Evolution of Salt Tolerance in *Arabidopsis Thaliana* on Siliceous Soils Does Not Convey Tolerance to Saline Calcareous Soils

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Research Article

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Abstract

Purpose Alkaline salinity constrains crop yield. Previously, we found local adaptation of *Arabidopsis thaliana* demes to saline-siliceous soils ($\text{pH} \leq 7$) and to non-saline carbonate soils. However, any 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 saline-alkaline conditions of *A. thaliana* demes differing in salinity and carbonate tolerance.

Methods *A. thaliana* native to saline-siliceous soils (G3), to non-saline carbonate soils (G1), or to soils with intermediate levels of these factors (G2) were cultivated in common gardens on saline-siliceous or saline-calcareous substrate. Hydroponics and irrigation experiments confirmed the phenotypes. Growth, mineral concentrations, genome differences, and expression of candidate genes were assessed in the different groups.

Results G3 performed best on saline-siliceous soil and in hydroponics with salinity ($\text{pH} 5.9$). However, G3 was more sensitive to saline-alkaline conditions than G1 and G2. Fitness under saline-alkaline conditions was $\text{G2} > \text{G1} > \text{G3}$ and G2 best maintained ion homeostasis under alkaline salinity. Whole genome scan did not differentiate among the groups, while distinctive patterns for *FRO2*, *NINJA*, and *CCB4* were found and confirmed by qPCR.

Conclusion In *A. thaliana*, salinity tolerance evolved on saline-siliceous soils does not provide tolerance to alkaline salinity. Plants from soils with intermediate conditions (G2) have more plasticity to adapt to alkaline salinity than those locally adapted to these individual stress factors. Higher expression of *NINJA* and *CCB4* may contribute to this better adaptation.

Introduction

Saline stress is one of the major constraints in agriculture. Soil salinization is frequently co-occurring with alkalinity, especially under arid and semi-arid climates, in which high evapotranspiration rates move an excess of soluble ions like Na^+ , Ca^{2+} , Mg^{2+} , K^+ , CO_3^{2-} and HCO_3^- to the superficial soil layers. Moreover, soil salinization may destroy soil structure and cause changes in the biological activity (Singh, 2021). Besides natural causes, human activities like crop irrigation are adding more soluble salts to the soils. Including both the natural and anthropogenic origin, it is estimated that 831 million hectares of the 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 of salts. Salinity causes both ion toxicity and osmotic effects. Plant adaptive mechanisms towards NaCl salinity are well established. Sodium enters by NNCs (non-specific channels) reducing the membrane potential. A huge cost of energy is spent to remove salt from the cytosol, store it in the vacuoles, or return it to the apoplast by *Salt Overly Sensitive 1 (SOS1)*. The *High Affinity Potassium transporter 1 (HKT1)* is an efficient transporter retrieving Na^+ from the xylem and limiting Na^+ transport to the shoots (Sandhu et al., 2017). Weak alleles of *HKT1* favor leaf Na^+

accumulation, which may be an advantage under fluctuating and moderate saline conditions through contribution to ABA signaling and osmotic adjustment (Busoms et al., 2018).

The presence of high carbonate and bicarbonate levels affects soil micronutrient availability, especially Fe and Zn (Marschner 1995); moreover, it alters the root cell membrane potential further enhancing nutrient deficiencies. Carbonate/bicarbonate stress causes an imbalance in the 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 a consequence (Alhendavi 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., 2018). Higher expression of *FRO2* and *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; Chumnakthong et al. 2019).

The mechanisms behind these synergistic effects are still not clearly established. Double stress has a strong influence on the root architecture. In comparison to NaCl stress alone, the root architecture of *Lotus tenuis* changed to a more herringbone phenotype under mixed salt-alkaline stress (Paz et al., 2012). Under alkaline-saline conditions a strong inhibition of nutrient uptake, especially 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. Enhancement of proline (Shi and Sheng, 2005), betaine, and soluble sugar concentrations are quantitatively the most important changes of 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). These extreme habitats are characterized by the presence of sodium carbonate, an extreme 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 up to 10 (Yang et al., 2007; Hajiboland et al., 2018). Such conditions are far beyond the tolerance limits of most crop plants and of the genetic model *A. thaliana* (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 to saline stress under alkaline conditions in crop plants. In *Oryza sativa*, relative expressions 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 that will be useful in crop breeding.

Differences in the tolerance to salinity and moderate carbonate levels as individual stress factors have been described among natural populations of *A. thaliana* in the NE part of Catalonia (Busoms et al. 2015; Terés et al. 2019). 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 the phenotype (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 substrate. Inland populations native to areas located close to limestone soils performed better on non-saline, 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 (unpublished data). 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. Nonetheless, the presence of tolerance mechanisms to moderate saline or alkaline conditions as individual stress factors in our *A. thaliana* populations makes these plants an ideal material for exploring the involvement of these tolerance mechanisms to alkaline salinity resistance.

Thus, the objective of this study was to examine the contribution of individual saline and alkaline tolerance mechanisms to the tolerance to the double stress. For this purpose, we analyzed germination, growth, and reproductive fitness of *A. thaliana* demes differing in saline and alkaline tolerance. Common gardens under greenhouse conditions using saline and saline-alkaline soils were performed. Plant responses using hydroponics and plates under controlled conditions were used to validate responses of soil-grown plants. The contribution of selected genes to differences in tolerance was approached by qPCR. These candidate genes were selected based on previous genome scans and GWAS experiments (unpublished data).

Materials And Methods

Plant and soil materials

In previous studies, natural variation of *Arabidopsis thaliana* populations from Catalonia was tested in a multi-year small-scale common garden 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 they were purchased from Nottingham Arabidopsis Stock Centre NASC (Scholl, May, and Ware 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 MΩmilli-Q water. Seeds were stratified for 4 days at 4°C to synchronize germination.

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 of 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

In order 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 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 carbonates (CaCO₃) (Ballabio et al., 2019).

Soil physical and chemical analyses

Six independent samples of each soil type were used. The soil characterization was performed on air-dried 2-mm fraction samples. For measurement of soil pH and electric conductivity (EC) 25 ml of 18 MΩ water were added to 10 g soil in a Falcon tube. After mixing at constant rotation for 30 min, pH measured using a pH-meter (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 was determined following the methods described by Porta (2006). Calcium carbonate content (%) was measured according to Loeppert et al., (1996).

To determine the available mineral nutrient concentrations, 5 grams of soil were dried at 60°C for 48 hours in 50-mL Falcon tubes. Each sample was diluted to 6.0 mL with DTPA-NH₄ and analyzed 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

Plant material was dried for 4 days at 60 °C. Approximately 0.1g was used to perform an 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 contrasted saline and saline-alkaline soil. 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, pictures of the entire rosette were taken. Siliques number 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 ($\frac{1}{2}$ MS, 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 + 10mM NaHCO₃, pH 8.3). For each deme and treatment, a total of 60 seeds were divided among 4 plates. All plates contained Phyto-agar 0.6% (Duchefa, Haarlem, The Netherlands), and solutions were buffered using different proportions of MES and BTP depending on 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 daily checked using a stereomicroscope (Model, Leica, Wetzlar, Germany) during the following 10 days.

Hydroponic experiment Sterilized seeds of demes from 6 coastal (G3), 4 intermediate (G2), 4 inland (G1) demes were sown in 0.2 mL tubes containing 0.6% agar prepared with nutrient solution $\frac{1}{2}$ strength-Hoagland ($\frac{1}{2}$ HS, 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 (48 hours approximately), the bottom of the tubes containing seedlings was cut off and the tubes were placed in 150 mL hydroponic containers with aerated nutrient solution $\frac{1}{2}$ HS (pH 5.9). 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 ($\frac{1}{2}$ HS, pH 5.9) and two alkaline-saline treatments ($\frac{1}{2}$ HS + NaCl 40 mM + 10mM NaHCO₃, pH 8.3) and ($\frac{1}{2}$ HS + NaCl 60 mM + 15 mM NaHCO₃, pH 8.3). Solutions were buffered with different proportions of MES and BTP. Control and treatments solutions were replaced every 3 days. Plants remained in these conditions for two weeks. Every third day, pictures of the entire plants were taken. 37-days old plants were harvested and leaves and roots were

pictured, weighted and stored at -80°C. 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 A1 (G1), T6 (G3), LG5 (G2), V1 (G2)V3 (G2) demes and Col-0 (REF) were cultivated individually in square pots of 10-cm diameter in sterilized quartz sand. 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 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 ¼ strength Hoagland at neutral pH 5.9 solution 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 (½ HS, pH 5.9), salt (½ HS + 75 mM NaCl, pH 5.9), alkaline (½ HS + 15 mM NaHCO₃, pH 8.3), and alkaline-saline conditions (½ HS + 60 mM NaCl + 15 mM NaHCO₃, pH 8.3). Solutions were buffered with different proportions of MES and BTP. After 2 weeks under treatment conditions, plant material was collected. Roots were carefully rinsed with deionized water and fresh weight of each organ was measured prior to store at -80°C. A detailed outline of the experimental designs is shown in Supplementary Fig. S1.

Gene expression analysis

Frozen leaf material of plants from the irrigation experiments was used for RNA extraction. Total RNA was extracted using PROMEGA RNA plant kit and following manufactured 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 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. Dilution of the cDNAs was performed 1:50 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: denaturalization 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 each 5 seconds. Plates were edited using the CFX manager version 3.1 software. Primers from selected genes were designed using NCBI primer blast tool (Ye et al., 2012). The sequences of primers used are detailed in Supplementary Dataset S1. The expression of target genes was normalized to the expression level 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 $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001).

Statistical analysis

For the hierarchical clustering of the Catalan *A. thaliana* demes, we generated a progressive alignment of 37574 SNPs for the whole-genome tree of 75 plants from (Busoms et al., 2018). For the clustering of *CCB4*, *NINJA*, *FRO2* and *SOS1* genes the alignment was generated with 134, 25, 37 and 28 SNPs respectively. Pairwise genetic distance between individuals and between demes was calculated using the Maximum Likelihood statistical method and the Jukes–Cantor substitution model.

Data normality was checked for all phenotypes and non-normal data were transformed before applying any parametrical 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 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. All the statistical analyses were performed using the 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 North-East 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 are placed 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 is 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 (Supplementary Fig. S2 B-D). Coastal populations only occur on siliceous substrate (Supplementary Fig. S2A). Previous work has provided evidence that *Arabidopsis* populations from this region harbor substantial genetic variability and 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 CaCO_3 concentrations of their native soils, the *A. thaliana* demes were split into three categories: G1-plants from soils with high CaCO_3 contents and low salinity (average values 9.4% CaCO_3 , 44 Na^+ mg g^{-1}); G2-plants native to soils with intermediate carbonate levels and salinity (5.7% CaCO_3 , 61 Na^+ mg g^{-1}); and G3-plants from soils with low CaCO_3 contents and high salinity (0.65% CaCO_3 , 129 Na^+ mg g^{-1}) (Fig. 1B).

Native soil parameters monitored during 2013 to 2015 (Busoms, 2015) were used as input to perform a Principal Component Analysis (PCA). Soil Na, Mg, Mo, chlorides and sulphates groups G3 demes, while Fe, Zn, K, P, pH, CaCO_3 and WHC differentiate G1 demes (Fig. 1C). Correlations between the different

parameters of the native soils (pH, OM, WHC and nutrients) can be found in Supplementary Dataset S3. Fig. 1D represents the mean relative values of available mineral nutrients in the native soil of each group. G1 soil was characterized by higher Na^+ , Mg and Cl concentrations, while higher K^+ concentrations were found in soils from G2 and G3. Phosphorus, Zn and Ni concentrations were higher in G3 than in G2 with intermediate levels of carbonate and salinity (Supplementary Dataset S3).

Hierarchical clustering of the whole-genome tree of 74 individuals did not reveal a clear population structure defining the three established groups (Fig. 1E). The native soils characteristics do not provide a genome-wide differentiation and several lineages are present in different habitats. Despite we do not observe genotypic differentiation by geography, the distance between some demes of the G2 (around 60 km apart) explains why they cluster in two different nodes.

Reciprocal soil transplant experiment

To reveal potential differences in fitness among demes in response to salinity on alkaline or siliceous substrate, a reciprocal soil transplant experiment was established in a greenhouse. Plants were grown from seeds in pots filled either with saline-siliceous or saline-alkaline soil. Both soils had a similar sandy-loam texture and salinity levels, but clearly differ 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 measuring rosette diameter of 5 weeks old plants (Fig. 2A) and the number of siliques per plant at maturity (Fig. 2B). On saline-siliceous soil, plants of G3, native to saline siliceous soils, performed better than plants from G1 or G2 (Fig. 2A, B). Contrastingly, on saline-alkaline soil best performance was observed for G2 plants, native to soils with intermediate levels of salinity and alkalinity. Plants adapted to saline-siliceous soils suffered severe reduction of growth and number of siliques when cultivated on saline-alkaline soil (Fig. 2A, B and Supplementary Dataset S5). The plasticity index based on rosette diameter shows that plasticity is a major factor responsible for the better growth of G2 plants on alkaline-saline soil. Contrastingly, G3 plants were maladapted to alkaline-saline conditions, while G1 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. 2E, F and Supplementary Dataset S6). Plants adapted to saline-siliceous soils (G3) were able to maintain higher K^+ concentrations on saline-siliceous soil than plants from G1 and G2, while concentrations of Mo and P were considerably lower in G3 plants (Fig. 2F). On the saline-alkaline soil, G2 plants maintained higher leaf concentrations of all analyzed nutrients (Fig. 2F). Sodium concentrations were similar in G2 and G3 plants; only G1 plants restricted better the Na transport to the leaves.

Saline-alkaline stress experiments

To further confirm the differential responses of the *A. thaliana* demes to saline-alkaline conditions, germination tests and hydroponic cultures were performed. Under control conditions (no NaCl, pH 5.9), the germination rates determined on agar plates did not differ among the three groups. Moderate salinity (50 mM NaCl) applied at slightly acidic pH (pH 5.9) somewhat reduced the germination rates in all three groups to similar extent. Contrastingly, under non-saline alkaline conditions (10 mM NaHCO₃, pH 8.3) germination rates were higher in demes native to soils with the highest carbonated level (G1) and lowest in demes locally adapted to salinity on siliceous soil (G3). The saline-alkaline treatment (40 mM NaCl + 10 mM NaHCO₃) almost completely inhibited germination of G3 plants, while in G1 and G2 demes germination rates were similar to those observed with NaCl salinity under slightly acidic pH conditions (Fig. 3A). The germination rates of the demes under the alkalinity treatment were positively correlated to the soil carbonate content in their native habitats, but negatively related to the corresponding soil Na⁺ contents. A negative correlation between germination rate and native soil Na⁺ was also observed for the alkaline-saline treatment (Supplementary Dataset S7).

For growth performance under saline alkaline conditions, two levels of stress were assayed: a moderate stress level with 40 mM NaCl + 10 mM NaHCO₃ (pH 8.3) and a strong level with 60 mM NaCl + 15 mM NaHCO₃ (pH 8.3). The strong treatment reduced root and leaf length in all three groups (Fig. 3B). Under the moderate saline-alkaline stress plants from G1, and G3 also suffered inhibition of root and leaf elongation, while G2 plants were hardly affected (Fig. 3B, Supplementary Dataset S7). These results confirm the better tolerance of G2 plants to saline-alkaline stress.

Irrigation experiments and molecular analyses

Further experiments using sand cultures irrigated with different treatment solutions confirmed the superior behavior of G2 plants under saline-alkaline conditions and the best performance of G3 plants under saline slightly acidic pH conditions (Fig. 4A; 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 G2; only the relative rosette diameter was more inhibited in Col-0.

Based on a previous GWAS performed with the *A. thaliana* HapMap cohort cultivated in the same saline-alkaline soil used here for the reciprocal transplant experiment (unpublished data), we explored the candidate genes associated with leaf Na⁺ and Fe concentrations. We generated a neighbor-joining (NJ) cladogram for each locus and we selected *NINJA* (*Novel Interaction of JAZ*) and *CCB4* (*Cofactor Assembly of Complex C*) because these were the only genes that clustered the majority of G2 plants in the same node (Fig. 4F, H). Moreover, we included in the analysis two genes, *SOS1* (*Salt Overly Sensitive 1*) and *FRO2* (*Ferric Reduction Oxidase 2*), with a well-established function in tolerance to salinity and Fe deficiency, respectively. The cladogram of *SOS1* revealed no genomic relationship between groups (Fig. 4B) but *FRO* clustering grouped all G2 plants and one G1 deme (SCF) in the same node (Fig. 4D), suggesting that *FRO2* can have a role in saline-alkaline tolerance.

Quantitative PCR analysis of the selected genes performed 10 days after exposure to the different treatment solutions confirmed that *SOS1* was enhanced by salinity stress in all plants (Fig. 4C). However, in T6 plants (G3) this gene was highly expressed in the plants submitted to salinity but not in the ones exposed to saline-alkaline stress. Interestingly, in the rest of plants *SOS1* expression was equally high in the plants submitted to the saline-alkaline treatment (Fig. 4C). Regarding *FRO2*, the alkalinity treatment enhanced the expression in all plants, but the salt-alkaline exposure only increased the expression of *FRO2* in the G2 plants (Fig. 4E). A similar pattern was observed for the *CCB4* gene. *CCB4* was significantly enhanced by saline-alkaline stress only in G2 plants (Fig. 4G). Alkalinity also raised the expression of *CCB4* in Col-0 and A1 (G1) plants, suggesting that this gene is activated due to the presence of bicarbonate. It is well-known that abiotic stresses activate plant defense hormones like jasmonic acid (JA). *NINJA* encodes part of a repressor complex that negatively regulates JA signaling (Acosta et al., 2013). We detected that *NINJA* was enhanced in G2 plants under all the treatments but especially under alkaline salinity (Fig. 4I).

Discussion

It is already established that saline-alkaline conditions are more stressful to plants than salinity under slightly acidic or neutral pH conditions (Shi et al., 2005; Gao et al. 2014). Our results confirm sensitivity to saline-alkaline conditions of both demes locally adapted to salinity on siliceous soils and those locally adapted to non-saline carbonated soil (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 (e.g. Julkowska et al., 2016; Liu et al., 2020), and studies with *A. thaliana* seldomly 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 have 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 (G3) occur all on siliceous saline soils. Soil carbonate (pH >7) is a further factor for local adaptation of *A. thaliana* at non-saline inland sites (G1 demes) (Terés et al., 2018) (Fig. 1A). Based on soil characteristics and phenotype data, here we defined a third group of plants (G3) 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 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 this region local adaptation to strong saline-alkaline conditions has still not evolved in *A. thaliana*.

Plants able to adapt to alkaline-saline conditions must efficiently manage multiple stress factors: high Na^+ , low osmotic potential, low availability of micronutrients, especially Fe and Zn, and imbalance of

carbon metabolism due to dark fixation of inorganic carbon (Poschenrieder et al., 2018). PCA analysis using physico-chemical characteristics of the native soils of the *A. thaliana* demes analyzed here confirm the clear separation of the three plant groups (Fig. 1C). Chloride content, sulfate and Mg, Mo and Na are correlated, and they overlap with the coastal populations, while Ca, Zn and P concentrations are correlated and are characteristic for the alkaline soils. Differences in the relative availability of soil nutrients among the habitats further characterize the three plants groups (Fig. 1D).

Although soil Na^+ and carbonate concentrations clearly distinguish G1 and G3 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., 2018), the whole-genome tree does not reveal a clear stratification by soil of the 3 groups (Fig. 1E). Contrastingly phenotype differences were evident both in greenhouse common gardens and hydroponics (Figs. 2 and 3).

Phenotype 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 such 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. A main obstacle for coastal demes from siliceous soils to colonize the nearby saline alkaline soils seems to be the strong inhibition of the germination rate (Fig. 3A). Synergistic effects of salinity and alkalinity on germination have previously been reported 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). All *A. thaliana* demes suffered inhibition of germination on alkaline-saline substrate. G3 plants were most severely inhibited, while G1 and G2 plants had similar germination rates (Fig. 3A).

Subsequent growth of germinated transplanted seedlings to hydroponics revealed better performance of G2 plants under saline-alkaline conditions. This was especially visible for the mild alkaline-saline treatment (40 mM NaCl + 10 μM NaHCO_3) which did not cause any reduction in rosette diameter and root length in the G2 plants native to soils with intermediate levels of salinity and alkalinity (Fig. 3B). Contrastingly, plants native to non-saline alkaline soil (G1) and those native to saline-siliceous soils (G3) suffered from growth inhibition even under the mild alkaline-saline stress treatment. Comparison of relative growth values (Fig. 4A) clearly demonstrates the best performance of G2 under saline-alkaline conditions in contrast to G3 plants, with best growth under saline, low pH conditions.

Higher performance of G2 under saline-alkaline stress was also confirmed by the common garden study (Fig. 2A-B). Under saline conditions, G3 plants were best maintaining high K^+/Na^+ tissue ratios (Fig. 2F). However, under saline-alkaline conditions G3 plants had the lowest relative K^+ concentrations. Contrastingly, G2 plants not only maintained high K^+/Na^+ ratios but also had higher relative concentrations of Fe and Zn, micronutrients that are often limiting growth of plants under alkaline conditions (Riaz et al., 2020). 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). Our results suggest that alkalinity may hamper the transport mechanisms that allow salt tolerant plants to maintain high K^+/Na^+ ratios under non-alkaline saline conditions.

Transcript expression of selected genes

SOS1 is required for salinity tolerance by maintaining Na^+ and K^+ homeostasis in plants exposed to salinity (Shi et al., 2000). Extreme increases of the Na^+/K^+ ratio in *sos1* rice mutants have been observed, especially in the xylem parenchyma (El Mahi et al., 2019). In our *A. thaliana* demes, *SOS1* expression was enhanced by both saline and saline-alkaline treatments in all demes to similar extent. Exceptions were T6 from G3 and V3 from G2, which had considerably higher *SOS1* expression under non-alkaline salinity (Fig. 4C). Contrastingly, in saline tolerant rice higher *SOS1* expression has been reported under pH 8 than under pH 7 (Chummnathong et al., 2019). 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 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*. *SOS1* gene expression is regulated by a complex signaling network (Ji et al., 2013). Recently it has been shown that AtbZIP62 negatively regulates both *SOS1* expression (Rolly et al., 2020) and systemic acquired pathogen defense probably mediated by salicylate; but an interplay with alkaline stress has not been shown so far.

FRO2, coding for ferric-chelate reductase plays an important role in plants iron acquisition both in roots and in leaves. The enzyme is responsible for the reduction of FeIII prior to transport into the root or leaf cells and its activity is substantially enhanced under Fe deficiency (Connolly et al., 2003). Alkalinity enhanced *FRO2* expression more in roots of carbonate tolerant than carbonate sensitive soybean varieties (Waters et al., 2018). Contrastingly, *FRO2* expression is decreased in chlorotic leaves of sensitive orange trees exposed to carbonate (Martínez-Cuenca et al., 2017). Under the alkaline non-saline conditions of this study, *FRO2* expression was enhanced in all demes; G2 plants achieved the highest values (Fig. 4E). Excepting the G2 deme LG5, *FRO2* expression under alkaline salinity was lower than under alkaline stress alone. However, under saline alkaline conditions all G2 plants maintained higher *FRO2* expression levels than the reference accession Col-0 and plants from G1 and G3 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 (Sathbai et al., 2017). Here we observed similar polymorphisms suggesting that *FRO2* might be under selection in our *A. thaliana* demes. Non-coding sequence variation of *FRO2* should be further explored to determine if the expression differences found in the saline-alkaline tolerant demes (G2) are associated with particular alleles.

Under saline alkaline stress, transcription of *NINJA* was characteristically enhanced in all demes, except in T6 adapted to non-alkaline salinity (Fig. 4I). This G3 deme showed extremely low expression of *NINJA* under all treatments. *NINJA* is a key factor in jasmonate signaling; the protein associates with the

transcription factor ERF19 inhibiting its transcriptional role (Huang et al., 2019). Interestingly, jasmonate has been found to inhibit the expression level of *FRO2* (Maurer et al., 2011). We selected *NINJA* for this expression analysis as a previous GWA study revealed an association between *NINJA* and the variance in leaf Na^+ concentrations (unpublished results). Results here indicate that a constitutively low expression of *NINJA* is associated with low Na^+ leaf concentrations under saline treatments in T6. Non-alkaline salinity increased *NINJA* expression in salt sensitive A1 of the G1 group and in Col-0, but not in plants of G2 (Fig. 4I). In these plants tolerant to alkaline-salinity, *NINJA* expression was substantially enhanced only under saline -alkaline conditions. *NINJA*, by assembly with FRS proteins, may act as a repressor of glucosinolate (GS) biosynthesis (Fernández-Calvo et al., 2020). In alkaline tolerant *A. thaliana* exposed to bicarbonate we have previously observed upregulation of GS biosynthesis related genes and we proposed that enhanced GS production may be a way to retrieve excessive organic carbon produced by dark fixation of inorganic carbon (Pérez-Martín et al., 2021). This seems not to apply to saline alkaline conditions in G2 plants where the upregulation of *NINJA* could repress this induction of GS synthesis. Excess dark-fixed carbon in G2 plants may be used to produce compatible solutes. Osmoprotectants like sugars and anthocyanins accumulate in *A. thaliana* in response to salinity (Leschevin et al., 2021). Moreover, *NINJA* plays a central role in leaf development (Baekelandt et al., 2018) and therefore the enhanced expression of *NINJA* in our G2 plants allows a better growth under stress conditions.

Under saline-alkaline stress the optimization of an efficient acquisition and use of nutrients is essential. Iron is especially critical (Li et al., 2016). Under these conditions, G2 plants were able to maintain better leaf Fe concentrations (Fig. 2F). Adjustment of number of photosynthetic cells to the available Fe and preferential allocation of Fe into the thylakoid membranes seem essential for avoidance of chlorosis development (Terry and Zayed, 1995). Heme proteins are a major sink for chloroplast Fe (Kroh and Pilon, 2020). Cytochrome b_6f , one of these heme proteins, is a major target for Fe deficiency (Hantzis et al., 2018). Cytochrome b_6f plays a key role not only in the linear electron transfer between PSII and PSI, but also is crucial in cyclic electron transport, in avoidance of ROS damage, and acting as redox sensor (Malone et al., 2021). Under alkaline-saline stress, here we found a considerable upregulation of *CCB4* expression specifically in plants of G2 (Fig. 4G). *CCB4* is a key protein in the formation of the functional cytochrome b_6f as it is required for incorporation of heme into the apoprotein (Schöttler et al., 2015). A key role for *CCB4* in alkaline saline tolerance of *A. thaliana* is further supported by the observation that the neighbor joining cladogram for *CCB4* clustered all G2 demes in the same group (Fig. 4F). Moreover, the truncated *CCB4* allele found in T6 may explain the lower expression of *CCB4* in these plants (Supplementary Fig. S5).

Plasticity

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). In NE Catalonia we have previously shown that G1 and G3 demes are locally adapted to non-saline alkaline soil and to non-alkaline saline soils, respectively. Here we found superior performance of G2 plants under moderate alkaline-saline conditions

in greenhouse and hydroponic experiments. However, these demes native to habitats with intermediate levels of carbonate and NaCl are not locally adapted to the harsh natural conditions of the alkaline-saline soils of the Catalanian coast. The analysis of plasticity index in the three plant groups clearly show that plasticity is a major factor responsible for the better growth of G2 plants on alkaline-saline soil (Fig. 2C). Contrastingly, G3 plants were maladapted to alkaline-saline conditions, while G1 plants revealed low plasticity. Plasticity is favored under conditions of large gene flow, while local adaptation is enhanced under limited gene flow (Scheiner, 2013). The demes adapted to non-alkaline salinity on the Catalanian coast are still under divergent selection (Busoms et al., 2015). It is likely that genes with unfavorable influence under alkaline salinity are present in G3 demes adapted to non-alkaline salinity. This view is supported by the observation of low expression of *SOS1* and *CCB4* under saline alkalinity in T6, a deme of G3. Further identification of genes responsible for the maladaptive behavior of G3 plants under alkaline salinity would be of great interest for crop breeding. The genetic basis of plasticity in plants is still poorly known (Laitinen and Nikoloski, 2019). A possible candidate for the adaptive plasticity based on rosette diameter in G2 could be *NINJA*. This gene regulating leaf morphology (Baekelandt et al., 2018) clustered almost all G2 plants in the same node and was significantly enhanced under saline-alkaline conditions (Fig. 4H-I).

In conclusion, *Arabidopsis thaliana* distribution in the north-east of 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 CaCO₃ (G2) were observed to be the most tolerant to alkaline salinity. The high sensitivity of G3 plants to saline-alkaline stress suggests that the evolved molecular mechanism conveying tolerance to salinity on siliceous substrates can be detrimental under alkaline conditions. G2 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*, *CCB4* and *NINJA*, seem to play a key role in the better tolerance to alkaline-saline stress in G2. This finding is especially relevant for breeding programs considering salinity tolerance in crops destined to saline soils on limestone parent material.

Declarations

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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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Figures

Figure 1

***Arabidopsis thaliana* distribution and soil characteristics.** (A) Geographic location of the Catalan *Arabidopsis* demes (black stars) and the two soil sampling points (purple circles) in the saline-alkaline soil map of Europe. (B) Carbonate (CaCO_3) vs Na^+ content, (C) PCA of soil parameters, and (D) normalized difference of 14 elements in the native soils of 19 *A. thaliana* demes. (E) Neighbour-joining cladograms of the whole-genome (37,574 SNP sites) among 74 *Arabidopsis* individuals from our study region and the reference Col-0. *A. thaliana* demes are classified in three groups according to its native soil characteristics in G1-plants (low Na^+ and high CaCO_3 , blue), G2-plants (intermediate levels of Na and CaCO_3 , green), and G3-plants (high Na and low CaCO_3 , red).

Figure 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 (G1=blue, G2=green, G3=red). **(C)** Mean growth $\pm$ 95% confidence intervals of G1, G2 and G3 plants cultivated in saline and saline-alkaline

soil in a greenhouse. Normalized difference of 12 elements in the leaves of plants from the three groups cultivated in (D) saline soil or (E) saline-alkaline soil in a greenhouse. Elements exhibiting significant differences (according to a t-test) are marked with an asterisk (*, $P < 0.05$).

Figure 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* demes groups (G1=blue, G2=green, G3=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.

Figure 4

Potential effects of saline-alkaline candidate genes. (A) Relative rosette diameter, root length and biomass ($X_{Treatment}/X_{Control}$) of G1-plants (blue), G2-plants (green), G3-plants (red), and Col-0 plants (purple) cultivated in 2:1 sand:perlite substrate irrigated with no NaCl or NaHCO₃ (Control, pH 5.9), with 50 mM of NaCl (Saline, pH 5.9), with 10 mM NaHCO₃, (Alkaline, pH 8.3), or with 40 mM NaCl + 10 mM NaHCO₃ (Sal-Alk treatment, pH 8.3) for 15 days. Letters indicate significant differences (Tukey's HSD, $P < 0.05$) between *A. thaliana* groups. Neighbour-joining cladograms of **(B)** *SOS1* allele (based on 28 SNPs), **(D)** *FRO2* allele (based on 37 SNPs), **(F)** *CCB4* allele (based on 134 SNPs), and **(H)** *NINJA* allele (based on 25 SNPs). Each individual is colored according to the *A. thaliana* demes groups (G1=blue, G2=green, G3=red, REF = purple). Relative transcript expression (treatment vs control) of **(C)** *SOS1*, **(E)** *FRO2*, **(G)** *CCB4*, and **(I)** *NINJA* in shoots of triplicate biological replicates of plants expose to Saline (red bars), Alkaline (blue bars), or Sal-Alk (green bars) treatments 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.

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