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Abscisic Acid Decreases Leaf Na^+ Exclusion in Salt-Treated *Phaseolus vulgaris* L.

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Abstract Previous results showed that in short-term NaCl-treated beans increased leaf abscisic acid (ABA) concentration was triggered by Na^+ but not by Cl^-. In this work, the specificity of ABA signaling for Na^+ homeostasis was studied by comparing the plant's responses to solutions that modified accumulation of ABA and/or Na^+ uptake and distribution, such as supplemental Ca^{2+}, increased nutrient strength, different isosmotic composition, application of exogenous ABA, fluridone (an ABA inhibitor) and aminooxiacetic acid (AOA, an ethylene inhibitor). After fluridone pretreatment, salt-treated beans had lower Na^+ uptake and higher leaf Na^+ exclusion capacity than non-pretreated plants. Moreover, Na^+ uptake was increased and leaf Na^+ exclusion was decreased by AOA and ABA. NaCl and KCl similarly increased leaf ABA and decreased transpiration rates, whereas supplemental Ca^{2+} and increased strength nutrient solution decreased leaf ABA and leaf Na^+. These results show (1) a non-ion-specific increase in ABA that probably signaled the osmotic component of salt, and (2) increased ABA levels that resulted in higher leaf Na^+ concentrations due to lower Na^+ exclusion or increased root-shoot Na^+ translocation.

Keywords Abscisic acid · Aminooxiacetic acid · Bean · Ethylene · Fluridone · Na^+ exclusion · *Phaseolus* · Salinity

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Introduction

Salinity causes important yield reduction in most cultivated plants due to the metabolic costs involved in the adaptation mechanisms to low soil water potential and the presence of high concentrations of toxic ions, principally Na^+ and Cl^-, in the soil solution. Even very salt-sensitive plants such as bean have maintained a certain capacity to respond to salinity. Beans have an efficient Na^+-exclusion mechanism that prevents toxic amounts of Na^+ from reaching their leaves (Jacoby 1999 and references therein).

Abscisic acid (ABA) is involved in the plant's adaptation to osmotic stresses, participating, for instance, in the signaling events that lead to stomatal closure (Borel and others 2000; Mustili and others 2002); however, its role in other adaptative responses such as leaf growth inhibition remains controversial (Voisin and others 2006). Although much information is available on the role of ABA in regulating ion fluxes in guard cells, little is known of ABA participation in the control of uptake and distribution of toxic ions such as Na^+. Previous results (Montero and others 1998; Sibole and others 1998) showed that in salt-treated beans there is a high positive correlation between leaf Na^+ and xylem and leaf ABA, whereas no relationship was found between leaf ABA and leaf Cl^- concentrations. Moreover, NaCl-pretreated bean plants showed improved growth when they were exposed to NaCl during later stages of their life cycle, and this improvement was related to their higher Na^+ exclusion capacity and lower leaf ABA concentration with respect to non-NaCl-pretreated plants (Montero and others 1997).

The aim of this work was to study the ABA signaling specificity for Na^+ and its relative importance in the leaf Na^+ exclusion mechanism of salt-treated bean plants by using different physiologic approaches. In the first series of

experiments, variations in growth and ABA concentration were studied in plants grown in NaCl, KCl, and isosmotic solutions of mannitol to evaluate whether there were any differences in the ABA response when the decrease in the water potential of the growing media was due to different causes: water deficit, ion excess, or ion excess due to different salts. Another approach was to examine the ABA response in plants grown in conditions that favored Na^+ exclusion such as in supplemental Ca^{2+} (LaHaye and Epstein 1971), or in a more concentrated nutrient solution that provided higher Ca^{2+}:Na^+ ratios than in solutions with supplemental Ca^{2+}, but without causing nutrient imbalance (Plaut and Grieve 1988).

To acquire additional evidence regarding the relationship between ABA and Na^+, a second series of experiments in which the plant ABA concentration was decreased with fluridone pretreatment was performed. Moreover, the interaction between ABA and ethylene, the latter being at times an antagonist for some ABA-regulated responses, was investigated by treating the plants with aminooxyacetic acid (AOA), an ethylene inhibitor.

Material and Methods

Plant Material and Culture Conditions

Phaseolus vulgaris cv. Contender seeds were germinated in vermiculite soaked with distilled water. After 8 days seedlings were individually transplanted into beakers filled with 300 ml of 25% modified Hoagland nutrient solution aerated using an air pump. Plants were supported by inserting them through holes drilled in styrofoam rings held in place by the beaker neck. Containers and tops were completely covered with aluminum foil to keep out light from the hydroponic culture. Plants were grown in natural light in a glasshouse under controlled conditions. Glasshouse maximum and minimum average air temperature and relative humidity during the experimental periods were 25.5 ± 3.2°C and 18.2 ± 1.2°C and 40–70%, respectively.

The following treatments were started 48 h after the plants were transplanted into the nutrient solution.

In the first series of experiments, plants were transferred at dusk to 25% modified Hoagland solution with 1 mM NaCl (C), 1 mM NaCl + 4 mM $CaCl_2$ (Ca), 50 mM NaCl (Na), 50 mM KCl (K), 50 mM NaCl + 4 mM $CaCl_2$ (CaNa), and 100 mM mannitol (Man) or to 100% modified Hoagland solution with 1 (FH) or 50 mM NaCl (FHNa). The following day plants were harvested and analyzed. Sampling took place from 12:00 until 16:00 p.m., and time-dependent measurements such as leaf expansion and transpiration rate were conducted from 09:00 to 16:00 to avoid excessive deviations because light conditions in the glasshouse during this period were the most stable.

In another series of experiments, half of the plants were transferred to beakers with 10 μM fluridone (1-methyl-3-phenyl-5-[3-trifluromethyl]-4-(14)-pyridinone) (SePRO, Carmel, IN) added to the solution culture for 24 h at a lab bench. Fluridone was dissolved in ethanol plus three to four drops of TWEEN 20 (Sigma, St. Louis, MO) (Ober and Sharp 1994). The same amount of ethanol and TWEEN 20 was added to the solution culture of non-fluridone-treated plants. Afterwards the roots were carefully rinsed with running tap water followed by rinsing three times in distilled water. During the fluridone treatment, plants were kept under dim light (150 μmol photons m^{-2} s^{-1}) during the day period.

The following treatments were set up at dusk using plants pretreated and non-pretreated with fluridone: 25% Hoagland solution (C) modified with 10 μM AOA, 50 mM NaCl (Na), or 10 μM AOA + 50 mM NaCl (Na + AOA). An additional group of plants that did not have the fluridone pretreatment were treated with 25% Hoagland solution modified with 1 μM ABA (±)-ABA (Na + ABA) (Sigma-Aldrich, St. Louis, MO). AOA is an inhibitor of 1-aminocyclopropane-1-carboxylic acid (ACC) synthase, a key enzyme of ethylene synthesis (Abeles and others 1992). The following day, plants were harvested and analyzed. Sampling took place as stated above for the first series of experiments.

Leaf Area, Tissue Fresh and Dry Weights, and Water Content

Leaf area was determined at 9:00 a.m. and before plant harvest (from 12:00 to 16:00 p.m.) as the product of measured length and width according to the following equation: LA (cm^2) = L × W × 0.86. The 0.86 coefficient was calculated as the mean ratio of the difference between the maximum length × width of 20 individual primary leaves and their actual area measured using a leaf area meter (Δ-T Area Meter, Delta-T Devices, Burwell, Cambridge, UK) (CV = 2.3%). Leaf expansion rate was calculated as the hourly increment in leaf surface area. Starting at 12:00 p.m. and following a sequence of one plant from each treatment group until there were four to six replications, plants were transferred to the lab bench where leaves, petioles, and stems were excised and their fresh weight immediately recorded. Roots were rinsed three times with distilled water and carefully dampened using tissue paper before their fresh weight was recorded. Tissue was frozen immediately afterward in liquid N_2, then

freeze-dried and finely ground with a mortar and pestle. Leaf water content was calculated as follows: (FW − DW)/DW.

Transpiration Rate

Total plant transpiration rate was determined by recording the weight loss of the plant culture solution system (beaker + plant) from 9:00 a.m until plant harvest. To prevent direct evaporation, beaker tops were covered by plastic wrap. Weight was always recorded using the same balance (readability = 0.01 g) both times following the same sequential order, one plant from each treatment group until four to six replications. Total plant transpiration is expressed as weight loss per hour and leaf area unit. Differences between plants due to differences in the sampling period are included in the standard deviation of each mean shown in Fig. 1.

Tissue Ion Concentration

Forty milligrams of freeze-dried leaf tissue were digested in 15 ml of 0.1 M nitric acid for 12 h and filtered for cation analysis. Ca^{2+}, Mg^{2+}, K^{+}, and Na^{+} tissue and xylem sap contents were determined by inductively coupled plasma (ICP) spectroscopy (Plasma-2000, PerkinElmer, Waltham, MA).

Tissue ABA Concentrations

Forty milligrams of freeze-dried tissue were extracted overnight in the dark at 4°C with 2 ml of distilled water. ABA was quantified by radioimmunoassay (RIA) (Quarrie and others 1988). No nonspecific interferences in this RIA were found in bean tissues (Trejo and Davies 1991).

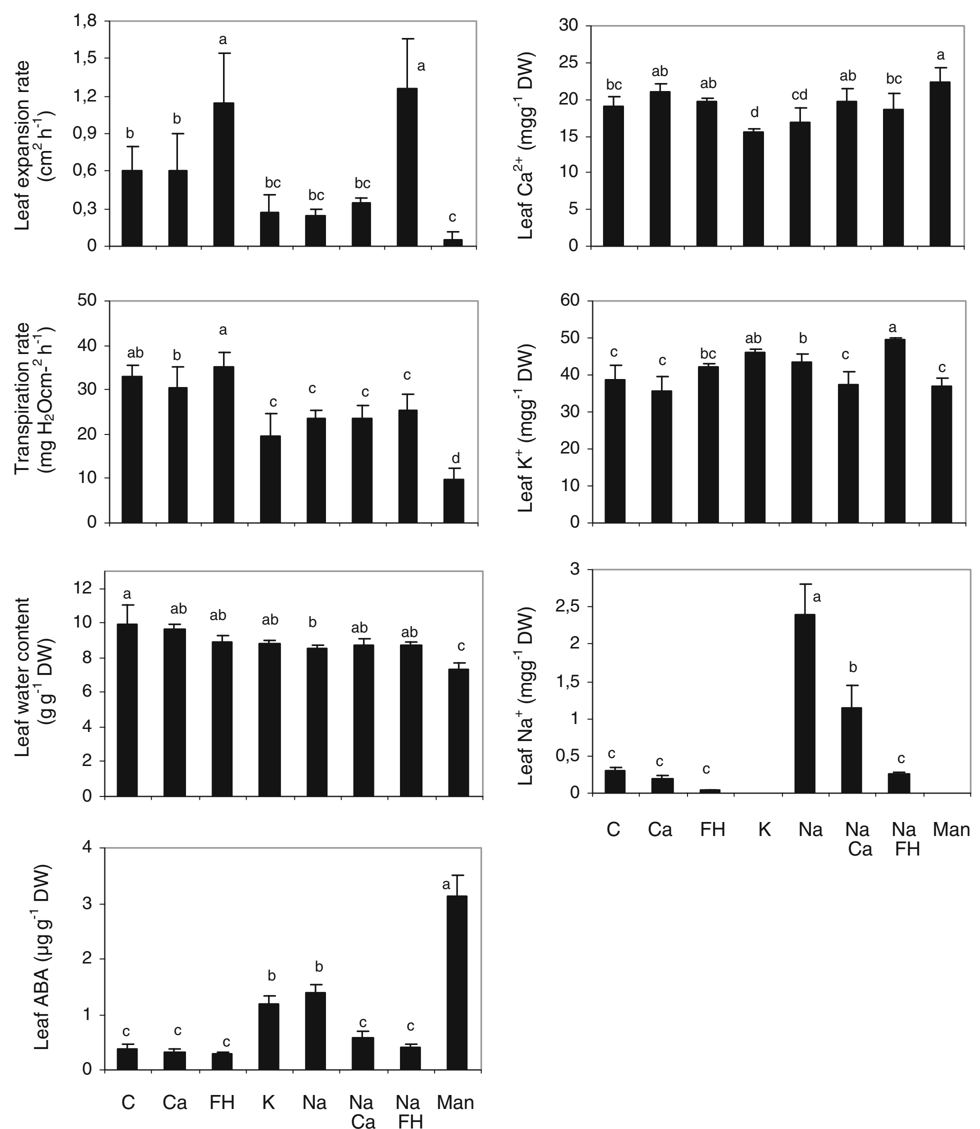

Fig. 1 Leaf expansion and transpiration rates, leaf water content, leaf ABA, Ca^{2+}, K^{+}, and Na^{+} concentrations in beans grown for 16–20 h in 25% modified Hoagland solution with 1 mM NaCl (C), 1 mM NaCl + 4 mM $CaCl_2$ (Ca), 50 mM KCl (K), 50 mM NaCl (Na), 50 mM NaCl + 3 mM $CaCl_2$ (CaNa), or 100 mM mannitol (Man) or in full Hoagland solution (FH) modified with 50 mM NaCl (FHNa). Values with the same letter are not significantly different ($P > 0.05$)

Statistics

Data are presented as the mean ± SD (standard deviation) for each treatment ($n = 4$–6). Significant differences between treatments were analyzed by one-way analysis of variance (ANOVA). Least significant difference (LSD) values were calculated at the $P < 0.05$ probability level (Statview v4.0, Abacus Concepts, Berkeley, CA).

Results

Bean plants treated with 100 mM mannitol (Man) showed a 26% decrease in leaf water content and significant reductions in leaf expansion and transpiration rate along with an increase in leaf ABA concentration in which values were two times higher than those found in plants treated with isosmotic saline concentrations (Fig. 1).

Plants grown in a 25% Hoagland solution modified with either 50 mM NaCl or KCl showed a decrease in leaf expansion and transpiration rate and an increase in leaf ABA concentration, with no significant differences between treatments (Fig. 1). Moreover, both salts reduced Ca^{2+} uptake and translocation to the leaves. Total plant Ca^{2+} was 20 and 26.7% and leaf Ca^{2+} 10.8 and 18.3% lower in NaCl- and KCl-treated plants, respectively, than in controls. Potassium uptake increased by 8.7% in KCl-treated plants and decreased by 7.4% in NaCl-treated plants. Leaves of plants treated with NaCl or KCl showed higher K^+ concentrations than controls (17.8% in NaCl-treated and 20.6% in KCl-treated plants) (Fig. 1). NaCl-treated plants supplemented with 4 mM Ca^{2+} showed a decrease in leaf Na^+ and ABA concentrations, although no significant changes were found in leaf expansion and transpiration rates with respect to NaCl-treated plants not supplemented with Ca^{2+}. Plants grown in full Hoagland solution with 50 mM NaCl had increased leaf expansion rates compared to NaCl-treated plants grown in 25% Hoagland solution; however, no differences in transpiration rates were noted between the two treatments. Moreover, in NaCl-treated plants grown in full Hoagland solution, leaf Na^+, K^+, and ABA concentrations were significantly lower, whereas leaf Ca^{2+} was higher than in the NaCl-treated plants grown in 25% Hoagland solution.

Fluridone pretreatment increased leaf K^+ in controls (C) and decreased this cation in salinized plants treated with AOA (Na + AOA) (Fig. 2), yet significantly increased leaf Ca^{2+} in the saline (Na) and saline-supplemented-with-AOA plants (Na + AOA). Leaf ABA significantly decreased in the fluridone pretreated plants in all treatments, whereas it logically increased in the ABA-treated plants.

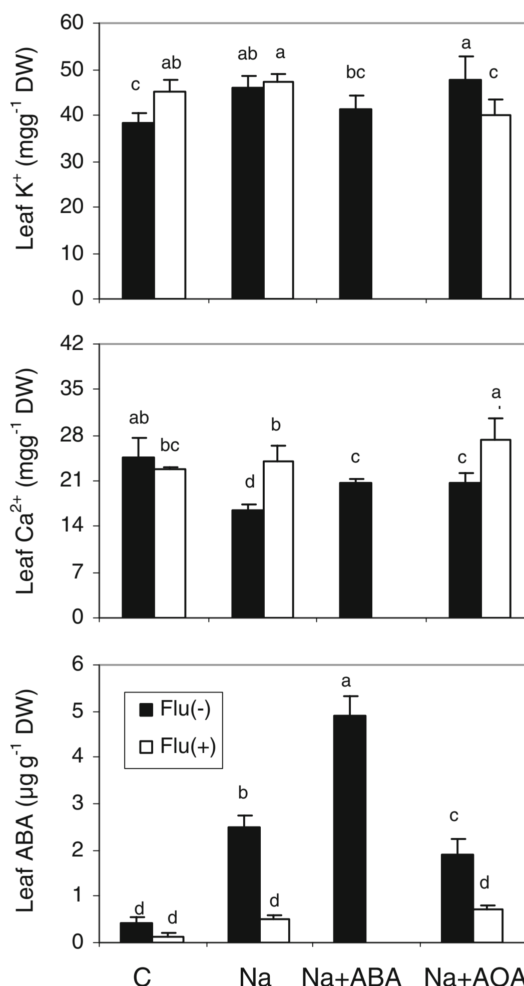

Fig. 2 Leaf K^+, Ca^{2+}, and ABA concentrations in beans not pretreated (*black columns*) or pretreated with fluridone (*white columns*) grown for 16–20 h in 25% Hoagland solution (C), modified with 50 mM NaCl (Na), 50 mM NaCl + 1 µM ABA (Na + ABA), or 50 mM NaCl + 10 µM AOA (Na + AOA). Values with the same letter are not significantly different ($P > 0.05$)

Fluridone pretreatment of salt-treated plants significantly decreased Na^+ uptake and translocation to the leaves. Total plant Na^+ and leaf Na^+ were, respectively, 19 and 85% lower when salt-treated plants were pretreated with fluridone (Fig. 3). External ABA (Na + ABA) increased Na^+ uptake by 9% and especially favored Na^+ translocation to the stems (38%) and the leaves, and these latter organs had a 77% higher translocation than the NaCl-treated plants (Na). A marked increase in Na^+ uptake and translocation to the leaves (36.3 and 60.5% higher, respectively) was also found in salt-treated plants when they were supplemented with 10 µM of AOA (Na + AOA). When salt-treated plants were supplemented with AOA, pretreatment with fluridone decreased both Na^+ uptake by 27.3% and more markedly Na^+ translocation to stems (56.5%) and leaves (97.2%).

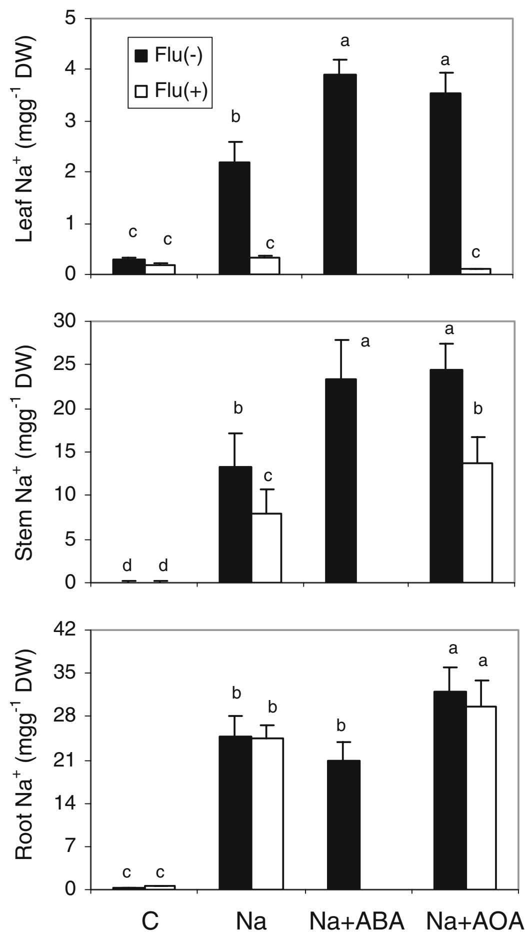

Fig. 3 Leaf, stem, and root Na^+ concentrations in beans not pretreated (*black columns*) or pretreated with fluridone (*white columns*) grown for 16 h in 25% Hoagland solution (C), modified with 50 mM NaCl (Na), 50 mM NaCl + 1 μM ABA (Na + ABA), or 50 mM NaCl + 10 μM AOA (Na + AOA). Values with the same letter are not significantly different ($P > 0.05$)

Discussion

We have used different physiologic approaches to modify Na^+ uptake and/or translocation to examine the signaling specificity and relative importance of ABA in the short-term leaf Na^+ exclusion capacity of bean plants.

The increase in leaf ABA in NaCl-treated beans was quantitatively less marked than the increase of this hormone in response to isosmotic concentrations of mannitol. This higher ABA concentration in mannitol-treated plants was probably partly caused by the significantly lower values in leaf water content induced by the nonpermeable osmoticum compared to salt-treated plants, because the decrease in leaf water is one of the most important factors that switches on ABA synthesis (Popova and others 2000).

It has been long known that supplemental Ca^{2+} favors leaf Na^+ exclusion in bean plants (LaHaye and Epstein 1971), and in some species it ameliorates plant growth under salt stress (Cramer 2002). In the present study, the Na^+:Ca^{2+} ratio of salinized plants grown in 25% Hoagland solution was 50, much greater than the ratios found for salt-treated plants grown in full Hoagland solution (12.5) and in 25% Hoagland solution supplemented with Ca^{2+} (10). Therefore a low Na^+:Ca^{2+} ratio could at least partly explain the decrease in Na^+ uptake in salinized plants grown in full Hoagland and Ca^{2+}-suppplemented nutrient solution.

Leaf ABA concentration depends on the ionic composition of the nutrient solution. Previous results showed that leaf ABA was higher in response to NaCl than to isosmotic concentrations of concentrated Hoagland solution (Montero and others 1997). In the present work, leaf ABA was lower when salinized plants were grown in full Hoagland or Ca^{2+}-supplemented nutrient solution than when grown in 25% Hoagland solution. Moreover, in the latter treatments, leaf Na^+ was also lower and pointed to a possible role of ABA signaling in Na^+ homeostasis, which is in agreement with previous studies (Montero and others 1997) that reported that the increase in ABA in NaCl-treated beans was specific for Na^+ and not for Cl^-. However, our results showed similar ABA responses in leaves of KCl- and NaCl-treated plants and, therefore, it cannot be concluded that the increase in leaf ABA found in salt-treated beans was specific for Na^+.

On the other hand, although salt-treated plants supplemented with Ca^{2+} or grown in full Hoagland had lower leaf Na^+ and ABA concentrations, neither treatment group showed ameliorated transpiration rates so therefore no simple relationship can be established between bulk leaf ABA and stomatal conductance.

Several reports have shown that ABA regulates K^+ channel activity in roots of different species (Roberts and Snowman 2000). However, considering the results found in the two experimental approaches used in this work, no relationship could be drawn between ABA and K^+ concentrations. Nonetheless, leaf Na^+ showed a positive relationship with leaf ABA, suggesting a possible specific effect of this hormone on the leaf Na^+ exclusion mechanism. As cytosolic Na^+ negatively affects many physiologic processes, Na^+ transport must be finely regulated, and specific signaling pathways for Na^+ toxicity have been described in plants. In *A. thaliana* the SOS pathway regulates sodium homeostasis in response to Na^+ toxicity (Chinnusamy and others 2004).

Our results showed that fluridone pretreatment significantly reduced Na^+ uptake and translocation to the leaf, whereas plants in which the nutrient solution was supplemented with 1 μM ABA showed a significant increase in both Na^+ uptake and leaf translocation. These results suggest that ABA could directly or indirectly participate in the upregulation of transporters involved in Na^+ uptake

and xylem loading and/or the downregulation of transporters involved in leaf Na^+ exclusion. Although the complete nature of the Na^+ transporters that mediate the cell's Na^+ homeostasis is not yet well defined, HKT1 functions as a potential Na^+ uptake pathway, whereas members of the NHX family contribute to Na^+ exclusion into the vacuole and the apoplast; therefore, the upregulation of *HKT1* and downregulation of *NHX* isoforms could greatly contribute to increasing the Na^+ load on plant tissue (Maathuis 2006).

This ABA effect on Na^+ uptake and translocation was antagonized by ethylene because the AOA treatment greatly increased Na^+ uptake and transport to the leaves. This response was annulled when ABA synthesis was inhibited. Although ethylene synthesis is not triggered by osmotic stress (Morgan and others 1990), different plant responses result from the interaction between the ABA and the ethylene signaling cascades. Our results suggest that ethylene counteracts the ABA effect on Na^+ uptake and leaf translocation and therefore participates in the regulation of Na^+ homeostasis in beans.

To conclude, our results showed that in short-term salinized beans there is a non-ion-specific increase in ABA which probably signals the osmotic component of salinity. However, the transpiration rate was found to be not directly related to bulk leaf ABA. Moreover and surprisingly, our results showed an ABA-specific effect on Na^+ uptake and transport which resulted in an increase in leaf Na^+ concentration.

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