Universitat Autònoma de Barcelona

Physiological, molecular and genetic mechanisms of adaptation to alkaline and saline soils in Arabidopsis thaliana populations

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Physiological, molecular and genetic mechanisms of adaptation to alkaline and saline soils in *Arabidopsis thaliana* populations

Doctoral Thesis **by Laura Pérez Martín**

Doctoral Program in **Plant Biology and Biotechnology**

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Master's in Plant Biology and Biotechnology by Universitat Autònoma de Barcelona

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2021

Acknowledgments

This work was funded by the Spanish government MICINN projects BFU2013-42839-R and BFU2016-75176-R and PID2019-104000RP-100. This thesis covers a broad spectrum of subjects and would not have been possible without the help of many people. Firstly, I would like to thank my supervisors Charlotte Poschenrieder and Roser Tolrà for the Ph.D. opportunity, their guidance, and many learning opportunities during these years. Secondly, I would like to express my gratitude to my lab mates and professors from the Plant Physiology unit as Rosa Padilla, Joan Barceló, Benet Gunsé, Mercè Llugany and Josep Allué. To my close lab mates, Soledad Martos, Joana Terés, Silvia Busoms, Nicole Azagury, Eliana Bianucci and Maria Almira for these coffees in the morning, constant interactions and for helping each other every time. Thanks to the Master and Bachelor students: Laia Moles, Miquel Bistuè, Margalida Vidal and Sharmila Gosh, Hilal Nur Akpnar, Katarina Hrovat, and Xiandan Zhen for making my English much better and for always being super positive and hard-working. A special acknowledgment to Carol Joana Ramirez Diaz, for encouraging me to apply for a PhD grant. Also, I would like to express my gratitude to Salvador Bartolomé from Biochemistry Unit, explaining so many molecular techniques to me. To Marina Palmero from CREAF for helping me with GIS issues and being a good friend. To Alba Lazaro, for introducing me to the amazing R programming world. Many thanks to Lourdes Rubio and José Antonio Fernández, from Málaga University, for teaching me a lot of things about plant electrophysiology by stopping their jobs to help. Thanks to “Doctorands en Lluita” for fighting to improve the working rights of the next PhD students and for dreaming that a quality public university is possible.

I would like to express my gratitude to Professor Mark Aarts, from the Genetics Laboratory at Wageningen University -WUR, for accepting my request and allowing me to complete part of my research in the genetics group. Also, at WUR I’ve found kind and helpful people as Puong Nguyen and Jitpanu Yamjabok (thank you!). To my Droevendaal housemates from 51 and 107, specially Arwin and Haribo, for making me happy. And a special acknowledgment to Victoria Miles, Martí Vidal, Lindy Schneider, Jorge Hernández, Eva Fernández and the Helix team, for making me enjoy a nice 2019 Dutch summer.

Quiero también agradecer a mi familia y amigos por el apoyo recibido. Andrea, Sandra y Laura, gracias por ser un equipo indestructible y sincronizar agendas para quedar. A Belén por convencerme de hacer el máster de Biología y Biotecnología Vegetal y siempre escucharme. Yaya, ojalá estuvieras aquí para verlo. A mis tíos Jose y Emi, por escucharme y ayudarme a resolver mis

problemas y los de mis plantas. A mi mamá y mi papá, por intentar entender qué estoy haciendo exactamente con mi vida y hacerse cargo de Coco cuando yo no podía. Por último, a Paquita y José Luis por animarme a seguir. A Carlos, por supervisar mis escritos en inglés y tratar de calmarme con sus bromas cuando estaba nerviosa o preocupada. Finalmente, a Coco, por estar siempre disponible para salir a la calle, caminar y caminar.

Laura Pérez Martín

Abstract

Saline-alkaline soils produce more harmful effects in plants than neutral salts, severely reducing plant productivity. This reduction in fitness is opposite to the agronomic objective of this century, which consists of increasing agricultural productions to feed a growing population. This study aims to explore the physiological, molecular, and genetic mechanisms underlying the differences in plant performance under alkaline-saline conditions using natural populations of *A. thaliana* from Catalonia, on one hand, and the worldwide distributed stock center collection on the other.

In chapter I, physiological responses from a moderate-carbonate tolerant A1 $_{(c+)}$ and a sensitive T6$_{(c-)}$ *A. thaliana* deme (small strands) from Catalonia were determined in plants submitted to alkaline treatments (high pH *vs* bicarbonate) under hydroponic conditions. In both demes the $NaHCO_3$ treatment was more harmful than alkaline pH alone achieved by organic buffer. The tolerant deme translocated more nutrients, maintained higher chlorophyll levels, had better growth performance and consequently had a higher siliqua production under alkalinity than the sensitive deme. A quick activation of genes related to iron uptake was found in the roots of the tolerant line.

Differences in physiological traits under alkaline treatment indicate differences at the transcriptomic level. In the second chapter of this dissertation, RNA-sequencing in leaves and microarray in roots were performed in plants exposed for 3h or 48h to either pH stress alone (pH 5.9 *vs* pH 8.3) or pH and alkalinity (10 mM $NaHCO_3$ at pH 8.3). Differences in plant responses to high pH *vs* bicarbonate were detected. Our results indicate that leaves of carbonate-tolerant plants do not sense iron deficiency as fast as sensitive ones. In A1$_{(c+)}$ leaves, the activation of other genes related to stress perception, signal transduction, glucosinolates, sulfur acquisition, and cell cycle yield an efficient response to bicarbonate stress and precedes the induction of iron homeostasis mechanisms.

Across the Catalonia populations, plant tolerance to calcareous soils was found to be driven by the native soil $CaCO_3$ content. To see whether this also applies to a worldwide distribution scale, in Chapter III, 360 *A. thaliana* populations were sown in natural soils with contrasted $CaCO_3$ concentrations. Plants with higher relative growth on calcareous soil were able to take up more sulfur, zinc and phosphorous. These phenotypes were used to perform a Genome Association Study. Candidate gene validation was performed using T-DNA lines in Col-0 background grown again in

contrasted calcareous soils from the study area. The Zn phenotype, points to a malate vacuolar transporter, TDT, as an important negative regulator of mineral nutrient content and pH regulation on calcareous soils.

Chapter IV addresses the fact that the disruption of *A. thaliana* distribution along the Catalan coast overlaps with the presence of alkaline-saline soil spots, suggesting that tolerance mechanisms to saline calcareous soils are not present in this plant species. To test this hypothesis, local A. *thaliana* populations were tested in multiyear small-scale common gardens and greenhouse experiments using natural alkaline-saline soil. Overall, germination was severely inhibited, especially in plants with low $CaCO_3$ content in their native soil. Nutrition status and silique number were higher in demes from regions with moderate levels of both salinity and bicarbonate in their native soils.

In conclusion, differences in plant responses were found between high pH and bicarbonate treatments. Under bicarbonate exposure, shoots of sensitive plants detect nutrient deficiency earlier than tolerant ones as tolerants maintain more efficient transport mechanisms. Alkaline-saline stress produces more deleterious effects than the single stress factors. Germination is severely reduced by alkaline salinity, especially in coastal populations adapted to saline siliceous soils. This could be a possible explanation for absence of *Arabidopsis thaliana* on coastal alkaline soils in NE Catalonia.

Resum

Els sòls salins-alcalins produeixen efectes més perjudicials que les sals neutres, reduint severament la productivitat de les plantes. Contràriament, l'objectiu agronòmic d'aquest segle consisteix a augmentar les produccions agrícoles per alimentar a una població creixent. Aquest estudi té com a objectiu explorar els mecanismes fisiològics, moleculars i genètics subjacents a les diferències en el rendiment de les plantes en condicions alcalines-salines utilitzant poblacions naturals d' *A. thaliana* de Catalunya i del món.

En el capítol I, les respostes fisiològiques d' ecotips catalans d' *A. thaliana* contrastats, $A1_{(c+)}$ tolerant als carbonats i $T6_{(c-)}$ sensible, es van sotmetre a tractaments alcalins (alt pH *vs* bicarbonat) sota condicions hidropòniques. En ambdues poblacions, el tractament de $NaHCO_3$ és més perjudicial que el pH alcalí aconseguit per amortidor orgànic. La població tolerant, transloca més nutrients, manté nivells més alts de clorofil·la, creix més i produeix més síliqües que la població sensible sota condicions d'alcalinitat.

En el segon capítol, l'anàlisi del transcriptoma es va realitzar utilitzant plantes $A1_{(c+)}$ i $T6_{(c)}$ exposades durant 3h o 48h a l'estrès per pH (pH 5.9 *vs* pH 8.3) i alcalinitat (10 mM $NaHCO_3$ a pH 8.3). Els nostres resultats indiquen que les plantes presenten diferències en la resposta de pH elevat *vs* HCO_3^-. A més, les fulles de plantes tolerants al carbonat no presenten una deficiència de ferro tan ràpidament com les sensibles. En les fulles $A1_{(c+)}$, l'activació d'altres gens relacionats amb la percepció de l'estrès, la transducció del senyal, els glucosinolats, l'adquisició de sofre i el cicle cel·lular donen una resposta eficient a l'estrès per bicarbonats i precedeix la inducció dels mecanismes d'homeòstasi de ferro.

En les poblacions de Catalunya, la tolerància de les plantes als sòls calcaris és conduïda pel contingut de $CaCO_3$ al sòl natiu. Per tal de testar si és aplicable a nivell mundial, en el capítol III, 360 poblacions d' *A. thaliana* es van cultivar en sòls amb nivells de $CaCO_3$ contrastats. Les plantes amb un creixement relatiu més alt en sòls calcaris són capaces de prendre més sofre, zinc i fòsfor. Aquests fenotips es van utilitzar per realitzar un estudi d'associació del genoma. La validació de gens candidats es va realitzar utilitzant línies de T-ADN utilitzant el genoma de referència Col-0 conreades de nou en sòls calcaris contrastats. El fenotip Zn, apunta a un transportador vacuolar malat, TDT, com un important regulador negatiu del contingut de nutrients minerals i de la regulació del pH en sòls calcaris.

El capítol IV aborda la interrupció de la distribució d'*A. thaliana* al llarg de la costa catalana, que es superposa amb la presència de sòls alcalino-salins, suggerint que els mecanismes de tolerància envers als sòls alcalino-salins no es troben presents en aquesta espècie. Per provar aquesta hipòtesi, les poblacions locals de *A. thaliana* es van testar en jardins comuns durant diversos anys. Tanmateix, es van repetir els experiments a hivernacle utilitzant sòls alcalins naturals. La germinació va ser severament inhibida, especialment en plantes amb baix contingut de $CaCO_3$ en el seu sòl natiu. L' estat nutricional i el nombre de síliqües eren més alts en les poblacions de regions amb nivells moderats de salinitat i bicarbonat en els seus sòls natius.

En conclusió, les plantes responen diferencialment entre els tractaments d'alt pH i bicarbonat. Les línies sensibles detecten ràpidament deficiència de nutrients sota tractaments alcalins. L'estrès alcalí-salí produeix efectes més severs que l'estrès salí, i la germinació es veu fortament afectada especialment en les poblacions costaneres adaptades a sols salins silícics. Això podria ser una possible explicació en la manca de presència i distribució d'*Arabidopsis thaliana* en sòls alcalins dins la costa catalana.

Resumen

Los suelos salinos-alcalinos producen efectos más perjudiciales en las plantas que las sales neutras, reduciendo severamente la productividad de las plantas. Contrariamente al objetivo agronómico de este siglo que consiste en aumentar las producciones agrícolas debido a una demanda creciente. Este estudio tiene como objetivo explorar los mecanismos fisiológicos, moleculares y genéticos subyacentes a las diferencias en el rendimiento de las plantas en condiciones alcalinas-salinas utilizando poblaciones naturales de *A. thaliana* de Cataluña y del mundo.

En el capítulo I, las respuestas fisiológicas de ecotipos catalanes de *A. thaliana* contrastados, $A1_{(c+)}$ tolerante a los carbonatos y $T6_{(c-)}$ sensible, se sometieron a tratamientos alcalinos (alto pH *vs* bicarbonato) bajo condiciones hidropónicas. En ambas líneas, el tratamiento de $NaHCO_3$ es más perjudicial que el pH alcalino. La población tolerante, transloca más nutrientes, mantiene niveles más altos de clorofila, crece más y produce más silicuas que la población sensible bajo condiciones alcalinas.

En el segundo capítulo, el análisis del transcriptoma se realizó utilizando plantas $A1_{(c+)}$ y $T6_{(c-)}$ expuestas durante 3h o 48h al estrés por pH (pH 5.9 *vs* pH 8.3) y alcalinidad (10 mM $NaHCO_3$ a pH 8.3). Nuestros resultados indican las plantas presentan diferencias en la respuesta pH elevado *vs* HCO_3^-. Además, las hojas de plantas tolerantes con el carbonato no presentan una deficiencia de hierro tan rápidamente como las sensibles. En las hojas $A1_{(c+)}$, la activación de otros genes relacionados con la percepción del estrés, la transducción de la señal, los glucosinolatos, la adquisición de azufre y el ciclo celular dan una respuesta eficiente al estrés por bicarbonatos y precede la inducción de los mecanismos de homeostasis de hierro.

En las poblaciones de Cataluña la tolerancia de las plantas a los suelos calcáreos es conducida por el contenido de $CaCO_3$ al suelo nativo. Con el fin de testar si es aplicable a nivel mundial, en el capítulo III, 360 poblaciones de *A. thaliana* se sembraron en suelos con niveles de $CaCO_3$ contrastados. Las plantas con un crecimiento relativo más alto en suelo calcáreo eran capaces de tomar más azufre, zinc y fósforo. Estos fenotipos se utilizaron para realizar un estudio de asociación del genoma. La validación de genes candidatos se realizó utilizando líneas de T-ADN utilizando el genoma de referencia de Col-0 cultivadas de nuevo en suelos calcáreos contrastados. El fenotipo

Zn, apunta a un transportador vacuolar malato, TDT, como un importante regulador negativo del contenido de nutrientes minerales y la regulación de pH en suelos calcáreos.

El capítulo IV aborda la interrupción de la distribución de *A. thaliana* a lo largo de la costa catalana, que se superpone con la presencia de suelos alcalino-salinos, sugiriendo que los mecanismos de tolerancia para suelos alcalino-salinos no están presentes en esta especie. Para probar esta hipótesis, las poblaciones locales de *A. thaliana* se testaron en jardines comunes durante varios años. Los experimentos se repitieron en invernadero utilizando suelos alcalinos naturales. La germinación fue severamente inhibida, especialmente en plantas con bajo contenido de $CaCO_3$ en su suelo nativo. El estado nutricional y el número de silicuas eran más altos en las demás regiones con niveles moderados de salinidad y bicarbonato en sus suelos nativos.

En conclusión, las plantas responden diferencialmente entre los tratamientos de alto pH y bicarbonato. Las líneas sensibles detectan rápidamente deficiencia de nutrientes bajo tratamientos alcalinos. El estrés alcalino-salino produce efectos más severos que el estrés salino, y la germinación se ve fuertemente afectada especialmente en la población costera adaptada a suelos salinos silíceos. Esto podría ser una posible explicación de la falta de distribución de *Arabidopsis thaliana* en suelos alcalinos en la costa catalana.

List of abbreviations

ABA	Abscisic acid
AMM	Accelerated mixed model
ANOVA	Analysis of variance
AZ	Acetazolamide
Bic	10 mM $NaHCO_3$ pH 8.3
BTP	1,3-Bis[tris(hydroxymethyl)methylamino]propane
CA	Carbonic anhydrase
cDNA	Complementary deoxyribonucleic acid
CEC	Cation exchange capacity
C_{inorg}	inorganic carbon
DGE	Differential gene expressed
EC	Electric conductivity
ESDAC	The European Soil Data Centre
FDR	Fold discovery rate
FR	Ferric reductase
Fv/Fm	Quantum yield of photosystem II
GIS	Geographical information system
GO	Gene Ontology
GS	Glucosinolate
GST	Gluthatione-S-transferase
GWAS	Genome-Wide Association Studies
HKT1	High Potassium transporter 1
ICP -OES	Induced Coupled plasma optical emission spectroscopy
JA	Jasmonic acid
KEGG	Kyoto Encyclopedia of Genes and Genomes
LD	Linkage disequilibrium

LFC	Log2 fold change
MATE	Multidrug and Toxic Compound Extrusion
MDA	Malondialdehyde
MES	(2-(N-morpholino) ethanesulfonic acid hydrate 4-morpholineethanesulfonic acid)
MIR	Mechanism of iron reduction
NADPH	Nicotinamide adenine dinucleotide phosphate
NCBI	National Center for Biotechnology Information
NMC	Nutrient mineral content
NNCs	Non-specific channels
OAs	Organic acids
PCA	Principal component analysis
qPCR	Quantitative Polymerase Chain Reaction
RD	Rosette diameter
RL	Root length
RE	Relative expression
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SA	Salicylic acid
SDM	Specie Distribution Model
SNP	Single nucleotide polymorphism
SOS1	Salt Overly Sensitive 1
TAIR	The Arabidopsis Information Resource
T-DNA	Transfer DNA
TF	Transcription factor
USDA	United States Department of Agriculture
WRB	World Reference Base

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General introduction, hypothesis and aims.

1. Opening consideration

The world population is increasing in a climate change scenario. Ensuring quality and food security is one of the main objectives of this century, especially in arid and semi-arid places where the salinization of calcareous soils can affect crop production [1]. The speed of climate change may be too fast for the adaptation of agricultural species to the new climatic conditions challenging the agricultural production [2]. In plants, high Na^+ concentrations can cause severe injury like a reduction in the uptake of essential nutrients, the production of reactive oxygen species and membrane damages [3]. On the other hand, high pH from calcareous soils reduces nutrient availability, especially iron, zinc an phosphorus [4]. Saline stress in plants is well studied and nowadays molecular and physiological information regarding different species is available [5][6][7]. However, concerning stress factors on calcareous soils, many authors focused on pH effects and consequently iron deficiency responses [8][9], but few studies consider carbonate and bicarbonate stress. Furthermore, most studies have been performed under controlled laboratory conditions and can underestimate the diversity of stimuli and environmental factors that plants face under natural conditions. Studying physiological and molecular mechanisms in response to altered ionomic profiles on saline-alkaline soils in model plants may provide useful knowledge for breeding programs in species of agronomic interest. The research into plants sensitivity and tolerance mechanisms in response to salinity-alkalinity can have both direct and indirect social and economic effects in areas severely affected by climate change and high population density [10].

In this sense, this thesis is focused on detecting responses to salinity and alkalinity conditions at molecular and physiological levels using local populations of *Arabidopsis thaliana*. The work integrates several scientific areas to achieve specific aims as Ecology, Soil Science, Plant Physiology, Molecular Biology, Genetics, and Bioinformatics.

The introduction to this multidisciplinary approach is divided into four parts. The first part is focused on the description of carbonated and saline soils, the salinization process in the Mediterranean areas and their implications at the agricultural level. Adaptative mechanisms and molecular responses of plants under alkaline-saline stress are exposed. Then, *Arabidopsis thaliana* and its implication in science, emphasizing the diversity of natural populations, local adaptation events and previous studies performed are analyzed. Finally, a summary of techniques used in this thesis is exposed.

2. Calcareous and saline soils

2.1 Origin, distribution, and effects on crop production

Calcareous soils occupy 30% of the earth's surface and are located in the arid, semi-arid, and subtropical climates of both hemispheres. According to USDA United States Department of Agriculture, calcareous soil can be classified in Aridisols, related to arid climates, while for the Word Reference Base Soil (WRB) they should be classified into Calcicosols (Figure 1A). Lime-rich soils are defined by more than 15 % of $CaCO_3$ parental rock material in different forms such as powder, nodules, and crust [4]. Some ions as Ca^{2+} and $Mg^{2+}$are presented in excess. Furthermore, carbonate dissolution in soils produces a bicarbonate concentration between 5-35 mmol L^{-1} [11] which buffers soil pH into a range from 7.5 to 8.5 [12].

Calcareous soils can occur due to natural processes or by anthropic activities. Under natural conditions, two different processes can lead to the appearance of calcareous soils depending on climate: limestone material can come from many organisms that use calcium carbonate to build their mineral skeleton; after the death of these organisms, the accumulation of these minerals remains in many environments in such quantities that they become sediments, origin of most existing limestone. In primary formation, hydration of the bedrock material rich in limestone $CaCO_3$ or dolomite $CaMg(CO_3)_2$ occurs, producing bicarbonates that are soluble in water. Under dry climates, carbonate salts are moved by capillary action towards the surface, where they are deposited in layers. The secondary formation is produced in more humid climates, where constant hydration of carbonates leads to its solubilization, transport, and deposition in low areas or in basins [4] . Besides natural processes of formation of calcareous soil, anthropogenic activities such as crop irrigation with bicarbonate-rich waters or hydration of deposited carbonates can increase the HCO_3^- levels in the soil (Figure 1C).

Although saline soils are present in a wide range of climates, salt-affected soils are more frequent under arid and semi-arid climates, where the evapotranspiration volume is higher than the precipitation volume throughout the year. For this reason, countries more affected by saline soil include Pakistan, China, United States, India, Argentina, and Sudan. (Figure 1B). Saline soils contain high quantities of soluble salts as Na^+, Ca^+, Mg^+, K^-, Cl^-, SO_4^{-2} and CO_3^{-2}, producing an electrical conductivity of more than 4 dS/cm at 25 °C in a saturated soil paste in a neutral pH range from 6 to 7 [5], [13]. Solonchark and Solonetz from WRB classification are used for soils with high sodium surface accumulation, while soils prone to salinize are Acrisol, Alisol, Fluviosol, Greysol, Luviosol and

Vertisol [14]. Saline soils refer to an accumulation of soluble salts, while sodic soils refer to these soils with high concentrations of Na^+ as the main soluble ion. Therefore, saline-sodic soils are those having soluble salt excess, being Na^+ most predominant [15].

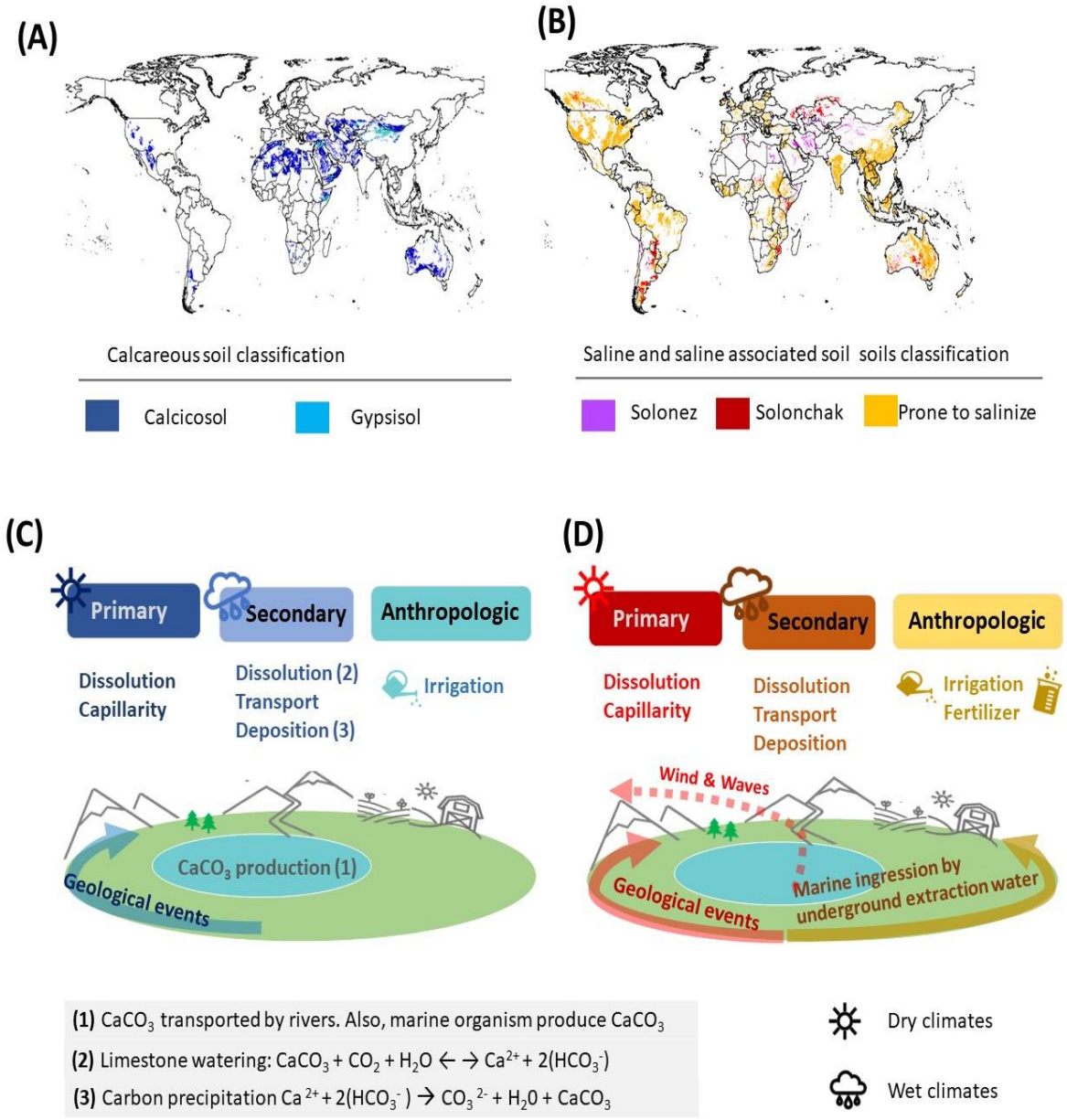


Figure 1. Calcareous and saline soil worldwide distribution map and soil origin schema. **(A)** Calcareous and Gypsum soils worldwide distribution map. Gypsisol distribution is indicated in light blue and Calcisol distribution is indicated in dark blue color. **(B)** Saline-associated soils worldwide distribution map. Saline soils (Solonez and Solonchak) are indicated in purple and red, respectively. Soils prone to salinize are colored in orange: Acrisol, Alisol, Fluviosol, Greysol, Luviosal and Vertisol. Maps were constructed using Harmonized World Soil Database Viewer from FAO. Scheme of natural and anthropologic soil origin of **(C)** Calcareous soil and **(D)** Saline soil. Inspired by Daliakopoulos et al. 2016 [14] with several modifications.

Soil salinity can be originated by natural events such as saline bedrock, geological past events, maritime aerosols transported by the wind, marine inclusions in coastal areas, or specific events such as tsunamis. The arid climate favors the capillarization of soluble salts and the transport to superficial layers. Furthermore, carbonate dissolution from crop irrigation can also cause soil salinization. Other anthropogenic activities increasing the concentration of salt in the soil are the inappropriate use of irrigation water, massive extraction of water from aquifers promoting sea water intrusion, excessive use of fertilizers and use of high water demanding plants in locations with water scarcity [14]. See Figure 1D.

Soil salinization can deteriorate and erode soils to different degrees. Excess of Na^+ salts in the soil can produce loss of nutrients by sodium replacing calcium, thus inhibiting clay flocculation and causing severe deterioration of the soil structure. Clay particle dispersion can result in a reduction of porosity, permeability and hydraulic conductivity [16]. Soil organisms, like worms, micro invertebrate, fungus, or bacteria can be replaced or change their proportion in soils that are becoming more and more saline [17].

Besides salinity, the cultivation of calcareous soils presents many challenges at nutrient and soil structure levels [18]. Excess of $CaCO_3$ in soil could produce poor structure, deep cracks and surface crusting which causes low water holding capacity, high infiltration rate loss of nutrients via leaching or deep percolation and poor aeration [19]. High soil pH jointly with low organic matter content can produce a reduction in the availability of macro and micronutrients such as nitrogen (N) and phosphorus (P) zinc (Zn), and iron (Fe). Also, nutritional imbalances are frequently observed mainly affecting alkaline and alkaline earth elements like potassium (K), magnesium (Mg) and calcium (Ca) [20]–[22].

Plant nutrition in high pH soil can be a difficult task. Although iron is the fourth most abundant element in the earth's crust, high $CaCO_3$ content in the soils controls the pH in a range of 7.5 - 8.5 [12]. Under these conditions, iron solubility decreases 1000 times for each unit of pH increase, thus reducing the concentration of soluble Fe to values lower than 10^{-20} M. Clearly, it is an insufficient concentration for optimal plant growth because plants require soluble Fe in a concentration range of 10^{-9} and 10^{-4} M [23]. Therefore, low Fe availability in the soil produce Fe deficiency in susceptible to the plants.

2.2 Calcareous soils prone to salinization: focusing on Mediterranean areas

In Europe, calcareous soils are present in the Mediterranean basin, due to the dry climate and presence of lime parental rock [24]. Figure 2A shows that Catalonia has mostly carbonate-rich limestone rather than siliceous substrate. For instance, most of the Pre-Pyrenean Mountains, in the north part of Catalonia, are formed by compact calcareous rock [25]. In arid and semi-arid locations, due to climatic conditions co-occurrence of calcareous and saline soils are frequent (Figure 2B). Moreover, climate change and human activities are accelerating undesirable soil salinization. Constant irrigation with poor-quality water is one of the main causes of salinization. Saline-influenced areas are expanding every year, especially in arid and semi-arid areas, mainly due to human activities affecting [26]. In the case of the Mediterranean coast, soil salinization is a major cause of desertification and a major factor limiting crop production and land development [13], [27]–[29].

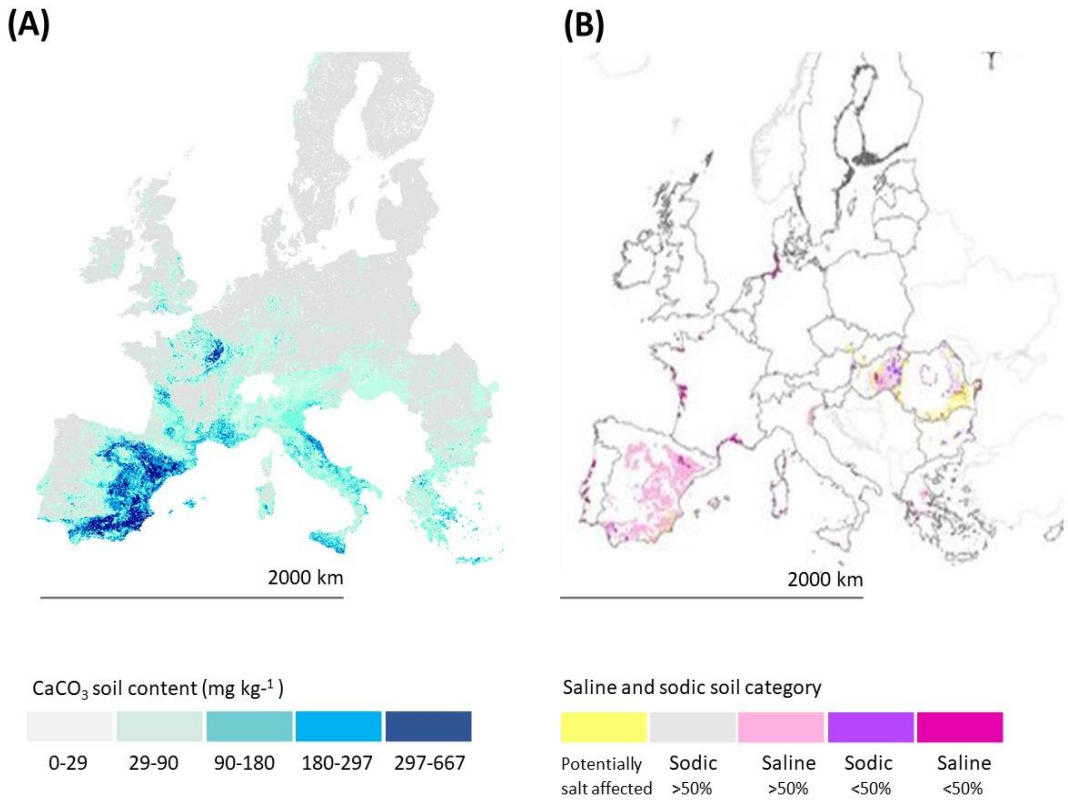


Figure 2. Calcareous and saline-associated soil maps in Europe. (A) European calcareous soil map. **(B)** Saline and sodic soil category. Data were downloaded from European Soil Data Centre (ESDAC) [30].

2.3 Biotechnological approaches of soil amendment

Adequate soil and water management practices can be effective to avoid undesirable ion accumulation in alkaline-saline soil. The soil structure can be improved by tillage, which increases aeration and reduces superficial crust formation. Legume rotation for improving nitrogen fixation, organic amendments and supply of beneficial microflora can help to favor nutrient solubilization in alkaline soils [14][31]. However, water management is a key factor to avoid or reduce salinization. Built-up excess of soluble salts in soils can be prevented or removed by lixiviation using water with low salt content. Unfortunately, high-quality water is a main limiting factor in most of the affected regions. Therefore, irrigation methodologies need to be modernized controlling the watering frequency and the quantity and quality of the water used. Irrigation efficiency can be further improved by selecting crops with hydric requirement adapted to the regional climate [2].

The selection of suitable crops is a decisive element that can minimize yield loss under saline-alkaline conditions. In this context, plant science studies focusing on the tolerance of alkaline saline soil conditions are crucial for the development of both more productive crop varieties and the enhancement of their nutritional value. For such an increase in crop productivity, different approaches can be applied.

3. Physiological and molecular effects of saline calcareous soils in plants

Many studies related to saline stress in plants have been reported. However, less information on bicarbonates and carbonate is available. This may be because most of the research attraction has been classically focused on iron deficiency. High pH in soils reduces the solubility of several nutrients, especially iron (Fe), therefore growth on these soils may cause symptoms of Fe deficiency. In agriculture, a visible phenomenon is chalk chlorosis caused by iron deficiency. Some studies have already pointed to the possibility that the responses of plants to iron deficiencies may not be similar nor comparable to those of high pH or bicarbonates. Under natural conditions, calcareous soils contain large amounts of OH^-, HCO_3^- and CO_2^{-2} [32] [33]. Trying to mimic natural conditions in the laboratory can provide more realistic plant responses and, in a near future, this new information can be applied to genetic improvement programs. Unfortunately, until now the mechanisms of carbonate tolerance remain largely unknown [34].

As a general overview, bicarbonate imposes huge stress on plant performance. Common features are morpho-anatomical changes in roots, disbalance in nutrient uptake, reduction of total photosynthetic pigments and photosynthetic activity, accumulation and exudation of organic acids and activation of the biosynthesis of antioxidant enzymes [35]. Excess of Na^+ in plants produces deleterious effects like ionic toxicity and osmotic effects. Osmotic stress in plants causes disturbance of water relations, stomatal closure, and accumulation of sugars [7]. Cytotoxic effects can also be observed at increasing ROS, producing membranes injury and inhibiting cytosolic reactions. Osmotic and toxic stress leads to inhibition of germination and survival rate, reduction of chlorophyll content, decrease of growth affecting both leaf area and biomass, and, in consequence, yield production [36][37].

The combination of two major abiotic stresses such as salinity and carbonate in soils is a key threat for agriculture worldwide, especially in semi-arid areas [38]. For this reason, renewed interest has appeared during the last years and several studies focused on crop tolerance to alkaline - saline stress. Differences in physiological adaptive mechanisms to saline stress and alkaline stress were found in several species like *Glycine soja* [39], *Medicago ruthenica* [40], *Solanum lycopersicum* [41], *Helianthus annuus* [42], *Avena sativa* [43], *Medicago sativa* [44]**,** *Alba morus* [45], or *Actinidia sp. Lindl* [46].

These studies mostly use a salinity treatment and visualize the effects at different pH values achieved either by a buffer or in combination with carbonates or a mixture of bicarbonates. The used concentrations vary depending on the target species and the developmental stage. As an example, 25 mM of $NaHCO_3$ were applied as treatment in a germination test in *Medicago* [40]. In *Triticum aestivum* [47] and *Glycine max* plants, physiological responses towards 100 mM of neutral salt versus alkaline salts were compared for 10 and 5 days while in *Kochia sieversiana*, 400mM of $NaHCO_3$ and NaCl were applied during 6 weeks [48]. According to present knowledge, plant tolerance varies depending on species and its development stage. These tolerance differences among species allow detecting differential mechanisms among lines or ecotypes.

3.1 Ecological classification

(I) Calcifuge plants are those not found on soils rich in calcite ($CaCO_3$) or dolomite ($MgCa(CO_3)_2$) [49]. These plants are sensitive to the prevalent properties of these soils. According to the main hypothesis, iron deficiency rather than carbonate or hydroxide ions *per se* is the principal limiting factor for their distribution [50]. Under alkaline conditions, iron becomes less soluble and calcifuges plants develop iron deficiency symptoms. Contrastingly, calcicole species are mainly found on calcium carbonate-rich soils and are tolerant to the properties of these soils

(II) In the case of salinity, the most extreme, contrasting behaviors can be found in halophytes and glycophytes. While glycophytes are extremely sensitive to the presence of Na^+ in soils, halophytes are exclusively or predominantly found on saline soils. They have developed different tolerant strategies focused to prevent or palliate both the osmotic and cytotoxic effects of high NaCl concentrations [51].

3.2 Effects on germination, morphology, growth, and yield production

Seedling emergence is a crucial step for plant establishing. Plant tolerance can vary depending on its development state, being seedling one of the most vulnerable stages [52]. Salt stress affects seed germination and seedling establishment through osmotic stress. Furthermore, more severe germination inhibition was observed under alkaline-salinity than in neutral salt treatments in several plant species like *Leymus chinensis* [52], *Triticum aestivum* [53], *Medicago ruthurnica* [40] and *Helianthus annuus* [42]**.**

Under alkaline stress, pH *per se* can affect root growth by inhibiting cell division and elongation [54]. In sensitive rice plants, root growth inhibition has been related to ROS accumulation. [55]. Anatomical changes have been detected in *Melilotus officinalis* subjected to 200mM $NaHCO_3$. Compared to controls, xylem vessels, epidermis cells, palisade and spongy mesophyll cells were reduced. These modifications could be a strategy for thylakoid integrity protection [56]. In kiwi plants (*Actinidia Lindl*) with leaves exposed to 20 mM NaCl for 35 days, plasmolysis and chloroplast irregularities were found [46]. Field experiments comparing the responses to saline-alkaline stress of contrasted *Prunus persica* lines revealed more severe chloroplast damage, in the form of dispersed grana structure, in the sensitive lines [57].

3.3 Adaptation mechanisms

Adjusting internal pH and root exudation: One way for plants to adapt to high pH is to accumulate small metabolites with buffering function, especially organic acids for adjusting the internal pH value. However, the adjusting of external and internal pH is a process that consumes energy and reduces plant growth simultaneously. Carbonic anhydrase (CA) can contribute to buffering the cytosol by catalyzing the reversible conversion of HCO_3^- to CO_2. In cells, pH homeostasis is crucial to develop chemical reactions and an optimal enzyme function. Although CAs are crucial for aquatic plants through the mechanism of carbonate concentration, their role in land plants under carbonate stress has not yet been elucidated. [34]**.** In alkaline-saline stress, the production of organic acid has been found in several plant species like wheat [58], sunflower [42], or oat [52] and in the alkaline-saline tolerant *Kochia sieversiana* [48]. The production of organic acids is a common response in plants exposed to alkaline conditions. The role of organic acids has classically been attributed to intracellular pH homeostasis. However, several authors further relate organic acid production under alkaline stress to ionic balance. Excess Na^+ produces ionic imbalances in plants; organic acids acting as organic anions contribute to the maintenance of the cation/anion balance, in addition to sustaining a stable intracellular pH. In *Avena sativa* under saline stress, inorganic anions Cl^-, NO_3^-, $H_2PO_4^-$, and SO_4^{2-} are increased to neutralize the high concentrations of cations while organic acids were accumulated under alkaline saline stress to face high Na^+ cations [43]**.** Oxalate, malate and citrate are mostly enhanced under alkaline stress [34] [59].

*Ion toxicity, oxidative stress, and osmotic stress***:** Soil Na^+ enters roots by non-selective ion channels causing ionic stress. Excess Na^+ uptake causes intracellular toxicity. High intracellular concentrations of Na^+ damage proteins, lipids and membranes and reduce the activity of several enzymes producing an increase of reactive oxygen species (ROS). Contrastingly, the osmotic effect is a consequence of the low osmotic potential of saline soils hampering water availability and causing drought stress in sensitive plants. Osmotic and ionic stress affect plant aerobic metabolism and induce the accumulation of reactive oxygen species beyond the plant's capacity for cellular oxidant detoxification [27]. Under saline stress, activation of enzymatic and non-enzymatic antioxidants is frequently found.

To overcome these effects plants must both accumulate solutes for osmotic regulation and detoxify intracellular Na^+ either by limiting its accumulation or by compartmentalizing Na^+ into the vacuole [5][7]**.** Under saline-alkaline stress, higher leaf Na^+ levels have been observed than in leaves

exposed to saline stress alone. Several hypotheses have been proposed to explain this phenomenon [37] [60].

(I) Sodium accumulation in leaves is related to the decreased exclusion of Na^+. Sodium exclusion depends on the activity of a Na^+/H^+ antiporter. Salt overly sensitive 1 (SOS1), exchanges cytoplasmic Na^+ for external H^+. Under alkaline stress, a lack of external protons might weaken the exchange activity of the Na^+/H^+ antiporter in the root plasma membrane [61].

(II) Reductions in root K^+ content might be attributable to an inhibitory effect of high pH on K^+ absorption, which relies on the transmembrane proton gradient.

(III) Alkaline stress might weaken the mechanisms controlling absorption or transport of Na^+. A sharp increase in Na^+ content in leaves and stems may disrupt the ionic balance or pH homeostasis in the tissue.

The need for osmotic adjustment when Na^+ accumulates in plants under alkaline-saline stress may explain the higher production of soluble sugars, proline, betaine, and organic acid. Also, higher levels of malonyl dialdehyde (MDA) were found in double stress indicating stronger cell damage [42][45][52][58].

Variation in ionomic content: High levels of bicarbonate have been associated with an increase of the pH in the apoplast; this neutralizes the protons pumped out of the cytosol reducing the absorption of nitrates by the cotransport H^+/NO_3^-. Bicarbonate produces Fe accumulation in the apoplast. This apoplastic Fe, mainly in the oxidized form, is not physiologically available to the plant. Furthermore, the inhibition of the Fe-reducing capacity of the roots decreases Fe uptake and translocation to the shoots [62]. At the physiological level, salinity imposes osmotic stress that limits water uptake and ion toxicity causing nutrition deficiencies (N, Ca, K, P, Fe, Zn) and oxidative stress [60]. Potassium is the most affected ion under saline stress due to the similarity between K^+ and Na^+ that favors the competition for K^+ entrance sites in the root. Also, in the cytoplasm, Na^+ competes with K^+. Na^+ only can substitute for K^+ in osmotic functions, but not in its essential metabolic roles. Therefore, this competition produces inhibition of several metabolic and enzymatic reactions in the cell including photosynthesis and CO_2 assimilation. Alkalinity aggravates these negative effects of salinity on plant mineral nutrition. In *Kochia sieversiana*, Na^+ accumulation in leaves was enhanced, while K^+ and anion concentrations (Cl^-, SO_3^{-2}, NO_3^- and HPO_3^-) were reduced under alkaline-saline stress in

comparison to salt stress alone. The decrease of inorganic anions in the plants was balanced or adjusted by organic anions, mainly organic acids. [48]. A similar situation was found in *Medicago sativa*. Cations Na^+, Ca^+, and Mg^+ accumulated in roots, while a decrease of Fe^+ and K^+ concentration was observed in roots [44]. In *Avena sativa*, contents of Na^+, SO_4^{2-} increased while K^+, NO_3^-, and $H_2PO_4^-$ decreased under alkaline stress [43]. In wheat, the level of Fe in plants was higher under alkaline-saline stress in roots [47]**.** Preferential translocation to the root vacuoles of organic acids chelating Fe may be responsible for this root accumulation and reduced export to the shoots.

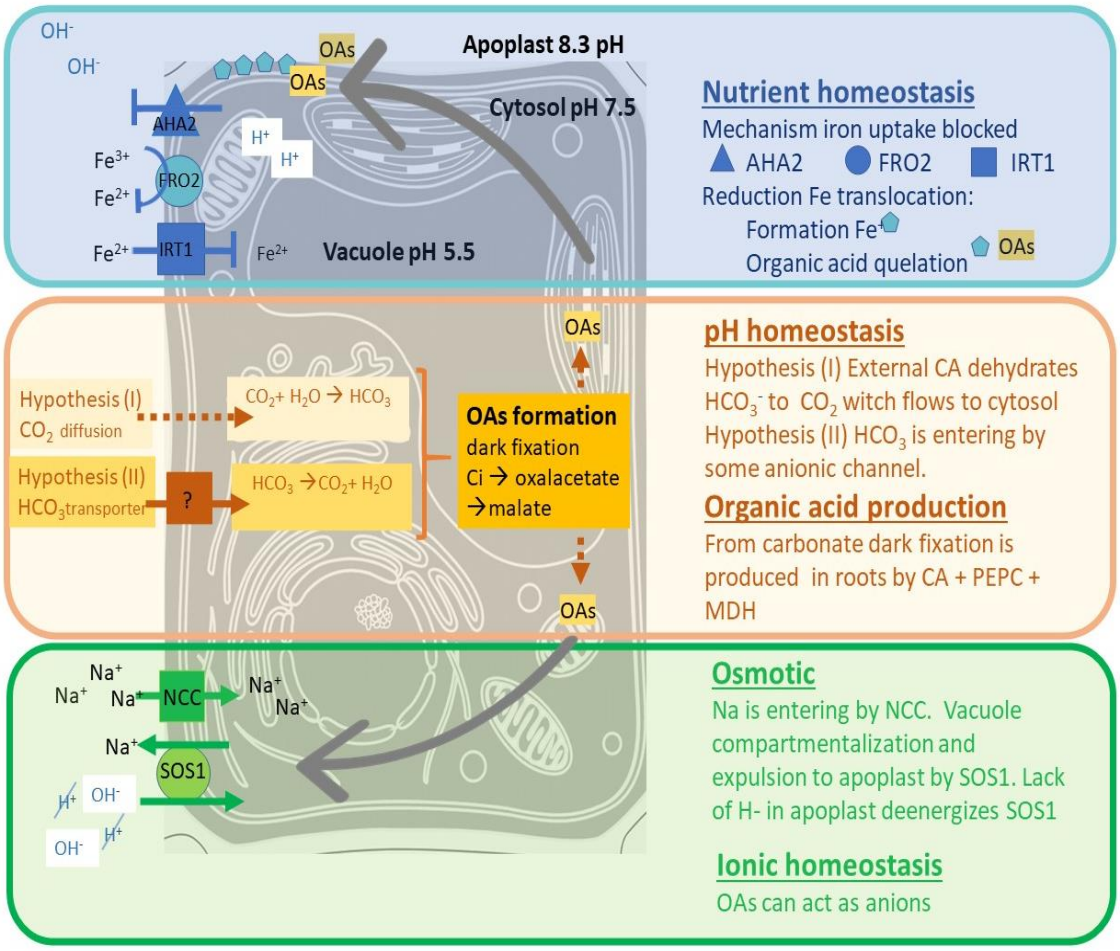


Figure 3. Scheme of physiological responses and adaptive mechanisms in plants under alkaline-saline stress.

3.4 Molecular changes

Biological pathways activated under alkali-saline stress

In *Arabidopsis thaliana*, few investigations address transcriptomic responses to alkaline-saline conditions. However, recent papers were focused to detect specific HCO_3^- responses in roots from Col-0 after 4 days using a mild concentration (10 mM $NaHCO_3$) [63]. These authors revealed that brassinolides signaling (EBS1) is specifically required for Arabidopsis tolerance to bicarbonate. Moreover, exogenous abscisic acid (50 µmol·L^{-1}) sprayed on wheat seedlings showed an alleviation of the harmful effect of 100 mM $NaHCO_3$ treatment. Reduction in Na content and increase in biomass were the main finding in external ABA application [64]. Hormones play an important role in many stresses, still much needs to be explored in hormone regulation under alkaline-saline stress. Transcriptomic experiments under 30 mM $NaHCO_3$ in *Ziziphus acidojujuba* roots after 1 and 12 h exposure indicate differences in the over-expression of genes related to sensing and signal transduction pathways like calmodulin-like proteins, serine/threonine protein kinases and cysteine-rich receptor-like protein kinases [65]. Similar results were found in wheat roots under 100 mM mixed salts ($NaHCO_3$:Na_2CO_3). After 9 days under treatment, main biological functions were clustered into transcription regulation, signal transduction and protein modification categories [47]. In *Glycine soja* root submitted to 50mM $NaHCO_3$ for 24 hours, transcriptomic responses were related to transcriptional regulation, redox processes, binding and response to stress categories [66]. After 24 hours of exposure to 90 mM $NaHCO_3$, soja genes were clustered into categories related to phenylpropanoid biosynthesis and phenylalanine metabolism, transcription factors, and ion transporters [67]. In leaves, the transcriptome of *Xanthoceras sorbifolia* under 150 mM Na_2CO_3 at 4, 12, 24 and 48 h reveals changes in genes involved in carbon metabolism, biosynthesis of amino acids, starch, sucrose metabolism, reactive oxygen species, signaling networks and transport [68]. Similarly, in *Beta vulgaris* seedlings exposed to 75mM mixed alkaline salt Na_2CO_3:$NaHCO_3$ for 3 and 7 days, carbon fixation and photosynthesis were the main affected pathways [69]. Proteomic studies performed in *Solanum lycopersicum* comparing saline and alkaline stress at 50 mM during 72 h revealed downregulated proteins accounting for metabolism and energy conversion, while novel upregulated proteins were involved in signaling or transport [41]. In *Triticum aestivum* roots submitted to 50 mM $NaHCO_3$ for 2 days, upregulated proteins were involved in ion homeostasis and osmotic regulation: superoxide dismutase, malate dehydrogenases, and V-ATPase. Meanwhile, downregulated proteins were malic enzyme and tricarboxylic acid cycle (TCA) enzymes, which may be related to enhanced accumulation of malate [70]. *Helianthus tuberosus* submitted to alkaline

stress (50 mM) for 7 days reported enhancement of glycolysis, TCA cycle, PSI system, ROS scavenging and signal transduction [71]. In field experiments, a comparative transcriptome between two *Prunus persica* cultivars on saline-alkaline soil showed alteration in photosynthesis, antioxidation and ion metabolism. In the tolerant cultivar, an activatione of cell wall degradation, secondary metabolism and starch degradation routes were observed [57].

Transcription factor:

Transcription factors (TFs) orchestrate the regulation of plant development and the responses to environmental factors. Detecting differences in TF regulation under alkaline and or saline stress can provide genomic candidates for crop breeding. P2/ERF, HD-ZIP, bHLH, MYB, WRKY, NAC, C2H2, HB, and TIFY are the main TFs activated by double stress in *Ziziphus acidojujuba* plants [65]. In *Xanthoceras sorbifolia*, fewer TFs were involved in the alkaline-saline response, namely bHLH, C2H2, bZIP, NAC, and ERF [69]. Transcriptome analysis comparing two peach cultivars pointed to a role for the WRKY family in the alkaline-saline tolerant cultivar, while MYB was involved in the sensitive one [57]. Studies with maize on the natural variation of a 4-bp deletion located in the 3'UTR of *ZmNSA1* (EF-hand Ca^{2+}-binding protein-coding gene) revealed increased root Na^+ efflux promoting shoot Na^+ exclusion and saline-alkaline tolerance [72]. Also, from a genome-wide association study (GWAS) of 295 japonica rice varieties at the seedling stage under 15% $NaHCO_3$, several candidate genes were obtained. Most interesting, OsIRO3 contained a 7-bp deletion in the starting position of the 5'UTR. A previous study indicated that OsIRO3 is a negative regulator of the Fe-deficiency response in rice. Reduction in *OsIRO3* expression produces phenotypes with less sensitivity to iron deficiency [73]

Transporters

From current transcriptomic studies, we can conclude that alkaline-saline treatments alter ion transport and that different genes are regulated to maintain ion homeostasis. Among anion transporters, gene families such as *ALMT, NRT/POT* and *SLAHs* are related to bicarbonate response in *Glycine max.* Moreover, in *Glycine soja* transcriptomics, differentially expressed genes were related to ion transporters such as ABC transporters and glutamate receptors under 90 mM $NaHCO_3$ after 24 hours [67]. The overexpression of the anion transporters *GsBOR1* (boron efflux transporter) and *GsSLAH* (efflux of uncharacterized inorganic or organic anions) are also involved in HCO_3 tolerance. GsCHX19.3, a member of the cation/H^+ exchanger superfamily from wild soybean contributes to high salinity and carbonate alkaline tolerance. *GsCHX19.3* transgenic lines showed lower Na^+ in alkaline-saline conditions producing less delirious effects for plants.

4. *Arabidopsis thaliana*: model plant in molecular biology

Arabidopsis thaliana L. is an annual herbaceous plant from the Brassicaceae family. In this family, there are several examples of species with economic importance as radish, cabbage, and broccoli. Although *A. thaliana* has no commercial interest, it has been accepted by the scientific community as a genetic model for dicotyledoneous plants.

4.1 Botanical description, geographical distribution, and habitat

Arabidopsis thaliana at the vegetative state is forming a rosette. When starting the mature state, from the center of the rosette a stem begins to emerge. This stem grows up to approximately 10 to 30 cm. At the upper extreme of the stem, inflorescences appear in bunches. The hermaphroditic flowers, with a diameter of about 5 mm, are composed of 4 white petals and sepals. When one ovule is pollinated, the fruit starts to appear in the center of the flower. The fruit is a silique of about 3 cm in length which contains two cavities bearing around 30 seeds in each silique. Mature seeds have an orange color and measure about half a millimeter. According to Kramer 2015 [74] life cycle can be completed in 60 days.

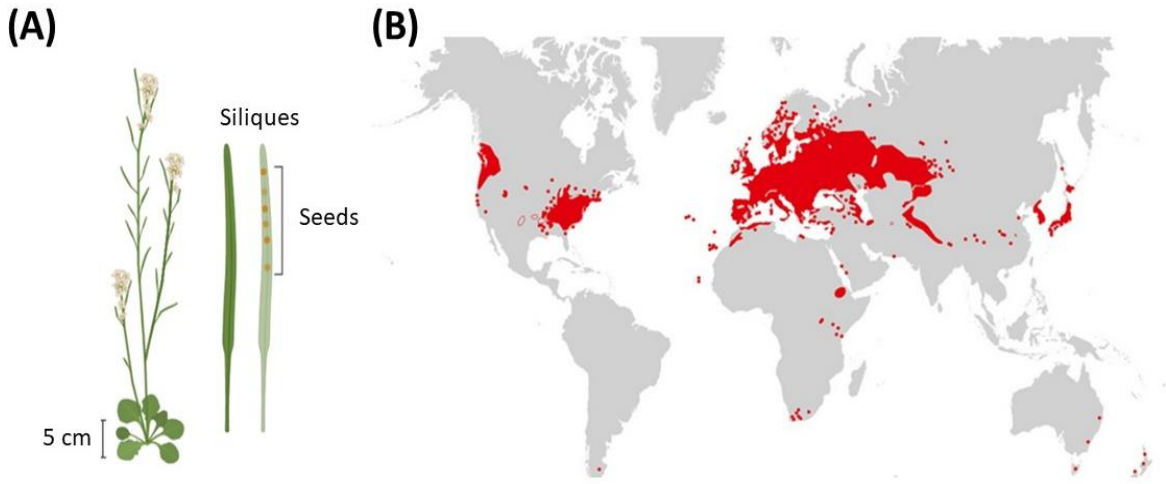


Figure 4. *Arabidopsis thaliana* and its native distribution. (A) *Arabidopsis thaliana* at mature state and silique production. **(B)** Native distribution colored in red from Kramer 2015.

Arabidopsis thaliana is native to Europe and Central Asia and is now naturalized in many parts of the world. It has a wide climatic distribution and for this reason, *A. thaliana* plants can be found from sea level up to 4250 m, and from 68º N to 0º of latitude [75]. *Arabidopsis thaliana* occupies open or disturbed habitats which indicates that it is a rapid colonization plant and poor competitor

in dense vegetation [76]. The species can often be found on sandy or loamy soils such as riverbanks, roadsides, rocky slopes, wasteland, and cultivated grounds [74].

4.2 Evolutionary origin

The current genetic variation is not balanced across its distribution. Several investigations conclude that after the last glaciation, the southern region became a refuge for biodiversity and the population spread to northern latitudes, experiencing successive bottlenecks. As a result, the populations of the Scandinavian areas present less diversity than the African populations [77]–[79]. Besides successive bottlenecks, local adaptation phenomena were reported to different climatic conditions across Eurasia producing a genetic and phenology diversity [80]. In this sense, large differences in life cycles due to seed dormancy, vernalization requirement, or late/early flowering time can be observed due to differences in climatic conditions across their natural habitat. For instance, northern Europe accession are late flowering and typical winter annuals [81], while southern populations can be both summer and winter annuals.

4.3 *Arabidopsis thaliana* as the model system

A. thaliana has been adopted as a reference plant, especially suitable for different areas of biology research such as genetics, molecular biology, and ecology. Some of the main reasons are:

- From the biological perspective: The small size of the species favors working with several replicates in restricted spaces. The short life cycle takes around 6 weeks to produce more than 10.000 seeds per plant. In addition, this plant adapts to different cultivation techniques such as pots, hydroponics, and agar plates [74].
- From the genetic perspective: *A. thaliana* has a small genome size, 120 Mb, distributed in 5 chromosomes and around 27.000 genes. Lines are typically inbred because the species is predominantly selfing, but also crosses between lines are easy and possible [76]. Mutant lines production can be done by efficient transformation methods utilizing *Agrobacterium tumefaciens* as a vector. Extensive native population collections are distributed along different ecological ranges, making *A. thaliana* a perfect specie for ecological studies across different climate conditions [80], [82], [83]. Using genome variations, these genetic and phenotypic variation is a powerful tool for discovering novel alleles involved in adaptive traits.

- From the molecular perspective: the multinational research community accepts *A. thaliana* as the model in dicotyledonous plants. From Genome Initiative in 2000, the genome from Columbia (Col-0) accession was completely sequenced [84]. Several efforts from public and private institutions are directed to support seed stock centers like NASC and ABCR, databases like TAIR, Thalemine and Signal Salk, and to update new analysis tools like BAR (Bio-Analytic Resource for Plant Biology) or repositories as Arapheno [85].

The knowledge generated in Arabidopsis has helped to discover the fundamentals of plant biology in various areas, especially plant development, cell biology, metabolism, physiology, genetics and epigenetics. All this knowledge has been integrated thus unrevealing interesting molecular mechanisms. Gene interactions, biosynthetic pathways, signaling events, immunity responses, and biotic and abiotic stress responses are of special interest. Research carried out in Arabidopsis has facilitated quick applications in different crop breeding programs. The huge gap of knowledge in physiological responses and the molecular bases of tolerance towards bicarbonates and double stress caused by salinity under alkaline conditions promotes this thesis taking advantage of the large diversity of the natural population of *A. thaliana* in Catalonia evolved and adapted to different types of soils.

4.4 Previous studies in Catalan natural population of *A. thaliana*

In previous studies on *Arabidopsis thaliana* in Catalonia (NE Spain) a distribution model of the species was performed using 20 geographically referenced environmental variables in combination with the location of 36 *A. thaliana* demes. The model predicted that *A. thaliana* would not be found on limestone-derived calcareous soil. Surveys performed from 2011 to 2015 confirmed the absence of *A. thaliana* on calcareous soils and only a few inland populations were found on soils close to calcareous zones with carbonate, but at low concentrations. Populations from coastal habitats were locally adapted to moderate soil salinity levels [86], [87]. Several reciprocal transplant experiments comparing inland and coastal populations of *A. thaliana* also identified soil Na^+, as the driving factor for local adaptation to salinity [86]. Different frequencies of polymorphisms at loci related to mineral nutrition (*FPN2*, *MOT1* and *HKT1*), were found among lines and hydroponic experiments with different levels of salinity demonstrated the role of the weak allele of HKT1 as potentially adaptive to moderate and fluctuating soil salinity [86].

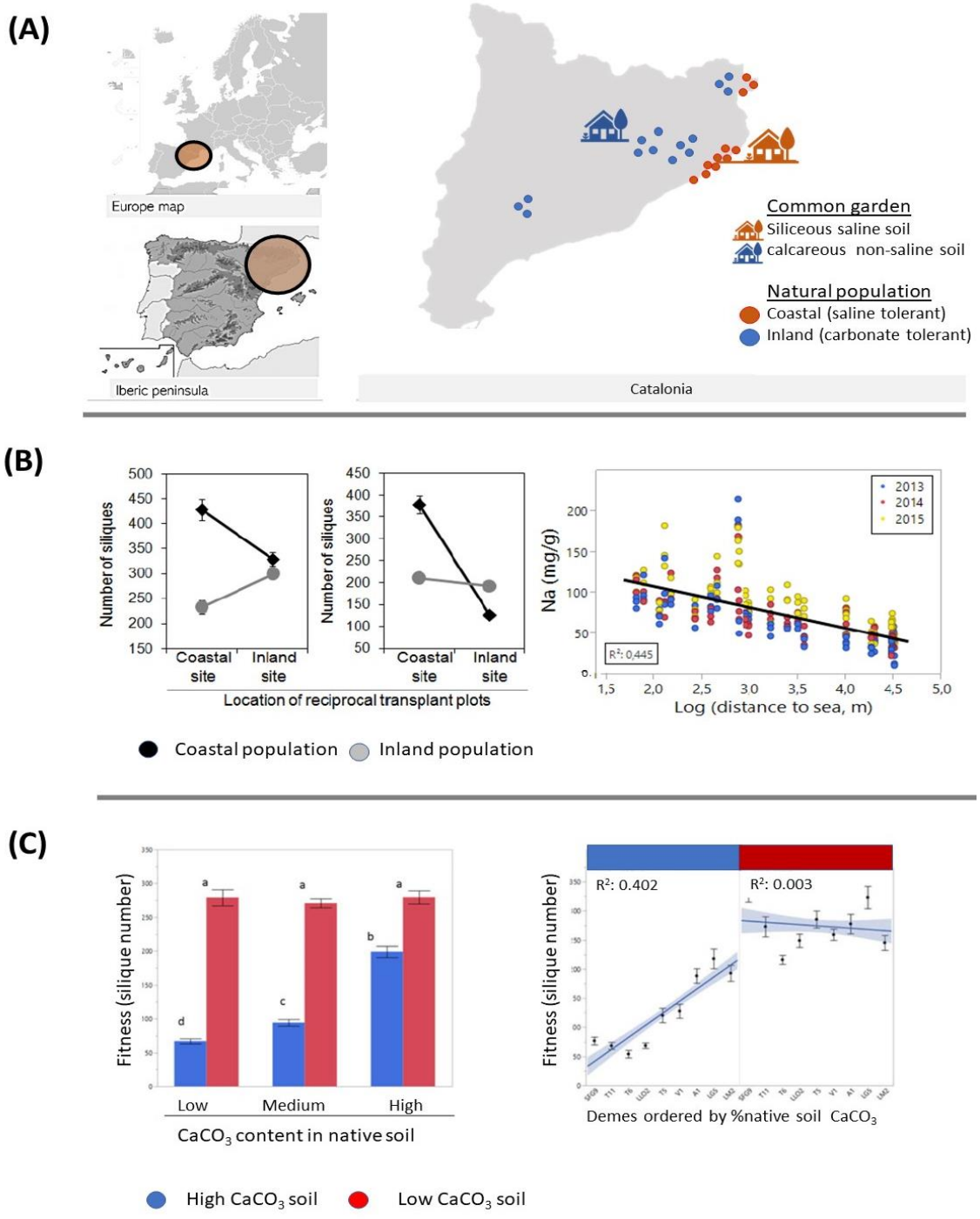


Figure 5. Catalan natural population location and main results from previous studies. **(A)** Catalonian natural populations. Demes from coastal soils are marked in red while inland demes were colored in blue. Greenhouse experiments were performed in saline and alkaline soils from 2013 to 2015. **(B)** Deme fitness in a common garden experiment. Sodium soil content and log distance to the sea correlation. Both figures were extracted from Busoms 2015 [86]. **(C)** Bar plot fitness from plants growing in calcareous and control soil. Fitness *vs* native $CaCO_3$ soil correlation. Extracted from Terés 2019 [19].

In the study area of this thesis, edaphic conditions have been pointed as a driving factor for phenotypic and genetic diversity leading to local adaptation phenomena in plants. Busoms [86] reported that, on the coast and adjacent areas to inland habitats of northeastern Spain, *A. thaliana* demes (multiple small stands of plants) are locally adapted to the local conditions of the areas. The observed local adaptation is due to ongoing divergent selection driven by the differential soil salinity content at the original sites where these demes occur (coastal or inland).

Also, according to the distribution data of *A. thaliana* demes from Catalonia and those from all over the world, this species preferably grows on siliceous soils and can be considered a calcifuge species. However, some local adaptations to moderately carbonated soils (less than 30%) have been also described [19]. Local adaptation to granite-derived *versus* limestone-derived soil has been examined by performing common garden field experiments at two locations - one on the coast (siliceous soil) and another on an inland site rich in carbonate. These experiments revealed better fitness on carbonate-rich soils of demes originally coming from soils closer to the limestone soil area than of demes native to siliceous soil or coastal areas. Hydroponic experiments confirmed higher root exudation of coumarins from the carbonate tolerant line as a mechanism to better acquire iron under conditions of high substrate pH [19] [88].

5. Biotechnological approaches in *A. thaliana* natural variation

This section aims to introduce the theoretical framework of the techniques used in the following chapters (Figure 6). At a physiological level, many techniques are available to determine morphological changes, chemical compounds, and substances distribution in tissues and over time. Here, the ionome concept is explored because plant nutrition is a key point affecting other plant processes such as photosynthesis, growth and fitness performance. The ionome technique has been used in chapters 1, 3 and 4. Furthermore, plant physiology responses can be explained by underlying molecular mechanisms. Current technology allows us to elucidate transcriptomic changes and visualize complex biological responses as shown in chapter 2. Moreover, natural genetic variation can be used to detect genomic regions involved in differences at the physiological level. Here, in chapter 3, GWAS was performed using growth and ionome data as input. Finally, in chapter 4, common garden experiments were used to determine differences in plant performance on saline-alkaline soil.

As shown in Figure 6, different scientific areas can be integrated to elucidate plant responses to altered conditions. This is especially important in a climate crisis scenario where plants are pressured in different ways. Increasing crop production is needed to nourish a constantly growing human population by a reduction in resources and environmental impact [89]. Meanwhile, climate change is forcing faster plant adaptations to a new environment [89]. The future of biology and plant biology is to have a holistic vision to understand plant processes from different perspectives. This integrative knowledge may help scientists to predict plant responses in a near future to develop tools that allow us to optimize crops to new climatic conditions [90].

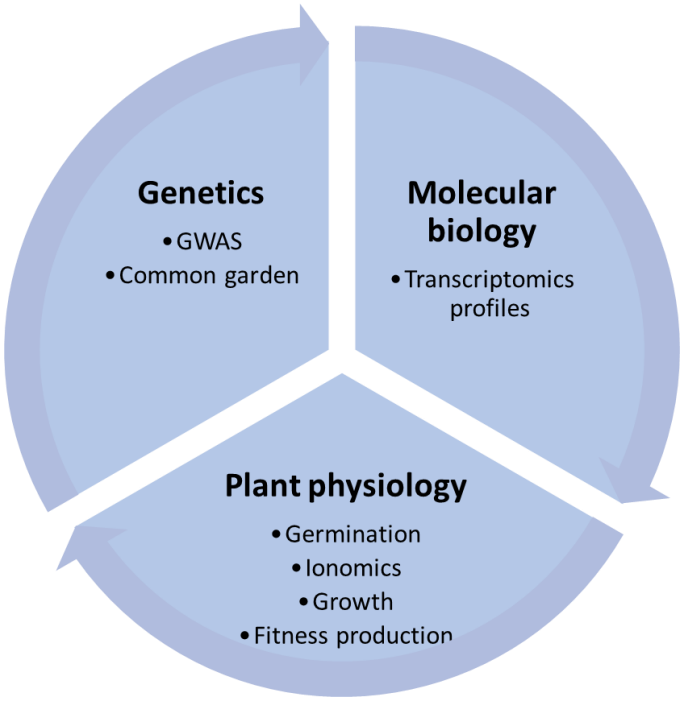


Figure 6. Different scientific areas used in this thesis.

5.1 Ionomics

Nutrient profiles or ionomes were defined by Salt [91] as the mineral nutrient and trace element composition of an organism representing the inorganic components of cellular and organismal systems. Under natural conditions, soils frequently present high heterogeneity regarding mineral nutrient composition and availability. Some elements are present at low or high concentrations causing deficiency or toxicities in plants. Plant toxicity or deficiency at a given level of substrate availability depends on the developmental stage, the plant species, and synergic or antagonism phenomena between nutrient during absorption [92]. For terrestrial plants, as sessile organisms, the maintenance of nutrient homeostasis under heterogeneous edaphic conditions is crucial for survival. Plant homeostasis includes several critical steps like uptake, transport, and storage. Ion homeostasis is regulated by many genes coding for proteins that are actuating in this process as transporters, channels, chelator biosynthesis, or involved in mechanisms of sensing, signal transduction regulation [93]. Therefore, the ionomic profile of plants grown in a common environment may reflect adaptations to their native local environment [94].

The natural biodiversity that exists within the *A. thaliana* species*,* in addition to the artificially produced mutants, like knockouts or overexpressing genes in a Col-0 background, can be used to investigate regulatory mechanisms of individual elements and their interactions and to identify genes important for these processes. The identification of such natural or artificial allelic variants may allow a new approach to investigate the molecular basis of adaptation to native soils [95].

5.2 Transcriptomics

Microarray and RNA seq can be used for analyzing the influence of environmental factors on gene expression profiles in plants. Microarray technology consists of a two-dimensional array on a solid substrate which can be used as a simultaneous study of the expression levels of thousands of predefined genes at one time. Commercials array for *Arabidopsis* covers around 25.000 genes from 27.000 predicted genes [96].

RNA-sequencing is a technique that quantifies and sequences transcriptomes for detecting gene expression or identifying splicing events in a biologic sample using next-generation sequencing (NGS) [96]. One of the principal RNA-seq advantages is the possibility to study novel gens because the technique does not depend on previously known mRNA. Consequently, transcriptomic studies can be done in non-reference genome species [97]. During the last 10 years, RNA-seq publications are steadily increasing because of a reduction in sequencing cost and an increase of bioinformatic tools [98].

After library preparation and sequencing, quality control of Fastq files is performed. The alignment procedure consists of mapping reads into a more similar region on the genome using Software tools like Bowtie [99] and TopHat. Quantifying gene expression is achieved by counting the number of reads aligned to each gene. Based on the number of reads, the expression level of the study genes can be estimated. Normalization of the number of reads per sample and number of reads per gene is applied, considering gene length FPKM = normalized estimation of gene expression-based in RNA-seq data [100]. Transcriptomic differences between samples or between treatment *vs* control are the main goal of transcriptomic experiments. Commonly used tools include DESeq, edgeR, and limma, which are available through R/Bioconductor [101][102][103][104] Different tools use regression or non-parametric statistics to identify differentially expressed genes. After differential expression analysis, candidate genes can be classified into biological pathways. There are several web-based tools and databases like *Keeg pathway* [105], *DAVID database* [106]

and AgriGO [107] that calculate how many genes in the study set overlap with gene sets of signaling pathways. Protein-Protein interaction network can be visualized by STRING [108].

5.3 Genome-Wide Association Studies (GWAS)

Understanding the genetic basis of fitness variation across different environmental conditions is of major importance when studying the genetic mechanisms of adaptation [109]. Genome-Wide Association Studies (GWAS) are a powerful approach to identify loci associated with fitness. Although GWAS has primarily been used for human diseases, like chron disease, schizophrenia, or diabetes II [110]–[112], it has also been successful in mapping causal variants in many other organisms, including *A. thaliana*. Recent reviews point to GWA studies as an increasingly successful analysis in the identification of key genes that are useful for crop improvement [113].

GWA studies reveal single-nucleotide polymorphisms (SNPs) responsible for certain phenotypic traits employing natural variation in a population of the studied species [114]. GWAS starts with phenotypic and genotyping data from characterized populations. Genotyping technologies such a chip-based microarray technology can provide a 250K SNP dataset [115] [116] while next-generation sequencing technologies full sequenced genome can be obtained [117]. In recent years, sequenced genomes are becoming more and more available due reduction of sequencing costs, better tools and new algorithms.

To detect statistically significant differences between a quantitative phenotype and genetic polymorphisms tested in different individuals, data from genetic markers and observed phenotypes from each tested individual tested are required. Before performing the association study, the Kinship matrix (relatedness matrix) reduces the population structure and eliminates a high number of false positives that appear only for the proximity of the populations. To perform statistical GWAS studies different models' area available. Election depends on data. A nonparametric Wilcoxon rank-sum test (Wilcoxon, 1945), a simple linear regression (LM), or an accelerated mixed model (AMM) can be used. Different repositories and tools have been developed to perform GWA studies [118] [119] [120] Nowadays, different platforms are available to perform a genome-wide association study. Examples are R scripts, plink [121] using command lines developed by, tassel [122] and GWA portal web interface from the Max Plank Institute [118].

5.4 Common garden experiments

Plants are sessile organisms exposed to diverse stress in their local habitat. As a result, different populations of a species are subject to different selection pressures leading to adaptation to local conditions and intraspecific divergence. Ecology and evolutionary biology are focused on detecting phenotypic and genetic variation in populations and the adaptative mechanisms that force plants to adapt to their native environment [90]. A common garden experiment is a good approach to test the effect of the environment by moving individuals from their native environments into a common environment. Transplant experiments have the advantages of being simple and requiring little technology. On the other hand, they may require considerable time and labor. Advances in molecular biology have provided researchers with the ability to study genetic variation more directly [123].

In the literature, several common gardens were conducted with different plant species, being *A. thaliana* one of the best candidates due to its large distribution under different climatic and edaphological conditions. At the European scale, several studies were focused on the detection of differences across northern and southern edges of the native range concluding strong adaptive differentiation [124] due to climate, differ in seedling responses to high-temperature stress [125] and flowering time [126]. Also, adaptation to edaphic conditions was found at both large scale and small scale [87][127].

Hypothesis and aims

Catalan *Arabidopsis thaliana* populations are locally adapted to soil salinity and clear genetic differences among populations evolved under low, moderate, or strong salinity conditions under acid or near to neutral pH [86], [87]. Most saline-adapted populations come from the coastal part where salinity is higher than in non-coastal locations. Although *A. thaliana* is a calcifuge specie, inland populations are present in locations with moderate carbonate levels. Inland populations present local adaptation to moderate carbonate soil levels in non-saline conditions [19].

The gap of knowledge and controversial information found regarding bicarbonate stress in plants impulse us to detect genetic, molecular and physiological responses using extreme demes found in previous experiments [19]. After that, the apparent lack of natural *A. thaliana* populations in coastal habitats on saline calcareous soils impels the hypothesis that there are specific mechanisms of tolerance required for fitness on soils combining both stress factors. According to that, saline-alkaline conditions are going to be studied by exploiting the natural variation among all Catalan demes.

General aim

The general aims of this thesis were to identify specific physiological, molecular and genetic mechanisms that are involved in alkaline and alkaline-saline stress responses using natural populations of *A. thaliana*. This general aim is divided into four chapters.

Specific aims

Chapter 1: Physiological responses from contrasted Catalan *Arabidopsis thaliana* demes under alkaline stress: In previous studies, native populations of Catalonia were tested under calcareous common gardens in field conditions, obtaining lines with contrasting tolerance to carbonates. The main aim of this chapter is to find a mild $NaHCO_3$ concentration for hydroponic or plate culture in order to avoid higher mortalities and simulate soil experiments. Also, detection of differences in plant responses focus on germination, growth, nutrition, oxidative stress, and silique production under two alkaline treatments (high pH *vs* $NaCO_3$) in the plate or hydroponic culture. Finally, assess gene expression involved in iron uptake mechanism and pH homeostasis in plants at a short time to detect first stages responses.

Chapter 2: Transcriptomics responses from contrasted Catalan *Arabidopsis thaliana* demes under alkaline stress. This chapter aims to reveal biological pathways involved in plant responses towards two types of alkaline stresses (high pH achieved by organic buffer *vs* bicarbonate). Transcriptomic profiles from contrasted tolerant demes are compared in order to detect specific early responses from each deme towards alkaline stress. For these reasons, transcriptomic analysis using contrasted demes $A1_{(c+)}$ carbonate tolerant and $T6_{(C-)}$ carbonate sensitive -was performed at short term and medium-term responses - 3h and 48 h, respectively.

Chapter 3: Genome Wide Association Studies and candidate genes validation of *Arabidopsis thaliana* natural population growth in carbonate soil. The natural worldwide distribution of *Arabidopsis thaliana* was used to perform a GWA study to elucidate novelty genomic regions involved in growth and plant nutrition on calcareous soil. Candidate genes validation and its role on plant tolerance to salinity and carbonates were performed.

Chapter: 4 Catalan *A. thaliana* population screening on alkaline-saline soil Catalan natural populations were used to perform common garden experiments under alkaline-saline stress to detect extreme phenotypes of plant performance in alkaline saline soil under semi-controlled conditions (greenhouse). Plant performance (germination, growth, nutrition, and fitness) were scored in controlled environment to validate plant response in field conditions.

Chapter 1: Physiological responses from contrasted *Arabidopsis thaliana* demes under alkaline stress

1 Introduction

Calcareous soils are present mostly under semiarid and arid climates, where high evapotranspiration and low rainfall produce calcium carbonate and bicarbonate accumulation in the upper soil layers. Excess of CO_3^{2-} and HCO_3^- anions, from 5 to 35 mM [11], buffer soils in a pH range from 7.5 to 8.5 [12]. High pH affects around 30 % of the cultivable land and modifies physical, chemical, and biological soil properties [19].

High pH stress effects in plants depend on the development stage and the plant species. Notable differences can be observed among ecotypes. Bicarbonate imposes huge stress on plant performance such as germination delay or inhibition [49] [128] and morpho-anatomical changes in roots. Enhanced lateral root development and increase of root hair density often occur to increase nutrient uptake surface, leading to the so-called herringbone phenotype [129]. HCO_3^-, is known to buffer or neutralize the extrusion and accumulation of H^+ and acidic compounds reducing nutrient uptake by roots and their transport toward shoots [32][130]. Antioxidant enzyme activation and organic acid biosynthesis and exudation to adjust the internal pH value and anion balance [35] are frequently observed. All these adjusting processes consume energy and reduce plant growth simultaneously [131]. Also, imbalance in nutrient uptake [44] may cause reduction of photosynthetic pigments and decrease of photosynthetic activity. Several studies have shown that plants perceive and respond in different ways towards high pH, excess of HCO_3^-, or iron deficiency [32][132], displaying different responses to each stress regarding mineral nutrition and organic acid exudation.

Nutrient deficiencies (Fe, Zn, P, N) are common problems for crop plants on calcareous soils. Although iron is the fourth most abundant element in the earth's crust, high soil pH reduces iron solubility to concentrations below the threshold for optimal plant growth (10^{-9} and 10^{-4} M) [23]. Iron is an essential micronutrient required for multiple metabolic processes. Most crops are highly sensitive to low iron availability [133]. This fact has prompted many efforts to unrevealing the mechanisms of Fe uptake, transport and regulation, and the tolerance to Fe deficiency [8], [134], [135]. Several studies on Fe uptake mechanism revealed strategy I for all plants excepting most members of the *Poaceae* family. The strategy I of iron acquisition consists of three main steps:

(I) Acidification of the rhizosphere by organic acids and extrusion of H^+ by ATPase (AHA2) increasing Fe^{3+} solubility and producing an optimal pH for the activity of the ferric reductase enzyme.

(II) Reduction of Fe^{3+} to Fe^{2+} at the root surface by a plasma membrane-bound ferric-chelate reductase (FRO2).

(III) Uptake of Fe^{2+} into root cells by the Fe high-affinity transporter Iron-Regulated Transporter (IRT1).

Under iron deficiency, Strategy I related genes are activated. However, a different situation is observed under bicarbonate stress [136][137]. Another interesting point is the plants' use of Fe under bicarbonate stress. Under bicarbonate exposure, a strong Fe accumulation in the roots and an inhibition of Fe translocation to the shoots is frequently observed. This inability to translocate iron to the shoots and to manage it adequately can cause a malfunction of the iron-dependent proteins. Among others, Fe participates in the activity of important proteins related to the photosynthetic apparatus, Fv/Fm and chlorophyll content, as well as to essential components of the electron transport chains in chloroplasts and mitochondria [129].

Another enzyme, carbonic anhydrase (CA), which catalyzes the interconversion of CO_2 to bicarbonate, can be involved in bicarbonate stress responses in inland plants [34]. Seagrasses access HCO_3^- for photosynthesis by 2 mechanisms, apoplastic carbonic anhydrase-mediated dehydration of HCO_3^- to CO_2 and direct HCO_3^- uptake [138]. In higher plants, no selective HCO_3^- transporter or channel has been characterized at the molecular level and the role of CAs in roots under bicarbonate stress is still unknown [34].

High pH soil is a worldwide problem with direct affectation in agriculture production. However, the current understanding of plant response to bicarbonate stress is limited and few investigations are focused on unraveling physiological responses to bicarbonate stress. Most studies have been performed on crop species such as *Glycine max*, *Oryza sativa*, and *Solanum lycopersicum* while *Arabidopsis thaliana* has deserved less interest. The large genotypic and phenotypic diversity of natural populations of the genetic model plant *A. thaliana* provides an ideal experimental scenario for exploring such mechanisms. In previous studies, we identified two contrasting demes of *A. thaliana* with differential tolerance to moderate soil alkalinity: the sensitive deme $T6_{(c-)}$ and the

tolerant $A1_{(c+)}$. The better performance of $A1_{(c+)}$ under iron deficiency conditions was mainly attributed to enhanced root release of coumarin-type phenolics [19].

2. Aims

- To select an adequate $NaHCO_3$ concentration for studying alkaline stress in *Arabidopsis thaliana* species, avoiding distress and allowing to distinguish differences in tolerance among demes in hydroponic culture.
- To assess phenotypic markers from the alkaline stress response using yield components: germination, growth, silique production and nutrient profiles.
- To quantify plant chlorophyll content and photosynthetic rate under control and bicarbonate stress conditions.
- To visualize reactive oxygen species, a proxy of membrane damage, and to quantify membrane damage caused by bicarbonate stress.
- To determine the influence of HCO_3^- on membrane potential and carbonic anhydrase activity in roots.
- To characterize iron uptake mechanisms after short-term exposure to bicarbonate.

3 Material and Methods

3.1 Plant material

In previous studies, natural variation of *Arabidopsis thaliana* populations from Catalonia [86] were tested in a multi-year small-scale common garden experiment under carbonate conditions [19]. Seeds of extreme phenotype lines from $A1_{(c+)}$, an alkaline-moderate tolerant deme, and $T6_{(c-)}$, an alkaline sensitive deme, were collected from the last reciprocal transplant experiment performed in 2015 and stored under fresh (4 ºC) and dry conditions until the beginning of the experiments. Col-0 seeds from Nottingham Arabidopsis Stock Centre (NASC) [139] were included as a reference genome.

3.2 Growth conditions

3.2.1 Seed sterilization: Selected 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.

3.2.2 Hydroponic culture: Seeds were sown in 0.2 mL tubes containing 0.6% agar prepared in nutrient solution ½ Hoagland (pH 5.9). Seeds were kept at 4 °C for 7 days in the dark to synchronize germination. Tubes containing seeds were placed in the growth chamber (12 h light/12 h dark, 150 µmol $cm^{-2} \cdot s^{-1}$, 40% humidity and 25 °C). After root emergence, the bottom of the tubes containing seedlings was cut off and the tubes were placed in 150 mL hydroponic containers with aerated nutrient solution ½ Hoagland (pH 5.9). Seedlings of 15 days old, were separated into different sets and the following treatments were applied: control (½ Hoagland solution at pH 5.9), high pH (½ Hoagland solution at pH 8.3), and bicarbonate (½ Hoagland solution at pH 8.3 with 10 mM $NaHCO_3$). Solutions were buffered with different proportions of MES (2-(N-morpholino) ethanesulfonic acid hydrate, 4-morpholineethanesulfonic acid) and BTP 1,3-bis[tris (hydroxymethyl)methylamino] propane) depending on the desired final pH. Solutions were continuously aerated. The selected bicarbonate concentration has previously been shown to discriminate between the carbonate-tolerant and sensitive deme [19]. To check whether this concentration allows reproductive growth in the tolerant accession, we performed $A1_{(c+)}$ preliminary essays with bicarbonate concentrations ranging from 0 to 20 mM (pH 8.3). Rosette diameter and length of the longest root were monitored every week by image analysis [140], [141].

3.2.3 Plate culture: For germination assays, 45 seeds from each deme were sown in plates under a flow cabinet with sterile material. Plates contained 3 treatments: control (½ MS at pH 5.9), high pH (½ MS at pH 8.3), and bicarbonate (½ MS at pH 8.3 and 10 mM $NaHCO_3$). All plates contained Phyto-agar 0.6% (Duchefa, Haarlem, The Netherlands), and solutions were buffered using different proportions of MES and BTP depending on the desired final pH. Plates with seeds were kept at 4 °C for synchronizing germination. After 7 days under stratification treatment, plates were moved to a growth chamber (12 h light/12 h dark, 150 µmol $cm^{-2} \cdot s^{-1}$, 40% humidity and 25 °C). Germination and radicle emergence was daily checked during the following 10 days.

3.3 Physiological trait measurements

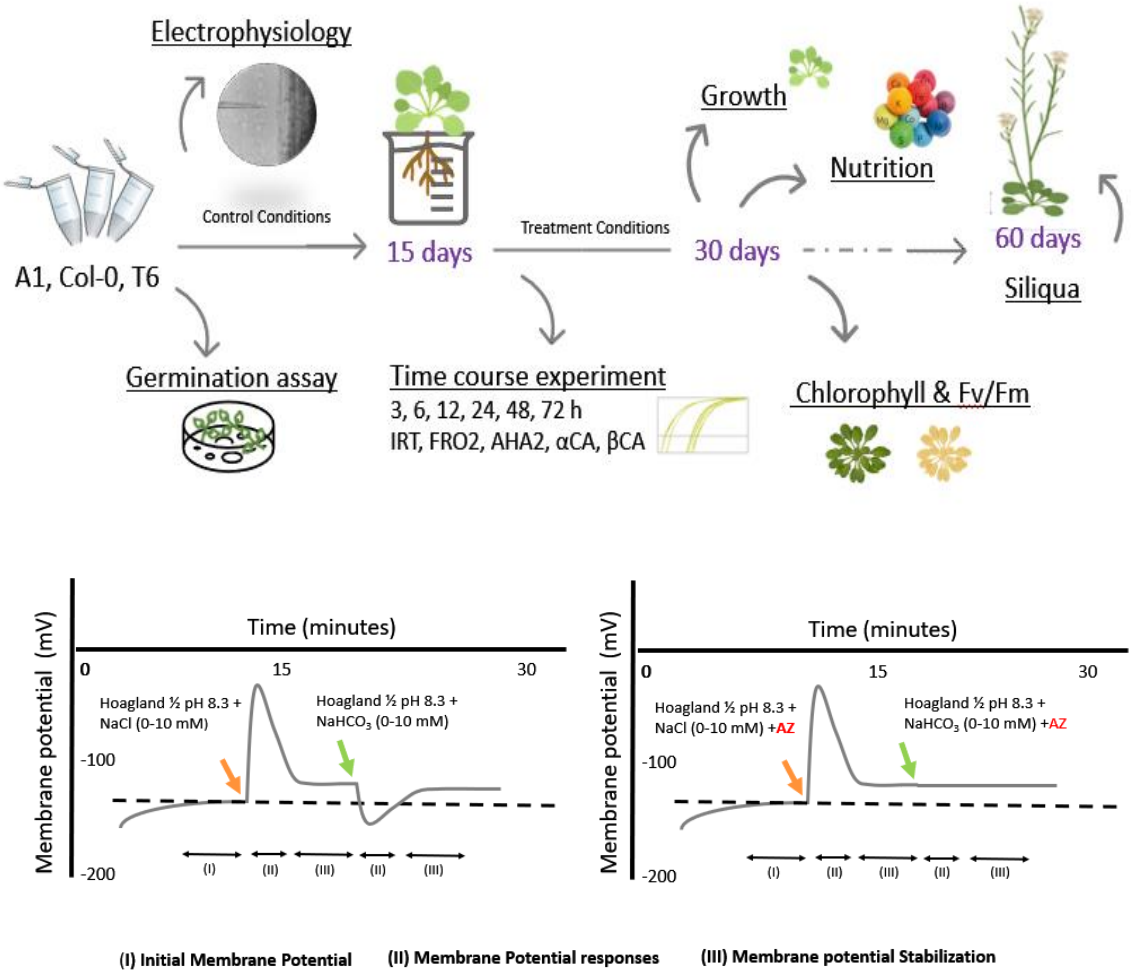


Figure 0. Experimental design scheme and detailed electrophysiologic experiments.

3.3.1 Growth and silique production:

Plants from tolerant $A1_{(c+)}$, sensitive $T6_{(c-)}$, and Col-0 were used to test fitness in hydroponic conditions. Every week, pictures of the entire plants were taken. The photographs were used to measure the length of the largest root and the rosette diameter (n= 9–12) using ImageJ software [140], [141]. We did not use a dye before the image acquisition. Silique production was counted at maturity (n= 9).

3.3.2 Nutrient mineral content:

The leaf nutritional state of plants was assessed in plants submitted for 15 days to treatment conditions. Roots were carefully washed with 18 MΩ water to remove adhering nutrients. Plant

material was dried for 4 days at 60 ºC. Approximately 0.1g was used to perform and open-air digestion in Pyrex tubes using 0,7 mL concentrated HNO_3 at 110 ºC 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) *(n=4)* [142].

3.3.3 Chlorophyll content and photosynthetic efficiency:

Chlorophyll contents and photosynthetic efficiency (Fv Fm^{-1}) were measured in leaves under different treatments. Leaf chlorophyll concentrations were obtained using a SPAD chlorophyll meter (CCM-200, Opti-Science, Hudson, USA). The maximum PSII quantum yield (Fv Fm^{-1}) determination was performed in young leaves previously dark-adapted at least for 30 min and measured with a PAM fluorometer (SHIMADZU RF-551, Ontario, Canada) *(n=20).*

3.3.4 Reactive Oxygen Species (ROS) visualization and MDA:

O_2^- histochemical *in situ* detection in leaves: Leaves were incubated in 1mM nitroblue tetrazolium (NBT) previously prepared in sodium citrate buffer (10 mM pH 6), in darkness at room temperature on a shaker for 8h. Then leaves were blanched with ethanol 96º. NBT reacts with $O2^{\cdot-}$ to form a dark blue insoluble compound [143].

H_2O_2 histochemical *in situ* detection in leaves: Leaves were incubated with 1mg mL^{-1} of 3,3-diaminobenzidine (DAB)-HCl pH 3.8 at room temperature, on a shaker for 8 hours. Stained leaves were bleached in ethanol 96º. DAB reacts with H_2O_2 in presence of peroxidases forming brown deposits with were observed and pictured [144].

MDA: Fresh plant material (0.1 g) was ground into a powder using liquid nitrogen, and then put into a tube containing 1 ml 0.1% (w/v) TCA. The solution was centrifuged at 10,000 *x g* for 10 min. The supernatant (1mL) was transferred to a new tube containing 4 ml of 20% TCA with 0.5% TBA. The mixture was heated at 95 °C for 15 min and quickly cooled on ice. The resulting solution was measured with a spectrophotometer at 532 nm. The standard curve was generated using a serial concentration (1-50 µM) of MDA [145].

3.3.5 Relative gene expression

Time-course experiments were displayed to detect differences in genes involved in the mechanism of iron uptake at a short time. Plants were grown in hydroponics under the same

conditions explained in the section *3.2.2.* The root material was extracted at 0, 3, 6, 24, and 48 hours. Three plants were pooled to perform a replicate and 3 replicates were taken from each deme and treatment. Root material was rinsed with deionized water and immediately frozen at -80 ◦C. RNA was extracted using Maxwell plant RNA kit (Promega Corporation, Madison, WI, USA). After quality and quantity control, RNA was transformed to cDNA using iScriptTM cDNA Synthesis Kit (Bio-Rad, USA). 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 10'' 95◦ C followed by annealing and extension 30'' 60◦ C. A total of 40 cycles were run. A melting curve was performed, increasing from 65.0◦ C to 95.0◦ C by 0.5◦ C each 5 seconds. Primers for *FRO2*, *IRT1, AHA2*, *αCAs* (1-8) and *βCAs* (1-6) were designed using NCBI primer blast tool [146] and they were purchased from Biolegio (Nijmegen, The Netherlands). Actin was used as a housekeeping gene [147]. Sequence of the primers are shown in Annex tables 26 and 27 and in Supplementary Figure 6 and 7 respectively. Plates were edited using the CFX manager version 3.1 software. The expression of the target gene relative to the expression of the reference gene was calculated using the $2^{-\Delta\Delta Ct}$ method [148].

3.3.6 Electrophysiological assay

Seeds were sterilized and stratified (see section *3.2 Growth conditions)* in plates containing control media conditions (MS ½ pH 5.70) for 7 days. Electrodes were fabricated using a Pull 100 puller (WPI Europe, Hertfordshire, UK). The reference electrode was filled with 0.5 M KCl + agar 0.03% while the measurement electrode was filled with 0.5 M KCl. Both electrodes were connected to a high-impedance differential amplifier (FD223a). The signals from differences in root membrane potential were monitored on a chart recorder (Linseis L250E). Electrophysiological experiments were performed on roots. The measurement chamber was open on both sides allowing the approach of several electrodes to the root. A gravity-based flow-through system permitted controlled changes of the medium at a rate of 10 ml/min. This system kept the temperature, ionic concentration, and gases constant during the experiments. Microscope light (150 µmol photons m^2/s) was on during the experiments. To detect changes in membrane potential produced by HCO_3^- application, a solution of NaCl pH 8.3 buffered with MES and BTP was used as a control to account for the effect of Na^+. Dose experiments were performed from 0 to 10 mM. Furthermore, inhibition

of external anhydrase was performed using 10 mM acetazolamide (AZ) solution diluted in 0.005 N NaOH [138]. See Figure 0.

3.4 Statistical analysis

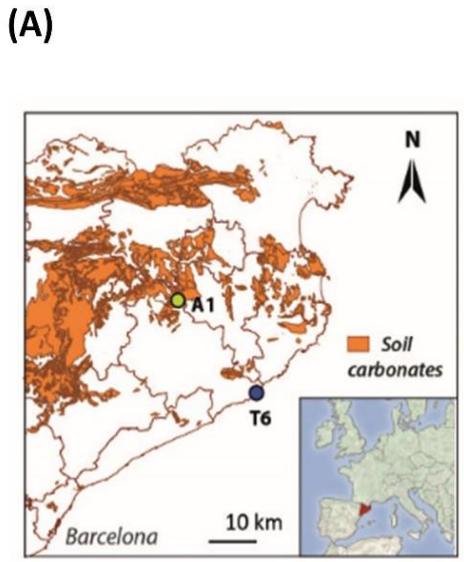
In physiological measurements, the normal distribution of data was confirmed by Levene's test. Treatments and population effects were analyzed by two-way ANOVA. Post-hoc analyses for significance of differences were realized using the Tukey test. Mineral nutrient data were standardized (X- mean)/(min-max) to avoid bias due to different orders of magnitude order. A radial plot was used to visualize differences among treatments in each deme. Statistical analyses and plots were performed using JMP13 software. (JMP, Version 13. SAS Institute Inc., Cary, NC, USA, 1989–2019).

4 Results & discussion

4.1 Selection of an adequate $NaHCO_3$ concentration for discriminating between sensitive and tolerant *A. thaliana* demes in hydroponics.

In previous studies, we identified two contrasting *A. thaliana* demes with differential tolerance to moderate soil alkalinity: the sensitive $T6_{(c-)}$ and the tolerant $A1_{(c+)}$ [19]. $A1_{(c+)}$ is native to areas close calcareous parental material, and soil $CaCO_3$ content and pH in its habitat are higher than in native soils of $T6_{(c-)}$, which are derived from siliceous parental rocks (Figure 1A, B).

To ascertain the bicarbonate concentration that efficiently discriminates both demes under hydroponic conditions, we performed preliminary studies using a concentration range from 0 to 20 mM $NaHCO_3$. Rosette diameter and root length growth of both demes were monitored for 5 weeks (Figure 1 C, D, and Supplementary F1). Concentrations higher than 10 mM caused high mortality before ending the life cycle. This contrasts with more carbonate-tolerant crop plants such as *Glycine max*, where 50 mM $NaHCO_3$ has been established as a mild stress situation avoiding high mortalities [149]. In the wild type of *A. thaliana*, plant growth was tested in a dose-response assay performed in plates containing incremental $NaHCO_3$ and NaOAc (sodium acetate) treatments. Plant growth was attenuated between concentrations of 1.5 mM–8 mM indicating more sensitivity to $NaHCO_3$ [63]. According to our results, a concentration of 10 mM $NaHCO_3$ was selected as an adequate discriminatory treatment in all subsequent experiments.



(B)

	A1(c+)
pH	7.36 ± 0.09
%CaCO3	12.13 ± 4.89

	T6(c-)
pH	6.55 ± 0.22
%CaCO3	0.81 ± 0.55

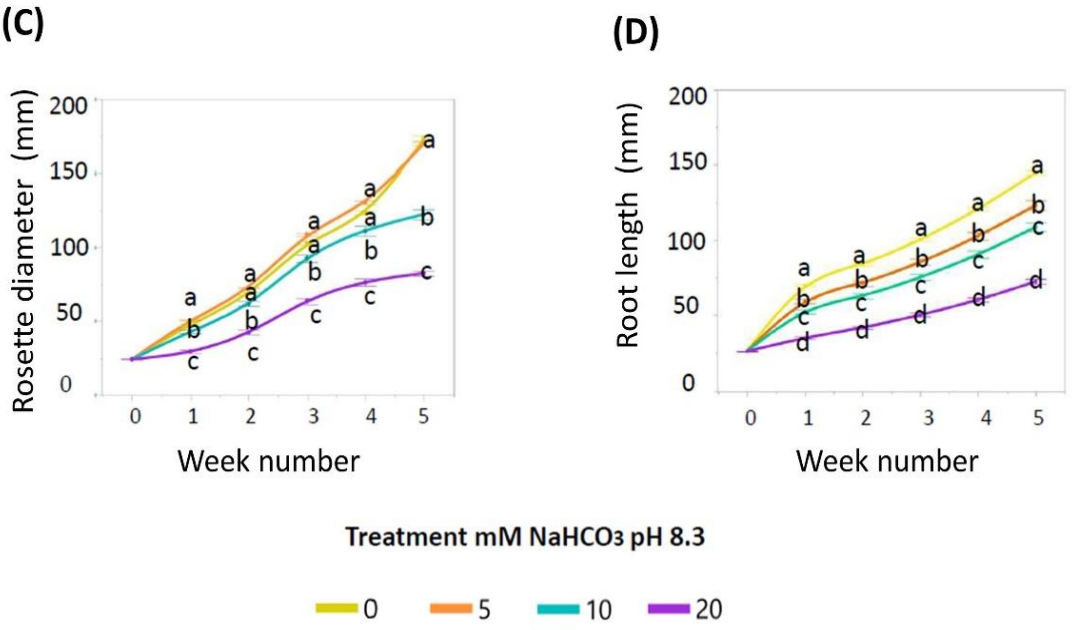


Figure 1. Location, soil parameters, and growth responses of moderate bicarbonate tolerant *A. thaliana* deme under incremental $NaHCO_3$ concentrations. (A) Location of *Arabidopsis thaliana* natural populations $A1_{(c+)}$ and $T6_{(c-)}$ on the soil map of Catalonia. Orange areas represent calcareous soils and white areas non-calcareous soils. **(B)** Soil carbonate and pH levels (means ± Standard Error; *n* = 9) in the natural habitat of $A1_{(c+)}$ and $T6_{(c-)}$ populations **(C)** Rosette diameter (cm) and **(D)** root length (cm) of $A1_{(c+)}$ plants grown in hydroponics for 5 weeks. Letters indicate significant differences between treatment (*n= 9*; $p < 0.05$ according to Tukey's HSD).

4.2 Fitness characterization *A. thaliana* plants under high pH and bicarbonate stress.

The ability to germinate, i.e., root emergence, is a prerequisite for efficient plant establishment on problem soils, and seed germination rate under stress conditions have frequently been used to assess plant tolerance to soil constraints [49]. Here, seed germination tests were performed, comparing the contrasting natural demes with the model accession Col-0. Seed germination tests revealed, for all accessions, a delay in germination in the high-pH treatments (pH 8.3 and bicarbonate) in comparison to pH 5.9. However, 10 days after sowing the germination rate was hardly affected in Col 0. $A1_{(c+)}$ seeds showed better germination rates under the high pH treatments than under pH 5.9. In contrast, both alkaline stresses reduced the germination rate of $T6_{(c-)}$ (Figure 2A). Accordingly, $A1_{(c+)}$ and Col-0 are more tolerant to alkaline conditions than $T6_{(c-)}$. However, seed germination does not guarantee further growth, survival, and reproductive fitness under alkaline stress [49].

Hydroponic studies using solutions with control pH (pH 5.9), high pH (pH 8.3), and bic (10 mM $NaHCO_3$, pH 8.3) confirmed the higher tolerance of $A1_{(c+)}$ to both pH 8.3 and bicarbonate. Col-0 exhibited an intermediate response, while $T6_{(c-)}$ was most sensitive. Both alkaline treatments, pH 8.3 and bic, induced severe chlorosis in leaves of $T6_{(c-)}$ (Figure 2B). Bicarbonate treatment also caused stronger chlorosis symptoms in Col-0 than in $A1_{(c+)}$, while pH 8.3 had little effect in both accessions.

Rosette diameter and root length data measured 15 days after the start of treatments and number of siliques further revealed alkaline sensitivity of both $T6_{(c-)}$ and Col-0 and confirmed tolerance of $A1_{(c+)}$ (Figure 2 C, D, E, Supplementary F2). Interestingly, while in the sensitive accession bicarbonate was more stressful than pH 8.3, the alkaline-tolerant $A1_{(c+)}$ performed better under bicarbonate than under pH 8.3 (Figure 1F). The observation that in sensitive plants bicarbonate is more stressful than pH 8.3 is in line with the inhibitory effects of bicarbonate on essential metabolic processes in plants [47]. Especially affected by bicarbonate are mechanisms activated under Fe deficiency conditions such as riboflavin biosynthesis genes and NRAMP1 in roots or bHLH38 and IRT1 in shoots, as shown in melon plants exposed to either Fe deficiency alone or in combination with bicarbonate [150].

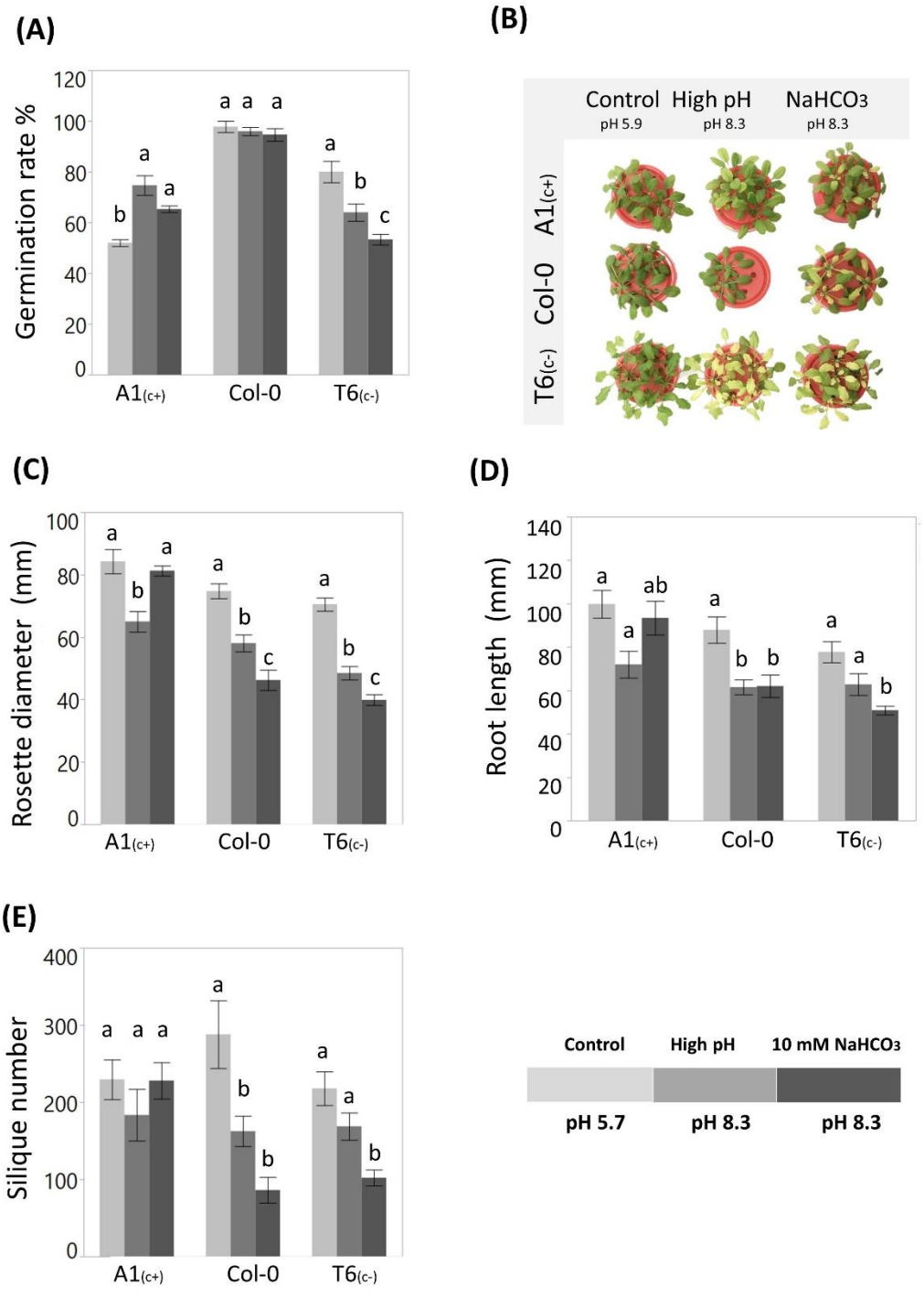


Figure 2. Physiological characterization. **(A)** Germination rate **(B)** Picture of 30-day-old Col-0, $A1_{(c+)}$, and $T6_{(c-)}$ plants under different alkaline treatments for 15 days, **(C)** rosette diameter and **(D)** root length **(E)** Silique number at maturity. $A1_{(c+)}$, $T6_{(c-)}$, and Col-0 plants submitted to control (pH 5.9, light-grey bars), high pH (pH 8.3, medium grey bars), or bic (pH 8.3 with 10 mM of $NaHCO_3$, dark grey bars) in plates or hydroponic culture. Values are means ± SE; *n*= 45, *n*= 12, *n*= 12, *n*= 9, respectively; letters indicate significant differences ($p < 0.05$, Tukey's honestly significant difference HSD per deme).

4.3 Nutrient content profile: $A1_{(c+)}$ better nutrition under HCO_3^- stress.

Differences in tolerance to $pH_{8.3}$ and bic treatments were also reflected in the plants' ionomic profiles. Nutrient concentrations in Col-0 were more affected by bic than by $pH_{8.3.}$ In general, the tolerant deme $A1_{(C+)}$ maintained ion homeostasis better than the sensitive plants in leaves (Figures 3 A, B, C, Supplementary F3). Moreover, maintenance of leaf ion homeostasis in $A1_{(C+)}$ was better under the bic treatment than under pH 8.3. Only P, Ca and Mn concentrations were significantly reduced by bic in all demes. The statistical significance of treatment *vs* genotype interactions (p value<0.05) is shown in Supplementary F3. The contrary situation is observed in roots, where $A1_{(c+)}$ and Col-0 show a general reduction of nutrient concentrations under alkaline treatments while sensitive lines tend to accumulate ions in bic treatments. (Figures 3 D, E, F and Supplementary F3)

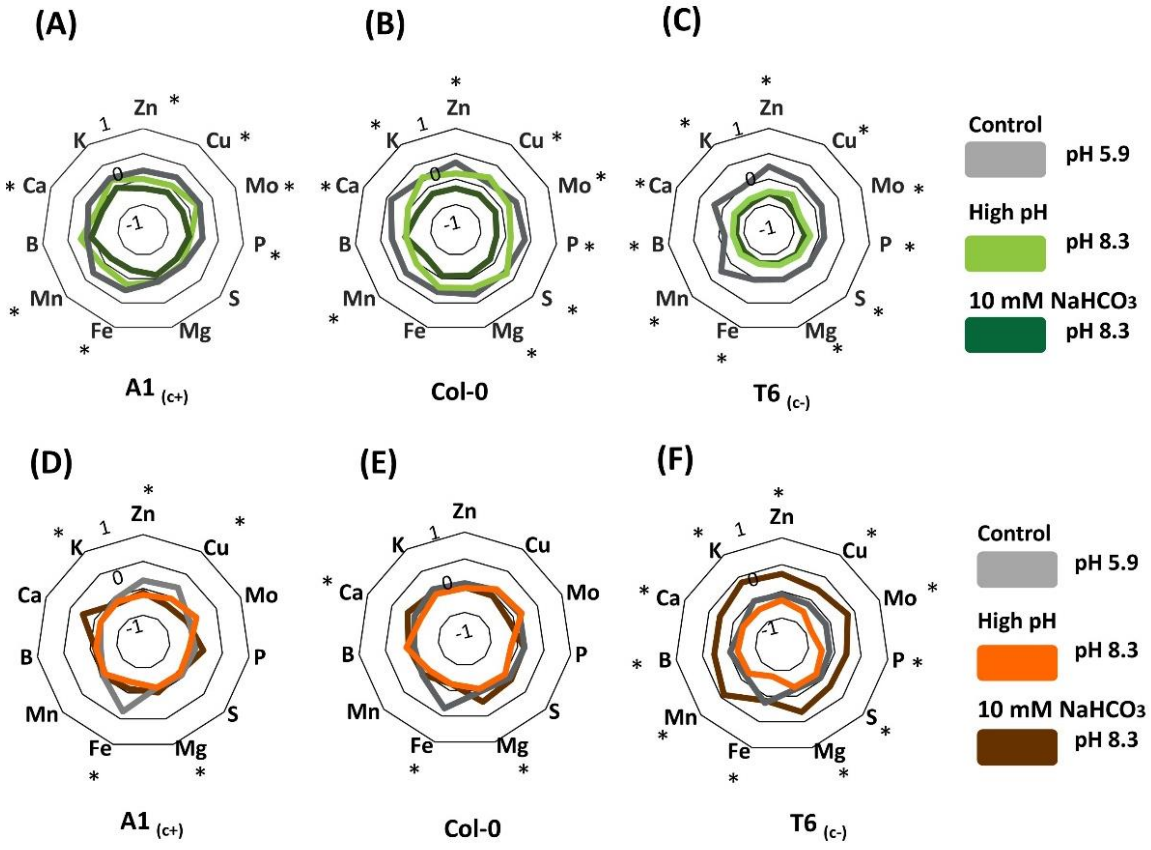


Figure 3. Nutrient mineral profiles. Standardized nutrient mineral content of 15-day old plants $A1_{(c+)}$, $T6_{(c-)}$, and Col-0 plants submitted to control (pH 5.9), high pH (pH 8.3) or bic (pH 8.3 with 10 mM of $NaHCO_3$) in hydroponic culture for 15 days. **(A-C)** The radial plots from leaves data while **(D-F)** roots. Asterisk indicates statistical significance *(n=4)*

However, all tissue concentrations in $A1_{(C+)}$ exposed to bic remained above the deficiency thresholds for Brassicaceae species [151]. Especially relevant is the ability of $A1_{(C+)}$ to maintain leaf Fe concentrations under bicarbonate stress. According to previous investigations, this is due to the ability of this deme to mobilize Fe by the exudation of coumarins [19] acting as Fe reductants and chelators [152].

The analysis of fitness parameters and ionomic data revealed clear differences in the tolerance of *A. thaliana* plants to alkaline pH *per se* and bicarbonate. In the sensitive accessions, Col-0 and $T6_{(c-)}$, bic was more toxic than pH 8.3. $T6_{(c-)}$ evolved on siliceous soil with extremely low carbonate content. Contrastingly, $A1_{(c+)}$ which has evolved on a moderately carbonated soil (12.13% carbonate content) with pH 7.36 was more tolerant to bic (pH 8.3) than to pH 8.3 achieved by organic buffer. Soil conditions can act as driving force for local adaptation [19] [86] and the carbonate/bicarbonate concentration in the original soil of $A1_{(c+)}$ was strong enough to allow this accession to withstand the bicarbonate concentration provided in the nutrient solution (10 mM) yielding a pH of 8.3. However, this pH value achieved by organic buffer was harmful. It has previously been shown that buffers used to simulate alkaline stress may injure plants due to high salinity levels [53]. This was not the case here. The electric conductivity of both solutions was similar. Moreover, bicarbonate was supplied as sodium salt and $A1_{(c+)}$ that under bic better maintained growth and nutrient homeostasis had higher Na tissue contents than the plants from the pH 8.3 treatment (data not shown). We hypothesize that in the bicarbonate tolerant deme the buffer may not be as efficient as bicarbonate in triggering the expression of key genes required for nutrient acquisition and growth under bicarbonate stress.

4.4 Efficiently use of Fe favors maintaining photosynthetic pigments and PSII efficiency

Iron is an essential micronutrient for plant growth that gets poorly available under carbonate conditions [133]. The total iron content was reduced in all accession under bicarbonate stress, but Fe translocation in tolerant plants was higher than in sensitive plants. (Figure 4A, Supplementary F4). The same nutrition profile has been found in contracted *Lotus japonicus* plants where tolerant lines efficiently translocate Fe to the upper part to maintain photosynthetic and metabolic activities, while sensitive lines accumulate huge amounts of iron in roots while Fe translocation to the shoots was inhibited [129]. Bicarbonate not only interferes with the Fe reducing capacity of the roots [137] but also specifically inhibits the translocation from roots to shoots [62]. This inhibition of Fe translocation has classically been related to enhanced root production of organic acids due to dark

fixation of inorganic carbon (C_{inorg}), especially citrate, a strong ligand for Fe which may favor Fe sequestration in root vacuoles [21]. Moreover, accumulation of iron precipitates can reduce and slow down iron transport [32][62]. More recently, studies with bic-exposed Kiwi plantlets related bic-induced inhibition of Fe translocation to the leaves with an apoplastic accumulation of water-soluble Fe in the cell wall apoplast and the imbalance of nitrogen and carbon metabolism. Authors from this work hypothesize root tissue alkalinization produced by surrounding HCO_3^- may favor iron to precipitate as Fe III. This precipitation can retain Fe in the root cell wall compartment and reduce shoot transport and produce translocation inhibition [62].

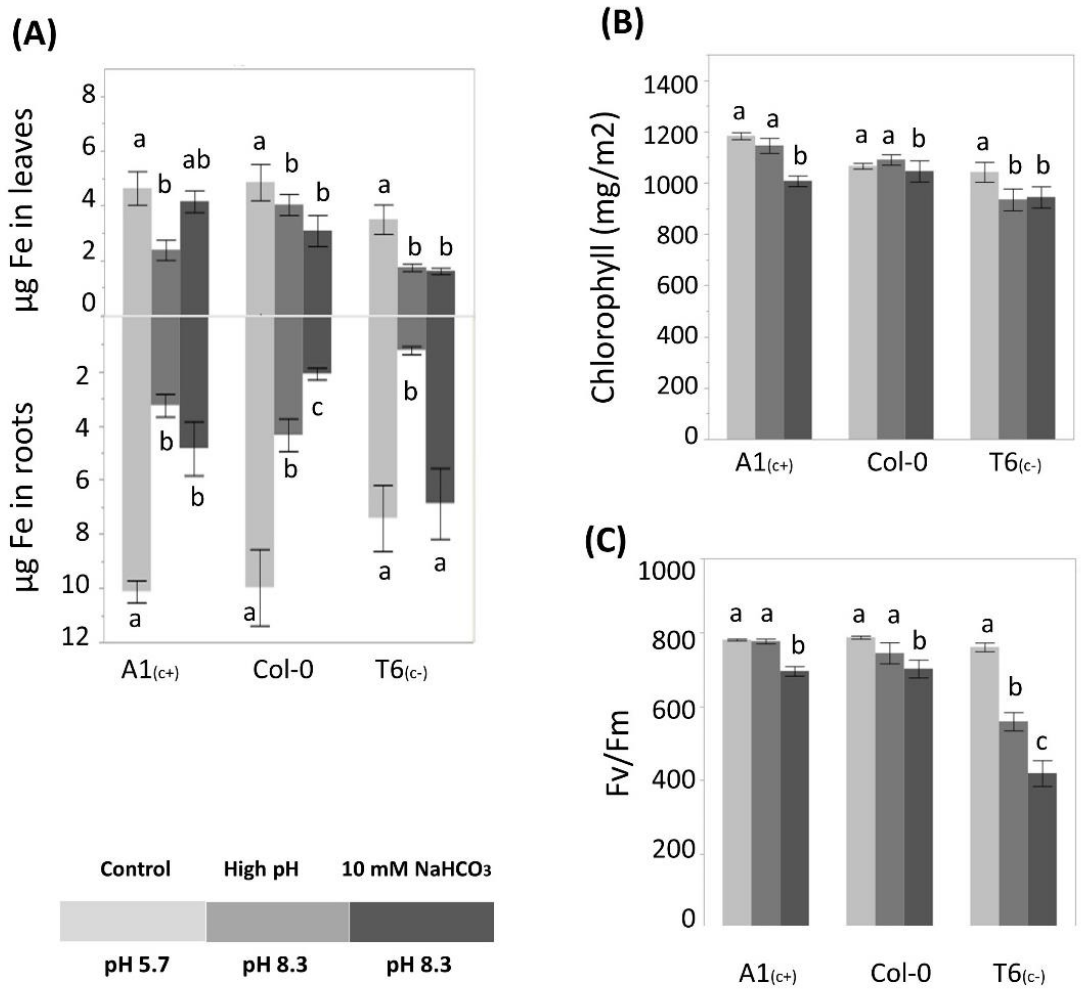


Figure 4. Content and use of iron. (A) Iron content **(B)** photosynthetic pigments **(C)** efficiency of the photosystem II. $A1_{(c+)}$, $T6_{(c-)}$, and Col-0 plants submitted to control (pH 5.9, light-grey bars), high pH (pH 8.3, medium grey bars), or bic (pH 8.3 with 10 mM of NaHCO3, dark grey bars) in hydroponic culture. Values are means ± SE; *n = 4*, *n=20*, *n=20* respectively

Fe participates in important proteins related to photosynthesis [129]. For this reason, the total chlorophyll and efficiency of the photosystem were determined. Photosynthetic pigments were reduced in all accession under bic treatment. However, tolerant lines maintain the same efficiency of the photosystem II as under the control conditions after 15 days of exposure. Contrastingly, in Col-0 and $T6_{(c-)}$ the reduction of pigments was accompanied by a reduction in photosynthetic rates. (Figure 4 B, C, Supplementary F4). Fv Fm^{-1} is the most used chlorophyll fluorescence parameter because it is a non-invasive technique that permits the assessment of photosynthetic performance *in vivo.* In tolerant plants maintenance of higher Fv/Fm under stress conditions can be achieved by higher ROS scavenging and more efficient use of nutrients, especially iron [129].

4.5 Reactive oxygen species under alkaline stress

Imbalance in ROS production can damage cell structures, inhibition of essential enzymatic reactions and decrease plant metabolism[153]. MDA content is widely used as an indicator of damage in plant membranes [145]. Also, $O_2 \cdot^-$ (blue) and H_2O_2 (brown) histochemical visualizations can corroborate ROS accumulation in plant tissue. Both alkaline treatments produced significant lipid peroxidation in plant cells as indicated by MDA. This cell damage was especially high in roots. Tolerant plants maintained lower tissue levels of MDA than the sensitive deme (Figure 5 A, B, Supplementary F5). From previous works performed by Terés [19], it is known that tolerant lines under alkaline treatment present higher catalase and SOD activity than sensitive lines helping to reduce ROS levels.

According to leaf staining, bicarbonate stress produced more H_2O_2 than alkaline pH condition in both demes (Figure 5C). In tolerant lines intense H_2O_2 acumulation was found in the petiole, the central vein and the external part of the leaves leaving central areas with low acumulation. In sensitive lines, H_2O_2 staining was uniformly spred over the entire leaves surface. The $O_2 \cdot^-$ staining revealed severe acumulation under alkaline pH in both demes while under bic stress intense acumulation was observed in the sensitive $T6_{(c-)}$. This acumulation patter fits with previos MDA results. Previos studies report changes in ROS status in plant under nutrient deficiency [55].

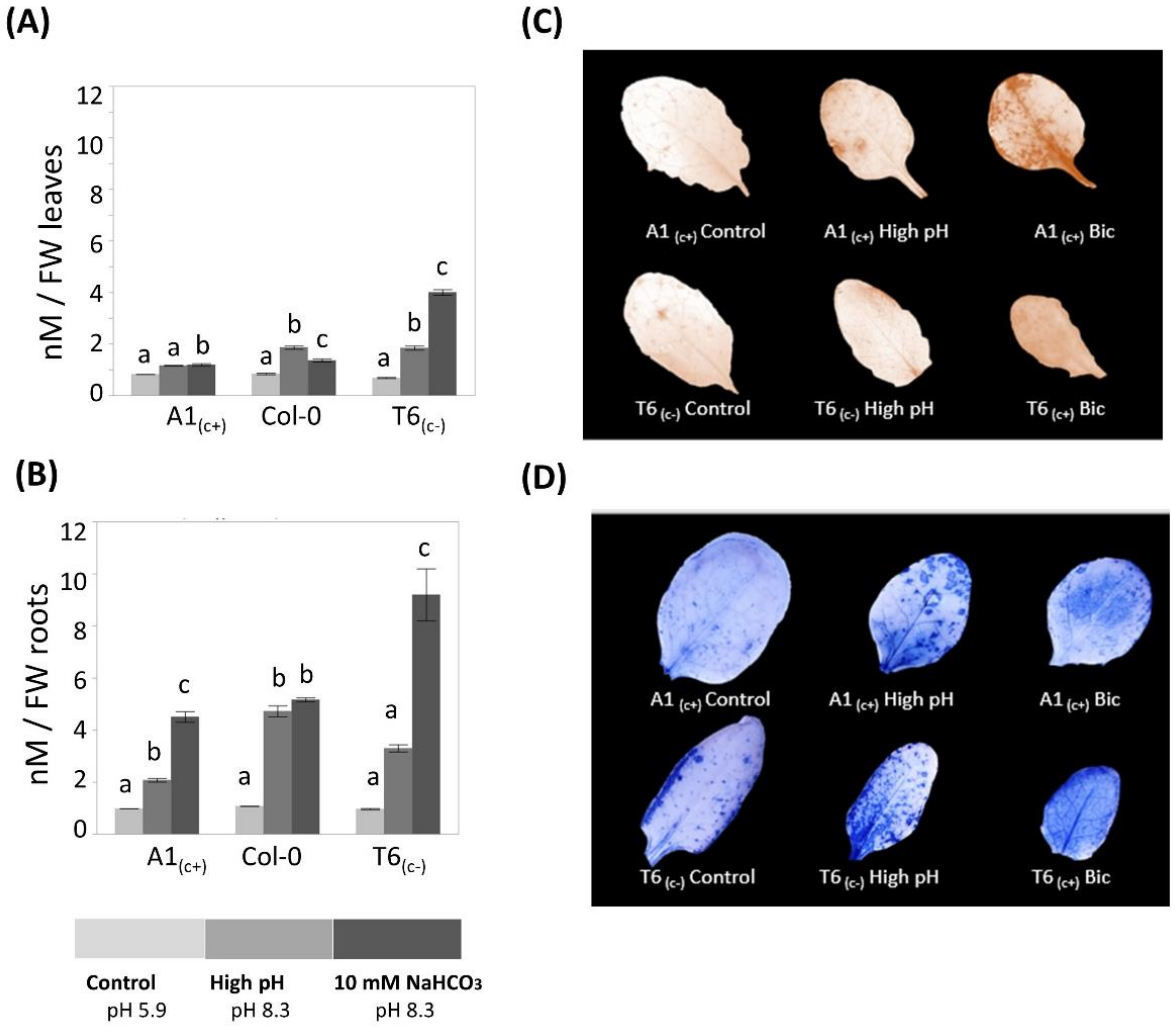


Figure 5. Redox status of 30- old day plants under different alkaline treatments for 15 days (A) MDA of leaves **(B)** MDA from roots Control (pH 5.9, light-grey bars), high pH (pH 8.3, medium grey bars), or bic (pH 8.3 with 10 mM of $NaHCO_3$, dark grey bars) in plates or hydroponic culture. Values are means ± SE; *n* = 4; letters indicate significant differences ($p < 0.05$, Tukey's honestly significant difference HSD per deme. **(C)** Peroxide leaves stain **(D)** Superoxide anion leaves stain.

4.6 Electrophysiological assays

As differences in root MDA between the tolerant and sensitive deme indicate better membrane stability as a tolerance trait, the membrane potential was measured to further characterize these differences between tolerant and sensitive demes under $NaHCO_3$ treatment. Incremental solutions from 0 to 10 mM of NaCl at pH 8.3 were used as control treatment to detect specific responses to the HCO_3^- anion. In general, $T6_{(c-)}$ had a more negative membrane potential indicating more cationic proteins (-221 mV ± 5.6) while the tolerant line had a membrane potential of around (-199 mV ± 7.8). Sensitive deme $T6_{(c-)}$ is native to coastal sites and saline soils. A possible strategy of local adaptation can be an elevated membrane potential to deal with the excess of Na^+ causing osmotic and toxic effects. Membrane depolarization under saline stress exposure is a result frequently reported in the literature [154]. However, several researchers pointed out that saline-tolerant genotypes have mechanisms to avoid Na^+ entering in plant cells [37]. In our study, a contrary response was observed. Nonetheless, an increment of the number of replicates is essential and further electrophysiology experiments are required to confirm this result.

Under NaCl at high pH treatment, membrane potential depolarized in a range of 0-30 mM while in HCO_3^- treatment a hyperpolarization was observed in both demes. However, the tolerant deme exhibited a more intense response (Figure 6A, 6B).

In aquatic plants, two contrasting strategies in response to bicarbonate have been reported (I) External carbonic anhydrase (CA) which decomposes HCO_3^- into CO_2 and water or (II) Direct HCO_3^- uptake into the cells by anionic channels [138]. Membrane depolarization might indicate the presence of external CA catalyzing the transformation of HCO_3^- into CO_2 and water. The gas may flow to cytosol producing acidification. Contrastingly, hyperpolarization can indicate HCO_3^- entrance into the cytosol by some anionic channels [138]. However, up to date no specific bicarbonate transporter in terrestrial plants has been characterized [34]. To test which strategy may operate in our *A. thaliana* plants, we used as a control treatment, NaCl + AZ (an inhibitor of external anhydrase) at pH 8.3. After that, the solution was replaced by HCO_3^-. Interestingly, no response was observed (Figure 6C). This inhibition of the hyperpolarization response to bic after AZ application suggests the possible presence of an external carbonic anhydrase. This supports the second hypothesis; however, more studies are needed like measuring cytosol pH or CO_2 root production for further confirmation.

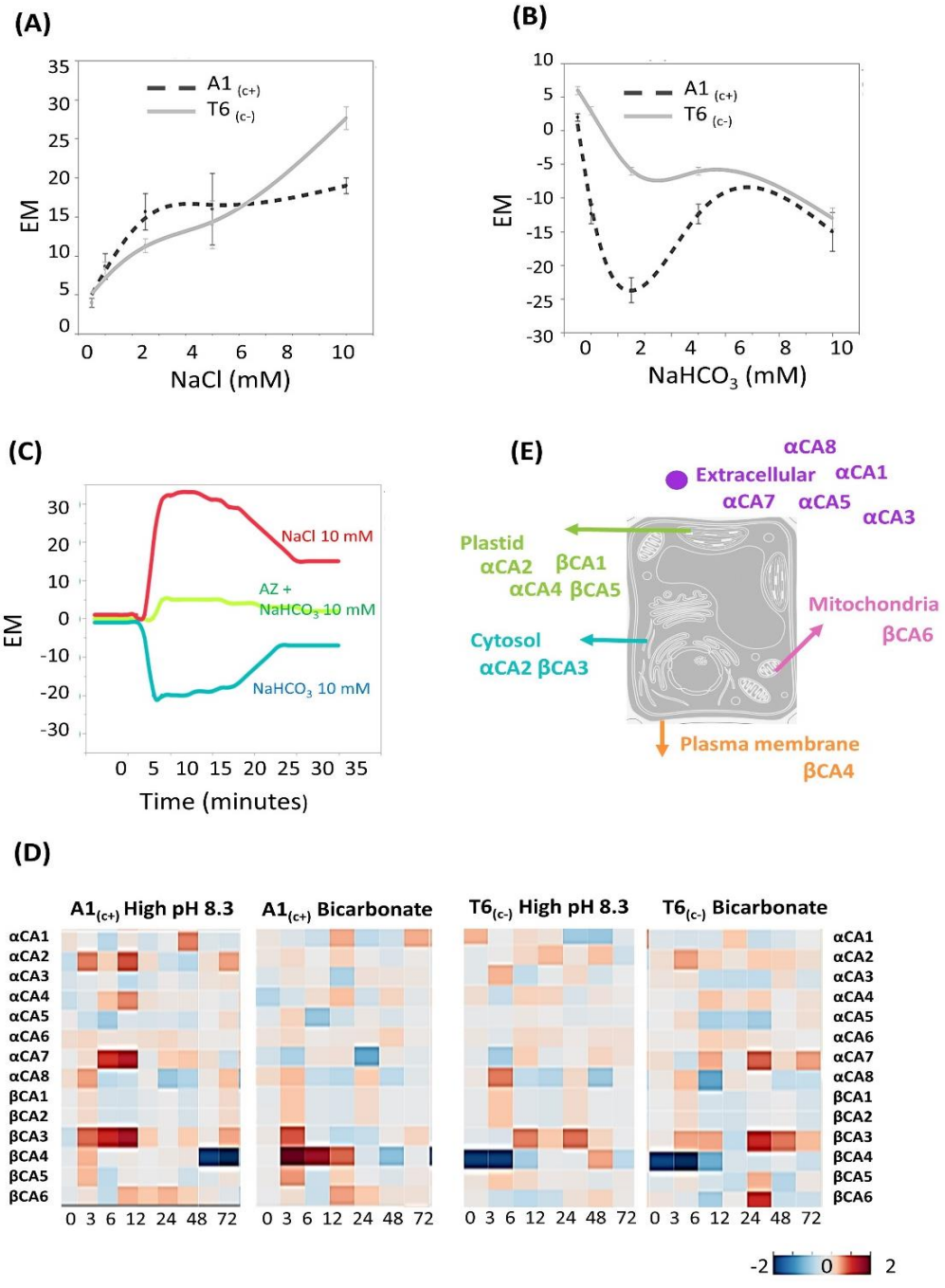


Figure 6. Electrophysiological experiments and the role of carbonic anhydrases in roots (A) Membrane potential depolarization at increasing NaCl pH 8.3 concentration. **(B)** Membrane potential hyperpolarization at increasing $NaHCO_3$ pH 8.3 concentration. **(C)** $A1_{(c+)}$ average responses in 10mM of NaCl, 10 mM $NaHCO_3$ and AZ + 10mM . All treatments were performed at pH 8.3. Mean and SE. **(D)** Relative expression heatmap plot of αCAS and βCAS in a timecourse experiment (0 to 72 hours). In each time point, data was relative to control.

To further explore a possible role for CA in bicarbonate tolerance, the expression of different CAs was followed in a time-course experiment from 0 to 72 hours (Figure 6D). Relative expression was visualized in a Heatmap and predicted gene location was checked using Biogrid. (Figure 6E). The main results are that in both alkaline treatments from 6 to 24 hours $A1_{(C+)}$ activates *βCAs* (1-6) more than $T6_{(C+)}$. Also, αCA7 was upregulated in $A1_{(C+)}$ only in high pH treatments. Under bicarbonate stress, *βCA3 and even more βCA4* were activated in $A1_{(C+)}$ while its expression was reduced in $T6_{(C-)}$. The localization of the different CAs in *A. thaliana* is shown in figure 6E. While αCA7 is located in the apoplast, *βCA3* is found in the cytosol and *βCA4* is located in the plasma membrane.

In *A. thaliana* guard cells *βCA4* has been identified to act along with the aquaporin PIP2.1 as CO_2/HCO_3^- sensor activating SLAC1 (*SLOW ANION CHANNEL-ASSOCIATED1*) anion channel and stomatal closure [155]. The opposite expression of *βCA4* in the roots of our *A .thaliana* demes differing in bicarbonate sensitivity suggests an important role for *βCA4* in the early tolerance response to bicarbonate in $A1_{(c+)}$. Further studies are needed to determine the possible interaction of βCA4 with aquaporins and SLACs in the bicarbonate tolerance mechanism in roots.

4.7 Mechanisms of iron uptake at short time

Genes related to the mechanism of iron uptake were selected to perform a time course experiment from 0 to 48 hours. The most notorious result from qPCR was the fast activation of *AHA2* and *FRO2* after the first 24 hours in $A1_{(C+)}$ (Figure 7 A,C) while $T6_{(C-)}$ activates *IRT1* (Figure 7F). Also, in sensitive demes, ferric reductase activity was decreased after the first 6 hours (Figure 7E). Selected genes show a quick response from 0 to 24 hours, as a possible adjusting mechanism while responses decayed after 48 hours. According to Lucena 2007 [137], under bicarbonate stress genes related to the mechanism of iron uptake were downregulated at 72 hours using Col.0. According to our experiments, the main peak response was performed within 0 and 48 hours in all analyzed genes and the downregulation of *AHA2* after 48 hours was observed both in the sensitive and the tolerant deme.

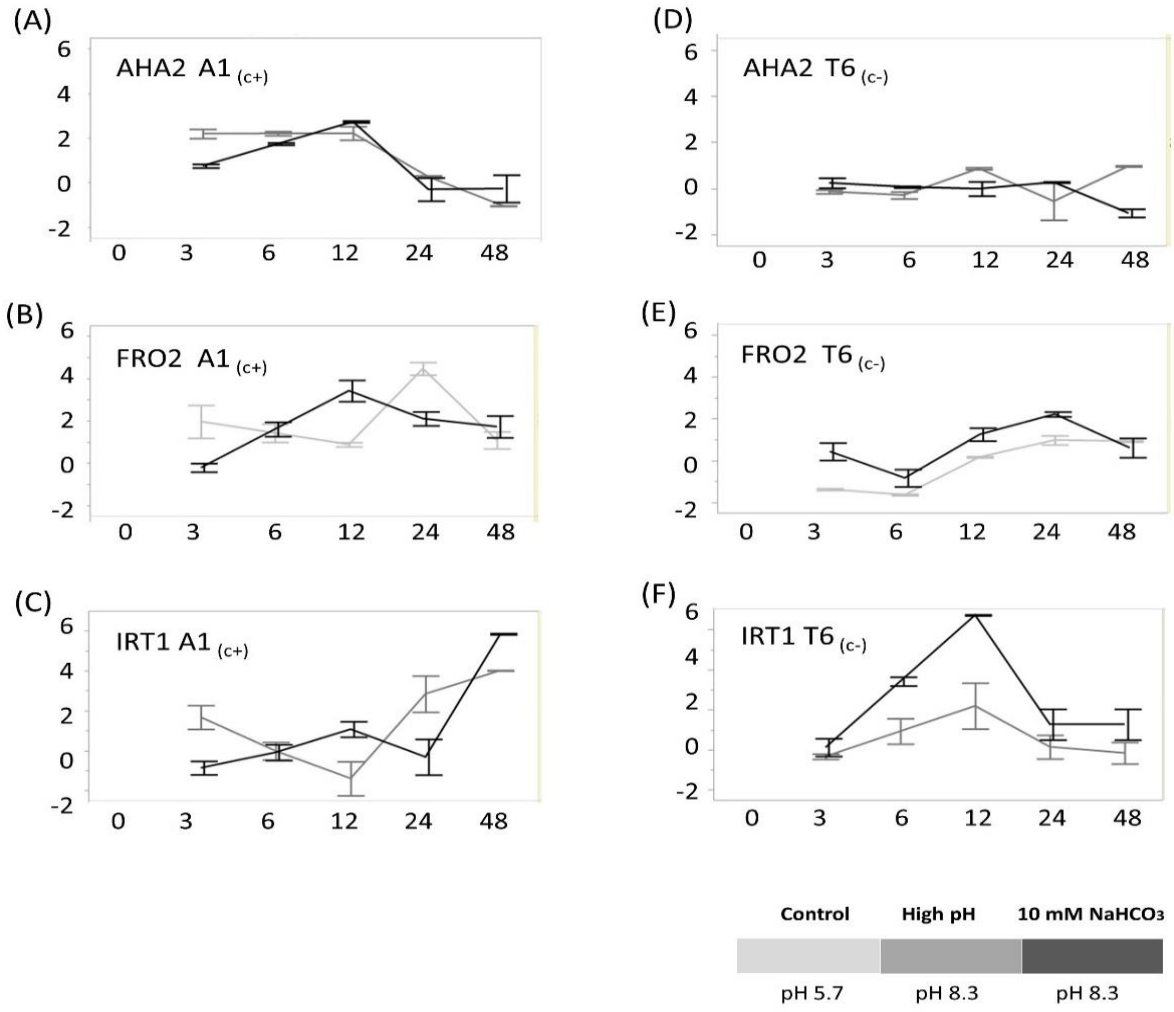


Figure 7. Time course experiment of genes related to iron uptake. Relative expression of selected genes was assessed in 15-day old roots exposed to control, high pH and bicarbonate. As a tolerant line $A1_{(C+)}$ and sensitive to calcareous $T6_{(C-)}$

5 Conclusions

The main conclusions from this chapter are:

- The concentration of 10 mM $NaHCO_3$ is a proper mild treatment that allows distinguishing between tolerant and sensitive demes in *Arabidopsis thaliana*.
- At the physiological level, plants responses to HCO_3^- differ from those caused by high pH alone. At equal pH, bicarbonate is more toxic to sensitive $T6_{(C-)}$ and Col-0 than an alkaline solution with organic buffer. Otherwise, in the tolerant $A1_{(C+)}$ the opposite was found.
- In general, the tolerant deme $A1_{(C+)}$ maintains ion homeostasis better than the sensitive plants. In the sensitive deme the nutrient profiles reveal accumulation in roots, while the tolerant deme maintains better nutrient translocation to the leaves under bicarbonate exposure.
- Higher translocation in tolerant demes and more efficient use of Fe in the plant could maintain a better photosynthetic rate and producing fewer ROS.
- Preliminary electrophysiological studies indicate a possible role of external carbonic anhydrase in bicarbonate tolerance of $A1_{(C+)}$. This is further supported by enhanced expression in $A1_{(C+)}$ of carbonic anhydrase genes located in the apoplast and the plasma membrane.
- Relative expression of genes related to mechanisms of iron uptake indicates differential strategies in both demes. $A1_{(C+)}$ activates *AHA2* and *FRO2*, while $T6_{(C-)}$ activates *IRT1* expression. Strong differences are observable across time-course indicating a quick and medium-term response.

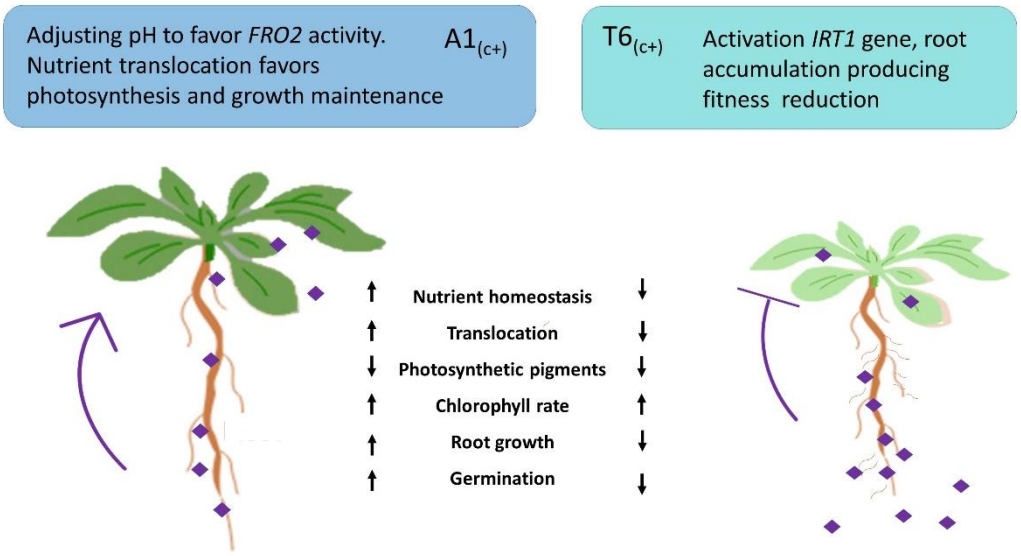


Figure 8. Schematic conclusions

Chapter 2: Transcriptomics responses from contrasted Catalan *Arabidopsis thaliana* demes under alkaline stress

1. Introduction

Several constraints affect the growth, physiology, and metabolism of crops cultivated on calcareous/alkaline soils of arid and semi-arid regions over the world. The main anions present in calcareous soils are HCO_3^- and CO_3^{2-} [15] in concentrations between 5-35 mmol L^{-1} [12] yielding pH ranges from 7.5 to 8.5. Soil carbonates act as pH buffers and play an important role in rhizosphere processes especially affecting nutrient availability to plants. The high pH surrounding the plant roots can disrupt the membrane potential and inhibit the absorption of essential ions [156]. Moreover, high pH lowers the availability of nitrogen (N); phosphorous (P); and micronutrients such as iron (Fe), zinc (Zn), and manganese (Mn), producing nutrient deficiencies in sensitive plants [157]. At the molecular level, several anion transporters and gene families such as *ALMT, NRT/POT*, and *SLAHs* are related to bicarbonate response in *Glycine max* [67] [158]. The overexpression of the anion transporters *GsBOR1* (boron efflux transporter) and *GsSLAH3* (efflux of uncharacterized inorganic or organic anions) are also involved in HCO_3^- tolerance.

Transcriptomic experiments under alkaline stress in *Tamarix hispida*, *Ziziphus acidojujuba*, and *Glycine max* identified gene families and transcription factors (TFs) involved in $NaHCO_3$ stress responses. These genes can be clustered in sensing and signal transduction pathways as calmodulin-like proteins, serine/threonine protein kinases, and cysteine-rich receptor-like protein kinases. *P2/ERF, HD-ZIP, bHLH*, *MYB, WRKY*, *NAC, C2H2, HB*, and *TIFY* are the main TFs [65][149][159]. Moreover, there is increasing evidence for both quick shoot responses and shoot to root signaling under iron deficiency or bicarbonate stress [160][161]. Proteomic studies performed in *Solanum lycopersicum* comparing saline and alkaline stress further revealed downregulated proteins accounting for metabolism, energy conversion, and novel upregulated proteins involved in signaling or transport [41].

Nonetheless, the current understanding of plant response to bicarbonate stress is limited. Most studies have been performed on roots of crop species such as *Glycine max* or *Oryza sativa* [162][55]. Less information is available regarding the molecular-genetic mechanisms underlying naturally selected tolerance and fast bicarbonate responses at the gene expression level in shoots and roots. The large genotypic and phenotypic diversity of natural populations of the genetic model plant *Arabidopsis thaliana* provides an ideal experimental scenario for exploring such mechanisms. In previous studies, we identified two contrasting demes of *A. thaliana* with differential tolerance

to moderate soil alkalinity: the sensitive deme $T6_{(c-)}$ and the tolerant $A1_{(c+)}$. The better performance of $A1_{(c+)}$ under iron deficiency conditions was mainly attributed to enhanced root release of coumarin-type phenolics [19]

2. Aims

- To detect differential expressed genes (DGEs) from moderate tolerant $A1_{(C+)}$ and sensitive $T6_{(c-)}$ in two alkaline treatments: (I) Bic ($NaHCO_3$ pH 8.3) (II) High pH (pH 8.3 achieved by organic buffer) performed in hydroponics at 3 h and 48h.
- To cluster DGEs into functional analysis, gene Ontology biological processes, molecular function and cellular locations) and KEEG Pathway terms.
- To predict putative relations among DGEs in each treatment using protein-protein interaction analysis from genes specific from bicarbonate response.

3. Materials and Methods

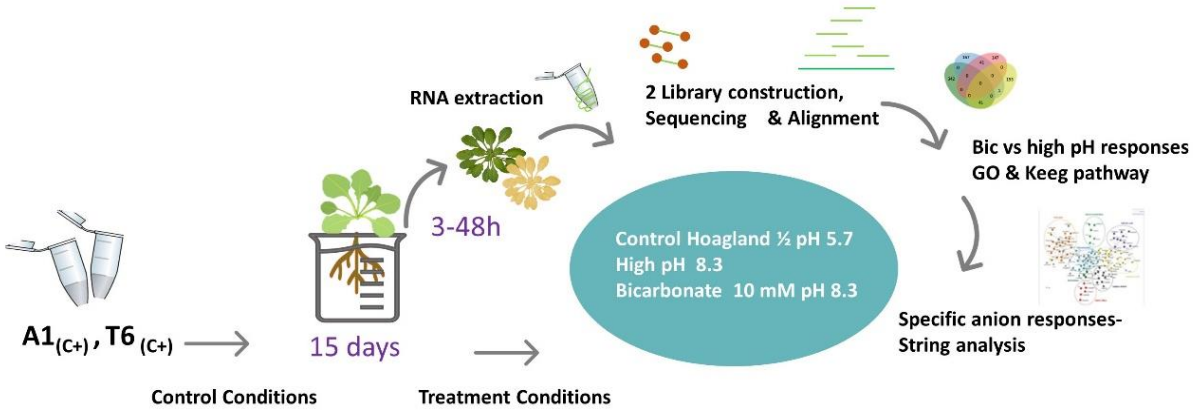


Figure 0. Transcriptomic experimental design scheme

3.1 Plant Material

In previous studies, natural populations of *Arabidopsis thaliana* from Catalonia [86] were tested in a multi-year small-scale common garden under carbonated conditions [19]. Seeds of extreme phenotype lines $A1_{(c+)}$, a moderately alkaline-tolerant deme, and $T6_{(c-)}$, an alkaline-sensitive deme, were collected from the last reciprocal transplant experiment performed in 2015 and stored under fresh (4 °C) and dry conditions until the beginning of the experiments.

3.2 Growth Conditions

3.2.1 Seed sterilization: Selected 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 MΩ (milli-Q) water.

3.2.2 Hydroponic culture: Seeds were sown in 0.2 mL tubes containing 0.6% agar prepared in nutrient solution ½ Hoagland (pH 5.9). Seeds were kept at 4 °C for 7 days in the dark to synchronize germination. Tubes containing seed were placed in the growth chamber (12 h light/12 h dark, 150 µmol $cm^{-2}{\cdot}s^{-1}$, 40% humidity and 25 °C). After root emergence, the bottom of the tubes containing seedlings was cut off and the tubes were placed in 150 mL hydroponic containers with aerated nutrient solution ½ Hoagland (pH 5.9). When 15 days old, the seedlings were separated into different sets and the following treatments were applied: control (½ Hoagland solution at pH 5.9), high pH (½ Hoagland solution at pH 8.3), and bicarbonate (½ Hoagland solution at pH 8.3 with 10 mM $NaHCO_3$). Solutions were buffered with different proportions of MES (2-(N-morpholino) ethanesulfonic acid hydrate, 4-morpholineethanesulfonic acid) and BTP (1,3-Bis[tris (hydroxymethyl) methylamino] propane), depending on final pH in continuous aeration.

3.3 RNA isolation and quality control

Leaf and root material from plants cultivated under hydroponic conditions was recollected 3 and 48 h after starting the treatments. Twelve plants per line and treatment were pooled to perform 3 biological replicates. Leaves were immersed in liquid nitrogen, homogenized to a fine powder, and stored at –80 °C. The total RNA of 100 mg of leaf powder for each biological replicate was extracted using the Maxwell® plant RNA kit (Promega Corporation, WI, USA) following the manufacturer's instructions. For each sample, approximately 2 µg of total RNA was used for quality evaluation. Total RNA was quantified using a Qubit 2.0 Q32866 (Life Technologies, Carlsbad, CA, USA) to prepare the complementary deoxyribonucleic acid (cDNA) library.

3.4 Microarray technology

After quality control, GeneChip® 3' IVT Express Kit (Affymetrix) was used to tag, hybridized and cleaned total RNA. The library was composed of 24 samples producing 2 biological replicates in each deme, treatment and timepoint After library production, microarray expression analysis was performed by Affymetrix GeneChip™ Arabidopsis Genome ATH1. The function gcrma from was used to adjust background intensities, removing optical noise and non-specific binding from Affymetrix array data.

3.5 RNA-seq technology: Library Construction and Sequencing

The library was composed of 72 samples, collected from $A1_{(c+)}$ and $T6_{(c-)}$ plants exposed for 3 h (quick response) or 48 h (medium-term response) to the different treatments (2 biological replicates). Library preparation was performed using Novogene protocol. The cDNA library was sequenced at the Illumina NovaSeq 6000 using standard procedures to generate paired-end reads of 150 bp and number of analyzed read 30-47 million pairs (Novogene, Sacramento, CA, USA).

Clean reads were obtained by removing reads containing adapters, reads containing poly-N, and low-quality reads from raw data using Trim Galore. Simultaneously, Q20, Q30, and GC content of the clean data were calculated. After filtering, clean reads on average had 35.7 million reads, which occupied 98% of the total reads of all libraries. In all samples, the Q30 value was over 89%, and the GC content was 44% (Supplementary F1). All downstream analyses were based on clean data with high-quality reads. Reference genome and gene model annotation files were downloaded from the genome website directly. Index of the reference genome was built using Bowtie v2.2.3 (John Hopkins University, Baltimore, MD, USA) and paired-end clean reads were aligned to the reference genome using TopHat v2.0.12 (John Hopkins University, Baltimore, MD, USA).

3.6 Differential expression analysis

3.6.1 RNA-seq: Differential expression analysis of samples was performed using a model based on the negative binomial distribution[102] and the DESeq R package (1.18.0) (www.Bioconductor.org). The resulting *p*-values were adjusted using Benjamini and Hochberg's approach [163] for controlling the false discovery rate (FDR). To perform the final list of DEGs, we filtered genes by adjusted *p*-value < 0.05 and LFC > 1 and LFC < −1. Raw reads and normalized gene expression of RNA-seq analysis were deposited in the Gene Expression Omnibus (GEO) database

and the National Center for Biotechnology Information's (NCBI) Sequence Read Archive (GSE164502).

3.6.1 Microarray: The R package limma was used to test the expression data in search of differentially expressed genes. A model matrix was defined by specifying which sample belongs to each treatment. Using *lmFit* and *contrasts.fit* function, fold changes and confidence statistics associated with the comparisons of interest were obtained. The p-value was adjusted for multiple testing adj. p-value, using Benjamini and Hochberg's method to control the false discovery [163]. The R library *ath1121501.db* was used to include names and descriptions of the genes associated with the microarray's features probed.

3.7 Gene Ontology (GO), KEGG Pathway, and Functional Protein Association Network Analysis (STRING) of DEGs.

Gene Ontology (GO) enrichment analysis of DEGs was implemented by AgriGO V2 (GO Analysis Toolkit and Database for Agricultural Community (cau.edu.cn) [107]. Significant GO terms ($p < 0.05$) were classified into 3 categories: biological function, molecular process, and cellular component. KEGG pathway and STRING version 11.0 were used to understand the high-level function and gene interaction network of differential expressed genes [108].

3.8 Relative Expression validation

To produce cDNAs from RNAs, we used the iScriptTM 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 MiniTM, Bio-rad, Hercules, CA, USA) at 5 min 25 °C, 30 s 42 °C, and 5 s 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 10'' 95 °C followed by annealing and extension 30'' 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. Extremely expressed genes across lines at 3 h were chosen as candidate genes to validate transcriptomic experiments using qPCR. Primers from selected genes were designed using NCBI primer blast tool [146] (Biolegio, Nijmegen, the Netherlands). The

sequences of primers used are detailed in Dataset S0. The expression of target genes was normalized to the expression level of the Actin and Tubulin genes of *A. thaliana* [147]. The relative expression (RE) of each gene was calculated in comparison to the control treatments (pH 5.9) at each time point. The expression of the target gene relative to the expression of the reference gene was calculated using the $2^{-\Delta\Delta Ct}$ method [148].

4. Results & Discussion

4.1. Shoot RNA-Seq and root microarray data analysis

RNA-seq analysis in leaves and microarray in roots were performed to characterize, at the gene expression level, the mechanisms underlying the differential response to bicarbonate of the two naturally selected accessions. Early responses were assessed by using samples after 3 and 48 h exposure when the plants still did not exhibit any foliar symptoms of the stress treatments.

After sequencing and bioinformatic quality control (Supplementary F1, annex table 1) annotated genes were filtered by log fold change (LFC) > 1 and LFC <-1 and adjusted *p*-value < 0.05. We performed pairwise comparation to understand the differences between $A1_{(c+)}$-tolerant and $T6_{(c-)}$-sensitive lines in response to different treatments (pH 8.3 *vs* pH 5.9 and bic *vs* control pH 5.9) at two-time points (3 h and 48 h). Eight genes with highly different expression levels in both demes after 3 h were selected. The expression levels of the selected genes were quantified using qPCR. Results obtained from both techniques were compared and we obtain a high correlation between the expression level of the genes tested (Supplementary F1, annex 2). After filtering, a total of 6163 differentially expressed genes (DEGs) were identified, considering accession, time, and treatment in leaves while 4788 DGEs were found in roots (Figure 1). In general, the bicarbonate treatment caused a higher number of DEGs than the high pH treatment (5202 *vs* 2420 genes in leaves and 3226 genes *vs* 1351 genes in roots). This indicates that bicarbonate stress involves more complex processes than simply the specific responses to alkaline pH. Similar results were found in transcriptomics by other authors using 125 mM $NaHCO_3$ during 12 *h in Gossypium hirsutum* seedlings and NaOH at pH 8.3 as a control treatment [164] or ten-day-old *Arabidopsis thaliana* seedlings exposed to 3 mM $NaHCO_3$ and 4mM NaOAc [63].

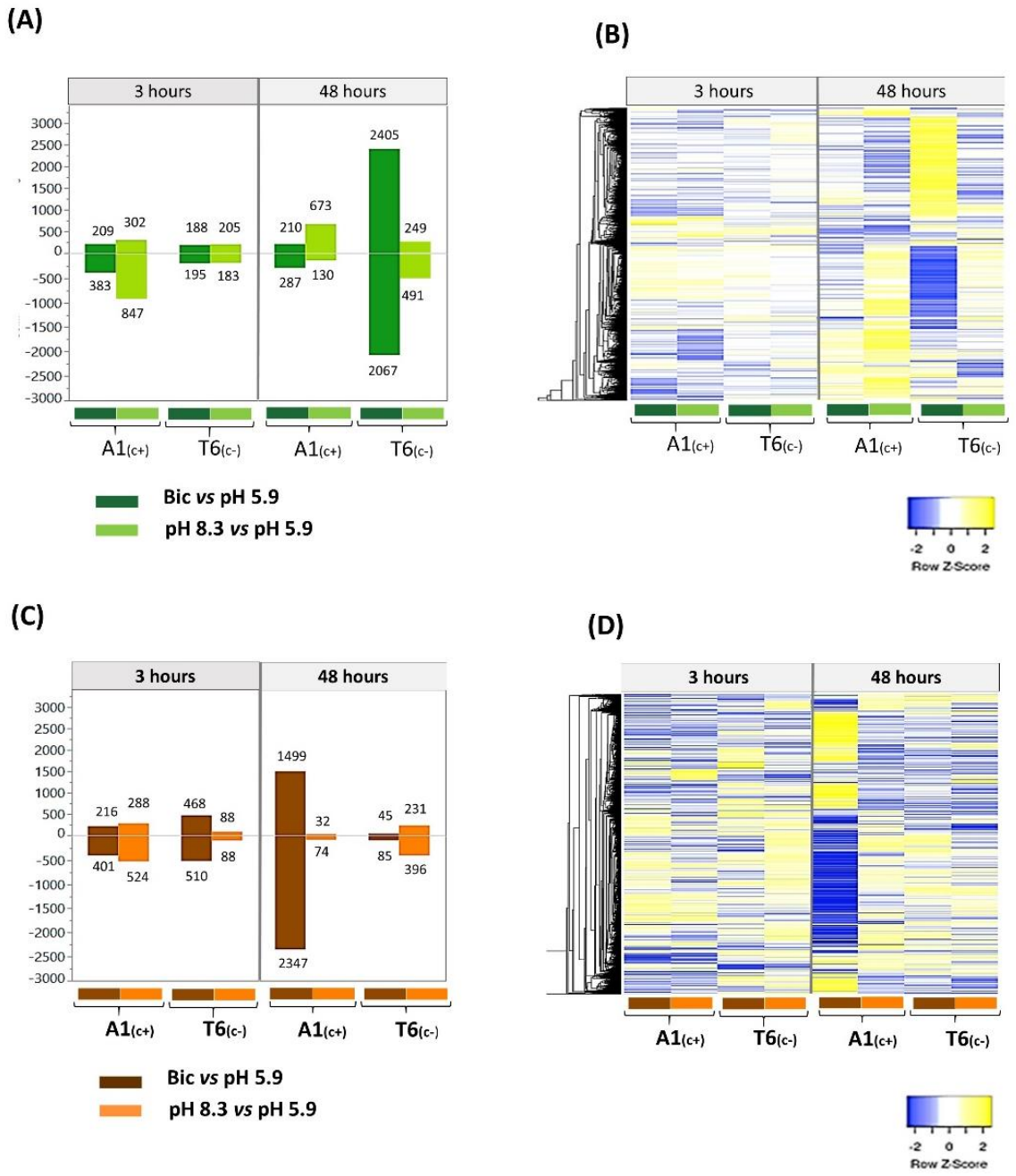


Figure 1. Compared transcriptomic profiles under different alkalinity treatments at two-time points. Differentially expressed genes (DEGs) visualized in bar plot **(A)** in leaves and **(C)** in roots and heatmap profiles in (**B**) leaves and **(D)** roots. DGEs were obtained from pairwise comparison pH 8.3 *vs*. pH 5.9 and bic *vs*. pH 5.9 in $A1_{(c+)}$ and $T6_{(c-)}$ demes. DEGs were filtered at log fold change (LFC) > 1, LFC < −1, and adjusted *p*-value < 0.05. Yellow areas indicate upregulated genes and blue areas indicate downregulated genes.

Here we found that the exposure for 3h to bic induced differential expression of a higher number of genes in $A1_{(c+)}$ than in $T6_{(c-)}$ in leaves. The highest number of DEGs in leaves was observed in the carbonate-sensitive accession $T6_{(c-)}$ when exposed for 48 h to bic (Figure 1A). This situation is opposite in roots; moderate tolerant $A1_{(c+)}$ activates fewer genes than $T6_{(c-)}$ after 3 hours while a huge response is observed after 48 hours under bicarbonate in roots of $A1_{(c+)}$ (Figure 1C). Gene expression profiles are compared in the heatmap plots shown in Figures 1B and 1D. Many of these DEGs that were either upregulated or downregulated by bic were not, or were only slightly, affected by pH 8.3. These differences in DGE number can indicate contrasted deme strategies toward bicarbonate stress. The Veen diagrams are shown in Supplementary F1, annex 3.

Main transcriptomic responses are happening in roots in the tolerant line while in the sensitive line more DGEs are found in leaves after 48h under bicarbonate stress. Our results indicate a possible event of root-shoot plant communication. Nonetheless, this conclusion must be taken with care because two different methods were used to analyze transcriptomics in roots and shoots. Although several articles have found correlations of up to 70% between both techniques, especially in high abundance genes [165], little information is available on this correlation under alkaline conditions [47]. For this reason, we are not combining both techniques and in further analysis, “leaves” and “roots” will be treated as separated variables.

4.2. Gene Ontology (GO) under bicarbonate stress

The general overview of differential gene expression comparing bic versus pH 8.3 reveals that a greater number of genes and pathways were specifically implicated in the bic responses. Moreover, $A1_{(c+)}$ and $T6_{(c-)}$ showed contrasted reactions toward bicarbonate stress. For this reason, we further focused our analysis on the DEGs and pathways found in the plants exposed to the bic treatment. DEGs from each time point, treatment, and deme were mapped into the KEGG pathway and Gene Ontology (GO) terms to analyze their biological functions, molecular functions, and cell localization (Figure 2). GO from bic and pH treatment can be found in Supplementary F2 in a table format.

The highest log fold discovery rate (FDR) and number of DEGs corresponded to 48 h bic exposure of $T6_{(C-)}$ leaves. Most up-regulated genes were involved in response to stimulus, catalytic activity, transport and transcription while down-regulated genes were related to electron carrier

activity. (Figure 2B) In leaves of $A1_{(c+)}$, genes of similar pathways were differentially up-and downregulated. These genes were mainly related to response to stimulus and catalytic activity (Figure 2B). In roots of $A1_{(c+)}$ main pathways activated after 48 h were related to activation of biological processes and metabolism located in the extracellular region. Also, enzymatic regulatory activity, transport and transcription factor. (Figure 2D) were altered. In $T6_{(c-)}$ only up and down modifications in catalytic activity were found.

After 3 h exposure to bic the number of DGEs classified in each category was almost 10-fold lower in comparison to the 48 h treatment (110 to 1120). The short time response in leaves of $A1_{(c+)}$ consisted in up and down modifications in the categories of response to stimulus and catalytic activity, while more changes were observed in $T6_{(c-)}$ (Figure 2A). In roots, both accessions shared similar trends involving response to stimulus and electron carrier activity (Figure 2C). The main categories found in the GO analysis have previously been found by other authors under alkaline stress [47][69][166] confirming their role in plant response to alkaline stress.

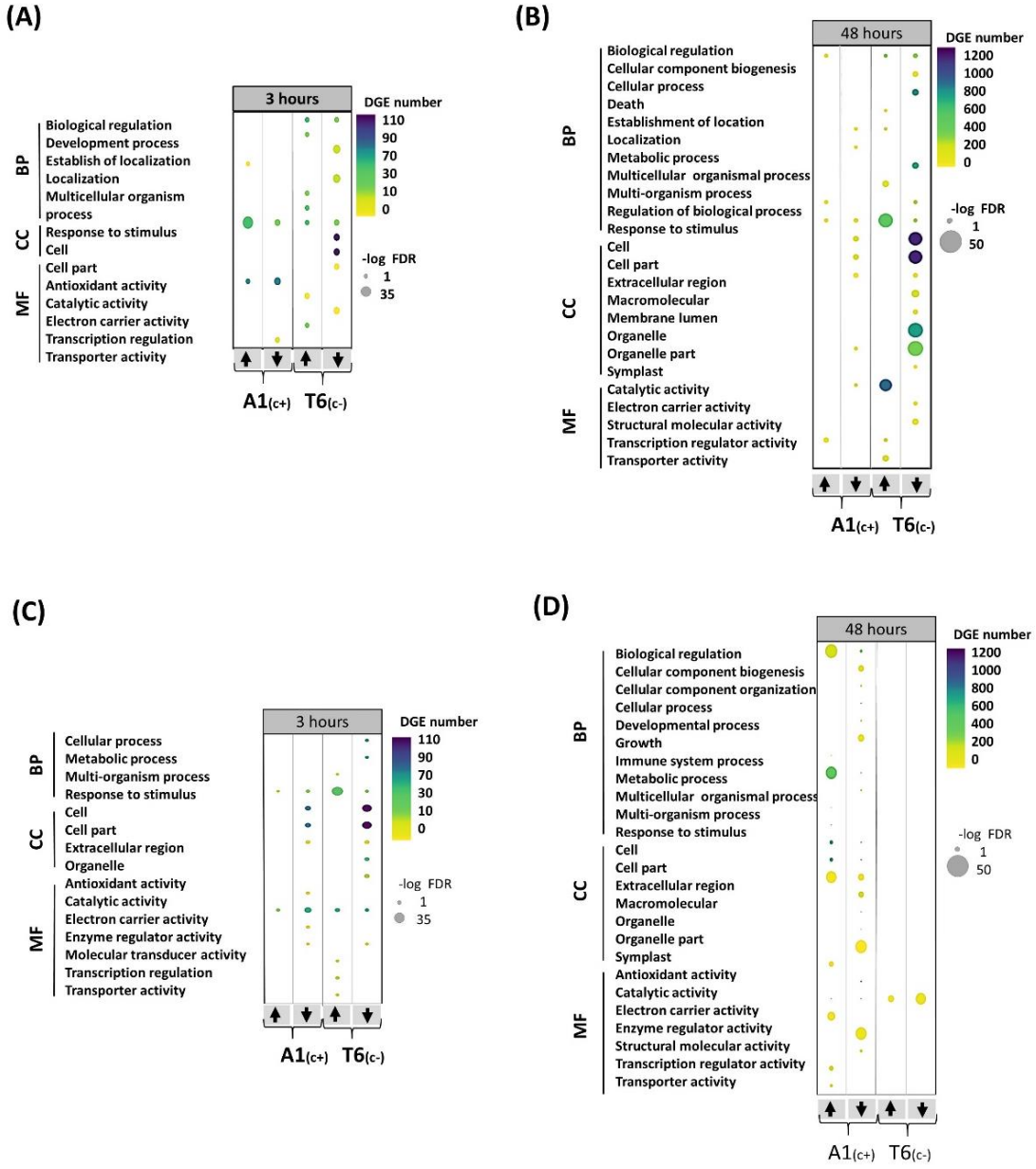


Figure 2. Gene Ontology (GO) of bic *vs.* pH 5.9 treatments DEGs at two-time points. Bubble plots indicating significant GO analysis of differentially expressed genes in bic *vs*. pH 5.9 comparison between $A1_{(c+)}$ and $T6_{(c-)}$. **(A)** 3h leaves **(B)** 48h leaves **(C)** 3h roots **(D)** 48 h roots**.** GO were filtered to adjusted *p*-value < 0.05. Scale colors indicate numbers of DEGs while bubble size indicates -log of the adjusted *p*-value. GO terms were separated into biological function, cellular component, and molecular function. Arrows indicate up or downregulated genes. BP, biological process; CC, cellular component; MF, molecular function.

4.3. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways under bicarbonate stress

After 3 h exposure in $T6_{(c-)}$, genes related to fatty acid elongation and cutin, suberin, wax biosynthesis and sugar metabolism were distinctive in the sensitive deme. Contrastingly, in the

tolerant $A1_{(c+)}$, genes related to 2-oxocarboxylic acid, alpha-linoleic acid, flavonoid biosynthesis, and glyoxylate and dicarboxylate metabolism were differentially regulated after 3 h exposure to bic. (Figure 3A, 3C). After 48 h, genes related to photosynthesis, plant hormone nitrogen metabolism, and carbohydrate metabolism were mostly affected (Figure 3B, 3D). KEEG from bic and pH treatment can be found in Supplementary F3 in a table format.

Most notably, in the tolerant $A1_{(c+)}$ leaves, a 30-fold enrichment in the upregulation of genes involved in glucosinolate biosynthesis was observed. Enrichments between 5- to 15-fold in upregulated genes related to 2-oxocarboxylic acids; alpha-linolenic acids; fatty acid elongation; and valine, leucine and isoleucine were found (Figure 3A). This last category was also present in roots after 3 h (Figure 3C). Downregulation in $A1_{(c+)}$ leaves after 3h mostly affected genes related to base excision repair (Figure 3A) while in roots the changes were related to the biosynthesis of secondary metabolites, pentose interconversion and phenylpropanoid pathway (Figure 3C). In the sensitive $T6_{(c-)}$, the most conspicuous enrichment after 3h concerned the upregulation of genes related to fatty acid elongation, fatty acid degradation, and galactose metabolism (Figure 3A). Both upregulation and downregulation of genes related to secondary metabolism were observed. Downregulation also affected genes related to phenylpropanoid biosynthesis and plant hormone signal transduction (Figure 3A). In roots of the sensitive $T6_{(c-)}$ downregulated genes were related to carbon fixation and photosynthesis while activated pathways were involved in sugar metabolism and plant pathogen relations (Figure 3C).

Categories from our analysis are shared by other transcriptomics experiments in other plant species in monocotyledons and dicotyledonous in experiments performed within the range from 10 to 75 mM $NaHCO_3$ and with pH ranges from 7.5 to 9.5within a time frame between 1 to 72 hours [47][66], [69]. Also, results from proteomic experiments performed in *A. thaliana* cells exposed to 3 mM of $NaHCO_3$ point to these categories [167]. In *Solanum lycopersicum* [41] and *Triticum aestivum* [70] comparisons between saline and alkaline stress responses were related to carbohydrate metabolism, energy conversion, nitrogen metabolism, transporters and ROS scavengers.

In our study, a huge number of DEGs were detected in leaves of $T6_{(c-)}$ after 48 h exposure to bic (Figure 3B), while in $A1_{(c+)}$ most changes were detected in roots. This activation of a large number of DGEs in the leaves of $T6_{(c-)}$ indicates that this sensitive deme suffered from severe stress, leading to multiple secondary stress responses affecting almost all cellular and metabolic processes, even

before visible foliar symptoms were detectable. As our aim was to detect primary stress responses, our further analytical efforts were focused on the differential responses between demes after only 3 h of exposure to bic.

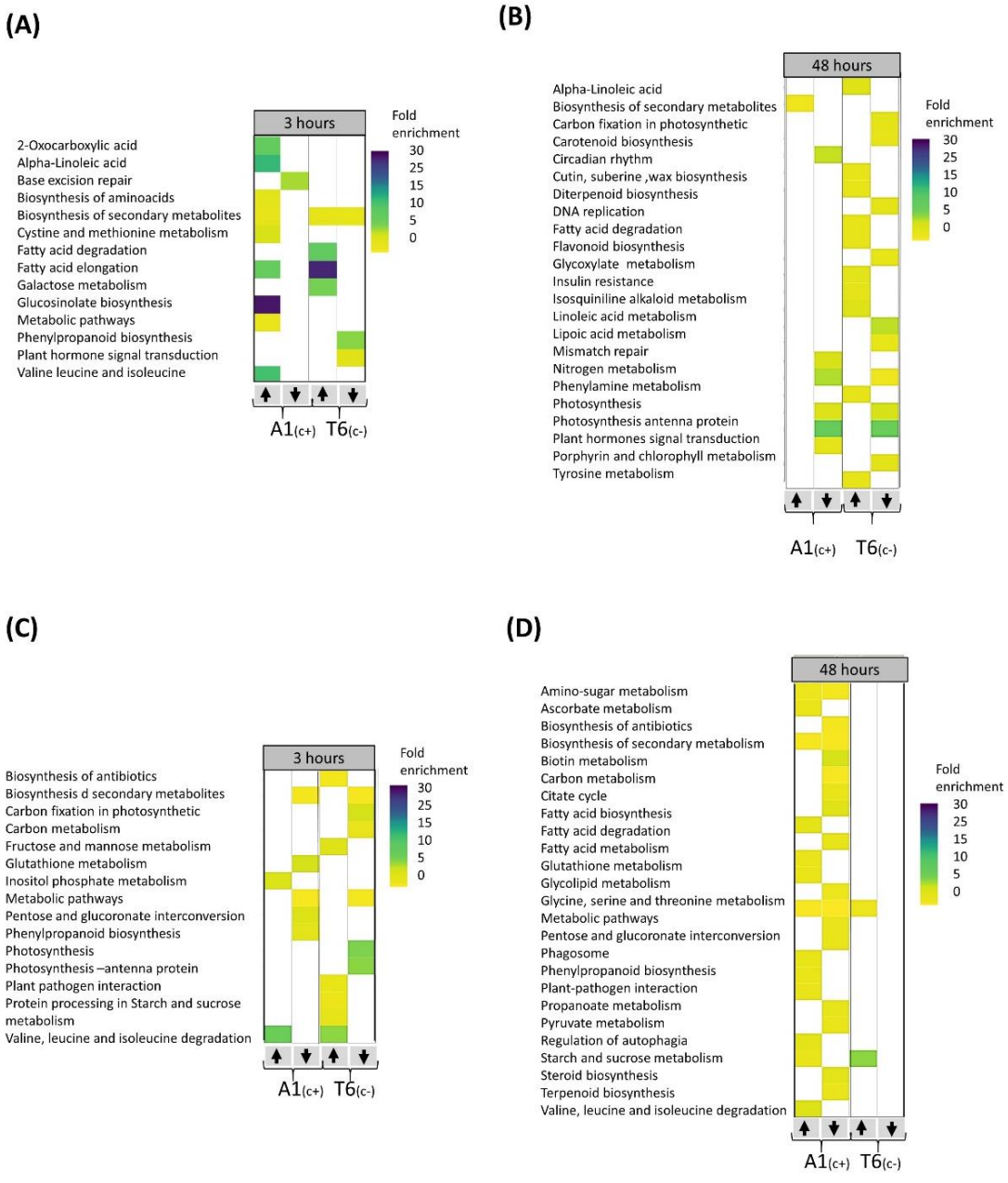


Figure 3. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway of bic vs. pH 5.9 treatments DEGs at two-time points. Heatmaps of KEGG pathway analysis from DEGs in bic 8.3 vs. pH 5.9 comparison between $A1_{(c+)}$ and $T6_{(c-)}$ **(A)** at 3 h leaves **(B)** 48 h leaves **(C)** at 3 h roots **(D)** 48 h roots. KEGG pathway terms were filtered by *p*-value < 0.05. Scale colors indicate pathway fold enrichment.

4.4 Protein-Protein Interaction Network Functional Enrichment Analysis (STRING) with specific bic-induced genes

The analysis of the gene ontology and Keeg pathways reveals different deme strategies at 48 hours, impelling the possibility of differences in signal perceiving and transduction in the short term. To further explore these differential mechanisms, we performed protein-protein interaction network functional enrichment analysis with the STRING database using specific bicarbonate genes for each deme at 3 hours (Figures 4 and 5). After 3 h exposure to bic, 273 genes were differentially expressed in $A1_{(c+)}$ and 123 genes in $T6_{(c-)}$. In both demes, part of the genes was not linked to others due to a lack of information on these protein interactions. Nonetheless, we could observe clear differences between the tolerant $A1_{(c+)}$ (Figure 4) and the sensitive $T6_{(c-)}$ (Figure 5).

STRING analysis A1 $_{(c+)}$ tolerant line

In $A1_{(c+)}$ leaves, many of the DEGs could be grouped into seven interconnected, functional categories related to salicylic acid (SA), jasmonic acid (JA), glutathion (GS), cell cycle, carbohydrate metabolism, sulfur deficit, antioxidants, and Multidrug and Toxic Compound Extrusion (MATE) efflux (Figure 4A). In roots, genes were related to response to stimulus, glucosinolates, cell wall modification, glycerophospholipids, auxin and alternative splicing and epigenetic modifications (Figure 4B).

In the tolerant $A1_{(c+)}$, bic enhanced SA, as shown by the upregulation of *SARD1* [168] and several SA-induced genes such as *WAK1* and *At2g25510*. Salicylic acid, in turn, induced *WRKY70*, a key regulator of SA-induced genes and a repressor of JA-responsive genes [169]. In our tolerant plants, bic exposure caused downregulation of *ORA59*, which is essential for the integration of JA/ethylene transduction pathways. *ORA59* is required for the expression of defensin *PDF 1.2*, a typical JA signaling gene [170]. Contrastingly, *VSP2*, another JA-responsive marker gene, as well as *AOS*, *AOC1,* and *OPR3* involved in JA biosynthesis, were upregulated (Figure 4A).

STRING connected AOS and OPR3 to GGP1, a γ-glutamyl peptidase that catalyzes the hydrolysis of the γ-glutamyl residue of GSH-conjugated glucosinolates. GSH is the sulfur donor for GS biosynthesis and GGP1 is a key enzyme in this process [171]. Upregulation of *BCAT4*, *FMOGS-OX1*, *AKHSDH1*, *IMD1*, and *GSTU20* is in line with a bic-induced enhancement of aliphatic GS. There is a close connection between mineral nutrient supply and GS biosynthesis [172]. Sulfur deficit

specifically reduces the production of aliphatic GS. Here, we observed an upregulation of *SDI1* (*Sulfur Deficit Induced 1*) and *LSU2* (*Response to Low Sulfur*). *SDI1* has been reported to promote the release of sulfur from internal storage sites [173]. *LSU2* is upregulated not only under S-deficiency, but also by Fe deficiency, high pH, and Cu toxicity [174]. Interestingly, *BGLU28* was downregulated. This myrosinase gene usually is upregulated under sulfur deficiency, while *bglu28* mutants display high GS levels [175]. Accordingly, our data indicate that in the tolerant deme $A1_{(c+)}$ bic does not directly cause S deficiency but specifically induces GS production and, consequently, the upregulation of S-deficiency-related genes to cope with the higher S-demand. Whether an enhanced sulfate uptake may antagonize HCO_3^- uptake in the tolerant $A1_{(c+)}$ deserves further analysis.

STRING connected GS genes to cell cycle and DNA-repair related genes by *AK3* and *WEE1* (Figure 4A). *AK3* codes for an aspartate kinase catalyzing the first step in aspartate-derived amino acids [176]. WEE1 is a kinase involved in cell cycle inhibition related to DNA damage and repair [177]. Surprisingly, exposure to bic downregulated many genes involved in DNA repair, such as *XR1*, *BUB32*, *BRAC1*, and *MND1*. DNA damage repair is usually upregulated in plants exposed to different abiotic and biotic stresses [178]. Downregulation of *WEE1* together with enhancement of *FIB* coding for tryptophan aminotransferase, a key enzyme in auxin biosynthesis [179], supports the view that leaf cell growth was activated after 3 h exposure to bic. These results further indicate that in the tolerant $A1_{(c+)}$ adaptive shoot growth events are activated in response to bic. However, RNA-seq only provides a snapshot of gene expression at a given time point; here, 3 h. This growth promotion may be a consequence of an earlier cell cycle arrest and upregulation of DNA repair in response to stress, followed by a downregulation after successful correction. To prove this attractive hypothesis, one must perform an expression analysis after a shorter exposure time. In addition, STRING connected GS biosynthesis to carbohydrate metabolism via adenosine 5'-phosphosulfate kinase (APK) and *SEX*1, an α-glucan water dikinase required for starch degradation. Enhanced starch degradation is also supported by the upregulation of *BAM6* coding for ß-amylase, while *BMY3* coding for α-amylase was downregulated. Glucose has been identified as a positive regulator of GS biosynthesis [180]. Furthermore, GS biosynthesis was related to organic carbon from 2-oxocarboxylic acid metabolism via *AK3*.

DTX/MATE efflux carrier expression was downregulated by bic exposure. STRING connected downregulated *DTX1* and *DTX3* (*At2g04050*) to upregulated *DHAR1* (deshydroxyascorbate reductase) by means of the glucosyltransferase gene *UGT74E2*. Ascorbic acid not only plays a key role in the protection against oxidative stress but also improves internal Fe availability[181]. The functions of DTX efflux carriers are still poorly established. *DTX1* has been involved in cadmium tolerance [182]. *DTX3* may facilitate membrane transport of xenobiotics; both DTX3 and DTX4 (*At2g04070*) seem to be efflux proteins with broad substrate specificity [183]. Downregulation of these DTXs could be related to the reduced efflux of some organic compounds acting as a strong ligand for Fe, thus facilitating Fe availability.

We further used bic-related genes that were exclusively modified in $A1_{(c+)}$ roots as input to STRING analysis Figure 4B). These DEGs can be grouped into functional categories related to response to stimulus, glutathione, cell wall modification, alternative splicing and DNA modification (Figure 4B). Only, response to stimulus, glycerophospholipid (included in membrane modification), glutathione and sulfur were statistically significant (Supplementary F4). Despite the fact that bic modified different genes in roots and leaves, similar pathways seem to be involved in bic specific tolerance in both plant organs.

The ethylene response factor (ERF) family in *Arabidopsis thaliana* comprises 122 members in 12 groups, yet the biological functions of the majority remain unknown. Downregulation was observed in root ERF5. Both, ERF5 and ERF6 may act as positive regulators of JA-mediated defense and potentially overlap in their function [184] while ERF6 also are found in adaptative growth under water scarcity [185]. The Arabidopsis genome codes for 53 GSTs, divided into seven subclasses which have catalytic activity, and the main functions are related to detoxification of electrophilic xenobiotics and peroxides. Unexpectedly, both GSTU1 and GSTU7 were downregulated. These genes encode glutathione transferase belonging to the tau class of GSTs. Changes in external pH caused large effects on genes involved in cell wall modification [186]. In $A1_{(c+)}$ cell wall modifications were related to main family genes for xyloglucans, galactans and root hair function.

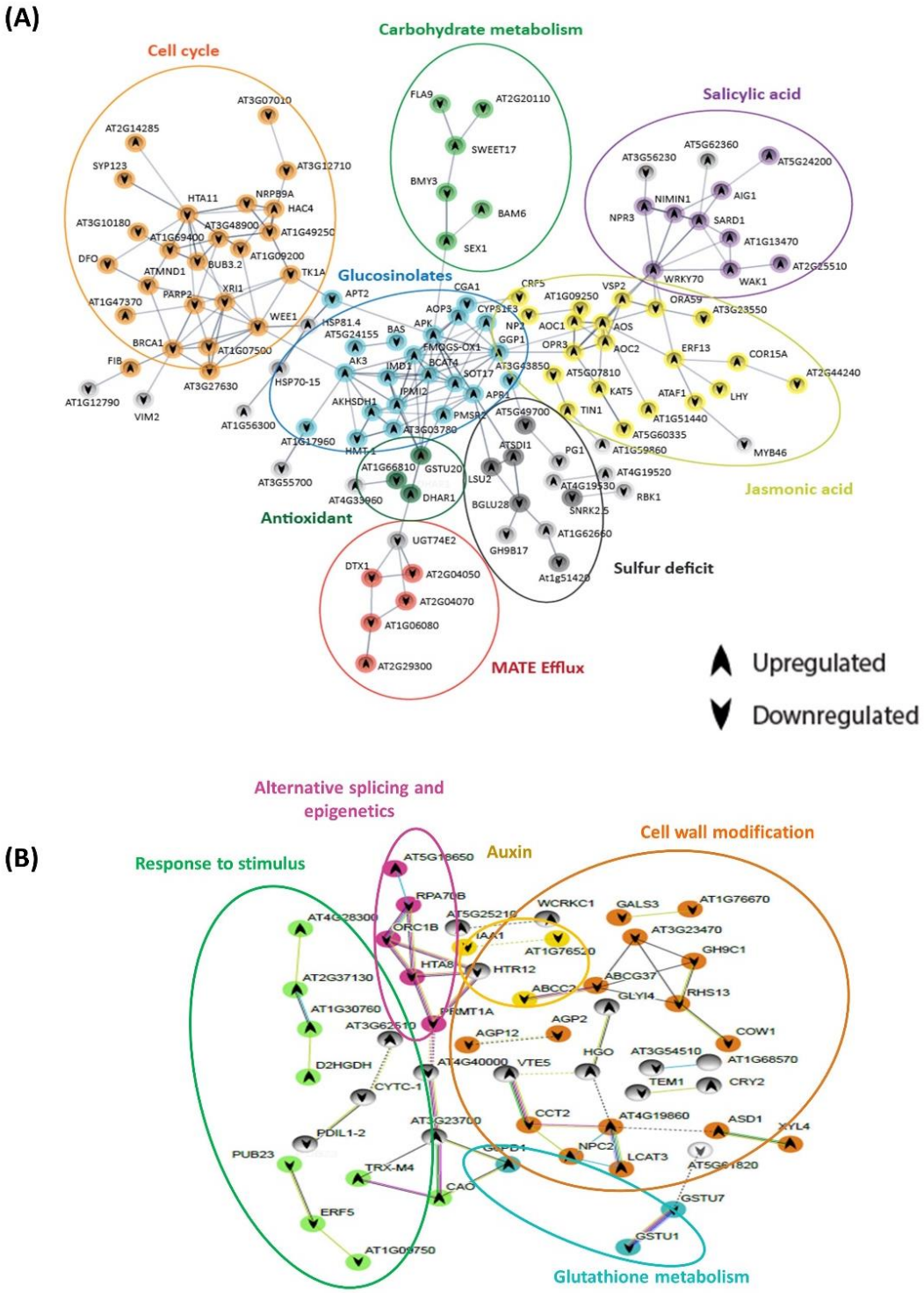


Figure 4. Protein-protein interaction network functional enrichment analysis of specific genes derived from the response to bicarbonate of A1$_{(c+)}$. Gene protein interaction network of A1$_{(c+)}$ exclusive DEGs from bic *vs*. pH 5.9 comparison after 3 h exposure **(A)** in leaves **(B)** in roots. Each sphere corresponds to one gene and nodes represent protein interactions. Gene pathways are shown in different colors. Arrows indicate up- or downregulation of the genes.

STRING analysis T6 $_{(c+)}$ sensitive line

Contrastingly, in the sensitive $T6_{(c-)}$, the main DEGs were grouped into five categories: Fe homeostasis (up); abiotic and biotic stress response; cuticular wax, nutrient, and transport activity; lipid transfer; and oxidative stress response. Only nutrient transport activity, lipid transfer, and oxidative stress response were connected by STRING (Figure 5A, Supplementary F5). Shen *et al.* (2016) [187] reported that Fe deficiency induces SA accumulation, which enhances auxin and ethylene signaling and activates Fe transport by *bHLH38/39* regulation of downstream iron genes. In fact, in the sensitive deme $T6_{(C-)}$, both the *FIT*-dependent [188] and the *FIT*-independent pathways [189] for iron homeostasis were activated by bic, as indicated by the upregulation of *bHLH39* and *bHLH100*, respectively. Upregulation of *NAS4* coding for nicotianamine synthase and *NRAMP4* coding for Fe transporter exporting Fe from vacuoles further confirms the quick activation of Fe mobilizing mechanisms in the bic-sensitive deme. Induction of Fe deficiency is among the best-known consequences of bicarbonate toxicity in sensitive plants. The quick upregulation of Fe homeostasis-related genes in leaves of $T6_{(c-)}$ exposed to bic (Figure 5A) is a clear sign of the difficulty of these sensitive plants to maintain sufficient Fe transport to the leaves.

Bicarbonate not only interferes with the Fe-reducing capacity of the roots [190] but also specifically inhibits the translocation from roots to shoots [62]. This inhibition of Fe translocation has classically been related to enhanced root production of organic acids, especially citrate. Citrate is a strong ligand for Fe and may favor Fe sequestration into root vacuoles [21]. More recently, studies with bic-exposed kiwi plantlets supplemented with ^{57}Fe related bic-induced inhibition of Fe translocation to the leaves with apoplastic accumulation of water-soluble Fe in the cell wall apoplast and the imbalance of nitrogen and carbon metabolism [181]. In fact, due to carbonic anhydrase activity, HCO_3^- supply favors dark fixation of inorganic C [34], leading to enhanced organic acid biosynthesis. Moreover, in $T6_{(c-)}$, bic exposure led to DEGs involved in nutrient balance and transport activities that were not directly related to Fe deficiency DEGs (Figure 5A).

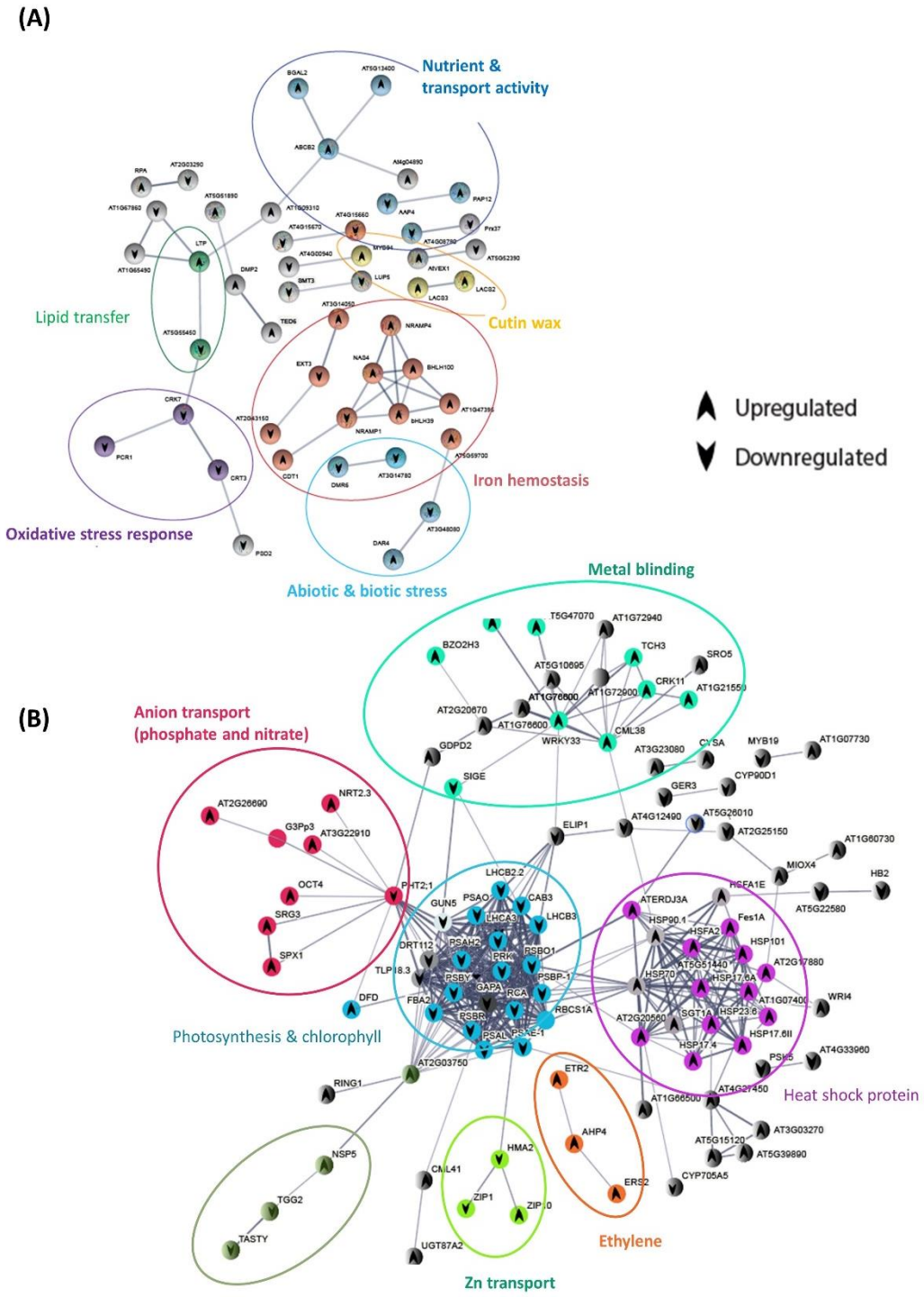


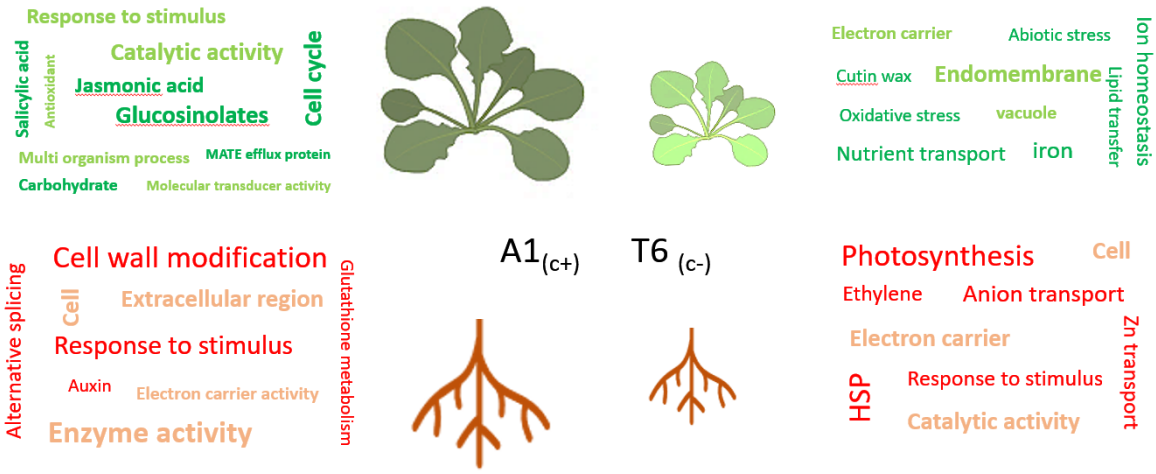
Figure 5. Protein-protein interaction network functional enrichment analysis of specific genes derived from the response to bicarbonate of T6 $_{(c-)}$. Gene protein interaction network of T6$_{(c-)}$ exclusive DEGs from bic *vs*. pH 5.9 comparison after 3 h exposure (A) in leaves and (B) in roots. Each sphere corresponds to one gene and nodes represent protein interactions. Gene pathways are shown in different colors. Arrows indicate up or downregulation of the genes

In roots, specific bicarbonate responses after 3 hours in the sensitive deme were clustered in Heat Shock Proteins (HSP) activation and photosynthetic gene suppression (Figure 5B). Heat Shock Proteins are a group of proteins highly conserved in all organisms. These proteins are activated under different stress conditions [191]. Similar to our results, previous studies with *Tamarix hispida* roots under $NaHCO_3$ treatments also have observed a huge activation of Heat shock protein, proline and Late Embryogenesis Abundant (LEA) proteins [192]

Although root cells are photosynthetically inactive, previous work showed suppression of photosynthetic genes under restricted P in the root in *A. thaliana* [193]. In the carbon fixation process, a large amount of Pi is necessary. Reduction of photosystem under low Pi can be an adaptive strategy to manage Pi sink in the plant. In our experiments reported in chapter 1, phosphate (P) content in the plant was determined. Under the control conditions bic tolerant $A1_{(c+)}$ plants have higher P tissue concentrations than $T6_{(c-)}$. Under bicarbonate treatment, $A1_{(c+)}$ reduces the total P concentration by around 28% while a 41 % decrease was observed in $T6_{(c-)}$ comparing treatment versus control conditions. Furthermore, a greater reduction in P translocation to the shoots was found in $T6_{(c-)}$.

5. Conclusions

- Our results demonstrate that the transcriptomic effects of bicarbonate on *A. thaliana* differ from those caused by high pH alone
- At equal pH, bicarbonate is more toxic than a solution with organic buffer in both demes.
- Differential gene expressed were identified under $NaHCO_3$ alkaline stress in both demes across time.
- After medium-term exposure (48h), tolerant lines responses are mainly produced in roots while in sensitive lines main changes are produced in shoots. These differences can impel the possibility of differences in signal perception and transduction in a short time.
- Specific bicarbonate responses after short time exposure (3h) show contrasted pathways involved in both demes. In the tolerant deme, DGEs are classified in glucosinolates, hormones, antioxidants, cell wall modification and genomic events as DNA repair, alternative splicing and epigenetics.
- Specific bicarbonate responses after short-time exposure in the sensitive deme are related to nutrient acquisition, especially iron deficiency in leaves. Phosphate deficiency pathways are clearly activated in roots. The fast upregulation of Fe-deficiency related genes in $T6_{(c-)}$ does not palliate the Fe deficiency condition and rather is a sign of Fe inefficiency under bicarbonate than an effective adaptation strategy.



F6. Graphical conclusions

Chapter 3: Genome Wide Association Studies and candidate genes validation of *Arabidopsis thaliana* natural population growth in carbonate soil.

1. Introduction

Calcareous soils are defined by high concentrations of $CaCO_3$ leading to high HCO_3^- activity and alkaline pH in the soil solution. The high HCO_3^- concentration in the rhizosphere produces a buffer effect maintaining high pH. Under dry weather, the excess of soil $CaCO_3$ tends to form a superficial crust which reduces aeration and water retention while high pH reduces nutrient availability limiting yield production [4]. Moreover, elevated OH^- in the apoplast of plant roots decreases the membrane potential hampering the ion uptake capacity. Adaptative strategies in plants involve the production of organic acids to neutralize the excess of HCO_3^-. However, the energy cost of maintaining pH homeostasis is detrimental to growth and fitness in plants [34]

As sessile organisms, maintaining nutrient homeostasis in the heterogeneous edaphic condition is important for plant survival. Plant homeostasis includes several critical steps like ion uptake, transport, and storage. Several proteins are involved in the plant nutrition process as transporters, channels, chelators, sensors, signal transducer and regulators [91][194]**.** Plants that are sensitive to calcareous soil conditions, called calcifuges, hardly maintain ion homeostasis due to their limited ability to take up and use iron (Fe), zinc (Zn), nitrogen (N), and phosphorus (P) when growing on these soils [195]. Excess uptake of Ca may cause further constraints to the performance of calcifuges on calcareous soils. The main hypothesis to explain this fact points to difficulties in Ca storage or immobilization [195]. The high bicarbonate concentration can strongly interfere with the plants' mechanisms for acquiring and use Fe and Zn [196]. Root proton extrusion for maintenance of a slightly acidic rhizosphere and apoplastic environment is neutralized by HCO_3^-. Bicarbonate enhances the accumulation of both soluble Fe and Fe precipitations in the apoplast. Bicarbonate-induced release of organic acid production can chelate Fe; however, in a situation of apoplast alkalinization Fe becomes less soluble, producing precipitates. Fe deficiency in the upper part of the plant contrasts with Fe accumulation in roots due to a reduction in nutrient translocation [62]. Thus, maintaining nutrient homeostasis when facing calcareous stress is a key point in the performance of tolerant plants.

Genomic Wide Association Studies (GWAS) are genetic tools allowing to explore the genetic architecture of traits relevant to agriculture and ecology. Natural genetic variation can be produced by SNP, indels, CNV or epigenetic markers [113]. Such differences in DNA can produce changes in the transcriptome and, consequently, in the proteome. DNA variations can produce differences in plant performance while different local climatic and edaphic conditions can pressure allele fixation.

Nowadays, genome sequencing is becoming an affordable technology and BSA-seq or GWA studies are now viable. During the last years, several authors were interested in combining nutrient profiles or metabolomics as a phenotype to get insight into the underlying genetic architecture. *Arabidopsis thaliana* is a suitable specie to perform GWAS due to its wide range of distribution and its growth in different habitats, including continuous ecological gradients as latitude, longitude and altitude, or heterogeneous categories as soil types [74][119]. Several studies have demonstrated local adaptation of *A. thaliana* populations to their native environment by fixing or enriching beneficial alleles in a habitat type. This natural variation has been exploited in different studies both using the European or local distribution scales of population of *A. thaliana* [82][94][197].

Remarkable advances have been done in mineral nutrient profiling of *A. thaliana* allowing the identification of metal transporters from several families. High heritability of ionomic traits makes them perfect candidates for GWA studies [92]**.** Several examples are available in the literature where data of plant mineral nutrient concentrations from natural populations have been explored by GWAS. As a result, transporters related to nutrient levels in plants have been elucidated. High-affinity K^+ transporter (HKT1) for sodium [87], [94], heavy metal ATPase (HMA3) for cadmium [198] and high arsenic content (HAC1) for arsenate [199]. Several studies focused on toxic or sub-optimal growth conditions to study the genetic architecture of ionome related traits. In a magnesium supply experiment, simulating serpentine soil conditions, a single strong peak of SNPs associated with Ca concentration was found corresponding to candidate gene *NRX1* (*AT1G60420),* which codes for a nucleoredoxin in *Arabidopsis thaliana* [127]. Also, a north European subpopulation was tested under low iron conditions in plates using root length as phenotypic trait for GWAS. Results pointed to *FRO2* as a candidate gene. Accessions with long roots under iron deficiency showed higher *FRO2* expression, more FRO2 enzymatic activity and higher root Fe concentrations [200].

Several alkaline stress studies have been performed using *Oryza sativa* and *Glycine max* at the seedling stage. Such studies test important agriculture traits, as germination and biomass production [73] [201] under either natural conditions or iron deficiency in hydroponics in order to detect potential tolerance genes. In comparison to other stress factors, tolerance to calcareous soil conditions has deserved less attention. Contrastingly, effects of salinity in plant performance and mechanisms of salinity tolerance have been investigated in depth. Salinity has been identified as a driver of local adaptation [94] at the European scale. In native Catalan *Arabidopsis thaliana* demes, HKT1 weak allele has been identified as responsible for local adaptation to soils with largely

fluctuating sodium concentrations located at an intermediate distance from the sea (1 to 1.5 km) [87]. Moreover, local adaptation to moderately carbonated soils was detected at a small geographical scale by means of testing demes from the same native Catalan population in a multi-year small-scale common garden experiment [19].

Lack of studies exploiting natural variation of traits of interest under alkaline soils to find local adaptation at a large geographical scale impelled us to perform a GWAS study on natural carbonated and control soils from the study area. A set of 360 natural accessions of *A. thaliana* was used to perform a Genome-Wide Association Study (GWAS) on two soils differing in their carbonate content to identify candidate genes under carbonate stress. The main goals are detecting QTL involved in mineral homeostasis and growth parameter in calcareous soils and validate these genes using knockout T-DNA lines

2. Aims

- The overall aim here was to detect differences in genomic traits underlying differences in plant fitness on soils with contrasting $CaCO_3$ concentrations.
- For this purpose, the first aim was to obtain the parameters of physic-chemical soil characteristics from the native population using soil datasets, in order to select an appropriate contrasting soil for the GWAS experiment.
- Second aim was to acquire phenotype data measuring rosette growth and nutritional profiles for the detection by GWAS of candidate regions involved in differences in plant tolerance to soil carbonate.
- Then local adaptation at a European scale was tested using native soil data and plant performance.
- Final aim was to validate the candidate genes using TDNA lines and assess plant performance under calcareous soil.

3. Material and methods

This study was performed in close collaboration with Joana Terés from our lab at the UAB. . Field soils and their properties were as reported by [88]. Phenotyping of 360 lines grown in contrasted $CaCO_3$ soils, GWAS analyses and gene validation were performed at the UAB. All procedures and results are presented in this thesis for an easy comprehension. The contribution of each author to the work is properly specified along the manuscript.

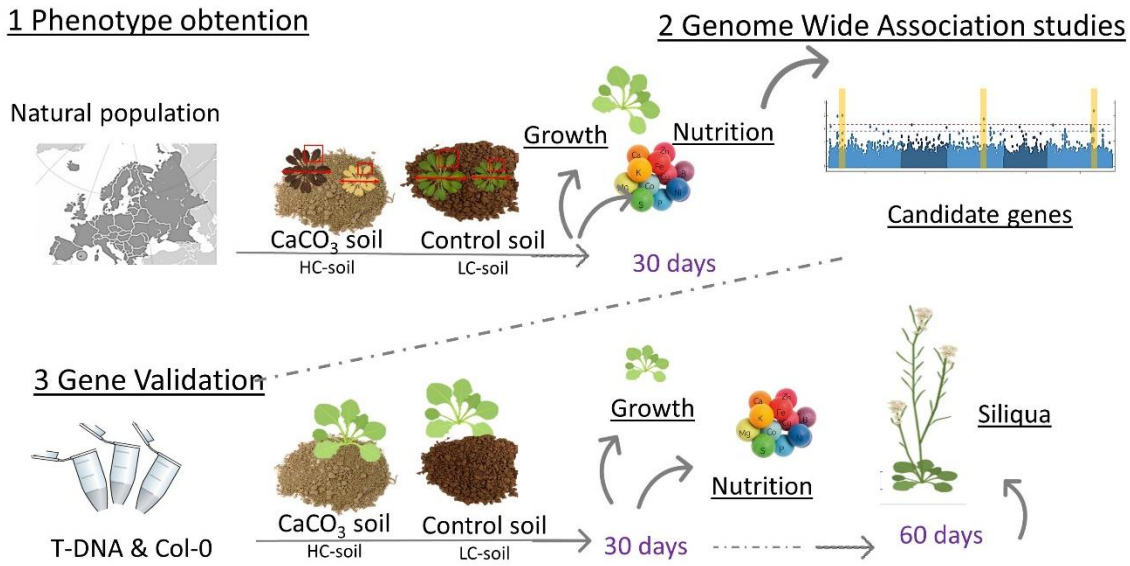


Figure 0. Experimental set-up

3.1 Soils analysis

Origin of soils and their analysis were as described by Terès [89]. Briefly, contrasted $CaCO_3$ soils were excavated from two different locations from Mallorca Island. For the control treatment, low $CaCO_3$ content soil was used located in x: 516699, y: 4392835 coordinates. A high carbonate soil was used as treatment located in x: 505185, y: 4390717 UTM coordinates in ETRS89 31S projection. Six independent samples from each location have taken between 0 and 30 cm depth. Trace Elements were determined by microwave digestion with *aqua regia* [133] and quantification 5300DV Optima ICP-OES (Perkin-Elmer, Massachusetts). Total organic carbon [202], $CaCO_3$ content using a Bernard calcimeter [203], cation exchange capacity using ammonic acetate protocol [204], and texture and particle size by [203].

3.2 Plant material and growth conditions

3.2.1 Plant material: In this study, we included a set of 360 natural accessions of *A. thaliana* (L.) Heynh from the HapMap population (Supplementary material and methods). For multiplying and genotyping these lines, seeds were stratified for three days at 4 ºC in the dark and then transferred to grow on nutrient-supplied rockwool in a greenhouse at 70 % humidity 16h/8h light/dark 150 mmol/m^2s of light intensity, and 20/18C day/night temperatures. T-DNA insertion lines were genotyped to select or confirm homozygote plants. Primers for T-DNA insertion mutant lines genotyping were designed using Salk institute primers (www.signal/salk/tdnaprimers). Confirmed homozygous T-DNA insertion mutants were grown in carbonate soil and control soil as described above (Supplementary material Fig 4). *Arabidopsis natural* population and T-DNA insertion lines of candidate genes were ordered from Nottingham Arabidopsis purchased from Nottingham *A. thaliana* Stock Centre (NASC, Nottingham, UK) [139].

3.2.2 Seed sterilization: Seeds were sterilized with bleach solution 30% for 10 minutes in constant movement. Bleach solution has been removed and seeds have been rinsed with MQ water six times.

3.2.3 Soil culture: Arabidopsis thaliana HapMap cohort (360 accessions) were cultivated in two soil types with contrasted $CaCO_3$ concentrations. Control and carbonated soils were mixed with perlite (2:1) to increase soil aeration. Before germination, seeds were stratified for 7 days in the dark. Plants were grown for 4 weeks in controlled chamber conditions set at 70% humidity 12/12 hours light/dark cycle at 150 mmol/m_2.s^{-1} of light intensity. Plants were watered with MQ water as needed.

3.3 Phenotyping

3.3.1 Rosette Diameter (RD): Rosette diameter from 360 accessions was measured in carbonate and control soils every week during 4 weeks with ImageJ software. The growth rate of each accession was calculated as $RD_{(CaCO3\text{-}soil/control\text{-}soil\ )}$ from each week's measure.

3.3.2 Nutrient mineral content (NMC): Plants at 4 weeks old from the two different soils were sampled by removing 2–3 leaves (1–5 mg dry weight) and washed with 18 MΩ water before placing into Pyrex digestion tubes. Sampled plant material was dried for 42 hours at 60 ºC and weighed before open-air digestion in Pyrex tubes using 0,7 mL concentrated HNO_3 (Mallinckrodt AR select grade) at 110 ºC for 5 h. Each sample was diluted to 6.0 mL with 18 MΩ water and analyzed for Ca,

Co, Cu, Fe, K, Mg, Mn, Mo, Na, P, S, and Zn content in (ppm) on an Elan DRCe ICP-MS (PerkinElmer Sciex). NIST traceable calibration standards (ULTRAScientific, North Kingstown RI) were used for the calibration [142]. The relative nutrient mineral content of each accession was calculated as NMC $_{(CaCO3\text{-}soil/Control\text{-}soil)}$.

3.3.3 Silique's production: At the end of the mature state, silique production was counted.

3.4 GWAS analysis

To identify genomic regions underlying $CaCO_3$ tolerance, we conducted GWAS on several phenotypes: (I) Leaf ionome content and (II) and rosette diameter from 4-week-old plants grown in control and $CaCO_3$ soil; (III) Relative growth rate RD $_{(CaCO3\text{-}soil/control\text{-}soil)}$ and (IV) relative nutrient mineral content rate $NMC_{(CaCO3\text{-}soil/control\text{-}soil)}$.

Genome-Wide Association Studies analysis was performed using the GWA-portal web application (https://gwas.gmi.oeaw.ac.at/) [118]. Each phenotype was run in a 250 K SNP dataset in an Accelerate Mixed Model (AMM) to reduce false positives because of population structure [120][205]. Bonferroni multiple testing procedure was used to control the false discovery rate. Assuming arbitrary dependence between SNPs, the 5% false discovery rate (FDR) threshold was plotted as a dashed horizontal line. Only the SNPs with a higher value of 6 were considered. Linkage disequilibrium (LD) structure could be also detected with GWA-portal by calculating genome-wide r^2 values between the selected SNP and all other displayed SNPs and color-coding them in the Manhattan plot.

3.5 Gene ontology (GO)

All GO annotations were downloaded from TAIR (www. Arabidopsis.org). The gene ontology enrichment analysis was performed for three categories: cellular component, molecular function, and biological process [106].

3.6 Geographical information Program (GIS) data extrapolation

To estimate the edaphic parameters of each *A. thaliana* natural populations native soil, coordinate locations and public maps from the European Soil Data Centre (ESDAC) database [30] were combined using Q-GIS (http://qgis.osgeo.org). Natural populations coordinates were extracted from GWAPP (http://gwapp.gmi.oeaw.ac.at/)[118] in WGS84 system latitude and longitude. (Supplementary material and methods). Maps of soil properties at European scale, based

on Lucas 2009/2012 topsoil data, were used to extract the following variables: pH (measured in H_2O), Cation Exchange Capacity (CEC), Calcium carbonates ($CaCO_3$) [30].

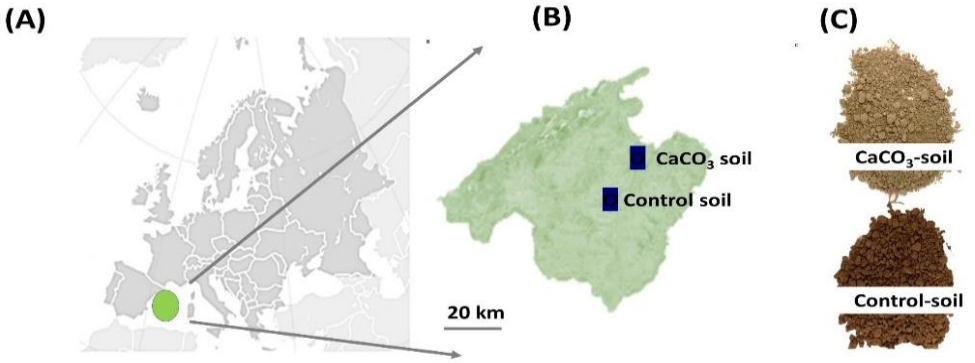
3.7 Statistics

Data normality was checked for all phenotypes and non-normal data were transformed before applying any parametrical tests. Ionome data from plants growing in carbonated soil and non-carbonated soil was used to perform a Principal Component Analyses (PCA) using varimax correction. To avoid bias, from differences among macronutrients and micronutrients, ionome values were standardized using Z value (value-mean)/(max-min). Radial plots were constructed from the nutrition of 25 % tail distribution of RD $_{(CaCO3\text{-}soil/control\text{-}soil)}$ to detect differences in nutrition in high and low-growing plants. Also, mean-standardized was applied. To test for correlations between two variables a Bivariate Fit was conducted. One-way ANOVA was used to test for significant differences ($p\text{-}value < 0.05$) between genotypes growth in contrasting soils. To perform multiple comparisons of group means we used Tukey's HSD. All the statistical analyses were conducted using JMP software (JMP, Version 13. SAS Institute Inc., Cary, NC, USA, 1989–2019).

4. Results & Discussion

4.1 Suitability of selected soils for differential tolerance assessment

To investigate the effect of calcareous soil on natural accessions of *Arabidopsis thaliana*, 360 accessions constituting the HapMap Panel [115] were cultivated in a $CaCO_3$-enriched soil excavated from Mallorca Island. Also, populations were cultivated in a control low carbonate soil with similar characteristics (Figure 1A, B, C). Physic-chemical analysis from contrasted soils supports their suitability for studying plant performance in $CaCO_3$ natural soils. As shown in Figure 1D, both soils display similarities in physical properties and chemical element composition. Main statistical differences among soils are only present in $CaCO_3$ content, pH, Ca, and Mn content, which are higher in $CaCO_3$ soil (Figure E). Furthermore, the analysis validates the classification of the $CaCO_3$ soil as calcareous soil based on its high content of $CaCO_3$ and alkaline pH [203].



(D)

	Carbonated Soil	Control Soil
WRB (2014)	Haplic Calcicosol	Chromic Endoleptic Luvisol
Soil taxonomy (2014)	Chromic Luvisol	Typic Haploxeraf
CaCO3 (g/kg)	664	1.5
Active Lime (g/kg)	259	0
CEC (mmol/kg)	167	170.5
EC 1:5 (µS/cm)	116	33
pH H20	8.5	7.1
pH KCL	7.4	5.3
Field capacity (g/kg)	227.3	204.9

(E)

	Carbonated soil		Control soil				
Element	Mean(µg/g)	SD	N	Mean(µg/g)	SD	F ratio	Prob
Na	108	28.1	6	147	40.5	3.7	0.0833
Mg	235	36.1	6	307	78.4	4.2	0.0666
K	34.1	22.4	6	76.8	55.1	3.3	0.0992
Ca	720	35.4	6	628	45.3	14.5	0.0041
Mn	32.8	5.2	6	77.7	4.9	4.9	0.0495
Fe	10.8	1.5	6	12.8	2.9	2.2	0.1682
Co	0.1	0.08	6	0.09	0.05	0.007	0.9352
Ni	0.13	0.03	6	0.013	0.03	0.07	0.8047
Cu	2.65	1.36	6	6.55	5.64	2.7	0.1308
Zn	19.5	5.9	6	29.2	12.8	2.8	0.1242
Mo	0.04	0.001	6	0.05	0.01	4.8	0.0515

Figure 1: **Location and physical and chemical parameters of the soils used in the GWAS experiment**. **(A)** Mallorca island is marked in green on the Europe map. **(B)** Zoom in Mallorca Island and location of carbonate soil and non-carbonated soil **(C)** Picture from both soils. **(D)** Physical and chemical parameters from contrasted soils. *n=6*. Soils excavated at 10 cm depth. Data obtained from doctoral thesis Teres 2017 [89].

4.2 Carbonate soil effect on natural populations of *A. thaliana*

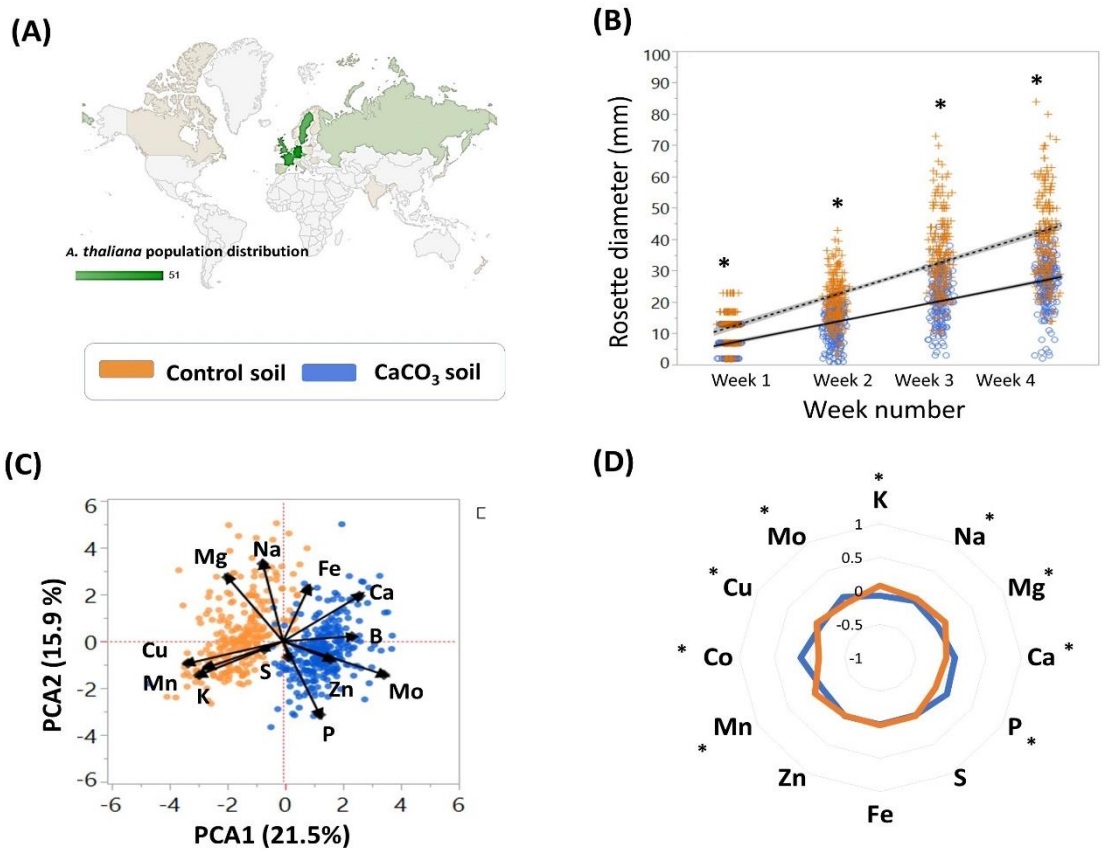
Plants were growing in soils with contrasted carbonate levels for 4 weeks. Rosette diameter (RD) was assessed every week while leaves nutrient mineral content (B, Ca, Co, Cu, Fe, K, Mg, Mo, Mn, Na, S and Zn) was determined by ICP-Ms at the end of the experiment. From the 360 natural population sown in contrasted soils (Figure 2A), 255 populations were able to germinate and survived more than 4 weeks in control soils while 271 populations survived on the calcareous soils. A total of 222 populations were overlapping and survived on both soils. Phenotype data from these were used as input to calculate relative growth $RD_{(CaCO3\text{-}soil/\ control\text{-}soil)}$ and relative nutrition mineral content $NMC_{(CaCO3\text{-}soil/Control\text{-}soil)}$ (Supplementary material and methods).

Rosette Diameter of 4weeks old plants varied significantly among all the accessions under both control and $CaCO_3$ conditions. Figure 2B. However, the RD of almost all the accessions under $CaCO_3$ stress was reduced by 37.04%

To detect differences in nutrient mineral content between treatments, plant ionome was taken as an input to perform a Principal Component Analysis (PCA). Nutrient mineral concentrations were clustered in two groups corresponding to plant nutrition in $CaCO_3$-soil and Control-soil. Most plant ionome variations were explained by PCA1 (21.5%) while PC2 represented a 15.9% of the variation. PCA1 separates the data according to the treatment. Nutrients with the highest relative contribution to PCA1 are Co>Ca>B>Mo while negative contributions are K>Cu>Mn. In principal component 2 (PCA2) the higher contributions are Na>Mg>Fe and negative contributions by P>Zn. Figure 2C. The nutrient mineral content of 4-week-old plants showed a remarkable reduction of K, Mg, Cu, Mn and Na concentrations and an increased uptake of Co, B, Mo, P and Ca in $CaCO_3$-soil. Figure 2D. Similar ionome results and biomass decrease were found in different plant species such as *Avena sativa* and *Medicago sativa* [44][43].

Although differences in soil nutrient concentrations between $CaCO_3$-soil *vs* Control-soil were statistically different only for Mn (-41%) and Ca (+14%), the entire plant ionome was strongly altered in plants growing on $CaCO_3$-soil. This supports the view that the $CaCO_3$ content is the modifying agent in plant ionome. Micronutrient availability decreases in alkaline soil, especially in the case of Zn, Fe, Cu and Mn. In contrast, B and Mo are more available in high pH soil [4]. These trends were also observed in our plant ionomic analyses. Regarding P, the solubility of phosphate usually increases rather than decreases with pH in the range of 6 to 8. However, P deficiency is common in

plants on $CaCO_3$ soils, mainly because of low total P content and low soil moisture [196]. This was not the case here as the experimental plants were irrigated and, in fact, plants on $CaCO_3$-soil had higher P concentrations than control-soils plants. Higher tissue Ca concentrations are a typical plant response to calcareous soil conditions. In our experiment, Ca increase in plants was accompanied by a reduction of K and Mg. Noteworthy was the lower Na concentration in plants growing in $CaCO_3$ soil when compared to control. This can be explained by the overall low concentration of soluble salts ($CE_{1:5}$ 116 µS/cm) in the experimental soils, corresponding to a concentration of about 1.16 mM. Contrastingly, on alkaline-saline soil, plants show higher Na uptake probably due to the reduction of the membrane potential and consequently a decrease of Na extrusion capacity [42]. The imbalance in essential nutrients in plants growing on saline-alkaline soils containing high concentrations of sodium carbonate is causing more severe growth reduction [59] than the constraints of calcium carbonate-rich soils with low Na content.



(E)

Mallorca carbonate					Mallorca control						
Phenotype	Mean (µg/DW)	Max	Min	SD	Phenotype	Mean (µg/DW)	Maximun	Min	SD	% variation	Prob > F
B	195.35	615.49	66.92	72.68	B	152.37	356.75	53.05	47.95	28.21	<.0001
Ca	30767.63	52687.30	21666.81	4712.64	Ca	25842.92	49662.45	12263.90	5756.84	19.06	<.0001
Co	7.79	21.20	2.61	2.58	Co	2.47	8.02	0.59	1.10	215.91	<.0001
Cu	6.52	28.59	3.60	1.71	Cu	8.33	13.39	4.32	1.55	-21.69	<.0001
Fe	99.21	469.75	53.05	46.68	Fe	103.54	386.46	52.68	49.04	-4.17	0.3023
K	27976.44	58674.74	8962.67	6927.63	K	36031.96	61171.03	8353.29	8712.51	-22.36	<.0001
Mg	1903.22	3859.00	877.47	422.12	Mg	2321.81	4279.98	1174.15	475.05	-18.03	<.0001
Mn	87.96	147.07	38.17	18.24	Mn	110.48	211.55	54.10	26.67	-20.39	<.0001
Mo	0.82	4.03	0.08	0.64	Mo	0.41	3.39	0.07	0.34	102.45	<.0001
Na	1230.28	14401.69	89.93	1698.61	Na	2136.69	20019.69	92.85	2753.36	-42.42	<.0001
P	5084.81	9420.15	1393.20	1554.43	P	3483.09	6989.77	914.83	1201.77	45.99	<.0001
S	7748.32	15450.61	3542.97	1652.91	S	7936.40	15637.51	4137.80	1692.96	-2.37	0.1993
Zn	85.71	854.74	27.37	79.78	Zn	76.93	389.95	33.91	41.22	11.42	0.118

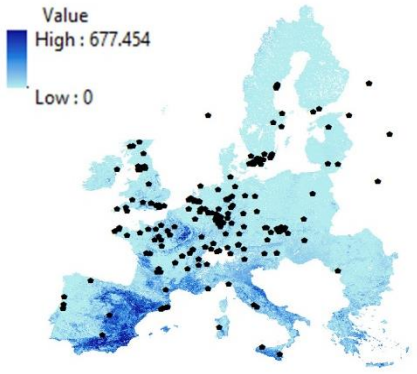
Mallorca carbonate					Mallorca control						
Phenotype	Mean	Maximun	Min	SD	Phenotype	Mean	Maximun	Min	SD	% variation	Prob > F
RD1	7.20	7.75	6.64	4.09	RD1	11.64	12.33	10.94	5.05	-38.14	<.0001
RD2	12.40	13.12	11.68	5.32	RD2	21.32	22.30	20.34	7.15	-41.84	<.0001
RD3	21.96	23.01	20.91	7.74	RD3	35.49	37.15	33.82	12.06	-38.11	<.0001
RD4	26.34	27.40	25.29	7.78	RD4	41.27	43.14	39.41	13.48	-36.17	<.0001

Figure 2 Natural population origin map, growth and nutrition parameters in contrasted $CaCO_3$ soil. **(A)** Hapmap population worldwide distribution map. **(B)** Rosette diameters (RD) increment for 4 weeks. **(C)** Principal component analysis and **(D)** Radial plot from Hapmap population grown in contrasted $CaCO_3$ soil for 4 weeks. **(E)** Statistics table. Asterisk indicates significant differences p-value>0.05. Data obtained from doctoral thesis Teres 2017 [89].

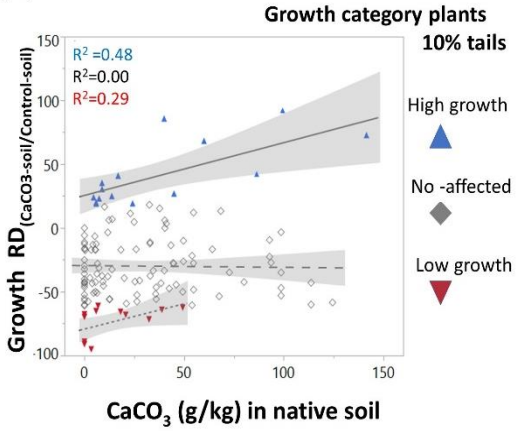
4.3 Native soils of *A. thaliana* on the European scale to detect local adaptation

Examining the European soil map [30], a pattern of soil $CaCO_3$ can be observed involving latitude (Figure 3A). In southern latitudes, pH values (r^2 = -0.43) and $CaCO_3$ (r^2 = -0.47) contents tend to be higher than in northern latitudes producing a cline. Also, pH and $CaCO_3$ were highly correlated with CE (See Figure 3B). Under arid and semi-arid climates soils are prone to accumulate soluble salts in the superficial layers due to low rainfall. If these salts contain high concentrations of HCO_3^- the soil pH is increased. Latitude makes a difference in climate, especially in Europe. For this reason, most carbonate soils are found in the south [4].

(A)



(C)



(B)

Var 1	Var 2	Correlation	p-value
Latitude	$CaCO_3$ (g/kg)	-0.47	1.10E-07
Latitude	CEC (cmol/kg)	-0.17	6.76E-02
Latitude	pH H_3O	-0.43	1.47E-06
$CaCO_3$ (g/kg)	CEC (cmol/kg)	0.43	9.11E-07
$CaCO_3$ (g/kg)	pH H_2O	0.62	1.06E-13
CEC (cmol/kg)	pH H_2O	0.39	1.07E-05

(D)

Var1	Var2	Correlation	p-value
$RD_{(CaCO3/control)}$	Latitude	0.05	0.56
$RD_{(CaCO3/control)}$	CaCO3 (g/kg)	0.06	0.55
$RD_{(CaCO3/control)}$	CEC (cmol/kg)	0.00	0.99
$RD_{(CaCO3/control)}$	pH H2O	0.13	0.16

Figure 3 Correlation between 360 accessions Hapmap native habitat *vs* fitness in a common garden experiment.
(A) Natural populations distribution in the European calcareous soil map. Ecotypes are indicated as a dot. **(B)** Latitude, $CaCO_3$, CEC and pH correlation and p-value table. **(C)** Relative plant growth correlated with native $CaCO_3$ soil. Relative growth was categorized by using a 10% tails distribution. Blue arrows indicate plants with better growth in $CaCO_3$ conditions; grey diamond plants with few effects observed and red arrows plants with higher reduction in plant fitness. **(D)** Relative rosette diameter *vs* latitude, $CaCO_3$, CEC and pH correlations and p-value table.

Although *Arabidopsis thaliana* has a calcifuge distribution some local adaptation has been observed among the *A.thaliana* populations in NE Catalonia [19]. To see whether such local adaptation can also be confirmed on a European scale here we put our rosette diameter data of the hap map population grown in the GWAS experiment on the European map and overlapped it with European pH, CEC and $CaCO_3$ maps. No linear correlation was found when using the whole hap map population. Figure 3D. For this reason, relative growth was separated into categories. These categories were built from relative growth distribution and a 10% tail was used. The top distribution is formed by better performance on calcareous soils of plants whose native $CaCO_3$ content ranges from 0 to 150 g Kg^{-1}. The category in between distribution tails does not show any correlation to the native $CaCO_3$ soil. The bottom category was configured by the 10% of plants with worse performance when compared to control. In these group, native soil $CaCO_3$ content ranges from 0 to 50 g Kg^{-1}. Figure 3C.

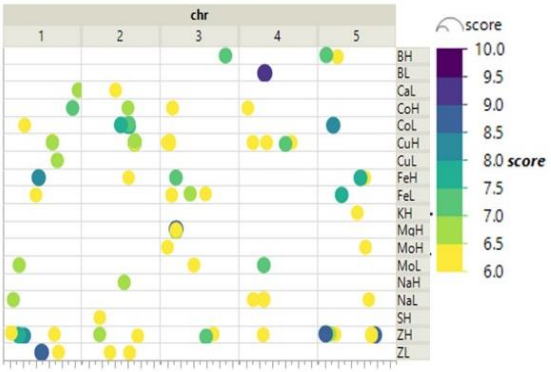
4.4 Detection of SNPs associated with nutrition in high growth on $CaCO_3$ soil

An important condition for natural selection to act upon a trait is that this trait has a phenotypic variation that is genetically determined. Regions with specific carbonate tolerance phenotypes and a molecular mechanism involved haven't been well elucidated yet. To identify genomic regions underlying tolerance to $CaCO_3$, we conducted GWAS on several phenotypes: (I) rosette diameter (RD) and mineral nutrient concentrations (MNC) of plants grown in control and $CaCO_3$ soils; (II) relative growth ($RD_{CaCO3\text{-}soil/Control\text{-}soil}$) and mineral nutrient concentration ($NMC_{CaCO3\text{-}soil/Control\text{-}soil}$) of 4 weeks old plants. After running GWAS, using a 250K SNP dataset in an accelerated mixed model (AMM) to avoid false positives, gene position and associate p-values from each phenotype were downloaded. All phenotypes were filtered by -log10 (adjusted p-value) > 6 to produce a panel shown in Figure 4 A, B.

The fact that several phenotypes gave significant peaks made candidate selection difficult. (Figure 4 A, B and Supplementary F4). For this reason, phenotype data from mineral nutrients and growth were combined. First of all, the effect on the rosette diameter under control conditions was checked. As sown in Figure 4C, rosette diameter under control conditions does not predict growth on $CaCO_3$-soil. Relative growth $RD_{(CaCO3\text{-}soil/Control\text{-}soil)}$ distribution were used again; as in Figure 3, top 10% tails were used to configurate groups of plants with "higher growth" or "lower growth". Figure 4D. Nutrient mineral content profiles from these two groups in calcareous soil were calculated and visualized in a radial plot. From the radial plot we can detect that "higher growth" plants can take

up more Zn, S and P than those showing low relative growth, in calcareous soil treatment (Figure 4E and Supplementary F4). Zn peaks were studied in detail because plant Zn uptake is a major constraint for plants on calcareous soils and Zn efficiency has been identified as an important adaptive trait in *Phaseolus vulgaris* [206].

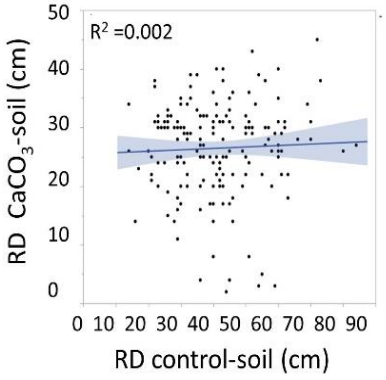
(A)



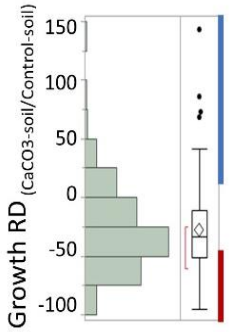
(B)

Phenotype	Carbonated soil	Control soil	% Difference
B	3	3	3
Ca	0	2	9
Co	4	5	1
Cu	29	1	0
Fe	6	6	4
K	1	0	0
Mg	4	0	0
Mn	0	0	0
Mo	2	3	3
Na	1	7	3
P	0	0	0
S	1	0	0
Zn	17	7	0
D4	0	0	0

(C)



(D)



(E)

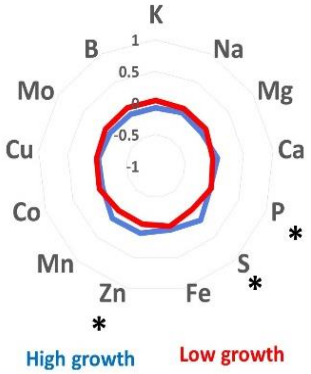


Figure 4. GWAS output and nutrition corresponding with "higher and lower growth" group plants in $CaCO_3$ soil.
(A) GWAS output panel. Several phenotypes are disposed in rows indicated in the y-axis while the x-axis indicates chromosome and SNP position. Phenotypes were filtered to -log p-value >6 and significant peaks were plotted using color code shown in the legend. **(B)** Summary table of the number significant SNP from each phenotype **(C)** Correlation plot from plant rosette diameter growth in control *vs* $CaCO_3$ soil. **(D)** Distribution of relative rosette diameter growth $RD_{(CaCO3\text{-}soil/control\ soil)}$. **(E)** Nutrient mineral content from "high growth" and "low growth" plant group plant. Radial plot was used to visualized ionomic data. Asterisk indicates significant differences p-value>0.05.

4.5 Selection of candidate genes responsible for higher growth on calcareous soil.

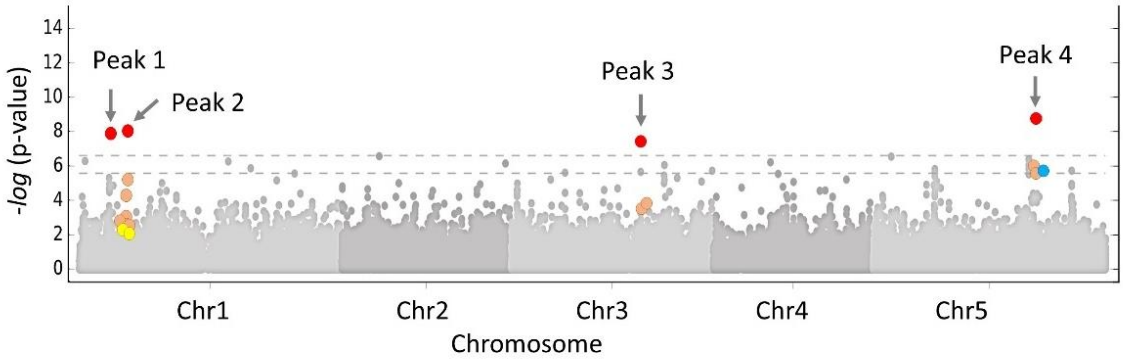
Few SNPs were found for S and P, making the Zn concentration phenotype with four main peaks the best candidate; these were considered for detailed analysis. The four major peaks observed for shoot Zn concentrations were located in chromosomes 1, 3 and 5. We also explored the LD region associated with the highest score SNP, giving a total of 18 SNPs linked to 15 genes (Figure 5). A total of 12 T-DNA Col-0 background lines were tested. For all the SNPs, haplotype block was studied (Supplementary F5). The function, localization, and expression of these genes were examined.

Candidate genes were involved in membrane signal reception and transport. The first peak was composed of two genes: cyclin-dependent kinase inhibitor AT1G10690, involved in negative regulation of mitotic nuclear division, regulation of DNA endoreduplication and located in the nucleus and AT1G10700, which encodes a P-independent phosphoribosyl pyrophosphate (PRPP) synthase (Araport 11).

In the second peak from chromosome 1, several genes were in LD. Three candidate genes belong to WAK-like kinase (WAKL), a subfamily of receptor-like kinase (RLK) with a cytoplasmic Ser/Thr protein kinase domain and an extracellular domain with EGF-like repeats. WAKL subfamily is composed of 26 members in *Arabidopsis thaliana*, their main function is related in signaling molecules that communicate between the cell wall and the cytoplasm protein [207]. Our results show WAKL 6, 1 and 4 as putative candidates in these family (Figure 5B). WAKL4 is involved in Arabidopsis root mineral responses to Zn, Cu, K, Na, and Ni [208]. WAK1 is an aluminum early responsive gene and its overexpression results in aluminum tolerance [209]. Also, in chromosome 2 there are genes related to molecule transporters. Organic cation/carnitine transporter 3 (AT1G16390) has carbohydrate transmembrane transporter activity and is mainly expressed in siliques [210], while a member of Na^+/H^+ antiporter family (AT1G16380) may mediate K^+ transport and pH homeostasis [210], and have been localized to intracellular and plasma membrane [211].

In peak 3, only one gene was in the LD region; AT3G42970 codes for an unknown protein and mutant lines were not available. In chromosome 5, four candidate genes were included in the same peak: a member of Heat Shock Protein family (HSP) AT5G47590 involved in the response to many environmental stress factors and highly conserved in plants; a transmembrane protein with unknown function (AT5G47580), finally a tonoplast dicarboxylate transporter (TDT) AT5G47560 which is involved in the uptake of malate and fumarate to the vacuole and pH homeostasis [212].

(A)



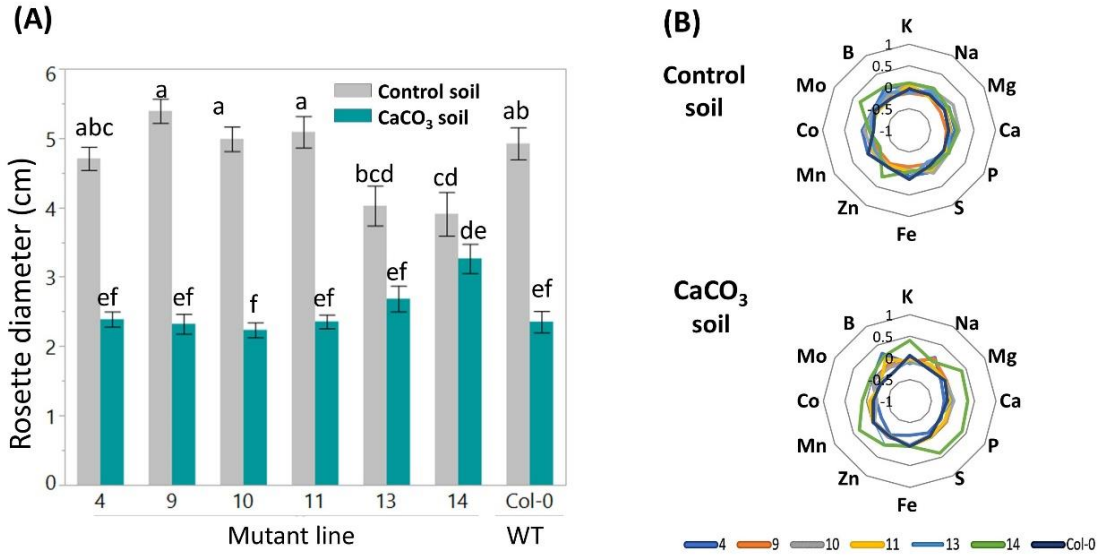
(B)

	Chromosom	Position	Score	Effect	Gene	Function	T-DNA line
Peak 1	Chr1	3551054	7.94	Intergenic			
					AT1G10690	Cyclin-dependent kinase inhibitor	CLON SALK_074523.37.40.x
					AT1G10700	Phosphoribosyl pyrophosphate (PRPP) synthase 3	not available
Peak 2	Chr1	5520641	8.07	UTR_3'	AT1G16110	Wall associated kinase-like 6	not availabe
		5522235	2.99	Intergenic	AT1G16120	Wall associated kinase-like 1	CLON SAIL_912_E08
		5533737	3.00	Synonymous	AT1G16150	Wall associated kinase-like 4	CLON SALK_043295.56.00.x
		5543599	4.17	Intron	AT1G16190	Rad23 UV excision repair protein family	CLON SAIL_199_F08
		5545724	4.19	Intron			
		5546768	5.25	Intron	AT1G16210	Coiled-coil protein	CLON SALK_138693.39.50.x
		5572032	3.02	Synonymous	AT1G16290	Transglycosylase	CLON SALK_105517.27.40.x
		5599695	2.5	Non-syn	AT1G16380	Cation/hydrogen exchanger family protein	CLON SAIL_187_F10
		5604901	2.53	Intergenic	AT1G16390	Organic cation/carnitine transporter 3	CLON SAIL_681_C09
Peak 3	Chr3	15023629	7.50	Intergenic			
		15024837	3.27	Non-syn	AT3G42970	Unknown protein	not available
		15062847	2.90				
Peak 4	Chr5	19292148	8.79	Intergenic			
		19291992	5.82	Intergenic	AT5G47590	Heat shock protein HSP20/alpha crystallin	CLON SALK_072401.55.50.x
		19291111	2.60	Intergenic	AT5G47580	Transmembrane protein	CLON SALK_005475.29.99.f
		19291111	2.60	Intergenic	AT5G47570	NADH dehydrogenase ubiquinone 1 beta subcomplex subunit	CLON SAIL_8_F03
		19288458	1.29	Synonymous	AT5G47560	Tonoplast dicarboxylate transporter	CLON SAIL_681_C09

Figure 5. Manhattan plots and candidates genes (A) Manhattan plot with a significant peak for SNP associations to leaf Zn content. The horizontal lines correspond to a nominal 0.05 significance threshold after Bonferroni correction and Benjamini Hochberg. **(B)** Position, score (p-value), effect and genes in high LD found in significant peaks **.** SNPs are color-coded to show their LD relationships with the top SNP (blue = > 0.4 r^2 ; yellow = 0.4 r^2 < 0.6; y orange = 0.6 r^2 < 0.8; red = r^2 > 0.8).

4.6 Validation of candidate genes by assessing phenotypic responses to high $CaCO_3$

To verify if these genes were responsible for the observed associations, we obtained the knockout mutant lines of these genes. The same pot experiment conducted for the GWA study was performed. Additionally, we included the reference Col-0, which exhibited intermediate levels of leaf Zn and growth rates (Figure 6). A total of 11 T-DNA insertion mutant lines built in the WT Col-0 background were tested. From these lines, 7 were able to reach 4 weeks old at the necessary biological replicates number to obtain reliable results.



(C)

GENE	Arbitrary number	Symbol	Description (Araport11)
AT5G47580	1	ASG7	Transmembrane protein
At1G10690	2	AT1G10690	Cyclin-dependent kinase inhibitor
AT1G16290	4	AT1G16290	Transglycosylase
AT1G16190	5	RAD23A	Rad23 UV excision repair protein family
AT1G16390	8	OCT3	Organic cation/carnitine transporter 3
AT5G47570	9	AT5G47570	NADH dehydrogenase ubiquinone
AT5G47590	10	AT5G47590	Heat shock protein HSP20/alpha crystallin family
AT1G16150	11	WAKL4	Wall associated kinase-like 4
AT1G16210	13	AT1G16210	Coiled-coil protein
AT5G47560	14	TDT	Tonoplast dicarboxylate transporter
AT1G16380	15	ATCHX1	Cation/hydrogen exchanger family protein

Figure 6. Validation of candidate genes. Mutants and wt were sown in soil pot experiment using contrasted $CaCO_3$ soil. After 4 weeks rosette diameter was scored, and leaves were used to determine ionome profiles **(A)** Rosette diameter from mutants and wt **(B)** leaf ionome radial plot from Col-0 and the selected mutant lines. **(C)** Table including gene, an arbitrary number used in bar plots and description for each mutant line. Blue arrows are indicating validated lines.

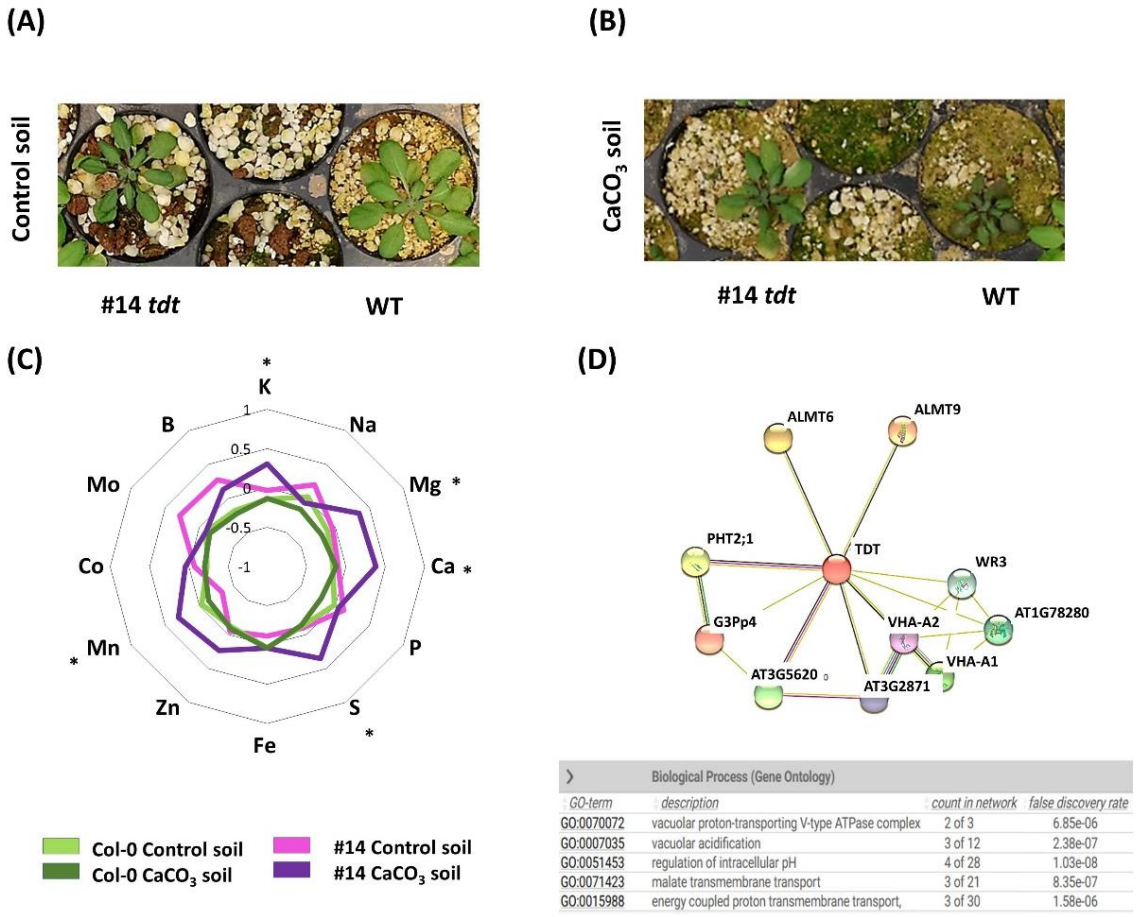
Rosette diameters were scored four weeks after sowing (Fig. 6-A). In control conditions, mutant lines designed as 13 and 14 showed a significant reduction in rosette diameter. In $CaCO_3$ soil, only these same lines achieved higher growth than Col-0. Ionome profiles were also analysed. In control conditions, all lines showed similar ionomic profile, while in $CaCO_3$ conditions mutant line 14 showed a higher nutrient translocation to the upper part of the plant (Fig 6-B).

As specified in Figure 6C, line 14 corresponds to the knock-out mutant line of the gene AT5G47560, located in plant tonoplast and with dicarboxylate transporter function (TDT). This gene was found in LD with the mapped SNP in peak 4, corresponding to 19288458 position and 1.29 LOD score producing a synonymous change. However, the mutant line performed better on calcareous soil than wt (Figure 7 A, B). A clear increase in S, Mg, Zn, Mg, Ca and K accumulation in aerial parts is observed when comparing only mutant 14 and WT Col-0 lines grown on calcareous soil (Figure 7C & Supplementary F7). Protein-protein interaction analysis shows this gene is related to ALMT6, ALMT9 (malate transporters) and a vacuolar ATP-ase VH-A1 and VH-A2. Moreover, they are involved in pH regulation and malate transporter (Figure 7D).

Previous studies performed in discs of *A. thaliana* leaves using wt and *tdt* lines grown at a pH range from 4 to 7 found the mutant line reduced the capacity to generate OH^- to overcome acidification. Changes in malate and citrate rates were found and *tdt* line had more citrate and less malate accumulation [213]. This research tested plants in a pH range lower than we tested in our experiment. On the other hand, the study was performed on seedlings, while in this study 4-week-old plants are used. Therefore, results and conclusions from both studies might not be clearly comparable. In the study by Hurth et al (2005) [213] with lower pH the control mutant lines did not differ in rosette diameter with Col-O. Contrastingly, in our study, significant differences were observed. The higher sensitivity of the *tdt* mutant line under acidity could be in line with a higher tolerance of the same line to alkalinity.

More studies are required testing malate and citrate contents in WT and *tdt* lines to determine differences in natural populations of *A. thaliana.* Moreover, validation of the candidate genes OCT*3* and *CHX1* would be of great interest. Knock-out mutant lines could not be tested properly in a pot experiment due to low replicates. *OCT3* is an organic cation transporter that also was upregulated in the transcriptomic analysis described in Chapter 2. Also, recent research in *Glycine soja* pointed to members of the CHX cation/H^+ exchanger superfamily as contributors to high salinity and carbonate alkaline tolerance [214]. ASG7 is a transmembrane protein that may be

involved in the role of nutrient homeostasis. However, no further information about these genes was found to date.



Biological Process (Gene Ontology)			
GO-term	description	count in network	false discovery rate
GO:0070072	vacuolar proton-transporting V-type ATPase complex	2 of 3	6.85e-06
GO:0007035	vacuolar acidification	3 of 12	2.38e-07
GO:0051453	regulation of intracellular pH	4 of 28	1.03e-08
GO:0071423	malate transmembrane transport	3 of 21	8.35e-07
GO:0015988	energy coupled proton transmembrane transport,	3 of 30	1.58e-06

Figure 7. Validation of candidate genes. Col-0 and *tdt* lines were sown in a soil pot experiment using soils with contrasted $CaCO_3$ content. After 4 weeks rosette diameter was scored, and leaves were used to determine ionome profiles **(A)** Aerial picture from Col-0 (wt) *tdt* mutant (#14) **(C)** Leaf ionome radial plot from Col-0 and *tdt* grown on control (light pink and light green lines) and calcareous soil (purple and dark green lines). **(D)** Protein-protein interaction analysis of TDT gene. Asterisks represent statistical significances in ANOVA 2 ways (treatment* line)

5.Conclusions

- Physic-chemical analyses of soils from the study areas confirm significant differences in carbonate concentrations and their suitability as test substrates for studying differential plant adaptation to carbonate.
- The negative influence of high soil carbonate on plant growth was confirmed using rosette diameters an indicator.
- Plants grown on calcareous soil suffer alterations in their mineral nutrient content profile.
- $CaCO_3$ content in soils can drive local adaptation at a small geographical scale but this trend is not observed at large-scale when using the whole HapMap collection.
- Accessions showing better growth performance on calcareous soil were those able to maintain the uptake and translocation of P, S and Zn to aerial parts.
- The *tdt* knock-out mutant line presents a better phenotype than WT on calcareous soil. Thus, TDT is pointed as a negative regulator of Zn homeostasis under carbonate stress, involved in pH adjustment and nutrient homeostasis.
- Further studies addressing the relation between dark fixation of inorganic C, the production and storage of organic acids in the root vacuoles and the inhibition of Zn and Fe translocation to the shoots are needed to determine the role of TDT under alkaline stress.

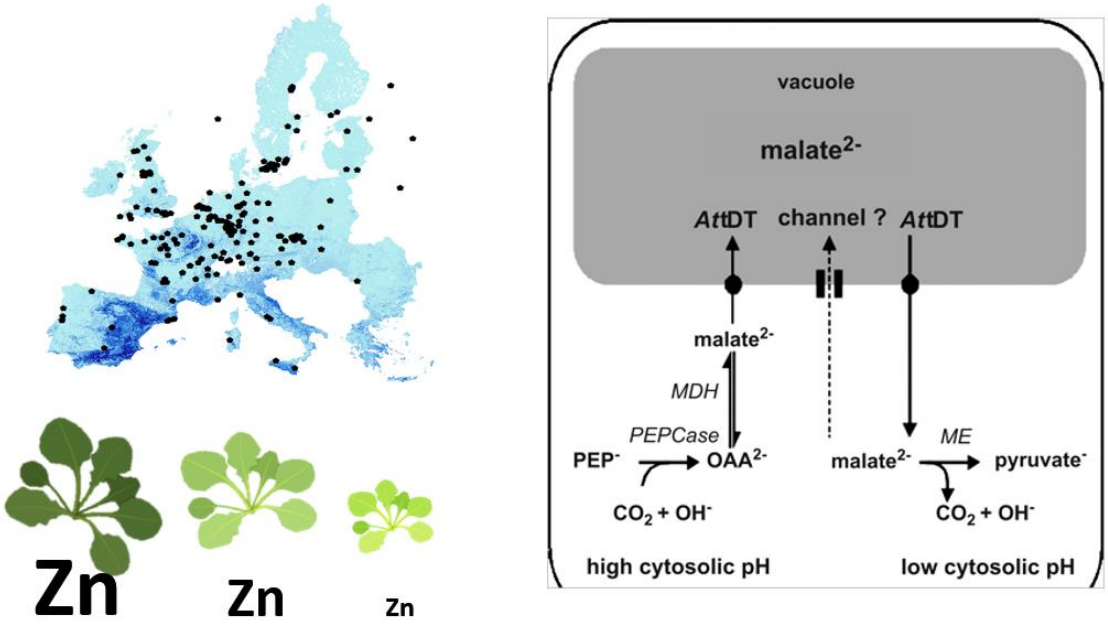


Figure 8: Graphical conclusions. Natural populations and *tdt* mutants that are able to maintain Zn translocation to the shoots perform better on calcareous soils. Right part of the figure taken from *Martinoia et al.* 2006 [215]

Chapter 4: Catalan *A. thaliana* population screening on alkaline-saline soil

1.Introduction

Saline stress is one of the majors constrains in agriculture. Soil salinization is often co-occurring with alkalinity, especially in arid and semi-arid climates. In these climates, the high evapotranspiration moves and deposits in the superficial soil layer excess of soluble salts like Na^+, Ca^+, Mg^+, K^+, CO_3^{2-} and HCO_3^-. Soil salinization often includes structure degradation, alteration in chemical composition and changes in biological activity [10]. Besides natural causes, anthropic activities like crop irrigation are adding more soluble salts into soils. Including both the natural and anthropogenic origin, it is estimated that 831 million hectares of the Earth are covered by saline-alkaline soils. [4].

On these soils, plants need to cope with the deleterious effects of high pH and excess salts. The high alkalinity affects the cell membrane potential producing nutrient deficiency. Salinity causes both ion toxicity and osmotic problems. Several studies performed with different plant species show more destructive effects of salinity under high pH than under neutral conditions [216] [55]. In comparison to saline stress alone, under alkaline-saline conditions plants take up more cations, especially Na^+, while a reduction of inorganic anions is replaced by organic acids (OAs) [43]. These changes in OA composition differ in roots compared to leaves showing different strategies in each organ. Under either salinity or alkaline-saline stress the enhancement of proline [42] and betaine concentrations are quantitively the most important changes in soluble sugars [217].

Other research was focused on differences between tolerant and sensitive plant species and varieties. Tolerant plants are able to maintain growth, translocate more nutrients showing less damage in cell structure. Plant adaptative mechanisms toward salinity are well established. Sodium enters by NNCs (non-specific channels) reducing 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)* mechanisms. The *High Affinity Potassium transporter 1 (HKT1)* is as an efficient transporter retreaving Na from the xylem and limiting Na transport to the shoots [218]. In *Oriza sativa*, relative expression of *HKT1* and *SOS1* were assessed between two contrasted lines towards alkaline-saline stress. Tolerant lines were able to activate both genes more than tolerant lines limiting Na^+ accumulation in the leaf. High expression of *SOS1* in roots is removing salt from cytosol to apoplast. Furthermore, Fe acquisition and rhizosphere acidification genes were highly induced to maintain plant nutrient homeostasis [216].

Halophytes are also included as interesting plants to study adaptative responses towards double stress. These experiments are testing halophyte plants in upper ranges of 400 mM [48]. Unfortunately, mechanism founds in halophyte plants are difficult to apply in non-tolerant crop species. *Arabidopsis thaliana,* as many economical interesting plant species, is a calcifuge glycophyte. However, tolerance differences among natural populations have been described by Busoms 2015 [86] and Terés 2019 [19]. This indicates that *A. thaliana* may be used as a useful model for analyzing the molecular genetic mechanisms underlying possible stress responses to alkaline/saline conditions.

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. For example, a multi-year large-scale common garden performed in the northern edge of the native range (Sweden) and one near the southern edge (Italy) revealed fitness tradeoffs providing evidence of adaptive differences in tolerance to freezing at the northern site [80]. In other studies in the Pyrenees (northeastern Spain) altitudinal clines were used as a common garden to reveal adaptive responses to spring heat and drought over several hundred kilometers from the coast [219]. In previous studies of our research team, Catalan populations were sown in a coastal saline common garden for 3 years. From these small-scale common gardens, the main conclusion is that *A. thaliana* native from the Catalan coast present local adaptation to saline soils [87]. Also, the same protocol was followed to test local adaptation to a calcareous soil. Population established on a moderately carbonate soil (non-coastal) presented better performance to a calcareous soil than coastal demes located on siliceous soil [19].

According to that, coastal populations present better performance toward salinity while inland or non-coastal populations present a better performance in calcareous soil. However, the species distribution model elaborated by Busoms in 2015 predicted the lack of *A. thaliana* in saline-alkaline soil and several surveys in different years confirmed this model prediction [86]. The distribution of *A. thaliana* was disrupted at a coastal location containing lime-rich soils. The main hypothesis is *A. thaliana* has no specific mechanism to colonize saline-alkaline soils.

2. Aims

The objective of this chapter is to perform a screening to test the fitness of the Catalan natural population using natural alkaline-saline soils. Plant response using hydroponics and plates at controlled conditions was performed to validate field responses.

3. Material and methods

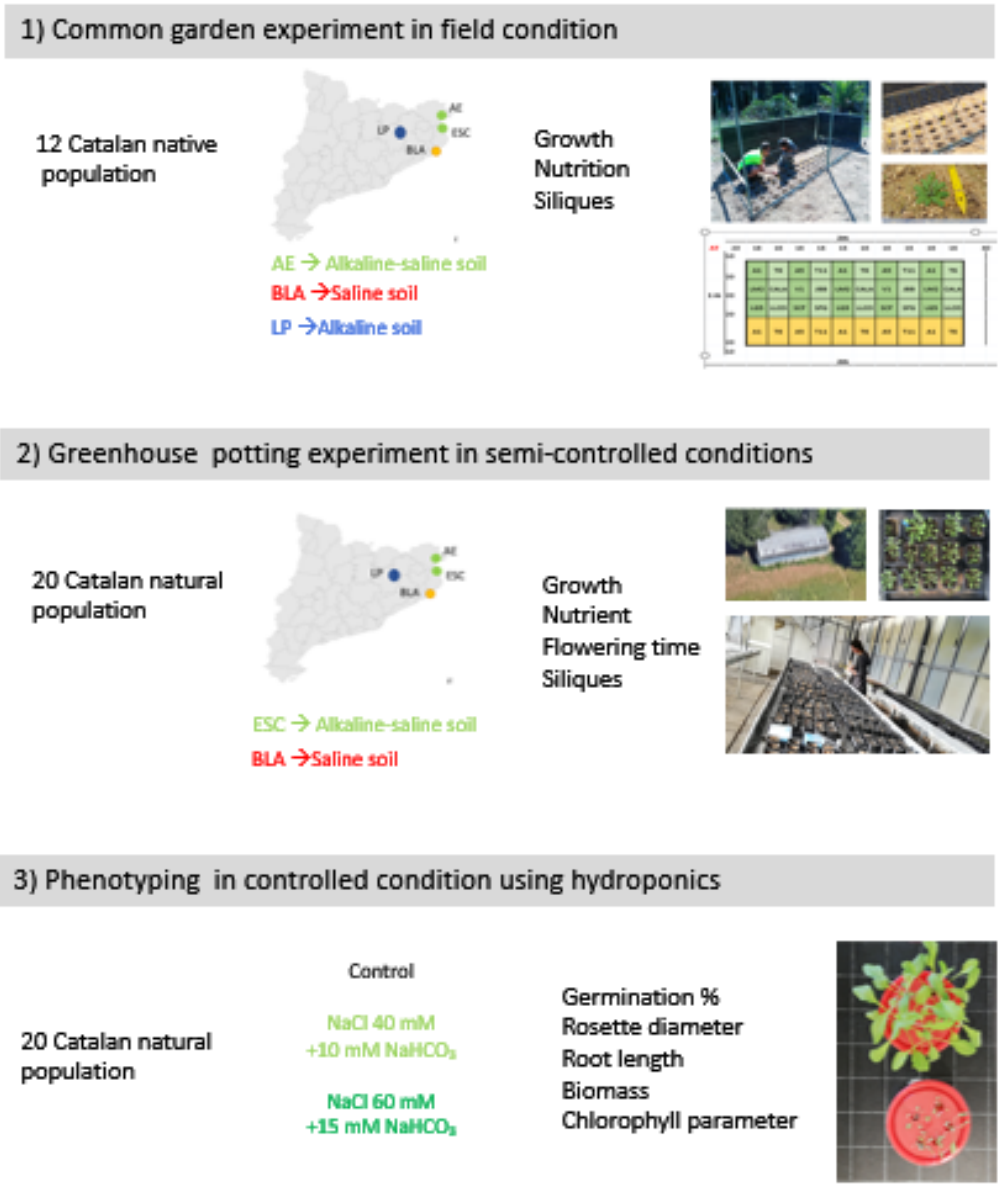


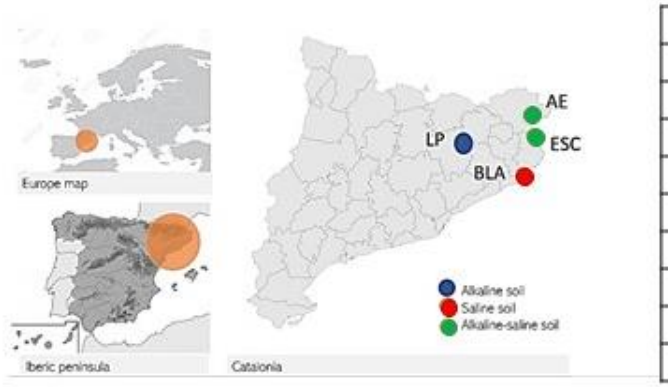
Figure: 0 Experimental design

3.1 Soil and plant material

3.1.1 Soil material:

Catalan natural populations of *Arabidopsis thaliana* were cultivated in soils with contrasted $CaCO_3$ and salinity concentrations. For the two-years field experiments (2017- 2018), the saline common garden was located Jardí Botànic de Blanes named BLA (41°53'42.4"N 3°01'11.7"E), the calcareous common garden was in Les Planes d'Hostoles (LP) (42°06'43.1"N 2°54'51.9"E) while alkali-saline was located inside Parc Natural Aiguamolls de l'Empordà (AE) (42°23'66.6" 3°09'94.2"). For 2020 greenhouse experiments located in UAB, *A. thaliana* native population were sown in pots containing soil excavated from two different locations Catalan Coastal location. The saline siliceous soil was excavated from Jardí Botànic de Blanes while saline-alkaline soil was selected after a characterization of soils from different locations from L'Escala (ESC). Six independent samples from each location were taken from a depth between 0 and 30 cm. Finally, ESC1 was taken as a representative of alkali-saline soil (See Figure 1) .

(A) **(B)**



Soil	Latitude	Longitude
LP	42°06'71"N	2°54'03"E
AE	42°23'66"N	3°09'94" E
BLA	41°53'42"N	3°01'11"E
ESC1	42°13'03"N	3°11'30"E
ESC2	42°53'88"N	3°11'51"E
ESC3	42°13'38"N	3°10'09"E
ESC4	42°11'66"N	3°15'67"E
ESC5	42°11'48"N	3°15'67"E
ESC6	42°11'48"N	3°16'06"E

Figure 1: Soil's location map and georeference table. The map indicates common garden used in field experiments 2017-2018 and soil excavated for greenhouse potting experiment 2020. Blue color indicates alkaline soil LP (Les Planes d'Hostoles). Orange color represents saline soil BLA (Jardí Botànic de Blanes) while alkaline-saline soil is colored in green AE (Aiguamolls de l'Empordà) and ESC (L'Escala).

3.1.2 Soil physical, chemical and geological analysis: To analyze the soil's physical parameters, six independent samples of each soil type were used. The soil characterization was performed on the 2-mm fraction sample. Soil Electric Conductivity (EC) was measured using a conductimeter (Hanna, Woonsocket, Rhode Island, USA in saturated paste extract using 18 MΩ water. For soil pH measurements, 25 ml of 18 MΩ water were added in a falcon tube which contained 10 g of soil.

Before measure, samples were mixed in constant rotation for 30 min. After that, pH measurement was performed using a pH-meter (Basic 20+, Crison, Barcelona. Spain). The texture was estimated using soil size particle, water holding capacity (WHC) was calculated by soil water retention percentage after 5 minutes. Organic material was oxidized with $K_2Cr_20_7$ and determination was performed by Mohr salt (1M) and diphenylamine as an indicator. All procedures were performed following methods described by [203].

A geologic map from Catalonia (Mapa Geològic 1:25.000) from Institut Cartogràfic i Geològic de Catalunya (ICGC) was used to determine parental rock from common garden soil. Georeferenced positions were overlapped with the Catalan geological map using Miramon (v8. CREAF, UAB).

To determine the available mineral nutrient concentrations, 5 grams of soil were dried for 42 h at 60 °C in 50-mL Falcon tubes. Each sample was diluted to 6.0 mL with DTPA-NH_4 and analyzed for As, B, Ca, Cd, Co, Cu, Fe, K, Li, Mg, Mn, Mo, Na, Ni, P, Rb, S, Se, Sr, 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 [142].

3.1.3 Plant material: In previous studies, natural variation of *Arabidopsis thaliana* populations from Catalonia were tested in a multi-year small-scale common garden under saline and carbonated conditions [86] [19]. Seeds from reciprocal transplant experiment performed in 2015 and stored under cold (4 ºC) and dry conditions until the beginning of the experiments (See Supplementary Material and Methods). Col-0 seeds were included as a reference genome and they were purchased from Nottingham Arabidopsis Stock Centre NASC [139].

3.2 Growth conditions and plant phenotyping

3.2.1 Seed sterilization: Selected 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 in 0.1% agar solution to synchronize germination.

3.2.2 Field common garden experiments: To detect differences in plant tolerance among the native population of Catalan *A. thaliana* a common garden experiments were designed in 2017 and 2018. As representative location we selected coastal environment BLA, calcareous soil LP and representative of saline-alkaline soil AE. The same common garden design was reproduced at both sites. The common garden was 2 × 6 m in the native soil at each site, and each garden was covered

with a shading mesh that reduced irradiation by 70%. Ten replicates from 12 native populations were sown at random position in November. Fitness was assessed from February to June. Every week, pictures of the entire plants were taken. The photographs were used to measure the rosette diameter (n = 9–12) using ImageJ software (Schneider, Rasband and Eliceiri, 2012). The leaf nutritional levels were assessed in 30 days plants after seedling emergence. Plant material was dried for 4 days at 60 ºC. Approximately 0.1g was used to perform and open-air digestion in Pyrex tubes using 0,7 mL concentrated HNO_3 at 110 ºC 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) *n=10* [142]. As a proxy to fitness, the number of siliques per plant was counted at maturity *(n= 10)*.

3.2.3 Greenhouse experiments (Semi-controlled conditions) : To detect differences in plant tolerance among the native population of Catalan *A. thaliana* a common garden experiment in pots was designed. Soils were taken from the Marimurtra Botanic Garden, Blanes (BLA) (41º 40’ 37,64”; 2º 48’ 3,86”), a representative coastal environment, and a representative of saline-alkaline soil from L’Escala. In November 2019, each deme was sown in 15 pots for each soil distributed randomly (in each replicated plot each deme had a different position). Two weeks after germination, in February 2020 we left 1 plant in each square. Siliques number was counted at plant maturity, around June 2020 (*n=10*). The greenhouse was located in the experimental field facilities at Universitat Autònoma de Barcelona. Air temperature and humidity and sun incidence were monitored but not modified. Irrigation was applied twice a week. Every week, pictures of the entire plants were taken. The photographs were used to measure the rosette diameter (*n= 9–12*) using ImageJ software [140], [141]. The leaf nutritional state was assessed in plants 30 days after seedling emergence. Plant material was dried for 4 days at 60 ºC. Approximately 0.1g was used to perform and open-air digestion in Pyrex tubes using 0,7 mL concentrated HNO_3 at 110 ºC 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) (*n=5)* [142]. Silique’s production was counted at maturity around June 2020 (*n= 15*).

3.2.4 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 (½ MS at pH 5.9), neutral salinity (½ MS NaCl 50 mM pH 5.9), alkalinity (½ MS 10 mM $NaHCO_3$ pH 8.3), and alkaline-saline treatment (½ MS NaCl 40 mM + 10mM $NaHCO_3$ pH 8.3). For each deme and treatment, a total of 60 seeds were divided among 4 plants to perform different biological replicates. 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 µmol $cm^{-2}{\cdot}s^{-1}$, 40% humidity and 25 °C). Germination and radicle emergence were daily checked during the following 10 days. Non-germinated seeds at 10 days old were removed to control conditions to assess germination percentage

3.2.5 Hydroponic experiment: Seeds were sown in 0.2 mL tubes containing 0.6% agar prepared in nutrient solution ½ Hoagland (pH 5.9). Seeds were kept at 4 °C for 7 days in the dark to synchronize germination. Tubes containing seed were placed in the growth chamber (12 h light/12 h dark, 150 µmol $cm^{-2}{\cdot}s^{-1}$, 40% humidity and 25 °C). After root emergence, the bottom of the tubes containing seedlings was cut off and the tubes were placed in 150 mL hydroponic containers with aerated nutrient solution ½ Hoagland (pH 5.9). When 15 days old, the seedlings were separated into different sets and the following treatments were applied: control (½ MS at pH 5.9) and two alkaline-saline treatments (½ MS NaCl 40 mM + 10mM $NaHCO_3$ pH 8.3), and (½ MS NaCl 60 mM + 15 mM $NaHCO_3$ pH 8.3). Solutions were buffered with different proportions of MES (2-(N-morpholino) ethanesulfonic acid hydrate, 4-morpholineethanesulfonic acid) and BTP 1,3-bis [tris (hydroxymethyl) methylamino] propane). The hydroponic solution was changed every three days to maintain a constant concentration of nutrients in the solution. Plants remained in these conditions for two weeks. We harvested 30-days old plants. Every tree day, pictures of the entire plants were taken. The photographs were used to measure the length of the largest root and the rosette diameter (n = 4-6) using ImageJ software [141].

3.3 Statistics

Data normality was checked for all phenotypes and non-normal data were transformed before applying any parametrical tests. Ionome data from Catalan *A. thaliana* native soil was used to perform a Principal Component Analyses (PCA) with varimax correction. To avoid bias, from

differences among macronutrients and micronutrients, ionome values were standardized using Z value (value-mean)/(max-min). Radial plots were constructed for soil and plant ionome visualization. Also, mean-standardization was applied. To test for correlations between two variables a bivariate fit was conducted. One-way ANOVA was used to test for significant differences (*p-value < 0.05*) between genotypes growth in contrasting soils. To perform multiple comparisons of group means we used Tukey's HSD. All the statistical analyses were conducted using JMP software JMP, Version 13. SAS Institute Inc., Cary, NC, USA, 1989–2019).

4 Results & Discussion

4.1 Climatic and soil study from native Catalan *A. thaliana* distribution

In previous studies, fitness from Catalan native populations was tested in a saline common garden unraveling local adaptation to saline soil. In other words, plants native to higher saline soils were able to tolerate salinity better than those populations with low Na^+ content in their native habitat. Also, several surveys and the Specie Distribution Model (SDM) confirm that Catalan populations are mainly distributed on siliceous on soil with slightly acidic or neutral pH [86]. However, some population were found near to calcareous soil and for this reason, local adaptation to calcareous soil have been tested [19]. At this point, we have some populations identified with high tolerance to saline which follow soil native patterns but without tolerance to calcareous soil. On the other hand, calcareous tolerant plants have low abilities to grow in saline soil. Supplementary Material and Methods. The objective here was to explore the responses of our natural demes to combined stress, salinity under alkaline pH conditions.

Before starting to test the demes in alkaline-saline conditions, soil and climatic factors from the native population were re-analyzed. All Catalan demes were placed in littoral and pre-littoral climatic regions. As can be seen in Figure 2A, the Catalan *A thaliana* population are located in Mediterranean coastal climate. The population distribution on the coast is clearly interrupted in areas with alkaline-saline soils (Figure 2B). As the main hypothesis, we consider that alkaline-saline soil makes a huge constraint eliminating the possibility of *A. thaliana* establishment.

According to the agent which drives local adaptation found in previous studies, the Catalan population was plotted by its Na^+ and $CaCO_3$ content in the native soil. As shown in Figure 2C, the Catalan population were split into three categories according to native soil: **G1**-Plant from high $CaCO_3$ content and low salt (9.45% $CaCO_3$, 44.33 Na^+ mg/g); **G2**-Plant with intermediate salt and carbonate exposure (5.7% $CaCO_3$ and 60.65 Na^+ mg/g) and **G3**-Plants with high saline exposure and low $CaCO_3$ contents (0.65% and 129.44 Na^+ mg/g). These groups are fitting with previous research performed by Busoms [86] and Terés [19].

Soil nutrients, organic material (OM), water hold capacity (WHC), pH and $CaCO_3$ parameters monitored during 2013 to 2015 by Busoms and during 2017 to 2019 by the author were used as input to perform a Principal Component Analysis (PCA) showed in Figure 2D. Natural population groups were separated into PCA coordinates. In this analysis, PCA1 explains 34.4 % of the variation.

Distance to the sea seems to be correlated with high pH, $CaCO_3$ and WHC, Zn and P. A possible explanation is coastal demes are located in a sandy location with low WHC and slightly acidic or neutral pH due to granitoid parental rock, while inland populations are located in lime soils producing high pH and $CaCO_3$ content. Also, high clay in inland soil produces more WHC. Chloride content, sulfate and Mg, Mo and Na are correlated, and they overlap with the coastal population while Ca, Zn and Pare correlated and are characteristic from an alkaline soil. Correlations from native soil parameter analyzed (pH, OM, WHC and nutrients) can be found in Supplementary F2.

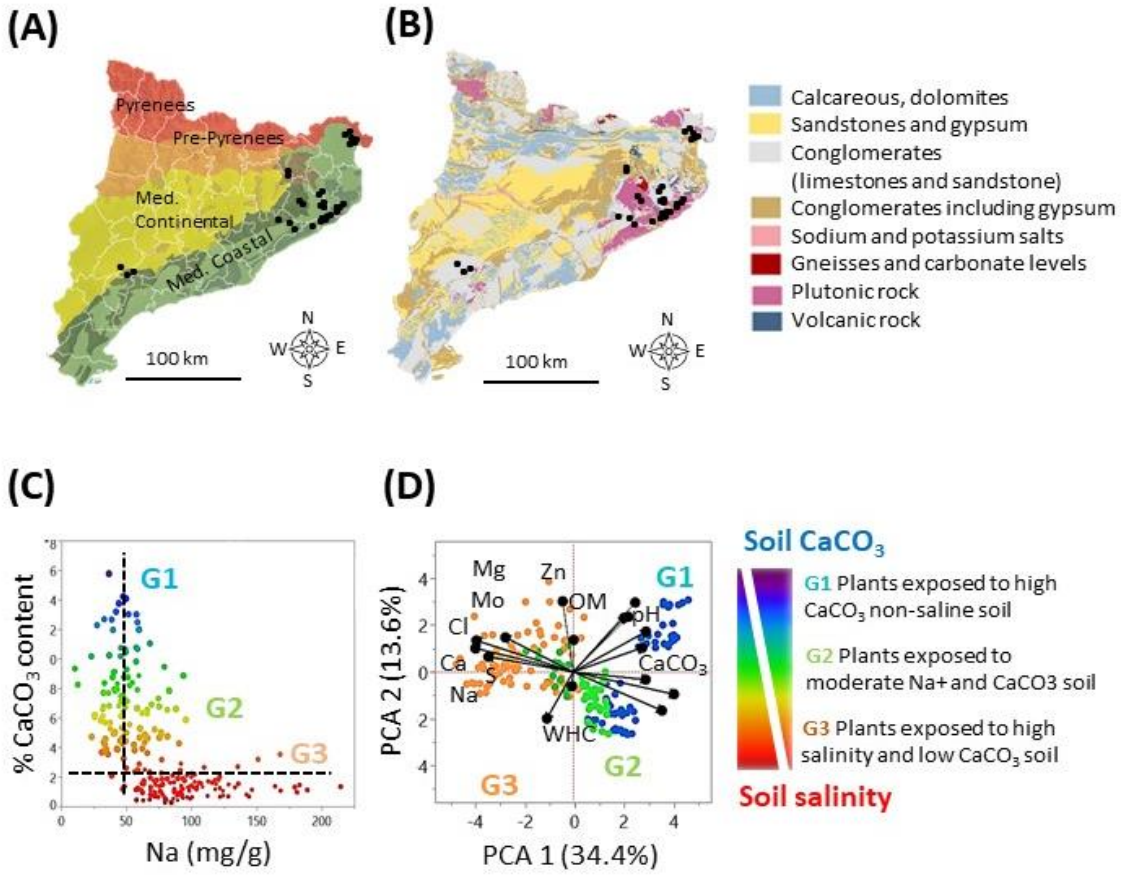


Figure 2: Climatic and soil factors from native Catalan *A. thaliana* demes. (A) Demes located on Catalonia climatic map from Meteocat.com **(B)** Geological map from Institut Cartogràfic i Geològic de Catalunya (ICGC). Black dots represent exact deme glocalization. **(C)** $CaCO_3$ and Na^+ native soil content from Catalan demes x-y plot. **(D)** Principal component analysis using soil parameters from native populations. Different colors indicate different plant groups. Dark blue indicates more $CaCO_3$ soil content while red indicates more Na^+ content in native soil.

4.2 Analysis of common garden soils used for field experiments

Plant fitness was assessed in field common garden located in LP (alkaline), BLA (saline) and AE (alkaline-saline) common garden using 12 demes representing groups previously made G1-G3. Saline common gardens have an EC around 2 dS/cm while alkaline common gardens have higher pH than saline and approximately 18% $CaCO_3$ content (Figure 3A).

Available soil fraction from the common garden was analyzed showing statistical differences in each element Figure 3B and Supplementary F3. Characteristically, higher Na^+ concentration were detected in the saline common gardens (AE 160 ug g^{-1}; BLA 124 ug g^{-1}) than in alkaline soil. Moreover, reduced Fe and higher Ca concentrations were observed in alkaline soil in comparison to saline common gardens. Ionome profile together with pH, EC and $CaCO_3$ contents validate our soils as representative candidates to perform experiments under saline (BLA), alkaline (LP) and alkaline saline (AE) conditions.

(A)

Soil location	pH (1:5)	EC dS/cm	%$CaCO_3$
AE	8.16 ± 0.27	2.12 ± 0.08	20.18 ± 1.19
BLA	6.30 ± 0.33	1.93 ± 0.18	2.00 ± 1.25
LP	7.93 ± 0.20	0.89 ± 0.28	16.4 ± 1.58

Soil location	Geology	Texture
AE	Limestone	Clay-loam
BLA	Granitoid	Sandy-loam
LP	Limestone	Clay-loam

(B)

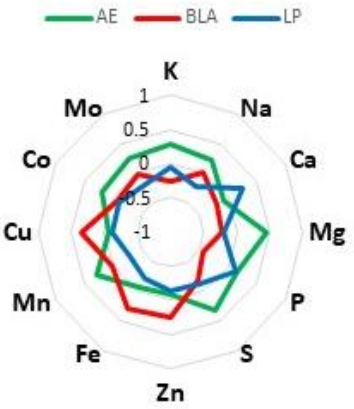


Figure 3: Physical, chemical, and geological soil analysis from common garden. (A) pH, EC, %$CaCO_3$ content, parental rock and texture **(B)** Ionomoic analysis of available nutrients in the soils. Soils were analyzed in 2017 and 2018. *n=10*. Red color indicates BLA as representant of saline soil, blue LP as calcareous soil and green AE as alkaline-saline soil.

4.3 Fitness assessment in common garden experiments under field conditions

Different Catalan demes were sown in November 2016 and 2017 to mimic natural conditions. In February, seed germination was observed. In AE common garden, adverse climatic condition like flooding and dry periods in the germination stage, and herbivory at seedling stage produced mortalities in plants making difficult to assess plant performance in this location. Also, elevated clay content in AE produced high water retention during early spring causing flooding stress to plants. Contrastingly, in late spring under high evapotranspiration days the soil cracked causing both drought and mechanic stress to the plants.

For these reasons, only a few data were recorded in 2017 at AE (see Figures 4 and 5). This obviously hampered our aim to screen for the behavior of populations in an alkaline-saline common garden under field conditions. However, we did not desist, and we used plant performance in saline and alkaline common gardens in the greenhouse to test 3 plant groups previously made based on the results got in the common garden experiment performed 2017-2018 under field conditions. In the greenhouse, rosette diameter as a proxy of growth recorded every week for 8 weeks. In the saline common garden, plants from the native saline habitat G3 perform better than G2 or G1. Contrarily, plants native from moderate calcareous content, perform better in the calcareous common garden. Figure 4 A, C, and Supplementary F4. The last measure of rosette diameter was correlated with the content of Na^+ and $CaCO_3$ in native soil. (Figure 4B and Supplementary F4). According to Busoms [86], plants from siliceous coastal location (G3) were able to growth better in neutral pH saline common garden showing a positive correlation with sodium soil content in their native habitat. On the other hand, in our research, inland populations (G1) were able to growth better in calcareous common gardens and rosette diameter presented a positive correlation with the $CaCO_3$ content in native soil in the years 2017 and 2018. These results differ from those of Terés, [88] who reports less obvious correlations between rosette diameter and CaCO3 content of native soils.

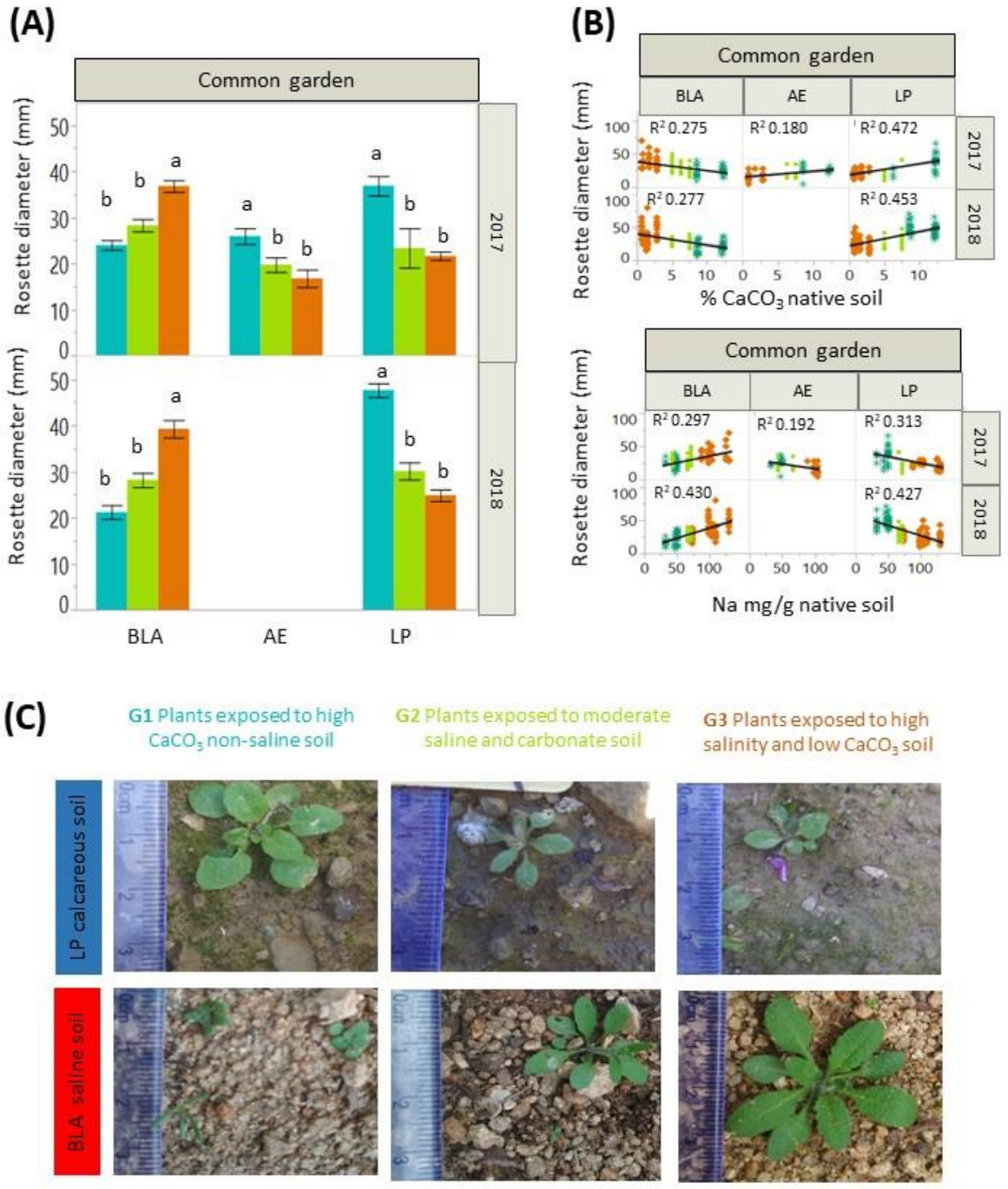


Figure 4: Plant growth in common garden experiment 2017-2018. (A) Rosette diameter bar plot separated by common garden and plant group. As a common garden soil: BLA saline; LP calcareous; AE Alkaline saline. As a plant group: G1_Plant tolerant to carbonate in blue; G2 Plants intermediate tolerance in green; G3 Plants tolerate to salinity in orange. **(B)** Plant rosette diameter *vs* native $CaCO_3$ and Na^+ soil content linear regression. Different colors are indicated plant group G1, G2 G3. **(C)** Picture of different plant groups in BLA and LP common garden.

Although some differences in the production of siliqua number were observed between the years 2017 and 2018, the trend is similar. In the saline common garden, plants from the native saline habitat G3 performed better than G2 or G1. Contrarily, plants native from soils with moderate calcium carbonate contents performed better in the calcareous common garden (see Figure 5A). Few individuals survived in AE common garden in 2017 showing no significant differences among groups. Siliqua numbers, a proxy of reproductive fitness was correlated with the content of Na^+ and $CaCO_3$ in the native soil (Figure 5B and Supplementary F5). According to Busoms [86], coastal plants (G3) were able to produce higher offspring than inland plants (G1) in siliceous saline common garden demonstrating an event of local adaptation driven by soil sodium contents in their native habitat. Furthermore, according to Terés, [88] inland populations (G1) were able to produce more siliqua in calcareous common gardens presenting positive correlation with native $CaCO_3$.

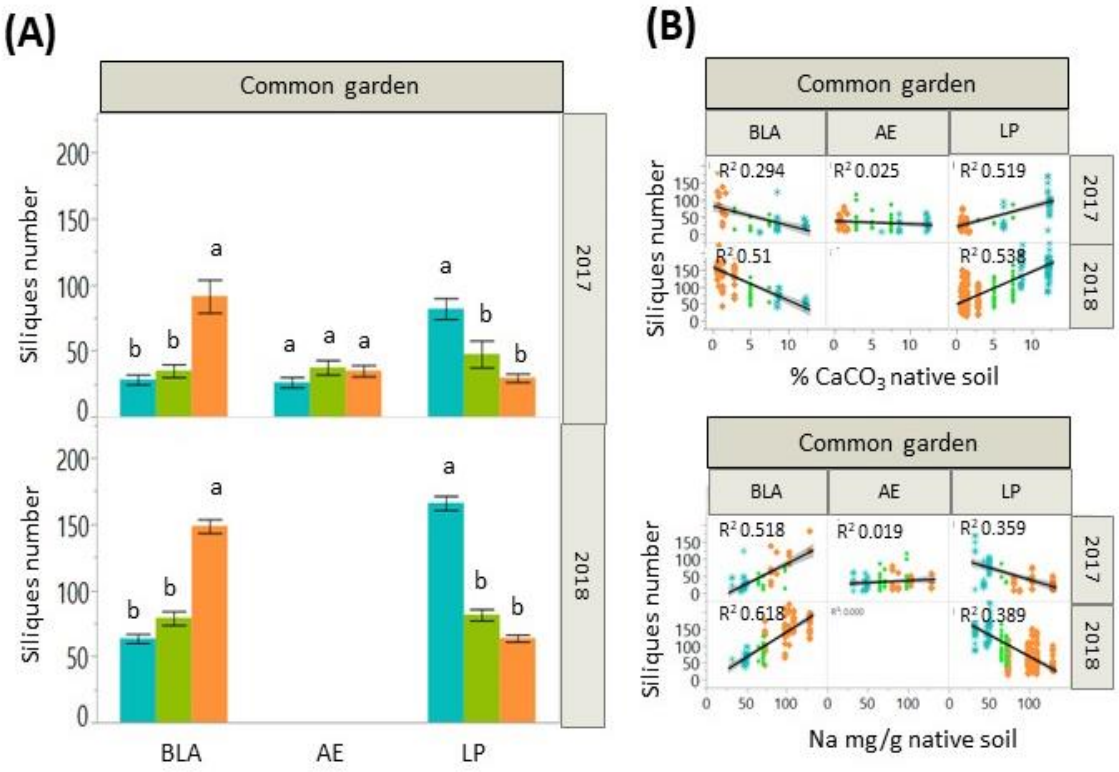


Figure 5: Fitness performance in common garden experiment 2017-2018. (A) Silique's production bar plot is separated by common garden and plant group. As common garden soil: BLA saline; LP calcareous; AE Alkaline saline. As a plant group: G1_Plant tolerant to carbonate in blue; G2 Plants intermediate tolerance in green; G3 Plants tolerate to salinity in orange. **(B)** Siliqua production fitted by linear regression into native $CaCO_3$ and Na^+ in soil. Different color indicates plant group G1, G2 G3.

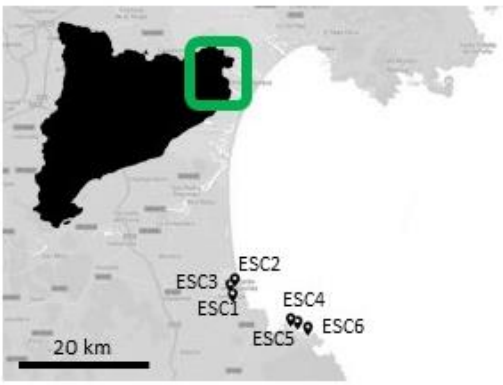
4.4 Analysis and selection of alkaline saline soils for greenhouse experiments

Difficulties in detecting plant performance in the saline-alkaline common garden experiment in the seasons 2017-2018 made us to a re-formulate experimental set-up. A pot experiment was designed using native soil in a greenhouse structure installed at the UAB. Universitat Autònoma de Barcelona is located 20 km from the Mediterranean Sea and is located in the same climatic region as BLA and ESC, being a suitable location (Figure 6A). In the greenhouse, plants were protected from herbivory, watered as they needed, climatic conditions were not controlled to perform a semi-controlled potting experiment. In this point, we chose to focus on the saline *vs* saline-alkaline perspective. Eliminating calcareous soil common garden soil allowed us to increase the number of replicates.

Although AE apparently is a good representative for saline-alkaline soil, elevated content in clay makes it difficult to compare with saline sandy location of BLA. For these reasons, a preliminary analysis of soil characteristics was done to select a potentially adequate saline-alkaline soil with lower clay content. Soils were excavated at 10 cm depth at different points at L'Escala (Girona). Soil determination standard methods were used to detect carbonate content, salinity, pH, and texture to select the best location. (Figure 6A-B). As control soil we used silicon-rock-derived soil from Blanes (Marimurtra Botanical Garden) already used in previous experiments [86] .

The finally selected soil, representative for the coastal limestone-derived calcareous soils was ESC1 located in the coastal region of L'Escala Alt Empordà (Girona Province). This soil was selected because of its texture and EC are similar to those of the Blanes soil (Figure 5C). The main differences between BLA *vs* ESC1 soil are soil pH (6.5 *vs* 8.4) and $CaCO_3$ content (2% *vs* 18%) (Figure 6C). According to these differences, we can classify BLA as saline soil and ESC1 as a proper candidate for alkaline-saline soil. Alkaline-saline soil selected (ESC1) have a similar sandy soil texture to saline soil (BLA) which can be related to other chemical and physical soil parameters. Sandy soils have high aeration, low water holding capacity, and low CEC because big particles retain fewer nutrients. Available nutrients from selected soil and control soil were determined. As we expected in ESC a reduction of nutrients can be caused by its high pH. In fact, only Mg, P, Fe, Mn and Zn show a statistically reduction in ESC1 soil. Figure 6D and Supplementary F6. The previous author performed a geological characterization of Empordà Basin [220] analyzing pH, CEC and $CaCO_3$ content in the parameter analyzed in this thesis is in the range found by this author.

(A)



(B)

	pH $_{(1:5)}$	EC (dS/cm)	% $CaCO_3$
ESC1	8.44 ± 0.05	1.8 ± 0.21	18.86 ± 1.50
ESC2	8.02 ± 0.09	1.2 ± 0.13	15.02 ± 3.50
ESC3	7.96 ± 0.10	1.3 ± 0.19	9.43 ± 0.60
ESC4	8.22 ± 0.10	1.2 ± 0.15	6.31 ± 2.40
ESC5	8.55 ± 0.18	2.37 ± 0.10	6.27 ± 3.10
ESC6	7.52 ± 0.38	2.8 ± 0.40	2.0 ± 1.20

ESC1 ESC2 ESC3 ESC4 ESC5 ESC6

(C)

Soil location	pH $_{(1:5)}$	EC (dS/cm)	% $CaCO_3$
BLA	6.30 ± 0.33	1.93 ± 0.18	2.00 ± 1.25
ESC1	8.44 ± 0.05	1.8 ± 0.21	18.86 ± 1.50

Soil location	Geology	Texture
BLA	Granitoid	Sandy-loam
ESC1	Limestone	Sandy-loam

(D)

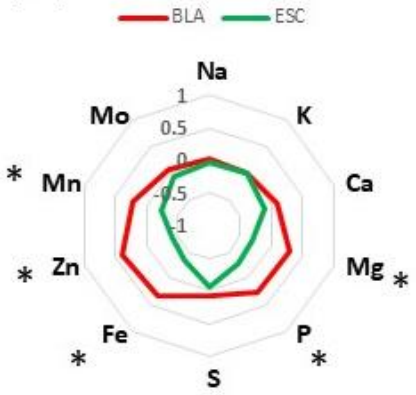


Figure 6: Alkaline-saline soil selection. (A) Candidate soils map located in L'Escala village **(B)** Picture from candidate soil location. **(C)** Soil pH, EC and % $CaCO_3$ average and standard deviation table. **(D)** Available nutrient radial plot. Statistical differences between nutrients in both soils are indicated by an asterisk (*n=6*).

4.5 Fitness assessment under semi-controlled conditions (greenhouse)

Two coastal soils influenced by salinity from the sea with contrasting $CaCO_3$ content were selected to test Catalan *A. thaliana* natural variation performance under saline neutral and saline-alkaline stress. These soils were excavated from their native location and used as a treatment in a semi-controlled experiment. Plant material for the saline-alkaline common garden includes contrasting Catalan natural populations which are shown to be tolerant to either salinity or moderate carbonate. We hypothesized that some extreme accession with high tolerance to $CaCO_3$ or Na^+ content could perform better than others under double stress.

Sowing was performed in November 2019 to mimic natural germination conditions. Three seeds were sown in each plot, 15 pots were used for each deme in two contrasting soils: saline and saline-alkaline. Seeds started to germinate in February and all demes were growing in March 2019. In alkaline-saline soil, germination delay was observed for all demes. Unfortunately, the exact delay was not properly recorded. Rosette diameter was recorded every week and when plants were 5 weeks old two or three leaves from each deme and soil were removed to analyze nutrient concentrations. Siliqua production was counted at maturity between April and May 2020.

Plant growth and siliqua production on alkaline-saline soil was lower than on saline soil. In saline soils, plants which come from coastal location grew better and produced more siliqua than those from inland origin. This agrees with previous work from Busoms 2015 and Terés 2019. The opposite situation was found in the alkaline-saline soil treatment. Extreme tolerant demes from saline or carbonate grew less and produce fewer siliques in comparison to non-adapted demes. Demes without local adaptation or intermediate demes belonging to G2 showed better performance than demes from tolerant to alkali or salinity stress alone (G1 and G3) (Figure 7 A-C. Supplementary 9 and 10). When plant performance (growth and siliqua number) was plotted according to the corresponding soil $CaCO_3$ or Na^+ contents a higher correlation was found for siliqua production, while few correlations were found in ESC common gardens. In saline common gardens we observed correlations similar to those reported in field experiments by Silvia Busoms from 2013 to 2015 [86] (Figure 7 B-D. Supplementary datasets 11 and 12).

In our initial hypothesis, we considered that extreme tolerance to saline or calcareous may be related to better performance on alkaline-saline soil. Interestingly, however, we observed that the demes with intermediate tolerance were fittest on alkaline-saline soil. Saline-alkaline stress

cause more deleterious effects than single stress. It can be explained by the synergetic effect from double stress in plants [43]. Local adaptation is a fixed genetic variation that provides an advantage to one specific climatic and/or edaphic condition [109][221]. In this case, alkaline-saline stress imposes severe constrains for plants adapted to saline (G3) or alkaline (G1). Meanwhile, G2 which shows low signature of local adaptation to calcareous or Na soil, shows better plant performance in alkaline -saline soil. This may be due to the observation that group 1 was adapted to calcareous soil with low EC content, while G3 was adapted to high salinity on siliceous parental rock. Plants from group 2, were able to resist the combination of both stresses but only at a mild level under semi-controlled greenhouse conditions. This results clearly indicates that different traits must be responsible for tolerance to saline and alkaline saline soils.

To further characterize these differences, leaf concentrations of selected mineral nutrients grown on saline (BLA) and alkaline-saline soil (ESC) were analyzed in common gardens, and a radial plot was drawn. Although Figure 6F shows lower levels of available nutrients in soils from ESC *vs* BLA (statistically significant for Mn, Zn, Fe, P, Mg) the differences in plant nutrient levels were relatively small. On ESC soil plants had lower leaf concentrations of P, Mn, K and Ca (Figure 6E + Supplementary F6). In the radial plot presentation, the intermediated group, G2, shows higher nutrient uptake than G1 or G3. Elements statistically significant were S, Fe, Zn, Na and Ca (Figure 6F + Supplementary F6). The ability of G2 plants to maintain higher leaf concentrations of Zn, P, and Fe, nutrient typically limiting under alkaline conditions, in addition to maintenance of Ca and K concentrations important for the maintenance of K^+/Na^+ and Ca^{2+}/Na^+ ratios and ion balance under salinity seem to be key traits for their better performance under alkaline saline soil conditions.

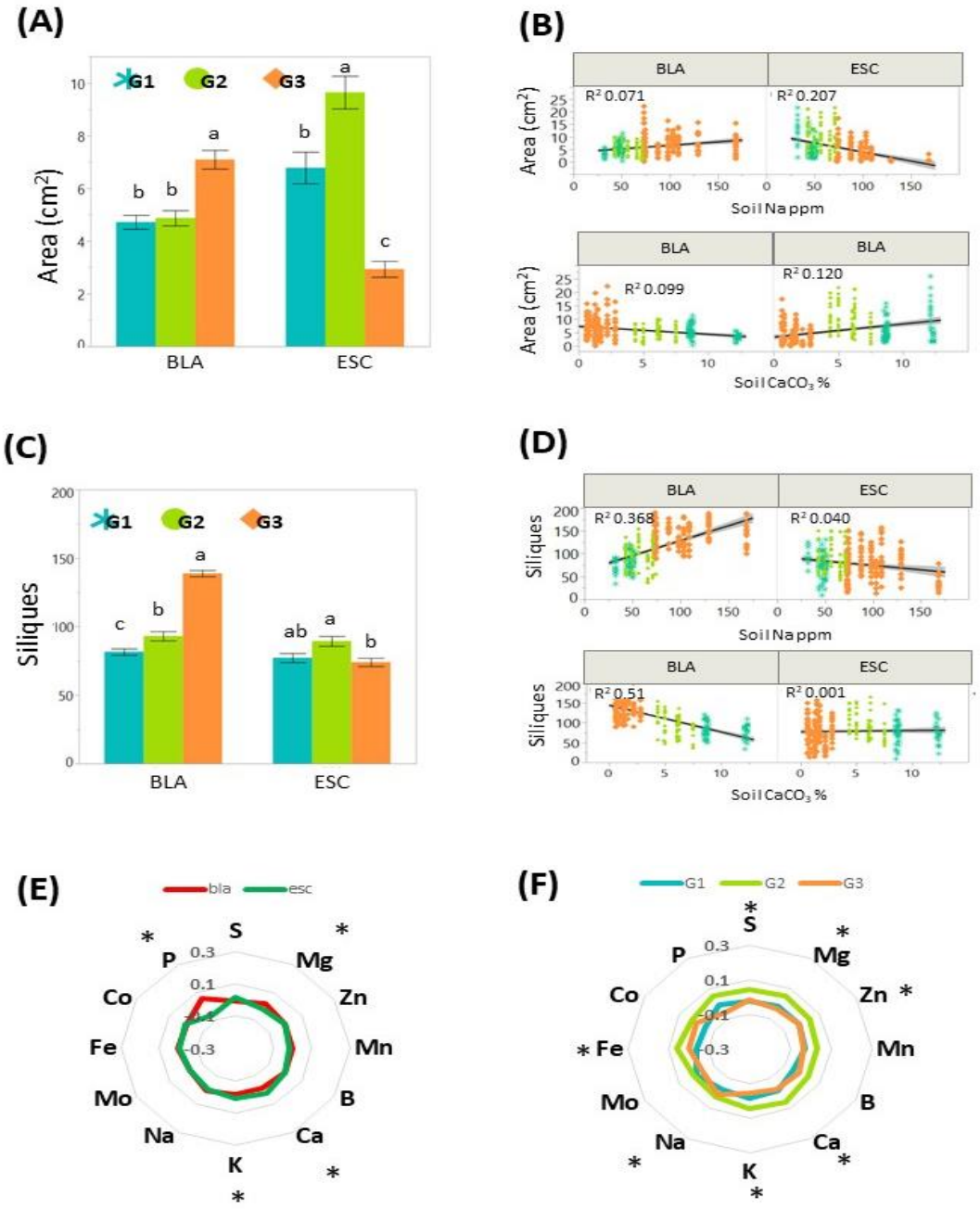


Figure 7: Plant growth in a potting greenhouse experiment 2020. As a common garden soil: BLA saline; LP calcareous; ESC1 Alkaline saline. As a plant group: G1_Plant tolerant to carbonate in blue; G2 Plants intermediate tolerance in green; G3 Plants tolerate to salinity in orange. **(A)** Rosette diameter bar plot separated by common garden and plant group. **(B)** Plant rosette diameter *vs* native $CaCO_3$ and Na^+ soil content linear regression. **(C)** Silique bar plot separated by common garden and plant group. **(D)** Siliqua *vs* native $CaCO_3$ and Na^+ soil content linear regression. **(E)** A radial plot from Ionomic available soil fraction. Soils were analyzed in 2019. *n=6*. The red color indicates BLA as a representant of saline soil and dark green ESC1 alkaline-saline soil. **(F)** A radial plot showing ionomic data from leaves cultivated in BLA or ESC soil in greenhouse 2020 experiments. Asterisks indicate statistical significances p-value<0.05

4.6 Fitness assessment under controlled conditions (hydroponic and plates)

In the greenhouse experiment under semi-controlled conditions, germination delay in alkaline saline soil were observed for all demes. Germination is a key step in plant establishment, for this reason, germination assays were repeated under controlled conditions using plates to score differences observed in greenhouse experiments. In the germination assays different treatments were tested: control conditions (pH 5.9), alkaline 10mM $NaHCO_3$ pH 8.3; slightly acidic saline conditions 50mM NaCl pH 5.9 and alkaline saline conditions 40 mM NaCl + 10 mM $NaHCO_3$ pH 8.3 (Figure 8A).

Lines from G1 present a cline, showing a progressive reduction in germination in comparison to group G2 and G3 in plates with alkaline conditions. A different situation is observed in saline plates. All demes had lower germination rates under stress than under control conditions. No differences among groups were observed under saline stress. Contrastingly a strong germination inhibition was found in the G3 group in the alkaline-saline plates (Figure 8A + Supplementary F8). After 10 days, G3 seeds were transfers to control conditions and almost 50% were able to recover the germination ability. Germination is a critical step in plant establishment. In plate experiments, a strong germination inhibition is found especially in G3 under alkaline and alkaline-saline stress. This can explain the disruption in the coastal distribution of natural *A. thaliana* demes in the area with alkaline saline soils (Figure 8 B). Coastal plants may have severe problems establishing under natural alkaline-saline conditions. This can be explained by low $CaCO_3$ content and the slightly acidic to neutral pH of the soils in their native habitat [86].

Few local adaptation studies are performed in early-life plant stages. However, Postma and Agreen 2016 [222] have demonstrated that adaptive differentiation during early life stages can be important for understanding the ecology and genetics of local adaptation. In our case, any of our stocks is native to alkaline-saline soil. The conditions of alkaline-saline soils are too harsh for successful *A. thaliana* seedling establishment.

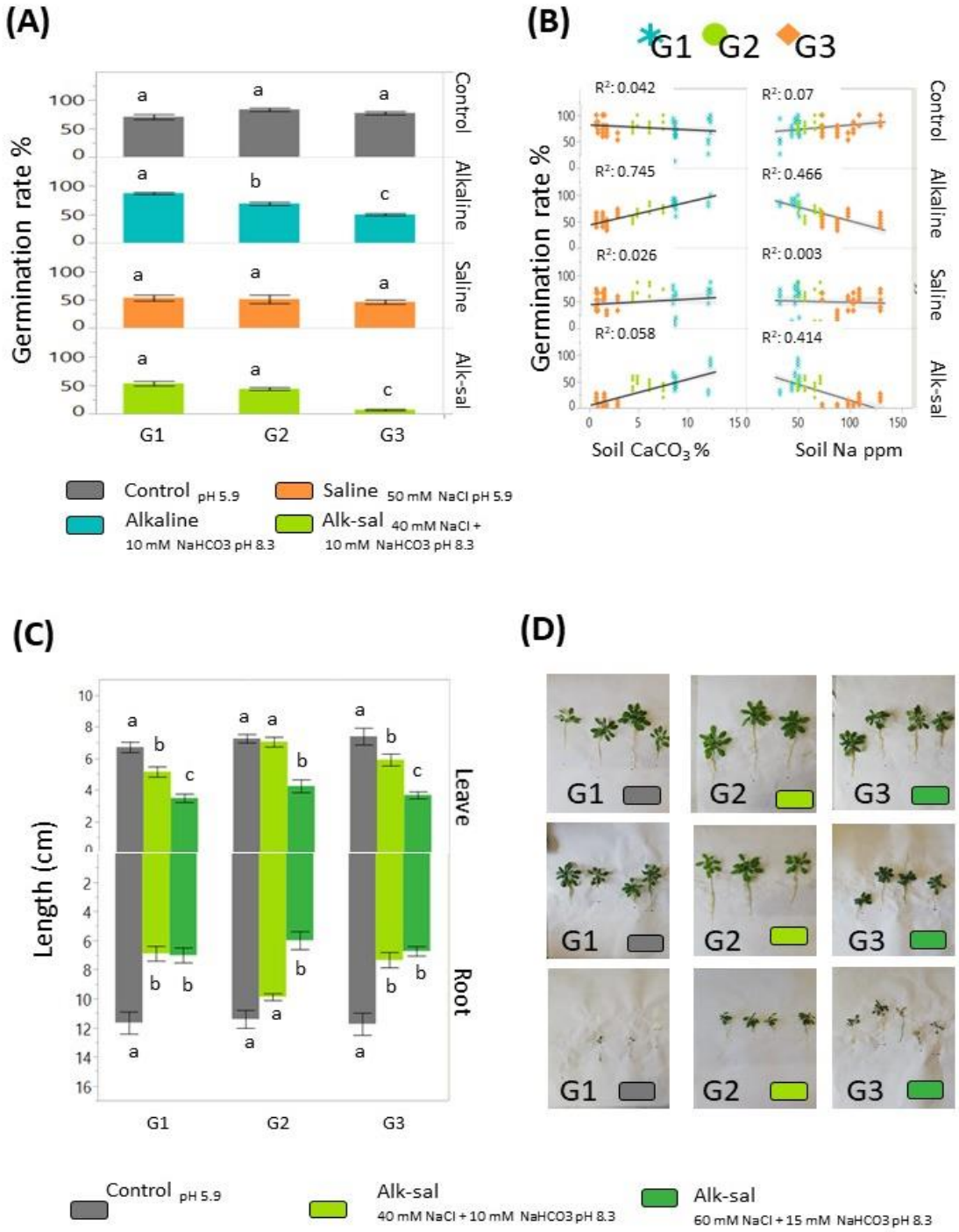


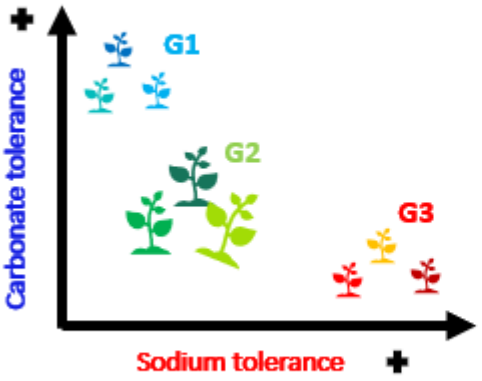
Figure 8: Plant growth in hydroponic and plate culture. Treatment: control conditions pH 5.9; alkaline 10mM $NaHCO_3$ pH 8.3; neutral salinity 50mM NaCl pH 5.9 and alkaline saline 40 mM NaCl + 10 mM $NaHCO_3$ pH 8.3. As a plant group: G1_Plant tolerant to carbonate in blue; G2 Plants intermediate tolerance in green; G3 Plants tolerate to salinity in orange. **(A)** Germination rate (%) bar plot separated by treatment and plant group. **(B)** Germination rate (%) *vs* native $CaCO_3$ and Na^+ soil content linear regression. **(C)** Leaves and root length bar plot separated by treatment and plant group. In this case treatments are control conditions pH 5.9; alkaline-saline 40 mM NaCl + 10 mM $NaHCO_3$ pH 8.3 and alkaline-saline 60 mM NaCl + 15 mM $NaHCO_3$ pH 8.3 **(D)** Picture from plants 30 day old summited to treatment for 15 days.

The hydroponic culture in the growth chamber was used to remove any climatic and soil-derived factors that may alter plant response and confirm phenotype from the greenhouse. Plants were cultures for 15-days under control condition (Hoagland ½ pH 5.9) and alkaline- saline treatment 40 mM NaCl + 10 $NaHCO_3$ and 60 mM + 15 mM $NaHCO_3$. Treatment solutions were renewed every 3 days and rosette diameter and root length were measured. After 15 days in treatment, fresh weight was measured. As the main results, a huge reduction in all parameters was detected under both alkaline-saline treatments (Figure 8C). Higher mortalities were produced in G1 lines, especially in 60 + 15 mM treatments. This can be because in hydroponic we added a total of 50- or 75-mM Na^+ favoring plant responses from salt-tolerant lines (Figure 8D).

In general, results obtained in hydroponics were similar to those got in the greenhouse. The observed differences between greenhouse and hydroponic can be due to the fact in the soil experiment plants germinated under treatment conditions while in hydroponic experiments seeds germinated under control conditions and 15-day old seedlings were transplanted to treatment solutions. Moreover, salinity stress was higher in the hydroponic treatment than in the soil experiment with a moderate electric conductivity around 2 dS/cm. The higher salinity applied in hydroponics favored demes from the coastal location. Another important difference comparing soil and hydroponic approaches is the lower incidence of nutrient availability constraints in hydroponics. Regardless these differences, some lines showed tolerant and sensitive phenotypes in both culture media. These lines were selected to perform further experiments.

5. Conclusions

- Germination of all-natural *A. thaliana demes* is severely delayed and the seedling establishment in most demes is aborted under alkaline- saline soil conditions. Demes from moderate calcareous soil present less reduction in germination. This indicates that alkaline tolerance is more relevant property for tolerance to alkaline saline conditions than salinity tolerance evolved on siliceous saline soils with slightly acidic or near neutral pH conditions.
- This difficulty to germinate or to establish a viable seedling is a key factor for understanding the discontinuity in the coastal distribution of *A. thaliana* in the NE Catalonian coast where alkaline-saline soil occurs.
- Alkaline-saline conditions impose a more severe stress on growth, mineral nutrition and siliqua production than the single factors individually.
- Plants from locations with moderate contents of $CaCO_3$ and Na^+ show better performance under alkaline-saline stress than plants native to soils with either high $CaCO_3$ or high Na^+ content.
- Field responses are reproducible under controlled conditions in hydroponics to a certain extent. However, the concentrations of stress factors (alkalinity, salinity) must be explored in detail and adjusted to mimic the natural conditions.



Plant Group	G1	G2	G3
Germination	X	X	
Growth		X	X
Nutrition		X	
Siliqua		X	

F9. Graphical conclusions. Designed by Maria Almira modified by the author.

General discussion

Agriculture in the 21st century faces multiple challenges. Anthropic climate change is favoring the alterations of the soil surface chemistry which can be faster than the adaptation ability of plants to the new situation. Crop yield reduction can produce a food supply problem within a near future. The world population is expected to reach 2.3 billion humans by 2050. Enhancing food production using fewer natural resources is the greatest challenge in this century [89]. Although, climate change is a global problem, in arid and semi-arid areas consequences can be more intense than in other latitudes. In the Mediterranean areas, soil salinization can be aggravated by the presence of lime-stone-derived soils. Moreover, these areas are densely inhabited. These conditions may aggravate the impact of climate as agriculture decay, compromising food security, depress the local economy, and increase social problems and migratory crisis [223][224].

Alkaline-saline soil reduces severely crop productivity. Sodium excess produces osmotic and toxic effects while high pH reduces essential nutrient uptake availability and cause metabolic imbalances [225]. The current understanding of plant response to bicarbonate stress is limited and little research has been performed using *A. thaliana.* To date, there is no specific mechanistic model for bicarbonate stress in terrestrial plants [34]. At this point, more funding and efforts are needed to determine the specific adaptative mechanism of plants under bicarbonate stress and alkaline-saline stress.

The major impact of this project is its contribution to the knowledge of physiology, molecular and genomic basis of alkaline and alkaline-saline responses in *Arabidopsis thaliana.* This new knowledge can be included in breeding programs to optimize crops to the new climatic conditions. Understand plant biological mechanisms may give scientists new tools and the possibility of predict plant behavior in new climate conditions, having a huge socioeconomic impact [90].

Differences in plant pH and bicarbonate perception

High pH soils reduce nutrient availability to plants [4]. Iron, among others, is one of the most affected elements in alkaline soil. Iron chlorosis is one of the major abiotic stresses affecting fruit trees like Citrus and other crops on calcareous soils [226]. For these reasons, several studies have been performed to determine the specific adaptative plant responses to iron deficiency. However, responses under field conditions are more complex.

Here in chapters 1-2, we elucidated physiological and molecular plant responses differentiating high pH effects from those induced by 10 mM bicarbonate. Clear differences between both stress factors were found. In the moderately bicarbonate tolerant plants, high pH produces more deleterious effects than bicarbonate and in transcriptomics, sensitive and tolerant demes show different profiles in the high pH *vs* bicarbonate comparison. Similar results heve been reported by other authors. At the physiological level, the extremely calcicole plant *Parietaria diffusa* has been submitted to (-Fe) and (-Fe+bic) conditions. Under -Fe conditions it has been observed that the mechanisms of iron uptake are more activated than under +bic treatment while Parietaria showed a greater production and exudation of phenolics and organic acids when grown in the presence of bicarbonate [32]. This also agrees with Lucena (2007), who using *A. thaliana* detected a bicarbonate-induced reduction in the relative expression of genes related to iron uptake mechanism [137]. Furthermore, recent multi-omics research in *Oryza sativa* under different pH ranges (4 to 8), concludes that high pH modifies oxidative status in plants and that a root barrier is produced to avoid undesirable toxic elements [227]. In *A. thaliana*, specific bicarbonate signatures have been followed using sodium bicarbonate and sodium acetate treatments in plate culture. These authors revealed differential responses between both stresses and proposed EBS1 related to brassinolide signaling as a positive regulator of bicarbonate tolerance in *A. thaliana* [63]. Another possible candidate GsBOR2 overexpressed in Arabidopsis exhibited enhanced alkaline tolerance compared to wild-type plants in $KHCO_3$ treatment but not to high-pH stress [158].

Higher toxicity of alkaline pH, achieved with organic buffers, than of bicarbonate may be due to the observation that chemical buffers have some undesirable properties such as chemical reactivity, stability and osmotic potential, which can have negative effects on plants. This deleterious side effect can be reduced by reducing buffer amount and changing nutrient solution more often. In all case, pH of the nutrient solution needs to be increased before HCO_3^- or $CaCO_3$ addition. In our experiments, all treatments had a mix of buffers. Depending on the final pH buffer proportion was changed but the same final concentration was maintained. Therefore, we consider that the better performance of bicarbonate tolerant plants under bicarbonate exposure than under the high pH treatment was rather due to a better activation of tolerance mechanisms by bicarbonate than by the organic buffer alone.

In fact, the quickest differential response to bicarbonate exposure in tolerant and sensitive *A. thaliana* was the hyperpolarization of the root plasma membrane potential (Chapter 1 Figure 6).

This hyperpolarization after only 5 minutes of bicarbonate exposure was much more intense in the tolerant $A1_{(C+)}$ than in the bicarbonate sensitive deme. As this response was abolished by addition of AZ, an inhibitor of external carbonic anhydrase (CA), we can speculate that membrane hyperpolarization is a fast mechanism of bicarbonate perception in the tolerant plant. This perception, in the tolerant $A1_{(C+)}$ leads to a quick (3h), bicarbonate-specific upregulation of apoplastic *βCA3* and of *βCA4*, the membrane located CA which in the sensitive deme was strongly downregulated within the same time frame (Chapter 1 Figure 6D). The joint action of both CAs may lead to a fast dehydration of bicarbonate in the apoplast and a hydration of CO_2 in the cytosol. The resulting bicarbonate may fixed by PEP carboxylase leading to oxaloacetate and enhancing organic acid production. Further research is required to confirm this way of CO_2/HCO_3^- processing in roots of bicarbonate tolerant $A1_{(C+)}$.

Germination, a tolerance key point in alkaline-saline stress

Germination is a key point in plants establishment which is strongly regulated to select the best moment to initiate germination [228]. Seed dormancy is a protective mechanism that prevents germination in an unfavorable environment or avoids intraspecies competition being an important trait for adaptive evolution [229][230]. Calcifuge plants have difficulties in germination and plant establishment on calcareous soils [49] and iron deficiency seems to be one of the factors which increase seed dormancy period in plants [50]. Previous studies with *Leymus chinensis* under different neutral and alkaline salts have reported strong interactions between salinity and alkaline pH on seed germination. Alkalinity in absence of salinity (low EC) poorly affected germination, while strong negative effects on germination have been observed under combined stress [54]. Here we detected a strong germination inhibition in seeds of *A. thaliana* exposed to alkaline treatments, especially in demes native from habitats with low soil $CaCO_3$ contents (see Chapter 1 Figure 2 and Chapter 4 Figure 8). Germination inhibition was further potentiated in alkaline-saline soil, and this inhibitory effect was also related to the soil carbonate concentration in the plants' native habitats. In coastal plants native to habitats with extremely low soil $CaCO_3$ soil germination was most inhibited. Luckily, however, some demes were able to recover their germination ability when transferred to control conditions.

In NE Catalonia, *A. thaliana* is distributed all along the Mediterranean coast excepting a disruption in areas with alkaline-saline soils as for example in the Baix and Alt Empordà region with soils classified as xerothents [231]. This thesis covers the study of physiological plant stages

(germination, growth, nutrition and reproduction) under alkaline-saline conditions (Chapter 4) to find an ecological response to this absence. Strong germination inhibition of coastal plants from saline-siliceous soils submitted to alkaline-saline stress may explain the lack of distribution in alkaline-saline soils. Low germination rates in alkali-saline soils reduce plant establishment and colonization of these sites with alkaline coastal soils in NE Catalonia. If this germination inhibition is scalable worldwide, it could explain the calcifuge distribution of *A. thaliana* population observed in Chapter 3 [88].

Nutrition, translocation and efficient use

Nutrition under alkaline conditions is a big challenge for plants [133]. High pH treatments reduce micronutrient availability producing a severe constraint affecting plant performance [40][43][52]. Inhibition of nutrient translocation under alkaline treatment has previously been described in several plant species. For example, in *Actinidia lindl* exposed for 28 days to 20 mM $CaCO_3$-$NaHCO_3$. inhibition of Fe translocation to shoots by the accumulation water-soluble apoplastic Fe has been observed. In this situation, the high tissue content of Fe is unavailable for biochemical processes [62]. In Chapter 1, we found that bicarbonate tolerant demes were able to translocate more nutrients to upper parts when submitted to 10 mM bicarbonate in hydroponic solution. Opposite, the bicarbonate sensitive lines accumulate more nutrients in roots due to an inhibition of their translocation capacity. Under alkaline-saline conditions (Chapter 4 Figure 7), however, neither plants with highest carbonate tolerance nor those with highest salt tolerance evolved on siliceous substrate performed best. Surprisingly, plants from group G2, with moderate tolerance to both stress factors had highest reproductive fitness and best nutrient profiles. This clearly demonstrates that salt tolerance mechanisms evolved in coastal siliceous habitats with slightly acidic to neutral soils does not confer tolerance to alkaline-saline conditions.

Bicarbonate blocks the expression of genes related to iron uptake in Arabidopsis [190] and *Citrus limon* (L.) [130]. In this thesis, time course of relative expression of genes related to the iron uptake (Chapter 1 Figure 7) revealed different strategies. The bicarbonate tolerant line $A1_{(c+)}$ activates *AHA2* to decrease the pH in the apoplast thus providing a better environment for the activity of FR (Terés). Contrastingly, T6, the sensitive line activates *IRT1*. Notorious differences were found after only 3 hours exposure impelling the possibility of interesting short-term responses. After 48 hours, this bicarbonate induced plant response decay, as has previously been shown by Lucena [190].

Maintenance of Fe translocation under bicarbonate stress is a key factor for plant performance on calcareous soil; but Fe is not the only critical factor. Leaf ionome profiles (Chapter 1 Figure 3) revealed that the tolerant line also takes up and translocates more P and S than $T6_{(c-)}$ under bicarbonate exposure. Transcriptomic data (Chapter 2), showed the activation of multiple genes involved in glucosinolate production in the tolerant line which is accompanied by high demand of S production [172]. In sensitive lines, root microarray data pointed to a typical P deficiency response [232]. In Chapter 3, using worldwide population, plants with higher relative growth under bicarbonate were able to translocate more Zn, P, S (Chapter 3, Figure 4). Phosphorous and Sulphur are macronutrients involved in many metabolic processes like photosynthesis, respiration, biosynthesis of primary and secondary metabolites [233], while Zn is a micronutrient required in multiple enzymes. From previous research, interactions between S-Fe and P-Fe-Zn were studied. Sulfur scarcity inhibits part of the mechanism of iron uptake under Fe deficiency. This process limits the unspecific transport into the root of potentially toxic divalent cations by IRT1 and NRAMP1 [234]. This agrees with our results, *IRT1* upregulation is the strategy of the sensitive lines under bicarbonate stress (Chapter 1, Figure 7). Phosphate starvation modifies the expression of genes involved in the maintenance of metal homeostasis such as Zn and Fe [235]. The Integration of P, S, Fe, and Zn nutrition signals in *Arabidopsis thaliana revealed the* potential involvement of PHOSPHATE STARVATION RESPONSE 1 (PHR1) PHR1 has been proposed as a regulator of responses not only to P scarcity but also micronutrient deficiency [236].

Taken together this information indicates that in addition to Fe, the ability to maintain nutrient homeostasis, especially of S, P and Zn under bicarbonate can be a good marker in breeding programs addressing plant tolerance to alkaline stress. In this case, plants with a higher ability to uptake and translocate P and S can be a good strategy.

Organic acids as osmotic, ionic and pH homeostasis.

Although no specific bicarbonate transporters or channels have so far been characterized in higher land plants, the possibility of membrane transport of bicarbonate by specific or unspecific anion transporting proteins cannot be excluded [34]. The high availability of large HCO_3^- soil concentration may compete with other anions for anion transporter proteins in plants. Under exposure to high HCO_3^- (from 5 mM to 20 mM), a strong inhibition of nitrate, sulfate, and phosphate uptake by roots has been observed. Such inhibition could be caused by competition between HCO_3^- and other anions in low anion specificity transport mechanisms [237].

Aquatic plants use bicarbonate as a main carbon source. In these planta two contrasted strategies are unrevealed (I) direct uptake by bicarbonate transporters and (II) CO_2 entrance by previous HCO_3^- hydrolysis mediated by external carbonic anhydrase [138]. These two strategies contrast in electrophysiological response and cytosolic pH. Several authors hypnotize the possible use of bicarbonate as a source of dark fixation in plants. Bicarbonate entering directly into the roots or produced from CO_2 by symplastic CA can be transformed into oxaloacetate (OAA) by PEP carboxylase. OAA may either be reduced to malate by malate dehydrogenase or enter directly into the Krebs cycle yielding different organic acids. A high organic acid production under alkaline treatments has frequently been observed [35] [58]. Several authors indicate that organic acid production can help nutrient uptake by acidifying apoplast and producing a favorable pH for ferric reductase enzyme. Moreover, organic acids can be involved in osmotic adjustment and anion/cation balance. Under conditions causing the inhibition of uptake of inorganic anions, enhanced production of organic acids can contribute to anion/cation balance [43]. However, an excess of organic acid production due to dark fixation of inorganic carbon in roots under bicarbonate exposure has classically been made responsible for the inhibition of Fe and or Zn translocation to the shoots in bicarbonate sensitive plants. Citrate and malate are strong ligands for Fe and Zn. Interestingly, high citrate concentrations can cause severe toxicity in yeast in an iron-dependent manner [238]. Under excess Fe or Zn, a preferential storage of Fe or Zn along with organic acids in root vacuoles has been an important mechanism for preventing toxicity [239]. Under sufficient or deficient micronutrient supply, however, vacuolar storage of organic acids may limit export to the xylem causing deficiency in the shoots. Interestingly, our transcriptomic analysis under bicarbonate exposure points to the regulation of several genes coding for proteins involved in the transport of organic acids and sugars (Chapter 2). Moreover, the candidate gene from GWAS analysis performed on plants growing on bic-rich soil was the *Tonoplast Dicarboxylate Transporter TDT*. The observation that the *tdt* knock out line performed better under bic stress than the wild type (Chapter 3, Figure 6A) supports the view that restriction of tonoplast transport of dicarboxylic acids in roots may favor the availability these chelators for Fe and or Zn transport to the shoots in the bicarbonate tolerant *A. thaliana*.

Transcriptomics

In Chapter 2, transcriptomic experiments were developed comparing plant responses to pH *vs* bicarbonate. Transcriptomic analysis after short exposure times (3 hours and 48) allowed us to detect stress induced changes in gene expression before any visible stress symptoms were

detectable. The main categories of genes responsive to 10 mM $NaHCO_3$ *vs* pH stress were oxidoreductases, pH homeostasis, transport and secondary metabolism activation. Similar alterations have previously been described by others [47][67][69] mostly in experiments performed with a concentration higher than 50 mM $NaHCO_3^-$. This difference further supports the view of the relatively high sensitivity of *A. thaliana* to bicarbonate. Moreover, it has to be considered that alkaline treatments using 50 mM, or even higher, $NaHCO_3^-$ actually represent saline-alkaline stress treatments because such solutions have an electric conductivity of at least 5 $dS.cm^{-1}$ [241].

Genome Wide Association Studies

Several previous genome-wide association studies performed with native populations of *A. thaliana* and the nutrient profiles under control conditions as a phenotype have revealed interesting genes responsible for nutrient transport. Examples are *HKT1* [94] involved in Na^+ transport, *HAC1* for arsenate reduction facilitating arsenite efflux [199] and *HMA3* involved in Cd uptake [198]. Here (Chapter 3) we performed a GWAS approach using a worldwide population of *A thaliana* submitted to soils with contrasting carbonate contents. Phenotype input data were physiological changes related to growth and nutrient profiles. Such traits have previously been observed as reliable indictors of differences in sensitivity/tolerance to alkaline conditions [39][41][62][70]. Local adaptation to calcareous soil conditions was more evident in Catalonian populations [19] than in the collection of European populations. One possible explanation might be a bias produced by the European soil dataset [24] as it does not include all *A. thaliana* population from this study and consequently excludes some points.

Also, different analytical methods may have an influence. Nowadays new technologies such as remote sensing techniques are developed to detect soil elements like Na^+ in soil [240]. However, mapping remains challenging due to soil heterogeneity and variations in ion concentrations along the year. In Mediterranean areas, high evapotranspiration increases soluble salt in the upper soil layers during summer, while autumn and early spring rains cause considerable leakage; thus huge differences across the year can be observed [10] [220]. International cooperation is required to have reliable soil worldwide datasets in different seasons using the same measurement protocol.

Another explanation may be that native population from Catalonia have similar climatic conditions while heterogenous edaphic condition. Consequently, events of local adaptation in local *A. thaliana* from Catalonia are mainly driven by soil properties[19][86]. However, on the much larger

European scale processes of local adaptation to soil factors can be overridden due to huge differences in climate conditions and photoperiods affecting germination [230], flowering, and plant fitness [84].

Certain local adaptation was found in plants from Chapter 3, considering the extreme values of relative growth data (rosette diameter in carbonate soil *vs* rosette diameter in control soil). Contrastingly, nutrient leaf concentrations using data from the whole population were not statistically different comparing control *vs* treatment plants. However, statistically significant differences were found intragroup for Zn, P and S in leaves comparing high *vs* low relative growth groups on calcareous soil. Zn peak analysis of the Manhattan plots revealed WAKL 6, 1 and 4 as putative candidates in this family. Wall-Associated Kinases (WAK), a subfamily of receptor-like kinases, are associated with the cell wall. These genes have been suggested as sensors of the extracellular environment and implicated in intracellular signals [209]. WAKL4 is involved in Arabidopsis root mineral responses to Zn, Cu, K, Na, and Ni [208]. WAK1 is an aluminum early responsive gene and its overexpression results in aluminum tolerance [209]. These genes were also differentially expressed in microarray and transcriptomics from Chapter 2. Although no differences were found in WAKL mutant knock-out lines, more studies are needed to discard gene function duplicity by testing double mutants. As already commented above, a further candidate gene revealed by GWAS was a tonoplast dicarboxylate transporter (TDT, AT5G47560) which is involved in the uptake of malate and fumarate to the vacuole and pH homeostasis [212]. Previous studies performed in discs of *A. thaliana* leaves using wt and *tdt* lines grown at a pH range from 4 to 7 found the mutant line reduced the capacity to generate OH^- to overcome acidification. Therefore, the observed higher sensitivity of *tdt* mutant line to acidity could be in synch with a higher tolerance of the same line to alkalinity. More studies are required testing malate and citrate contents in WT and *tdt* lines to determine differences in natural populations of *A. thaliana* [213].

Common garden experiments using Catalan demes of *Arabidopsis thaliana* under alkaline-saline stress:

The climatic crisis is forcing plants to adjust to a new environment more quickly than they did before [90]. The determination of climatic or soil factors which are driving evolutionary processes is a key point to predicting responses to the new scenario. To detect phenomena of local adaptation or plant plasticity, genetics, ecology and plant physiology are needed [241]. Also, evolutionary genomics can improve the prediction of species' responses to climate change [90].

Across *A thaliana* distribution, local adaptation is happening on both short and long scales. This includes a huge range of biologic, climatic and soil factors as seed establishment [230], heat and drought [242] among others. Unraveling selecting pressure factors that are driven by subpopulations can allow us to predict plant responses in future scenarios [243]. In previous research, the Iberic peninsula was pointed out as a hotspot of *A thaliana species* diversity [78]. This area became a fauna and flora refuge during the Pleistocene. Iberic accessions have more genetic variation than those from northern latitudes due to successive bottlenecks produced by specie recolonization [244]. Catalonia is a small region located in southern edges of *A. thalian distribution has a* with a high heterogeneity of altitudes and geological substrates. In a relatively small area, high genotypic and phenotypic variations are available and Catalonia is becoming a natural laboratory for local adaptation studies [19], [86], [87]. I would like to empathize the importance of these native population for both basic studies into the genetic mechanisms of tolerance to environmental factors and as a source of genetic information for crop breeding. In this context, stock centers are key institutions allowing to preserve all this genetic diversity which is continuously threatened by human activities.

Catalan coastal populations of *A. thaliana* have been found tolerant to moderate saline conditions, and a process of local adaptation was confirmed [86], [87]. Also, inland populations with moderate $CaCO_3$ concentrations in their native soil were able to tolerate more $CaCO_3$ than coastal populations native to siliceous soil [19]. Despite the fact that *A. thaliana* has evolved tolerance to moderate alkalinity and salinity, we could not find *A. thaliana* in alkaline-saline areas. To further characterize this apparent difficulty to adapt to salinity under alkaline conditions, in this thesis, we tested local *A. thaliana* demes in different common gardens. In our initial hypothesis, we expected that those plants with highest tolerance to either alkalinity or salinity would also better tolerate the combination of both stress factors. Our results clearly contradict this hypothesis, and plants with intermediate degree of alkaline or saline tolerance performed best on alkaline-saline soil. This result indicates that mechanisms evolved in plants that allow them to better adapt to alkaline or saline conditions and to be competitive under these circumstances are highly specific to either soil conditions. Salinity tolerance evolved on siliceous soil may even be disadvantageous for performance under alkaline-saline conditions. Plants with intermediate tolerance were able to grow in common gardens on alkaline saline soil under semi-controlled conditions. However, in the real field scenario no natural populations on saline-alkaline soil were detected. Although divergent selection in the study area is still in progress, it is possible that *A. thaliana* lacks the genetic

background for adaptation to the extremely harsh conditions on alkaline-saline soils. Other Brassicaceae species, like *Brassica fructiculosa* or *Matthiola sinuata* (unpublished results) have this ability and deserve further investigations.

Our study highlights the importance of conservation of natural variation in both non-commercial plants and crops. Climate change maybe too fast for plants' adaptability and for this reason highly specific local adaptation mechanisms may become a disadvantageous strategy in the soon future [245]. Maintenance of plant genetic and phenotypic variety is crucial for finding biotechnological solutions for crop production in the near future. International consortia are collaborating in the production, management and share of resources for the research community. In the case of *A. thaliana* this huge effort allows increasing our speed in performing experiments and produce new technologies and tools. Evolutionary genomics can improve the prediction of species' responses to climate change [90]. For this scenario, more experiments under real field conditions must be performed. Experimentation under natural conditions is expensive, takes more time, is hampered by higher variability and therefore needs increased sample size. However, experiments into the mechanisms of tolerance to specific stress factors alone or in combination under natural conditions provide us more truthful responses because interacting natural inputs as light, herbivory, changing conditions are taken into consideration.

Future work

A doctoral thesis has to be performed within a certain time frame and several aspects of this work require future work to be allow further conclusions. Among those we can identity in the physiological chapter, the role of carbonic anhydrases in bicarbonate-carbonate stress responses. We only performed a relatively small number of membrane potential measurements. More replicates are needed not only to confirm the differences between *A. thaliana* demes in bicarbonate-induced changes of the membrane potential, but also measurements of cytosolic pH and root ion flux responses by electrophysiological technique are required. Furthermore, measurements after the application of inhibitors of internal and external carbonic anhydrases may help to elucidate the specific mechanism of bicarbonate handling in roots of plants differing in bicarbonate tolerance.

In the second chapter, a primary objective of future research is to explore molecular mechanisms under bicarbonate and high pH after short-term exposure. Besides transcriptomics,

other genetic events like SNP, indel, and deletion should be explored. Also, phenomena in alternative splicing and epigenetics are an unexplored field in bicarbonate stress areas. New technologies nowadays available and the improved computing power allows the combination of different omics which can help the researcher to understand biological functions. Furthermore, inclusion of results under non-optimal pH stress in *A. thaliana* abiotic stress database could be a great challenge to detect the specific signature of alkaline stress response in plants.

In the GWAS chapter, *tdt* (AT5G47560) as a candidate gene was detected. This gene encodes for tonoplast dicarboxylate transporter which is involved in the uptake of malate and fumarate to the vacuole and pH homeostasis [212]. In a near future, we plan to use *tdt* overexpressing mutants to assess plant performance under calcareous soil conditions. The combination of knock-out and over-expressing mutants will help us to complete the putative role of this gene in calcareous soil and its possible role in pH homeostasis in plants. Besides soil culture, hydroponic culture can be used to test plants responses under more controlled conditions. Finally, soil data from native populations (pH, CEC and EC) can be run in a genotype-environment Association (GEA) to detect genes with ecological importance [246], [247].

From common garden experiments, in the near future, F1 hybrids between salt-tolerant plants and moderate carbonate plants will be tested to detect genes involved in tolerance to alkaline-salt stress. In addition, Catalan populations with identified polymorphism are going to be used. These alleles are potentially related to tolerance/sensitivity to salt or carbonate and occur spontaneously in the study region. These genes are transporters HKT1 (potassium and sodium), MOT1 (molybdenum) and FPN2 (divalent, especially iron). Apart from crosses and alleles, more research is required using populations that have demonstrated tolerance/sensitivity to double stress. These studies can target Na and Ca compartmentalization by fluorescence microscopy.

Final conclusions

- Bicarbonate treatments produce more delirious effect than pH. An adequate concentration for *A. thaliana* species in a mild treatment is 10 mM. Under bicarbonate stress, tolerant plants can maintain better rates of germination, growth, and siliqua production. This can be explained by better nutrient translocation, more efficient use of iron, maintaining better photosynthetic efficiency. Moreover, a lower content of reactive oxygen species is observable in tolerant lines. Variation in carbonic anhydrase relative expression and differences in membrane potential in response to alkali stress indicates a putative mechanism of HCO_3^- uptake also in terrestrial plants.

- Transcriptomic responses show that fast activation responses (3 h) are detectable in roots of tolerant lines, while sensitive lines suffered severe reactions after 48 h in leaves. Responses in the tolerant line were focused on the glucosinolate pathway in response to C/N imbalance under carbonate stress. Main genes were related to stress sensing, signal transduction and TF responses. Contrastingly sensitive lines responses focused on iron uptake.

- The European populations, in general present a reduction in rosette diameter and changes in nutritional profiles on carbonate rich soil. Plant with higher relative growth can translocate more Zn, P and S. Signal of local adaptation in European population was only present when the extremes of relative growth data were compared. Plant tolerance to calcareous soils is multigenic as shown by the huge amount of candidate SNP from growth and ionome data from plants growing o the carbonate-rich calcareous soil. Candidate genes from GWAS using as an input mineral nutrient concentration in leaves are related to ion transport like CHX1 and OPT3, detoxification and cell walls. Validation of candidate genes using T-DNA lines shows a tonoplast dicarboxylate transporter (TDT) as a negative regulator involved in better growth and nutrition acquisition on calcareous soil.

- No natural population of *A. thaliana* was present on alkaline saline soil indicating the possibility that the specific mechanism required for performance on such soils are not established in this specie. Saline-alkaline stress produces more delirious effect in plants than neutral salt. Germination of *A. thaliana seeds* both on plates and in common gardens is strongly inhibited under alkaline-saline stress. At the seedling stage, plant with low local adaptation to carbonates or salinity present better performance under alkaline- saline stress than demes with high levels of tolerance to either carbonate or salinity. These intermediate population presents higher fitness under saline-alkaline conditions.

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Programs used

Geographic Information System

Harmonized World Soil Database Viewer from FAO V12; https :// iiasa.ac.at /web /home /research /researchPrograms /water/HWSD.html

Miramon; https://www.miramon.cat/Index_es.htm

ArcGIS; https://www.arcgis.com/index.html

R Maptools; https://cran.r-project.org/web/packages/maptools/maptools.pdf

Genetic & Molecular databases and tools

GWA-portal; https://gwas.gmi.oeaw.ac.at/ [118]

Arapheno; https://arapheno.1001genomes.org/ [85]

The Arabidopsis Information Resource (TAIR); https://www.arabidopsis.org/

Thalemine; https://bar.utoronto.ca/thalemine/begin.do

David Database; https://david.ncifcrf.gov/

AgriGO V2.0; http://systemsbiology.cau.edu.cn/agriGOv2/ [107]

Toronto bar; http://bar.utoronto.ca/

STRING V11; https://string-db.org/ [108]

Salk institute Genomic Analysis Laboratory SiGnAL ; http://signal.salk.edu/

Statistics and data visualization

JMP version 13; https://www.jmp.com/en_us/software.html

Microsoft (Excel 2016 and PowerPoint 2016); https://www.microsoft.com

RStudio V4.0; https://www.rstudio.com

Illustrator CC2019; https://www.adobe.com/es/products/illustrator.html

Reference's manager Mendeley Desktop; https://www.mendeley.com/download-desktop-new/

English corrector Grammarly; https://app.grammarly.com/

Supplementary material

Supplementary material: Chapter 1

Supplementary F1

Annex Table 1: Rosette diameter RD (mm) ANOVA factor analysis from tolerant to calcareous soil $A1_{(c+)}$ plants cultivated in a $NaHCO_3$ incremental concentration (0, 5, 10, 20 mM) in hydroponics for 5 weeks. *n=10*

Factor	DF	F value	Prob > F
Week number	5	2170.48	<0.001
Treatment	3	612.65	<0.001
Week number*Treatment	15	68.70	<0.001

Annex Table 2: Rosette diameter RD (mm) Mean ± Standard error, ANOVA and Tukey test from tolerant to calcareous soil $A1_{(c+)}$ plants cultivated in an incremental concentration of $NaHCO_3$ (0, 5, 10, 20 mM) in hydroponics for 5 weeks. *n=10*

Week number	$NaHCO_3$ (mM)	Mean RD (mm)	Std Error RD	F value	Prob > F	Tukey test
0	0	24.11	0.29	0	1	A
0	5	24.11	0.29			A
0	10	24.11	0.25			A
0	20	24.11	0.25			A
1	0	47.77	1.08	90.88	<.0001	A
1	5	50.45	1.08			A
1	10	42.96	0.93			B
1	20	29.45	0.93			C
2	0	68.78	1.52	94.33	<.0001	A
2	5	72.65	1.52			A
2	10	61.87	1.32			B
2	20	42.41	1.32			C
3	0	103.17	2.26	96.22	<.0001	A
3	5	108.97	2.26			A
3	10	92.8	1.96			B
3	20	63.62	1.96			C
4	0	123.81	2.7	96.7	<.0001	A
4	5	130.77	2.7			A
4	10	111.36	2.34			B
4	20	76.34	2.34			C
5	0	173.11	2.69	300.88	<.0001	A
5	5	170	2.69			A
5	10	122.5	2.33			B
5	20	82.5	2.33			C

Annex Table 3: Root length RL (mm) ANOVA factor analysis from tolerant to calcareous soil $A1_{(c+)}$ plants cultivated in an incremental concentration of $NaHCO_3$ (0, 5, 10, 20 mM) in hydroponics for 5 weeks. *n=10*

Factor	DF	F value	Prob > F
Week number	5	1344.4	<.0001
Treatment	3	748.55	<.0001
Week number*Treatment	15	41.68	<.0001

Annex Table 4: Root length RL (mm) Mean ± Standard error, ANOVA and Tukey test from tolerant to calcareous soil $A1_{(c+)}$ plants cultivated in an incremental concentration of $NaHCO_3$ (0, 5, 10, 20 mM) in hydroponics for 5 weeks. *n=10*

Week number	$NaHCO_3$ (mM)	Mean RL (mm)	Std Error RL	F value	Prob>F	Tukey test
0	0	26.12	0.29	0	1	A
0	5	26.12	0.29			A
0	10	26.12	0.25			A
0	20	26.12	0.25			A
1	0	69.93	1.28	155.5737	<.0001	A
1	5	59.48	1.28			B
1	10	52.45	1.11			C
1	20	34.96	1.11			D
2	0	83.91	1.52	157.9938	<.0001	A
2	5	71.37	1.52			B
2	10	62.93	1.32			C
2	20	41.96	1.32			D
3	0	100.69	1.82	159.7192	<.0001	A
3	5	85.65	1.82			B
3	10	75.52	1.57			C
3	20	50.35	1.57			D
4	0	120.83	2.17	160.9397	<.0001	A
4	5	102.78	2.17			B
4	10	90.63	1.88			C
4	20	60.42	1.88			D
5	0	145	2.6	161.7983	<.0001	A
5	5	123.33	2.6			B
5	10	108.75	2.25			C
5	20	72.5	2.25			D

Supplementary F2

Annex Table 5: Germination inhibition (%) ANOVA factor analysis from $A1_{(c+)}$, Col-0 $_{(wild\ type)}$, and $T6_{(c-)}$ seeds sown in each treatment plate. As a deme, $A1_{(c+)}$ tolerant and $T6_{(c-)}$ sensitive to a calcareous soil. Control treatment: MS ½ pH 5.9. High pH treatment: MS ½ pH 8.3 and Bicarbonate treatment: MS ½ pH 8.3 + 10 mM $NaHCO_3$. Each deme and treatment *n=45*

Factor	DF	F value	Prob > F
Deme	2	80.47	<.0001
Treatment	2	4.39	0.02
Deme*Treatment	4	17.01	<.0001

Annex Table 6: Germination inhibition (%) Mean ± Standard error, ANOVA and Tukey test from $A1_{(c+)}$, Col-$0_{(wild\ type)}$ and $T6_{(c-)}$ seeds sown in each treatment plate. As a deme, $A1_{(c+)}$ tolerant and $T6_{(c-)}$ sensitive to a calcareous soil. Control treatment: MS ½ pH 5.9. High pH treatment: MS ½ pH 8.3 and Bicarbonate treatment MS ½ pH 8.3 + 10 mM $NaHCO_3$. Each deme and treatment *n=45*

Deme	Treatment	Mean	Std Error	N	F value	Prob > F	Tukey test
$A1_{(C+)}$	T1	51.93	1.32	5	21.03	0	B
	T2	74.67	3.89	5			A
	T3	65.33	1.33	5			A
Col-$0_{(wt)}$	T1	97.78	2.22	5	0.44	0.66	A
	T2	96	1.63	5			A
	T3	94.67	2.49	5			A
$T6_{(c-)}$	T1	80	4.22	5	16	0	A
	T2	64	3.4	5			B
	T3	53.33	2.11	5			B

Annex Table 7: Rosette diameter RD (mm) ANOVA factor analysis from $A1_{(c+)}$, Col-$0_{(wild\ type)}$ and $T6_{(c-)}$ 2 weeks old plants summited in different hydroponic treatments for 2 weeks. As a deme, $A1_{(c+)}$ tolerant and $T6_{(c-)}$ sensitive to a calcareous soil. **(T1)** Control treatment: Hoagland ½ pH 5.9. **(T2)** High pH treatment: Hoagland ½ pH 8.3 and **(T3)** Bicarbonate treatment Hoagland ½ pH 8.3 + 10 mM $NaHCO_3$. Each deme and treatment *n=12*

Factor	DF	F value	Prob > F
Deme	2.00	63.90	<.0001
Treatment	2.00	56.49	<.0001
Deme*Treatment	4.00	11.23	<.0001

Annex Table 8: Rosette diameter RD (mm) Mean ± Standard error, ANOVA and Tukey test from $A1_{(c+)}$, Col-$0_{(wild\ type)}$ and $T6_{(c-)}$ 2 weeks old plants summited in different hydroponic treatments for 2 weeks. As a deme, $A1_{(c+)}$ tolerant and $T6_{(c-)}$ sensitive to a calcareous soil. **(T1)** Control treatment: Hoagland ½ pH 5.9. **(T2)** High pH treatment: Hoagland ½ pH 8.3 and **(T3)** Bicarbonate treatment Hoagland ½ pH 8.3 + 10 mM $NaHCO_3$. Each deme and treatment *n=12*

Deme	Treatment	Mean	Std Error	N	F value	Prob > F	Tukey test
$A1_{(C+)}$	T1	84.25	3.87	12	11.32	0	A
	T2	65	3.3	12			B
	T3	81.25	1.6	12			A
Col.0 $_{(wt)}$	T1	74.75	2.39	12	25.93	<.0001	A
	T2	58.08	2.72	12			B
	T3	46.25	3.26	12			C
$T6_{(C-)}$	T1	70.5	2.09	12	63.21	<.0001	A
	T2	48.5	2.13	12			B
	T3	39.92	1.7	12			C

Annex Table 9: Root length RL (mm) ANOVA factor analysis from $A1_{(c+)}$, Col-$0_{(wild\ type)}$, and $T6_{(c)}$ 2 weeks old plants summited in different hydroponic treatments for 2 weeks. As a deme, $A1_{(c+)}$ tolerant and $T6_{(c-)}$ sensitive to a calcareous soil. **(T1)** Control treatment: Hoagland ½ pH 5.9. **(T2)** High pH treatment: Hoagland ½ pH 8.3 and **(T3)** Bicarbonate treatment Hoagland ½ pH 8.3 + 10 mM $NaHCO_3$. Each deme and treatment *n=12*

Factor	DF	F value	Prob > F
Deme	2	13.9	<.0001
Treatment	2	15.8536	<.0001
Deme*Treatment	4	1.9287	0.122

Annex Table 10: Root length RL (mm) Mean ± Standard error, ANOVA and Tukey test from $A1_{(c+)}$, Col-$0_{(wild\ type)}$ and $T6_{(c-)}$ 2 weeks old plants summited in different hydroponic treatments for 2 weeks. As a deme, $A1_{(c+)}$ tolerant and $T6_{(c-)}$ sensitive to calcareous soil **(T1)** Control treatment: Hoagland ½ pH 5.9. **(T2)** High pH treatment: Hoagland ½ pH 8.3 and **(T3)**Bicarbonate treatment Hoagland ½ pH 8.3 + 10 mM $NaHCO_3$. Each deme and treatment *n=12*

Deme	Treatment	Mean	Sdt error	N	F value	Prob > F	Tukey test
$A1_{(C+)}$	T1	99.88	1.7	12	4.7878	0.0247	A
	T2	71.95	2.01	12			B
	T3	93.41	2.93	12			AB
Col-0 (wt)	T1	87.93	0.18	12	9.0646	0.0026	A
	T2	65.11	0.11	12			B
	T3	62.01	0.1	12			B
$T6_{(C-)}$	T1	77.75	0.22	12	10.3329	0.0015	A
	T2	62.84	0.12	12			A
	T3	50.89	0.31	12			B

Annex Table 11: Fitness reproduction (siliqua number) ANOVA factor analysis from $A1_{(c+)}$, Col-$0_{(wild\ type)}$ and $T6_{(c-)}$ 2 weeks old plants summited in different hydroponic treatments. As a deme, $A1_{(c+)}$ tolerant and $T6_{(c-)}$ sensitive to a calcareous soil. (T1) Control treatment: Hoagland ½ pH 5.9. (T2) High pH treatment: Hoagland ½ pH 8.3 and (T3) Bicarbonate treatment Hoagland ½ pH 8.3 + 10 mM $NaHCO_3$. Each deme and treatment *n=9*

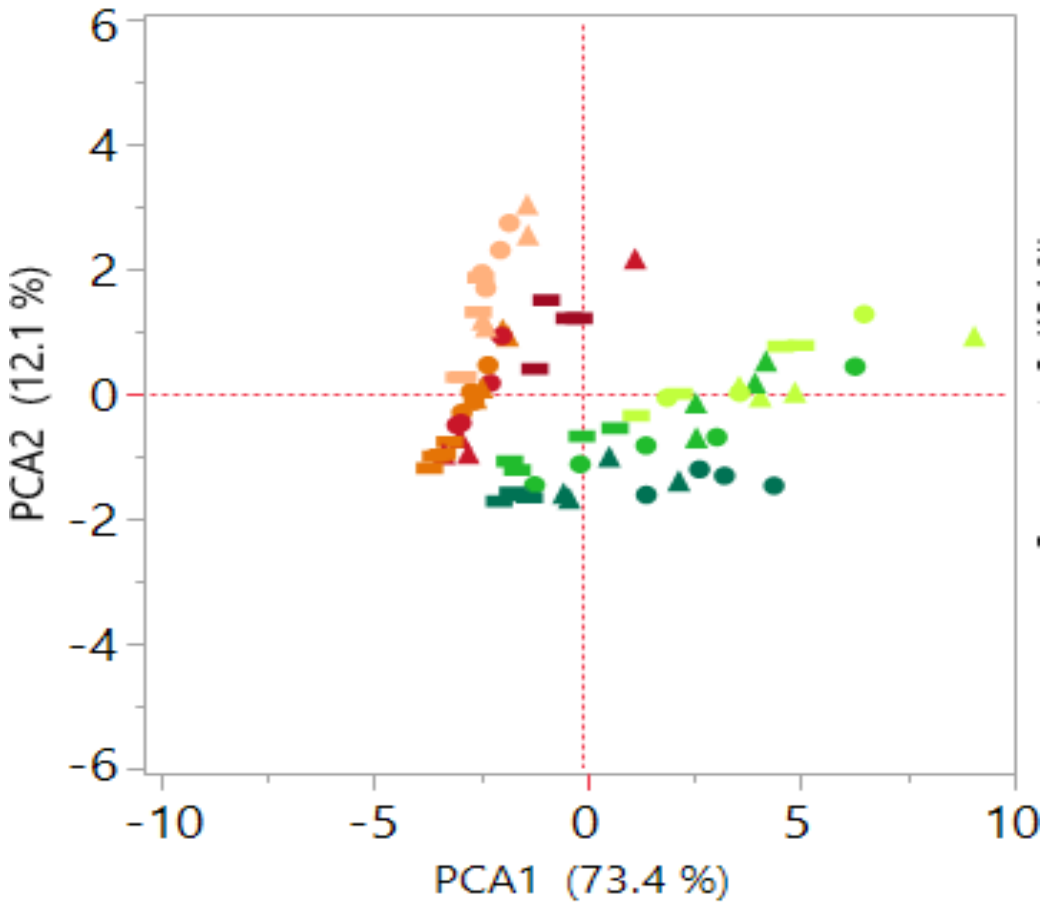
Factor	DF	F value	Prob > F
Deme	2.00	2.93	0.06
Treatment	2.00	12.59	<.0001
Deme*Treatment	4.00	3.80	0.01

Annex Table 12: Fitness reproduction (siliqua number) Mean ± Standard error, ANOVA and Tukey test from $A1_{(c+)}$, Col-$0_{(wild\ type)}$ and $T6_{(c-)}$ 2 weeks old plants summited in different hydroponic treatments. As a deme, $A1_{(c+)}$ tolerant and $T6_{(c-)}$ sensitive to a calcareous soil. **(T1)** Control treatment: Hoagland ½ pH 5.9. **(T2)** High pH treatment: Hoagland ½ pH 8.3 and **(T3)** Bicarbonate treatment Hoagland ½ pH 8.3 + 10 mM $NaHCO_3$. Each deme and treatment *n=9*

Deme	Treatment	Mean	Std Error	N	F value	Prob > F	Tukey test
$A1_{(C+)}$	T1	229.56	25.78	9	0.87	0.43	A
	T2	183.56	33.53	9			A
	T3	228.11	23.65	9			A
Col-0 (wt)	T1	287.89	43.93	9	9.05	0	A
	T2	162.57	19.73	7			B
	T3	86.17	16.76	6			B
$T6_{(C-)}$	T1	217.89	21.8	9	12.16	0	A
	T2	168.71	17.61	7			A
	T3	102.22	10.27	9			B

Supplementary F3

Annex Figure 13: Total nutrient mineral content Principal Component Analysis (PCA) using total nutrient mineral content. Orange color indicates roots nutrition while green color indicates leaves nutrition. Control treatment (Hoagland ½ pH 5.9) indicated in a light color. High pH treatment(Hoagland ½ pH 8.3) indicated in medium color and Bicarbonate treatment (Hoagland ½ pH 8.3 + 10 mM $NaHCO_3$) indicate in dark colors. Each deme and treatment *n=4*



Annex Table 14: Leave total nutrient content ANOVA factor analysis from $A1_{(c+)}$, Col-$0_{(wild\ type)}$ and $T6_{(c)}$ 2 weeks old plants summited in different hydroponic treatments for 15 days. As a deme, $A1_{(c+)}$ tolerant and $T6_{(c-)}$ sensitive to a calcareous soil. **(T1)** Control treatment: Hoagland ½ pH 5.9. **(T2)** High pH treatment: Hoagland ½ pH 8.3 and **(T3)** Bicarbonate treatment Hoagland ½ pH 8.3 + 10 mM $NaHCO_3$. Each deme and treatment *n=4*

Element µg	**Factor**	**DF**	**F value**	**Prob > F**
Zn	Deme	2	3.5	0.04
	Treatment	2	31.06	<.0001
	Deme*Treatment	4	2.93	0.04
Cu	Deme	2	3.98	0.03
	Treatment	2	12.13	0
	Deme*Treatment	4	3.95	0.01
Mo	Deme	2	15.45	<.0001
	Treatment	2	23.43	<.0001
	Deme*Treatment	4	4.93	0
P	Deme	2	7.94	0
	Treatment	2	34.27	<.0001
	Deme*Treatment	4	1.72	0.17
S	Deme	2	11.62	0
	Treatment	2	10.01	0
	Deme*Treatment	4	3.26	0.03
Mg	Deme	2	16.62	<.0001
	Treatment	2	10.37	0
	Deme*Treatment	4	2.51	0.07
Fe	Deme	2	11.98	0
	Treatment	2	10.78	0
	Deme*Treatment	4	2.45	0.07
Mn	Deme	2	5.41	0.01
	Treatment	2	35.02	<.0001
	Deme*Treatment	4	1.94	0.13
B	Deme	2	19.66	<.0001
	Treatment	2	3.4	0.05
	Deme*Treatment	4	1.42	0.26
Ca	Deme	2	8.32	0
	Treatment	2	45.08	<.0001
	Deme*Treatment	4	1.89	0.14
K	Deme	2	17.28	<.0001
	Treatment	2	9.06	0
	Deme*Treatment	4	3.23	0.03
Na	Deme	2	17.8	<.0001
	Treatment	2	125.55	<.0001
	Deme*Treatment	4	13.6	<.0001

Annex Table 15: Leave total nutrient content Mean ± Standard error, ANOVA and Tukey test from $A1_{(c+)}$, Col-$0_{(wild\ type)}$ and $T6_{(c-)}$ 2 weeks old plants summited in different hydroponic treatments. As a deme, $A1_{(c+)}$ tolerant and $T6_{(c-)}$ sensitive to a calcareous soil. **(T1)** Control treatment: Hoagland ½ pH 5.9. **(T2)** High pH treatment: Hoagland ½ pH 8.3 and **(T3)** Bicarbonate treatment Hoagland ½ pH 8.3 + 10 mM $NaHCO_3$. Each deme and treatment *n=4*

Deme	Element	Treatment	Mean (µg)	Std error	F value	Prob > F	Tukey test
$A1_{(c+)}$	B	T1	4.55	0.57	0.82	0.4689	A
$A1_{(c+)}$	B	T2	3.94	0.74			A
$A1_{(c+)}$	B	T3	4.99	0.36			A
$A1_{(c+)}$	Ca	T1	1458.28	218.01	6.76	0.0161	A
$A1_{(c+)}$	Ca	T2	692.06	130.82			B
$A1_{(c+)}$	Ca	T3	867.11	82.95			AB
$A1_{(c+)}$	Cu	T1	0.28	0.05	3.07	0.0964	A
$A1_{(c+)}$	Cu	T2	0.16	0.03			B
$A1_{(c+)}$	Cu	T3	0.22	0.02			AB
$A1_{(c+)}$	Fe	T1	4.64	0.61	6.31	0.0194	A
$A1_{(c+)}$	Fe	T2	2.38	0.37			B
$A1_{(c+)}$	Fe	T3	4.16	0.4			AB
$A1_{(c+)}$	K	T1	2332.52	240.7	2.22	0.1651	A
$A1_{(c+)}$	K	T2	1631.26	278.73			A
$A1_{(c+)}$	K	T3	2057.52	182.94			A
$A1_{(c+)}$	Mg	T1	146.27	21.29	1.41	0.2923	A
$A1_{(c+)}$	Mg	T2	108.4	16.56			A
$A1_{(c+)}$	Mg	T3	136.91	9.87			A
$A1_{(c+)}$	Mn	T1	2.88	0.4	9.03	0.0071	A
$A1_{(c+)}$	Mn	T2	1.13	0.21			B
$A1_{(c+)}$	Mn	T3	1.79	0.24			AB
$A1_{(c+)}$	Mo	T1	0.45	0.04	6.51	0.0179	A
$A1_{(c+)}$	Mo	T2	0.22	0.05			B
$A1_{(c+)}$	Mo	T3	0.39	0.04			AB
$A1_{(c+)}$	Na	T1	76.69	13.92	55.59	<.0001	B
$A1_{(c+)}$	Na	T2	65.12	4.37			B
$A1_{(c+)}$	Na	T3	710.1	84.49			A
$A1_{(c+)}$	P	T1	396.37	42.32	7.86	0.0106	A
$A1_{(c+)}$	P	T2	213.22	42.18			B
$A1_{(c+)}$	P	T3	228.92	19.02			B
$A1_{(c+)}$	S	T1	317.77	45.69	2.87	0.1086	A
$A1_{(c+)}$	S	T2	204.83	30.55			A
$A1_{(c+)}$	S	T3	267.16	18.03			A
$A1_{(c+)}$	Zn	T1	2.74	0.31	9.13	0.0068	A
$A1_{(c+)}$	Zn	T2	1.24	0.23			B
$A1_{(c+)}$	Zn	T3	1.92	0.2			AB

Annex Table 15: Leave total nutrient content Mean ± Standard error, ANOVA and Tukey test from $A1_{(c+)}$, Col-$0_{(wild\ type)}$ and $T6_{(c-)}$ 2 weeks old plants summited in different hydroponic treatments. As a deme, $A1_{(c+)}$ tolerant and $T6_{(c-)}$ sensitive to a calcareous soil. **(T1)** Control treatment: Hoagland ½ pH 5.9. **(T2)** High pH treatment: Hoagland ½ pH 8.3 and **(T3)** Bicarbonate treatment Hoagland ½ pH 8.3 + 10 mM $NaHCO_3$. Each deme and treatment *n=4*

Deme	Element	Treatment	Mean	Std error	F value	Prob > F	Tukey test
$Col.0_{(wt)}$	B	T1	5.27	0.69	2.63	0.1262	A
$Col.0_{(wt)}$	B	T2	4.02	0.33			A
$Col.0_{(wt)}$	B	T3	3.8	0.36			A
$Col.0_{(wt)}$	Ca	T1	1809.12	197.7	22.38	0.0003	A
$Col.0_{(wt)}$	Ca	T2	966.99	76.94			B
$Col.0_{(wt)}$	Ca	T3	555.87	98.58			B
$Col.0_{(wt)}$	Cu	T1	0.24	0.02	10.03	0.0051	A
$Col.0_{(wt)}$	Cu	T2	0.27	0.02			A
$Col.0_{(wt)}$	Cu	T3	0.14	0.02			B
$Col.0_{(wt)}$	Fe	T1	4.86	0.66	2.59	0.0129	A
$Col.0_{(wt)}$	Fe	T2	4.04	0.38			B
$Col.0_{(wt)}$	Fe	T3	3.09	0.56			B
$Col.0_{(wt)}$	K	T1	2198.73	356.43	6.24	0.0199	A
$Col.0_{(wt)}$	K	T2	2256.96	74.05			A
$Col.0_{(wt)}$	K	T3	1273.87	116.79			B
$Col.0_{(wt)}$	Mg	T1	189.69	23.3	6.41	0.0186	A
$Col.0_{(wt)}$	Mg	T2	162.01	8.68			AB
$Col.0_{(wt)}$	Mg	T3	109.84	12.3			B
$Col.0_{(wt)}$	Mn	T1	2.62	0.36	11.26	0.0036	A
$Col.0_{(wt)}$	Mn	T2	1.54	0.19			B
$Col.0_{(wt)}$	Mn	T3	0.94	0.14			B
$Col.0_{(wt)}$	Mo	T1	0.47	0.05	8.12	0.0097	A
$Col.0_{(wt)}$	Mo	T2	0.37	0.03			AB
$Col.0_{(wt)}$	Mo	T3	0.23	0.04			B
$Col.0_{(wt)}$	Na	T1	28.9	8.77	37.27	<.0001	B
$Col.0_{(wt)}$	Na	T2	80.34	5.65			B
$Col.0_{(wt)}$	Na	T3	370.39	51.18			A
$Col.0_{(wt)}$	P	T1	481.4	64.65	15.35	0.0013	A
$Col.0_{(wt)}$	P	T2	298.59	27.02			B
$Col.0_{(wt)}$	P	T3	149.23	22.32			B
$Col.0_{(wt)}$	S	T1	392.67	61.86	5.88	0.0232	A
$Col.0_{(wt)}$	S	T2	389.7	30.69			A
$Col.0_{(wt)}$	S	T3	212.18	26.05			B
$Col.0_{(wt)}$	Zn	T1	3.14	0.45	10.55	0.0044	A
$Col.0_{(wt)}$	Zn	T2	2.19	0.16			AB
$Col.0_{(wt)}$	Zn	T3	1.24	0.19			B

Annex Table 15: Leave total nutrient content Mean ± Standard error, ANOVA and Tukey test from $A1_{(c+)}$, Col-$0_{(wild\ type)}$ and $T6_{(c-)}$ 2 weeks old plants summited in different hydroponic treatments. As a deme, $A1_{(c+)}$ tolerant and $T6_{(c-)}$ sensitive to a calcareous soil. **(T1)** Control treatment: Hoagland ½ pH 5.9. **(T2)** High pH treatment: Hoagland ½ pH 8.3 and **(T3)** Bicarbonate treatment Hoagland ½ pH 8.3 + 10 mM $NaHCO_3$. Each deme and treatment *n=4*

Deme	Element	Treatment	Mean	Std error	F value	Prob > F	Tukey test
$T6_{(c-)}$	B	T1	3.1	0.37	6.98	0.0148	A
$T6_{(c-)}$	B	T2	2.22	0.16			AB
$T6_{(c-)}$	B	T3	1.86	0.1			B
$T6_{(c-)}$	Ca	T1	1325.46	178.34	25.49	0.0002	A
$T6_{(c-)}$	Ca	T2	446.44	76.45			B
$T6_{(c-)}$	Ca	T3	266.39	12.3			B
$T6_{(c-)}$	Cu	T1	0.25	0.03	11.37	0.0034	A
$T6_{(c-)}$	Cu	T2	0.16	0.03			AB
$T6_{(c-)}$	Cu	T3	0.08	0.01			B
$T6_{(c-)}$	Fe	T1	3.5	0.54	10.37	0.0046	A
$T6_{(c-)}$	Fe	T2	1.74	0.14			B
$T6_{(c-)}$	Fe	T3	1.61	0.12			B
$T6_{(c-)}$	K	T1	1633.23	190.14	10.49	0.0044	A
$T6_{(c-)}$	K	T2	986.05	164.29			B
$T6_{(c-)}$	K	T3	706.9	37.71			B
$T6_{(c-)}$	Mg	T1	126.67	15.38	11.28	0.0035	A
$T6_{(c-)}$	Mg	T2	70.9	9.75			B
$T6_{(c-)}$	Mg	T3	60.32	2.55			B
$T6_{(c-)}$	Mn	T1	2.44	0.34	22.15	0.0003	A
$T6_{(c-)}$	Mn	T2	0.85	0.18			B
$T6_{(c-)}$	Mn	T3	0.48	0.03			B
$T6_{(c-)}$	Mo	T1	0.38	0.05	28.09	0.0001	A
$T6_{(c-)}$	Mo	T2	0.12	0.01			B
$T6_{(c-)}$	Mo	T3	0.09	0			B
$T6_{(c-)}$	Na	T1	56	2.02	54.22	<.0001	B
$T6_{(c-)}$	Na	T2	41.5	7.17			B
$T6_{(c-)}$	Na	T3	266.48	28.67			A
$T6_{(c-)}$	P	T1	320.58	43.83	13.76	0.0018	A
$T6_{(c-)}$	P	T2	167.91	31.35			B
$T6_{(c-)}$	P	T3	93.19	5.03			B
$T6_{(c-)}$	S	T1	283.05	37.5	8.87	0.0074	A
$T6_{(c-)}$	S	T2	164.11	24.22			B
$T6_{(c-)}$	S	T3	138.21	5.01			B
$T6_{(c-)}$	Zn	T1	2.85	0.36	17.64	0.0008	A
$T6_{(c-)}$	Zn	T2	1.11	0.25			B
$T6_{(c-)}$	Zn	T3	0.89	0.04			B

Annex Table 16 Root total nutrient content ANOVA factor analysis from $A1_{(c+)}$, $Col\text{-}0_{(wild\ type)}$ and $T6_{(c-)}$ 2 weeks old plants summited in different hydroponic treatments for 15 days. As a deme, $A1_{(c+)}$ tolerant and $T6_{(c-)}$ sensitive to a calcareous soil. **(T1)** Control treatment: Hoagland ½ pH 5.9. **(T2)** High pH treatment: Hoagland ½ pH 8.3 and **(T3)** Bicarbonate treatment Hoagland ½ pH 8.3 + 10 mM $NaHCO_3$. Each deme and treatment *n=4*

Element μg	**Factor**	**DF**	**F value**	**Prob > F**
B	Deme	2	5.10	0.0139
	Treatment	2	5.79	0.0086
	Deme*Treatment	4	2.58	0.0618
Ca	Deme	2	0.48	0.6218
	Treatment	2	14.34	<.0001
	Deme*Treatment	4	0.80	0.5345
Cu	Deme	2	0.72	0.4969
	Treatment	2	1.31	0.2855
	Deme*Treatment	4	4.84	0.0045
Fe	Deme	2	0.77	0.4749
	Treatment	2	23.42	<.0001
	Deme*Treatment	4	2.25	0.0904
K	Deme	2	2.75	0.0818
	Treatment	2	3.84	0.0341
	Deme*Treatment	4	4.05	0.0107
Mg	Deme	2	2.97	0.068
	Treatment	2	12.95	0.0001
	Deme*Treatment	4	2.18	0.0977
Mn	Deme	2	1.07	0.3557
	Treatment	2	11.16	0.0003
	Deme*Treatment	4	6.45	0.0009
Mo	Deme	2	0.80	0.4602
	Treatment	2	0.54	0.5875
	Deme*Treatment	4	7.06	0.0005
Na	Deme	2	0.96	0.3956
	Treatment	2	21.43	<.0001
	Deme*Treatment	4	1.57	0.2106
P	Deme	2	0.63	0.54
	Treatment	2	9.24	0.0009
	Deme*Treatment	4	0.94	0.4568
S	Deme	2	3.66	0.0393
	Treatment	2	3.09	0.0618
	Deme*Treatment	4	1.57	0.2111
Zn	Deme	2	0.32	0.7286
	Treatment	2	4.49	0.0207
	Deme*Treatment	4	4.66	0.0055

Annex Table 17: Root total nutrient content (mean ± Standard error), ANOVA and Tukey test from $A1_{(c+)}$, Col-$0_{(wild\ type)}$ and $T6_{(c-)}$ 2 weeks old plants summited to different hydroponic treatments. As a deme, $A1_{(c+)}$ tolerant and $T6_{(c-)}$ sensitive to a calcareous soil. **(T1)** Control treatment: Hoagland ½ pH 5.9. **(T2)** High pH treatment: Hoagland ½ pH 8.3 and **(T3)** Bicarbonate treatment Hoagland ½ pH 8.3 + 10 mM $NaHCO_3$. Each deme and treatment *n=4*

Deme	Element	Treatment	Mean (µg	Std Error	F value	Prob > F	Tukey test
$A1_{(c+)}$	B	T1	0.54	0.07	0.21	0.8158	A
$A1_{(c+)}$	B	T2	0.57	0.08			A
$A1_{(c+)}$	B	T3	0.6	0.06			A
$A1_{(c+)}$	Ca	T1	62.47	4.98	5.41	0.0287	B
$A1_{(c+)}$	Ca	T2	62.96	7.46			B
$A1_{(c+)}$	Ca	T3	221.42	67.67			A
$A1_{(c+)}$	Cu	T1	0.21	0.01	25.32	0.0002	A
$A1_{(c+)}$	Cu	T2	0.14	0.01			B
$A1_{(c+)}$	Cu	T3	0.1	0.01			B
$A1_{(c+)}$	Fe	T1	10.12	0.4	29.19	0.0001	A
$A1_{(c+)}$	Fe	T2	3.24	0.43			B
$A1_{(c+)}$	Fe	T3	4.83	1			B
$A1_{(c+)}$	K	T1	235.47	12.21	5.24	0.0309	A
$A1_{(c+)}$	K	T2	201.06	16.56			A
$A1_{(c+)}$	K	T3	178.04	7.4			B
$A1_{(c+)}$	Mg	T1	10.85	0.67	37.02	<.0001	B
$A1_{(c+)}$	Mg	T2	10.54	0.19			B
$A1_{(c+)}$	Mg	T3	19.08	1.19			A
$A1_{(c+)}$	Mn	T1	0.33	0.04	0.56	0.5888	A
$A1_{(c+)}$	Mn	T2	0.26	0.07			A
$A1_{(c+)}$	Mn	T3	0.34	0.05			A
$A1_{(c+)}$	Mo	T1	0.16	0.02	0.9	0.4414	A
$A1_{(c+)}$	Mo	T2	0.2	0.04			A
$A1_{(c+)}$	Mo	T3	0.15	0.03			A
$A1_{(c+)}$	Na	T1	35.6	4.8	13.84	0.0018	B
$A1_{(c+)}$	Na	T2	36.23	5.02			B
$A1_{(c+)}$	Na	T3	99.09	15.5			A
$A1_{(c+)}$	P	T1	80.07	6.23	2.31	0.1554	A
$A1_{(c+)}$	P	T2	56.4	3.99			A
$A1_{(c+)}$	P	T3	112.82	31.45			A
$A1_{(c+)}$	S	T1	49.75	4.56	3.77	0.0645	A
$A1_{(c+)}$	S	T2	41.8	2.32			A
$A1_{(c+)}$	S	T3	38.28	1.11			A
$A1_{(c+)}$	Zn	T1	1.25	0.14	5.73	0.0248	A
$A1_{(c+)}$	Zn	T2	0.66	0.1			B
$A1_{(c+)}$	Zn	T3	0.76	0.16			AB

Annex Table 17: Root total nutrient content (mean ± Standard error), ANOVA and Tukey test from $A1_{(c+)}$, Col-$0_{(wild\ type)}$ and $T6_{(c-)}$ 2 weeks old plants summited to different hydroponic treatments. As a deme, $A1_{(c+)}$ tolerant and $T6_{(c-)}$ sensitive to a calcareous soil. **(T1)** Control treatment: Hoagland ½ pH 5.9. **(T2)** High pH treatment: Hoagland ½ pH 8.3 and **(T3)** Bicarbonate treatment Hoagland ½ pH 8.3 + 10 mM $NaHCO_3$. Each deme and treatment *n=4*

Deme	Element	Treatment	Mean (µg	Std Error	F value	Prob > F	Tukey test
$Col.0_{(wt)}$	B	T1	0.72	0.1	0.95	0.4272	A
$Col.0_{(wt)}$	B	T2	1.03	0.07			A
$Col.0_{(wt)}$	B	T3	1.27	0.58			A
$Col.0_{(wt)}$	Ca	T1	131.53	3.92	1.23	0.3359	B
$Col.0_{(wt)}$	Ca	T2	74.47	6.94			C
$Col.0_{(wt)}$	Ca	T3	192.94	8.94			A
$Col.0_{(wt)}$	Cu	T1	0.18	0.02	0.27	0.7668	A
$Col.0_{(wt)}$	Cu	T2	0.18	0.02			A
$Col.0_{(wt)}$	Cu	T3	0.14	0.07			A
$Col.0_{(wt)}$	Fe	T1	9.97	1.41	4.66	0.0409	A
$Col.0_{(wt)}$	Fe	T2	4.33	0.6			B
$Col.0_{(wt)}$	Fe	T3	2.32	1.12			C
$Col.0_{(wt)}$	K	T1	328.19	52.58	0.15	0.8636	A
$Col.0_{(wt)}$	K	T2	264.88	19.79			A
$Col.0_{(wt)}$	K	T3	297.41	130.47			A
$Col.0_{(wt)}$	Mg	T1	21.98	4.62	1.17	0.03537	B
$Col.0_{(wt)}$	Mg	T2	17.21	1.38			B
$Col.0_{(wt)}$	Mg	T3	38.98	1.68			A
$Col.0_{(wt)}$	Mn	T1	0.4	0.07	0.98	0.4133	A
$Col.0_{(wt)}$	Mn	T2	0.21	0.03			A
$Col.0_{(wt)}$	Mn	T3	0.38	0.16			A
$Col.0_{(wt)}$	Mo	T1	0.22	0.03	1.16	0.356	A
$Col.0_{(wt)}$	Mo	T2	0.23	0.03			A
$Col.0_{(wt)}$	Mo	T3	0.15	0.06			A
$Col.0_{(wt)}$	Na	T1	28.74	4.58	2.45	0.1415	A
$Col.0_{(wt)}$	Na	T2	25.36	5.49			A
$Col.0_{(wt)}$	Na	T3	139.49	71.51			A
$Col.0_{(wt)}$	P	T1	111.49	17.2	1.26	0.3296	A
$Col.0_{(wt)}$	P	T2	58.56	6.87			A
$Col.0_{(wt)}$	P	T3	109.53	42.47			A
$Col.0_{(wt)}$	S	T1	84.44	10.35	0.35	0.7113	A
$Col.0_{(wt)}$	S	T2	62.11	2.3			A
$Col.0_{(wt)}$	S	T3	93.26	45.53			A
$Col.0_{(wt)}$	Zn	T1	1.03	0.06	0.3	0.7508	A
$Col.0_{(wt)}$	Zn	T2	0.82	0.09			A
$Col.0_{(wt)}$	Zn	T3	0.86	0.33			A

Annex Table 17: Root total nutrient content (mean ± Standard error), ANOVA and Tukey test from $A1_{(c+)}$, Col-$0_{(wild\ type)}$ and $T6_{(c-)}$ 2 weeks old plants summited to different hydroponic treatments. As a deme, $A1_{(c+)}$ tolerant and $T6_{(c-)}$ sensitive to a calcareous soil. **(T1)** Control treatment: Hoagland ½ pH 5.9. **(T2)** High pH treatment: Hoagland ½ pH 8.3 and **(T3)** Bicarbonate treatment Hoagland ½ pH 8.3 + 10 mM $NaHCO_3$. Each deme and treatment *n=4*

Deme	Element	Treatment	Mean(µg)	Std Error	F value	Prob > F	Tukey test
$T6_{(c-)}$	B	T1	0.77	0.14	27.11	0.0002	B
$T6_{(c-)}$	B	T2	0.46	0.07			B
$T6_{(c-)}$	B	T3	1.41	0.03			A
$T6_{(c-)}$	Ca	T1	40.12	4.49	34.28	<.0001	B
$T6_{(c-)}$	Ca	T2	27.47	2.85			B
$T6_{(c-)}$	Ca	T3	235.19	34.04			A
$T6_{(c-)}$	Cu	T1	0.12	0.01	10.9	0.0039	B
$T6_{(c-)}$	Cu	T2	0.08	0.01			B
$T6_{(c-)}$	Cu	T3	0.22	0.04			A
$T6_{(c-)}$	Fe	T1	7.4	1.23	10.96	0.0039	A
$T6_{(c-)}$	Fe	T2	1.2	0.15			B
$T6_{(c-)}$	Fe	T3	6.87	1.31			A
$T6_{(c-)}$	K	T1	248.98	39.17	17.82	0.0007	B
$T6_{(c-)}$	K	T2	135.4	18.35			B
$T6_{(c-)}$	K	T3	490.48	60.54			A
$T6_{(c-)}$	Mg	T1	8.35	0.93	77.79	<.0001	B
$T6_{(c-)}$	Mg	T2	5.08	0.54			B
$T6_{(c-)}$	Mg	T3	46.69	4.41			A
$T6_{(c-)}$	Mn	T1	0.22	0.02	18.61	0.0006	B
$T6_{(c-)}$	Mn	T2	0.13	0.02			B
$T6_{(c-)}$	Mn	T3	0.89	0.17			A
$T6_{(c-)}$	Mo	T1	0.15	0.02	30.79	<.0001	B
$T6_{(c-)}$	Mo	T2	0.06	0.01			A
$T6_{(c-)}$	Mo	T3	0.28	0.03			B
$T6_{(c-)}$	Na	T1	28.89	1.12	172.06	<.0001	B
$T6_{(c-)}$	Na	T2	25.49	0.81			A
$T6_{(c-)}$	Na	T3	198.67	13			B
$T6_{(c-)}$	P	T1	62.67	10.06	28.14	0.0001	B
$T6_{(c-)}$	P	T2	31.97	3.34			A
$T6_{(c-)}$	P	T3	131.11	12.73			B
$T6_{(c-)}$	S	T1	50.44	7.7	15.85	0.0011	B
$T6_{(c-)}$	S	T2	28.67	4.37			A
$T6_{(c-)}$	S	T3	102.19	13.84			B
$T6_{(c-)}$	Zn	T1	0.79	0.09	9.12	0.0068	BC
$T6_{(c-)}$	Zn	T2	0.56	0.1			C
$T6_{(c-)}$	Zn	T3	1.63	0.29			A

Supplementary F4

Table 18: Chlorophyll determination (mg/m^2) ANOVA factor analysis from A1$_{(c+)}$, Col-0$_{(wild\ type)}$ and T6$_{(c-)}$ 2 weeks old plants summited to different hydroponic treatments. As a deme, A1$_{(c+)}$ tolerant and T6$_{(c-)}$ sensitive to a calcareous soil. **(T1)** Control treatment: Hoagland ½ pH 5.9. **(T2)** High pH treatment: Hoagland ½ pH 8.3 and **(T3)** Bicarbonate treatment Hoagland ½ pH 8.3 + 10 mM $NaHCO_3$. Each deme and treatment *n=18-12*

Factor	DF	F value	Prob > F
Deme	2	14.8071	<.0001
Treatment	2	3.1097	0.0482
Treatment*Deme	4	1.6293	0.1713

Table 19: Chlorophyll determination (mg/m^2) Mean ± Standard error, ANOVA and Tukey test from A1$_{(c+)}$, Col-0$_{(wild\ type)}$ and T6$_{(c-)}$ 2 weeks old plants summited on different hydroponic treatments. As a deme, A1$_{(c+)}$ tolerant and T6$_{(c-)}$ sensitive to a calcareous soil. **(T1)** Control treatment: Hoagland ½ pH 5.9. **(T2)** High pH treatment: Hoagland ½ pH 8.3 and **(T3)** Bicarbonate treatment Hoagland ½ pH 8.3 + 10 mM $NaHCO_3$. Each deme and treatment *n=18-12*

Deme	Level	Mean Chlorophyll	Std Error	N	F value	Prob	Tukey test
A1$_{(C+)}$	T1	1183.5	13.67	18	17.2853	<.0001	A
	T2	1145.64	29.79	14			A
	T3	1007.83	20.72	12			B
Col-0 $_{(wt)}$	T1	1066.29	11.27	14	5.1096	0.011	A
	T2	1090.86	20.3	14			A
	T3	994.83	31.43	12			B
T6$_{(C-)}$	T1	1042.53	38.55	15	7.8237	0.0013	A
	T2	877.63	28.05	16			B
	T3	907.86	26.5	14			B

Table 20: Photosystem II Efficiency (*Fv/Fm*) ANOVA factor analysis from $A1_{(c+)}$, $Col\text{-}0_{(wild\ type)}$ and $T6_{(c-)}$ 2 weeks old plants summited in different hydroponic treatments. As a deme, $A1_{(c+)}$ tolerant and $T6_{(c-)}$ sensitive to a calcareous soil. **(T1)** Control treatment: Hoagland ½ pH 5.9. **(T2)** High pH treatment: Hoagland ½ pH 8.3 and **(T3)** Bicarbonate treatment Hoagland ½ pH 8.3 + 10 mM $NaHCO_3$. Each deme and treatment *n=20*

Factor	DF	F value	Prob > F
Deme	2.00	106.37	<.0001
Treatment	2.00	83.47	<.0001
Treatment*Deme	4.00	24.67	<.0001

Table 21: Photosystem II efficiency (*Fv/Fm*) Mean ± Standard error, ANOVA and Tukey test from $A1_{(c+)}$, $Col\text{-}0_{(wild\ type)}$ and $T6_{(c-)}$ 2 weeks old plants summited to different hydroponic treatments. As a deme, $A1_{(c+)}$ tolerant and $T6_{(c-)}$ sensitive to a calcareous soil. **(T1)** Control treatment: Hoagland ½ pH 5.9. **(T2)** High pH treatment: Hoagland ½ pH 8.3 and **(T3)** Bicarbonate treatment Hoagland ½ pH 8.3 + 10 mM $NaHCO_3$. Each deme and treatment *n=20*

Deme	Treatment	Mean (Fv/Fm)	Std Error	N	F value	Prob > F	Tukey test
$A1_{(C+)}$	T1	0.78	0	21	43.12	<.0001	A
	T2	0.78	0.01	22			A
	T3	0.7	0.01	27			B
$Col\text{-}0_{(wt)}$	T1	0.79	0	18	3.5	0.04	A
	T2	0.74	0.03	19			AB
	T3	0.7	0.02	19			B
$T6_{(C-)}$	T1	0.76	0.01	26	59.54	<.0001	A
	T2	0.56	0.02	21			B
	T3	0.42	0.04	20			C

Supplementary F5

Table 22: Leaves MDA (nM/FWg) ANOVA factor analysis from $A1_{(c+)}$, Col-0 $_{(wild\ type)}$ and $T6_{(c-)}$ 2 weeks old plants exposed to different hydroponic treatments. As a deme, $A1_{(c+)}$ tolerant and $T6_{(c-)}$ sensitive to a calcareous soil. **(T1)** Control treatment: Hoagland ½ pH 5.9. **(T2)** High pH treatment: Hoagland ½ pH 8.3 and **(T3)** Bicarbonate treatment Hoagland ½ pH 8.3 + 10 mM $NaHCO_3$. Each deme and treatment *n=4*

Factor	DF	F ration	Prob
Treatment	2	441.2664	<.0001
Deme	2	296.8201	<.0001
Treatment*Deme	4	242.5468	<.0001

Table 23: Roots MDA (nM/FWg) ANOVA factor analysis from $A1_{(c+)}$, Col-0 $_{(wild\ type)}$ and $T6_{(c-)}$ 2 weeks old plants exposed to different hydroponic treatments. As a deme, $A1_{(c+)}$ tolerant and $T6_{(c-)}$ sensitive to a calcareous soil. **(T1)** Control treatment: Hoagland ½ pH 5.9. **(T2)** High pH treatment: Hoagland ½ pH 8.3 and **(T3)** Bicarbonate treatment Hoagland ½ pH 8.3 + 10 mM $NaHCO_3$. Each deme and treatment *n=4*

Factor	DF	F value	Prob > F
Treatment	2	169.34	<.0001
Deme	2	23.57	<.0001
Treatment*Deme	4	21.29	<.0001

Table 24: Leaves MDA (nM/FWg) Mean ± Standard error, ANOVA and Tukey test from $A1_{(c+)}$, Col-$0_{(wild\ type)}$ and $T6_{(c-)}$ 2 weeks old plants exposed to different hydroponic treatments. As a deme, $A1_{(c+)}$ tolerant and $T6_{(c-)}$ sensitive to a calcareous soil. **(T1)** Control treatment: Hoagland ½ pH 5.9. **(T2)** High pH treatment: Hoagland ½ pH 8.3 and **(T3)** Bicarbonate treatment Hoagland ½ pH 8.3 + 10 mM $NaHCO_3$. Each deme and treatment *n=4*

Deme	Treatment	Mean nmol/FW	Std Error	N	F value	Prob > F	Tukey test
$A1_{(C+)}$	T1	0.83	0.00	4	56.4919	0.0001	A
$A1_{(C+)}$	T2	1.17	0.01	4			A
$A1_{(C+)}$	T3	1.19	0.02	4			B
Col-$0_{(wt)}$	T1	0.84	0.01	4	85.1966	<.0001	A
Col-$0_{(wt)}$	T2	1.87	0.03	4			B
Col-$0_{(wt)}$	T3	1.36	0.03	4			C
$T6_{(C-)}$	T1	0.69	0.04	4	448.1997	<.0001	A
$T6_{(C-)}$	T2	1.85	0.14	4			B
$T6_{(C-)}$	T3	4.00	0.19	4			C

Table 25: Roots MDA (nM/FWg) Mean ± Standard error, ANOVA and Tukey test from A1(c+) , Col-0(wild type) and T6(c-) 2 weeks old plants exposed to different hydroponic treatments. As a deme, A1(c+) tolerant and T6(c-) sensitive to a calcareous soil. (T1) Control treatment: Hoagland ½ pH 5.9. (T2) High pH treatment: Hoagland ½ pH 8.3 and (T3) Bicarbonate treatment Hoagland ½ pH 8.3 + 10 mM NaHCO3. Each deme and treatment n=*4*

Deme	Treatment	Mean nmol/FW	Std Error	N	F Ratio	Prob > F	Tukey test
A1 $_{(C+)}$	T1	0.981	0	4	219.9647	<.0001	A
	T2	2.07	0.02	4			B
	T3	4.51	0.09	4			C
Col.0$_{(wt)}$	T1	0.98	0	4	309.311	<.0001	A
	T2	2.07	0.09	4			B
	T3	4.5	0.03	4			B
T6$_{(C-)}$	T1	0.96	0.01	4	52.7542	0.0002	A
	T2	3.3	0.06	4			B
	T3	9.19	0.43	4			C

Supplementary F6

Table 26: Primer list for transcript quantification of target genes. Specific primers were designed for *A.thaliana* using NCBI tool primer blast. All primers in this table have a primer efficiency from 90 to 110% .

Gene name	Tair ID	Forward sequence	Reverse sequence	Base pair
αCA1	AT3G52720	ACCCTGCTTTGTACGGTGAG	GGCATCTCCTGGCTGAAAGT	116
αCA2	AT2G28210	ACGCAAAATGTTATTTGGACCGT	CTGAATTATCGTGAACCGCCA	94
αCA3	AT5G04180	CCTCCTTGCACGGAAGATGT	CGTTCAGAGGTTGAGCTGGT	136
αCA4	AT4G20990	GCACTGAAGGCGTCATTTGG	ATCGTCAACGGCTTGCCTAA	89
αCA5	AT1G08065	AGTTGAGGACGAAACGCAGT	ACATTGCCCACTCTGGCTTT	89
αCA6	AT4G21000	CAGAGAAAGGACCAGCGGAA	TGAATTACAGCCGAAGCTGGT	160
αCA7	AT1G08080	AACGTTACTTGGAGCGTCGT	GCACTATGCGCTTGTTGGTT	133
αCA8	AT5G56330	TCCGAGGAACATATTCGCACT	AAGCATCGTGTACAGCCACA	70
βCA1	AT3G01500	ACCCTGCTTTGTACGGTGAG	GGCATCTCCTGGCTGAAAGT	116
βCA2	AT5G14740	CTCTTCGTCCCTCTCATCGC	TTGAAGTGGCTGAAGCAGGT	100
βCA3	AT1G23730	AGCTCCCACTAAGACCGAGT	CACGGCTTCCTTCTCACAGT	130
βCA4	AT1G70410	ATGGCTCCTGCATTCGGAAA	TCCTTTAATGGCGGCTTCG	102
βCA5	AT4G33580	TGCTGTCCTGGGATTCCAAC	CCACACCGGCTATGACCAAT	165
βCA6	AT1G58180	GCTCGTCGTCTAGTCTCTGC	GCTTCACCAAGCTGAAACCG	98
ACT2	AT3G18780	TCGCTGACCGTATGAGCAAA	TTGGAGATCCACATCTGCTG	151

Supplementary F7

Table 27: Primer list for transcript quantification of target genes. Specific primers were designed for *A.thaliana* using NCBI tool primer blast. All primers in this table have a tested primer efficiency from 90 to 110 %.

Gene	Tair ID	Forward sequence	Reverse sequence	Base pair
IRT1	AT4G19690	TGGGTCTTGGCGGTTGTATC	TCCAGCGGAGCATGCATTTA	185
FRO2	AT1G01580	CGACCGTCCATCTCCAACAT	CCACACTCGAACCTTCCACA	165
AHA1	AT4G30190	CAAAGACGCAAACCTCGCAT	ACATCACCACCTTTGCAATGAA	145
ACT2	AT3G18780	TCGCTGACCGTATGAGCAAA	TTGGAGATCCACATCTGCTG	151

Supplementary material: Chapter 2

Supplementary F1

Annex Table 1. Transcriptomic sequencing quality control table. Tolerant to calcareous soil $A1_{(C+)}$ and sensitive deme $T6_{(C-)}$. Plants 15-day old were submitted to different treatments. Control (Hoagland ½ pH 5.9); High pH (Hoagland ½ pH 8.3) and Bic (Hoagland ½ + 10 mM $NaHCO_3$. Leaves were extracted at 3 hours and 48 hours.

Deme	Treatment	Extraction (hours)	Raw Reads	Clean Reads	Effective Rate (%)	Error rate(%)	Gc Content(%)
$A1_{(c+)}$	pH 8.3	3 hours	35933740	35214764	98.00	0.01	46.20
$A1_{(c+)}$	pH 8.3	3 hours	33163439	32527812	98.08	0.01	46.14
$A1_{(c+)}$	pH 8.3	3 hours	37318125	36597109	98.07	0.01	46.30
$A1_{(c+)}$	pH 8.3	3 hours	33542467	32954568	98.25	0.01	46.36
$A1_{(c+)}$	pH 8.3	3 hours	39261311	38483922	98.02	0.01	46.45
$A1_{(c+)}$	pH 8.3	3 hours	34582220	34081441	98.55	0.01	46.41
$T6_{(c-)}$	pH 8.3	3 hours	35111264	34431789	98.06	0.01	46.01
$T6_{(c-)}$	pH 8.3	3 hours	38050838	37425783	98.36	0.01	45.99
$T6_{(c-)}$	pH 8.3	3 hours	34548309	33845369	97.97	0.02	45.97
$T6_{(c-)}$	pH 8.3	3 hours	36608898	35633337	97.34	0.01	46.33
$T6_{(c-)}$	pH 8.3	3 hours	41293496	40523696	98.14	0.01	46.00
$T6_{(c-)}$	pH 8.3	3 hours	42044151	41204164	98.00	0.01	46.08
$A1_{(c+)}$	pH 8.3	48 hours	34702956	34071859	98.18	0.01	45.72
$A1_{(c+)}$	pH 8.3	48 hours	34731325	34164549	98.37	0.01	45.76
$A1_{(c+)}$	pH 8.3	48 hours	31799679	31155790	97.98	0.01	45.66
$T6_{(c-)}$	pH 8.3	48 hours	38272541	37685089	98.47	0.01	46.30
$T6_{(c-)}$	pH 8.3	48 hours	32711245	31898297	97.51	0.01	46.20
$T6_{(c-)}$	pH 8.3	48 hours	31896502	31129936	97.60	0.01	46.38
$T6_{(c-)}$	pH 8.3	48 hours	33865023	33279183	98.27	0.02	46.36
$T6_{(c-)}$	pH 8.3	48 hours	34723668	34028222	98.00	0.01	46.08
$T6_{(c-)}$	pH 8.3	48 hours	31727150	30826825	97.16	0.01	45.91
$A1_{(c+)}$	pH 8.3	48 hours	32063259	31353472	97.79	0.01	45.93
$T6_{(c-)}$	pH 8.3	48 hours	33198566	32524909	97.97	0.01	46.29
$T6_{(c-)}$	pH 8.3	48 hours	35327496	34517496	97.71	0.01	46.06
$A1_{(c+)}$	Bic pH 8.3	3 hours	36451911	35127820	96.37	0.01	45.62
$A1_{(c+)}$	Bic pH 8.3	3 hours	34856531	34046945	97.68	0.01	45.61
$A1_{(c+)}$	Bic pH 8.3	3 hours	33856443	33390820	98.62	0.01	45.53
$A1_{(c+)}$	Bic pH 8.3	3 hours	39440568	38809209	98.40	0.01	45.79
$A1_{(c+)}$	Bic pH 8.3	3 hours	37129932	36546008	98.43	0.01	45.66
$A1_{(c+)}$	Bic pH 8.3	3 hours	34360293	33643478	97.91	0.01	45.70
$T6_{(c-)}$	Bic pH 8.3	3 hours	32744409	32322618	98.71	0.01	45.95
$T6_{(c-)}$	Bic pH 8.3	3 hours	33736467	33099086	98.11	0.02	45.62
$T6_{(c-)}$	Bic pH 8.3	3 hours	31986784	31051480	97.08	0.01	46.00

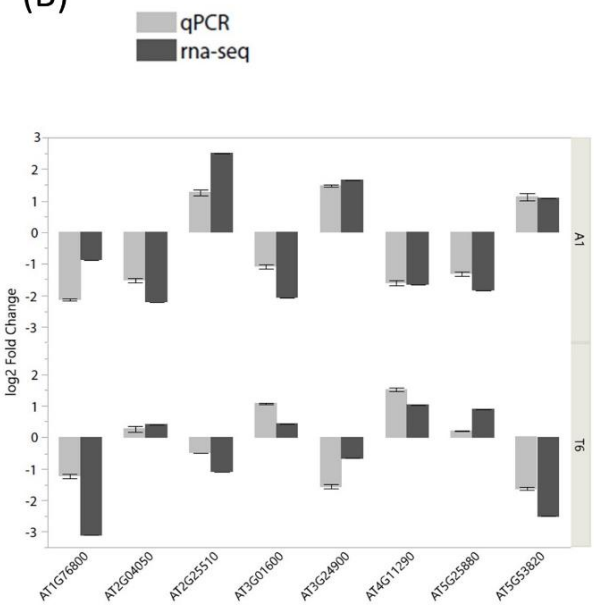
Deme	Treatment	Extraction (hours)	Raw Reads	Clean Reads	Effective Rate (%)	Error rate(%)	Gc Content(%)
$T6_{(c-)}$	Bic pH 8.3	3 hours	44216188	43481215	98.34	0.01	46.14
$T6_{(c-)}$	Bic pH 8.3	3 hours	40844404	40046068	98.05	0.01	46.11
$T6_{(c-)}$	Bic pH 8.3	3 hours	46227608	45492551	98.41	0.01	46.09
$A1_{(c+)}$	Bic pH 8.3	48 hours	30587655	30123526	98.48	0.02	46.21
$A1_{(c+)}$	Bic pH 8.3	48 hours	35688752	34940621	97.90	0.01	45.63
$A1_{(c+)}$	Bic pH 8.3	48 hours	37191854	36576721	98.35	0.01	45.58
$A1_{(c+)}$	Bic pH 8.3	48 hours	34469103	33962564	98.53	0.01	45.89
$A1_{(c+)}$	Bic pH 8.3	48 hours	33871219	33150723	97.87	0.01	45.51
$A1_{(c+)}$	Bic pH 8.3	48 hours	39121598	38441977	98.26	0.01	45.64
$T6_{(c-)}$	Bic pH 8.3	48 hours	35564223	34837492	97.96	0.01	45.36
$T6_{(c-)}$	Bic pH 8.3	48 hours	31939415	31485918	98.58	0.01	45.40
$T6_{(c-)}$	Bic pH 8.3	48 hours	33658474	32936561	97.86	0.01	45.22
$T6_{(c-)}$	Bic pH 8.3	48 hours	35807375	35087922	97.99	0.01	45.52
$T6_{(c-)}$	Bic pH 8.3	48 hours	36093112	35388493	98.05	0.01	45.30
$T6_{(c-)}$	Bic pH 8.3	48 hours	38343297	37666744	98.24	0.01	45.28
$A1_{(c+)}$	Control	3 hours	39307542	38543930	98.06	0.01	45.20
$T6_{(c-)}$	Control	3 hours	44280984	43622220	98.51	0.01	45.59
$T6_{(c-)}$	Control	3 hours	42268333	41466513	98.10	0.01	45.57
$T6_{(c-)}$	Control	3 hours	36352909	35884916	98.71	0.01	45.33
$A1_{(c+)}$	Control	3 hours	37646457	37109557	98.57	0.01	45.20
$A1_{(c+)}$	Control	3 hours	43952965	42970391	97.76	0.01	45.23
$A1_{(c+)}$	Control	3 hours	40408022	39754488	98.38	0.01	44.77
$A1_{(c+)}$	Control	3 hours	40225028	39053703	97.09	0.01	44.63
$A1_{(c+)}$	Control	3 hours	38789649	37761913	97.35	0.01	44.81
$T6_{(c-)}$	Control	3 hours	37084287	36482399	98.38	0.01	45.70
$T6_{(c-)}$	Control	3 hours	41150472	40435366	98.26	0.01	45.48
$T6_{(c-)}$	Control	3 hours	43250954	42384915	98.00	0.01	45.68
$A1_{(c+)}$	Control	48 hours	37221689	36665710	98.51	0.01	46.03
$A1_{(c+)}$	Control	48 hours	37999990	37233892	97.98	0.01	46.09
$A1_{(c+)}$	Control	48 hours	36965820	36322600	98.26	0.01	46.07
$A1_{(c+)}$	Control	48 hours	35386031	34663850	97.96	0.01	45.87
$A1_{(c+)}$	Control	48 hours	37796463	36575803	96.77	0.01	46.00
$A1_{(c+)}$	Control	48 hours	34040485	33509492	98.44	0.01	45.95
$T6_{(c-)}$	Control	48 hours	39976924	39115634	97.85	0.01	45.88
$T6_{(c-)}$	Control	48 hours	32393051	31840626	98.29	0.01	45.91
$T6_{(c-)}$	Control	48 hours	34561483	33843547	97.92	0.01	45.85
$T6_{(c-)}$	Control	48 hours	31772025	30976707	97.50	0.01	45.82
$T6_{(c-)}$	Control	48 hours	35606892	34935997	98.12	0.01	45.58
$T6_{(c-)}$	Control	48 hours	32969520	32287118	97.93	0.01	45.94

Annex Figure and Table 2. qPCR validation (A) Primer sequence from 8 selected genes used for RNA-seq expression results validation **(B)** Bar plot from RNA-seq (dark grey) and qPCR (light grey) expression results from the selected genes after 3 hours of exposure to bic and pH. Results are expressed in log2fold change; bars indicate standard error.

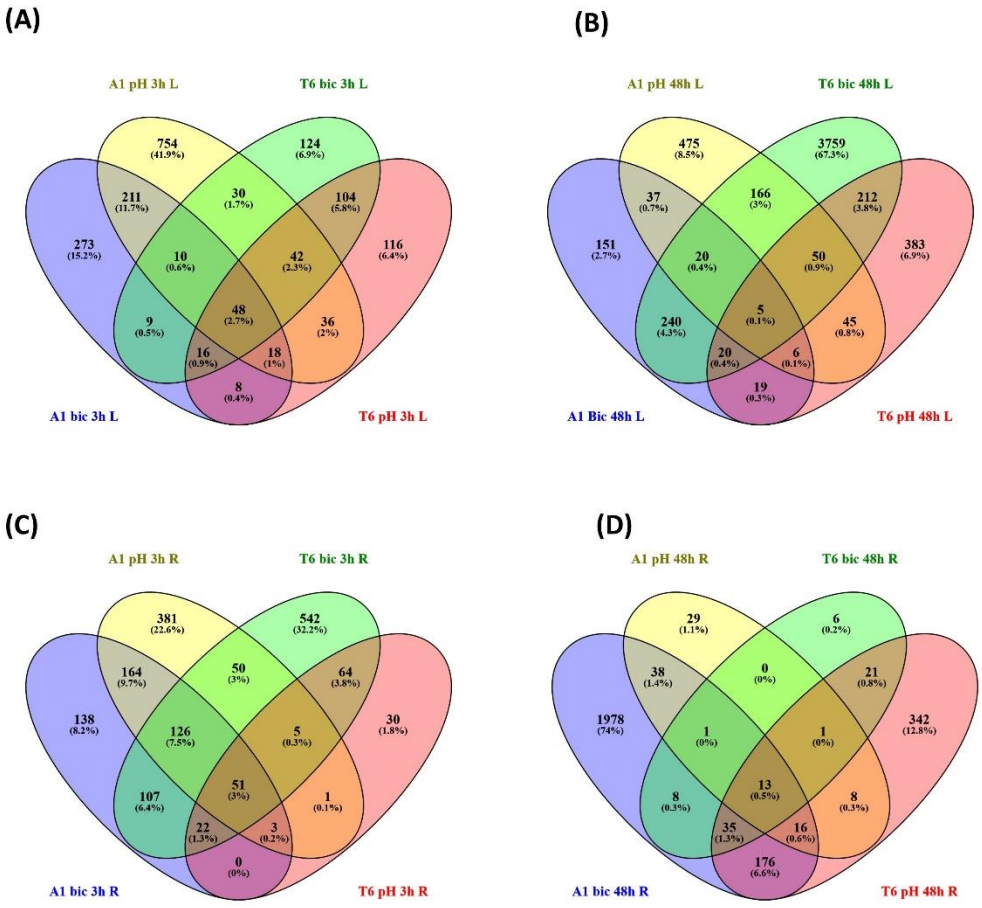
(A)

Gene Name	TAIR id	LFC_3h A1(c+) bic	LFC 3h T6(c-) bic	Forward sequence	Reverse sequence
Vacuolar iron transporter (VIT) family protein	AT1G76800	-0.88	-3.13	TTTCCGCAGGTGCGATTGTA	CCTAGCCGATGACCTAACCG
MATE efflux family protein	AT2G04050	-2.23	0.40	CTCTTCACATGCCGAAACATT	TGCCAACCACTTCCCCTTG
Transmembrane protein	AT2G25510	2.50	-1.10	AGGCACAAACGAACCTGGTC	TTATAGCGGCCGTTCGACAA
NAC domain (NAC044)	AT3G01600	-2.08	0.42	GTGGGATTGGTGGCTCTGAA	ATGCCTGCACTGTCTTGTGA
Receptor like protein 39(RLP39)	AT3G24900	1.65	-0.67	TCCTTGGCTTCAAGCTTCTTCT	TATCGCACCACACCCCATTC
Peroxidase superfamily protein	AT4G11290	-1.67	1.04	TTGGTGGACCGACATGGAAC	TGGGCACCGGATAACAAGAC
NADP-malic enzyme 3(NADP-ME3)	AT5G25880	-1.86	0.90	TCGTGTCCGTGATGACATGC	GATTCGATGCCAGTCCGAGT
Late embryogenesis abundant protein (LEA)	AT5G53820	1.07	-2.52	CAGAGCATGAGCTTCAACGC	ATGGTCTCACTCGCACCTTG

(B)



Annex Figure 3. Venn diagram bic-control *vs* pH 8.3 *vs* control treatment between contrasted demes ($A1_{(c+)}$ tolerant, $T6_{(c-)}$ sensitive) **(A)** leaves 3 hours **(B)** leaves 48 hours **(C)** roots 3 hours **(D)** roots 48 hours. DEGs were filtered at log fold change (LFC) > 1, LFC < −1, and adjusted *p*-value < 0.05. Blue color indicates tolerant line **$A1_{(C+)}$ bic *vs* control treatment (left down)**; yellow tolerant line **$A1_{(C+)}$ pH 8.3 *vs* control treatment (left up)**; green color is sensitive line **$T6_{(c-)}$ bic *vs* control (right up)** while red is showing sensitive line **$T6_{(c-)}$ at high pH *vs* control treatment (right down).**



Supplementary F2

Annex Table 4 Slim GO from DGEs (LFC>|1| & adj p-value < 0.05) in pairwise comparison treatments Bic 8.3 *vs* pH 5.9 and pH 8.3 *vs* pH 5.9 at different time points (3h and 48h) from $A1_{(c+)}$ and $T6_{(c-)}$ demes in leaves. BP= Biological process.

Deme	Treatment	Hour	GO	GO code	GO Name	DGE number	Fold Discovery	-log(FDR)
$A1_{(c+)}$	Bic	3h	BP	GO:0050789	Regulation of biological process	80	2.30E-03	2.64
$A1_{(c+)}$	Bic	3h	BP	GO:0065007	Biological regulation	89	2.10E-02	1.68
$A1_{(c+)}$	Bic	3h	BP	GO:0050896	Response to stimulus	118	2.40E-08	7.62
$A1_{(c+)}$	Bic	48h	BP	GO:0050896	Response to stimulus	96	2.10E-05	4.68
$A1_{(c+)}$	Bic	48h	BP	GO:0065007	Biological regulation	79	1.70E-03	2.77
$A1_{(c+)}$	Bic	48h	BP	GO:0050789	Regulation of biological process	70	2.40E-02	1.62
$A1_{(c+)}$	Bic	48h	BP	GO:0051234	Establishment of localization	40	3.30E-02	1.48
$A1_{(c+)}$	pH 8.3	3h	BP	GO:0032501	Multicellular organismal	86	3.00E-02	1.52
$A1_{(c+)}$	pH 8.3	3h	BP	GO:0050789	Regulation of biological process	152	7.60E-04	3.12
$A1_{(c+)}$	pH 8.3	3h	BP	GO:0065007	Biological regulation	167	1.10E-02	1.96
$A1_{(c+)}$	pH 8.3	3h	BP	GO:0050896	Response to stimulus	163	1.00E-03	3
$A1_{(c+)}$	pH 8.3	48h	BP	GO:0016043	Cellular component	57	8.30E-06	5.08
$A1_{(c+)}$	pH 8.3	48h	BP	GO:0050789	Regulation of biological process	115	4.30E-04	3.37
$A1_{(c+)}$	pH 8.3	48h	BP	GO:0065007	Biological regulation	125	9.80E-04	3.01
$A1_{(c+)}$	pH 8.3	48h	BP	GO:0009987	Cellular process	287	1.10E-02	1.96
$A1_{(c+)}$	pH 8.3	48h	BP	GO:0032501	Multicellular organismal	65	2.70E-02	1.57
$T6_{(c-)}$	Bic	3h	BP	GO:0051234	Establishment of localization	44	1.20E-05	4.92
$T6_{(c-)}$	Bic	3h	BP	GO:0051179	Localization	45	1.20E-05	4.92
$T6_{(c-)}$	Bic	3h	BP	GO:0050896	Response to stimulus	76	8.60E-05	4.07
$T6_{(c-)}$	Bic	48h	BP	GO:0050896	Response to stimulus	765	3.50E-35	34.46
$T6_{(c-)}$	Bic	48h	BP	GO:0008152	Metabolic process	1409	6.80E-08	7.17
$T6_{(c-)}$	Bic	48h	BP	GO:0065007	Biological regulation	600	6.30E-06	5.2
$T6_{(c-)}$	Bic	48h	BP	GO:0051704	Multi-organism process	144	5.00E-05	4.3
$T6_{(c-)}$	Bic	48h	BP	GO:0009987	Cellular process	1487	1.10E-04	3.96

Deme	Treatment	Hour	GO	GO code	GO Name	DGE number	Fold Discovery	-log(FDR)
$T6_{(c-)}$	Bic	48h	BP	GO:0050789	Regulation of biological process	516	5.50E-04	3.26
$T6_{(c-)}$	pH 8.3	3h	BP	GO:0032502	Developmental process	39	2.20E-02	1.66
$T6_{(c-)}$	pH 8.3	3h	BP	GO:0032501	Multicellular organismal	36	2.50E-02	1.6
$T6_{(c-)}$	pH 8.3	3h	BP	GO:0050789	Regulation of biological process	56	2.50E-02	1.6
$T6_{(c-)}$	pH 8.3	3h	BP	GO:0065007	Biological regulation	73	2.50E-03	2.6
$T6_{(c-)}$	pH 8.3	3h	BP	GO:0051234	Establishment of localization	43	6.90E-05	4.16
$T6_{(c-)}$	pH 8.3	3h	BP	GO:0051179	Localization	44	6.90E-05	4.16
$T6_{(c-)}$	pH 8.3	3h	BP	GO:0050896	Response to stimulus	77	1.40E-05	4.85
$T6_{(c-)}$	pH 8.3	48h	BP	GO:0050896	Response to stimulus	136	7.90E-07	6.1
$T6_{(c-)}$	pH 8.3	48h	BP	GO:0065007	Biological regulation	110	1.20E-02	1.92
$T6_{(c-)}$	pH 8.3	48h	BP	GO:0050789	Regulation of biological process	98	1.70E-02	1.77

Annex Table 5: Slim GO from DGEs (LFC>|1| & adj p-value < 0.05) in pairwise comparison treatments Bic 8.3 vs pH 5.9 and pH 8.3 *vs* pH 5.9 at different time points (3h and 48h) from $A1_{(c+)}$ and $T6_{(c-)}$ demes in leaves. CC=cellular component.

Deme	Treatment	Hour	GO	GO code	GO Name	DGE number	Fold Discovery Rate	-log(FDR)
$A1_{(c+)}$	pH 8.3	3h	CC	GO:0005623	Cell	504	2.60E-03	2.59
$A1_{(c+)}$	pH 8.3	3h	CC	GO:0044464	Cell part	504	2.60E-03	2.59
$A1_{(c+)}$	pH 8.3	3h	CC	GO:0005576	Extracellular region	19	2.60E-03	2.59
$T6_{(c-)}$	pH 8.3	3h	CC	GO:0005623	Cell	186	6.50E-03	2.19
$T6_{(c-)}$	pH 8.3	3h	CC	GO:0044464	Cell part	186	6.50E-03	2.19
$T6_{(c-)}$	pH 8.3	3h	CC	GO:0005623	Cell	189	3.70E-03	2.43
$T6_{(c-)}$	pH 8.3	3h	CC	GO:0044464	Cell part	189	3.70E-03	2.43
$A1_{(c+)}$	Bic	48h	CC	GO:0005623	Cell	238	1.50E-03	2.82
$A1_{(c+)}$	Bic	48h	CC	GO:0044464	Cell part	238	1.50E-03	2.82
$A1_{(c+)}$	Bic	48h	CC	GO:0005576	Extracellular region	12	7.80E-03	2.11
$T6_{(c-)}$	pH 8.3	48h	CC	GO:0005623	Cell	2115	4.20E-27	26.38
$T6_{(c-)}$	pH 8.3	48h	CC	GO:0044464	Cell part	2115	4.20E-27	26.38
$T6_{(c-)}$	pH 8.3	48h	CC	GO:0044422	Organelle part	422	4.50E-11	10.35
$T6_{(c-)}$	pH 8.3	48h	CC	GO:0043226	Organelle	1143	4.80E-10	9.32
$T6_{(c-)}$	pH 8.3	48h	CC	GO:0005576	Extracellular region	59	9.30E-04	3.03
$T6_{(c-)}$	pH 8.3	48h	CC	GO:0005623	Cell	330	3.60E-02	1.44
$T6_{(c-)}$	pH 8.3	48h	CC	GO:0044464	Cell part	330	3.60E-02	1.44
$T6_{(c-)}$	pH 8.3	48h	CC	GO:0005576	Extracellular region	14	4.30E-02	1.37

Annex Table 6: Slim GO from DGEs (LFC>|1| & adj p-value < 0.05) in pairwise comparison treatments Bic 8.3 *vs* pH 5.9 and pH 8.3 vs pH 5.9 at different time points (3h and 48h) from $A1_{(c+)}$ and $T6_{(c-)}$ demes in leaves. MF=molecular function.

Deme	Treatment	Hour	GO	GO code	Complete GO	DGE number	FDR	-log(FDR)
$A1_{(c+)}$	Bic	3h	MF	GO:0005488	Binding	180	2.30E-02	1.64
$A1_{(c+)}$	Bic	48h	MF	GO:0003824	Catalytic activity	164	2.40E-03	2.62
$A1_{(c+)}$	Bic	48h	MF	GO:0030528	Transcription regulator activity	55	2.60E-03	2.59
$A1_{(c+)}$	pH 8.3	48h	MF	GO:0030528	Transcription regulator activity	118	1.30E-05	4.89
$A1_{(c+)}$	pH 8.3	48h	MF	GO:0003824	Catalytic activity	250	9.30E-04	3.03
$A1_{(c+)}$	pH 8.3	48h	MF	GO:0030234	Enzyme regulator activity	20	3.40E-03	2.47
$A1_{(c+)}$	pH 8.3	48h	MF	GO:0005488	Binding	278	7.50E-03	2.12
$T6_{(c-)}$	Bic	3h	MF	GO:0005215	Transporter activity	33	2.50E-03	2.6
$T6_{(c-)}$	Bic	3h	MF	GO:0016209	Antioxidant activity	9	3.00E-03	2.52
$T6_{(c-)}$	Bic	3h	MF	GO:0003824	Catalytic activity	133	2.50E-03	2.6
$T6_{(c-)}$	Bic	3h	MF	GO:0009055	Electron carrier activity	15	2.10E-04	3.68
$T6_{(c-)}$	pH 8.3	3h	MF	GO:0005215	Transporter activity	36	2.70E-04	3.57
$T6_{(c-)}$	pH 8.3	3h	MF	GO:0030528	Transcription regulator activity	47	1.10E-03	2.96
$T6_{(c-)}$	pH 8.3	3h	MF	GO:0003824	Catalytic activity	127	8.20E-03	2.09
$T6_{(c-)}$	Bic	48h	MF	GO:0003824	Catalytic activity	1434	1.43E-24	23.84
$T6_{(c-)}$	Bic	48h	MF	GO:0009055	Electron carrier activity	66	5.70E-04	3.24
$T6_{(c-)}$	Bic	48h	MF	GO:0030528	Transcription regulator activity	354	8.70E-04	3.06
$T6_{(c-)}$	Bic	48h	MF	GO:0005215	Transporter activity	224	6.40E-03	2.19
$T6_{(c-)}$	pH 8.3	48h	MF	GO:0003824	Catalytic activity	274	3.80E-10	9.42
$T6_{(c-)}$	pH 8.3	48h	MF	GO:0030528	Transcription regulator activity	80	2.10E-04	3.68
$T6_{(c-)}$	pH 8.3	48h	MF	GO:0016209	Antioxidant activity	11	7.50E-03	2.12

Annex Table 7: Slim GO from DGEs (LFC>|1| & adj p-value < 0.05) in pairwise comparison treatments Bic 8.3 vs pH 5.9 and pH 8.3 vs pH 5.9 at different time points (3h and 48h) from $A1_{(c+)}$ and $T6_{(c-)}$ demes in roots . BP= Biological process.

Deme	Treatment	Hour	Go	GO Name	Num	FDR	log
$A1_{(c+)}$	Bic	3h	BP	Developmental process	120	1.00E-02	2
$A1_{(c+)}$	Bic	3h	BP	Multicellular organismal process	116	2.10E-02	1.67
$A1_{(c+)}$	Bic	3h	BP	Cell wall organization or biogenesis	48	2.40E-05	4.62
$A1_{(c+)}$	Bic	3h	BP	Cellular process	362	6.20E-04	3.21
$A1_{(c+)}$	Bic	3h	BP	Metabolic process	349	2.00E-04	3.7
$A1_{(c+)}$	Bic	3h	BP	Establishment of localization	114	1.80E-03	2.74
$A1_{(c+)}$	Bic	3h	BP	Localization	116	4.40E-03	2.36
$A1_{(c+)}$	Bic	3h	BP	Growth	36	3.50E-02	1.46
$A1_{(c+)}$	Bic	3h	BP	Response to stimulus	205	2.10E-07	6.68
$A1_{(c+)}$	pH 8.3	3h	BP	Developmental process	179	3.80E-08	7.42
$A1_{(c+)}$	pH 8.3	3h	BP	Multicellular organismal process	165	9.00E-06	5.05
$A1_{(c+)}$	pH 8.3	3h	BP	Cell wall organization or biogenesis	61	6.00E-07	6.22
$A1_{(c+)}$	pH 8.3	3h	BP	Biological regulation	238	1.90E-06	5.72
$A1_{(c+)}$	pH 8.3	3h	BP	Cellular process	479	2.30E-07	6.63
$A1_{(c+)}$	pH 8.3	3h	BP	Metabolic process	445	2.30E-05	4.63
$A1_{(c+)}$	pH 8.3	3h	BP	Growth	54	6.90E-05	4.16
$A1_{(c+)}$	pH 8.3	3h	BP	Response to stimulus	223	1.00E-03	3
$A1_{(c+)}$	Bic	48h	BP	Regulation of biological process	427	1.80E-02	1.74
$A1_{(c+)}$	Bic	48h	BP	Biological regulation	513	2.60E-05	4.58
$A1_{(c+)}$	Bic	48h	BP	Multi-organism process	118	9.00E-04	3.05
$A1_{(c+)}$	Bic	48h	BP	Cellular process	1330	8.60E-09	8.07
$A1_{(c+)}$	Bic	48h	BP	Metabolic process	1224	5.20E-09	8.28
$A1_{(c+)}$	Bic	48h	BP	Response to stimulus	627	1.90E-24	23.72
$T6_{(c-)}$	pH 8.3	3h	BP	Single-organism process	103	8.40E-10	9.08
$T6_{(c-)}$	pH 8.3	3h	BP	Cellular process	101	7.80E-03	2.11
$T6_{(c-)}$	pH 8.3	3h	BP	Metabolic process	102	5.10E-04	3.29
$T6_{(c-)}$	pH 8.3	3h	BP	Growth	11	4.90E-02	1.3
$T6_{(c-)}$	pH 8.3	3h	BP	Response to stimulus	70	5.00E-06	5.3
$T6_{(c-)}$	Bic	3h	BP	Signaling	143	7.70E-09	8.11
$T6_{(c-)}$	Bic	3h	BP	Regulation of biological process	239	3.00E-05	4.52
$T6_{(c-)}$	Bic	3h	BP	Immune system process	67	1.10E-05	4.95
$T6_{(c-)}$	Bic	3h	BP	Cellular component organization	138	1.90E-02	1.72
$T6_{(c-)}$	Bic	3h	BP	Biological regulation	308	1.10E-10	9.96
$T6_{(c-)}$	Bic	3h	BP	Positive regulation of biological process	48	3.10E-02	1.51
$T6_{(c-)}$	Bic	3h	BP	Death	38	3.10E-04	3.51
$T6_{(c-)}$	Bic	3h	BP	Multi-organism process	109	1.10E-06	5.96
$T6_{(c-)}$	Bic	3h	BP	Cellular process	620	4.10E-15	14.39
$T6_{(c-)}$	Bic	3h	BP	Signaling process	90	2.70E-03	2.57
$T6_{(c-)}$	Bic	3h	BP	Metabolic process	583	9.20E-13	12.04

Deme	Treatment	Hour	Go	GO Name	Num	FDR	log
$T6_{(c-)}$	Bic	3h	BP	Establishment of localization	201	1.00E-09	9
$T6_{(c-)}$	Bic	3h	BP	Localization	203	1.20E-08	7.92
$T6_{(c-)}$	Bic	3h	BP	Response to stimulus	392	9.00E-34	33.05
$T6_{(c-)}$	pH 8.3	48h	BP	Response to stimulus	93	4.20E-02	1.38

Annex Table 8: Slim GO from DGEs (LFC>|1| & adj p-value < 0.05) in pairwise comparison treatments Bic 8.3 vs pH 5.9 and pH 8.3 vs pH 5.9 at different time points (3h and 48h) from A1(c+) and T6(c-) demes in roots. CC=cellular component.

Deme	Treatment	Hour(h)	Process	Name	Num	FDR	-log(FDR)
$A1_{(c+)}$	Bic	3h	CC	Extracellular region	102	5.70E-05	4.24
$T6_{(c-)}$	Bic	3h	CC	Cell	798	5.60E-03	2.25
$T6_{(c-)}$	Bic	3h	CC	Cell part	798	5.60E-03	2.25
$T6_{(c-)}$	Bic	3h	CC	Symplast	52	1.10E-03	2.96
$T6_{(c-)}$	Bic	3h	CC	Extracellular region	133	1.30E-03	2.89
$T6_{(c-)}$	Bic	3h	CC	Organelle part	152	3.00E-03	2.52
$A1_{(c+)}$	pH 8.3	3h	CC	Extracellular region	140	5.40E-09	8.27
$T6_{(c-)}$	pH 8.3	3h	CC	Extracellular region	40	4.40E-04	3.37
$A1_{(c+)}$	Bic	48h	CC	Cell	1894	1.40E-37	36.86
$A1_{(c+)}$	Bic	48h	CC	Cell part	1894	1.40E-37	36.85
$A1_{(c+)}$	Bic	48h	CC	Extracellular region	57	8.60E-05	4.06
$A1_{(c+)}$	Bic	48h	CC	Organelle	941	5.90E-07	6.23
$T6_{(c-)}$	pH 8.3	48h	CC	Cell	298	1.60E-04	3.8
$T6_{(c-)}$	pH 8.3	48h	CC	Cell part	298	1.60E-04	3.8
$T6_{(c-)}$	pH 8.3	48h	CC	Cell part	1	6.50E-01	1

Annex Table 9: Slim GO from DGEs (LFC>|1| & adj p-value < 0.05) in pairwise comparison treatments Bic 8.3 vs pH 5.9 and pH 8.3 vs pH 5.9 at different time points (3h and 48h) from $A1_{(c+)}$ and $T6_{(c-)}$ demes in roots. MF=molecular function.

Deme	Treatment	Extraction	Process	Name	Num	FDR	-log(FDR)
$A1_{(c+)}$	Bic	3h	MF	antioxidant activity	10	4.30E-02	1.37
$A1_{(c+)}$	Bic	3h	MF	catalytic activity	281	2.10E-12	11.68
$A1_{(c+)}$	Bic	3h	MF	electron carrier activity	24	3.30E-02	1.49
$A1_{(c+)}$	pH 8.3	3h	MF	antioxidant activity	15	1.90E-03	2.72
$A1_{(c+)}$	pH 8.3	3h	MF	catalytic activity	347	2.30E-12	11.63
$A1_{(c+)}$	Bic	3h	MF	enzyme regulator activity	22	3.90E-02	1.41
$A1_{(c+)}$	Bic	48h	MF	transporter activity	204	1.60E-04	3.8
$A1_{(c+)}$	Bic	48h	MF	antioxidant activity	33	3.10E-03	2.51
$A1_{(c+)}$	Bic	48h	MF	catalytic activity	1374	1.00E-48	48
$A1_{(c+)}$	Bic	48h	MF	binding	1230	1.30E-04	3.89
$A1_{(c+)}$	Bic	48h	MF	enzyme regulator activity	56	4.90E-02	1.31
$A1_{(c+)}$	Bic	48h	MF	electron carrier activity	55	1.10E-03	2.96
T6	pH 8.3	3h	MF	catalytic activity	83	5.70E-04	3.24
T6	Bic	3h	MF	transporter activity	71	1.10E-02	1.96
T6	Bic	3h	MF	transcription regulator activity	84	3.50E-02	1.46
T6	Bic	3h	MF	catalytic activity	387	5.20E-07	6.29
T6	Bic	3h	MF	binding	440	6.60E-03	2.18
T6	Bic	3h	MF	enzyme regulator activity	26	3.50E-02	1.46
T6	Bic	48h	MF	catalytic activity	54	7.00E-04	3.15
T6	pH 8.3	48h	MF	antioxidant activity	11	4.10E-03	2.39
T6	pH 8.3	48h	MF	catalytic activity	221	6.70E-07	6.17
T6	pH 8.3	48h	MF	binding	220	1.10E-02	1.96
T6	pH 8.3	48h	MF	electron carrier activity	15	7.10E-03	2.15

Supplementary F3

Annex Table 10: KEEG pathway from DGEs (LFC> |1| & p-value < 0.05) in pairwise comparation treatments Bic vs pH 5.9 and pH 8.3 *vs* pH 5.9 at different time points (3h and 48h) from $A1_{(c+)}$ in leaves.

Deme	Treatment	Hour	Term	Count	%	p-value	Fold Enrichment	FDR
$A1_{(c+)}$	Bic	3h	alpha-Linolenic acid metabolism	6	1	5.30E-04	8.7	2.60E-02
$A1_{(c+)}$	Bic	3h	2-Oxocarboxylic acid metabolism	7	1.2	2.60E-03	4.9	6.80E-02
$A1_{(c+)}$	Bic	3h	Glucosinolate biosynthesis	3	0.5	2.10E-02	13	4.00E-01
$A1_{(c+)}$	Bic	3h	Fatty acid metabolism	5	0.9	4.30E-02	3.7	6.40E-01
$A1_{(c+)}$	Bic	48h	Pentose and glucuronate interconversions	7	1.4	2.90E-03	4.8	1.90E-01
$A1_{(c+)}$	Bic	48h	Plant hormone signal transduction	12	2.4	1.00E-02	2.4	3.50E-01
$A1_{(c+)}$	Bic	48h	Circadian rhythm - plant	4	0.8	2.90E-02	5.8	4.90E-01
$A1_{(c+)}$	Bic	48h	Starch and sucrose metabolism	7	1.4	3.00E-02	2.9	4.90E-01
$A1_{(c+)}$	Bic	48h	Nitrogen metabolism	4	0.8	4.40E-02	5	5.30E-01
$A1_{(c+)}$	pH 8.3	3h	Pentose and glucuronate interconversions	8	0.7	9.20E-03	3.3	3.90E-01
$A1_{(c+)}$	pH 8.3	48h	Homologous recombination	10	1.3	2.10E-06	8.3	1.30E-04
$A1_{(c+)}$	pH 8.3	48h	Pyrimidine metabolism	8	1	9.60E-03	3.3	3.00E-01
$A1_{(c+)}$	pH 8.3	48h	Ubiquitin mediated proteolysis	8	1	3.90E-02	2.5	7.70E-01
$A1_{(c+)}$	pH 8.3	48h	Mismatch repair	4	0.5	5.00E-02	4.8	7.70E-01
$T6_{(c-)}$	Bic	3h	Fatty acid elongation	6	1.6	2.20E-05	16.7	1.30E-03
$T6_{(c-)}$	Bic	3h	Biosynthesis of secondary metabolites	22	5.8	6.20E-04	2	1.80E-02
$T6_{(c-)}$	Bic	3h	Phenylpropanoid biosynthesis	7	1.9	5.20E-03	4.2	1.00E-01
$T6_{(c-)}$	Bic	48h	Photosynthesis - antenna proteins	20	0.5	4.20E-12	5.1	3.80E-10
$T6_{(c-)}$	Bic	48h	Photosynthesis	39	0.9	1.70E-10	2.8	6.20E-09
$T6_{(c-)}$	Bic	48h	Carbon fixation in photosynthetic organisms	29	0.7	6.40E-06	2.4	1.20E-04
$T6_{(c-)}$	Bic	48h	Glyoxylate and dicarboxylate metabolism	28	0.6	8.80E-05	2.1	1.40E-03
$T6_{(c-)}$	Bic	48h	Carotenoid biosynthesis	15	0.3	1.50E-04	2.9	2.10E-03
$T6_{(c-)}$	Bic	48h	alpha-Linolenic acid metabolism	17	0.4	1.80E-04	2.7	2.20E-03
$T6_{(c-)}$	Bic	48h	Porphyrin and chlorophyll metabolism	17	0.4	6.70E-03	2	5.70E-02
$T6_{(c-)}$	Bic	48h	Nitrogen metabolism	15	0.3	1.10E-02	2	8.00E-02
$T6_{(c-)}$	Bic	48h	Insulin resistance	13	0.3	2.20E-02	2	1.40E-01
$T6_{(c-)}$	Bic	48h	Diterpenoid biosynthesis	9	0.2	3.00E-02	2.3	1.70E-01
$T6_{(c-)}$	Bic	48h	Isoquinoline alkaloid biosynthesis	9	0.2	3.90E-02	2.2	1.70E-01
$T6_{(c-)}$	Bic	48h	Ascorbate and aldarate metabolism	14	0.3	2.10E-02	1.9	1.40E-01
$T6_{(c-)}$	Bic	48h	Insulin resistance	13	0.3	2.20E-02	2	1.40E-01
$T6_{(c-)}$	Bic	48h	DNA replication	16	0.4	2.30E-02	1.8	1.40E-01
$T6_{(c-)}$	Bic	48h	Diterpenoid biosynthesis	9	0.2	3.00E-02	2.3	1.70E-01
$T6_{(c-)}$	Bic	48h	Biosynthesis of amino acids	58	1.3	3.00E-02	1.3	1.70E-01
$T6_{(c-)}$	Bic	48h	Alanine, aspartate and glutamate metabolism	15	0.3	3.50E-02	1.8	1.70E-01
$T6_{(c-)}$	Bic	48h	Phenylalanine, tyrosine and tryptophan biosynthesis	17	0.4	3.60E-02	1.7	1.70E-01
$T6_{(c-)}$	Bic	48h	Phenylpropanoid biosynthesis	38	0.9	3.70E-02	1.4	1.70E-01
$T6_{(c-)}$	Bic	48h	Isoquinoline alkaloid biosynthesis	9	0.2	3.90E-02	2.2	1.70E-01

Deme	Treatment	Hour	Term	Count	%	p-value	Fold Enrichment	FDR
$T6_{(c-)}$	Bic	48h	Arginine and proline metabolism	16	0.4	3.90E-02	1.7	1.70E-01
$T6_{(c-)}$	Bic	48h	Tropane, piperidine and pyridine alkaloid biosynthesis	12	0.3	4.30E-02	1.9	1.80E-01
$T6_{(c-)}$	Bic	48h	Fatty acid degradation	13	0.3	4.80E-02	1.8	2.00E-01
$T6_{(c-)}$	pH 8.3	3h	Fatty acid elongation	7	1.9	5.10E-07	21.6	2.50E-05
$T6_{(c-)}$	pH 8.3	3h	Cutin, suberine and wax biosynthesis	4	1.1	1.90E-03	15.6	4.60E-02
$T6_{(c-)}$	pH 8.3	48h	Phenylpropanoid biosynthesis	15	2.1	2.20E-06	4.7	1.60E-04
$T6_{(c-)}$	pH 8.3	48h	Flavonoid biosynthesis	5	0.7	7.00E-04	11.7	1.70E-02
$T6_{(c-)}$	pH 8.3	48h	ABC transporters	4	0.5	1.20E-02	8.2	2.10E-01
$T6_{(c-)}$	pH 8.3	48h	Phenylpropanoid biosynthesis	15	2.1	2.20E-06	4.70E+00	1.60E-04
$T6_{(c-)}$	pH 8.3	48h	Biosynthesis of secondary metabolites	39	5.3	3.10E-05	1.90E+00	1.10E-03
$T6_{(c-)}$	pH 8.3	48h	Flavonoid biosynthesis	5	0.7	7.00E-04	1.17E+01	1.70E-02
$T6_{(c-)}$	pH 8.3	48h	ABC transporters	4	0.5	1.20E-02	8.20E+00	2.10E-01
$T6_{(c-)}$	pH 8.3	48h	Metabolic pathways	48	6.6	2.10E-02	1.30E+00	3.00E-01

Annex Table 11: Keeg pathway from DGEs (LFC> |1| & p-value < 0.05) in pairwise comparation treatments Bic *vs* pH 5.9 and pH8.3 *vs* pH 5.9 at different time points (3h and 48h) from $A1_{(c+)}$ in roots. Met: metabolism.

Deme	Treatment	Extraction	Term	Count	%	P-Value	Fold Enrichment	FDR
$A1_{(c+)}$	pH	3h	Glutathione met	11	1.4	1.80E-04	4.3	1.40E-02
$A1_{(c+)}$	pH	3h	Phenylpropanoid biosynthesis	13	1.7	1.00E-03	3	3.90E-02
$A1_{(c+)}$	pH	3h	Amino sugar and nucleotide sugar met	10	1.3	1.00E-02	2.7	2.10E-01
$A1_{(c+)}$	pH	3h	Biosynthesis of secondary metabolites	40	5.2	1.10E-02	1.4	2.10E-01
$A1_{(c+)}$	pH	3h	Pentose and glucuronate interconversions	7	0.9	1.60E-02	3.4	2.50E-01
$A1_{(c+)}$	Bic	3h	Glutathione met	11	1.8	1.70E-04	4.4	1.20E-02
$A1_{(c+)}$	Bic	3h	Metabolic pathways	67	11.1	1.50E-03	1.3	5.50E-02
$A1_{(c+)}$	Bic	3h	Valine, leucine and isoleucine degradation	6	1	6.70E-03	4.9	1.30E-01
$A1_{(c+)}$	Bic	3h	Phenylpropanoid biosynthesis	11	1.8	9.10E-03	2.6	1.30E-01
$A1_{(c+)}$	Bic	3h	Biosynthesis of secondary metabolites	40	6.6	9.30E-03	1.4	1.30E-01
$A1_{(c+)}$	Bic	3h	Pentose and glucuronate interconversions	7	1.2	1.60E-02	3.4	1.90E-01
$A1_{(c+)}$	Bic	3h	Nitrogen met	5	0.8	2.50E-02	4.4	2.60E-01
$A1_{(c+)}$	Bic	48h	Biosynthesis of secondary metabolites	221	6	2.60E-10	1.4	2.80E-08
$A1_{(c+)}$	Bic	48h	Fatty acid biosynthesis	19	0.5	6.00E-06	3.1	3.20E-04
$A1_{(c+)}$	Bic	48h	Valine, leucine and isoleucine degradation	20	0.5	1.00E-05	2.9	3.70E-04

Deme	Treatment	Extraction	Term	Count	%	P-Value	Fold Enrichment	FDR
$A1_{(c+)}$	Bic	48h	Biosynthesis of antibiotics	99	2.7	1.70E-05	1.5	4.60E-04
$A1_{(c+)}$	Bic	48h	Metabolic pathways	326	8.8	5.20E-05	1.2	1.10E-03
$A1_{(c+)}$	Bic	48h	Fatty acid met	24	0.6	1.50E-04	2.3	2.70E-03
$A1_{(c+)}$	Bic	48h	Phenylpropanoid biosynthesis	41	1.1	4.20E-04	1.7	6.00E-03
$A1_{(c+)}$	Bic	48h	Pyruvate met	26	0.7	4.50E-04	2	6.00E-03
$A1_{(c+)}$	Bic	48h	Amino sugar and nucleotide sugar met	36	1	6.80E-04	1.8	8.00E-03
$A1_{(c+)}$	Bic	48h	Steroid biosynthesis	14	0.4	8.40E-04	2.7	9.00E-03
$A1_{(c+)}$	Bic	48h	Starch and sucrose met	33	0.9	1.40E-03	1.7	1.40E-02
$A1_{(c+)}$	Bic	48h	Carbon met	58	1.6	2.10E-03	1.5	1.90E-02
$A1_{(c+)}$	Bic	48h	Biotin met	8	0.2	2.30E-03	3.8	1.90E-02
$A1_{(c+)}$	Bic	48h	Pentose and glucuronate interconversions	22	0.6	3.40E-03	1.9	2.60E-02
$A1_{(c+)}$	Bic	48h	Glycine, serine and threonine met	20	0.5	9.00E-03	1.8	6.40E-02
$A1_{(c+)}$	Bic	48h	Glutathione met	24	0.6	9.90E-03	1.7	6.60E-02
$A1_{(c+)}$	Bic	48h	Propanoate met	9	0.2	1.20E-02	2.7	7.20E-02
$A1_{(c+)}$	Bic	48h	Glyoxylate and dicarboxylate met	20	0.5	1.20E-02	1.8	7.20E-02
$A1_{(c+)}$	Bic	48h	Phagosome	22	0.6	1.40E-02	1.7	7.60E-02
$A1_{(c+)}$	Bic	48h	Nitrogen met	13	0.4	1.90E-02	2	9.50E-02
$A1_{(c+)}$	Bic	48h	Phenylalanine met	13	0.4	1.90E-02	2	9.50E-02
$A1_{(c+)}$	Bic	48h	Tyrosine met	12	0.3	3.10E-02	2	1.50E-01
$A1_{(c+)}$	Bic	48h	Tryptophan met	13	0.4	3.70E-02	1.9	1.70E-01
$A1_{(c+)}$	Bic	48h	Fatty acid degradation	12	0.3	3.70E-02	1.9	1.70E-01
$A1_{(c+)}$	Bic	48h	Histidine met	7	0.2	4.30E-02	2.6	1.90E-01
$A1_{(c+)}$	Bic	48h	Glycolysis / Gluconeogenesis	25	0.7	4.90E-02	1.5	2.00E-01
pH_48h	pH 8.3	48h	Amino sugar and nucleotide sugar met	8	1.3	4.20E-02	0	8.30E-01
$T6_{(c-)}$	Bic	3h	Photosynthesis	17	1.8	3.30E-07	4.7	2.70E-05
$T6_{(c-)}$	Bic	3h	Metabolic pathways	118	12.4	8.60E-06	1.4	3.50E-04
$T6_{(c-)}$	Bic	3h	Valine, leucine and isoleucine degradation	10	1	1.80E-04	4.7	5.00E-03
$T6_{(c-)}$	Bic	3h	Carbon fixation in photosynthetic organisms	12	1.3	3.00E-04	3.7	6.20E-03
$T6_{(c-)}$	Bic	3h	Carbon met	26	2.7	4.40E-04	2.1	7.00E-03
$T6_{(c-)}$	Bic	3h	Fructose and mannose met	11	1.2	5.10E-04	3.8	7.00E-03
$T6_{(c-)}$	Bic	3h	Biosynthesis of secondary metabolites	65	6.8	4.90E-03	1.4	5.70E-02
$T6_{(c-)}$	Bic	3h	Biosynthesis of antibiotics	33	3.5	6.80E-03	1.6	6.90E-02
$T6_{(c-)}$	Bic	3h	Nitrogen met	7	0.7	1.20E-02	3.5	1.10E-01
$T6_{(c-)}$	Bic	3h	Glycolysis / Gluconeogenesis	12	1.3	1.60E-02	2.3	1.30E-01
$T6_{(c-)}$	Bic	3h	Photosynthesis - antenna proteins	5	0.5	1.80E-02	4.8	1.30E-01
$T6_{(c-)}$	Bic	3h	Starch and sucrose met	12	1.3	3.10E-02	2	2.10E-01

Deme	Treatment	Extraction	Term	Count	%	P-Value	Fold Enrichment	FDR
$T6_{(c-)}$	Bic	3h	Pentose phosphate pathway	7	0.7	3.90E-02	2.8	2.40E-01
$T6_{(c-)}$	Bic	3h	Ascorbate and aldarate met	6	0.6	4.10E-02	3.1	2.40E-01
$T6_{(c-)}$	Bic	48h	Metabolic pathways	16	12.9	2.40E-03	1.9	7.10E-02
$T6_{(c-)}$	Bic	48h	Amino sugar and nucleotide sugar met	4	3.2	2.10E-02	6.4	3.00E-01
$T6_{(c-)}$	pH 8.3	3h	Valine, leucine and isoleucine degradation	9	5.2	3.60E-08	16.6	1.70E-06
$T6_{(c-)}$	pH 8.3	3h	Biosynthesis of antibiotics	20	11.5	2.80E-07	3.8	6.60E-06
$T6_{(c-)}$	pH 8.3	3h	Metabolic pathways	39	22.4	8.90E-06	1.8	1.40E-04
$T6_{(c-)}$	pH 8.3	3h	Carbon fixation in photosynthetic organisms	7	4	1.40E-04	8.4	1.70E-03
$T6_{(c-)}$	pH 8.3	3h	Carbon met	12	6.9	2.00E-04	3.8	1.90E-03
$T6_{(c-)}$	pH 8.3	3h	Biosynthesis of secondary metabolites	25	14.4	2.60E-04	2	2.10E-03
$T6_{(c-)}$	pH 8.3	3h	Glycolysis / Gluconeogenesis	7	4	2.00E-03	5.1	1.40E-02
$T6_{(c-)}$	pH 8.3	3h	Glyoxylate and dicarboxylate met	5	2.9	1.10E-02	5.6	6.60E-02
$T6_{(c-)}$	pH 8.3	3h	Nitrogen met	4	2.3	1.30E-02	7.9	7.00E-02
$T6_{(c-)}$	pH 8.3	3h	Tryptophan met	4	2.3	1.70E-02	7.2	8.00E-02
$T6_{(c-)}$	pH 8.3	48h	Phenylpropanoid biosynthesis	13	2.1	3.20E-04	3.4	2.50E-02
$T6_{(c-)}$	pH 8.3	48h	Biosynthesis of secondary metabolites	40	6.6	9.10E-04	1.6	3.60E-02
$T6_{(c-)}$	pH 8.3	48h	Carbon met	13	2.1	2.20E-02	2.1	5.70E-01
$T6_{(c-)}$	pH 8.3	48h	Amino sugar and nucleotide sugar met	8	1.3	4.20E-02	2.5	8.30E-01

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Annex Table 12: GO terms of exclusive genes involved in Bic response at 3 hours in $A1_{(c+)}$ deme in leaves. DGEs filtered (LFC> |1| & adj p-value < 0.05).

#term ID	term description	observed gene count	background gene count	strength	false discovery rate
GO:0044272	sulfur compound biosynthetic process	12	140	0.94	3.61E-05
GO:0006950	response to stress	57	2932	0.3	2.10E-04
GO:0006790	sulfur compound metabolic process	15	335	0.66	7.00E-04
GO:0016144	S-glycoside biosynthetic process	6	37	1.22	1.00E-03
GO:0019761	glucosinolate biosynthetic process	6	37	1.22	1.00E-03
GO:0019760	glucosinolate metabolic process	8	97	0.93	1.40E-03
GO:1901657	glycosyl compound metabolic process	11	211	0.73	1.40E-03
GO:1901659	glycosyl compound biosynthetic process	7	76	0.98	1.70E-03
GO:0000096	sulfur amino acid metabolic process	6	58	1.03	3.50E-03
GO:0009695	jasmonic acid biosynthetic process	4	16	1.41	3.50E-03
GO:0050896	response to stimulus	76	5064	0.19	4.10E-03
GO:0044550	secondary metabolite biosynthetic process	10	208	0.69	4.30E-03
GO:0000097	sulfur amino acid biosynthetic process	5	45	1.06	8.70E-03
GO:0033554	cellular response to stress	20	800	0.41	9.80E-03
GO:0043436	oxoacid metabolic process	22	973	0.37	1.73E-02
GO:0009610	response to symbiotic fungus	3	12	1.41	2.05E-02
GO:0009611	response to wounding	8	177	0.67	2.22E-02
GO:0051716	cellular response to stimulus	41	2428	0.24	2.22E-02
GO:1901607	alpha-amino acid biosynthetic process	8	175	0.67	2.22E-02
GO:0046394	carboxylic acid biosynthetic process	14	506	0.45	2.57E-02
GO:0006520	cellular amino acid metabolic process	11	354	0.5	3.60E-02
GO:0009067	aspartate family amino acid biosynthetic process	4	42	0.99	3.88E-02
GO:0019748	secondary metabolic process	11	365	0.49	4.28E-02
GO:0000075	cell cycle checkpoint	3	20	1.19	4.93E-02

Annex Table 13: KEEG pathway terms of exclusive genes involved in Bic response at 3 hours in $A1_{(c+)}$ deme in leaves. DGEs filtered (LFC> |1| & adj p-value < 0.05).

Term	Count	%	P-Value	Fold Enrichment	Benjamini	FDR
Alpha-Linolenic acid metabolism	5	1.90E+00	2.60E-04	1.53E+01	1.20E-02	1.20E-02
Biosynthesis of secondary metabolites	19	7.10E+00	1.50E-03	2.00E+00	3.60E-02	3.50E-02
Valine, leucine and isoleucine biosynthesis	3	1.10E+00	1.70E-02	1.43E+01	2.60E-01	2.50E-01
Metabolic pathways	24	9.00E+00	2.50E-02	1.40E+00	2.60E-01	2.50E-01
2-Oxocarboxylic acid metabolism	4	1.50E+00	2.70E-02	5.90E+00	2.60E-01	2.50E-01
Base excision repair	3	1.10E+00	5.50E-02	7.70E+00	3.90E-01	3.70E-01
Cysteine and methionine metabolism	4	1.50E+00	5.70E-02	4.40E+00	3.90E-01	3.70E-01
Biosynthesis of amino acids	6	2.30E+00	7.20E-02	2.60E+00	4.30E-01	4.20E-01
C5-Branched dibasic acid metabolism	2	8.00E-01	8.50E-02	2.20E+01	4.60E-01	4.40E-01

Annex Table 14: GO terms of exclusive genes involved in Bic response at 3 hours in $A1_{(c+)}$ deme in roots. DGEs filtered (LFC> |1| & adj p-value < 0.05).

GO term	Ontology	Description	Number in input	Number in BG/Ref	p-value	FDR
GO:0050896	Biological function	response to stimulus	46	5064	0.26	0.0198
GO:0005623	Cellular component	cell	84	12120	0.14	0.0082

Annex Table 15: KEEG pathway terms of exclusive genes involved in Bic response at 3 hours in $A1_{(c+)}$ deme in roots. DGEs filtered (LFC> |1| & adj p-value < 0.05).

#term ID	term description	observed gene count	background gene count	strength	false discovery rate
ath00480	Glutathione metabolism	4	98	0.91	0.049
ath00564	Glycerophospholipid metabolism	4	93	0.93	0.049
ath00920	Sulfur metabolism	3	41	1.16	0.049

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Annex Table 16 GO and terms of exclusive genes involved in Bic response at 3 h in $T6_{(c-)}$ deme in leaves. DGEs filtered (LFC> |1| & adj p-value < 0.05).

GO term	Ontology	Description	Number in input list	Number in BG/Ref	p-value	FDR
GO:0009055	Molecular function	electron carrier activity	7	294	5.40E-05	0.006
GO:0012505	Cellular component	endomembrane system	24	3416	0.0002	0.01
GO:0005773	Cellular component	vacuole	6	383	0.0016	0.04

Annex Table 17 KEEG pathway terms of exclusive genes involved in Bic response at 3 h in $T6_{(c-)}$ deme in leaves. DGEs filtered (LFC> |1| & adj p-value < 0.05).

#term ID	term description	observed gene count	background gene count	strength	false discovery rate
GO:0017001	antibiotic catabolic process	5	94	1.07	0.0476
GO:0050801	ion homeostasis	7	265	0.77	0.0476
GO:0051187	cofactor catabolic process	5	115	0.99	0.0476
GO:0055072	iron ion homeostasis	4	54	1.22	0.0476
GO:0055076	transition metal ion homeostasis	5	124	0.95	0.0476

Annex Table 18 GO and terms of exclusive genes involved in Bic response at 3 h in $T6_{(c-)}$ deme in roots. DGEs filtered (LFC> |1| & adj p-value < 0.05). BP: Biological process; MF: Molecular function; CC: Cellular component.

GO term	Ontology	Description	Number in input list	Number in BG/Ref	p-value	FDR
GO:0015979	BP	photosynthesis	19	215	1.19	1.05E-13
GO:0050896	BP	response to stimulus	66	5064	0.35	5.58E-09
GO:0042221	BP	response to chemical	45	2654	0.47	7.84E-09
GO:0009644	BP	response to high light intensity	10	76	1.36	1.54E-08
GO:0009642	BP	response to light intensity	12	142	1.17	1.73E-08
GO:0009266	BP	response to temperature stimulus	19	505	0.81	2.93E-08
GO:0009628	BP	response to abiotic stimulus	34	1699	0.54	2.93E-08
GO:0019684	BP	photosynthesis, light reaction	10	98	1.25	7.57E-08
GO:0006950	BP	response to stress	43	2932	0.41	6.89E-07
GO:0009408	BP	response to heat	11	184	1.02	1.60E-06
GO:0010033	BP	response to organic substance	31	1786	0.48	3.10E-06
GO:0009416	BP	response to light stimulus	17	585	0.7	5.45E-06

GO term	Ontology	Description	Number in input list	Number in BG/Ref	p-value	FDR
GO:0009765	BP	photosynthesis, light harvesting	6	35	1.47	8.33E-06
GO:0009768	BP	photosynthesis, light harvesting	5	19	1.66	1.33E-05
GO:0006091	BP	generation of precursor metabolites and energy	13	379	0.77	2.53E-05
GO:1901700	BP	response to oxygen-containing compound	24	1398	0.47	0.0001
GO:0034605	BP	cellular response to heat	5	32	1.43	0.00011
GO:0009617	BP	response to bacterium	12	385	0.73	0.00016
GO:0009725	BP	response to hormone	24	1502	0.44	0.00029
GO:0009409	BP	response to cold	11	347	0.74	0.00031
GO:1901615	BP	organic hydroxy compound metabolic process	9	248	0.8	0.00071
GO:0010218	BP	response to far red light	5	57	1.18	0.0011
GO:0010114	BP	response to red light	5	64	1.13	0.0017
GO:0042742	BP	defense response to bacterium	9	315	0.7	0.0038
GO:0010207	BP	photosystem II assembly	3	15	1.54	0.0046
GO:0018298	BP	protein-chromophore linkage	4	43	1.21	0.0048
GO:0006952	BP	defense response	19	1277	0.41	0.005
GO:0008152	BP	metabolic process	78	9671	0.15	0.0057
GO:0018171	BP	peptidyl-cysteine oxidation	2	2	2.24	0.0057
GO:0055062	BP	phosphate ion homeostasis	3	18	1.46	0.0061
GO:0009735	BP	response to cytokinin	7	212	0.76	0.0069
GO:0019253	BP	reductive pentose-phosphate cycle	3	20	1.42	0.0072
GO:0046475	BP	glycerophospholipid catabolic process	2	3	2.06	0.0072
GO:0070483	BP	detection of hypoxia	2	3	2.06	0.0072
GO:0019751	BP	polyol metabolic process	4	57	1.09	0.0086
GO:0009593	BP	detection of chemical stimulus	3	23	1.35	0.0087
GO:0006071	BP	glycerol metabolic process	3	25	1.32	0.0095
GO:0009769	BP	photosynthesis, light harvesting II	2	4	1.94	0.0095
GO:0016145	BP	S-glycoside catabolic process	3	24	1.34	0.0095
GO:0019762	BP	glucosinolate catabolic process	3	24	1.34	0.0095
GO:0042542	BP	response to hydrogen peroxide	4	63	1.04	0.01
GO:0071577	BP	zinc ion transmembrane transport	3	26	1.3	0.01
GO:0030643	BP	cellular phosphate ion homeostasis	2	5	1.84	0.0108
GO:0010035	BP	response to inorganic substance	13	795	0.45	0.0122
GO:0009605	BP	response to external stimulus	19	1488	0.35	0.0147
GO:0009697	BP	salicylic acid biosynthetic process	2	7	1.7	0.0158
GO:0009607	BP	response to biotic stimulus	16	1167	0.38	0.0174
GO:0048878	BP	chemical homeostasis	8	358	0.59	0.0174
GO:0001101	BP	response to acid chemical	15	1058	0.39	0.0175

GO term	Ontology	Description	Number in input list	Number in BG/Ref	p-value	FDR
GO:0009637	BP	response to blue light	4	80	0.94	0.0182
GO:0005975	BP	carbohydrate metabolic process	13	856	0.42	0.0198
GO:1901657	BP	glycosyl compound metabolic process	6	211	0.69	0.0204
GO:0006970	BP	response to osmotic stress	10	564	0.49	0.0222
GO:0016051	BP	carbohydrate biosynthetic process	7	295	0.61	0.0223
GO:0051707	BP	response to other organism	15	1140	0.36	0.0318
GO:0044281	BP	small molecule metabolic process	18	1503	0.32	0.0322
GO:0046364	BP	monosaccharide biosynthetic process	3	46	1.05	0.0322
GO:0098542	BP	defense response to other organism	13	919	0.39	0.0322
GO:0042592	BP	homeostatic process	9	514	0.48	0.0355
GO:0009636	BP	response to toxic substance	7	330	0.57	0.0361
GO:0001666	BP	response to hypoxia	3	51	1.01	0.0389
GO:0010205	BP	photoinhibition	2	14	1.39	0.0389
GO:0017000	BP	antibiotic biosynthetic process	2	14	1.39	0.0389
GO:0044237	BP	cellular metabolic process	65	8432	0.13	0.0389
GO:0051188	BP	cofactor biosynthetic process	6	253	0.61	0.0389
GO:0042493	BP	response to drug	9	533	0.47	0.0408
GO:0070838	BP	divalent metal ion transport	4	110	0.8	0.0408
GO:0043436	BP	oxoacid metabolic process	13	973	0.37	0.0429
GO:0002238	BP	response to molecule of fungal origin	2	16	1.34	0.0433
GO:0010200	BP	response to chitin	4	113	0.79	0.0433
GO:0009645	BP	response to low light intensity stimulus	2	17	1.31	0.0456
GO:0010105	BP	negative regulation of ethylene-	2	17	1.31	0.0456
GO:0009396	BP	folic acid-containing compound biosynthetic	2	18	1.29	0.0478
GO:0019904	MF	protein domain specific binding	8	71	1.29	4.68E-06
GO:0031409	MF	pigment binding	4	19	1.56	0.0011
GO:0016168	MF	chlorophyll binding	4	30	1.36	0.0036
GO:0008889	MF	glycerophosphodiester phosphodiesterase	3	13	1.6	0.0064
GO:0046872	MF	metal ion binding	33	2940	0.29	0.0076
GO:0097718	MF	disordered domain specific binding	2	3	2.06	0.012
GO:0043167	MF	ion binding	47	5070	0.21	0.0133
GO:0046906	MF	tetrapyrrole binding	8	299	0.67	0.0133
GO:0005385	MF	zinc ion transmembrane transporter activity	3	26	1.3	0.0157
GO:0017172	MF	cysteine dioxygenase activity	2	5	1.84	0.0157
GO:0051740	MF	ethylene binding	2	5	1.84	0.0157
GO:0005488	MF	binding	68	8611	0.14	0.0248
GO:0016701	MF	oxidoreductase activity,	3	37	1.15	0.0254

GO term	Ontology	Description	Number in input list	Number in BG/Ref	p-value	FDR
GO:0009521	CC	photosystem	13	87	1.41	2.18E-12
GO:0009535	CC	chloroplast thylakoid membrane	20	347	1	2.18E-12
GO:0009579	CC	thylakoid	21	483	0.88	2.23E-11
GO:0009522	CC	photosystem I	9	39	1.6	8.81E-11
GO:0010287	CC	plastoglobule	10	76	1.36	7.54E-10
GO:0009523	CC	photosystem II	8	62	1.35	6.67E-08
GO:0009570	CC	chloroplast stroma	18	649	0.68	5.32E-07
GO:0009941	CC	chloroplast envelope	16	584	0.68	3.04E-06
GO:0009507	CC	chloroplast	31	2026	0.42	4.03E-06
GO:0031984	CC	organelle subcompartment	24	1306	0.5	4.03E-06
GO:0005623	CC	cell	100	12120	0.16	6.63E-06
GO:0005737	CC	cytoplasm	71	7481	0.22	8.91E-06
GO:0005622	CC	intracellular	87	10570	0.15	9.95E-05
GO:0048046	CC	apoplast	12	463	0.65	9.95E-05
GO:0009538	CC	photosystem I reaction center	3	8	1.81	0.00013
GO:0043231	CC	intracellular membrane-bounded organelle	76	8914	0.17	0.00016
GO:0031977	CC	thylakoid lumen	5	70	1.09	0.00029
GO:0016020	CC	membrane	53	5592	0.22	0.00032
GO:0009654	CC	photosystem II oxygen evolving complex	3	22	1.37	0.0013
GO:0010319	CC	stromule	3	35	1.17	0.0043
GO:0016021	CC	integral component of membrane	33	3460	0.22	0.0076
GO:0031224	CC	intrinsic component of membrane	34	3602	0.21	0.0076
GO:0031969	CC	chloroplast membrane	6	250	0.62	0.0107
GO:0005576	CC	extracellular region	17	1502	0.29	0.0178
GO:0031012	CC	extracellular matrix	2	21	1.22	0.0206

Annex Table 19 KEEG pathway terms of exclusive genes involved in Bic response at 3 h in $T6_{(c-)}$ deme in roots. DGEs filtered (LFC> |1| & adj p-value < 0.05).

#term ID	term description	observed gene count	background gene count	strength	false discovery rate
ath00195	Photosynthesis	9	76	1.31	5.57E-08
ath04141	Protein processing in endoplasmic reticulum	9	205	0.88	7.90E-05
ath04626	Plant-pathogen interaction	7	165	0.87	0.00078
ath00196	Photosynthesis - antenna proteins	3	21	1.39	0.0032
ath01100	Metabolic pathways	23	1899	0.32	0.0046
ath00710	Carbon fixation in photosynthetic organisms	4	69	1	0.0051
ath00430	Taurine and hypotaurine metabolism	2	14	1.39	0.0196

Supplementary material : Chapter 3

Supplementary Material and Methods

Annex Table 1: Natural populations obtained from (http://bergelson.uchicago.edu/ wpcontent/ uploads/ 2015/ 04/ Justins-360-lines.xls). Green color indicates lines used for statistical and GWAS analyses in HN (nutrition in $CaCO_3$ soil), LN (nutrition in control soil,) HG (growth in $CaCO_3$ soil), LG (growth in control soil).

Native name	Ecotype	Collector	Lat	Lon	Country	$CaCO_3$ (g/kg)	CEC (cmol/kg)	pH H_2O	HN	LN	HG	LG
ALL1-2	1	Roux	45.27	1.48	FRA	0	10.98	5.07				
ALL1-3	2	Roux	45.27	1.48	FRA	0	10.98	5.07				
BUI	15	Roux	48.37	0.93	FRA	143.21	23.08	6.34				
CAM-16	23	Roux	48.27	-4.58	FRA	8.82	7.91	5.43				
CAM-61	66	Roux	48.27	-4.58	FRA	8.82	7.91	5.43				
CLE-6	78	Roux	48.92	-0.48	FRA	62.59	16.28	6.72				
CUR-3	81	Roux	45	1.75	FRA	46.12	14.37	6.43				
JEA	91	Roux	43.68	7.33	FRA							
LAC-3	94	Roux	47.7	6.82	FRA	8.84	11.35	6.16				
LAC-5	96	Roux	47.7	6.82	FRA	8.84	11.35	6.16				
LDV-14	104	Roux	48.52	-4.07	FRA							
LDV-25	116	Roux	48.52	-4.07	FRA							
LDV-34	126	Roux	48.52	-4.07	FRA							
LDV-58	149	Roux	48.52	-4.07	FRA							
MIB-15	166	Roux	47.38	5.32	FRA	92.89	18.2	7.2				
MIB-22	173	Roux	47.38	5.32	FRA	92.89	18.2	7.2				
MIB-28	178	Roux	47.38	5.32	FRA	92.89	18.2	7.2				
MIB-84	223	Roux	47.38	5.32	FRA	92.89	18.2	7.2				
MOG-37	242	Roux	48.67	-4.07	FRA	0	10.11	6.23				
PAR-3	258	Roux	46.65	-0.25	FRA	44.51	12.41	6.95				
PAR-4	259	Roux	46.65	-0.25	FRA	44.51	12.41	6.95				
PAR-5	260	Roux	46.65	-0.25	FRA	44.51	12.41	6.95				
ROM-1	267	Roux	45.53	4.85	FRA	107.93	15.32	6.84				
TOU-A1-115	281	Roux	46.67	4.12	FRA							
TOU-A1-116	282	Roux	46.67	4.12	FRA							
TOU-A1-12	286	Roux	46.67	4.12	FRA							
TOU-A1-43	321	Roux	46.67	4.12	FRA							
TOU-A1-62	328	Roux	46.67	4.12	FRA							

Native name	Ecotype	Collector	Lat	Lon	Country	$CaCO_3$ (g/kg)	CEC (cmol/kg)	pH H_2O	HN	LN	HG	LG
TOU-A1-67	333	Roux	46.67	4.12	FRA							
TOU-A1-96	357	Roux	46.67	4.12	FRA							
TOU-C-3	362	Roux	46.67	4.12	FRA							
TOU-E-11	366	Roux	46.67	4.12	FRA							
TOU-H-12	373	Roux	46.67	4.12	FRA							
TOU-H-13	374	Roux	46.67	4.12	FRA							
TOU-I-17	378	Roux	46.67	4.12	FRA							
TOU-I-2	379	Roux	46.67	4.12	FRA							
TOU-I-6	380	Roux	46.67	4.12	FRA							
TOU-J-3	383	Roux	46.67	4.12	FRA							
TOU-K-3	386	Roux	46.67	4.12	FRA							
VOU-1	390	Roux	46.65	0.17	FRA	191.38	19.86	7.55				
VOU-2	392	Roux	46.65	0.17	FRA	191.38	19.86	7.55				
LI-OF-095	641	Bossdorf	40.78	-72.91	USA							
Belmonte-4-94	957	Ã…gren	42.12	12.48	ITA	0	22.53	6.63				
KBS-Mac-8	1716	Byers	42.41	-85.4	USA							
MNF-Pot-48	1859	Byers	43.6	-86.27	USA							
MNF-Pot-68	1867	Byers	43.6	-86.27	USA							
MNF-Che-2	1925	Byers	43.53	-86.18	USA							
MNF-Jac-32	1967	Byers	43.52	-86.17	USA							
Map-42	2057	Byers	42.17	-86.41	USA							
Paw-3	2150	Byers	42.15	-86.43	USA							
Pent-1	2187	Byers	43.76	-86.39	USA							
SLSP-30	2274	Byers	43.67	-86.5	USA							
Ste-3	2290	Byers	42.03	-86.51	USA							
UKSW06-202	4802	Holub	50.4	-4.9	UK	0	13.45	5.52				
UKSE06-062	4997	Holub	51.3	0.5	UK	141.53	24.22	7.61				
UKSE06-192	5056	Holub	51.3	0.5	UK	141.53	24.22	7.61				
UKSE06-272	5116	Holub	51.3	0.4	UK	98.69	24.17	7.09				
UKSE06-278	5122	Holub	51.3	0.4	UK	98.69	24.17	7.09				
UKSE06-349	5158	Holub	51.3	0.4	UK	98.69	24.17	7.09				
UKSE06-351	5160	Holub	51.3	0.4	UK	98.69	24.17	7.09				
UKSE06-414	5202	Holub	51.3	0.4	UK	98.69	24.17	7.09				
UKSE06-429	5207	Holub	51.3	0.4	UK	98.69	24.17	7.09				
UKSE06-466	5232	Holub	51.2	0.4	UK	32.61	17.57	6.91				
UKSE06-482	5245	Holub	51.2	0.6	UK	3.9	18.9	6.58				

Native name	Ecotype	Collector	Lat	Lon	Country	$CaCO_3$ (g/kg)	CEC (cmol/kg)	pH H_2O	HN	LN	HG	LG
UKSE06-520	5264	Holub	51.3	1.1	UK							
UKSE06-628	5341	Holub	51.1	0.4	UK	15.21	16.96	6.07				
UKNW06-059	5380	Holub	54.4	-3	UK	0	12.04	5.23				
UKNW06-060	5381	Holub	54.4	-3	UK	0	12.04	5.23				
UKNW06-386	5565	Holub	54.6	-3.1	UK	6.85	12.71	5.66				
UKNW06-436	5606	Holub	54.7	-3.4	UK	0	16.88	5.72				
UKNW06-460	5628	Holub	54.7	-3.4	UK	0	16.88	5.72				
UKID22	5729	Holub	54.7	-3.4	UK	0	16.88	5.72				
UKID37	5742	Holub	51.3	1.1	UK							
UKID48	5753	Holub	54.7	-2.7	UK	0	18.64	5.89				
UKID80	5785	Holub	54.7	-2.9	UK	0	15.74	4.94				
UKID101	5805	Holub	53.2	-1.4	UK	22.47	14.94	6.44				
App1-16	5832	Nordborg	56.33	15.97	SWE	7.37	16.53	4.83				
Bor-1	5837	Nordborg	49.4	16.23	CZE	12.54	10.75	5.61				
DraIV 1-5	5887	Nordborg	49.41	16.28	CZE	0	11.53	5.95				
DraIV 1-7	5889	Nordborg	49.41	16.28	CZE	0	11.53	5.95				
DraIV 1-14	5896	Nordborg	49.41	16.28	CZE	0	11.53	5.95				
DraIV 6-16	5987	Nordborg	49.41	16.28	CZE	0	11.53	5.95				
DraIV 6-35	6005	Nordborg	49.41	16.28	CZE	0	11.53	5.95				
Duk	6008	Nordborg	49.1	16.2	CZE	13.91	17.83	6.37				
FjÃ¤1-2	6019	Nordborg	56.06	14.29	SWE	5.52	13.46	5.97				
FjÃ¤1-5	6020	Nordborg	56.06	14.29	SWE	5.52	13.46	5.97				
Hovdala-2	6039	Nordborg	56.1	13.74	SWE	21.05	12.87	5.63				
Lom1-1	6042	Nordborg	56.09	13.9	SWE	2.41	12.3	5.13				
LÃ¶v-5	6046	Nordborg	62.8	18.08	SWE							
Ã–r-1	6074	Nordborg	56.45	16.11	SWE	7.26	18.51	6.15				
Rev-2	6076	Nordborg	55.7	13.4	SWE	13.74	11.65	6.23				
Sparta-1	6085	Nordborg	55.71	13.05	SWE	5.24	16.02	6.81				
T1040	6094	Nordborg	55.65	13.21	SWE	0	8.09	5.78				
T1060	6096	Nordborg	55.65	13.22	SWE							
T1080	6098	Nordborg	55.66	13.22	SWE	0	12.07	6.36				
T1110	6100	Nordborg	55.6	13.2	SWE	6.25	10.67	6.58				
T1130	6102	Nordborg	55.6	13.2	SWE	6.25	10.67	6.58				
T510	6109	Nordborg	55.79	13.12	SWE	1.6	8.93	6.02				
T540	6112	Nordborg	55.8	13.1	SWE	0	13.26	6.16				
T620	6119	Nordborg	55.7	13.2	SWE	1.43	8.95	5.97				

Native name	Ecotype	Collector	Lat	Lon	Country	$CaCO_3$ (g/kg)	CEC (cmol/kg)	pH H_2O	HN	LN	HG	LG
T690	6124	Nordborg	55.84	13.31	SWE	10.07	9.84	5.88				
TÃ…D 01	6169	Nordborg	62.87	18.34	SWE	4.86	11.1	4.48				
TDr-1	6188	Nordborg	55.77	14.14	SWE	0.15	17.75	4.33				
TDr-3	6190	Nordborg	55.77	14.14	SWE	0.15	17.75	4.33				
TDr-8	6194	Nordborg	55.77	14.13	SWE	0	18.05	4.28				
TDr-17	6202	Nordborg	55.77	14.12	SWE	9.93	11.33	5.31				
TDr-18	6203	Nordborg	55.77	14.12	SWE	9.93	11.33	5.31				
Tomegap-2	6242	Nordborg	55.7	13.2	SWE	1.43	8.95	5.97				
Tottarp-2	6243	Nordborg	55.95	13.85	SWE	12.78	11.55	5.04				
Udul 1-34	6318	Nordborg	49.28	16.63	CZE	0	8.51	4.88				
Ull3-4	6413	Nordborg	56.06	13.97	SWE	5.43	16.98	4.84				
Zdrl 2-24	6448	Nordborg	49.39	16.25	CZE	11.74	9.73	6.01				
Zdrl 2-25	6449	Nordborg	49.39	16.25	CZE	11.74	9.73	6.01				
CIBC-2	6727	Crawley	51.41	-0.64	UK	0	10.77	5.69				
CIBC-4	6729	Crawley	51.41	-0.64	UK	0	10.77	5.69				
CIBC-5	6730	Crawley	51.41	-0.64	UK	0	10.77	5.69				
CSHL-5	6744	Weiss	40.86	-73.47	USA							
KNO-11	6810	Bergelson	41.28	-86.62	USA							
NFC-20	6847	Crawley	51.41	-0.64	UK	0	10.77	5.69				
Pu2-24	6953	Cetl	49.42	16.36	CZE	25.96	11.16	5.71				
Alst-1	6989	Koornneef	54.8	-2.43	UK	0	11.63	5.53				
Amel-1	6990	Koornneef	53.45	5.73	NED							
Ang-0	6992	Kranz	50.3	5.3	BEL	0	14.56	5.93				
Ann-1	6994	Koornneef	45.9	6.13	FRA	124.5	23.36	6.95				
An-2	6996	Kranz	51.22	4.4	BEL							
Arby-1	6998	Koornneef	59.43	16.8	SWE							
Aa-0	7000	Kranz	50.92	9.57	GER	46.02	10.44	4.83				
Baa-1	7002	Koornneef	51.33	6.1	NED	20.71	13.61	6.31				
Bs-2	7004	Kranz	47.5	7.5	SUI	13.82	16.69	6.73				
Benk-1	7008	Koornneef	52	5.68	NED	25.37	6.6	5.99				
Be-1	7011	Kranz	49.68	8.62	GER	72.74	13.5	7.36				
Ba-1	7014	Kranz	56.55	-4.8	UK	0	16.28	5.3				
Boot-1	7026	Koornneef	54.4	-3.27	UK	67.63	13.96	5.98				
Bsch-0	7031	Kranz	40.02	8.67	GER	93.65	17.92	7.25				
Blh-2	7035	Kranz	48	19	CZE	0	16.68	5.71				
Bu-8	7056	Kranz	50.5	9.5	GER	10.95	7.96	5.54				

Native name	Ecotype	Collector	Lat	Lon	Country	$CaCO_3$ (g/kg)	CEC (cmol/kg)	pH H_2O	HN	LN	HG	LG
Ca-0	7062	Kranz	50.3	8.27	GER	35.76	12.98	6.74				
Cnt-1	7064	Holub	51.3	1.1	UK							
Cha-0	7069	Kranz	46.03	7.12	SUI							
Chat-1	7071	Koornneef	48.07	1.34	FRA	47.82	13.63	7.07				
Cit-0	7075	Kranz	43.38	2.54	FRA	9.26	13.8	5.83				
Co-2	7078	Kranz	40.12	-8.25	POR	113.74	11.95	6.22				
Co-4	7080	Kranz	40.12	-8.25	POR	113.74	11.95	6.22				
Com-1	7092	Koornneef	49.42	2.82	FRA							
Da-0	7094	Kranz	49.87	8.65	GER	54.8	10.55	7.03				
Di-1	7098	Kranz	47	5	FRA	42.56	13.14	7.07				
Db-0	7100	Kranz	50.31	8.32	GER	38.86	11.26	5.86				
Do-0	7102	Kranz	50.72	8.24	GER	18.15	12.67	5.23				
Dra-2	7105	Kranz	49.42	16.27	CZE	0	10.34	5.58				
Ede-1	7110	Koornneef	52.03	5.67	NED	32.41	7.35	6.42				
Ep-0	7123	Kranz	50.17	8.39	GER	19.99	7.68	5.29				
Es-0	7126	Kranz	60.2	24.57	FIN	4.62	13.16	5.95				
Est-0	7128	Kranz	58.3	25.3	RUS	9.73	19.86	6.74				
Fr-4	7135	Kranz	50.11	8.68	GER							
Fi-1	7139	Kranz	50.5	8.02	GER	6.57	15.1	6.85				
Ga-2	7141	Kranz	50.3	8	GER	26.36	12.38	4.77				
Gel-1	7143	Koornneef	51.02	5.87	NED							
Ge-1	7145	Kranz	46.5	6.08	SUI							
Gie-0	7147	Kranz	50.58	8.68	GER	6.33	15.43	6.5				
Gu-1	7150	Kranz	50.3	8	GER	26.36	12.38	4.77				
Go-0	7151	Kranz	51.53	9.94	GER	36.46	10.82	6.65				
Gr-5	7158	Hauser	47	15.5	AUT	0	17.27	7.03				
Ha-0	7163	Kranz	52.37	9.74	GER							
Hau-0	7164	Kranz	55.68	12.57	DEN							
Hn-0	7165	Kranz	51.35	8.29	GER	80.1	14.42	6.23				
Hey-1	7166	Koornneef	51.25	5.9	NED	20.22	8.51	6.43				
Hh-0	7169	Kranz	54.42	9.89	GER	3.4	11.19	6.42				
Jm-1	7178	Kranz	49	15	CZE	9.53	11.65	4.95				
Je-0	7181	Kranz	50.93	11.59	GER	52.84	13.98	6.89				
Kn-0	7186	Kranz	54.9	23.89	LTU							
Kelsterbach-2	7188	Williams	50.07	8.53	GER	49.69	13.25	6.98				
Kl-5	7199	Kranz	50.95	6.97	GER							

Native name	Ecotype	Collector	Lat	Lon	Country	$CaCO_3$ (g/kg)	CEC (cmol/kg)	pH H_2O	HN	LN	HG	LG
Kr-0	7201	Kranz	51.33	6.56	GER							
Krot-2	7205	Clauss	49.63	11.57	GER	32.88	10.62	6.45				
Kro-0	7206	Kranz	50.07	8.97	GER	37.76	14.48	6.74				
Li-3	7224	Kranz	50.38	8.07	GER	5.85	12.54	6.45				
Li-5:2	7227	Kranz	50.38	8.07	GER	5.85	12.54	6.45				
Li-6	7229	Kranz	50.38	8.07	GER	5.85	12.54	6.45				
Li-7	7231	Kranz	50.38	8.07	GER	5.85	12.54	6.45				
Mnz-0	7244	Kranz	50	8.27	GER	64.19	15.74	7.33				
Mc-0	7252	Kranz	54.62	-2.3	UK	7.31	21.85	5.29				
Mh-0	7255	Kranz	50.95	7.5	POL	3.32	10.68	3.96				
Nw-0	7258	Kranz	50.5	8.5	GER	4.58	14.54	6.8				
Nw-2	7260	Kranz	50.5	8.5	GER	4.58	14.54	6.8				
Nz1	7263	Campanella	-37.79	175.28	NZL							
Nok-1	7270	Kranz	52.24	4.45	NED	44.95	6.95	6.38				
No-0	7275	Kranz	51.06	13.3	GER	32.95	13.18	6.61				
Ob-1	7277	Kranz	50.2	8.58	GER	40.36	8.57	6.51				
Old-1	7280	Kranz	53.17	8.2	GER	18.31	5.69	6.05				
Or-0	7282	Kranz	50.38	8.01	GER	1.66	11.66	5.84				
Ors-1	7283	Butnaru	44.72	22.4	ROU	53.99	22.46	6.92				
Ors-2	7284	Butnaru	44.72	22.4	ROU	53.99	22.46	6.92				
Pa-2	7291	Kranz	38.07	13.22	ITA	99.59	27.43	6.88				
Pla-0	7300	Kranz	41.5	2.25	ESP	142.89	14.48	6.83				
Pog-0	7306	Kranz	49.27	-123.21	CAN							
Pn-0	7307	Kranz	48.07	-2.97	FRA	43.43	14.62	6.6				
Pr-0	7310	Kranz	50.14	8.61	GER	54.41	15.94	6.99				
Rhen-1	7316	Koornneef	51.97	5.57	NED	11.62	8.21	5.35				
Rou-0	7320	Kranz	49.44	1.1	FRA	102.66	12.78	6.93				
Sapporo-0	7330	Hanzawa	43.06	141.35	JPN							
Sh-0	7331	Kranz	51.68	10.21	GER	2.76	9.43	6.4				
Sei-0	7333	Kranz	46.54	11.56	ITA	40.34	19.34	6.02				
Si-0	7337	Kranz	50.87	8.02	GER	68.43	13.95	6.22				
Sav-0	7340	Kranz	49.18	15.88	CZE	0.48	17.54	6.72				
Sp-0	7343	Kranz	52.53	13.18	GER							
Sg-1	7344	Kranz	47.67	9.5	GER							
Ste-0	7346	Kranz	52.61	11.86	GER	24.17	8.58	6.17				
Ty-0	7351	Kranz	56.43	-5.23	UK	18.37	16.19	5.77				

Native name	Ecotype	Collector	Lat	Lon	Country	$CaCO_3$ (g/kg)	CEC (cmol/kg)	pH H_2O	HN	LN	HG	LG
Tha-1	7353	Koornneef	52.08	4.3	NED	60	10.35	6.46				
Ting-1	7354	Koornneef	56.5	14.9	SWE	0	12.94	4.66				
Tiv-1	7355	Koornneef	41.96	12.8	ITA							
Tscha-1	7372	Koornneef	47.07	9.9	AUT	34.56	20.05	5.75				
Tsu-0	7373	Kranz	34.43	136.31	JPN							
Uk-1	7378	Kranz	48.03	7.77	GER	38.4	18.44	6.67				
Uk-2	7379	Kranz	48.03	7.77	GER	38.4	18.44	6.67				
Utrecht	7382	Willemsen	52.09	5.11	NED	53.48	9.9	6.33				
Ven-1	7384	Koornneef	52.03	5.55	NED	40.08	7.16	6.33				
Wag-3	7390	Koornneef	51.97	5.67	NED	36.02	11.38	6.39				
Wag-4	7391	Koornneef	51.97	5.67	NED	36.02	11.38	6.39				
Wag-5	7392	Koornneef	51.97	5.67	NED	36.02	11.38	6.39				
Wa-1	7394	Kranz	52.3	21	POL							
Ws	7397	Kranz	52.3	30	RUS							
Wc-2	7405	Kranz	52.6	10.07	GER	16.34	7.99	5.94				
Wt-3	7408	Kranz	52.3	9.3	GER	63.61	9.13	6.79				
Wl-0	7411	Kranz	47.93	10.81	GER	1.35	18.74	5.6				
Zu-1	7418	Kranz	47.37	8.55	SUI							
Jl-3	7424	Kranz	49.2	16.62	CZE							
Nc-1	7430	Kranz	48.62	6.25	FRA	12.29	14.53	6.68				
N4	7446	Savushkin	61.36	34.15	RUS							
N7	7449	Savushkin	61.36	34.15	RUS							
Da(1)-12	7460	Vizir	<Null>	<Null>	CZE	0	0	0				
S96	7472	Administrator	<Null>	<Null>	UNK	0	0	0				
WAR	7477	Pigliucci	41.73	-71.28	USA							
PHW-10	7479	Williams	51.29	0.06	UK	23.05	12.1	5.95				
PHW-13	7482	Williams	51.29	0.06	UK	23.05	12.1	5.95				
PHW-14	7483	Williams	51.29	0.06	UK	23.05	12.1	5.95				
PHW-20	7490	Williams	51.29	0.06	UK	23.05	12.1	5.95				
PHW-22	7492	Williams	51.42	-1.72	UK	16.89	14.51	6.64				
PHW-26	7496	Williams	50.67	-3.84	UK	5.36	11.74	5.81				
PHW-28	7498	Williams	50.35	-3.58	UK	24.94	14.59	5.72				
PHW-31	7502	Williams	51.47	-3.2	UK	41.07	17.35	7.02				
PHW-33	7504	Williams	52.25	4.57	NED	49.32	20.76	7.4				
PHW-35	7506	Williams	48.61	2.31	FRA	54.74	13.37	7.04				
PHW-36	7507	Williams	48.61	2.31	FRA	54.74	13.37	7.04				

Native name	Ecotype	Collector	Lat	Lon	Country	$CaCO_3$ (g/kg)	CEC (cmol/kg)	pH H_2O	HN	LN	HG	LG
PHW-37	7508	Williams	48.61	2.31	FRA	54.74	13.37	7.04				
RRS-7	7514	Dritz	41.56	-86.43	USA							
627ME-4Y1	7571	Bergelson	42.09	-86.36	USA							
Lis-2	8222	Nordborg	56	14.7	SWE							
BrÃ¶1-6	8231	Nordborg	56.3	16	SWE	0.28	10.77	5.95				
Gul1-2	8234	Nordborg	56.3	16	SWE	0.28	10.77	5.95				
Hod	8235	Nordborg	48.8	17.1	CZE	23.28	21.76	6.92				
HSm	8236	Nordborg	49.33	15.76	CZE	7.79	12.11	5.93				
KÃ¶ln	8239	Williams	51	7	GER							
Kulturen-1	8240	Nordborg	55.71	13.2	SWE	4.27	9.1	5.79				
Liarum	8241	Nordborg	55.95	13.85	SWE	12.78	11.55	5.04				
LillÃ¶-1	8242	Nordborg	56.15	15.78	SWE	12.64	14.78	6.14				
PHW-34	8244	Williams	48.61	2.31	FRA	54.74	13.37	7.04				
NC-6	8246	Bergelson	35	-79.18	USA							
Shahdara	8248	Vizir	38.35	68.48	TJK							
Ag-0	8251	Kranz	45	1.3	FRA	78.11	17.33	6.26				
Alc-0	8252	Roldan	40.31	-3.22	ESP	74.03	16.34	7.37				
An-1	8253	Kranz	51.22	4.4	BEL							
BÃ¥1-2	8256	Nordborg	56.4	12.9	SWE	0	9.23	4.46				
Bay-0	8260	Kranz	49	11	GER	28.84	22.28	5.29				
Bg-2	8261	Winterer	47.65	-122.31	USA							
Bla-1	8264	Kranz	41.68	2.8	ESP	185.5	18.22	7.53				
Blh-1	8265	Kranz	48	19	CZE	0	16.68	5.71				
Bor-4	8268	Nordborg	49.4	16.23	CZE	12.54	10.75	5.61				
Br-0	8269	Kranz	49.2	16.62	CZE							
Bu-0	8271	Kranz	50.5	9.5	GER	10.95	7.96	5.54				
Bur-0	8272	Kranz	54.1	-6.2	IRL	42.39	17.94	5.53				
C24	8273	Kranz	41.25	-8.45	POR	0	8.71	5.32				
Can-0	8274	Hauser	29.21	-13.48	ESP							
Cen-0	8275	Kranz	49	0.5	FRA	0	14.47	6.61				
CIBC-17	8276	Crawley	51.41	-0.64	UK	0	10.77	5.69				
Col-0	8279	Kranz	38.3	-92.3	USA							
Ct-1	8280	Kranz	37.3	15	ITA	190.67	20.72	8.17				
Cvi-0	8281	Kranz	15.11	-23.62	CPV							
Dra3-1	8283	Nordborg	55.76	14.12	SWE	14.69	11.53	5.91				
DraII-1	8284	Nordborg	49.41	16.28	CZE	0	11.53	5.95				

Native name	Ecotype	Collector	Lat	Lon	Country	$CaCO_3$ (g/kg)	CEC (cmol/kg)	pH H_2O	HN	LN	HG	LG
Eden-2	8287	Nordborg	62.88	18.18	SWE	0	9	4.27				
Edi-0	8288	Kranz	56	-3	UK							
Est-1	8291	Kranz	58.3	25.3	RUS	9.73	19.86	6.74				
FÃ¤b-4	8293	Nordborg	63.02	18.32	SWE							
Fei-0	8294	Alonso-Blanco	40.5	-8.32	POR	0	4.69	4.99				
Ga-0	8295	Kranz	50.3	8	GER	26.36	12.38	4.77				
Gd-1	8296	Kranz	53.5	10.5	GER	19.39	8.29	5.63				
Ge-0	8297	Kranz	46.5	6.08	SUI							
Got-7	8299	RÃ¶bbelen	51.53	9.94	GER	36.46	10.82	6.65				
Gr-1	8300	Hauser	47	15.5	AUT	0	17.27	7.03				
Gy-0	8302	Kranz	49	2	FRA	30.79	12.23	4.99				
Hi-0	8304	Kranz	52	5	NED	0	28.68	6.09				
Hov4-1	8306	Nordborg	56.1	13.74	SWE	21.05	12.87	5.63				
HR-5	8309	Crawley	51.41	-0.64	UK	0	10.77	5.69				
Hs-0	8310	Kranz	52.24	9.44	GER	92.85	20.06	6.61				
In-0	8311	Kranz	47.5	11.5	AUT	44.83	17.02	6.26				
Ka-0	8314	Kranz	47	14	AUT	35.98	19.54	5.97				
Kin-0	8316	Kranz	44.46	-85.37	USA							
Kno-18	8318	Bergelson	41.28	-86.62	USA							
Lc-0	8323	Kranz	57	-4	UK	10.97	20.37	5.16				
Ler-1	8324	Koornneef	47.98	10.87	GER	20.06	25.84	6.79				
Lip-0	8325	Kranz	50	19.3	POL	0	11.82	5.98				
Lis-1	8326	Nordborg	56	14.7	SWE							
LL-0	8328	Kranz	41.59	2.49	ESP	107.33	11.15	6.34				
Lm-2	8329	Kranz	48	0.5	FRA	1.88	8.91	5.84				
Lp2-2	8332	Cetl	49.38	16.81	CZE	1.56	15.33	5.88				
Lp2-6	8333	Cetl	49.38	16.81	CZE	1.56	15.33	5.88				
Lund	8335	Nordborg	55.71	13.2	SWE	4.27	9.1	5.79				
Lz-0	8336	Kranz	46	3.3	FRA	63.3	18.2	7.17				
Mr-0	8338	Kranz	44.15	9.65	ITA	76.06	20.24	6.72				
Mrk-0	8339	Kranz	49	9.3	GER	52.81	15.11	7.05				
Mt-0	8341	Kranz	32.34	22.46	LIB							
Mz-0	8342	Kranz	50.3	8.3	GER	5.79	10.72	4.68				
Na-1	8343	Kranz	47.5	1.5	FRA	32.98	10.6	5.27				
Nd-1	8344	Kranz	50	10	SUI	181.95	20.72	7.45				
NFA-10	8345	Crawley	51.41	-0.64	UK	0	10.77	5.69				

Native name	Ecotype	Collector	Lat	Lon	Country	$CaCO_3$ (g/kg)	CEC (cmol/kg)	pH H_2O	HN	LN	HG	LG
NFA-8	8346	Crawley	51.41	-0.64	UK	0	10.77	5.69				
Ã–mÃ¶2-1	8349	Nordborg	56.14	15.78	SWE							
Ost-0	8351	Kranz	60.25	18.37	SWE	3.4	30.01	4.95				
Oy-0	8352	Kranz	60.23	6.13	NOR							
Pa-1	8353	Kranz	38.07	13.22	ITA	99.59	27.43	6.88				
Per-1	8354	Kranz	58	56.32	RUS							
Petergof	8355	Vizir	59	29	RUS							
Pna-17	8359	Bergelson	42.09	-86.33	USA							
Pro-0	8360	Bergelson	43.25	-6	ESP	104.73	14.96	6.2				
Pu2-23	8361	Cetl	49.42	16.36	CZE	25.96	11.16	5.71				
Ra-0	8364	Kranz	46	3.3	FRA	63.3	18.2	7.17				
Rak-2	8365	Kranz	49	16	CZE	27.67	16.96	6.85				
Ren-1	8367	RÃ¶bbelen	48.5	-1.41	FRA	13.13	8.89	5.65				
Rmx-A180	8371	Bergelson	42.04	-86.51	USA							
RRS-10	8372	Dritz	41.56	-86.43	USA							
Rsch-4	8374	Holub	56.3	34	RUS							
Sanna-2	8376	Nordborg	62.69	18	SWE							
Sap-0	8378	Kranz	49.49	14.24	CZE	0	6.35	4.95				
Se-0	8379	Kranz	38.33	-3.53	ESP	10.21	14.64	6.89				
Sq-8	8385	Crawley	51.41	-0.64	UK	0	10.77	5.69				
St-0	8387	Kranz	59	18	SWE							
Ta-0	8389	Kranz	49.5	14.5	CZE	0	13.28	5.61				
Tamm-2	8390	Savolainen	60	23.5	FIN	5.95	15.47	5.11				
Ts-1	8392	Kranz	41.72	2.93	ESP							
Ull2-3	8396	Nordborg	56.06	13.97	SWE	5.43	16.98	4.84				
Ull2-5	8397	Nordborg	56.06	13.97	SWE	5.43	16.98	4.84				
Uod-7	8399	Koch	48.3	14.45	AUT	23.95	10.88	5.65				
Van-0	8400	Kranz	49.3	-123	CAN							
VÃ¥r2-1	8401	Nordborg	55.58	14.33	SWE							
Wei-0	8404	Holub	47.25	8.26	SUI							
Ws-0	8405	Kranz	52.3	30	RUS							
Wt-5	8407	Kranz	52.3	9.3	GER	63.61	9.13	6.79				
Yo-0	8408	Kranz	37.45	-119.35	USA							
Zdr-6	8410	Nordborg	49.39	16.25	CZE	11.74	9.73	6.01				
Sav-0	8412	Kranz	49.18	15.88	CZE	0.48	17.54	6.72				
Wil-1	8419	Kranz	54.68	25.32	LTU	0.28	5.69	4.82				

Native name	Ecotype	Collector	Lat	Lon	Country	$CaCO_3$ (g/kg)	CEC (cmol/kg)	pH H_2O	HN	LN	HG	LG
Kelsterbach-4	8420	Williams	50.07	8.53	GER	49.69	13.25	6.98				
FjÃ¤1-1	8422	Nordborg	56.06	14.29	SWE	5.52	13.46	5.97				
Kas-1	8424	Somerville	35	77	IND							
N13	8429	Savushkin	61.36	34.15	RUS							
Lisse	8430	Williams	52.25	4.57	NED	49.32	20.76	7.4				
11ME1.32	8610	Bergelson	42.09	-86.36	USA							
328PNA054	8692	Bergelson	42.09	-86.33	USA							
11PNA4.101	8796	Bergelson	42.09	-86.33	USA							

Supplementary F3

Annex Table 2: GWAS ionome results. The table indicates for each phenotype Chr (Chromosome), position, Score (-log p-value), MAF (Minor Allele Frequency), MAC (Minor Allele Count) and GVE (Genotype * Environment interaction). Scores were filtered by arbitrary number > 6. Phenotypes obtained from Control soil were represented by "L", $CaCO_3$ soil by "H" while relative nutrient mineral content $_{(CaCO3\ soil/Control)}$ were represented by "LHD"

Chr	Position	Score	MAF	MAC	GVE	Phenotype
2	10493484	6.29	0.19	49	0.1	CaL
1	10158240	6.69	0.06	14	0.1	FeL
1	10171691	6.35	0.06	15	0.1	FeL
3	1749881	6.42	0.17	43	0.1	FeL
3	8874490	6.89	0.2	50	0.11	FeL
3	14813232	6.21	0.11	27	0.09	FeL
5	6555809	7.51	0.11	29	0.12	FeL
1	18412150	6.77	0.11	27	0.1	CuL
1	3683490	6.89	0.08	20	0.11	MoL
3	10317806	6.44	0.06	14	0.1	MoL
4	6893508	7.43	0.06	16	0.11	MoL
1	5792665	6.45	0.06	15	0.1	CoL
2	12542961	7.8	0.09	23	0.12	CoL
2	15635227	7.26	0.09	22	0.11	CoL
2	15637905	7.99	0.11	27	0.12	CoL
5	3302831	8.2	0.06	16	0.13	CoL
1	26501594	6.75	0.07	17	0.1	CaL
1	1435846	6.66	0.27	69	0.1	NaL
4	2821561	6.28	0.15	39	0.1	NaL
4	6891043	6.05	0.23	57	0.09	NaL
4	6891119	6.26	0.23	57	0.1	NaL
4	6891572	6.21	0.25	62	0.09	NaL
4	6891823	6.22	0.22	56	0.09	NaL
5	17021448	6.02	0.06	15	0.09	NaL
4	7256304	6.48	0.16	41	0.1	BL
4	7269933	9.45	0.08	21	0.15	BL
4	7280653	6.06	0.11	28	0.09	BL
1	12353868	9.93	0.06	14	0.15	ZL
1	12357438	8.76	0.05	13	0.13	ZL
1	12360134	6.46	0.07	17	0.1	ZL
1	18855322	6.29	0.07	17	0.1	ZL
2	8357400	6.67	0.06	16	0.1	ZL
2	8360192	6.2	0.07	17	0.09	ZL
2	15912939	6.45	0.13	34	0.1	ZL

Chr	Position	Score	MAF	MAC	GVE	Phenotype
1	11219975	8.28	0.05	14	0.12	FeH
2	15525659	6.02	0.05	14	0.09	FeH
3	3391363	7.35	0.05	14	0.11	FeH
5	13789960	7.74	0.07	18	0.11	FeH
5	14132027	6.21	0.07	19	0.09	FeH
5	15460886	6.18	0.06	17	0.09	FeH
1	16487215	6.69	0.06	17	0.1	CuH
1	16503503	6.69	0.06	17	0.1	CuH
1	16503920	6.2	0.06	17	0.09	CuH
1	16509473	6.69	0.06	17	0.1	CuH
2	17834261	6.52	0.06	15	0.09	CuH
2	17834791	6.31	0.06	17	0.09	CuH
2	17834971	6.84	0.06	16	0.1	CuH
2	17847536	7.42	0.06	16	0.11	CuH
2	17853033	6.97	0.06	16	0.1	CuH
2	17853859	6.97	0.06	16	0.1	CuH
2	17854088	6.97	0.06	16	0.1	CuH
2	17854374	7.35	0.06	15	0.11	CuH
2	17854994	6.57	0.06	17	0.09	CuH
2	17856404	6.47	0.06	17	0.09	CuH
2	17860365	6.79	0.06	15	0.1	CuH
2	17861687	6.97	0.06	16	0.1	CuH
2	17861996	6.37	0.06	17	0.09	CuH
2	17862081	6.97	0.06	16	0.1	CuH
2	17862481	6.97	0.06	16	0.1	CuH
2	17865425	6.97	0.06	16	0.1	CuH
2	17866230	6.27	0.06	16	0.09	CuH
2	17870970	6.24	0.06	15	0.09	CuH
3	5819	6.2	0.07	18	0.09	CuH
3	948423	6.35	0.06	17	0.09	CuH
3	948608	6.37	0.07	18	0.09	CuH
4	2694229	6.3	0.05	14	0.09	CuH
4	7971311	6.45	0.07	18	0.09	CuH
4	15268789	7.24	0.06	16	0.1	CuH
4	17516527	6.28	0.09	24	0.09	CuH
3	105812	6.14	0.07	19	0.09	MoH
5	15767008	6.1	0.15	41	0.09	MoH
1	24329888	7.09	0.07	19	0.1	CoH
2	15281006	6.5	0.05	14	0.09	CoH
3	2078298	6.42	0.23	62	0.09	CoH
4	746534	6.18	0.05	14	0.09	CoH
3	3439072	6.36	0.13	34	0.09	MgH

Chr	Position	Score	MAF	MAC	GVE	Phenotype
3	3439481	6.84	0.08	22	0.1	MgH
3	3439730	6.05	0.13	36	0.09	MgH
3	3442703	8.62	0.14	39	0.12	MgH
2	4415096	6.16	0.05	14	0.09	SH
5	12587299	6.21	0.06	17	0.09	KH
2	13791424	6.69	0.06	15	0.1	NaH
3	22508719	7.17	0.08	21	0.1	BH
5	612834	7.47	0.06	17	0.11	BH
5	4933280	6.05	0.09	24	0.09	BH
1	669462	6.31	0.06	17	0.09	ZH
1	3551054	7.94	0.13	35	0.11	ZH
1	5520641	8.07	0.08	21	0.12	ZH
1	17278978	6.29	0.07	20	0.09	ZH
2	4362690	6.58	0.09	25	0.09	ZH
2	19044542	6.17	0.07	18	0.09	ZH
3	15023629	7.5	0.11	30	0.11	ZH
3	17773994	6.08	0.07	20	0.09	ZH
4	6657678	6.22	0.06	16	0.09	ZH
5	352142	8.54	0.05	14	0.12	ZH
5	2071431	6.55	0.12	33	0.09	ZH
5	4001555	6.16	0.06	15	0.09	ZH
5	18048832	6.39	0.12	32	0.09	ZH
5	18054746	6.05	0.12	33	0.09	ZH
5	18055570	6.39	0.12	32	0.09	ZH
5	18059206	6.39	0.12	32	0.09	ZH
5	19292148	8.79	0.11	29	0.13	ZH

Chr	pos	score	MAF	MAC	GVE	phenotype
1	21340739	6.52	0.11	25	0.11	FeHLD
2	14694655	6.22	0.05	13	0.11	FeHLD
5	13789960	7.15	0.06	13	0.12	FeHLD
5	15414186	6.05	0.06	13	0.15	FeHLD
1	11291745	6.61	0.08	17	0.11	MoHLD
4	9930184	6.7	0.06	14	0.12	MoHLD
5	6504971	6.87	0.06	13	0.12	MoHLD
4	5077876	7.34	0.14	30	0.13	CoHLD
1	26392275	6.22	0.1	22	0.11	CaHLD
5	8509007	6.27	0.08	17	0.11	CaHLD
4	5249059	6.23	0.12	27	0.11	CaHLD
4	5249165	6.48	0.12	27	0.11	CaHLD
4	5250587	6.57	0.16	36	0.1	CaHLD

Chr	pos	score	MAF	MAC	GVE	phenotype
4	5256418	7.48	0.12	26	0.13	CaHLD
4	5265661	6.75	0.12	27	0.12	CaHLD
4	5292175	6.16	0.16	36	0.11	CaHLD
5	17969356	6.12	0.11	25	0.1	CaHLD
3	8246346	31.45	0.16	36	0.47	NaHLD
4	9656689	32.57	0.23	51	0.48	NaHLD
4	16322002	33.23	0.07	16	0.49	NaHLD
3	16566264	7.68	0.08	18	0.14	BHLD
5	13860946	6.68	0.19	42	0.12	BHLD
5	13867993	6.06	0.15	35	0.15	BHLD

Supplementary F4

Annex Table 3: Nutrient mineral content, Mean ± Standard error, and t-test from high and low-performance plants sown in calcareous soil. Relative growth ($CaCO_3$-soil/ Control-soil) distributions were used to select 10% tails as high and low-performance plant groups.

	High relative growth plants group			Low relative growth plants group				
Phenotype	Mean	St error	N	Mean	St error	N	Ratio F	Prob > |t|
B11	208.18	13.24	24	185.43	14.77	23	1.15	0.2568
Ca43	30249.21	876.35	24	31153.67	945	23	-0.7	0.4859
Co59	7.14	0.37	24	8.28	0.48	23	-1.91	0.0631
Cu65	6.77	0.22	24	7.26	0.99	23	-0.49	0.6247
Fe57	95.88	6.5	24	95.69	8.58	23	0.02	0.9862
K39	27343.37	1269.2	24	29362.52	1861.21	23	-0.9	0.3713
Mg25	2026.39	85.78	24	2030.91	84.09	23	-0.04	0.9702
Mn55	89.62	4.37	24	81.21	3.17	23	1.55	0.1287
Mo98	0.79	0.15	24	1.03	0.19	23	-1.02	0.3129
Na23	1352.17	306.64	24	1387.17	600.79	23	-0.05	0.9583
P31	6012.78	321.1	24	4489.99	326.89	23	3.32	0.0018
S34	7908.62	282.88	24	6815.24	247.66	23	2.9	0.0058
Zn66	177.71	38.44	24	54.96	3.85	23	3.11	0.0032

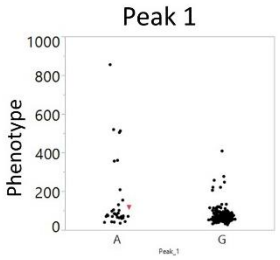
Supplementary F5

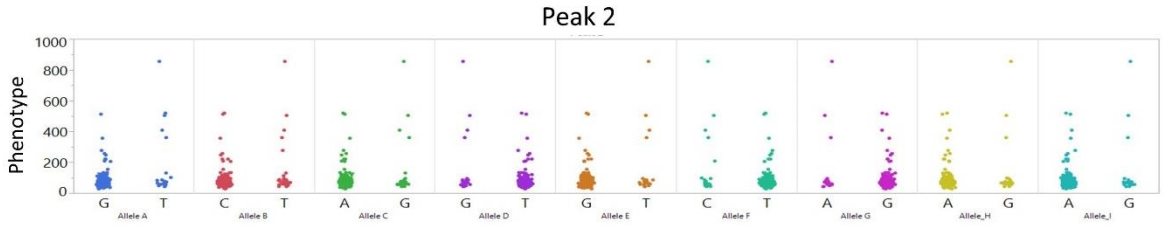
Annex Table 4: Primers list table used to confirm T-DNA insertion in Zn phenotype growth in $CaCO_3$-soils.

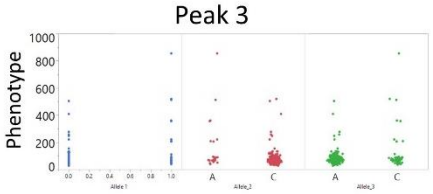
GENE	Arbitrary number	Description	LP	RP
AT5G47580	1	Transmembrane protein	GTGCAGGACAAAACTCGCTAG	TCGATTGGTATCTACGTTGCC
AT1G10690	2	Cyclin-dependent kinase inhibitor	CAACGGGTCAAACAAATCAAC	TGACGTAACTGCACTTCAACG
AT1G16290	4	Transglycosylase	TTTGATTCCATCTTGGATTCG	AGCTAAAGCATTGCCACACTG
AT1G16190	5	Rad23 UV excision repair protein family	GAAGCATCGAATGTTCAAAGC	TTGTGCTGACAACATCTTTGC
AT1G16390	8	Organic cation/carnitine transporter 3	TTGTGTGCTCGTTTCGTTATG	TAATCGCGGTGTTAGGACAAC
AT5G47570	9	NADH dehydrogenase ubiquinone	TGTTTGGCATTGGCTTATTTC	CCAAGCTCAACTCTCAGGTTG
AT5G47590	10	Heat shock protein	TGTTGTTCGAAATTAAAGCGG	GAACAAAACGCCATTTTTACG
AT1G16150	11	Wall associated kinase-like 4	GGATATCGGAGTTTAAAGCCG	TTATGAGTTTGTTCCGAACGG
AT1G16210	13	Coiled-coil protein	GAAACGTGTCTCTTGCCTCTG	ATGAGAGAATCATCGCGATTG
AT5G47560	14	Tonoplast dicarboxylate transporter	ATTTAATTTGTGCAAGTGCGG	TCGATGAGTAACGGAACCAAC
AT1G16380	15	Cation/hydrogen exchanger family protein	TGACACTACCGAGGAGCTACG	CTAATTCCGGACACTCTTCCC
			LBb1.3	ATTTTGCCGATTTCGGAAC

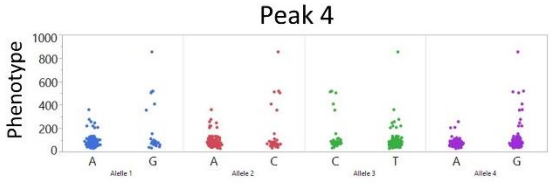
Annex Table 5: Haplotypes from different peaks. Table of candidates SNP and corresponding gene. Each plot represents haplotype groups (x-axis) of leaves Zn content (µg/g) phenotype from Hapmap population growth in $CaCO_3$ soil.

	Chromosome	Position	Score	Effect	Gene	Function
Peak 1	Chr1	3551054	7.94	Intergenic		
					AT1G10690	Cyclin-dependent kinase inhibitor
					AT1G10700	Phosphoribosyl pyrophosphate (PRPP) synthase 3
Peak 2	Chr1	5520641	8.07	UTR_3'	AT1G16110	Wall associated kinase-like 6
		5522235	2.99	Intergenic	AT1G16120	Wall associated kinase-like 1
		5533737	3.00	Synonymous	AT1G16150	Wall associated kinase-like 4
		5543599	4.17	Intron	AT1G16190	Rad23 UV excision repair protein family
		5545724	4.19	Intron		
		5546768	5.25	Intron	AT1G16210	Coiled-coil protein
		5572032	3.02	Synonymous	AT1G16290	Transglycosylase
		5599695	2.5	Non-syn	AT1G16380	Cation/hydrogen exchanger family protein
		5604901	2.53	Intergenic	AT1G16390	Organic cation/carnitine transporter 3
Peak 3	Chr3	15023629	7.50	Intergenic		
		15024837	3.27	Non-syn	AT3G42970	unknown protein
		15062847	2.90			
Peak 4	Chr5	19292148	8.79	Intergenic		
		19291992	5.82	Intergenic	AT5G47590	Heat shock protein HSP20/alpha crystallin family
		19291111	2.60	Intergenic	AT5G47580	Transmembrane protein
		19291111	2.60	Intergenic	AT5G47570	NADH dehydrogenase ubiquinone 1 beta subcomplex subunit
		19288458	1.29	Synonymous	AT5G47560	Tonoplast dicarboxylate transporter









Supplementary F6

Annex Table 6: Plant diameter at 4 weeks ANOVA factor analysis from mutant and Col-0 lines growing in contrasted $CaCO_3$ soil. *n=4*

Y	Factor	Sum of square	F value	Prob > F
Diameter week 4	Soil	141.63	438.4	<.0001
Diameter week 4	Line	2.56	1.32	0.2553
Diameter week 4	Soil*Line	19.45	10.03	<.0001

Annex Table 7: Plant diameter at week 4, Mean, Standard deviation, Standard error, ANOVA and Tukey test from Col-0 and mutant lines growing in calcareous soil and control soil.

Treatment	Line	Mean diameter (cm)	Std desv	Std Error	N	F value	Prob > F	Tukey test
Control soil	10	4.99	0.5	0.18	8	15.4577	0.0012	A
$CaCO_3$ soil	10	2.24	0.31	0.11	8			B
Control soil	11	5.09	0.68	0.23	9	2.8906	0.1112	A
$CaCO_3$ soil	11	2.36	0.28	0.1	8			B
Control soil	13	4.03	0.86	0.29	9	176.4895	<.0001	A
$CaCO_3$ soil	13	2.69	0.56	0.19	9			B
Control soil	14	3.91	0.89	0.31	8	111.6562	<.0001	A
$CaCO_3$ soil	14	3.27	0.59	0.21	8			A
Control soil	4	2.39	0.31	0.11	8	133.9754	<.0001	A
$CaCO_3$ soil	4	4.71	0.47	0.17	8			B
Control soil	9	5.39	0.52	0.17	9	185.8068	<.0001	A
$CaCO_3$ soil	9	2.32	0.43	0.14	9			B
Control soil	Col-0	4.92	0.65	0.23	8	85.3904	<.0001	A
$CaCO_3$ soil	Col-0	2.35	0.44	0.15	8			B

Annex Table 8: Nutrient mineral content, Mean, Standard deviation, Standard error, ANOVA and Tukey test from Col-0 and TDT mutant in calcareous soil and control soil.

TDNA	Element	Treatment	Mean	Std Desv	Std Error	N	F value	Prob > F	Tukey test
3	B	Control soil	17.73	19.07	9.53	4	6.25	0.041	B
3	B	$CaCO_3$ soil	101.39	63.89	28.57	5			A
3	Ca	Control soil	26347.39	19751.01	8832.92	5	6.10	0.0387	B
3	Ca	$CaCO_3$ soil	72835.82	37172.09	16623.87	5			A
3	Co	Control soil	29.14	26.92	12.04	5	0.10	0.7651	A
3	Co	$CaCO_3$ soil	19.70			1			A
3	Fe	Control soil	2692.96	4797.00	2145.28	5	0.09	0.7721	A
3	Fe	$CaCO_3$ soil	2028.16	1264.18	565.36	5			A
3	K	Control soil	17893.15	13668.23	6112.62	5	0.21	0.6603	A
3	K	$CaCO_3$ soil	14729.41	7320.99	3274.05	5			A
3	Mg	Control soil	2233.14	1660.39	742.55	5	0.03	0.87	A
3	Mg	$CaCO_3$ soil	2386.92	1176.19	526.01	5			A
3	Mn	Control soil	120.27	145.48	65.06	5	0.13	0.7247	A
3	Mn	$CaCO_3$ soil	95.13	50.79	22.71	5			A
3	Mo	Control soil	69.57	82.31	58.20	2	0.32	0.5966	A
3	Mo	CaCO3 soil	114.00	96.73	43.26	5			A
3	Na	Control soil	4716.21	2913.50	1302.96	5	0.06	0.8054	A
3	Na	$CaCO_3$ soil	4266.15	2668.07	1193.20	5			A
3	P	Control soil	1958.19	1659.95	742.35	5	0.41	0.5397	A
3	P	$CaCO_3$ soil	1412.60	933.55	417.50	5			A
3	Pb	Control soil	140.11	128.38	74.12	3	14.77	0.0184	B
3	Pb	$CaCO_3$ soil	797.06	266.84	154.06	3			A
3	S	Control soil	6757.21	5247.23	2346.63	5	0.08	0.7857	A
3	S	$CaCO_3$ soil	5978.67	3284.50	1468.87	5			A
3	Zn	Control soil	97.55	13.10	7.56	3	2.14	0.1941	A
3	Zn	$CaCO_3$ soil	182.21	96.69	43.24	5			A

TDNA	Y	Treatment	Mean	Std Desv	Std Error	N	F value	Prob > F	Tukey test
4	B	Control soil	169.36	167.44	83.72	4	0.20	0.6684	A
4	B	$CaCO_3$ soil	131.48	83.08	37.15	5			A
4	Ca	Control soil	54907.82	17272.08	7724.31	5	2.44	0.1568	A
4	Ca	$CaCO_3$ soil	33157.04	25894.45	11580.35	5			A
4	Co	Control soil	92.56	26.91	12.03	5	8.77	0.0415	A
4	Co	$CaCO_3$ soil	5.29			1			B
4	Fe	Control soil	2113.74	3392.95	1517.37	5	1.46	0.262	A
4	Fe	$CaCO_3$ soil	278.81	217.19	97.13	5			A
4	K	Control soil	32395.08	9742.95	4357.18	5	5.49	0.0472	A
4	K	$CaCO_3$ soil	15631.01	12685.08	5672.94	5			B

4	Mg	Control soil	4542.15	1068.72	477.95	5	12.31	0.008	A
4	Mg	$CaCO_3$ soil	1770.90	1405.80	628.69	5			B
4	Mn	Control soil	177.57	112.04	50.11	5	4.29	0.0771	A
4	Mn	$CaCO_3$ soil	51.73	49.14	24.57	4			A
4	Mo	Control soil	268.67	208.89	93.42	5	0.39	0.5538	A
4	Mo	CaCO3 soil	195.15	119.76	59.88	4			A
4	Na	Control soil	7682.10	5548.96	2481.57	5	0.06	0.8139	A
4	Na	$CaCO_3$ soil	6652.64	7663.16	3427.07	5			A
4	P	Control soil	4216.95	2078.97	929.74	5	2.76	0.1478	A
4	P	$CaCO_3$ soil	1761.65	1910.24	1102.88	3			A
4	S	Control soil	11550.49	4462.51	1995.70	5	10.33	0.0124	A
4	S	$CaCO_3$ soil	3385.56	3515.73	1572.28	5			B
4	Zn	Control soil	126.39	49.09	21.95	5	0.16	0.7001	A
4	Zn	$CaCO_3$ soil	147.05	102.63	51.31	4			A

TDNA	Y	Treatment	Mean	Std Desv	Std Error	N	F value	Prob > F	Tukey test
10	B	Control soil	71.63	68.15	30.48	5	0.29	0.6064	A
10	B	$CaCO_3$ soil	54.61	19.83	8.87	5			A
10	Ca	Control soil	62508.10	13639.11	6099.60	5	7.37	0.0265	B
10	Ca	$CaCO_3$ soil	93162.66	21254.74	9505.41	5			A
10	Co	Control soil	130.16	163.10	94.17	3			
10	Co	$CaCO_3$ soil				0			
10	Fe	Control soil	947.64	426.10	190.56	5	5.49	0.0471	A
10	Fe	$CaCO_3$ soil	2425.73	1344.33	601.20	5			A
10	K	Control soil	35647.97	9812.40	4388.24	5	11.81	0.0089	B
10	K	$CaCO_3$ soil	18675.98	5066.50	2265.81	5			B
10	Mg	Control soil	5219.65	1004.58	449.26	5	20.83	0.0018	A
10	Mg	$CaCO_3$ soil	3018.13	392.73	175.64	5			B
10	Mn	Control soil	134.53	20.48	9.16	5	0.70	0.4286	A
10	Mn	$CaCO_3$ soil	121.30	28.94	12.94	5			A
10	Mo	Control soil	400.73	214.24	123.69	3	2.78	0.1565	A
10	Mo	CaCO3 soil	177.74	143.38	71.69	4			A
10	Na	Control soil	12877.66	6972.59	3118.24	5	11.84	0.0088	A
10	Na	$CaCO_3$ soil	2043.42	985.62	440.78	5			B
10	P	Control soil	5361.15	3318.71	1484.17	5	5.42	0.0484	A
10	P	$CaCO_3$ soil	1788.44	877.90	392.61	5			B
10	S	Control soil	12162.09	3778.94	1690.00	5	8.25	0.0208	A
10	S	$CaCO_3$ soil	6793.08	1787.84	799.55	5			B
10	Zn	Control soil	140.67	38.56	17.25	5	11.79	0.0089	A
10	Zn	$CaCO_3$ soil	246.37	57.01	25.49	5			A

TDNA	Y	Treatment	Mean	Std Desv	Std Error	N	F value	Prob > F	Tukey test
6	B	Control soil	55.41	30.73	13.74	5	2.72	0.1377	A
6	B	$CaCO_3$ soil	109.33	66.32	29.66	5			A
6	Ca	Control soil	37307.25	18015.17	8056.63	5	0.14	0.7206	A
6	Ca	$CaCO_3$ soil	43502.06	32762.41	14651.79	5			A
6	Co	Control soil	52.10	24.96	11.16	5	2.78	0.1709	A
6	Co	$CaCO_3$ soil	6.54			1			A
6	Fe	Control soil	1044.90	825.48	369.17	5	0.31	0.5937	A
6	Fe	$CaCO_3$ soil	764.18	771.41	344.98	5			A
6	K	Control soil	24538.38	11917.52	5329.68	5	2.38	0.1614	A
6	K	$CaCO_3$ soil	13225.22	11255.30	5033.52	5			A
6	Mg	Control soil	2954.63	1469.06	656.98	5	1.15	0.3149	A
6	Mg	$CaCO_3$ soil	1960.32	1463.24	654.38	5			A
6	Mn	Control soil	84.89	50.21	22.46	5	0.03	0.8724	A
6	Mn	$CaCO_3$ soil	78.78	65.46	29.27	5			A
6	Mo	Control soil	27.85			1	0.68	0.4697	A
6	Mo	CaCO3 soil	289.76	283.83	141.92	4			A
6	Na	Control soil	3982.00	2248.73	1005.66	5	0.61	0.4579	A
6	Na	$CaCO_3$ soil	5864.35	4905.80	2193.94	5			A
6	P	Control soil	3384.00	2326.92	1040.63	5	5.60	0.0455	A
6	P	$CaCO_3$ soil	873.75	456.88	204.32	5			B
6	S	Control soil	7602.10	3752.21	1678.04	5	0.88	0.3751	A
6	S	$CaCO_3$ soil	5205.49	4298.17	1922.20	5			A
6	Zn	Control soil	79.87	39.86	17.83	5	0.84	0.3874	A
6	Zn	$CaCO_3$ soil	125.35	103.88	46.46	5			A

TDNA	Y	Treatment	Mean	Std Desv	Std Error	N	F value	Prob > F	Tukey test
9	B	Control soil	53.64	25.50	11.41	5	0.67	0.4375	A
9	B	$CaCO_3$ soil	64.41	14.78	6.61	5			A
9	Ca	Control soil	38906.08	18907.50	8455.69	5	15.90	0.004	B
9	Ca	$CaCO_3$ soil	90997.20	22272.10	9960.39	5			A
9	Co	Control soil	42.09	23.42	10.47	5			
9	Co	$CaCO_3$ soil				0			
9	Fe	Control soil	413.42	148.76	66.53	5	20.15	0.002	B
9	Fe	$CaCO_3$ soil	2504.33	1030.99	461.07	5			A
9	K	Control soil	24212.14	12683.22	5672.11	5	0.66	0.44	A
9	K	$CaCO_3$ soil	19495.30	2761.67	1235.06	5			A
9	Mg	Control soil	3078.85	1505.07	673.09	5	0.06	0.8098	A
9	Mg	$CaCO_3$ soil	3248.43	243.23	108.77	5			A
9	Mn	Control soil	88.07	44.69	19.99	5	2.61	0.1447	A

9	Mn	$CaCO_3$ soil	124.56	23.48	10.50	5			A
9	Mo	Control soil	133.14	117.47	58.73	4	0.00	0.9828	A
9	Mo	CaCO3 soil	135.19	149.57	66.89	5			A
9	Na	Control soil	6905.31	6003.66	2684.92	5	0.08	0.7897	A
9	Na	$CaCO_3$ soil	6120.70	2099.82	939.07	5			A
9	P	Control soil	4063.35	2797.17	1250.93	5	2.83	0.1307	A
9	P	$CaCO_3$ soil	1907.63	610.40	272.98	5			A
9	S	Control soil	7791.62	3983.78	1781.60	5	0.02	0.8904	A
9	S	$CaCO_3$ soil	7532.86	829.80	371.10	5			A
9	Zn	Control soil	83.05	51.52	25.76	4	18.28	0.0037	B
9	Zn	$CaCO_3$ soil	235.88	54.55	24.40	5			A

TDNA	Y	Treatment	Mean	Std Desv	Std Error	N	F value	Prob > F	Tukey test
11	B	Control soil	36.37	7.22	3.23	5	3.83	0.0862	A
11	B	$CaCO_3$ soil	99.27	71.53	31.99	5			A
11	Ca	Control soil	46078.93	1553.20	694.61	5	16.89	0.0034	B
11	Ca	$CaCO_3$ soil	72570.87	14329.43	6408.32	5			A
11	Co	Control soil	34.20	17.34	7.75	5	0.05	0.8235	A
11	Co	$CaCO_3$ soil	38.32	35.23	17.61	4			A
11	Fe	Control soil	1245.92	603.09	269.71	5	7.83	0.0233	B
11	Fe	$CaCO_3$ soil	2715.68	1007.73	450.67	5			A
11	K	Control soil	33605.71	1636.40	731.82	5	52.41	<.0001	A
11	K	$CaCO_3$ soil	20270.18	3780.04	1690.48	5			B
11	Mg	Control soil	3927.17	195.83	87.58	5	32.80	0.0004	A
11	Mg	$CaCO_3$ soil	2894.47	352.47	157.63	5			B
11	Mn	Control soil	129.86	20.13	9.00	5	1.21	0.303	A
11	Mn	$CaCO_3$ soil	142.16	14.81	6.63	5			A
11	Mo	Control soil	34.58	20.80	12.01	3	1.81	0.2495	A
11	Mo	CaCO3 soil	70.68	41.53	23.98	3			A
11	Na	Control soil	6269.55	1243.50	556.11	5	5.05	0.0548	A
11	Na	$CaCO_3$ soil	3422.02	2545.91	1138.57	5			A
11	P	Control soil	4244.40	480.11	214.71	5	79.04	<.0001	A
11	P	$CaCO_3$ soil	1629.03	449.67	201.10	5			B
11	S	Control soil	10592.57	637.31	285.01	5	1.97	0.1984	A
11	S	$CaCO_3$ soil	9172.09	2173.71	972.11	5			A
11	Zn	Control soil	111.49	9.21	4.12	5	29.78	0.0006	B
11	Zn	$CaCO_3$ soil	223.48	44.95	20.10	5			A

TDNA	Y	Treatment	Mean	Std Desv	Std Error	N	F value	Prob > F	Tukey test
13	B	Control soil	162.87	83.04	47.94	3	13.19	0.0109	B

13	B	$CaCO_3$ soil	29.83	18.08	8.08	5			A
13	Ca	Control soil	42829.16	5986.23	2677.12	5	7.41	0.0262	A
13	Ca	$CaCO_3$ soil	85072.70	34189.53	15290.02	5			A
13	Co	Control soil	60.15	43.59	25.17	3	0.55	0.5359	B
13	Co	$CaCO_3$ soil	22.85			1			B
13	Fe	Control soil	2645.76	2314.85	1035.23	5	0.05	0.8326	A
13	Fe	$CaCO_3$ soil	2371.05	1596.67	714.05	5			A
13	K	Control soil	24638.29	3607.48	1613.31	5	5.91	0.0411	B
13	K	$CaCO_3$ soil	19164.13	3509.98	1569.71	5			B
13	Mg	Control soil	3810.77	344.65	154.13	5	18.54	0.0026	A
13	Mg	$CaCO_3$ soil	2846.29	363.52	162.57	5			A
13	Mn	Control soil	192.72	73.88	42.66	3	2.48	0.1663	A
13	Mn	$CaCO_3$ soil	128.37	44.34	19.83	5			A
13	Mo	Control soil	346.61	469.65	234.82	4	0.09	0.7755	B
13	Mo	CaCO3 soil	233.42	268.86	190.11	2			A
13	Na	Control soil	12217.83	9146.55	4090.46	5	6.59	0.0333	A
13	Na	$CaCO_3$ soil	1692.27	633.45	283.29	5			A
13	P	Control soil	5546.30	2772.13	1239.74	5	9.74	0.0168	A
13	P	$CaCO_3$ soil	1139.72	296.59	148.29	4			A
13	S	Control soil	6898.22	3700.85	1655.07	5	1.39	0.2725	A
13	S	$CaCO_3$ soil	9160.38	2174.92	972.65	5			A
13	Zn	Control soil	166.41	96.96	43.36	5	1.48	0.2586	A
13	Zn	$CaCO_3$ soil	1450.58	2359.09	1055.02	5			A

TDNA	Y	Treatment	Mean	Std Desv	Std Error	N	F value	Prob > F	Tukey test
14	B	Control soil	149.10	83.14	37.18	5	0.86	0.3816	A
14	B	$CaCO_3$ soil	114.13	14.82	6.63	5			A
14	Ca	Control soil	59430.58	9174.47	4102.95	5	23.76	0.0012	B
14	Ca	$CaCO_3$ soil	176281.89	52808.17	23616.53	5			A
14	Co	Control soil	66.85	73.89	42.66	3	0.36	0.5795	A
14	Co	$CaCO_3$ soil	103.90	76.76	44.32	3			A
14	Fe	Control soil	1267.98	1167.27	522.02	5	3.79	0.0873	A
14	Fe	$CaCO_3$ soil	2751.52	1240.36	554.71	5			A
14	K	Control soil	35555.44	7749.11	3465.51	5	9.16	0.0164	B
14	K	$CaCO_3$ soil	56053.82	13007.63	5817.19	5			A
14	Mg	Control soil	4395.29	496.98	222.25	5	14.22	0.0055	B
14	Mg	$CaCO_3$ soil	8034.81	2100.45	939.35	5			A
14	Mn	Control soil	109.54	18.21	10.52	3	12.62	0.012	B
14	Mn	$CaCO_3$ soil	301.81	89.86	40.19	5			A
14	Mo	Control soil	1169.46	1115.60	498.91	5	2.73	0.1426	A
14	Mo	CaCO3 soil	224.41	195.60	97.80	4			A

14	Na	Control soil	14708.61	15400.81	6887.45	5	2.07	0.1878	A
14	Na	$CaCO_3$ soil	4729.25	1705.71	762.81	5			A
14	P	Control soil	5396.40	5476.72	2449.26	5	0.14	0.7194	A
14	P	$CaCO_3$ soil	4462.85	1210.08	541.16	5			A
14	S	Control soil	9815.57	5111.41	2285.89	5	13.49	0.0063	B
14	S	$CaCO_3$ soil	30208.15	11314.62	5060.05	5			A
14	Zn	Control soil	404.85	322.89	144.40	5	2.69	0.1398	B
14	Zn	$CaCO_3$ soil	1636.52	648.97	737.44	5			A

TDNA	Y	Treatment	Mean	Std Desv	Std Error	N	F value	Prob > F	Tukey test
Col-0	B	Control soil	37.27	9.35	4.18	5	2.30	0.1677	A
Col-0	B	$CaCO_3$ soil	25.60	14.43	6.45	5			A
Col-0	Ca	Control soil	42987.51	2569.67	1149.19	5	0.54	0.4832	A
Col-0	Ca	$CaCO_3$ soil	53195.98	30939.86	13836.73	5			A
Col-0	Co	Control soil	25.75	1.74	1.23	2	0.11	0.758	A
Col-0	Co	$CaCO_3$ soil	20.04	23.06	11.53	4			A
Col-0	Fe	Control soil	2689.27	2479.17	1108.72	5	0.00	0.9716	A
Col-0	Fe	$CaCO_3$ soil	2767.41	4053.88	1812.95	5			A
Col-0	K	Control soil	28907.67	3631.23	1623.94	5	0.03	0.8713	A
Col-0	K	$CaCO_3$ soil	29651.03	9252.31	4137.76	5			A
Col-0	Mg	Control soil	3773.61	118.41	52.95	5	5.93	0.0409	A
Col-0	Mg	$CaCO_3$ soil	2931.47	764.17	341.75	5			B
Col-0	Mn	Control soil	176.02	64.99	29.06	5	0.75	0.4106	A
Col-0	Mn	$CaCO_3$ soil	139.30	68.70	30.72	5			A
Col-0	Mo	Control soil	179.07	129.99	58.13	5	2.70	0.1442	A
Col-0	Mo	CaCO3 soil	61.58	62.92	31.46	4			A
Col-0	Na	Control soil	8278.08	2350.22	1051.05	5	43.75	0.0002	A
Col-0	Na	$CaCO_3$ soil	1289.70	241.72	108.10	5			B
Col-0	P	Control soil	3523.44	1412.66	631.76	5	11.02	0.0128	A
Col-0	P	$CaCO_3$ soil	1110.90	277.92	138.96	4			B
Col-0	S	Control soil	8632.11	1500.77	671.17	5	0.26	0.6265	A
Col-0	S	$CaCO_3$ soil	7917.46	2778.74	1242.69	5			A
Col-0	Zn	Control soil	151.71	54.65	24.44	5	1.22	0.3008	A
Col-0	Zn	$CaCO_3$ soil	263.06	218.33	97.64	5			A

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Annex Table 9: Plant nutrition at 4 weeks ANOVA two-way factor analysis from mutant 14 *tdt* and Col-0 lines growing in contrasted $CaCO_3$ soil. *n=4*

Element	Factor	Estimation	Std Error	t value	Prob > |t|
B	TDNA	50.09	9.64	5.20	<.0001
B	Treatment	-11.66	9.64	-1.21	0.2439
B	Treatment*TDNA	-5.83	9.64	-0.60	0.554
Ca	TDNA	34882.24	6925.27	5.04	0.0001
Ca	Treatment	31764.94	6925.27	4.59	0.0003
Ca	Treatment*TDNA	26660.71	6925.27	3.85	0.0014
Co	TDNA	31.24	16.40	1.90	0.0933
Co	Treatment	7.83	16.40	0.48	0.6457
Co	Treatment*TDNA	10.69	16.40	0.65	0.5328
Fe	TDNA	-359.29	564.37	-0.64	0.5334
Fe	Treatment	390.42	564.37	0.69	0.499
Fe	Treatment*TDNA	351.35	564.37	0.62	0.5424
K	TDNA	8262.64	2024.96	4.08	0.0009
K	Treatment	5310.43	2024.96	2.62	0.0185
K	Treatment*TDNA	4938.75	2024.96	2.44	0.0268
Mg	TDNA	1431.25	256.34	5.58	<.0001
Mg	Treatment	699.35	256.34	2.73	0.0149
Mg	Treatment*TDNA	1120.41	256.34	4.37	0.0005
Mn	TDNA	24.01	16.92	1.42	0.1779
Mn	Treatment	38.89	16.92	2.30	0.0375
Mn	Treatment*TDNA	57.25	16.92	3.38	0.0045
Mo	TDNA	288.30	144.16	2.00	0.0653
Mo	Treatment	-265.63	144.16	-1.84	0.0867
Mo	Treatment*TDNA	-206.89	144.16	-1.44	0.1732
Na	TDNA	2467.52	1752.41	1.41	0.1782
Na	Treatment	-4241.94	1752.41	-2.42	0.0278
Na	Treatment*TDNA	-747.75	1752.41	-0.43	0.6753
P	TDNA	1306.23	689.03	1.90	0.0774
P	Treatment	-836.52	689.03	-1.21	0.2435
P	Treatment*TDNA	369.74	689.03	0.54	0.5994
S	TDNA	5868.54	1432.31	4.10	0.0008
S	Treatment	4919.48	1432.31	3.43	0.0034
S	Treatment*TDNA	5276.81	1432.31	3.68	0.002
Zn	TDNA	456.65	173.92	2.63	0.0184
Zn	Treatment	385.75	173.92	2.22	0.0414
Zn	Treatment*TDNA	330.08	173.92	1.90	0.0759

Annex Table 10: Plant nutrition at 4 weeks Tukey test from mutant 14 *tdt* and Col-0 lines growing in contrasted $CaCO_3$ soil. *n=4*

Element	Effect	Level	Tukey test
B	Treatment*TDNA	Control,Col-0	B
B	Treatment*TDNA	Control,14	A
B	Treatment*TDNA	CaCO3, Col-0	B
B	Treatment*TDNA	CaCO3, 14	AB
Ca	Treatment*TDNA	Control,Col-0	B
Ca	Treatment*TDNA	Control,14	B
Ca	Treatment*TDNA	CaCO3, Col-0	B
Ca	Treatment*TDNA	CaCO3, 14	A
Co	Treatment*TDNA	Control,Col-0	A
Co	Treatment*TDNA	Control,14	A
Co	Treatment*TDNA	CaCO3, Col-0	A
Co	Treatment*TDNA	CaCO3, 14	A
Fe	Treatment*TDNA	Control,Col-0	A
Fe	Treatment*TDNA	Control,14	A
Fe	Treatment*TDNA	CaCO3, Col-0	A
Fe	Treatment*TDNA	CaCO3, 14	A
K	Treatment*TDNA	Control,Col-0	B
K	Treatment*TDNA	Control,14	B
K	Treatment*TDNA	CaCO3, Col-0	B
K	Treatment*TDNA	CaCO3, 14	A
Mg	Treatment*TDNA	Control,Col-0	B
Mg	Treatment*TDNA	Control,14	B
Mg	Treatment*TDNA	CaCO3, Col-0	B
Mg	Treatment*TDNA	CaCO3, 14	A
Mn	Treatment*TDNA	Control,Col-0	AB
Mn	Treatment*TDNA	Control,14	B
Mn	Treatment*TDNA	CaCO3, Col-0	B
Mn	Treatment*TDNA	CaCO3, 14	A
Mo	Treatment*TDNA	Control,Col-0	A
Mo	Treatment*TDNA	Control,14	A
Mo	Treatment*TDNA	CaCO3, Col-0	A
Mo	Treatment*TDNA	CaCO3, 14	A
Na	Treatment*TDNA	Control,Col-0	A
Na	Treatment*TDNA	Control,14	A
Na	Treatment*TDNA	CaCO3, Col-0	A
Na	Treatment*TDNA	CaCO3, 14	A
P	Treatment*TDNA	Control,Col-0	A
P	Treatment*TDNA	Control,14	A
P	Treatment*TDNA	CaCO3, Col-0	A

P	Treatment*TDNA	CaCO3, 14	A
S	Treatment*TDNA	Control,Col-0	B
S	Treatment*TDNA	Control,14	B
S	Treatment*TDNA	CaCO3, Col-0	B
S	Treatment*TDNA	CaCO3, 14	A
Zn	Treatment*TDNA	Control,Col-0	B
Zn	Treatment*TDNA	Control,14	B
Zn	Treatment*TDNA	CaCO3, Col-0	B
Zn	Treatment*TDNA	CaCO3, 14	A

Supplementary material: Chapter 4

Supplementary Material and Methods

Annex table 1: The table indicates the deme name, collector (Silvia Busoms, Kristen Bombiens and Joana Terés), UTM coordinates and deme origin group: G1-Plant from high $CaCO_3$ content and low salt native soil in average (9.45% $CaCO_3$, 44.33 Na^+ mg/g); G2- Plant with intermediate salt and carbonate exposure (5.7% $CaCO_3$ 60.65 Na^+ mg/g) and G3 - Plants exposed to high salts and low carbonates (0.65% $CaCO_3$ and 129.44 Na^+ mg/g). The plants used in Field Experiment (2017- 2018), Greenhouse (2020) and Hydroponics were marked with an "x". Lines used for the plate experiment were marked as a "y". The populations used in Greenhouse 2020 were colored in grey.

Deme ID	Source	UTM(x)	UTM(y)	Deme group	Field common garden 2017	Field common garden 2018	Greenhouse 2020	Hydroponic plates 2021
A1	S.Busoms	46.913	46.451	G1	x	x	x	x, y
A2	S.Busoms	46.913	46.451	G1				
A5	S.Busoms	47.043	46.439	G1	x		x	x, y
AM	S.Busoms	46.625	46.523	G1		x	x	x, y
C3	K.Bomblies	48.831	46.392	G2				
CALA	S.Busoms	49.558	46.199	G3	x			
JBB	K.Bomblies	48.391	46.14	G3	x	x	x	x, y
LG4	K.Bomblies	49.1	46.316	G2				
LG5	K.Bomblies	49.112	46.31	G2	x		x	x, y
LG6	S.Busoms	49.087	46.306	G2				
LG7	K.Bomblies	49.079	46.307	G2		x	x	x, y
LG8	K.Bomblies	49.126	46.302	G2				
LLM1	S.Busoms	48.835	46.412	G1				
LLO1	K.Bomblies	48.845	46.173	G3				
LLO2	S.Busoms	48.634	46.169	G3		x	x	x, y
LLO3	S.Busoms	48.508	46.157	G3	x			
LM11	S.Busoms	48.907	46.412	G1				
LM2	K.Bomblies	48.912	46.12	G1	x	x	x	x, y
O3	S.Busoms	46.772	46.446	G1		x	x	x
O4	S.Busoms	46.771	46.446	G1				
PA10	K.Bomblies	50.479	46.289	G3		x	x	x
PO1	anthos	51.347	46.862	G3	x		x	x

Deme ID	Source	UTM(x)	UTM(y)	Deme group	Field common garden 2017	Field common garden 2018	Greenhouse 2020	Hydroponic plates 2021
PR2	S.Busoms	33.046	45.79	G2				
PR3	S.Busoms	33.193	45.783	G2				
PR5	S.Busoms	33.173	45.771	G2				
RO2	Anthos	51.6	46.802	G3	x		x	x, y
RO3	Anthos	51.961	46.829	G2				
SCF	S.Busoms	47.722	46.347	G1	x	x	x	x
SFG9	S.Busoms	50.207	46.255	G3	x	x	x	x, y
T1	S.Busoms	49.412	46.184	G3				
T10	S.Busoms	49.385	46.183	G3				
T11	S.Busoms	49.217	46.189	G3	x		x	x, y
T12	S.Busoms	49.438	46.184	G3				
T13	S.Busoms	49.408	46.189	G3		x	x	x
T2	S.Busoms	49.411	46.184	G3				
T3	S.Busoms	49.412	46.184	G3				
T5	K.Bomblies	49.393	46.198	G3				
T6	K.Bomblies	49.393	46.198	G3	x	x	x	x, y
T7	K.Bomblies	49.399	46.196	G3				
T-ONI	J.Terés	49.412	46.184	G3				
V1	Anthos	51.247	46.862	G2	x		x	x, y
V2	S.Busoms	51.243	46.861	G2				
V3	S.Busoms	51.182	46.862	G2		x	x	x, y
V4	Anthos	51.071	46.873	G2				

Supplementary F2

Annex table 2: The table indicates soil from native Catalonia *A. thaliana* population Pearson correlation. Blue colors indicate positive correlation while red color negative correlation. Only, the colored numbers are statistically significant p-value >0.05

	Distance to sea (m)	pH	% CaCO3	WHC (mL/g)	Organic Matter (%)	Sulphate (mg/g)	Chloride	Na (mg/g)	K (mg/g)
Distance to sea (m)	1.000	0.904	0.988	0.915	0.742	-0.949	-0.962	-0.932	0.894
pH	0.904	1.000	0.877	0.783	0.771	-0.854	-0.845	-0.831	0.726
% CaCO3	0.988	0.877	1.000	0.959	0.721	-0.956	-0.979	-0.928	0.896
WHC (mL/g)	0.915	0.783	0.959	1.000	0.697	-0.944	-0.966	-0.906	0.854
Organic Matter (%)	0.742	0.771	0.721	0.697	1.000	-0.820	-0.742	-0.881	0.530
Sulphate (mg/g)	-0.949	-0.854	-0.956	-0.944	-0.820	1.000	0.965	0.952	-0.876
Chloride	-0.962	-0.845	-0.979	-0.966	-0.742	0.965	1.000	0.933	-0.882
Na (mg/g)	-0.932	-0.831	-0.928	-0.906	-0.881	0.952	0.933	1.000	-0.777
K (mg/g)	0.894	0.726	0.896	0.854	0.530	-0.876	-0.882	-0.777	1.000
Ca (mg/g)	-0.402	-0.408	-0.333	-0.229	-0.485	0.357	0.320	0.444	-0.399
Mg (mg/g)	-0.886	-0.793	-0.895	-0.871	-0.687	0.900	0.837	0.838	-0.808
P (mg/g)	0.701	0.669	0.651	0.509	0.557	-0.621	-0.619	-0.621	0.713
S (mg/g)	-0.039	-0.073	-0.046	0.005	-0.282	0.057	0.009	0.102	0.052
Mn (mg/g)	-0.171	0.054	-0.157	-0.125	0.022	0.119	0.210	0.175	-0.149
Fe (mg/g)	0.639	0.632	0.586	0.524	0.882	-0.703	-0.616	-0.743	0.509
Co (mg/g)	0.284	0.239	0.292	0.331	0.042	-0.309	-0.229	-0.196	0.457
Ni (mg/g)	0.712	0.735	0.660	0.532	0.753	-0.703	-0.601	-0.700	0.672
Cu (mg/g)	0.264	0.348	0.215	0.107	0.508	-0.334	-0.175	-0.316	0.296
Zn (mg/g)	0.696	0.741	0.641	0.500	0.786	-0.688	-0.609	-0.694	0.619
Mo (mg/g)	-0.341	-0.265	-0.394	-0.448	0.007	0.308	0.390	0.234	-0.310

	Ca (mg/g)	Mg (mg/g)	P (mg/g)	S (mg/g)	Mn (mg/g)	Fe (mg/g)	Co (mg/g)	Ni (mg/g)	Cu (mg/g)	Zn (mg/g)	Mo (mg/g)
Distance to sea (m)	-0.402	-0.886	0.701	-0.039	-0.171	0.639	0.284	0.712	0.264	0.696	-0.341
pH	-0.408	-0.793	0.669	-0.073	0.054	0.632	0.239	0.735	0.348	0.741	-0.265
% CaCO3	-0.333	-0.895	0.651	-0.046	-0.157	0.586	0.292	0.660	0.215	0.641	-0.394
WHC (mL/g)	-0.229	-0.871	0.509	0.005	-0.125	0.524	0.331	0.532	0.107	0.500	-0.448
Organic Matter (%)	-0.485	-0.687	0.557	-0.282	0.022	0.882	0.042	0.753	0.508	0.786	0.007
Sulphate (mg/g)	0.357	0.900	-0.621	0.057	0.119	-0.703	-0.309	-0.703	-0.334	-0.688	0.308
Chloride	0.320	0.837	-0.619	0.009	0.210	-0.616	-0.229	-0.601	-0.175	-0.609	0.390
Na (mg/g)	0.444	0.838	-0.621	0.102	0.175	-0.743	-0.196	-0.700	-0.316	-0.694	0.234
K (mg/g)	-0.399	-0.808	0.713	0.052	-0.149	0.509	0.457	0.672	0.296	0.619	-0.310
Ca (mg/g)	1.000	0.207	-0.726	-0.190	0.140	-0.536	0.013	-0.651	-0.614	-0.704	-0.220
Mg (mg/g)	0.207	1.000	-0.538	0.039	0.005	-0.580	-0.425	-0.674	-0.323	-0.598	0.421
P (mg/g)	-0.726	-0.538	1.000	-0.024	-0.181	0.676	0.086	0.842	0.591	0.817	0.070
S (mg/g)	-0.190	0.039	-0.024	1.000	-0.257	-0.238	0.080	-0.283	-0.272	-0.266	-0.402
Mn (mg/g)	0.140	0.005	-0.181	-0.257	1.000	-0.157	0.474	0.101	0.296	0.107	0.059
Fe (mg/g)	-0.536	-0.580	0.676	-0.238	-0.157	1.000	-0.032	0.797	0.535	0.762	0.173
Co (mg/g)	0.013	-0.425	0.086	0.080	0.474	-0.032	1.000	0.283	0.111	0.087	-0.139
Ni (mg/g)	-0.651	-0.674	0.842	-0.283	0.101	0.797	0.283	1.000	0.772	0.924	0.153
Cu (mg/g)	-0.614	-0.323	0.591	-0.272	0.296	0.535	0.111	0.772	1.000	0.832	0.271
Zn (mg/g)	-0.704	-0.598	0.817	-0.266	0.107	0.762	0.087	0.924	0.832	1.000	0.098
Mo (mg/g)	-0.220	0.421	0.070	-0.402	0.059	0.173	-0.139	0.153	0.271	0.098	1.000

Annex table 3: Available nutrient fraction from soils used as a common garden in field experiments 2017-2018. Statistical descriptors, ANOVA and Tukey test. The table indicates nutrient, common garden soil (AE alkaline saline; BLA saline; and LP calcareous soil), mean (ug/g), Std Dev (standard deviation), Std Error (standard error), N (replicate number), DF (degree freedom), F value and Prob> F from ANOVA test and Tukey test.

Nutrient	Soil	Mean (µg/g)	Std Dev	Std Error	N	DF	F value	Prob > F	Tukey test
	AE	275.66	38.65	12.22	10	2	12.69	0.0001	B
Ca	BLA	246.62	51.23	16.2	10				B
	LP	353.84	56.15	17.75	10				A
	AE	0.36	0.1	0.03	10	2	3.83	0.0344	A
Co	BLA	0.27	0.08	0.03	10				AB
	LP	0.26	0.07	0.02	10				B
	AE	2.41	0.1	0.05	10	2	99.85	<.0001	B
Cu	BLA	8.26	0.76	0.54	10				A
	LP	2.1	0.67	0.22	10				B
	AE	52.05	14.21	4.49	5	2	16.17	<.0001	B
Fe	BLA	77.97	18.86	5.96	10				A
	LP	40.85	10.73	3.39	10				B
	AE	98.83	28.29	8.95	10	2	22.96	<.0001	A
K	BLA	35.29	11.68	3.69	10				C
	LP	59.96	20.11	6.36	10				B
	AE	126.76	32.23	10.19	10	2	74.58	<.0001	A
Mg	BLA	30.17	8.56	2.71	7				B
	LP	33.9	9.62	3.04	10				B
	AE	62.36	18.28	5.78	10	2	20.06	<.0001	A
Mn	BLA	44.78	13.76	4.35	10				B
	LP	23.28	7.03	2.22	10				C
	AE	0.04	0.01	0.003	2	2	8.54	0.0013	A
Mo	BLA	0.03	0.01	0.002	10				B
	LP	0.02	0.01	0.003	10				B
	AE	166.54	42.71	13.51	10	2	15.02	<.0001	A
Na	BLA	124.53	35.85	11.34	10				B
	LP	79.15	26.56	8.4	10				C
	AE	42.2	12.53	3.96	10	2	23.02	<.0001	A
P	BLA	10.39	5.44	2.05	10				B
	LP	40.28	10.49	3.32	10				A
	AE	52.52	13.98	4.42	10	2	29.49	<.0001	A
S	BLA	20.68	7.21	2.28	10				B
	LP	25.53	7.21	2.28	10				B
	AE	5.42	1.95	0.62	9	2	5.91	0.0074	B
Zn	BLA	8.31	3.07	0.97	10				A
	LP	4.93	1.92	0.61	10				B

Supplementary F4

Annex table 4: Plant growth in field common garden experiment 2017-2018. Statistical descriptors, ANOVA and Tukey test. The table indicates common garden soil (AE alkaline saline; BLA saline; and LP calcareous soil), plant group (G1_G2_G3) mean (ug/g), Std Dev (standard deviation), Std Error (standard error), N (replicate number), DF (degree freedom), F value and Prob> F from ANOVA test and Tukey test.

Year	Common garden	Plant group	Rosette Mean (mm)	Std dev	Std error	N	F Value	Prob > F	Tukey test
2017	AE	G1	25.94	7.01	1.7	17	31.72	<.0001	B
2017	AE	G2	19.75	6.34	1.59	16			B
2017	AE	G3	16.76	7.74	1.88	17			A
2017	BLA	G1	24	7.47	1.06	50	7.44	0.0016	A
2017	BLA	G2	28.3	8.06	1.33	37			B
2017	BLA	G3	36.84	9.37	1.26	55			B
2017	LP	G1	36.88	12.38	2.12	34	21.19	<.0001	A
2017	LP	G2	23.38	12.08	4.27	8			B
2017	LP	G3	21.67	5.44	0.95	33			B
2018	BLA	G1	21.21	9.31	1.49	39	29.63	<.0001	B
2018	BLA	G2	28.2	6.83	1.53	20			B
2018	BLA	G3	39.32	13.54	1.91	50			A
2018	LP	G1	47.73	9.32	1.47	40	74.53	<.0001	A
2018	LP	G2	30.15	8.43	1.89	20			B
2018	LP	G3	24.88	8.88	1.26	50			C

Annex table 5: Linear correlation Plant growth in field common garden experiment 2017-2018 *vs* its native Na^+ and $CaCO_3$ soil content.

Year	Common garden	x-axis	y-axis	t Value	Prob > t	R^2
2017	AE	%$CaCO_3$	Rosette (mm)	10.55	0.0021	0.18
2017	BLA	%$CaCO_3$	Rosette (mm)	53	<.0001	0.28
2017	LP	%$CaCO_3$	Rosette (mm)	65.3	<.0001	0.47
2018	BLA	%$CaCO_3$	Rosette (mm)	41.07	<.0001	0.28
2018	LP	%$CaCO_3$	Rosette (mm)	89.3	<.0001	0.45

Year	Common garden	x-axis	y-axis	t Value	Prob > F	R^2
2017	AE	Na mg/g	Siliqua number	11.39	0.0015	0.19
2017	BLA	Na mg/g	Siliqua number	59.27	<.0001	0.3
2017	LP	Na mg/g	Siliqua number	33.21	<.0001	0.31
2018	BLA	Na mg/g	Siliqua number	41.07	<.0001	0.43
2018	LP	Na mg/g	Siliqua number	89.3	<.0001	0.43

Supplementary F5

Annex table 6: Plant siliques in field common garden experiment 2017 2018. Statistical descriptors, ANOVA and Tukey test. The table indicates common garden soil (AE alkaline saline; BLA saline; and LP calcareous soil), plant group (G1_G2_G3) mean (ug/g), Std Dev (standard deviation), Std Error (standard error), N (replicate number), DF (degree freedom), F value and Prob> F from ANOVA test and Tukey test.

Year	Common garden	Origen2	Mean (siliques)	Std Dev	Std Error	N	F Value	Prob > F	Letter
2017	AE	Group 1	26.136	18.3829	3.9192	33	15.6768	<.0001	A
	AE	Group 2	37.346	27.9169	5.47496	22			A
	AE	Group 3	34.875	21.0884	4.30466	32			A
	BLA	Group 1	28.212	21.2702	3.70267	19			B
	BLA	Group 2	34.894	21.2809	4.88217	26			B
	BLA	Group 3	91.235	51.331	12.449	5			A
	LP	Group 1	81.815	44.977	7.95	17			B
	LP	Group 2	47.4	22.589	10.102	24			A
	LP	Group 3	29.593	18.359	3.245	32			A
2018	AE	Group 1	0			26	73.9349	<.0001	
	AE	Group 2	0			1			
	AE	Group 3	0			82			
	BLA	Group 1	63.5	17.971	3.524	17			B
	BLA	Group 2	78.941	21.396	5.189	1			B
	BLA	Group 3	148.438	40.865	5.108	48			A
	LP	Group 1	165.939	48.102	5.312	64			A
	LP	Group 2	81.5	29.3641	4.238	1			B
	LP	Group 3	63.894	31.7674	2.6658	142			B

Annex table 7: Linear correlation plant siliques production in field common garden experiment 2017-2018 *vs* its native Na^+ and $CaCO_3$ soil content. Table indicates x-axis and y-axis, t-Student and correlation (R^2)

Year	Common garden	x-axis	y-axis	t Value	Prob > t	R^2
2017	AE	%$CaCO_3$	Silique number	1.7611	0.1888	0.025
2017	BLA	%$CaCO_3$	Silique number	27.966	<.0001	0.294
2017	LP	%$CaCO_3$	Silique number	72.429	<.0001	0.519
2018	BLA	%$CaCO_3$	Silique number	111.887	<.0001	0.51
2018	LP	%$CaCO_3$	Silique number	313.955	<.0001	0.5538

Year	Common garden	x-axis	y-axis	t Value	Prob > F	R^2
2017	AE	Na mg/g	Silique number	1.33	0.2528	
2017	BLA	Na mg/g	Silique number	72.028	<.0001	0.518
2017	LP	Na mg/g	Silique number	37.575	<.0001	0.359
2018	BLA	Na mg/g	Silique number	169.652	<.0001	0.618
2018	LP	Na mg/g	Silique number	171.924	<.0001	0.389

Supplementary F6

Annex table 8: Available nutrient fraction from selected soils used in greenhouse experiment 2020. Statistical descriptors and T-test. The table indicates common garden soil (ESC alkaline-saline; BLA saline), mean (ug/g), Std Error (standard error), N (replicate number), T value and Prob> T from the T-test.

	BLA		ESC				
Nutrient (µg/g Soil)	**Mean (µg/g Soil)**	**Std Error**	**Mean (µg/g Soil)**	**Std Error**	**N**	**T ratio**	**Prob>T**
Na	107	32.1	92.28	31.59	6	0.33	0.75
K	63	3.98	63.23	2.5	6	-0.05	0.96
Ca	414	10.87	395.91	13.4	6	1.05	0.32
Mg	62.29	2.81	27.02	4.4	6	6.75	<.0001
P	25.3	5.49	6.01	0.44	6	3.5	0.01
S	21.85	5.52	15.98	5.02	6	0.79	0.45
Fe	27.43	1.48	12.12	0.84	6	9	<.0001
Mn	21.33	4.2	9.65	0.38	6	2.77	0.02
Mo	0.02	0	0.02	0.01	6	0.81	0.44
Zn	6.26	0.29	0.92	0.06	6	18.25	<.0001

Supplementary F7

Annex table 9: Plant growth in greenhouse experiment 2020. Statistical descriptors, ANOVA and Tukey test. The table indicates common garden soil (ESC alkaline saline; BLA saline), plant group (G1_G2_G3) mean (ug/g), Std Dev (standard deviation), Std Error (standard error), N (replicate number), F value and Prob> F from ANOVA test and Tukey test.

Soil	Plant group	Mean Area cm^2	Std Dev	Std Error	N	F value	Prob > F	Tukey test
BLA	Group 1	4.72	2.28	0.26	75	16.9049	<.0001	B
BLA	Group 2	4.88	2.51	0.29	75			B
BLA	Group 3	7.1	4.13	0.36	135			A
ESC	Group 1	6.78	5.17	0.6	75	55.5851	<.0001	B
ESC	Group 2	9.64	5.36	0.62	75			A
ESC	Group 3	2.94	3.52	0.3	134			C

Annex table 10: Siliques in greenhouse experiments 2020. Statistical descriptors, ANOVA and Tukey test. The table indicates common garden soil (ESC alkaline-saline; BLA saline), plant group (G1_G2_G3) mean (ug/g), Std Dev (standard deviation), Std Error (standard error), N (replicate number), DF (degree freedom), F-value and Prob> F from ANOVA test and Tukey test.

Common garden	Plant group	Mean Siliques	Std dev	Std Error	N	F value	Prob > F	Tukey test
BLA	Group 1	81.63	19.18	2.33	68	146.642	<.0001	C
BLA	Group 2	93.14	28.53	3.32	74			B
BLA	Group 3	138.89	25.59	2.24	131			A
ESC	Group 1	77.25	27.43	3.23	72	5.7212	0.0037	AB
ESC	Group 2	89.44	30.59	3.58	73			A
ESC	Group 3	74.05	34	2.98	130			B

Annex table 11: Linear correlation plant area in greenhouse experiment 2020 *vs* its native Na^+ and $CaCO_3$ soil content. Table indicates x-axis and y-axis, t-Student

Soil	x-axis	y-axis	t value	Prob > t
BLA	%$CaCO_3$	Area	31.26	<.0001
ESC	%$CaCO_3$	Area	38.57	<.0001

Soil	x-axis	y-axis	t value	Prob > t
BLA	Na mg/g	Area	21.73	<.0001
ESC	Na mg/g	Area	73.48	<.0001

Annex table 12: Linear correlation plant siliques production in greenhouse experiment 2020 *vs* its native Na^+ and $CaCO_3$ soil content.

Soil	x-axis	y-axis	t value	Prob > t
BLA	%CaCO3	Siliques	-16.51	<.0001
ESC	%CaCO3	Siliques	0.54	0.5885

Soil	x-axis	y-axis	t value	Prob > t
BLA	Na mg/g	Siliques	12.57	<.0001
ESC	Na mg/g	Siliques	-3.38	0.0008

Annex table 13: Available soil fraction analysis from BLA *vs* ESC1. The table indicates common garden soil (ESC alkaline saline; BLA saline), mean (ug/g), Std Error (standard error), N (replicate number), t value and Prob> t from the t-test.

	BLA			ESC				
Soil Nutrient	**Mean µg/g**	**Std Error**	**N**	**Mean µg/g**	**Std Error**	**N**	**value t**	**Prob > |t|**
S	9027.17	152.77	105	10160	721.43	89	-1.66	0.0995
Mg	4880.36	92.91	105	4082.13	321.79	89	2.55	0.0115
Zn	165.82	27.85	105	145.1	10.69	88	0.65	0.5175
Mn	85.98	1.7	103	83.37	6.31	83	0.44	0.6619
B	61.78	10.05	94	56.03	4.2	82	0.5	0.6164
Ca	37843.6	702.19	105	50075.55	3729.41	89	-3.48	0.0006
K	27839.16	543.64	105	35432.42	3661.24	89	-2.22	0.0276
Na	14116.52	522.99	105	13360.6	840.6	89	0.79	0.4319
Mo	103.06	11.72	42	107.75	24.4	32	-0.19	0.8525
Fe	406.06	42.77	105	375.91	58.94	89	0.42	0.6734
Co	42.78	8.24	45	45.85	7.12	57	-0.28	0.7775
P	7362.84	152.74	105	4031.56	365.64	85	9	<.0001

Annex table 14: Leaf nutrient mineral content from plants grow in greenhouse 2020. The table indicates common garden soil (AE alkaline saline; BLA saline) plant group (G1_G2_G3) mean (ug/g), Std Dev (standard deviation), Std Error (standard error), N (replicate number), DF (degree freedom), F value and Prob> F from ANOVA test and Tukey test.

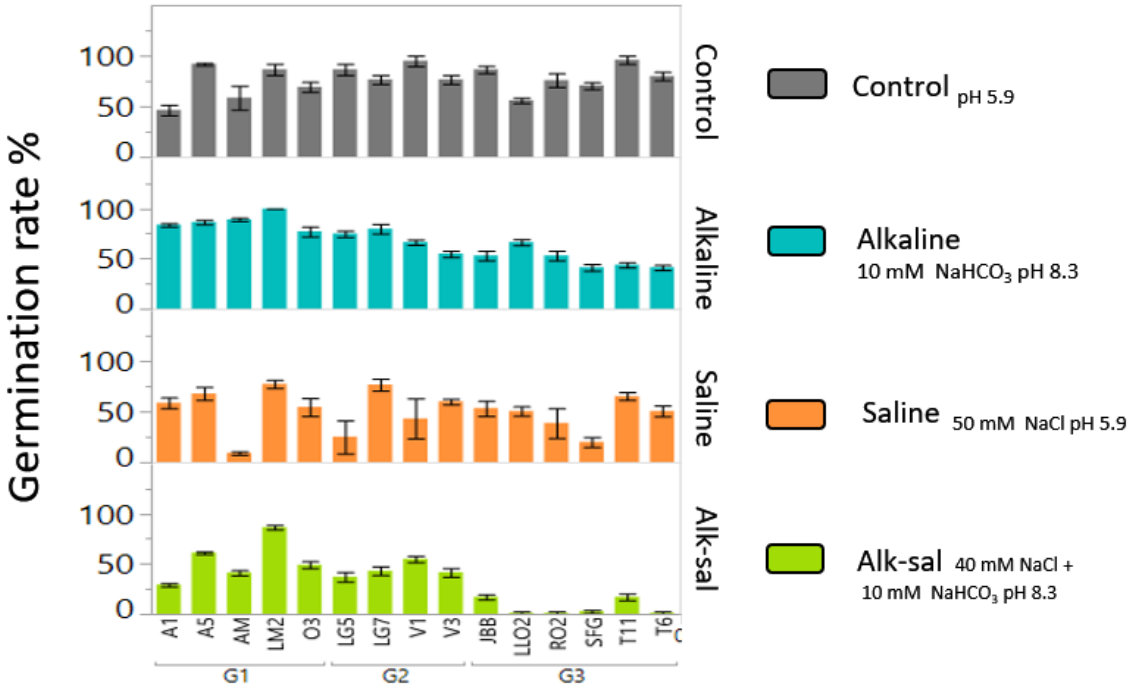
Soil	Plant group	Nutrient μg/g	Mean	Std Dev	Std Error	N	F value	Prob > F	Tukey test
BLA	Group 1	B	49.22	18.43	3.69	25	3.6196	0.0306	AB
BLA	Group 2	B	105.13	184.08	37.57	24			A
BLA	Group 3	B	43.7	28.71	4.19	47			B
BLA	Group 1	Ca	35881.23	3088.58	617.72	25	1.5774	0.2115	A
BLA	Group 2	Ca	37473.3	7428.45	1485.69	25			A
BLA	Group 3	Ca	38903.91	8243.38	1111.54	55			A
BLA	Group 1	Co	14.51	11.7	4.42	7	1.1213	0.3352	A
BLA	Group 2	Co	52.22	63.29	17.55	13			A
BLA	Group 3	Co	44.02	56.77	11.13	26			A
BLA	Group 1	Fe	336.2	137.28	27.46	25	2.9635	0.0561	A
BLA	Group 2	Fe	588.4	813.04	162.61	25			A
BLA	Group 3	Fe	354.93	217.78	29.37	55			A
BLA	Group 1	K	26539.88	5072.2	1014.44	25	8.3839	0.0004	B
BLA	Group 2	K	24927.54	6159.52	1231.9	25			B
BLA	Group 3	K	29753.21	4799.07	647.11	55			A
BLA	Group 1	Mg	4761.46	565.82	113.16	25	0.4432	0.6432	A
BLA	Group 2	Mg	5015.39	1351.77	270.35	25			A
BLA	Group 3	Mg	4873.03	881.04	118.8	55			A
BLA	Group 1	Mg/ca	0.13	0.01	0	25	0.8291	0.4393	A
BLA	Group 2	Mg/ca	0.13	0.02	0	25			A
BLA	Group 3	Mg/ca	0.13	0.02	0	55			A
BLA	Group 1	Mn	89.11	9.07	1.81	25	0.6755	0.5112	A
BLA	Group 2	Mn	86.5	30.72	6.27	24			A
BLA	Group 3	Mn	84.31	10.74	1.46	54			A
BLA	Group 1	Mo	125.76	78.78	21.85	13	0.8489	0.4356	A
BLA	Group 2	Mo	90.43	68.07	18.19	14			A
BLA	Group 3	Mo	95.17	81.05	20.93	15			A
BLA	Group 1	Na	12491.88	3457.55	691.51	25	2.9692	0.0558	B
BLA	Group 2	Na	16086.16	6880.63	1376.13	25			A
BLA	Group 3	Na	13959.7	5090.66	686.43	55			A
BLA	Group 1	Na/K	0.5	0.21	0.04	25	6.2517	0.0027	B
BLA	Group 2	Na/K	0.72	0.43	0.09	25			A
BLA	Group 3	Na/K	0.49	0.23	0.03	55			B
BLA	Group 1	P	7863.83	1601.59	320.32	25	1.8057	0.1696	A

Soil	Plant group	Nutrient µg/g	Mean	Std Dev	Std Error	N	F value	Prob > F	Tukey test
BLA	Group 2	P	7320.73	1411.77	282.35	25			A
BLA	Group 3	P	7154.26	1590.84	214.51	55			A
BLA	Group 1	Pb	475.66	319.51	66.62	23	1.5134	0.228	A
BLA	Group 2	Pb	326.46	186.95	49.97	14			A
BLA	Group 3	Pb	529.56	441.3	81.95	29			A
BLA	Group 1	S	8802.42	1333.27	266.65	25	0.4953	0.6109	A
BLA	Group 2	S	9245.17	1877.06	375.41	25			A
BLA	Group 3	S	9030.23	1522.67	205.32	55			A
BLA	Group 1	Zn	130.13	28.94	5.79	25	2.1292	0.1242	A
BLA	Group 2	Zn	267.44	576.92	115.38	25			A
BLA	Group 3	Zn	135.86	47.44	6.4	55			A
ESC	Group 1	B	47.23	30.56	6.11	25	2.6078	0.08	A
ESC	Group 2	B	49.52	24.02	4.9	24			A
ESC	Group 3	B	67.44	48.11	8.38	33			A
ESC	Group 1	Ca	52282.86	26990.49	5398.1	25	3.1269	0.0489	AB
ESC	Group 2	Ca	35951.99	14600.13	2920.03	25			B
ESC	Group 3	Ca	57714.17	45580.35	7298.7	39			A
ESC	Group 1	Co	28.28	16.86	5.33	10	2.222	0.1179	A
ESC	Group 2	Co	29.69	22.75	5.36	18			A
ESC	Group 3	Co	57.95	68.86	12.37	31			A
ESC	Group 1	Fe	247.95	150.62	30.12	25	6.6	0.0022	B
ESC	Group 2	Fe	153.17	77.17	15.43	25			B
ESC	Group 3	Fe	600.72	776.32	124.31	39			A
ESC	Group 1	K	39539.22	23417.73	4683.55	25	1.234	0.2962	A
ESC	Group 2	K	26272.04	11213.58	2242.72	25			A
ESC	Group 3	K	38671.9	47538.81	7612.3	39			A
ESC	Group 1	Mg	4265.91	1886.89	377.38	25			AB
ESC	Group 2	Mg	2900.85	1189.63	237.93	25	2.9264	0.0589	B
ESC	Group 3	Mg	4721.55	4103.56	657.09	39			A
ESC	Group 1	Mg/ca	0.08	0.01	0	25			A
ESC	Group 2	Mg/ca	0.08	0.01	0	25	0.999	0.3725	A
ESC	Group 3	Mg/ca	0.08	0.01	0	39			A
ESC	Group 1	Mn	86.84	46.37	9.27	25			A
ESC	Group 2	Mn	62.4	14.59	3.18	21	1.7254	0.1846	A
ESC	Group 3	Mn	90.48	75.52	12.25	38			A
ESC	Group 1	Mo	82.7	58.28	17.57	11			A
ESC	Group 2	Mo	93.8	100.85	50.42	4	0.3557	0.7037	A
ESC	Group 3	Mo	127.25	178.88	43.38	17			A
ESC	Group 1	Na	12610.66	7422.35	1484.47	25			AB
ESC	Group 2	Na	10273.36	4370.55	874.11	25	4.1612	0.0188	B

Soil	Plant group	Nutrient μg/g	Mean	Std Dev	Std Error	N	F value	Prob > F	Tukey test
ESC	Group 3	Na	15820.33	9269.67	1484.34	39			A
ESC	Group 1	Na/K	0.35	0.19	0.04	25			B
ESC	Group 2	Na/K	0.44	0.17	0.03	25	4.5866	0.0128	AB
ESC	Group 3	Na/K	0.53	0.28	0.05	39			A
ESC	Group 1	P	4339.64	2358.08	471.62	25			A
ESC	Group 2	P	3496.84	1463.78	292.76	25	0.4533	0.6371	A
ESC	Group 3	P	4193.43	4725.6	798.77	35			A
ESC	Group 1	Pb	449.38	330.28	67.42	24			A
ESC	Group 2	Pb	313.43	279.67	67.83	17	1.0738	0.3483	A
ESC	Group 3	Pb	468.93	417.83	91.18	21			A
ESC	Group 1	S	10305.29	5438.72	1087.74	25			AB
ESC	Group 2	S	7286.8	2479.3	495.86	25	3.74	0.0277	B
ESC	Group 3	S	11908.67	8724.78	1397.08	39			A
ESC	Group 1	Zn	152.37	90.5	18.1	25			AB
ESC	Group 2	Zn	99.26	44.35	8.87	25			B
ESC	Group 3	Zn	166.1	123.71	19.81	39	3.6896	0.029	A

Supplementary F8

Annex Figure 15: Germination rate % bar plot separated by treatment, deme plant group. As a treatment: control conditions pH 5.9; alkaline 10mM $NaHCO_3$ pH 8.3; neutral salinity 50mM NaCl pH 5.9 and alkaline saline 40 mM NaCl + 10 mM $NaHCO_3$ pH 8.3. As a plant group: G1_Plant tolerant to carbonate; G2 Plants intermediate tolerance; G3 Plants tolerate to salinity.



Annex Figure 16: The table indicates plate treatment and plant group (G1_G2_G3). As a treatment: control conditions pH 5.9; alkaline 10 mM $NaHCO_3$ pH 8.3; neutral salinity 50 mM NaCl pH 5.9 and alkaline saline 40 mM NaCl + 10 mM $NaHCO_3$ pH 8.3. As a plant group: G1_Plant tolerant to carbonate; G2 Plants intermediate tolerance; G3 Plants tolerate to salinity. Germination means (%), Std Dev (standard deviation), Std Error (standard error), N (replicate number), DF (degree freedom), F value and Prob> F from ANOVA test and Tukey test.

Treatment	Plant group	Mean Germination(%)	Std Dev	Std Error	N	F value	Prob > F	Tukey
Control	G1	70.67	22.03	4.41	25	2.838	0.0655	A
Control	G2	83.75	11.67	2.92	16			A
Control	G3	77.56	15.51	2.83	30			A
Alkaline	G1	87.47	9.29	1.86	25	80.746	<.0001	A
Alkaline	G2	69.17	11.64	2.91	16			B
Alkaline	G3	50	11.71	2.14	30			C
Saline	G1	53.6	26.7	5.34	25	0.55	0.5757	A
Saline	G2	51.25	30.76	7.69	16			A
Saline	G3	46.44	21.33	3.89	30			A
Alk-Sal	G1	53.6	20.5	4.1	25	81.04	<.0001	A
Alk-Sal	G2	43.92	10.56	2.56	17			A
Alk-Sal	G3	6.89	8.66	1.58	30			B

Annex table 17: Linear correlation germination *vs* its native Na^+ and $CaCO_3$ soil content. Table indicates x-axis and y-axis, t-Student and correlation (R^2). As a treatment: control conditions (Hoagland ½ pH 5.9); alkaline-saline (Hoagland ½ 40 mM NaCl + 10 mM $NaHCO_3$ pH 8.3) and (Hoagland ½ 60 mM NaCl + 15 mM $NaHCO_3$ pH 8.3)

Treatment	x	Y	F Value	Prob > F
Control	%$CaCO_3$	Germination %	2.991	0.0882
Saline	%$CaCO_3$	Germination %	1.829	0.1806
Alkaline	%$CaCO_3$	Germination %	201.203	<.0001
Alkaline-Saline	%$CaCO_3$	Germination %	96.540	<.0001

Treatment	X	Y	F Value	Prob > F
Control	Na ppm	Germination %	5.224	0.0253
Saline	Na ppm	Germination %	0.183	0.6704
Alkaline	Na ppm	Germination %	60.234	<.0001
Alkaline-Saline	Na ppm	Germination %	49.545	<.0001

Annex table 18: Leave diameter and root length in hydroponic conditions. The table indicates treatment and plant group . As a treatment: control conditions (Hoagland ½ pH 5.9); alkaline-saline (Hoagland ½ 40 mM NaCl + 10 mM $NaHCO_3$ pH 8.3) and (Hoagland ½ 60 mM NaCl + 15 mM $NaHCO_3$ pH 8.3). As a plant group: G1 Plant tolerant to carbonate; G2 Plants intermediate tolerance; G3 Plants tolerance to salinity. Leave diameter or root length mean, Std Dev (standard deviation), Std Error (standard error), N (replicate number), DF (degree freedom), F value and Prob> F from ANOVA test and Tukey test.

Organ	Treatment	Plant group	Mean (cm)	Std Dev	Std Error	N	F value	Prob > F	Tukey
Leave	Control	G1	6.71	1.48	0.33	20	23.9075	<.0001	A
Leave	40+10	G1	5.15	1.51	0.32	22			B
Leave	60+15	G1	3.49	1.07	0.27	16			C
Leave	Control	G2	7.26	1.26	0.27	21	20.9509	<.0001	A
Leave	40+10	G2	7.05	1.28	0.31	17			A
Leave	60+15	G2	4.25	1.28	0.41	10			B
Leave	Control	G3	7.39	2.37	0.53	20	25.9204	<.0001	A
Leave	40+10	G3	5.91	1.54	0.38	16			B
Leave	60+15	G3	3.68	1.09	0.22	24			C
Root	Control	G1	11.66	3.38	0.75	20	20.2695	<.0001	A
Root	40+10	G1	6.89	2.41	0.5	23			B
Root	60+15	G1	7.01	2.03	0.51	16			B
Root	Control	G2	11.41	2.81	0.61	21	20.221	<.0001	A
Root	40+10	G2	9.88	0.99	0.24	17			A
Root	60+15	G2	5.99	1.84	0.61	9			B
Root	Control	G3	11.73	3.4	0.76	20	25.5269	<.0001	A
Root	40+10	G3	7.34	2.11	0.53	16			B
Root	60+15	G3	6.73	1.64	0.33	25			B