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One-time abscisic acid priming induces long-term salinity resistance in *Vicia faba*: Changes in key transcripts, metabolites, and ionic relations

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Abstract

Abscisic acid (ABA) priming is known to enhance plant growth and survival under salinity. However, the mechanisms mediating this long-term acclimatization to salt stress are still obscure. Specifically, the long-term transcriptional changes and their effects on ion relations were never investigated. This motivated us to study the long-term (8 days) effect of one-time 24 h root priming treatment with 10 μM ABA on transcription levels of relevant regulated key genes, osmotically relevant metabolites, and ionic concentrations in *Vicia faba* grown under 50 mM NaCl salinity. The novelty of this study is that we could demonstrate long-term effects of a one-time ABA application. ABA-priming was found to prevent the salt-induced decline in root and shoot dry matter, improved photosynthesis, and inhibited terminal wilting of plants. It substantially increased the mRNA level of *AAPK* and *14-3-3* ABA inducible kinases and ion transporters (*PM H^+-ATPase*, *VFK1*, *KUP7*, *SOS1*, and *CLC1*). These ABA-induced transcriptional changes went along with altered tissue ion patterns. Primed plants accumulated less Na^+ and Cl^- but more K^+, Ca^{2+}, Zn^{2+}, Fe^{2+}, Mn^{2+}, NO_3^-, and SO_4^{2-}. Priming changed the composition pattern of organic osmolytes under salinity, with glucose and fructose being dominant in unprimed, whereas sucrose was dominant in the primed plants. We conclude that one-time ABA priming mitigates salt stress in *Vicia faba* by persistently changing transcription patterns of key genes, stabilizing the ionic and osmotic balance, and improving photosynthesis and growth.

1 | INTRODUCTION

Salinity is characterized by excessive soluble salts in the growth medium, which impose osmotic and ionic stress in plants (Munns & Tester, 2008). Key processes of photosynthesis, respiration, and transpiration are affected, thus limiting shoot and root growth (Munns & Tester, 2008). Field bean (*Vicia faba*) grown with an excess of sodium chloride (NaCl) has been reported to show severe growth retardation due to a high accumulation of Na^+ that interferes with K^+ uptake (Slabu et al., 2009). It possibly disrupts the regulation of cytoplasmic enzymes and stomatal conductance (Isayenkov & Maathuis, 2019). Moreover, in *Vicia faba* grown under NaCl stress, higher accumulation of chloride (Cl^-) in the chloroplast is thought to impair the photosynthetic machinery and thus to induce chloroplast degeneration leading to chlorosis or necrosis of the leaf tissue (Geilfus, 2018). To resist salt stress, plants have evolved mitigating mechanisms at different organizational levels. Many of these

mechanisms, for example, stomatal regulation, various morphological modifications, osmolyte accumulation, exclusion of salt ions by roots, and vacuolar partitioning of excess ions, are activated by salt stress, increasing resistance following the stress event. Hence, to pre-emptively strengthen the salt resistance in plants, it is vital to activate the resistance mechanisms before the onset of salt stress.

Priming represents a sustainable, fast, cost-effective, and environmental-friendly procedure for crop production under stressed environments (Tanou et al., 2012). The priming process is referred to as pre-treatment of plants, seeds, or roots, with mild stressor agents to activate resistance responses in plants to future stress exposure (Tanou et al., 2012). Priming induces physicochemical and transcriptional adaptations, which allow plants to react faster and more robustly after they encounter biotic or abiotic stress (Savvides et al., 2016). So far, numerous priming agents, such as salicylic acid, β-amino butyric acid, kinetin, or abscisic acid (ABA), have been used to enhance resistance against drought, cold, or salt stress (Savvides et al., 2016).

ABA is considered a stress hormone, and its rapid accumulation in plants under various abiotic stresses shows its involvement in stress resistance of plants (Geilfus et al., 2018; Zörb et al., 2013). Long-term root application of ABA to stressed plants has shown to improve drought resistance in rice (Teng et al., 2014), salt-resistance in potato (Etehadnia et al., 2008), and common bean (Khadri et al., 2007). These studies indicated that the improvement in growth of salt-stressed plants induced by exogenously applied ABA was related to decreased sodium (Na^+)-to-potassium (K^+) ratio and chloride (Cl^-) accumulation, and increased accumulation of proline or soluble sugars. Finkelstein (2013) reported that ABA regulates transcription of 1–10% of the *Arabidopsis* genome. Thus, we expect that ABA priming induces salt stress resistance in plants by changing the expression patterns of genes relevant to salt ion exclusion or inclusion. Previous studies on the effect of ABA priming on salinity resistance did not examine the expression of genes that are involved in salt resistance together with critical ionic load in the tissues. Thus, we hypothesized that a one-time 24 h ABA priming event induces long-term salt resistance via: (1) long-term effects on transcription of genes involved in salt stress signaling and ion regulation, (2) stabilizing ionic and osmotic relations, and (3) enhancing photosynthesis and plant growth. Therefore, we studied the long-term effects of short-term ABA priming on the mRNA levels of ABA-inducible kinases (ABA activated protein

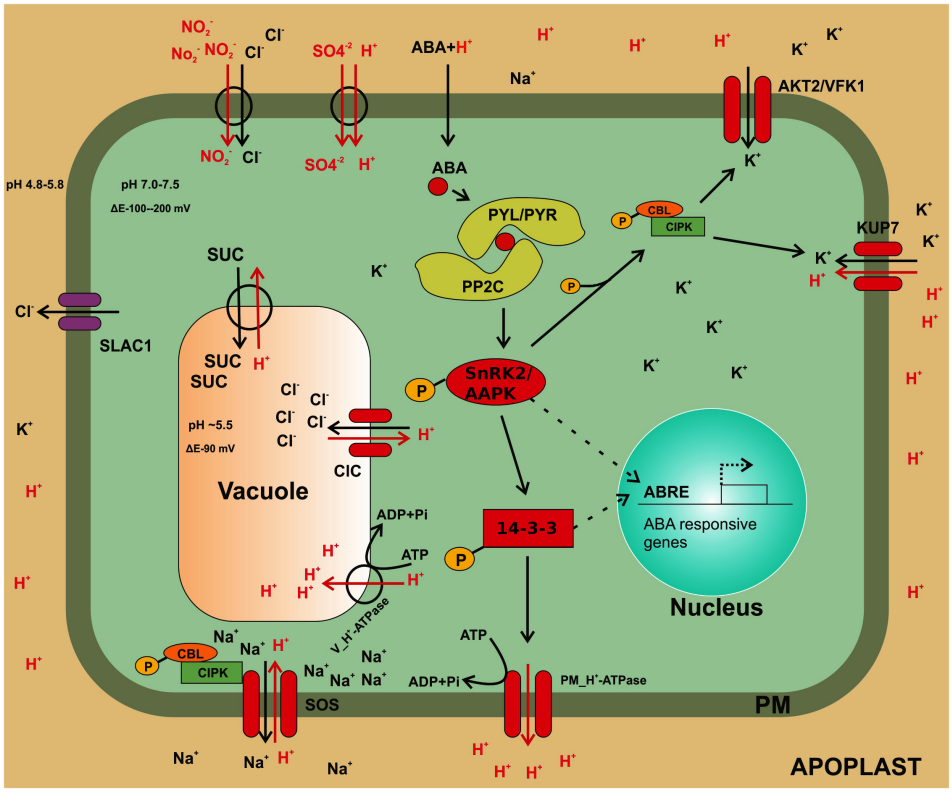

FIGURE 1 Schematic illustration of the current knowledge about transporters, ion channels, regulatory proteins, and signaling molecules are involved in ion regulation under salt stress and being affected by ABA priming. The increased expression after ABA priming is highlighted in red color. ABA receptors in the cytoplasm and nucleus activate ABA-dependent signaling cascades via primary enhanced expression and activation of AAPK/SnRK2 (ABA dependent kinase), which may subsequently phosphorylates other signaling kinases like 14-3-3, CIPK, CBL, CDPK, MAPK, etc. These components, along with SnRK2, may phosphorylate ABA-responsive elements like ABRE, ABF2 (transcription factors), which may further activate expression of stress response genes, and ion transporters for stress adaptation. Interaction of 14-3-3 with the plasma membrane (PM) H^+-ATPase may increase the proton-pumping activity and creates an electrochemical H^+ gradient across the PM, which may lead to membrane hyperpolarization, energizes secondary transport and activates voltage-dependent ion channels like CLC1, SLAC1, AKT2/3 for ion transport. The salt exclusion transporter SOS1 and the potassium transporter HAK/KUP is activated via phosphorylation by CIPK and CBL and by protonation. ABA priming is suggested to enable an increased activation of these interacting mechanisms helping plants to acclimate to salt stress

kinase [AAPK] and 14-3-3), which are important regulators of both ABA responsive genes and activators of transport proteins involved in ion regulation. The expression of important membrane ion transporter genes (viz. plasma membrane [PM] proton ATPase, potassium transporter, sodium antiporter and chloride channel) was also studied (for a visual explanation of the studied molecular mechanisms, see Figure 1). The priming-induced changes in ion transport expression were associated with ion relations and osmolyte composition under salinity stress. Based on this, we tested whether these changes were associated with a stabilized leaf water content, improved photosynthesis, and plant growth under salinity stress.

2 | MATERIAL AND METHODS

2.1 | Plant cultivation, ABA priming, and salinity treatment

Vicia faba L. (cv. Sirocco: NPZ GmbH, Hohenlieth, Germany) was cultivated under hydroponic culture condition in a controlled climatic chamber (14/10 h day/night; 20/15°C; 50/60% humidity). Seeds were surface sterilized with 0.5% sodium perchlorate for 3 min and then washed thoroughly with deionized water. Afterward, seeds were soaked in aerated 0.5 mM $CaSO_4$ solution overnight and sown in moist sterilized quartz sand for germination. After 1 week, uniform-sized seedlings were transferred to 2.5-L plastic pots containing a quarter strength of the nutrient solution (Geilfus & Mühling, 2013). To avoid any osmotic shock reaction, the concentration of the nutrient solution was progressively increased to ½, ¾, and full-strength on second, third, and fourth day after transplantation, respectively. The nutrient solution was changed after every 2 days to replenish the depleted nutrients. Unprimed and ABA-primed plants were grown without (0 mM) and with 50 mM sodium chloride (NaCl). Thus, the experiment had a two-factor factorial design in a completely randomized arrangement with four independent pot replicates for each treatment. Before induction of salinity treatment, 2 weeks old plants were pre-treated with ABA by adding the hormone to the nutrient solution (for the timing of treatments, see Figure 5).

For ABA priming, plants growing in nutrient solutions were supplied with 10 μM of ABA for 24 h, while the unprimed plants did not receive an ABA pre-treatment. Fifteen-day (fourth leaf stage) old unprimed and ABA-primed plants were subjected to 0 and 50 mM NaCl salinity in the nutrient solution. The salinity level of 50 mM was increased progressively for 2 days to avoid sudden osmotic shock. Thereafter, the nutrient solution, without (control treatment) and with the required concentration of NaCl salt (50 mM NaCl treatment), was changed after every day to replenish the depleted ions. After 8 days of growth under non-saline or saline conditions, plants were harvested, and roots and leaf samples were separately collected. Leaf and root samples were thoroughly washed with deionized water to remove the adhering ions from the surface, dried at 60°C for 72 h, and the dry weights were recorded. The dried materials were ground to a fine powder and used for mineral analysis. Fresh plant materials were used to analyze the abundance of certain transcripts. For this, the second batch of plants was grown at the same time under identical conditions. After washing with deionized water, fresh plant materials were shock-frozen in liquid N_2 and stored at −80°C until analysis.

2.2 | Gas exchange measurements

Gas exchange measurements were carried out on intact, fully expanded third-youngest leaves before harvesting. Photosynthetic rate (μmol CO_2 m^{-2} s^{-1}) and stomatal conductance (mol H_2O m^{-2} s^{-1}) were measured with an open flow portable photosynthesis system (LI 6400XT, Li-COR Biosciences Inc.). For measurements, leaves were placed across a 2 × 3 cm leaf chamber. The conditions for the measurements inside the leaf chamber were maintained identical to the external conditions of the climatic chamber. The light was provided by a LED red light source built into the top of the leaf chamber (250 μmol quanta m^{-2} s^{-1}), and the CO_2 concentration was controlled by Li-Cor LI-6400 CO_2 injection system (400 μmol CO_2 mol^{-1}) as identical to outside conditions.

2.3 | Non-invasive leaf water content (LWC) measurement

Non-invasive *in planta* measurement of leaf water content was started 2 days before the onset of ABA priming and continued till the day of harvest. A circular area of 2–3 cm diameter in the middle of the fourth leaf was selected for daily measurements. For the quantification of leaf water content, a ratiometric near-infrared (NIR) transmission setup was employed. The measurement uses the ratio in absorption at 1450 nm (strong absorption by liquid water) and 1050 nm (non-absorbing wavelength), which vary with leaf water content (Zhang et al., 2019). The custom-made water sensor (developed by H. Kaiser) device had a dual infrared LED emitter mounted on top of a leaf clip by a 45° angle and a photodiode collecting transmitted light below the leaf. A data logger was used to control the device and record data. For calibration of the water sensor, several detached *Vicia faba* leaves were placed in deionized water at 4°C for overnight. On the following day, NIR transmission ratio was determined at three-time intervals (0, 5, 10 min) during air drying of these leaves. Simultaneously, water content was measured by the gravimetric method. The linear relation between NIR transmission ratio and gravimetric water content was used to convert the sensor data into water content (g m^{-2}).

2.4 | Mineral analysis of plant tissue

For mineral analysis, oven-dried and finely ground leaf and root samples (200 mg) were digested with 10 ml of 69% HNO_3 (ROTIPURAN Supra for ICP, 69%) in a closed-vessels microwave digestion system (MARS 6 Xpress, CEM Corporation) adjusted to the following conditions: 2 min at 100°C, 1 min at 120°C, 20 min at 180°C and 20 min cooling time. Afterward, the digested samples were diluted with

Milli-Q water (18.2 MΩ cm conductivity) to 100 ml and stored at 4°C until further analysis. Concentrations of Na and macronutrients (K and Ca) and micronutrients (iron, Fe; manganese, Mn; zinc, Zn) were measured by inductively coupled plasma mass spectroscopy (ICP-MS; Agilent 7700, Agilent Technologies Inc.) as described by Jezek et al. (2015).

2.5 | Determination of free sugars and inorganic anions

Water-soluble sugars and free inorganic anions were extracted by hot water following the procedure of Cataldi et al. (2000) with minor modifications. Dried and powdered leaf and root samples (~ 20 mg) were dissolved and boiled for 5 min in 1.5 ml of sterile deionized water, mixed thoroughly by vortexing and immediately placed on an ice-water bath for 30 min. Then mixtures were centrifuged, and the supernatant was collected. For the precipitation of proteins, the supernatants were mixed with chloroform and centrifuged. Again, the supernatant was collected and cleaned by passing through strata C-18 columns (Phenomenex, Torrance). Afterward, both the anions and sugars were determined by isocratic ion chromatography (IC-5000 Capillary Reagent- Free IC System, Thermo Scientific).

2.6 | Primer design and Sanger sequencing

For analyzing the mRNA transcript level of ABA inducible kinases *AAPK* and *14-3-3*, and ion transporter genes PM H^+-ATPase *isogenes* (*vha2*, *vha4*, *vha5*), and *Vicia faba potassium channel1* (*VFK1*), primers were designed from sequences available on NCBI database for *Vicia faba*. Since genome sequences of salt overly sensitive1 (*SOS1*), *potassium uptake permease 7* (*KUP7*), and *chloride channel1* (*CLC1*) are not annotated for *Vicia faba*, *Fabaceae* family-specific primers were designed based on conserved regions: The coding sequences (CDSs) of genes of interest from *Glycine max*, *Vigna radiata*, *Cicer arietinum*, and *Medicago truncatula* were retrieved from the NCBI database (https://www.ncbi.nlm.nih.gov/nuccore/). Thereafter, multiple CDS sequences were aligned by using the CLC workbench tool (https://www.qiagenbioinformatics.com/products/clc-genomics-workbench/), and the homologous conserved region was chosen for primer design. A pair of primers were designed from the conserved aligned sequence for each gene by manual selection of oligonucleotide sequences on the CLC workbench tool. Characteristics of selected primers were checked and evaluated in silico by the online tool oligo calc (http://biotools.nubic.northwestern.edu/OligoCalc.html) and multiple primer analyzer tools provided by Thermo-Fisher. All primer oligos were purchased from Eurofins Genomics (Ebersberg, Germany). The details of the primers are listed in Table S1. All the primers were tested and validated by PCR amplification to ensure that (1) they work in *Vicia faba* and (2) there are specific to the targeted cDNA. The thermal-cycling program was performed as follows: initial denaturation at 95°C for 3 min, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 55–60°C for 30 s, and extension at 72°C for 30 s and final extension for 5 min at 72°C. PCR products obtained were run along with 1 kb DNA ladder (Gene ruler, Thermo Fisher Scientific) on 1% TBE agarose gels to ensure the correct length of the amplicon. Furthermore, amplification of the correct gene was confirmed by the sequencing of the amplicon (Sanger sequencing, Instituts für Klinische Molekularbiologie) and comparison with the BLASTN nucleotide collection database (Table S2).

2.7 | Gene expression analysis

Total RNA of leaves and roots were extracted using TRIzol (Invitrogen) according to the manufacture's protocol. The concentration and purity of RNA were determined by a NanoVue Plus Spectrophotometer (GE Healthcare Life Science), and integrity was checked by gel electrophoresis. Total coding RNA (1 μg) isolated was reverse transcribed following the manufacturer's instructions (Verso cDNA synthesis kit, Thermo Fisher Scientific, USA), including DNase I treatment. The quality of synthesized cDNA (1/10 dilution) was checked by standard PCR with the most stable housekeeping gene *Vf Cyclophilin* (*CYP*) (Gutierrez et al., 2011; Neuhaus et al., 2013). Quantitative RT-PCR was conducted by PowerUp™ SYBR™ Green Master Mix (Applied Biosystems) with primers shown in Table S1 on CFX96 Real-Time System (Bio-Rad Laboratories GmbH, München, Germany). Each reaction (20 μl) contains 100 nM of each primer, 2 μl of diluted cDNA templates, and other reaction components. After an initial denaturation step (95°C for 5 min), RT-qPCR was carried out over 40 cycles (95°C for 15 s, 60°C for 30 s, 72°C for 30 s), followed by a melt curve stage (95°C for 15 s, 60°C for 1 min, 95°C for 15 s, 24°C for 15 s). Three biological replicates and three technical replicates were used for each treatment. Transcript levels of gene were normalized with endogenous control (*Vf CYP*), and the expression changes of target mRNAs were determined using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001).

2.8 | Statistical analysis

Data were statistically analyzed following two-way ANOVA, which was performed using SPSS software (version 17.0). Significant differences among the means were determined by Tukey's HSD test at $P \leq 0.05$.

3 | RESULTS

3.1 | Growth, dry matter, and gas exchange attributes

Under saline condition, plant height and root growth were severely stunted in unprimed plants, while this effect was not observed for ABA-primed plants (Figure 2). Leaf dry matter of unprimed plants

decreased by 33% under saline condition as compared to non-saline condition (Figure 3A). However, ABA-primed plants did not show a decrease in leaf dry matter accumulation under saline condition. Similarly, reduction in root dry matter of unprimed plants under salinity was 21% as compared to non-saline condition. In contrast, salinity did not affect the root dry matter accumulation of ABA-primed plants (Figure 3B).

Under non-saline conditions, the photosynthetic rate of unprimed and ABA-primed plants was similar. Under saline condition, photosynthetic rate of unprimed plants declined by 45%, while that of ABA primed plants decreased by only 10% (Figure 4A). ABA priming under non-saline condition decreased stomatal conductance, measured after 4 h of light, by 27% as compared to unprimed plants (Figure 4B). However, under saline condition, ABA primed plants had a 53% higher stomatal conductance than unprimed plants.

3.2 | Leaf water content

In general, leaf water content (LWC) showed an increasing trend during the 8 days of observation, possibly due to ongoing maturation (Figure 5). ABA priming caused a significant extra increment of ca. 10% compared to the unprimed treatments. Under salinity, LWC in the unprimed plants increased at a high rate to a maximum on day 5, and onward to that rapidly decreased till the end of the experiment when visual wilting was observed. This decline was most likely caused by visual dieback of the fine roots, effectively impeding water supply to the shoot. The ABA-primed plants under salinity neither showed the large initial increase nor the later rapid decline in LWC; instead, they responded mainly parallel to the unprimed and primed non-saline treatments.

3.3 | Concentration of minerals in leaves and roots

Under saline conditions, Na^+ concentration increased 11- and 18-fold, respectively, in leaves and roots compared to the values recorded under non-saline conditions (Figure 6A,B). ABA priming under saline condition decreased leaf and root Na^+ concentration by a factor of 0.60 and 0.44, respectively. Potassium concentration in leaves and roots of unprimed plants showed a 17 and 30% decrease, respectively, under saline condition compared to non-saline condition (Figure 6C,D). ABA-primed plants did not show this salinity-induced decline in K^+ concentration in both the tissues. Salinity decreased Ca^{2+} concentration in leaves of both unprimed and ABA-primed plants by 28%, compared to non-saline unprimed plants (Figure 6E). Similarly, the root Ca^{2+} concentration declined by 25% under salinity in unprimed plants, in contrast to primed plants, which sustained their Ca^{2+} concentration (Figure 6F).

Salinity, ABA priming, and their interaction in some cases (Figure 7) significantly affected micronutrient (Zn^{2+}, Fe^{2+}, and Mn^{2+}) concentrations in leaves but not in roots. Under non-saline condition, unprimed and ABA-primed plants had similar Mn^{+2} concentration in both tissues, while Zn^{2+} and Fe^{2+} concentration in leaves were 16% and 32% higher in ABA-primed plants (Figure 7A,C,E,G). Salinity generally decreased micronutrient concentrations in leaves, but more so in unprimed than ABA-primed plants. Consequently, in leaves, ABA priming restored Zn^{2+} and Mn^{2+} (Figure 7A,E) and improved Fe^{2+}

FIGURE 2 Effect of ABA priming on plant height and root morphology of *Vicia faba* grown without or with 50 mM NaCl salinity. The 24-h lasting ABA priming with 10 μM ABA at the roots was done 1 day before application of salt stress and 8 days before pictures were taken

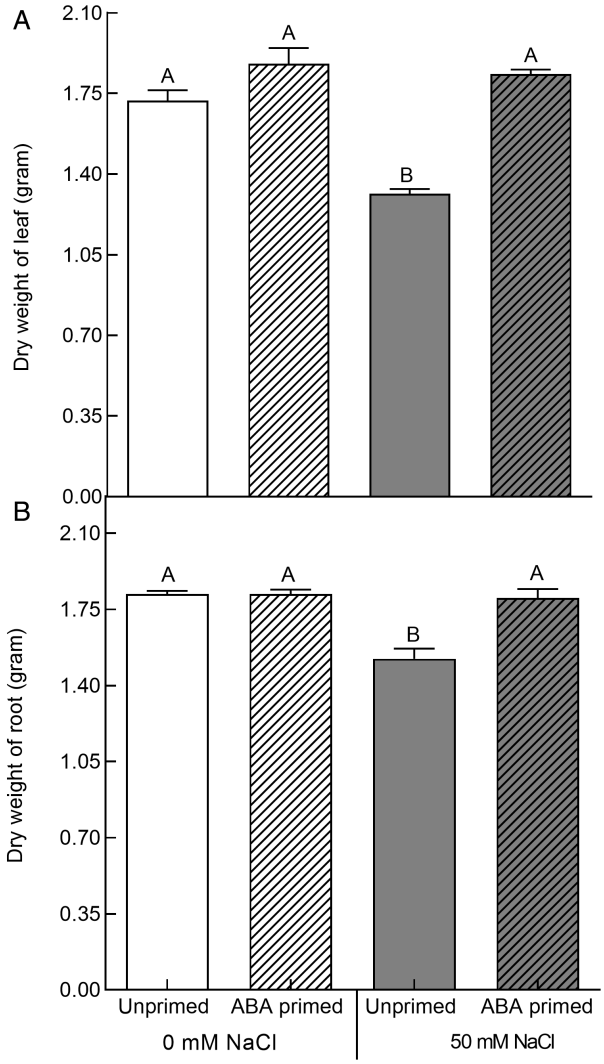

FIGURE 3 Effect of ABA priming on the leaf (A) and root (B) dry matter of *Vicia faba* L. grown without or with 50 mM NaCl salinity. The data ± SE are means of four independent pot replicates. Different letters on data bars indicate significant differences among the treatments at $P \leq 0.05$

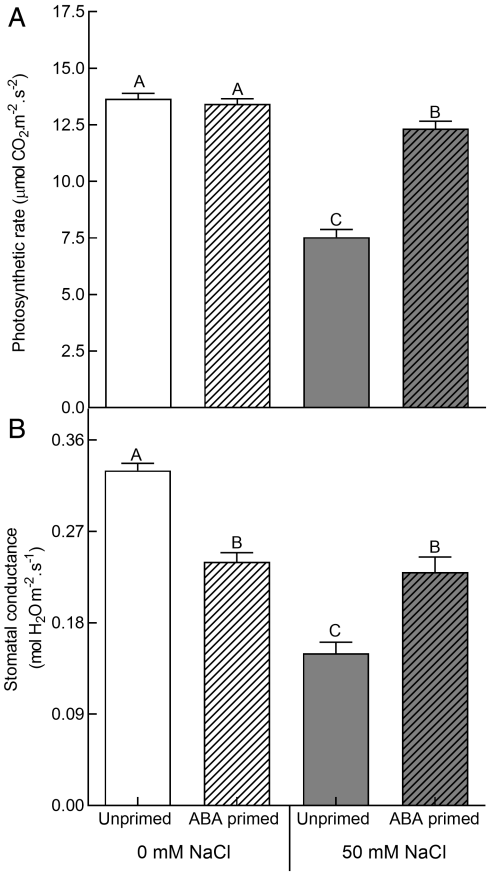

FIGURE 4 Effect of ABA priming on photosynthetic rate (A), and stomatal conductance (B) of *Vicia faba* L. grown without or with 50 mM NaCl salinity. The data ± SE are means of four independent pot replicates. Different letters on data bars indicate significant differences among the treatments at $P \leq 0.05$

concentration by 82%, as compared to unprimed plants under saline condition (Figure 7C).

Salinity increased Cl^- concentration in leaves and roots of unprimed plants by 4.6- and 5.1-fold, respectively (Figure 8A,B). ABA-priming decreased Cl^- concentration in leaves and roots by a factor of 0.60 and 0.53, respectively, compared to the unprimed plants (Figure 8A,B). Salinity decreased nitrate (NO_3^-) and sulfate (SO_4^{2-}) concentrations in leaves by 80 and 37%, respectively, in unprimed plants (Figure 7C,E). ABA priming doubled the NO_3^- and SO_4^{2-} concentrations in leaves as compared to unprimed plants under saline condition (Figure 8C,E). Under salinity, NO_3^- concentration in roots decreased by 69% in unprimed plants as compared to non-saline unprimed. In comparison, ABA priming improved NO_3^- concentration in roots by 150% as compared to saline unprimed plants (Figure 8D). Concentrations of SO_4^{2-} in roots were neither affected by salinity nor by ABA priming under saline condition (Figure 8F).

3.4 | Accumulation of organic osmolytes by leaves and roots

In leaves, the salinity response of unprimed plant was dominated by three- and twofold increase in glucose and fructose concentrations, respectively (Figure 9A,C). The sucrose concentration, however, remained unchanged. Priming inverted this pattern: it reduced the fructose and glucose concentrations back to the level of non-saline conditions and led to a ca. twofold increase in sucrose concentration, effectively shifting the dominant sugars in osmotic adjustment from fructose and glucose to sucrose (Figure 9A,C,E). A similar shift to sucrose as prevalent sugar was observed in the roots, where salinity halved the sucrose concentration in roots of unprimed plants and priming led to a slight increase under salinity. At the same time, the salinity-induced increase in glucose and fructose concentrations was a little (fructose) or absent in ABA-primed plants (Figure 9B,D,F).

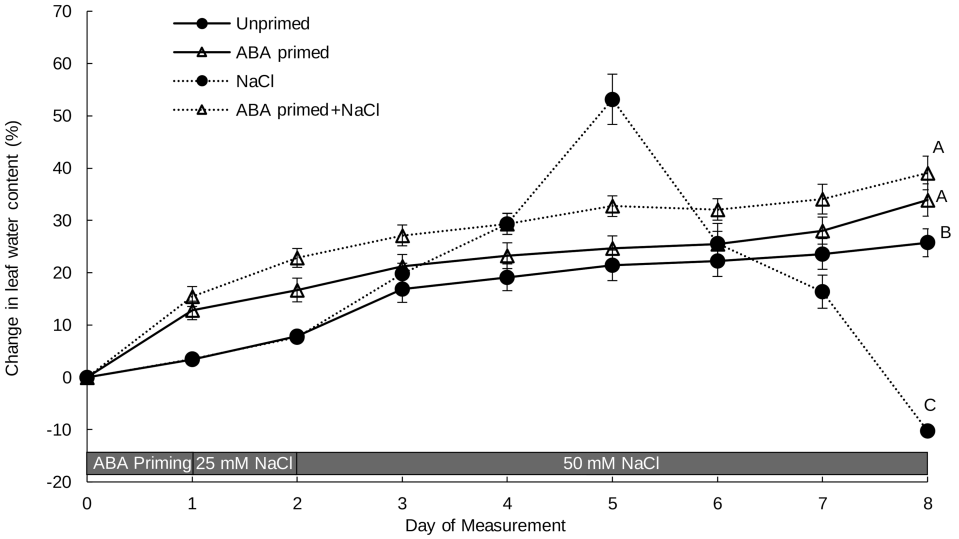

FIGURE 5 Change (%) in leaf water content relative to the initial LWC of *Vicia faba* L. grown under non-saline and 50 mM NaCl saline nutrient solution. The data ±SE are means of seven independent pot replicates. The different letters at the eighth day of measurement show significant differences at $P \leq 0.05$

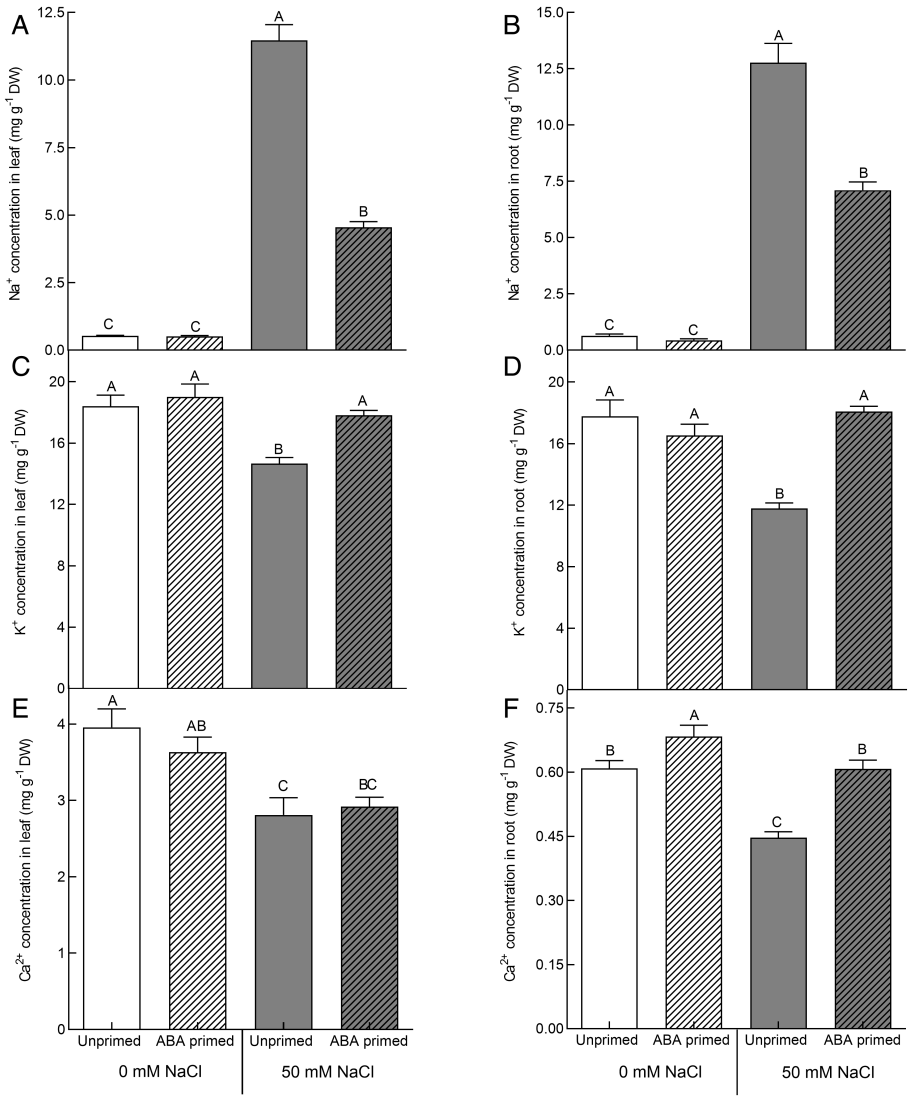

FIGURE 6 Effect of ABA priming on the concentration of Na^+ (A, leaf; B, root), K^+ (C, leaf; D, root), and Ca^{2+} (E, leaf; F, root) by *Vicia faba* L. grown under non-saline and 50 mM NaCl saline nutrient solution. The data ± SE are means of four independent pot replicates. Different letters on data bars indicate significant differences among the treatments at $P \leq 0.05$

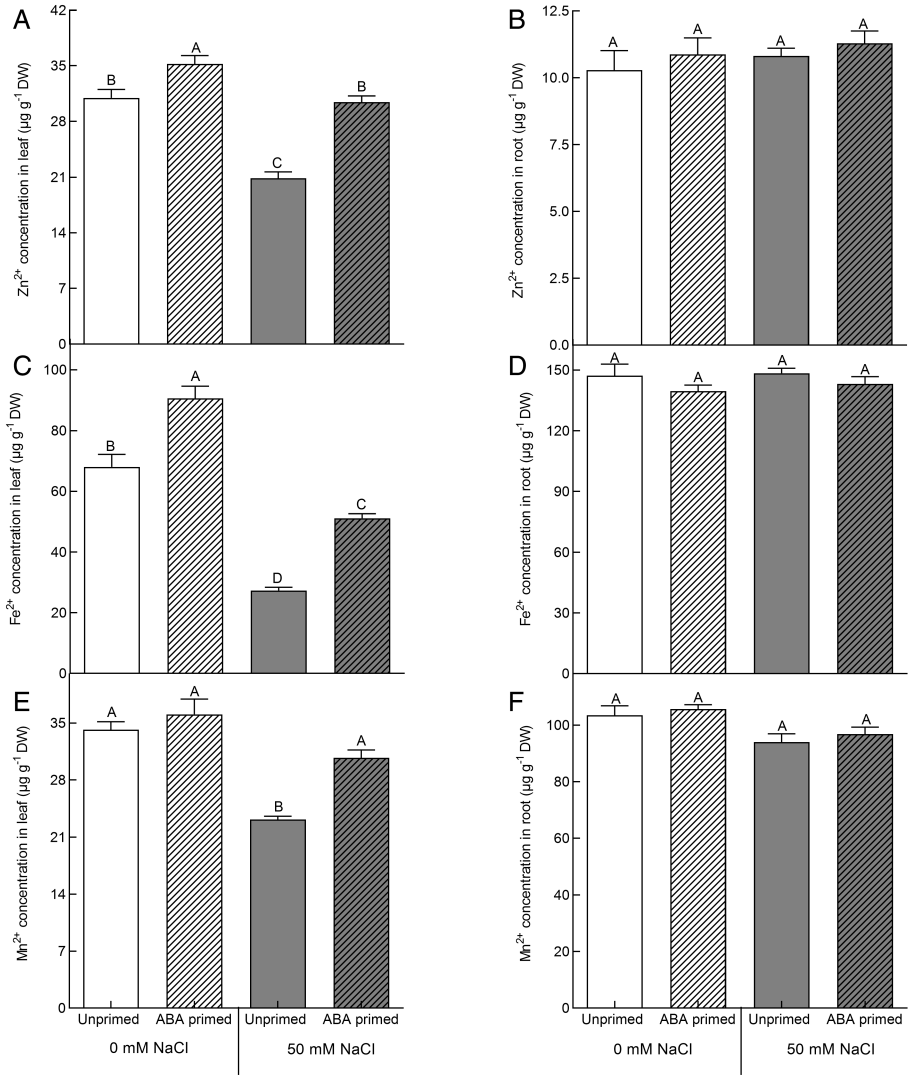

FIGURE 7 Effect of ABA priming on the concentration of Zn^{2+} (A, leaf; B, root), Fe^{2+} (C, leaf; D, root), and Mn^{2+} (E, leaf; F, root) by *Vicia faba* L. grown under non-saline and 50 mM NaCl saline nutrient solution. The data ±SE are means of four independent pot replicates. Different letters on data bars indicate significant differences among the treatments at $P \leq 0.05$

3.5 | Relative transcript abundance of ABA-inducible kinase and membrane transporters genes

Under saline conditions, ABA-primed leaves and roots showed significantly higher *AAPK* transcripts levels than unprimed plants (Figure 10A,B). Irrespective of ABA priming and salinity, 14-3-3 transcript remained unchanged in leaves (Figure 10C). However, in roots, ABA priming increased the mRNA expression of 14-3-3 by a factor of 3.2 under non-saline and 2.8 under saline condition compared to unprimed plants (Figure 10D).

The mRNA expression of membrane ion transporters *PM H^+-ATPase isogenes* (*vha2*, *vha5*), *KUP7*, *VFK1*, *SOS1*, *and CLC1* was decreased under saline condition (Figures 11 and 12). In leaves under saline condition, ABA priming clearly increased transcript levels, restoring those of *vha2* and *vha5* to non-saline levels and doubling the mRNA expression of *vha4* compared to unprimed plants (Figure 11A, C,E). Similarly, in roots, ABA-primed plants showed significantly higher mRNA expression of all three isogene transcripts than unprimed plants (Figure 11B,D,F). Under salt stress, the relative mRNA expression of *KUP7* was only affected in leaves, where unprimed showed suppression of transcript, while ABA-primed restored it to the level of non-saline treatment (Figure 12A,B). Moreover, 1.8-fold higher mRNA expression of *KUP7* in roots of ABA-primed versus unprimed plants was observed under non-saline condition only (Figure 12B). Additionally, *VFK1* mRNA abundance significantly increased by both salinity and ABA priming treatments in leaves (Figure 12C), 2.8-fold upregulation in *VFK1* was recorded in ABA-primed plants as compared to unprimed plants under non-saline condition. In roots, only ABA-primed plants showed significant upregulation compared to unprimed plants under saline condition (Figure 12D).

In leaves, *SOS1* mRNA expression was upregulated by salinity but not ABA priming (Figure 12E,F). In roots, ABA priming upregulated the mRNA expression of *SOS1* by 1.0- and 1.5-fold under saline and non-saline condition, respectively, than the unprimed counterparts. The relative mRNA expression of the *CLC1* was upregulated in ABA-primed plants in both tissues, but more prominently in the roots, 2.82- and 6.5-fold as compared to unprimed plants under non-saline and saline conditions, respectively.

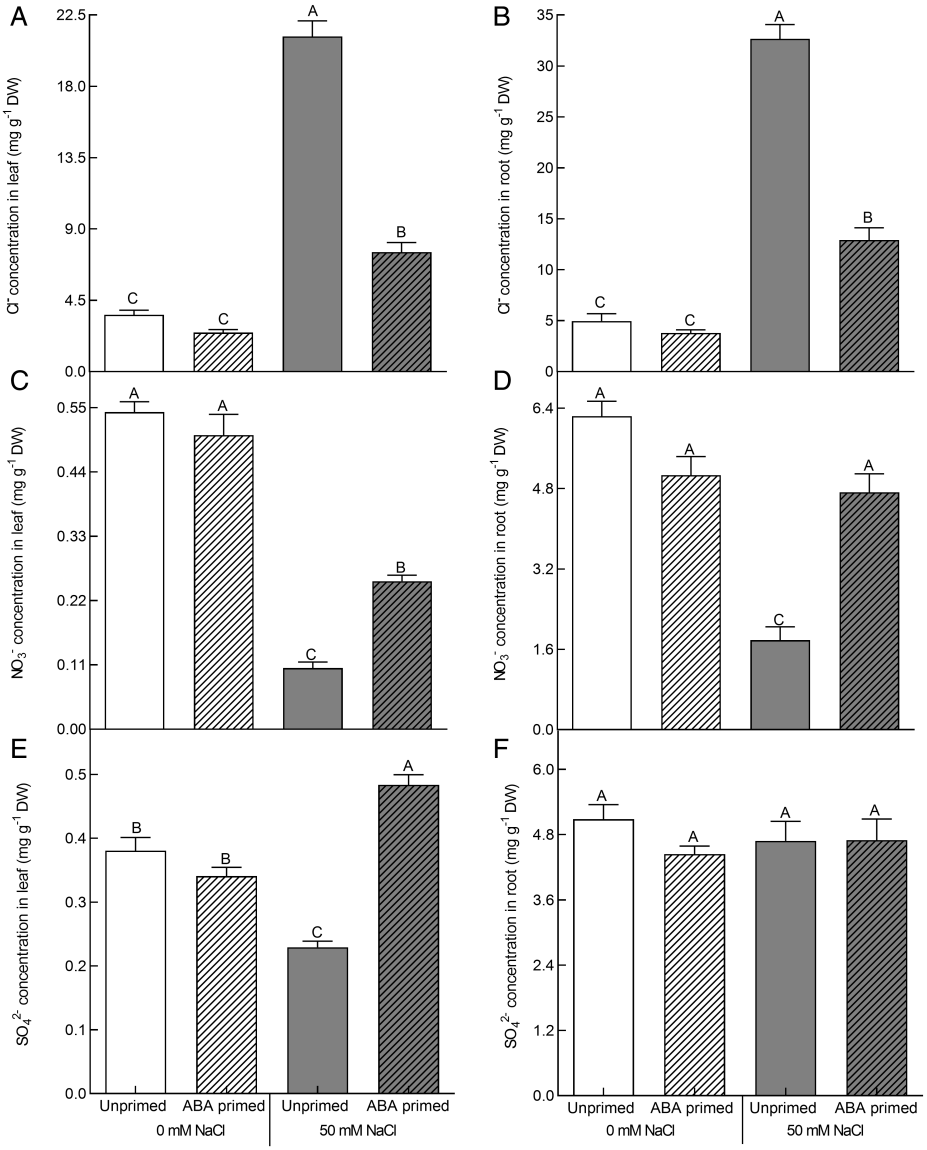

FIGURE 8 Effect of ABA priming on the concentration of Cl^- (A, leaf; B, root), NO_3^- (C, leaf; D, root), and SO_4^{2-} (E, leaf; F, root) by *Vicia faba* L. grown under non-saline and 50 mM NaCl saline nutrient solution. The data ± SE are means of four independent pot replicates. Different letters on data bars indicate significant differences among the treatments at $P \leq 0.05$

4 | DISCUSSION

Short-term ABA application substantially improved plant growth and excess salt ion exclusion for a long time (8 days). As a high turnover plant hormone, ABA is too short-lived (Ren et al., 2007; Yang & Zeevaart, 2006) to have any direct effect on plants 1 week after its application. Therefore, one can assume that the ABA-induced changes reflect the transformation into a more salt-resistant transcriptional and physiological state, a process usually referred to as "priming."

4.1 | ABA priming prevents salt-induced growth reduction

In our study, unprimed plants showed significant growth retardation under salt stress, which was mitigated by ABA priming (Figure 2). Higher dry matter accumulation by ABA-primed plants may be explained by attenuation of the inhibitory effects Na^+ and Cl^- on photosynthesis (Figure 4A) due to their substantially lower accumulation in primed as compared to unprimed plants (Figures 6A,B and 8A, B). Improved plant growth may also be linked to higher PM H^+-ATPase expression (Figure 11), which may facilitate (1) re-translocation of salt ions (Shabala et al., 2020), (2) apoplastic acidification for acid-induced growth (Cosgrove, 2005), or (3) higher auxin transport under water stress (Xu et al., 2013).

After 8 days of growth under saline condition, leaf water content (LWC) of unprimed plants decreased by 10% as compared to its initial value, whereas ABA-primed plants maintained a steady increase in LWC (Figure 5). The decrease in LWC of unprimed plants under saline condition can be explained by salinity induced root damage (Figures 2 and S1), which impaired the uptake of water, changed the pattern of osmolyte accumulation (Figure 9) and induced stomatal closure (Figure 4B). In contrast, the roots of ABA-primed plants had a normal length (Figure S1), and thus the shoot did not show a decline in LWC under saline condition (Figure S1). Higher sucrose concentrations in ABA-primed plants (Figure 9E) might also have contributed to higher

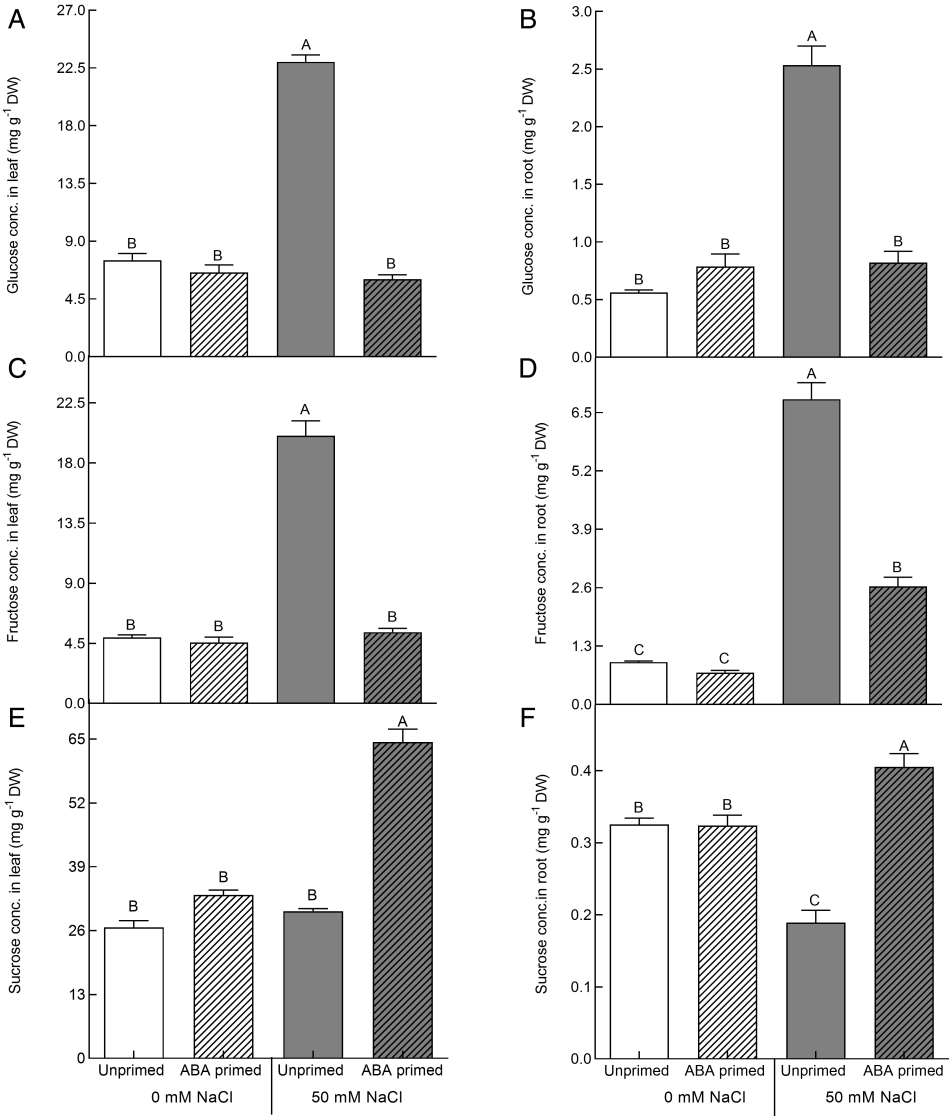

FIGURE 9 Effect of ABA priming on the concentration (conc.) of glucose (A, leaf; B, root), fructose (C, leaf; D, root), and sucrose (E, leaf; F, root) in *Vicia faba* L. grown under non-saline and 50 mM NaCl saline nutrient solution. The data ± SE are means of four independent pot replicates. Different letters on data bars indicate significant differences among the treatments at $P \leq 0.05$

LWC because of decreased osmotic potential. Moreover, ABA-primed plants accumulated more K^+ (Figure 6C,D) that helped in osmotic adjustments.

4.2 | ABA priming modulates ion concentrations under salt stress

As expected, plants that grew under saline conditions accumulated high quantities of Na^+ and Cl^- in roots and leaves; however, one-time ABA priming effectively reduced Na^+ and Cl^- an accumulation (Figures 6A,B and 8A,B). The lower accumulation of Na^+ in ABA-primed plants might be explained by the higher expression of *SOS1* (Na^+/H^+ antiporter) gene (Figure 12E,F), whose product efficiently excludes Na^+ as has been shown for some glycophytes such as *Arabidopsis* (Shi et al., 2002), white clover (Li et al., 2017), and black locust (Chen et al., 2017). The driving force for Na^+/H^+ antiport at plasma membrane is provided by the activity of the PM H^+-ATPases whose transcripts were also upregulated in primed plants under salt stress (Figure 11).

At the same time, higher K^+ concentration in ABA-primed plants (Figure 6C,D) indicates that Na^+ did not repress K^+ uptake. The enhanced expression of K^+ transporter (*KUP7*) and K^+ channel (*VFK1*) after ABA pre-treatment in roots under salt stress (Figure 12A,D) may have contributed to the increased K^+ uptake (Figure 6C,D). Higher transcript abundance of three isogenes of PM H^+-ATPase in ABA-primed plants observed in this study could also have contributed to higher K^+ accumulation, via hyperpolarization-activated opening of K^+ channel (Maathuis et al., 1997), and providing the driving force for H^+/K^+ cotransport (*KUP7*) and K^+ channel transport (*VFK1*) (Li et al., 2018; Maathuis et al., 1997). In ABA-primed plants, the enhanced accumulation of K^+ could have been one of the key mechanisms that helped avoid salt damage, as K^+ is a key osmoticum for osmotic adjustment and activator of various enzymes (Munns & Tester, 2008). Salinity significantly reduced Ca^{2+} in unprimed plants (Figure 6E,F), whereas ABA priming restored Ca^{2+} concentration in

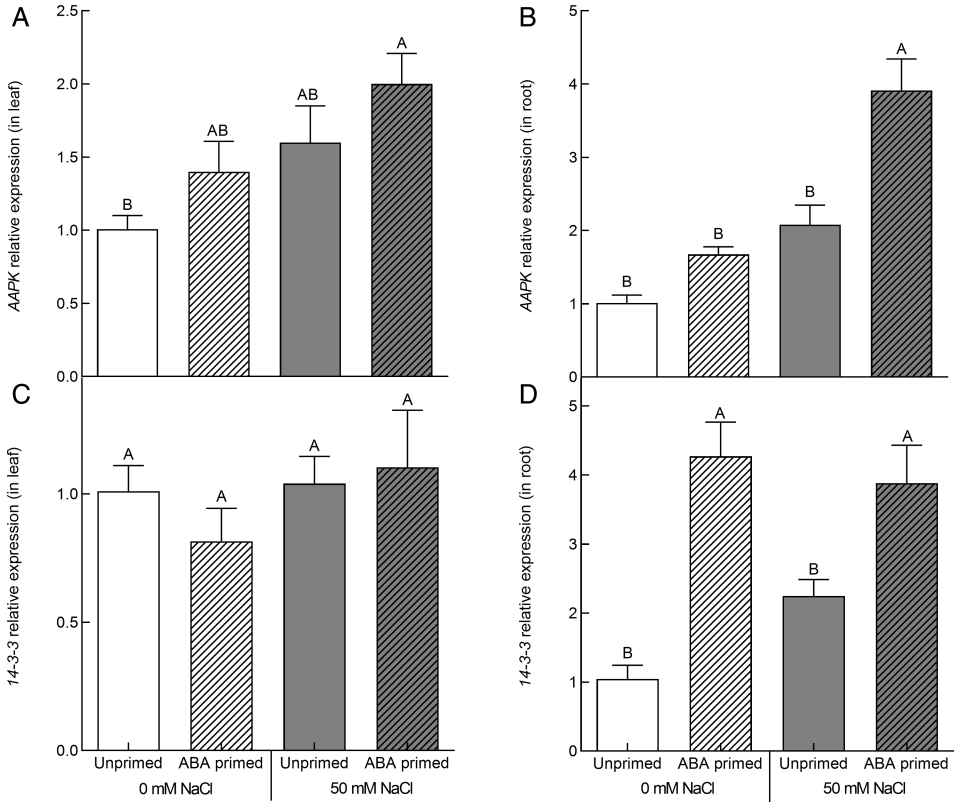

FIGURE 10 Effect of ABA priming on relative expression of ABA inducible kinase gene in *Vicia faba* L. grown under non-saline and 50 mM NaCl saline nutrient solution. The data ± SE are means of three independent pot replicates. Different letters on data bars indicate significant differences among the treatments at $P \leq 0.05$

roots (Figure 6F). Owing to the involvement of Ca^{2+} in various cell wall functions (Manishankar et al., 2018), its deficiency can be a problem under salinity, which could be mitigated by ABA priming. Another novelty of the present research is that ABA priming was also found to have beneficial or restorative effects on the concentration of micronutrients (Zn^{2+}, Fe^{2+}, Mn^{2+}) in both root and leaves. This effect may be explained by an upregulation of PM H^+-ATPase expression that is important for the establishment of the proton gradient across the plasma membrane and thus activating secondary transport of these micronutrients. The uptake of Fe^{+2}, Mn^{+2}, and Zn^{+2} is driven by this proton gradient (Gupta et al., 2016; Socha & Guerinot, 2014). Actually, Fe^{2+} and Zn^{2+} tissue concentration of unprimed plants under salinity fell below the threshold for a deficiency, which is about 30 μg g^{-1} for Fe^{+2} and 25 μg g^{-1} for Zn^{+2} for beans (Kosegarten et al., 1998; Rafique et al., 2015). This may have contributed to the limitation of photosynthesis (Figure 4A). Additionally, as these micronutrients (Zn^{+2}, Fe^{+2}, Mn^{+2}) serve as co-factors for various key metabolic enzymes, their lack in the unprimed salt-stressed plants may have contributed to growth retardation.

4.3 | ABA priming modulates anion contents

Chloride concentration under salt stress was much lower in primed than in unprimed plants (Figure 8A,B), indicating that ABA priming also induced Cl^- exclusion mechanisms as was found by Qiu et al. (2016) in *Arabidopsis*. The enhanced expression of *CLC1* in primed plants may have contributed to enhance the sequestration and compartmentation of Cl^- in the roots (Figure 12G,H). The limited root to shoot translocation of Cl^- due to the upregulation of *CLCs* has been confirmed by Wang et al. (2015) in transgenic *Arabidopsis* plants and by Wei et al. (2016) in soybean. Conversely, with increased Cl^- contents in unprimed plants under salinity, in our study, the uptake of NO_3^- and SO_4^{2-} was strongly reduced (Figure 8C–F). This might be due to the antagonistic interaction of Cl^- with these anions. Basically, Cl^- accumulation takes place at the expense SO_4^{2-} and, especially NO_3^- (Zhang et al., 2020) due to the sharing of the same transporters (Geilfus, 2018). ABA priming significantly increased the content of these anions under saline conditions (Figure 8). Together with a decreased Cl^- accumulation, this indicates that priming reduced the antagonistic effect of Cl^- (Qiu et al., 2016). Other ABA-induced mechanisms might also have contributed to increased SO_4^{2-} content. For example, Cao et al. (2014) reported that ABA application increased the transcript level of a SO_4^{2-}/H^+ co-transporter (SULTR3) and other S-metabolism-related genes, causing a higher SO_4^{2-} concentration.

4.4 | ABA priming modulates sizes of sugar pools

Sugar accumulation has diverse roles in salinity stress response. Sugars provide osmoprotection, ROS scavenging, energy, and act as signaling molecules (Halford et al., 2011). In the present study, we have found a peculiar change in sugar accumulation patterns between unprimed and primed plants under salinity. Under salt stress, unprimed plants showed a higher accumulation of hexoses (glucose and fructose, Figure 9A,D), while ABA priming enhanced the accumulation of sucrose (Figure 9E,F). Accumulation of hexoses has been found

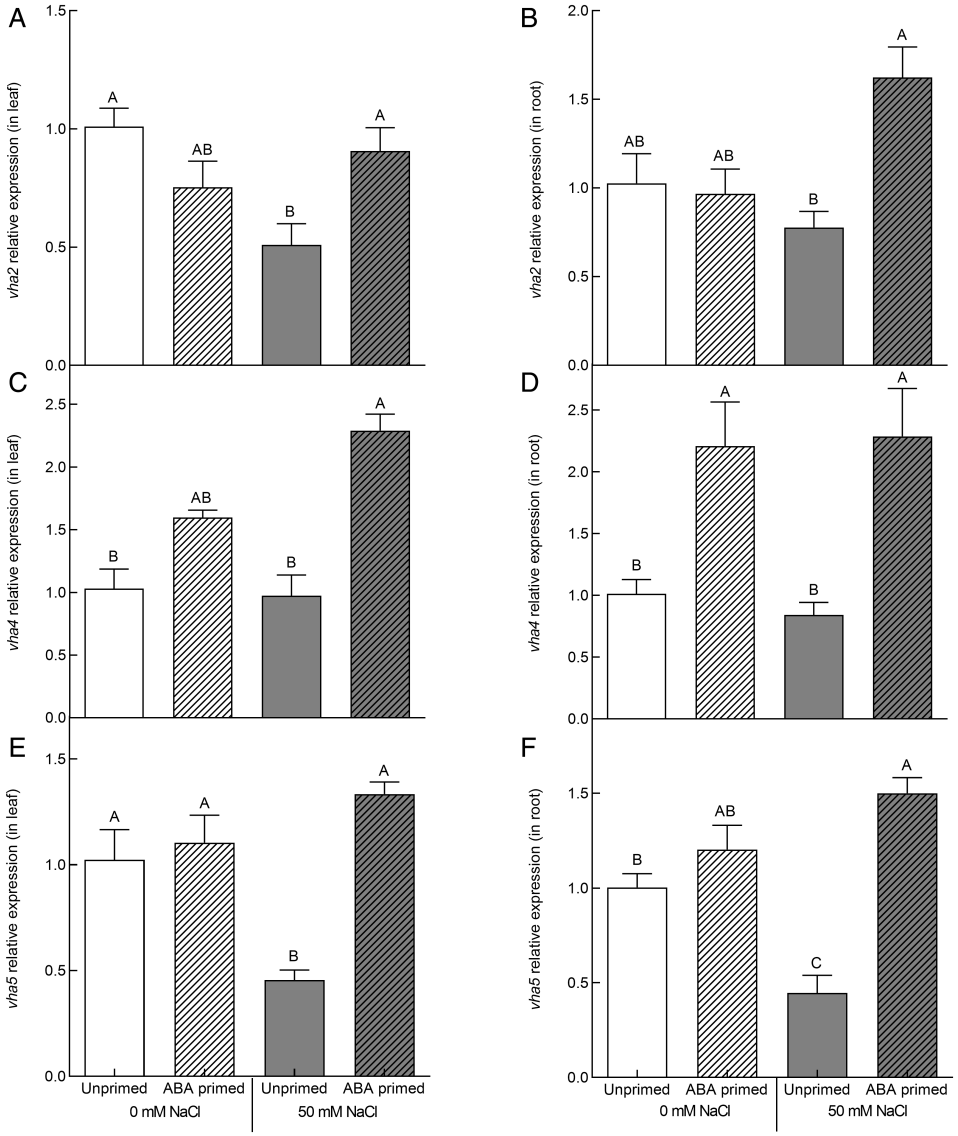

FIGURE 11 Effect of ABA priming on relative expression of plasma membrane H^+-ATPase isoforms in *Vicia faba* L. grown under non-saline and 50 mM NaCl saline nutrient solution. The data ± SE are means of three independent pot replicates. Different letters on data bars indicate significant differences among the treatments at $P \leq 0.05$

to induce enzymatic and transcriptional feedback inhibition of photosynthesis (Martínez-Carraseo et al., 1993; Paul & Pellny, 2003; Sehar et al., 2019), possibly being one cause for the lower photosynthetic rate of unprimed plant under salt stress (Figure 3A). Fivefold higher sucrose level in ABA-primed plants was observed, compared to the unprimed plants, under (Figure 9C,D). This could be attributed to a higher K^+/Na^+ ratio (Figure 6A–D), which removes Na^+ induced allosteric deactivation of various metabolic enzymes involved in sucrose synthesis (Hasanuzzaman et al., 2018) under salt stress. Sucrose is the only phloem mobile sugar; a high sucrose concentration may thus also improve the source-sink relations by providing carbon assimilates for growth and sufficient energy for ion homeostasis (Rosa et al., 2009).

4.5 | ABA priming modulates gene expression of ABA inducible kinases and membrane transporters

In the present study, we found a long-lasting effect of ABA priming on the expression of genes governing salt-resistance in plants, even though the ABA-priming application was done 8 days ago. Salt stress in unprimed plants had little or no effect on the expression of ABA-activated protein kinase (*AAPK*) in root and leaf tissues; however, ABA priming increased expression levels under salinity (Figure 10A,B). AAPK activates various downstream signal components such as CIPK, CDPK, 14-3-3, and other ABA-responsive elements via phosphorylation (Figure 1). Kulik et al. (2011) found that overexpression of *SnRK2.6*, an orthologue of *AAPK*, in transgenic *Arabidopsis* plants induced resistance to multiple stresses via higher expression of ABA-dependent stress-responsive genes, secondary root development, and improved osmotic potential. Hence, higher expression of *AAPK* in ABA-primed plants in this study could have induced salinity resistance through the same mechanisms. *SnRK2.6* has also been shown to be upregulated in pepper plants by exogenous application of ABA and salt treatment (Ruggiero et al., 2019). Moreover, elicitation of *14-3-3* by *AAPK*, in turn, facilitates the upregulation of ABA-responsive genes in an ABA-dependent manner (Takahashi et al., 2007; Yan et al., 2004) (Figure 1). Under salinity stress, we also observed a higher level of *14-3-3* mRNA transcript in roots of ABA-primed plants compared to

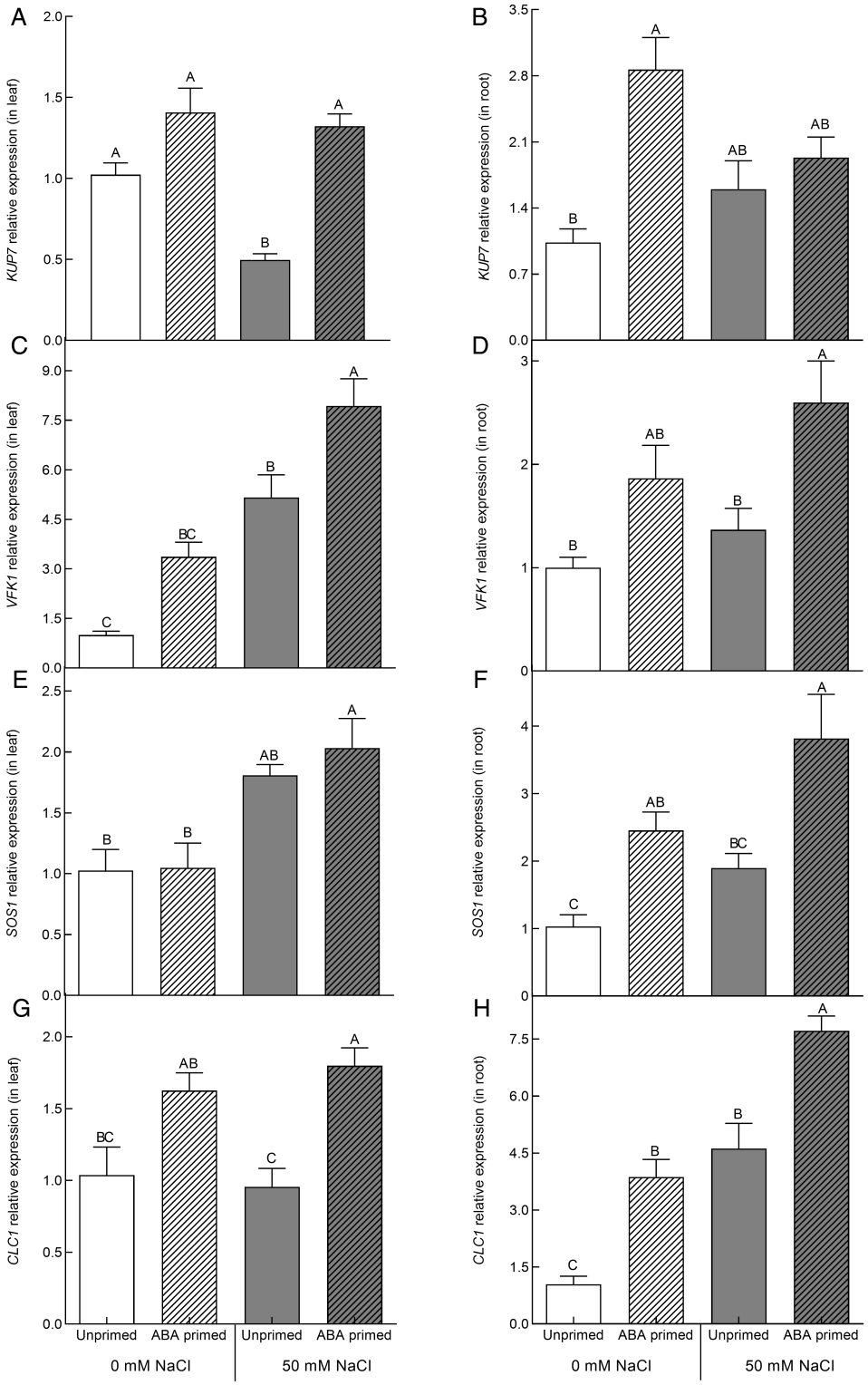

FIGURE 12 Effect of ABA priming on the relative expression of membrane ion channel and transporter in *Vicia faba* L. grown under non-saline and 50 mM NaCl saline nutrient solution. The data ± SE are means of three independent pot replicates. Different letters on data bars indicate significant differences among the treatments at $P \leq 0.05$

unprimed plants (Figure 10C,D). A previous study demonstrated that *14-3-3* expression was upregulated by exogenous application of ABA and salt in rice (Yashvardhini et al., 2018). Overexpression of *14-3-3* in transgenic cotton showed improved drought resistance by regulating metabolic processes and PM H^+-ATPase activity (Yan et al., 2004) (Figure 1). Our results thus suggest that ABA priming has a long-term effect on the expression of central ABA-dependent kinases.

Knowing that ABA priming reduces the effects of salinity on the ion composition, the question whether these changes in ionic relations functionally correlate with the changes in expression level of ion channels arose. Hence, we analyzed the expression patterns of different ion transporter genes in both root and leaf tissues. The expressions of three different isogenes of PM H^+-ATPase were upregulated by ABA priming under both normal and saline growth conditions

(Figure 11). Post-translationally, PM H^+-ATPases could have been activated by the increased levels of *14-3-3* and sucrose in ABA-primed plants (Figure 1), as both are activators of PM H^+-ATPases (Falhof et al., 2016; Okumura et al., 2016). Similarly, expressions of *KUP7* transporter and K^+ channel *VFK1* were upregulated in leaf and root tissues of ABA-primed plants under both non-saline and saline conditions (Figure 12A-D). Thus, the higher concentration of ions, particularly K^+, as observed in ABA-primed plants under salt stress (Figure 6C,D), could be attributed to the above transporter's upregulation. *KUP7* is located at the plasma membrane and is regarded as a high-affinity K^+/H^+ co-transporter for K^+ uptake. ABA-dependent SnRK2.6 kinases activate it, and its $K^{+/}H^+$ co-transport is energized by PM H^+-ATPase activity (Han et al., 2016; Li et al., 2018) (Figure 1). The observed upregulation by ABA priming of both *AAPK* and PM H^+-ATPases could activate and drive K^+ uptake by the more abundant *KUP7*. *VFK1* is a voltage-gated inward rectifying plasma membrane K^+ channel protein, an orthologue of *Arabidopsis* potassium channel 2/3 (*AKT2/3*), which plays a dual role in phloem cell of leaves and roots by loading K^+ from source tissues and unloading K^+ into sink organs (Dreyer & Uozumi, 2011; Gajdanowicz et al., 2011). ABA-induced hyperpolarization of the root plasma membrane was found to activate AKT2/3 (Roberts & Snowman, 2000). Thus, the higher expression of *VFK1* in ABA-primed roots and leaves could have contributed to the increased K^+ concentrations.

Our results show that *SOS1* transporter expression was upregulated in roots of ABA-primed plants (Figure 12E,F). SOS1 is the plasma membrane Na^+/H^+ antiporter, located in parenchyma cells at the xylem/symplast boundary in leaf, stem, and root cells. It plays a crucial role in Na^+ exclusion and governs its long-distance translocation (Munns & Tester, 2008; Shi et al., 2002). *SOS1* has been found to be upregulated via ABA-dependent CIPKs activation (Sripinyowanich et al., 2013; Zhao et al., 2019) or by ABA-responsive transcription factors via the ABA signaling pathway (Osakabe et al., 2014). Hence, the observed concomitant higher expression of *PM H^+-ATPase* and *SOS1* in ABA-primed plants might have increased the K^+/Na^+ ratio by expelling Na^+ from the cytoplasm.

Chloride exclusion and sequestration are important mechanisms in salt resistance. The vacuole sequestration depends on Cl^+/H^+ antiport by CLCs, which are voltage-gated vacuolar Cl^- channels. Gene expression of *CLC1*, an isoform of the *CLCs* (Diédhiou & Golldack, 2006), was upregulated by ABA priming in leaves and roots, and also by salinity (Figure 12G,H), possibly being a reason for the priming induced salt resistance. This suggestion is supported by an enhanced salt resistance after overexpression of *CLC1* in transgenic *Arabidopsis* (Wang et al., 2015) and soybean plants (Wei et al., 2016).

Taken together, we conclude that ABA priming generally increases the expression of the observed genes under salinity, leading to increased activity of kinases involved in stress signaling and important ion transport proteins, ultimately causing the observed improvement of salt resistance mechanisms in plants (Figure 1).

5 | CONCLUSION

This is the first study that thoroughly documents the long-term (8 days) benefits of a one-time 24-h ABA priming application. Long-lasting upregulation of genes for ABA inducible kinase AAPK, 14-3-3 protein, PM-ATPases, and ion transporters under saline conditions was the consequence of ABA priming. This may have helped ABA-primed plants to adapt to salt stress by limiting Na^+ and Cl^- uptake and translocation and favoring K^+, Ca^{2+}, Zn^{2+}, Fe^{2+}, Mn^{2+}, NO_3^-, and SO_4^{2-} accumulation. The observed lower Na^+ and Cl^- accumulation in leaves of ABA-primed plants is likely to have mitigated the salt-induced impairment of photosynthesis and may have improved plant growth under salinity. Furthermore, priming enhanced root growth and changed osmolyte composition by favoring sucrose and K^+ over hexoses, which could explain the stabilized leaf water content. Together, these results clearly show, for the first time, that short-term ABA priming generates long-lasting changes in interacting cellular processes on different organizational levels, which enhance salt resistance in plants. These results are essential for the understanding of mechanisms involved in priming by previous stresses. It could also lead to improved agricultural practices by inducing readiness in crops to resist salt, drought, and other environmental stresses.

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AUTHOR CONTRIBUTIONS

Amit Sagervanshi, Karl H. Mühling, and Hartmut Kaiser conceptualized and designed the experiments. Amit Sagervanshi performed experiments, analyzed data, and wrote initial draft of manuscript. Asif Naeem, Christoph-Martin Geilfus, and Hartmut Kaiser participated in data analysis, writing, and editing the paper. All the authors have read, edited, and approved the final manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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