



Evolution of salt tolerance in *Arabidopsis thaliana* on siliceous soils does not confer tolerance to saline calcareous soils

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Abstract

Purpose Alkaline salinity constrains crop yield. Previously, we observed local adaptation of *Arabidopsis thaliana* to saline-siliceous soils ($\text{pH} \leq 7$) and to non-saline carbonate soils. However, no natural population of *A. thaliana* was localized on saline-alkaline soils. This suggests that salinity tolerance evolved on saline-siliceous soils may not confer tolerance to alkaline salinity. This hypothesis was explored by addressing physiological and molecular responses to alkaline salinity of *A. thaliana* that differ in tolerance to either non-alkaline salinity or carbonate.

Methods *A. thaliana* native to saline-siliceous soils (high salinity, HS), non-saline carbonate soils (high alkalinity, HA), or soils with intermediate levels of these factors (medium saline-alkalinity, MSA) were cultivated in common gardens on saline-siliceous or saline-calcareous substrates. Hydroponics and irrigation experiments confirmed the phenotypes. The growth, mineral concentrations, proline content, osmotic potential, genetic variation distribution, and expression levels of selected genes involved in salinity and alkalinity tolerance were assessed.

Results HS performed best on saline-siliceous soil and in hydroponics with salinity ($\text{pH} 5.9$). However, HS was more sensitive to saline-alkaline conditions than HA and MSA. The fitness under saline-alkaline conditions was ranked according to $\text{MSA} > \text{HA} > \text{HS}$. Under alkaline salinity, MSA best maintained ion homeostasis, osmotic balance, and higher expression

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levels of key genes involved in saline or alkaline tolerance (*AHA1*, root *HKT1* and *FRO2*, and shoot *NHX1* and *IRT1*).

Conclusion In *A. thaliana*, salinity tolerance evolved on saline-siliceous soils does not provide tolerance to alkaline salinity. Plants native to intermediate conditions (MSA) have more plasticity to adapt to alkaline salinity than those locally adapted to these individual stress factors.

Keywords Saline-alkaline soils · *Arabidopsis thaliana* · Plasticity · Local adaptation · Ionome

Introduction

Saline stress is one of the major constraints in agriculture. Soil salinization frequently co-occurs with alkalinity, especially under arid and semiarid climates, in which high evapotranspiration rates move an excess of soluble ions such as Na^+ , Ca^{2+} , Mg^{2+} , K^+ , CO_3^{2-} and HCO_3^- to the superficial soil layers. Moreover, soil salinization may destroy the soil structure and cause changes in biological activity (Singh 2021). In addition to natural causes, human activities such as crop irrigation add more soluble salts to soils. Including both natural and anthropogenic origins, it is estimated that 831 million hectares of Earth are covered by saline-alkaline soils (FAO 1973). On these soils, plants need to cope with the deleterious effects of both high pH and excess salts. Salinity causes both ion toxicity and osmotic effects. Plant adaptive mechanisms towards NaCl salinity are well established. Sodium enters NSCC (nonselective cation channels), reducing the membrane potential. A huge energy cost is spent removing Na^+ from the cytosol, storing it in the vacuoles, or returning it to the apoplast by *salt overly sensitive 1* (*SOS1*) mechanisms. *High affinity potassium transporter 1* (*HKT1*) is an efficient transporter that releases Na^+ from the xylem and limits Na^+ transport to the shoots (Sandhu et al. 2017). Weak alleles of *HKT1* favour leaf Na^+ accumulation, which may be an advantage under fluctuating and moderate saline conditions through contributions to ABA signalling and osmotic adjustment (Busoms et al. 2018).

The presence of high carbonate and bicarbonate levels affects soil micronutrient availability, especially with respect to Fe and Zn (Marschner 1995).

Moreover, bicarbonate alters the root cell membrane potential, further enhancing nutrient deficiencies. Carbonate/bicarbonate stress causes an imbalance in plant carbon metabolism due to dark fixation of $\text{CO}_2/\text{HCO}_3^-$ (Poschenrieder et al. 2018). The observed enhancement of the production of organic acids and an inhibition of iron translocation from roots to shoots may be consequences of this carbon imbalance (Alhendawi et al. 1997). Better mobilization of Fe from alkaline soil by root exudation of catechol groups bearing phenolics has been identified as a key process in tolerance to alkaline soils in *A. thaliana* (Terés et al. 2019). Higher expression of *ferric reduction oxidase 2* (*FRO2*) and *iron-regulated transporter 1* (*IRT1*) conveys tolerance to iron deficiency (Connolly et al. 2003). Tolerance to alkaline salinity has been less explored. However, several studies performed with different plant species show more destructive effects of salinity under high pH than under neutral conditions (Gao et al. 2014; Zhang et al. 2017b; Chuamnakhong et al. 2019).

The mechanisms behind these synergistic effects are still not clearly established. Double stress exerts a strong influence on root architecture. In comparison to NaCl stress alone, under mixed salt-alkaline stress, the root architecture of *Lotus tenuis* changed to a more herringbone structure, a phenotype with a reduced number of lateral roots (Paz et al. 2012). Under alkaline-saline conditions, a strong inhibition of nutrient uptake, especially of nitrate and phosphate, in addition to micronutrient deficiencies and a high Na^+/K^+ ratio may injure sensitive plants more than saline stress caused by NaCl under neutral or slightly acidic conditions (Gao et al. 2014). As under saline stress alone, the capacity to accumulate compatible solutes is a key feature of tolerance to saline-alkaline conditions. Enhancements of proline (Shi and Sheng 2005), betaine, and soluble sugar concentrations are quantitatively the most important changes in osmotically active substances (Wang et al. 2018).

Halophytes have evolved extreme tolerance to saline conditions. Geochemical modelling using species distribution models for Australian grass species suggests a correlation between salt and alkalinity tolerance (Saslis-Lagoudatis et al. 2015). In fact, many well-known halophytes have evolved under extreme saline-alkaline conditions (Akhani 2006). Saline-alkaline habitats are characterized by the presence of sodium carbonate, an electrical conductivity of more

than 4 dS m⁻¹, and a percentage of exchangeable sodium higher than 15%. Lab studies with alkaline-tolerant halophytes have used experimental exposures up to 400 mM NaCl and pH values of 10 (Yang et al. 2007; Hajiboland et al. 2018). Such conditions far exceed the tolerance limits of most crop plants and of the *A. thaliana* genetic model (Hajiboland et al. 2018). Tolerance mechanisms revealed in studies with these halophytes are of interest for both basic plant biology and halophyte agriculture but may be less relevant for the improvement of major crop species, which are mainly glycophytes.

There is relatively little information on the mechanisms underlying tolerance differences with respect to saline stress under alkaline conditions in crop plants. In *Oryza sativa*, the relative expression levels of *HKT1* and *SOS1* were compared between two contrasting lines differing in alkaline-saline stress tolerance. Tolerant lines were able to more efficiently limit Na⁺ accumulation in the leaves by higher activation of both genes in comparison to sensitive lines. Furthermore, Fe acquisition and rhizosphere acidification genes were highly induced to maintain plant nutrient homeostasis (Chuamnakthong et al. 2019). Exploring tolerance mechanisms in natural populations of the model plant *A. thaliana* locally adapted to moderate levels of salinity and alkalinity may provide insights into the underlying genetic background of traits that will be useful in crop breeding, especially in a scenario of climatic change, where soil salinization may occur in the Mediterranean limestone basin (Tomaz et al. 2020).

Differences in tolerance to salinity and moderate carbonate levels as individual stress factors have been described among natural populations of *A. thaliana* in the NE region of Catalonia (Busoms et al. 2015; Terés et al. 2019; Pérez-Martín et al. 2021). Reciprocal transplant and common garden experiments comparing genetically distinct populations under identical environmental conditions are efficient tools to discriminate the effects of genetic and environmental variations on phenotypes (Johnson et al. 2021). Using such approaches, we have previously shown that coastal populations of *A. thaliana* perform better under salinity than inland populations. However, these studies were performed on siliceous substrates. Inland populations native to areas located close to limestone soils performed better on nonsaline calcareous soil with a moderate carbonate content than

coastal plants evolved on siliceous saline soils. The species distribution model elaborated by Busoms (2015) predicted the lack of *A. thaliana* in saline-alkaline soil from this region, and several surveys in different years confirmed this model prediction (Busoms 2015). The distribution of *A. thaliana* is disrupted at coastal locations with saline, lime-rich soils. This indicates that tolerance to saline-alkaline field conditions has still not evolved in this species in the region of NE Catalonia. Nonetheless, the presence of tolerance mechanisms to counteract moderate saline or alkaline conditions as individual stress factors in our *A. thaliana* populations makes these plants ideal for exploration of the involvement of these tolerance mechanisms to alkaline salinity resistance.

Thus, the primary objective of this study was to examine the contributions of individual saline and alkaline tolerance mechanisms to tolerance of double stress. For this purpose, we analysed the germination, growth, and reproductive fitness of *A. thaliana* demes (multiple small stands or local populations) native to different soils: nonalkaline saline soils (HS), nonsaline carbonated soils (HA) and soils with intermediate levels of salinity and carbonate (MSA). The experiments were performed in common gardens and controlled laboratory conditions using saline and saline-alkaline soils or solutions. Moreover, determination of the contributions of selected genes with an established role in the tolerance to alkalinity or salinity as individual stress factors was approached by qPCR.

Materials and methods

Plant and soil materials

In previous studies, natural variations in *A. thaliana* populations from Catalonia were tested in multiyear small-scale common gardens under saline and carbonated conditions (Busoms et al. 2015; Terés et al. 2019). Seeds from reciprocal transplant experiments performed in 2015 were stored under cold (4 °C) and dry conditions until the beginning of the experiments. Col-0 seeds were included as a reference genome and were purchased from Nottingham Arabidopsis Stock Centre NASC (Scholl et al. 2000). Prior to use, seeds were surface sterilized by soaking in 70% (v/v) ethanol for 1 min, suspended in 30% (v/v) commercial

Clorox bleach and 1 drop of Tween-20 for 5 min and rinsed 5 times in sterile 18 M Ω Milli-Q water. Seeds were stratified for 4 days at 4 °C to synchronize germination. The population coordinates and lines used in each experiment are detailed in Supplementary Dataset S1.

Soils from the native Catalan populations of *A. thaliana* were sampled, excavated at 10 cm depth and transported to the laboratory for further analysis. For the greenhouse experiments, soils from two different locations along the Catalan coast were excavated and transported to the greenhouse at the Universitat Autònoma de Barcelona (UAB). The saline siliceous soil was excavated at Blanes (41°53'42"N 3°01'11"E), while the saline-alkaline soil was obtained from l'Escala (42°13'03"N 3°11'30"E).

GIS data extrapolation

To estimate the geologic, edaphic and climatic parameters of each habitat of the *A. thaliana* natural populations, native soil, coordinate locations, and public maps from the Institut Cartogràfic i Geològic de Catalunya (ICGC) and the European Soil Data Centre (ESDAC) database (Panagos et al. 2012) were combined using Miramon v8 (Pons 2004) and Q-GIS (<http://qgis.osgeo.org>). Maps of soil properties at the European scale, based on Lucas 2009/2012 topsoil data, were used to extract the following variables: pH (measured in H₂O), cation exchange capacity (CEC), and calcium carbonate (CaCO₃) content (Ballabio et al. 2019). To determine whether the distribution based on these soil characteristics of *A. thaliana* in Catalonia matched the *A. thaliana* distribution on a larger scale, the HapMap population coordinates were used (<https://1001genomes.org>) to combine our data with the European maps (Supplementary Dataset S2).

Soil physical and chemical analyses

Six independent samples of each soil type were used for the analyses. The soil characterization was performed on air-dried 2-mm fraction samples. For the measurement of soil pH and electric conductivity (EC), 25 mL of 18 M Ω water was added to 5 g of soil in a Falcon tube. After mixing at constant rotation for 30 min, the pH was measured using a pH metre (Basic 20+, Crison, Barcelona, Spain), and CE was determined with a conductometer (Hanna,

Woonsocket, Rhode Island, USA) (Sonmez et al. 2008). Texture, water holding capacity (WHC) and organic matter were determined following the methods described by Porta et al. (1986). The calcium carbonate content (%) was measured according to Loepfert and Suarez (1996).

To determine the available mineral nutrient concentrations, 5 g of soil was dried at 60 °C for 48 h in 50-mL Falcon tubes. Each sample was diluted to 6.0 mL with DTPA-NH₄ and analysed for B, Ca, Co, Cu, Fe, K, Mg, Mn, Mo, Na, Ni, P, S, and Zn on an ELAN-DRCe ICP-MS instrument (PerkinElmer, SCIEX). National Institute of Standards and Technology (NIST) traceable calibration standards (ULTRA Scientific) were used for calibration (Soltanpour and Schwab 1977).

Plant ionome

The plant material was dried for 4 days at 60 °C. Approximately 0.1 g was used to perform open-air digestion in Pyrex tubes using 0.7 mL concentrated HNO₃ at 110 °C for 5 h in a hot-block digestion system (SC154-54-Well Hot Block™, Environmental Express, SC, Charleston, USA). The concentrations of the following elements (Ca, K, Mg, Na, P, S, B, Mo, Cu, Fe, Mn and Zn) were determined by inductively coupled plasma optical emission spectroscopy ICP-OES (Thermo Jarrell-Ash, Model 61E Polyscan, England) (Soltanpour and Schwab 1977).

Greenhouse experiment

In January 2019, 19 *A. thaliana* demes and Col-0 were sown in contrasting saline and saline-alkaline soils. Five seeds of each deme were sown in 30 pots (15 of each soil type) and distributed randomly in the greenhouse. Two weeks after germination, seedlings were thinned out so that only one plant per pot was left. Irrigation was applied twice a week. Every week, photographs of the entire rosette were taken. The number of siliques was counted at plant maturity. Air temperature, humidity and sun incidence were monitored throughout the experiment.

Salinity-alkalinity tolerance assays

Plate culture For germination assays, sterilized seeds from each deme were sown in plates under a

flow cabinet with sterile material. Plates contained 4 treatments: control ($\frac{1}{2}$ Murashige & Skoog media M5524 (MS), Sigma–Aldrich, pH 5.9), neutral salinity ($\frac{1}{2}$ MS NaCl 50 mM, pH 5.9), alkalinity ($\frac{1}{2}$ MS 10 mM NaHCO₃, pH 8.3), and alkaline-saline treatment ($\frac{1}{2}$ MS NaCl 40 mM+10 mM NaHCO₃, pH 8.3). For each deme and treatment, a total of 60 seeds were divided among 4 plates. All plates contained 0.6% Phyto-agar (Duchefa, Haarlem, The Netherlands), and solutions were buffered using different proportions of MES and BTP depending on the final pH. Plates with seeds were kept at 4 °C for synchronizing germination. After 4 days under stratification treatment, plates were moved to a growth chamber (12 h light/12 h dark, 150 $\mu\text{mol cm}^{-2}\cdot\text{s}^{-1}$, 40% humidity and 25 °C). Germination and radicle emergence were checked daily using a stereomicroscope (Zoom 2000, Leica, Wetzlar, Germany) during the following 10 days.

Hydroponic experiment Sterilized seeds of demes from 6 coastal (HS), 4 intermediate (MSA), and 4 inland (HA) demes were sown in 0.2 mL tubes containing 0.6% agar prepared with $\frac{1}{2}$ strength Hoagland nutrient solution ($\frac{1}{2}$ Hoagland, pH 5.9). Seeds were kept at 4 °C for 4 days in the dark to synchronize germination and placed in the growth chamber (12 h light/12 h dark, 150 $\mu\text{mol cm}^{-2}\cdot\text{s}^{-1}$, 40% humidity and 25 °C). After root emergence (approximately 48 h), the bottoms of the tubes containing seedlings were removed, and the tubes were placed in 150 mL hydroponic containers with aerated nutrient solution ($\frac{1}{2}$ Hoagland, pH 5.9). The nutrient solution was replaced every 2 to 3 days to maintain a constant concentration of nutrients in the solution. When 15 days old, the seedlings were separated into different sets. To avoid osmotic shock, the treatment solutions were gradually increased in each set to achieve the final treatment conditions after one week. The following treatments were applied: control ($\frac{1}{2}$ Hoagland, pH 5.9) and two alkaline-saline treatments ($\frac{1}{2}$ Hoagland + NaCl 40 mM+10 mM NaHCO₃, pH 8.3 and $\frac{1}{2}$ Hoagland + NaCl 60 mM+15 mM NaHCO₃, pH 8.3). Neutral pH solutions were buffered with 1:2 MES-BTP, while for high pH solution, 2:1 MES-BTP was used. The control and treatment solutions were replaced every 3 days. Plants remained under these conditions for two weeks. Every third day, photographs of the entire plant were

taken. Thirty-seven-day-old plants were harvested, and leaves and roots were photographed, weighed and stored at −80 °C. The rosette diameters were scored, and the length of the largest root of each plant was measured using ImageJ software (Bourne 2010; Schneider et al. 2012).

Irrigation experiment Plants from selected demes of all groups (HA, inland; MSA intermediate habitat; HS coast) were cultivated individually in square pots of 10-cm diameter in sterilized quartz sand. The selected demes were A1 (HA), T6 (HS), LG5 (MSA), V1 (MSA), V3 (MSA) and Col-0 as a reference. Sterilized seeds were sown on wet soil, and the pots were covered with polyvinyl chloride film until the seedlings had germinated. Pots with germinated seedlings were placed in a growth chamber with a 12-h light/12-h dark photoperiod, an irradiance of 150 $\text{mmol m}^{-2} \text{s}^{-1}$, and a constant temperature of 22 °C. Plants were watered with $\frac{1}{4}$ strength Hoagland solution at neutral pH 5.9 every 2 to 3 days. After 2 weeks, seedlings of each deme were split into 4 groups, and different treatments were applied ($n=8$). To avoid osmotic shock, the treatment solutions were gradually increased to achieve the final treatment conditions after one week. The applied treatments were control ($\frac{1}{2}$ Hoagland, pH 5.9), salt ($\frac{1}{2}$ Hoagland +75 mM NaCl, pH 5.9), alkaline ($\frac{1}{2}$ Hoagland +15 mM NaHCO₃, pH 8.3), and alkaline-saline conditions ($\frac{1}{2}$ Hoagland +60 mM NaCl +15 mM NaHCO₃, pH 8.3). Solutions at pH 5.9 were buffered with 1:2 MES-BTP, while for high pH solutions, 2:1 MES-BTP was used. After 2 weeks under treatment conditions, chlorophyll content was measured (see below), and plant material was collected and separated into roots and shoots. The roots were carefully rinsed with deionized water, and the fresh weights of the roots and shoots were measured prior to storage at −80 °C. Chlorophyll contents were measured in leaves under different treatments. Leaf chlorophyll concentrations were obtained using a SPAD chlorophyll metre (CCM-200, Opti-Science, Hudson, USA). To measure the osmotic potential, two full leaves were thawed and inserted into a 1 mL syringe stuffed with fibreglass. Sap samples (50 μL) were collected, and their osmolality was measured with a freezing-point depression osmometer (Osmostat 3000, Gonotec). Proline content was determined colorimetrically using a method adapted from Bates et al. (1973). Briefly, leaf

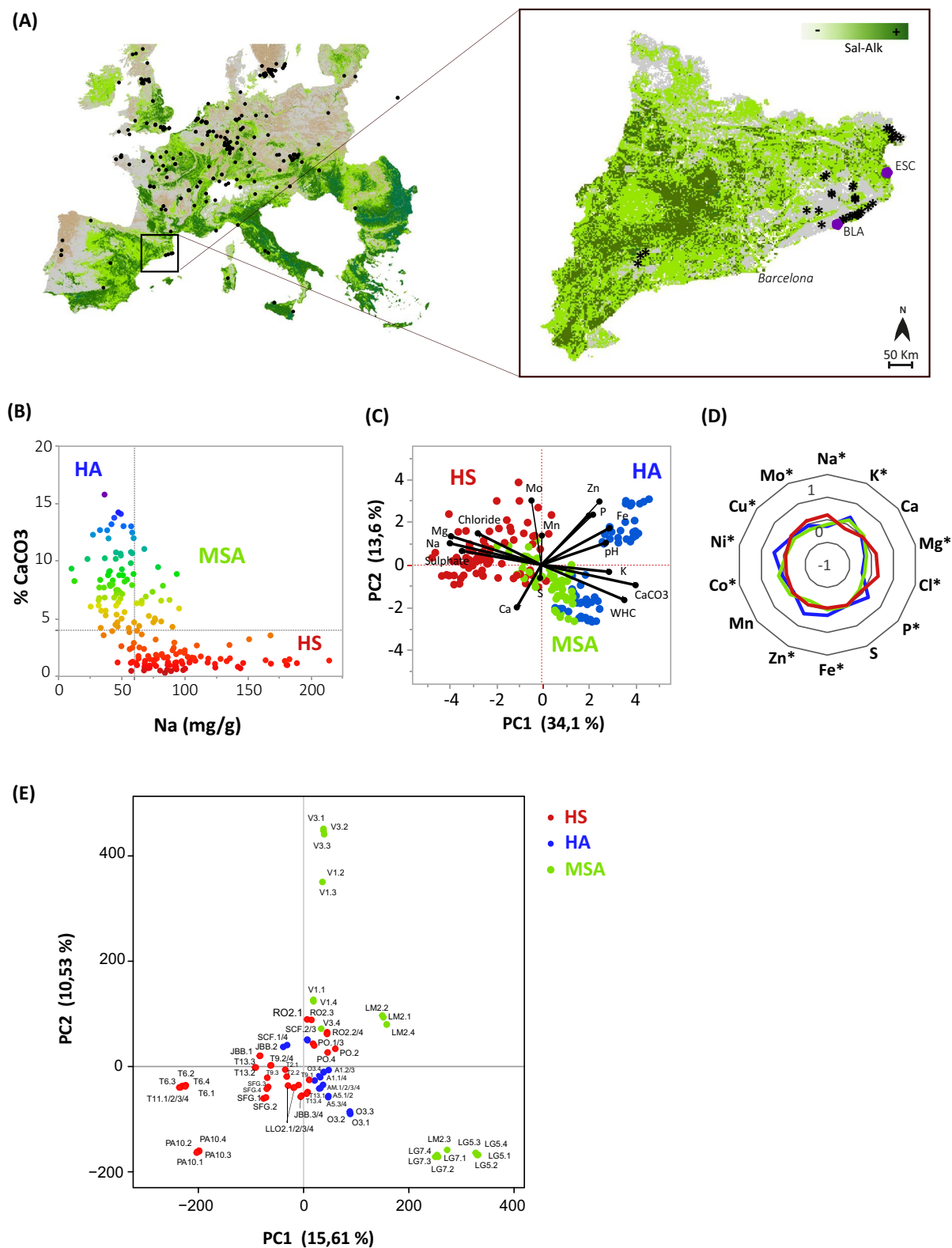


Fig. 1 *Arabidopsis thaliana* distribution and soil characteristics. **A** Geographic location of the European (black circles) and Catalan (black stars) *A. thaliana* demes and the two soil sampling points (purple circles) in the saline-alkaline soil map of Europe. **B** Soil carbonate (CaCO_3) vs soil Na^+ content, **C** PCA of soil parameters, and **D** normalized difference of 14 elements in the native soils of 19 *A. thaliana* demes. **E** Whole-genome PCA (37,574 SNP sites) of 76 *A. thaliana* individuals from our study region. Colors classify the *A. thaliana* demes in three groups according to its native soil characteristics: HA-plants (low Na^+ and high CaCO_3 , blue); MSA-plants (intermediate levels of Na^+ and CaCO_3 , green); and HS-plants (high Na^+ and low CaCO_3 , red)

material (50 mg) was homogenized in 1 mL 3% (w/v) sulfosalicylic acid and centrifuged at 12,000 rpm for 10 min at 4 °C. The supernatant was supplemented with ninhydrin (250 μL) and glacial acetic acid (250 μL) in a test tube. The mixture was heated in a water bath at 100 °C for 60 min, and then the reaction was stopped with ice. The mixture was extracted with a 0.3 volume of toluene, and the absorbance was read at 520 nm using a TECAN-Spark Reader ($n=4$ per deme per treatment).

A detailed outline of the experimental designs is shown in Supplementary Fig. S1; the demes used in each experiment are detailed in Supplementary Dataset S1.

Gene expression analysis

Frozen leaf material of plants from the irrigation experiments was used for RNA extraction. Total RNA was extracted using a PROMEGA RNA plant kit following the manufacturer's instructions. Total RNA was used to produce cDNAs using the iScript™ cDNA Synthesis Kit (Bio–Rad, Hercules, CA, USA) with 1 μL iScript Reverse Transcriptase +4 μL 5x iScript Reaction Mix + Sample + Molecular Water to obtain a 20 μL volume. Samples were run in a thermocycler (48-well MJ Mini™, Bio–Rad, Hercules, CA, USA) for 5 min at 25 °C, 30 s at 42 °C, and 5 s at 85 °C. Fifty-fold dilution of the cDNAs was performed with water (Molecular Biology Reagent, Sigma–Aldrich, St. Louis, MO, USA). Diluted cDNA (1:50) was used as a template for quantitative PCRs using iTaq Universal SYBR Green Supermix (Bio–Rad, Hercules, CA, USA). Real-time detection of fluorescence emission was performed on a CFX384 Real-Time

System (Bio–Rad, Hercules, CA, USA) using the following conditions: denaturation step for 10" at 95 °C followed by annealing and extension for 30" at 60 °C. A total of 40 cycles were run. A melt curve was performed, increasing from 65.0 °C to 95.0 °C by 0.5 °C every 5 s. Plates were edited using CFX manager version 3.1 software. Primers from selected genes were designed using the NCBI Primer-BLAST tool (Ye et al. 2012). The sequences of the primers used are detailed in Supplementary Dataset S10. The expression of target genes was normalized to the expression levels of the *Actin2* and *Tubulin* genes of *A. thaliana* (Dekkers et al. 2012). The relative expression (RE) of each gene was calculated in comparison to the control treatment. The expression of the target gene relative to the expression of the reference gene was calculated using the $2^{-\Delta\Delta\text{Ct}}$ method (Livak and Schmittgen 2001).

Statistical analysis

PCAs were performed on the genome-wide SNP data using the glPCA function of the adegenet R package (Jombart and Ahmed 2011). PCAs were visualized using ggplot2 (Wickham et al. 2016). The genome-wide SNP dataset was linkage disequilibrium-pruned using custom scripts and further filtered to include only putatively neutral fourfold degenerate sites (37,574 SNPs in total). PCAs were performed using 75 samples (4 plants of 19 demes except LLO2.1, which was excluded) from Busoms et al. (2018).

Data normality was checked for all phenotypes, and nonnormal data were transformed before applying any parametric tests. Mean-standardized values ($-1 < \text{value} < 1$) of elemental contents of soil and leaf material were used to represent the radar plots and compare each group. One-way or multivariate ANOVA was used to test for significant differences ($p \text{ value} < 0.05$) between means of data with respect to fitness, elemental contents of soil and leaf material, and gene expression. To test for correlations between two variables, a bivariate fit was applied. To perform multiple comparisons of group means, we used Tukey's HSD. The phenotypic plastic responses of different *A. thaliana* groups were expressed by their slopes of growth (rosette

diameter) in the soil reciprocal transplant, and their plasticity was shown by the absolute values of the slopes (Gao et al. 2018). All statistical analyses were performed using SAS Software JMP v.16.0 (https://www.jmp.com/es_es/home.html).

Results

Distribution of native *A. thaliana* demes according to climate, soil and genetic characteristics

The *A. thaliana* demes used in this study occur spontaneously in the northeast region of the Iberian Peninsula (Fig. 1A), which corresponds to the southern edge of the distribution area of this species (Krämer 2015). Almost all Catalan *A. thaliana* populations occur in the same climatic region, which corresponds to the Mediterranean coastal climate. Coastal and inland demes have been distinguished based on the salinity concentrations in the soils (Busoms et al. 2015). However, the distribution of *A. thaliana* demes was clearly interrupted in the saline-alkaline areas of the coast (dark green areas in Fig. 1A). Soils in these areas have high salinity, high pH and high carbonate contents. Coastal populations only occur on siliceous substrates with slightly acidic pH values (Supplementary Fig. S2 and Supplementary Dataset S2). Previous work has provided evidence that Arabidopsis populations from this region harbour substantial genetic variability and exhibit adaptive variation to elevated salinity (Busoms et al. 2015, 2018) and to moderate soil carbonate content (Terés et al. 2019). According to the Na⁺ and CaCO₃ concentrations of their native soils, the Catalan *A. thaliana* demes were split into three categories: HA plants from soils with high CaCO₃ contents and low salinity (average values 9.4% CaCO₃, 44 Na⁺ mg g⁻¹); MSA plants native to soils with intermediate carbonate levels and salinity (5.7% CaCO₃, 61 Na⁺ mg g⁻¹); and HS plants from soils with low CaCO₃ contents and high salinity (0.65% CaCO₃, 129 Na⁺ mg g⁻¹) (Fig. 1B).

Native soil parameters (organic matter (OM, %), pH, water holding capacity (WHC, mL/g), CaCO₃ (%), Ca (mg/g), chloride, Co (mg/g), Cu (mg/g), Fe (mg/g), K (mg/g), and Mg (mg/g)) monitored from 2013 to 2015 (Busoms 2015) were used as input to perform a principal component analysis (PCA). Soil Na, Mg, Mo, chloride and sulfate define the demes

of HS groups, while Fe, Zn, K, P, pH, CaCO₃ and WHC differentiate HA demes (Fig. 1C). Correlations between the different parameters of the native soils (pH, OM, WHC and nutrients) can be found in Supplementary Dataset S3. Figure 1D represents the mean relative values of available mineral nutrients in the native soil of each group. HS soil was characterized by higher Na⁺, Mg and Cl concentrations, while higher K⁺ concentrations were found in soils from MSA and HA. Phosphorus, Zn and Ni concentrations were higher in HA than in MSA with intermediate levels of carbonate and salinity (Supplementary Dataset S3).

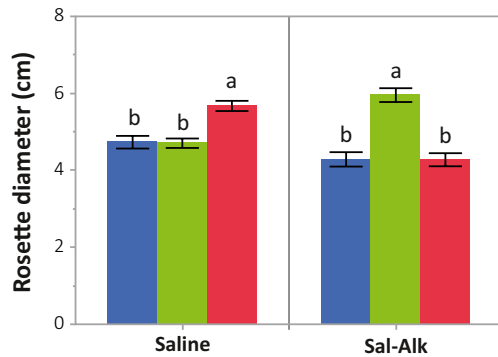
To gain an understanding of the demography of our demes, we performed a whole-genome PCA (Fig. 1E). Most of the demes are highly clonal, and there is no clear differentiation by location or geography. However, in general, MSA plants are the most divergent from the rest, suggesting that the genetic diversity of these demes might enclose interesting molecular signatures of selection.

Reciprocal soil transplant experiment

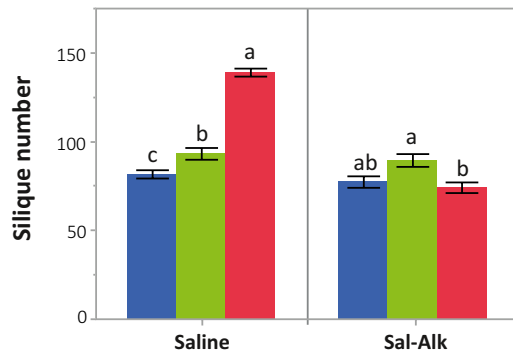
To reveal potential differences in fitness among demes in response to salinity on alkaline or siliceous substrates, a reciprocal soil transplant experiment was established in a greenhouse. Plants were grown from seeds in pots filled with either saline-siliceous or saline-alkaline soil. Both soils had a similar sandy-loam texture and salinity levels but clearly differed in pH (6.5 vs. 8.4) and carbonate content (2% vs. 18%) (Supplementary Fig. S3A). The nutrient availability of both soils was also considerably different. Higher Fe, Zn, Mn, and Mg concentrations were found in the saline-siliceous soil, while Na⁺ and K⁺ concentrations were similar in both (Supplementary Fig. S3B and Dataset S4).

Plant fitness was assessed by measuring the rosette diameter of 5-week-old plants (Fig. 2A) and the number of siliques per plant at maturity (Fig. 2B). On saline-siliceous soil, plants of HS, native to this soil type, performed better than plants from HA or MSA (Fig. 2A, B). In contrast, in saline-alkaline soil, the best performance was observed for MSA plants native to soils with intermediate levels of salinity and alkalinity. Plants adapted to saline-siliceous soils suffered a severe reduction in growth and number of siliques

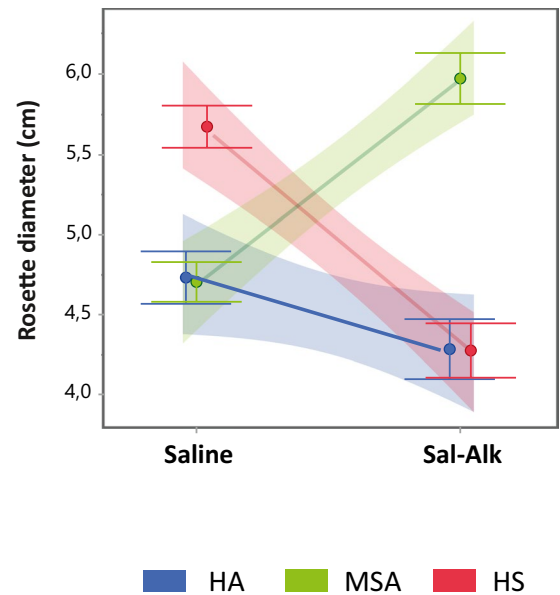
(A)



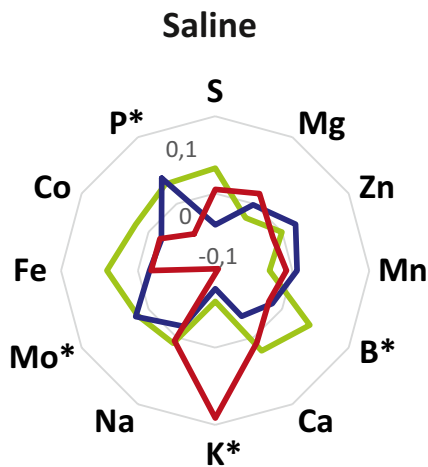
(B)



(C)



(D)



(E)

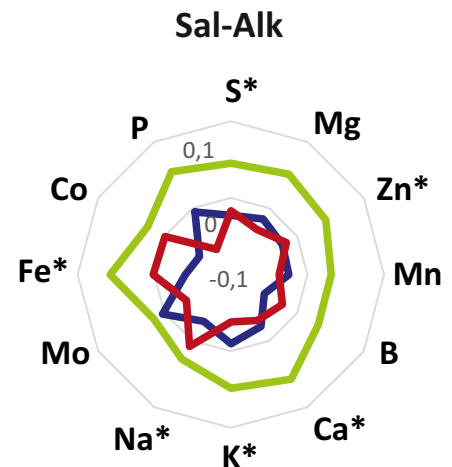


Fig. 2 Soil reciprocal transplant performance of *A. thaliana* groups. Mean \pm SE of **A** growth (rosette diameter, RD) and **B** fitness (number of siliques produced) of *A. thaliana* plants cultivated in saline (BLA site) and saline-alkaline (ESC site) soil in semi-controlled conditions (UAB greenhouse). Letters indicate significant differences (Tukey's HSD, $P < 0.05$) between *A. thaliana* demes groups (HA = blue, MSA = green, HS = red).

C Mean of growth (RD) \pm 95% confidence intervals of HA, MSA and HS plants cultivated in saline and saline-alkaline soil. Normalized differences of 12 elements in the leaves of plants from the three groups cultivated in **D** saline soil or **E** saline-alkaline soil. Elements exhibiting significant differences (according to a t-test) are marked with an asterisk (*, $P < 0.05$)

when cultivated on saline-alkaline soil (Fig. 2A, B and Supplementary Dataset S5).

The phenotypic plastic responses based on rosette diameters were calculated considering the absolute values of the slope (Gao et al. 2018) of the line connecting the values from saline to alkaline saline common gardens (Fig. 2C). The interactions between demes and growth conditions (soils) showed significant effects ($P < 0.05$) on rosette diameter and number of siliques, indicating significantly different reaction norms among plant groups (Supplementary Dataset S5). The plasticity index based on rosette diameter shows that plasticity is a major factor responsible for the better growth of MSA plants on alkaline-saline soil. In contrast, HS plants were maladapted to alkaline-saline conditions, while HA plants revealed low plasticity (Fig. 2C).

Plants from different groups also differed in the relative leaf ion concentrations when grown on the contrasting soils (Fig. 2D, E and Supplementary Dataset S6). Plants adapted to saline-siliceous soils (HS) were able to maintain higher K^+ concentrations on saline soil than plants from HA and MSA, while concentrations of Mo and P were considerably lower in HS plants (Fig. 2D). In the saline-alkaline soil, MSA plants maintained higher leaf concentrations of all analysed nutrients (Fig. 2E). Sodium concentrations were similar in MSA and HS plants; only HA plants better restricted Na^+ transport to the leaves.

Saline-alkaline stress experiments

To further confirm the differential responses of *A. thaliana* demes to saline-alkaline conditions, germination tests and hydroponic cultures were performed. Under control conditions, the germination rates determined on agar plates did not differ among the three groups. Moderate salinity (50 mM NaCl) applied at a slightly acidic pH (pH 5.9) somewhat reduced the germination rates in all three groups to a similar extent. In contrast, under nonsaline-alkaline conditions (10 mM $NaHCO_3$, pH 8.3), germination rates were higher in demes native to soils with the highest carbonate level (HA) and lowest in demes locally adapted to salinity on siliceous soil (HS). The saline-alkaline treatment (40 mM NaCl + 10 mM $NaHCO_3$) almost completely inhibited the germination of HS plants, while in HA and MSA, the germination rates

were similar to those observed with respect to NaCl salinity under slightly acidic pH conditions (Fig. 3A). The germination rates of the demes under the alkalinity treatment were positively correlated with the soil carbonate content in their native habitats but negatively correlated with the corresponding soil Na^+ contents. A negative correlation between the germination rate and native soil Na^+ was also observed for the alkaline-saline treatment (Supplementary Dataset S7).

For the evaluation of the growth performance under saline-alkaline conditions, two levels of stress were assayed: a moderate stress level with 40 mM NaCl + 10 mM $NaHCO_3$ (pH 8.3) and a strong stress level with 60 mM NaCl + 15 mM $NaHCO_3$ (pH 8.3). The strong treatment reduced root and leaf length in all three groups (Fig. 3B). Under moderate saline-alkaline stress, plants from HA and HS also suffered inhibition of root and leaf elongation, while MSA plants were hardly affected (Fig. 3B, Supplementary Dataset S7). These results confirm the superior tolerance of MSA plants to saline-alkaline stress.

Further experiments using sand cultures irrigated with different treatment solutions confirmed the superior behaviour of MSA plants under saline-alkaline conditions and the best performance of HS plants under saline, slightly acidic pH conditions (Fig. 3C; Supplementary Fig. S4). *A. thaliana* Col-0 plants were included here as a reference. Tolerance to the saline-alkaline treatment of the Col-0 plants was similar to that of MSA; only the relative rosette diameter was more inhibited in Col-0 (Fig. 3C).

To determine the physiological adaptive responses of the different plant groups under alkaline-saline conditions, chlorophyll, proline, osmotic potential and nutrient mineral content were determined. Plants of the HA group were the best at maintaining leaf chlorophyll concentrations under alkaline conditions, while HS plants were superior under high saline conditions (pH 5.9). Under alkaline-saline conditions, MSA maintained the highest chlorophyll concentrations. In contrast, Col-0 suffered a marked decrease in leaf chlorophyll under alkaline-saline conditions (Fig. 3D; Supplementary Dataset S8).

Leaf mineral nutrient concentrations of plants from the irrigation experiments were similar to those obtained under greenhouse conditions. Regarding the relative Na^+ content, in saline and alkaline conditions, all plant groups showed similar responses. However,

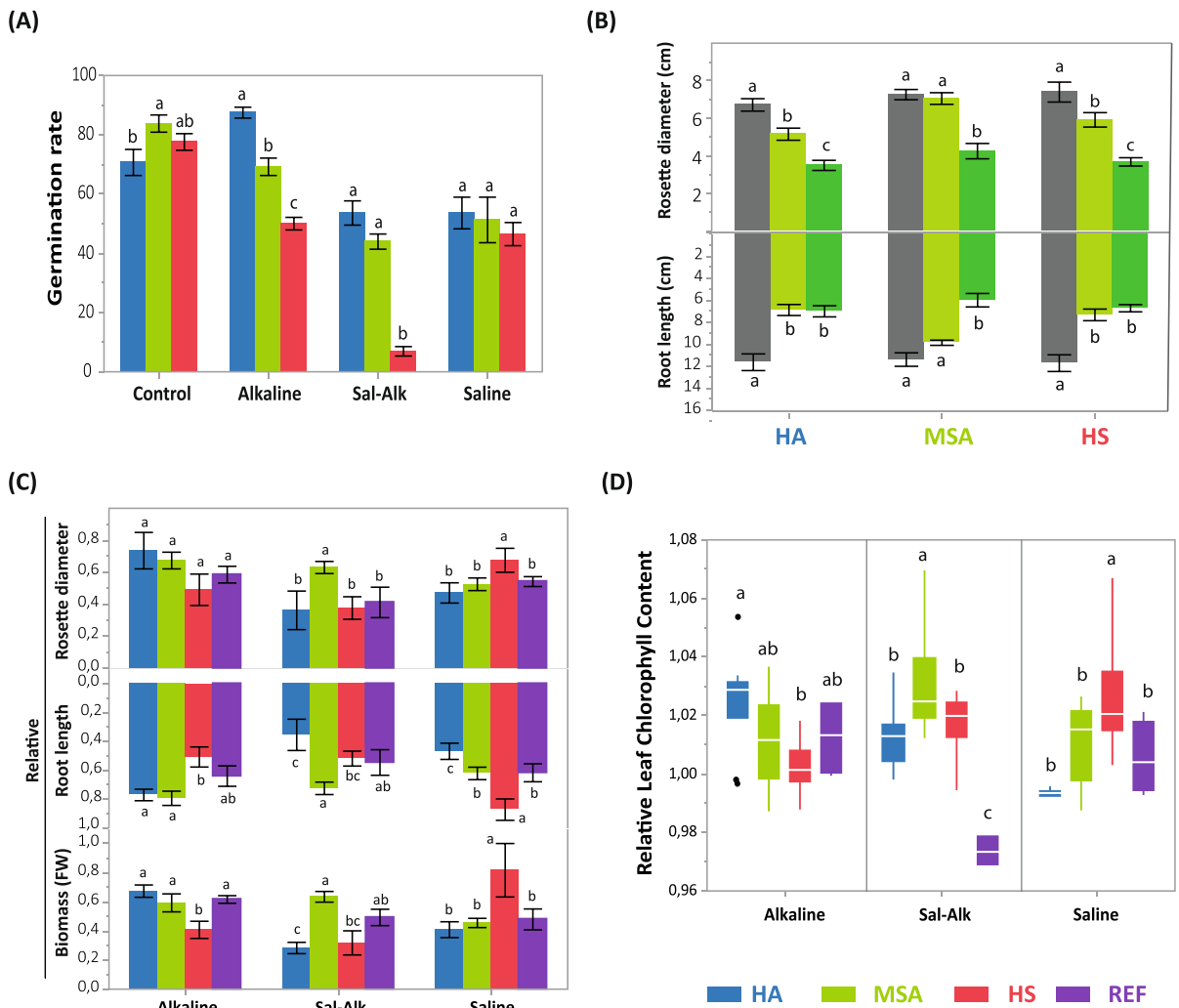


Fig. 3 Salinity-alkalinity tolerance of *A. thaliana* groups. **A** Germination rate (%) \pm SE of *Arabidopsis* seeds sown in MS plates with no NaCl or NaHCO₃ (Control treatment, pH 5.9), with 50 mM of NaCl (Saline treatment, pH 5.9), with 10 mM NaHCO₃ (Alkaline treatment, pH 8.3), or with 40 mM NaCl +10 mM NaHCO₃ (Sal-Alk treatment, pH 8.3). Letters indicate significant differences (Tukey's HSD, $P < 0.05$) between *A. thaliana* groups: HA=blue, MSA=green, HS=red. **B** Mean \pm SE of rosette diameter (cm) and root length (cm) of *A. thaliana* plants cultivated in hydroponics under Control (0 mM NaCl +0 mM NaHCO₃, pH 5.9, grey), Sal-Alk-1 treatment (40 mM NaCl +10 mM NaHCO₃, pH 8.3, light green),

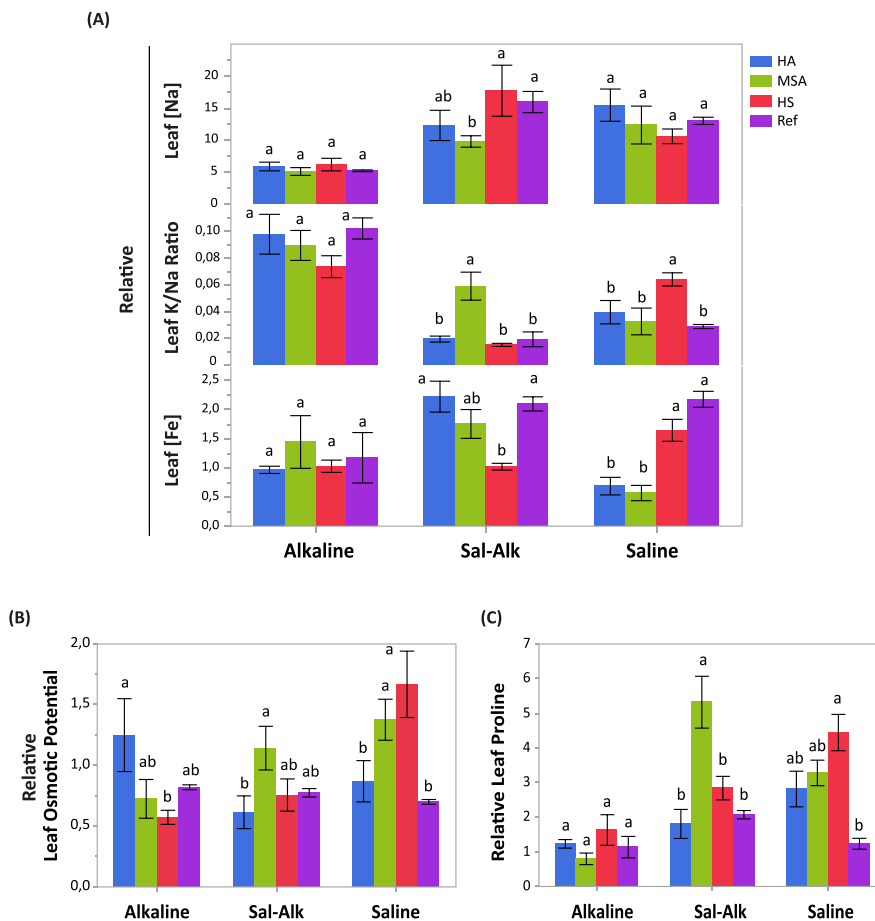
and Sal-Alk-2 treatment (60 mM NaCl +15 mM NaHCO₃, pH 8.3, dark green) for 15 days. Letters indicate significant differences (Tukey's HSD, $P < 0.05$) between treatments for each *A. thaliana* deme group. **C** Relative growth parameters (rosette diameter, root length and biomass) and **D** leaf chlorophyll of HA (blue), MSA (green), HS (red), and Col-0 (purple) plants cultivated in 2:1 sand:perlite substrate irrigated with no NaCl or NaHCO₃ (Control, pH 5.9), with 50 mM of NaCl (Sal, pH 5.9), with 10 mM NaHCO₃ (Alk, pH 8.3), or with 40 mM NaCl +10 mM NaHCO₃ (Sal-Alk, pH 8.3) for 15 days. Letters indicate significant differences (Tukey's HSD, $P < 0.05$) between *A. thaliana* groups for each treatment

under alkaline-saline conditions, leaf Na⁺ uptake in Col-0 and HS was higher than that in MSA. The HS group showed the highest K⁺/Na⁺ ratio under saline conditions, while the MSA group outperformed the HA and HS individuals under alkaline saline

conditions. Higher concentrations of leaf Fe were observed in HA plants under alkaline-saline and saline conditions (Fig. 4A; Supplementary Dataset S8).

Plants from the MSA group showed the highest relative osmotic potential under alkaline-saline

Fig. 4 Physiological plant responses of *A. thaliana* groups. **Relative leaf A** Na^+ , K^+ / Na^+ ratio and Fe nutrient content, **B** osmotic potential, and **C** proline content of HA (blue), MSA (green), HS (red), and Col-0 (purple) plants cultivated in 2:1 sand:perlite substrate irrigated with no NaCl or NaHCO_3 (Control, pH 5.9), with 50 mM of NaCl (Saline, pH 5.9), with 10 mM NaHCO_3 , (Alkaline, pH 8.3), or with 40 mM NaCl + 10 mM NaHCO_3 (Sal-Alk treatment, pH 8.3) for 15 days. Letters indicate significant differences (Tukey's HSD, $P < 0.05$) between *A. thaliana* groups for each treatment



conditions. Only plants from the HS group had similarly high values, but under nonalkaline salinity. HA individuals showed the highest osmotic adjustment capacity under the alkaline treatment (Fig. 4B; Supplementary Dataset S8). Under salt stress, proline accumulates as a major organic osmolyte in *A. thaliana* (Verbruggen et al. 1993). Here, we observed an increase in leaf proline concentrations in all individuals under saline (HS > MSA > HA) and saline-alkaline conditions (MSA > HS > HA), with MSA being the only group showing significantly higher relative proline content under saline-alkaline treatment (Fig. 4C; Supplementary Dataset S8).

Molecular analyses

Quantitative PCR analysis of the selected genes performed 10 days after exposure to the different treatment solutions revealed that *AHA1* was activated by alkaline-saline conditions, especially in the roots and

leaves of MSA plants (Fig. 5A). *SOS1* was enhanced by salinity stress in all plants, especially in the root tissue (Fig. 5B). However, in T6 individuals (HS), this gene was highly expressed in the plants submitted to nonalkaline salinity, but not when they were exposed to saline-alkaline stress. Interestingly, *SOS1* expression was less or equally induced in almost all the plants subjected to saline-alkaline treatment (Fig. 5B). *NHX1* was clearly activated by salinity in the roots and leaves of HS plants. Moreover, under alkaline-saline conditions, MSA plants also exhibited higher expression of *NHX1* in their aerial tissue (Fig. 5C). Nonalkaline salinity enhanced *HKT1* expression in the roots of HS, MSA and Col-0, while under alkaline salinity, only MSA plants were able to strongly upregulate *HKT1* (Fig. 5G).

Regarding *FRO2*, the alkalinity treatment enhanced the expression in all plants, but alkaline-saline exposure only increased the expression of *FRO2* in the MSA plants (Fig. 5D). The nonsaline

alkalinity treatment also induced the expression of *IRT1* in the roots of almost all the plants but only in the leaves of HA plants. Alkaline salinity did not significantly alter *IRT1* expression, except for enhanced expression in the leaves of MSA plants (Fig. 5E). *FPN2* was activated under alkaline conditions, especially in the roots of HA, MSA, and Col-0 plants. Nonalkaline salinity also caused enhanced expression of *FPN2* in MSA, while alkaline salinity did not enhance *FPN2* expression in any group (Fig. 5F).

Discussion

It is widely known that saline-alkaline conditions are more stressful to plants than salinity under slightly acidic or neutral pH conditions (Shi and Sheng 2005; Gao et al. 2014). Our results clearly demonstrate that *A. thaliana* demes locally adapted to salinity on siliceous soils (pH 5.9) are more sensitive to saline-alkaline conditions than plants adapted to soils with rather moderate levels of salinity and carbonate (Figs. 2 and 3). Even so, most studies addressing the molecular genetic mechanisms underlying salinity tolerance in *A. thaliana* are performed under slightly acidic pH (e.g., DeRose-Wilson and Gout 2011; Busoms et al. 2015); many authors do not even mention the pH conditions of the treatments applied (e.g., Julkowska et al. 2016; Liu et al. 2020), and studies with *A. thaliana* seldom include bicarbonate in the saline treatment solutions (e.g., Jia et al. 2017).

Local adaptation to soil conditions in NE Catalonia

In NE Catalonia, *A. thaliana* demes exhibit a particular distribution. In previous studies, we have shown that soil Na^+ drives divergent selection of coastal demes with higher tolerance to salinity (Busoms et al. 2015). These demes (HS) occur exclusively on siliceous saline soils. Soil carbonate (pH > 7) is a further factor for the local adaptation of *A. thaliana* at nonsaline inland sites (HA demes) (Terés et al. 2019), despite the rarity of finding wild *A. thaliana* demes on alkaline soils (Fig. 1A). Based on soil characteristics and phenotype data, we defined a third group of plants (MSA) from habitats with intermediate soil levels of Na^+ and carbonate (Fig. 1B). However, no *A. thaliana* demes were found under the harsh conditions of coastal saline-alkaline habitats with both

high Na^+ and carbonate concentrations. This is illustrated by the fact that the distribution of *A. thaliana* demes on the Catalan coast is restricted to saline-siliceous soils on plutonic rocks and interrupted characteristically on coastal calcareous dolomites, limestones, sandstones and gypsum (Fig. S2A). This distribution indicates that in our region, local adaptation to strong saline-alkaline conditions has not evolved in *A. thaliana*. Apparently, this also seems to be the case at the European scale because any of the georeferenced European ecotypes located in the saline soils ($\text{Na}^+ > 150 \text{ mg/g}$) exceed respective pH or CaCO_3 levels higher than 7.4 or 9% in coastal locations or 7.8 or 42% in inland sites (Supplementary Dataset S2).

Plants able to adapt to alkaline-saline conditions must efficiently manage multiple stress factors: high Na^+ , low osmotic potential, low availability of micro-nutrients, especially Fe and Zn, and imbalance of carbon metabolism due to dark fixation of inorganic carbon (Poschenrieder et al. 2018). PCA using physico-chemical characteristics of the native soils of the *A. thaliana* demes analysed here confirmed the clear separation of the three plant groups (Fig. 1C). Chloride content, sulfate and Mg, and Mo and Na are correlated, and they overlap with the coastal populations, while Ca, Zn and P concentrations are correlated and are characteristic of alkaline soils. Differences in the relative availability of soil nutrients among the habitats further characterize the three plant groups (Fig. 1D).

Although soil Na^+ and carbonate concentrations clearly distinguish HA and HS demes and we previously have shown that these soil factors drive local adaptation in *A. thaliana* demes in this region (Busoms et al. 2015; Terés et al. 2019), the whole-genome PCA does not reveal a clear stratification by soil of the 3 groups (Fig. 1E). Our previous studies (Busoms et al. (2018)) revealed that these Catalan demes are not Iberian relicts and that they share most of the genome with the European accession. Nonetheless, they harbour high genetic diversity when examining particular genes or regions that have been the target of selection agents. Here, we found contrasting phenotypic differences both in greenhouse common gardens and hydroponics under saline-alkaline conditions (Figs. 2 and 3), suggesting that the genetic variability present on these demes could have positive or negative consequences under this combined stress.

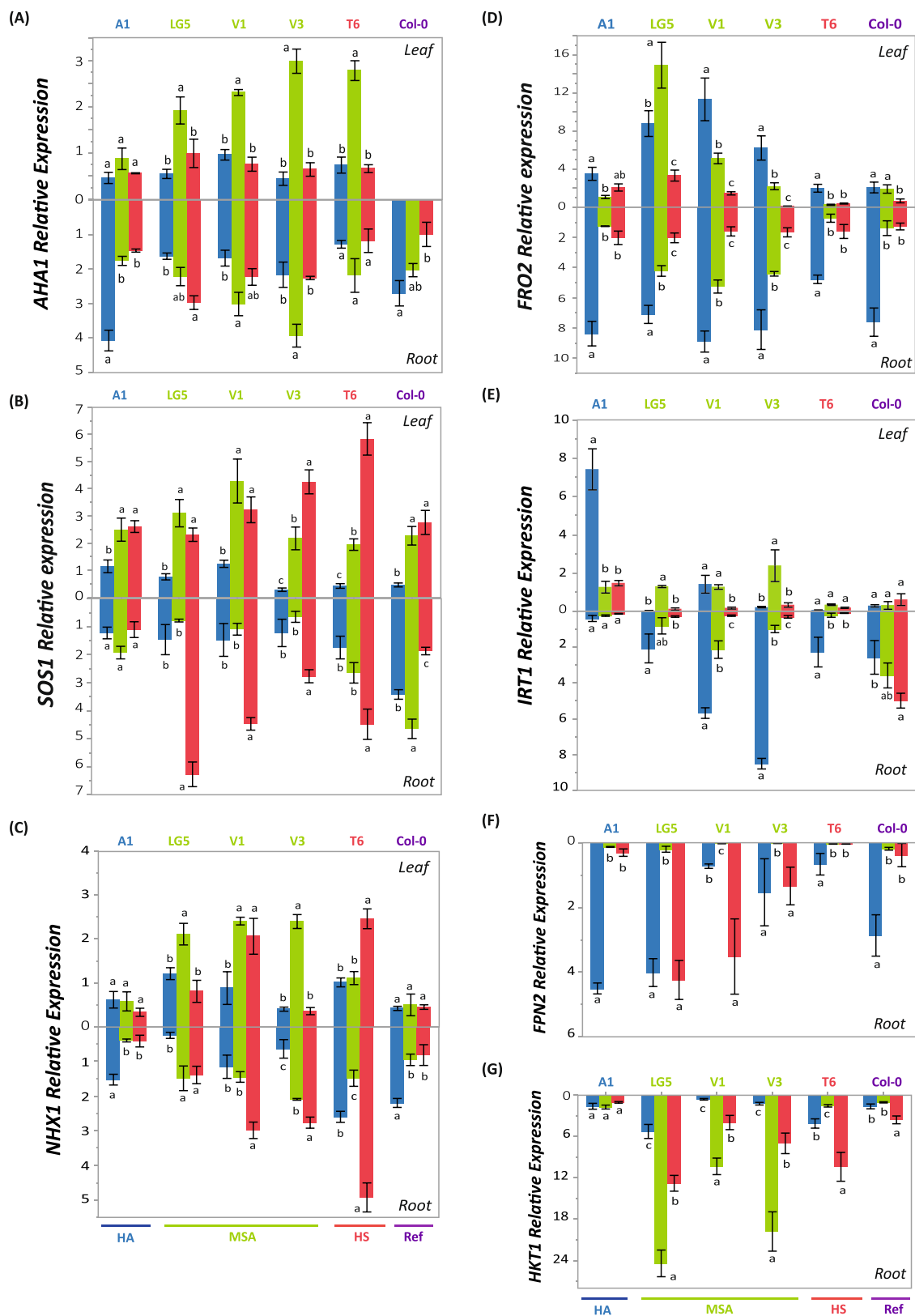


Fig. 5 Effects in expression of salinity and alkalinity responsive genes. Relative transcript expression (treatment vs control) of **A** *AHA1*, **B** *SOS1*, **C** *NHX1*, **D** *FRO2*, **E** *IRT1*, **F** *FPN2* and **G** *HKT1* in shoots and roots of triplicate biological replicates of plants from A1 (HA), LG5 (MSA), V1 (MSA), V3 (MSA), T6 (HS) demes and Col-0 (Ref) exposed to salinity (50 mM of NaCl, pH 5.9, red bars), alkalinity (10 mM NaHCO₃, pH 8.3, blue bars), or salinity-alkalinity (40 mM NaCl +10 mM NaHCO₃, pH 8.3, green bars) for 15 days. Data represent the mean \pm SE of three plants per accession. Letters indicate significant differences (Tukey's HSD, $P < 0.05$) between treatments for each *A. thaliana* deme

Phenotypic differences under saline-alkaline stress

Although *A. thaliana* is unable to colonize the saline-alkaline soils of the Catalanian coast, plants from all three groups were able to grow in common gardens on alkaline saline-soil under greenhouse conditions. The lack of competition in the experimental pots and the regular irrigation to field capacity provided less stressful conditions. The main obstacle for coastal demes from siliceous soils to colonize the nearby alkaline saline soils seems to be the strong inhibition of the germination rate. All *A. thaliana* demes inhibited germination on alkaline-saline substrates. HS plants were most severely inhibited, while HA and MSA plants had similar germination rates (Fig. 3A). Synergistic effects of salinity and alkalinity on germination have previously been reported in canola (Wang et al. 2022) and in alfalfa cultivars differing in alkaline-saline tolerance (Zhang et al. 2017a). Germination and early growth stages are also the most sensitive in wheat (Lin et al. 2012). Absciscic acid is a main inhibitor of germination in nondormant seeds, and NaCl exposure enhances seed ABA concentrations (Shu et al. 2017). The mechanisms behind the stronger inhibition of germination under alkaline salinity remain unknown.

Subsequent growth of germinated seedlings transplanted to hydroponics revealed better performance of MSA plants under saline-alkaline conditions. This was especially visible for the mild alkaline-saline treatment (40 mM NaCl +10 μ M NaHCO₃), which did not cause any reduction in rosette diameter or root length in the MSA plants native to soils with intermediate levels of salinity and alkalinity (Fig. 3B). In contrast, plants native to nonsaline-alkaline soil (HA) and those native to saline-siliceous soils (HS) suffered growth inhibition even under the mild alkaline-saline stress

treatment. Comparison of relative growth values clearly demonstrates the better performance of MSA under saline-alkaline conditions in contrast to HS plants, with the best growth observed under saline (pH 5.9) conditions (Fig. 3C). A decrease in chlorophyll is, among other factors, a marker of cell damage due to salt stress (Peharec Stefanic et al. 2013). Here, the negative effect of alkaline-salinity on chlorophyll concentrations in the MSA group was smaller than in HA and HS plants. In turn, alkalinity and salinity, as single stress factors, exerted less impact on chlorophyll loss in the HA and HS groups, respectively (Fig. 3D). These results further support the view of local adaptation of coastal (HS) and inland (HA) demes to their native habitats and the higher tolerance to combined saline and alkaline conditions in MSA individuals.

The greater performance of MSA under saline-alkaline stress was also confirmed by the common garden and irrigation studies (Figs. 2 and 4). Maintenance of a high K⁺/Na⁺ ratio is critical for salinity tolerance (Rubio et al. 2020). Alkaline salinity has an especially severe inhibitory effect on this parameter (Lin et al. 2012). Under saline conditions, HS plants were best at maintaining high K⁺/Na⁺ tissue ratios. However, under saline-alkaline conditions, HS plants had the lowest relative K⁺ concentrations (Fig. 2E and Fig. 4A). In contrast, MSA plants not only maintained high K⁺/Na⁺ ratios but also had higher concentrations of Fe and Zn, micronutrients that often limit the growth of plants under alkaline conditions (Riaz et al. 2020). Our results indicate that alkalinity hampers the transport mechanisms that allow salt-tolerant plants to maintain high K⁺/Na⁺ ratios under nonalkaline saline conditions. In fact, HS plants were unable to increase *HKT1* expression in roots when exposed to alkaline salinity (Fig. 5).

Saline stress in plants enhances proline production, reducing both osmotic and ionic stress. Contrasting information can be found regarding the role of proline under alkaline saline conditions. In *O. sativa* seedlings, the proline content was not correlated with plant tolerance (Lv et al. 2015), while the opposite was found in *Beta vulgaris* (Liu et al. 2013). In our system, alkaline saline conditions led to higher Na⁺ uptake and proline accumulation (Fig. 4C). Furthermore, the higher leaf proline content observed in plants of the MSA group under

saline-alkaline exposure was coincident with better growth maintenance, higher K^+/Na^+ ratios and higher chlorophyll retention than in HA and HS plants. Altogether, our results indicate that the ability to accumulate proline in response to alkaline salinity plays a crucial role in the tolerance of the MSA group.

Transcript expression of selected genes

In glycophytes, performance under saline conditions requires strict regulation of Na^+ and K^+ homeostasis. Different transporters and channels are responsible for this control (Wu et al. 2018). Under alkalinity, the ability to maintain the acquisition and transport of Fe is one of the critical factors (Terés et al. 2019). The observation here that plants of the HS and HA groups were more sensitive to alkaline salinity than plants from MSA suggests that alkaline salinity hampers the ion homeostasis and iron efficiency mechanisms evolved under natural conditions of nonalkaline salinity or nonsaline alkalinity. To explore this possibility, we focused on the expression of selected transporter genes involved in Na^+ and Fe homeostasis in *A. thaliana*: *AHA1*, *SOS1*, *NHX1*, *HKT1*, *FRO2* and *IRT1* (Fig. 5).

In plants, plasma membrane H^+ -ATPases are responsible for establishing the electrochemical proton gradient that maintains the intracellular and extracellular pH balance. In particular, the plasmalemma H^+ -ATPase *AHA1* provides the H^+ gradient required for the maintenance of high intracellular K^+ concentrations and Na^+ exclusion under salinity stress (Bose et al. 2015). Moreover, acidification of the rhizosphere can favour Fe availability under alkaline conditions, and several authors have reported that alkaline stress triggers signalling pathways that influence the expression of AHAs, increasing the alkalinity tolerance of the plants (Yang et al. 2019; Xie et al. 2022). In fact, alkalinity substantially enhanced *AHA1* expression in roots of HA, while both root and leaf expression of *AHA1* were increased by saline-alkalinity in MSA and HS plants (Fig. 5A). The overexpression of *AHA1* in response to all of the treatments supports the important role of these genes under single or combined stress factors.

SOS-, HKT- and NHX-type proteins are the key Na^+ transporters that modulate salt tolerance in plants. Although much is known about their

respective functions under salt conditions, few studies have examined their roles under saline-alkaline conditions. *SOS1* is required for maintaining Na^+ and K^+ homeostasis in plants exposed to salinity (Shi et al. 2000). Extreme increases in the Na^+/K^+ ratio in the xylem parenchyma of *sos1* rice mutants confirmed its function (El Mahi et al. 2019). In saline-tolerant rice, *SOS1* was reported to be more highly expressed at pH 8 than at pH 7 (Chuamnakthong et al. 2019). However, in our *A. thaliana* demes, root expression of *SOS1* was most enhanced by salinity (pH 5.9) in MSA and HS plants. Leaf *SOS1* expression was enhanced by both saline and saline-alkaline treatments to a similar extent. Exceptions were T6 from HS and V3 from MSA, which had considerably higher leaf *SOS1* expression under nonalkaline salinity (Fig. 5B). The *A. thaliana* T6 deme has evolved on saline, slightly acidic soil, and the highest expression of *SOS1* was obtained in slightly acidic saline solutions simulating the conditions of the native habitat. The lower expression under alkaline conditions suggests that alkaline salinity is either less perceived by these plants or that other constraints imposed by the alkaline-saline conditions inhibit the full expression of *SOS1*. The T6 deme also had lower expression of *NHX1* under alkaline salinity than under salinity (pH 5.9), while the MSA plants highly expressed *NHX1* under alkaline salinity (Fig. 5C). *AtNHX1* is a major player in the control of vacuolar pH and K^+ and Na^+ uptake (Bassil et al. 2019). Its activity is crucial in salinity tolerance, as this Na^+/H^+ exchanger brings Na^+ into the vacuole, decreasing the Na^+ levels in the cytosol. Salt tolerance has been found to be distinctively enhanced in *A. thaliana* overexpressing NHX from halophytes (Liu et al. 2017). Our results suggest that higher leaf expression of *NHX1* can enhance saline-alkaline tolerance by promoting Na^+ compartmentalization and osmotic adjustment.

HKT1 is mainly expressed in roots, where it functions to retrieve Na^+ from the xylem and reduce the transport of Na^+ to shoots (Rus et al. 2006). Here, plants from the HS group exhibited enhanced *HKT1* root expression only under salinity (pH 5.9). In contrast, MSA individuals showed the highest root *HKT1* expression levels under saline-alkaline conditions (Fig. 5G). Taken together, our results show that alkaline salinity induces higher levels of expression of genes involved in Na^+ and K^+ homeostasis in MSA plants than in plants naturally adapted to salinity on

siliceous soils or to nonsaline-alkaline soils. In HS plants, bicarbonate hampered the expression of these genes. The mechanisms underlying this inhibition clearly deserve further attention.

Enhancements of the expression levels of *FRO2*, *IRT1* and *FPN2* are important mechanisms for improving Fe acquisition in dicot plants exposed to low Fe availability, a common feature under alkaline soil conditions. In *A. thaliana*, rhizosphere Fe (III) is reduced by *FRO2* and then transported as Fe^{2+} into epidermal cells by the divalent metal transporter *IRT1* (Poschenrieder et al. 2018). *FPN2*, a protein with Fe, Co and Ni transporter activity, localizes to the vacuole. The protein is expressed in the two outermost layers of the root in response to iron deficiency, suggesting a role in buffering metal influx (Morrissey et al. 2009). In rice, higher expression levels of *IRT1* have been associated with better tolerance to alkaline salinity (Li et al. 2016). In our *A. thaliana* demes, *IRT1* and *FPN2* were mainly activated by alkaline stress (Fig. 5E). Under saline-alkaline conditions, *IRT1* expression was higher in MSA than in HA and HS. However, the expression levels did not follow a clear pattern either in roots or shoots, indicating that this gene is not directly regulated by this combined stress. Curiously, in the MSA demes, the nonalkaline salinity treatment intensified the expression of *FPN2*, while under alkaline salinity, *FPN2* was poorly expressed. Cobalt has been found to alleviate salinity stress in *Salvia officinalis* (Torun 2019). The crosstalk between Fe^{2+} , Co^{2+} and Na^+ may be relevant for establishing the mechanisms of saline-alkaline tolerance.

Alkalinity enhanced *FRO2* expression more in roots of carbonate tolerant than carbonate sensitive soybean varieties (Waters et al. 2018). In contrast, *FRO2* expression is decreased in chlorotic leaves of sensitive orange trees exposed to carbonate (Martínez-Cuenca et al. 2017). Under the alkaline nonsaline conditions of this study, *FRO2* expression was enhanced in all demes; MSA plants achieved the highest values (Fig. 5D). Except for MSA deme LG5, *FRO2* expression under alkaline salinity was lower than under alkaline stress alone. However, all MSA plants maintained higher *FRO2* expression levels than the reference accession Col-0 and plants from HA and HS demes. Natural genetic variants for *FRO2* have been reported in *A. thaliana* accessions native to soils differing in Fe availability in Sweden. Different polymorphisms in long- and short-root accessions

were found (Satbhai et al. 2017). Here, we observed similar polymorphisms, suggesting that *FRO2* might be under selection in our *A. thaliana* demes. Noncoding sequence variation of *FRO2* should be further explored to determine whether the expression differences found in saline-alkaline tolerant demes (MSA) are associated with particular alleles.

Plant plasticity under alkaline-saline conditions

Local adaptation is a fixed genetic variation that provides an advantage to one specific climatic and/or edaphic condition (Valladares et al. 2014; Josephs 2018). As an example, phenotypic plasticity in root development plays a role in adaptation to saline environments in salt-sensitive *Lotus glaber* plants (Echevarria et al. 2008). In NE Catalonia, we previously observed that HA and HS demes were locally adapted to nonsaline-alkaline soil and to nonalkaline saline soils, respectively. Here, we observed superior performance of MSA plants under moderate alkaline-saline conditions in greenhouse and hydroponic experiments. However, these demes are native to habitats with intermediate levels of carbonate and Na^+ and are not locally adapted to the harsh natural conditions of the alkaline-saline soils of the Catalan coast. The analysis of the plasticity index in the three plant groups clearly shows that plasticity is a major factor responsible for the better growth of MSA plants on alkaline-saline soil (Fig. 2C). In contrast, HS plants were maladapted to alkaline-saline conditions, while HA plants revealed low plasticity. Plasticity is favoured under conditions of large gene flow, while local adaptation is enhanced under limited gene flow (Scheiner 2013). The demes adapted to nonalkaline salinity on the Catalan coast are still under divergent selection (Busoms et al. 2015). It is likely that genes with unfavourable influences under alkaline salinity are present in HS demes adapted to nonalkaline salinity. This view is supported by the observation of low expression of *SOS1* and *HKT1* under saline alkalinity in T6, an HS deme. Further identification of genes responsible for the maladaptive behaviour of HS plants under alkaline salinity would be of great interest for crop breeding. Unfortunately, the genetic basis of plasticity in plants is still poorly understood (Laitinen and Nikoloski 2019).

In conclusion, the distribution of *A. thaliana* in northeastern Spain is disrupted in coastal areas with

saline-alkaline soils. Under less severe, controlled conditions, demes native to sites with intermediate levels of Na^+ and CaCO_3 (MSA) were identified as the most tolerant to alkaline salinity. The high sensitivity of HS plants to saline-alkaline stress indicates that the evolved molecular mechanisms conveying tolerance to salinity on siliceous substrates are not efficiently expressed or can even be detrimental under alkaline conditions. MSA plants with higher plasticity under saline alkalinity stress are able to maintain both higher germination rates and better ion homeostasis under alkaline-saline conditions. The higher expression of several key genes, such as *FRO2*, *HKT1* and *AHA1*, can serve a key role in the better tolerance of alkaline-saline stress in MSA. This finding is especially relevant for breeding programs considering salinity tolerance in crops intended for saline soils on limestone parent material. The mechanisms underlying the lower expression levels of *HKT1*, *NHX1* and *SOS1* in plants adapted to salinity on siliceous soil when exposed to alkaline salinity merit further investigation.

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Author contribution CP, SB, LP-M, JB and RT conceived the study. SB, LP-M, MAC, NA and JT performed the experiments. SB, LP-M and CP wrote the manuscript with inputs from all coauthors. All the authors gave final approval for publication.

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Code availability Not applicable.

Declarations

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Consent to participate Not applicable.

Consent for publication Not applicable.

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