+ levels are crucial for metabolic competence of a cell and the observed fluctuations in K+ content could thus have a predominant role in mediating the salt-sensitive response phenotype of vps9a-2 mutant.

Evaluation of endocytosis under low K+ conditions revealed significantly reduced FM4-64 uptake in the absence of vps9a (Figure 2d), that could be functionally complemented by expression of vps9a-GFP, thereby establishing the significant role vps9a in the K+ homeostasis pathway. Membrane trafficking pathways have an important role in controlling abundance of PM proteins during salt stress (Zwiewka et al., 2015). Alterations in abundance of dominant PM localized proteins such as aquaporin and K+ transporters are shown to be regulated by trafficking mechanisms (Hachez et al., 2014; Sutter et al., 2007). Our results suggest a similar role of vps9a in modulating proteins essential for the maintenance of K+ levels. 

In line with these results, the observed Gork transcript induction can cause the higher degree of K+ efflux observed in vps9a-2 mutant roots, correlating with lower root K+ levels under both control and NaCl stress conditions (Figures 1e and 3a). Similarly, 19-fold reduction in root Skor transcript levels under salt stress conditions (Figure 3c) and concomitant 32-fold and 45-fold Hak5 transcript induction in vps9a-2 mutant, was observed, under control and salt stress conditions, respectively (Figure 3e). It has been reported that increased acidification causes decreased Skor currents and pH modulations affect Skor-mediated regulation of K+ secretion into the xylem sap (Lacombe et al., 2000). Given the crucial role of AtHak5 encoded K+/H+ symporter in K+ acquisition especially under internally depleted K+ resources (typical of prolonged duration of stress) (Ahn et al., 2004; Armengaud et al., 2004; Gierth et al., 2005; Nieves-Cordones et al., 2010; Qi et al., 2008; Rubio et al., 2008); our results indicate that vps9a-2 mutant plants can efficiently turn on the high-affinity uptake system and perhaps over the induction of the Hak5 system could impact membrane potential and pH gradient maintenance. Together our results indicate that several of these molecular changes orchestrate vps9a-dependent K+ deficiency and disruption of H+ gradients.

Internal PM is usually negative as compared to extracellular space and intracellular pH gradient is shown to be relatively more alkaline (Sze & Chanroj, 2018). Conditions such as high external KCl and/or alkaline external pH, disrupt pH gradients and consequently membrane potential. aha2 mutant plants are reported to be sensitive to high KCl and alkaline pH (Haruta et al., 2010). Results from our study show that vps9a-2 mutant display higher root growth inhibition in response to both high KCl and alkaline pH. Together with transcript analysis results, this strongly indicates that the disruption of pH gradient could be a causative factor for the observed NaCl-induced sensitivity of vps9a-2 mutant. The absence of vps9a function leads to higher degree of membrane depolarization upon salt stress (Figure 4d), suggesting involvement of vps9a in regulating pH gradients under salt stress.

Many lines of evidence have established endosomal trafficking and vesicle-vacuole fusion to be an important aspect of cellular responses to abiotic stress (Hamaji et al., 2009; Mazel et al., 2003; Walker et al., 1996). Our results with reduced root growth phenotype observed in vps9a-2 mutant suggest that vacuolar expansion could be one such contributory factor. Increased activity of proton pumps such as vacuolar H+-ATPase (V-H+-ATPase) and H+-pyrophosphatase (V-H+-Pase), has been reported to result in enhanced vacuolar acidification under salt stress and application of NaCl (50/100 mM) is reported to result in pH acidification by 0.4–0.5 units (Zhang et al., 2012). Our experiments reveal that unlike WT cells, vps9a-2 mutant cells contain acidic vacuoles under control conditions, and this could be due to enhanced activity of one of the tonoplast pumps. While vacuolar pH in WT cells reduces by 0.3 units under salt stress, no such enhanced acidification was observed in vps9a-2 mutant following 24 hr exposure to salt stress (Figure 5d). AtNHX1 overexpression is reported to cause 2-fold
reduction in cytosolic K+ pools due to enhanced vacuolar K+ sequestration leading to increased Hak5-triggered, high-affinity K+ uptake, and sensitivity to low K+ (Bassil et al., 2018; Leidi et al., 2010; Regel et al., 2019). We observe similar increased Hak5 induction and low K+ sensitivity, together with low K+ content in vps9a-2 mutant. Based on similarly observed, presence of more acidic vacuoles in nhx1/2/3/4 quadruple mutant (Bassil et al., 2011) we speculate that the more acidic pH observed in vps9a-2 mutant is perhaps unrelated to nhx-inactivity, since vps9a-2 mutant also do not show increments in Na+ levels following salt stress. Our results showing differential vacuolar acidification capacities (Figure 5d,e), reveal H+ gradient disruption under salt stress that lead to differences in the extent of cytosolic pH acidification in vps9a-2 mutant. These results imply that aberrations in cytosolic acidification capacity upon the prolonged duration of stress render vps9a-2 mutant sensitive to salt stress.

In order to gain insight into early events upon stress exposure, we evaluated early morphological events that correlate with increased sensitivity and root growth inhibition following 24 hr-NaCl stress (Figure S5). Notably, in comparison to WT, vps9a-2 mutant lines display negligible expansion at the elongation zone (Figure 5a,b). Rates of cell division in the apical meristem and cell expansion in the elongation zone get impacted in response to environmental conditions and result in the inhibition of primary root. Polar auxin transport at the root tip influences primary root growth and is often impacted in adaptive responses to a variety of environmental stimuli such as cold, salinity, drought, metal ion response (Galvan-Ampudia et al., 2013; Grieneisen et al., 2007; Kopittke et al., 2015; Liu et al., 2015; Rahman, 2012; Remy et al., 2013; Ribba et al., 2020; Shibasaki et al., 2009; Sun et al., 2008; Velasquez et al., 2016; Zhang, et al., 2012). Earlier studies have reported that salt stress mediates auxin redistribution and consequent suppression of activity at root meristem (Liu et al., 2015; Zhao et al., 2010; Zhao, et al., 2010). Polar auxin transport (PAT) machinery constituted by AUX/LAX mediate acropetal, shoot to root flow of auxin via the stele and PIN efflux carriers moderate basipetal redistribution, to the root elongation zone (Müller et al., 1998; Rakusová et al., 2015; Zwiewka et al., 2019). PIN1, PIN2, PIN3, PIN4, and PIN7 facilitate cell to cell auxin transport in Arabidopsis roots. It is proposed that auxin delivery in the root cortical and epidermal regions of the elongation zone are largely mediated by PIN2 and mutations in pin2 is reported to cause inefficient basal transport (Billou et al., 2005). Under normal growth conditions polar PIN2 expression creates a differential expression pattern focal to root transition zone causing preferential IAA in the elongation zone, where the activation of H+−ATPase and consequent cell wall acidification promotes cell elongation and growth (Li et al., 2018; Zhang et al., 2017). Additionally, root surface alkalinization and inhibition of cell expansion due to external environmental stress has also been observed (Barbez et al., 2017; Liu et al., 2013; Staal et al., 2011).

Due to the imperative in vivo role of auxin distribution pathways, we first evaluated DR5 expression as a marker to visualize auxin response, to determine whether salt stress exposure is associated with generalized auxin attenuation in vps9a-2 mutant. In comparison to WT, DR5-YFP expression was reduced in vps9a-2 mutant under both control and NaCl treatment (Figure S6b,c). Notably, while there is a 20% reduction in DR5-YFP expression in salt-stressed WT plants, vps9a-2 mutant shows a reduction of 58% in comparison (**p < .01, Figure S6c). Although, these results implicate that despite inherent differences in vps9a-2 mutant, exposure to salt stress has a greater impact in the mutant; whether this is due to altered molecular changes or an outcome of the overall level of robustness of the vps9a-2 mutant remains a matter for further evaluation. Nonetheless, vps9a-2-mediated salt stress response appears to be more strongly impacted in comparison to WT.

Salt treatment is shown to reduce apical PIN2 localization in root epidermal cells due to increased endocytic rate and notably, other PM resident proteins such as PIN1, AUX1, PIN3 or PMA2 did not show any changes in either subcellular localization or internalization under salt stress (Adamowski & Friml, 2015). We observe that exposure to 100 mM NaCl stress for 24 hr resulted in the loss of polarity and reduced abundance of PIN2-GFP in vps9a-2 mutant (Figure 6b,c,e). While vps9a-2 mutants displayed an inherent reduction of PIN2-GFP (28%) (**p < .0001, Figure 6e) even under control conditions, the drop in PIN2-GFP levels following NaCl treatment in WT and vps9a-2 mutant were 20% and 36%, respectively (**p < .0001, Figure S6d). These results suggest that delivery of PIN2 to the PM could be differentially adjusted in vps9a-2 mutant and vps9a-mediated response to salt stress could utilize PIN2 as one of the factors to mediate cell elongation. Though our results indicate the contributory role of vps9a in modulating PIN2 abundance, the role of other PIN proteins like PIN1, PIN3, PIN4 or PIN7 as individual or in concert with PIN2 cannot be excluded. Furthermore, while salt stress exposure results in a more acute phenotype in vps9a-2 mutant, the distinction between this being an effect of co-ordinated reduction in molecular response or an outcome of inherently lower PIN2 level in mutant remain a matter for further evaluation.

Phenotypically, stress response to environmental changes is reflected as a decrease in primary root growth elongation. Specific mechanisms that facilitate PIN2 anchoring to PM and govern PIN2 abundance are not completely known (Łangowski et al., 2016). Our data reporting observations on altered PIN2 localization indicate an interesting aspect of vps9a-2 mutant, though molecular components downstream of vps9a, that link to the maintenance of PIN2 abundance under salt stress require further elucidation. Phosphorylation status and membrane sterol composition are described as essential for augmenting PIN2 polarity and function (Betts & Moore, 2003; Feraru et al., 2012; Nakamoto et al., 2015; Wang et al., 2019; Zhang et al., 2020). Specific enhancement in the
conversion of sitosterol to stigmasterol, under NaCl treatment has been reported in several plants and sterol pathways could link to vps9a-mediated response (Abooobucker & Suza, 2019). Interaction of Sand/Mon1 with Ccz1 generates the Mon1-Ccz1 (MC1) complex that functions as Rab7 GEF in yeast (Nordmann et al., 2010). Sand is an effector of GTP-bound Ara7 (Rab5) and together with Ccz1 leads to the activation of Rab7, RA3f in Arabidopsis (Singh et al., 2014). Rab7 activation is critical for vacuolar biogenesis and plant growth (Cui et al., 2014; Langemeyer et al., 2020). Several studies report Rab7 induction under salt stress and Rab7 over-expression renders salt tolerance capacity (Agarwal et al., 2007; Sui et al., 2017). Based on these and our results, it may be reasonable to speculate that lack of Rab7 activation could mediate salt-sensitive phenotype of vps9a-2 mutant.

As proposed by the "acid growth hypothesis" acidic cell wall pH has a positive impact on cell expansion capacity and is greatly influenced by auxin levels (Cosgrove, 2015; Dolan & Davies, 2004). Cellular response to auxin that regulates growth under abiotic stress conditions, involves active apoplastic acidification and alkalization and reduction in auxin levels, perception, or signaling abolish both apoplastic acidification and cellular expansion, thus impacting growth (Barbez et al., 2017; Felle, 2001). Our results reveal cell wall alkalization in vps9a-2 mutant indicates the role of vps9a in auxin-mediated root growth reduction under salt stress. A biphasic response in roots comprising of transient apoplastic alkalization followed by the restoration of acidic pH is reported and cells unable to undergo transient alkalization also fail to display growth inhibition (Barbez et al., 2017). We found that by 6 hr of exposure to 100 mM NaCl, vps9a-2 mutant shows greater degree of apoplastic pH alkalization in comparison to WT plants (Figure 6f,G) which extends to growth inhibition phenotype following 24 hr exposure (Figure 55b).

In summary, our studies reveal the important role of vps9a in maintaining K+ levels in root and shoot tissues through pathways modulating pH gradient. At the cellular level, NaCl stress imposed, root growth retardation phenotype associates with compromised elongation ability of the root elongation zone. Correlating with the lack of cell growth is a reduction in DR5 expression and loss of PIN2 polarity concomitant with increased cell wall alkalization in vps9a-2 mutant. Our results thus emphasize the importance of K+ homeostasis mechanisms, identify the role of H+ gradient maintenance, and suggest altered DR5 expression and PIN2 polarity as one of the functional components in vps9a-mediated response.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

AUTHOR CONTRIBUTIONS

D.R. performed experiments and analyzed data. D.R. and M.K.M. conceptualized, the project, wrote and edited the manuscript.

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