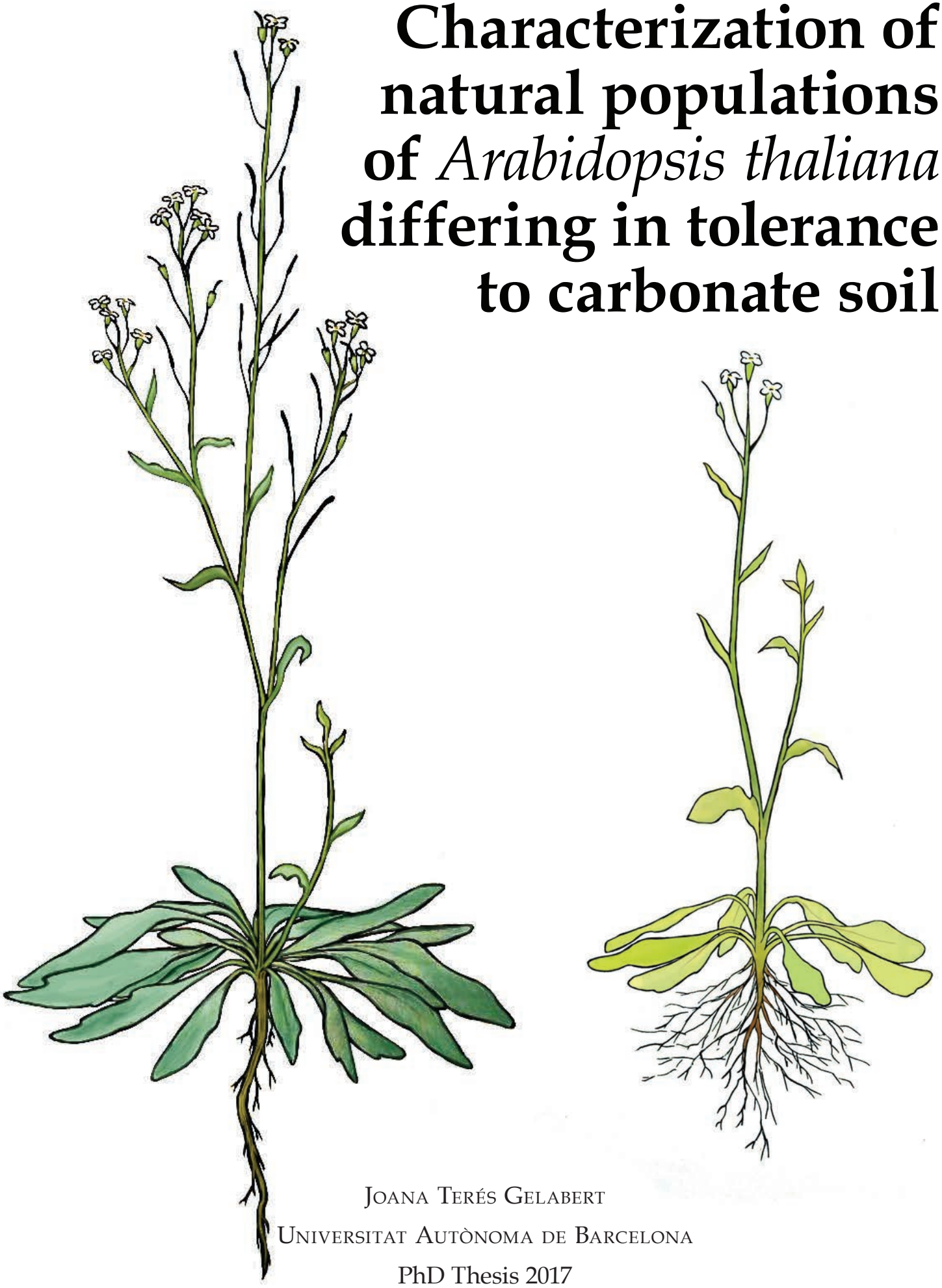
Universitat Autònoma de Barcelona

Characterization of natural populations of Arabidopsis thaliana differing in tolerance to carbonate soil

joana Terés Gelabert

Characterization of natural populations of *Arabidopsis thaliana* differing in tolerance to carbonate soil



Joana Terés Gelabert

Universitat Autònoma de Barcelona

PhD Thesis 2017

An expert is a man who has made all the mistakes which can be made, in a narrow field.

Niels Bohr, 1930

Agraïments

En primer lloc voldria agrair a les meves directores de Tesis Charlotte Poschenrieder, Roser Tolrà i al director David Salt, la oportunitat de fer un doctorat a la unitat de fisiologia vegetal.

Infinites gràcies als tots els companys del departament i la secretaria del BABVE, a la Sole per tants consells acadèmics i morals, a la Maite per l'excel·lent companyia, a la Rosa per les xarrades de primera hora, en especial a la Laura i a la Laia que m'han ajudat sense condicions en tot, i que entre cafè i cafè han fet que el darrer tram d'aquesta etapa fos menys feixuc, al Miquel pel seu bon humor constant i a la Silvia, que des del principi fins al final m'ha fet costat, m'ha acollit a casa i ha suportat amb molta paciència els meus micro-drames.

També vull donar les gracies als companys dels laboratoris de fora, especialment al XinYuan per la seva professionalitat i l'Edu, per la seva ajuda al lab i per obrir-me les portes de casa seva, a l'Adrián i l'Ana per fer que la meva estada a Zaragoza fos 100% profitosa tant a nivell acadèmic com gastronòmic, a la Cata i en Jaume per facilitar-me sòl de Mallorca i els anàlisis corresponents i a la Imelda per crear una convivència tan agradable a Aberdeen.

Gràcies a la Fiona i a la Simone per les correccions gramaticals i preparar-me per la presentació. I a la Laura per tota la composició artística que vesteix aquesta tesis.

M'agradaria donar les gràcies a la família, al meu pare per tota la ajuda moral i tecnològica aportada des de sempre i la seva implicació en la redacció d'aquesta tesis i al Mamut per cuidar-me infinit en tots els aspectes. A la Júlia per no dubtar quan li he demanat ajuda i fer-me riure tantíssimes vegades. A la padrina i a la iaia per les seves trucades de "i això quant s'acaba?". Al iaio i al padrí també els hauria agradat veure'm arribar fins aquí.

Estic molt agraïda al Toni, per ser-hi sempre, i seguir la tesis amb màxima implicació d'inici a final. Per ajudar-me quan ho he necessitat i mimar-me quan potser no m'ho mereixia.

Gràcies a la Marta i a la Cèlia, per la seva amistat incondicional, per fer de la nostra casa la millor del món, per escoltar amb ganes les meves presentacions i suportar els meus dies de mal humor.

Als amics i amigues per la seva alegria, per donar-me suport i obligar-me a sortir de casa a fer el vermut, en especial gràcies a la Laura, a la Lara, a l'Anna, al David, a la Neus i al Holger.

A la Charlotte i en Joan, per confiar en mi, molt abans de que jo ho fes, i brindar-me la possibilitat de sentir-me part de la seva família, ajudar-me en tot moment, tenir la porta sempre oberta a més preguntes i encoratjar-me en situacions crítiques, gràcies per fer-me sentir tan estimada.

Per últim, vull esmentar que el doctorat ha estat finançat amb una beca PIF (personal investigador en formació); les estades a l'estranger es va rebre el suport econòmic de la beca de mobilitat de la UAB. Aquest doctorat s'emmarca dins dels projectes MICINN (BFU2013-42839-R i BFU2016-75176-R) sense els quals no hauria estat possible.

Resum

S'han investigat les poblacions naturals d'*Arabidopsis thaliana* de Catalunya per identificar mecanismes d'adaptació local als sòls carbonatats. Després de caracteritzar les propietats químiques dels sòls natius, petits rodals de poblacions biològiques d' *A. thaliana* (que anomenem "demes"), es van realitzar experiments anuals de camp, en parcel·les amb diferents nivells de carbonats al sòl, per avaluar l'aptitud diferencial entre els "demes". La progènie d'aquests demes va mostrar una millor aptitud en el sòl control, sense carbonats. Les diferencies en l'aptitud dels demes per créixer l sòl carbonatat està associada amb el percentatge de $CaCO_3$ dels sòls natius. Aquest fet confirma que el nivell de carbonats al sòl és el factor limitant per a l'adaptació local.

Els demes contrastants A1 (moderadament tolerant) i T6 (sensible) es varen seleccionar per analitzar les característiques fisiològiques. Quan creixen bé en presencia de carbonats o bé amb deficiència de ferro s'observen diferencies en el contingut de clorofil·les, l'activitat de SOD i el perfil de compostos fenòlics exsudats per les arrels.

El deme tolerant A1 presenta l'al·lel *AtFPN2* com el genotip de referència Col.0. Per contra s'ha trobat que el deme sensible T6, presenta la seqüència de l'al·lel com Ts-1. La presencia de l'al·lel dèbil de *AtFPN2* de Ts-1 podria justificar l'elevada acumulació de metalls divalents a la fulla del deme sensible T6.

Creuaments realitzats entre demes tolerants i sensibles revelen l'herència d'aquests trets. En la F3 es van seleccionar famílies tolerants i sensibles per fer l'anàlisi de "bulk-segregation", que encara esta en procés de realització.

Per tal d'identificar gens candidats per a l'adaptació als sòls carbonatats, s'ha utilitzat una col·lecció HAP-MAP provinent de NASK. Aquestes accessions van ser plantades en sol carbonatat i sòl control i es va dur a terme un seguiment del creixement i un anàlisis ionòmic de la part aèria. El *Genome wide association analysis* (*GWAS*) ens ha proporcionat una llista de gens potencialment relacionats amb la tolerància als sòls carbonatats.

En conclusió, els nostres resultats demostren que les plantes d'*A. thaliana* que tenen com a habitat natural sòl amb concentracions moderades o baixes de carbonat són més tolerants als carbonats que les plantes que viuen en sòls sense carbonats. Aquesta "tolerància" és heretable i el GWAS ha revelat múltiples gens candidats a ser els responsables d'aquesta tolerància al sòl carbonat. Els resultats que s'obtindran amb el BSA-seq anàlisis proporcionaran informació útil i addicional per a la identificació dels gens clau involucrats en aquesta tolerància.

Abstract

Natural populations of *Arabidopsis thaliana* from Catalonia were investigated to identify mechanisms of local adaptation to carbonate soils. After characterizing the chemical properties of the native soils of multiple small stands of *A. thaliana* (called "demes"), multi-year common garden experiments, based on contrasting soil carbonate levels, were performed to identify differential fitness among demes. Progenies from these demes performed better on control soil without carbonate. However, fitness differences among demes on carbonate soils were associated with the percentage of $CaCO_3$ in the native soils. This confirms that the soil carbonate level is a driving factor for local adaptation.

Contrasting demes A1 (moderately tolerant) and T6 (sensitive) were selected for analyzing physiological traits. When growing either with carbonate or under iron deficiency both demes differed in chlorophyll content, SOD activity, and the profile of phenolic compounds in root exudates.

Tolerant deme A1 has the *AtFPN2* allele like the reference genotype Col.0. Contrastingly, in sensitive deme T6 the allele sequence is as in Ts-1. The presence of the weak allele of *AtFPN2* of Ts-1 could justify the higher accumulation of divalent metals in the leaf of deme T6.

Crosses between tolerant and sensitive demes revealed heritability of these traits. In F3, tolerant and sensitive families were selected for bulk segregation analysis, which is still under progress.

For further identifying candidate genes for adaptation to carbonate soil, a Hap-Map collection from NASK was used. Different accessions were grown in carbonate and control soils. Growth and shoot ionome was compared to plants growing on non-carbonate soil. Genome wide association analysis (GWAS) provided a list of genes potentially related with plant tolerance to carbonate soils.

In conclusion, our results demonstrate that *A. thaliana* plants naturally adapted to soil with moderate-low carbonate concentrations are more carbonate tolerant than plants from soils without carbonate. This tolerance is inheritable and GWAS revealed multiple candidate genes responsible for tolerance to carbonate soil. BSA-seq results will provide further useful information for the identification of the key genes involved.

Contents

Acronyms

ANOVA	Analysis of variance
BSA	Bulk Segregation Analysis
DNA	Deoxyribonucleic acid
FCR	Ferric Chelate Reductase
GWAS	Genome wide association Study
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
LD	linkage disequilibrium
LP	Les Planes d'Hostoles
O.M.	Organic Matter
PS I/II	Photosystem I/II
QTLs	Quantitative trait locus
QTGs	Quantitative trait genes
ROS	Reactive oxygen species
SDM	Species Distribution Model
SCF	Santa Coloma de Farners
WHC	Water Hold capacity

GLOBAL INTRODUCTION

Work context and Introduction

A major challenge for biologists is to understand the mechanisms, which enable a plant to adapt to its environment and to perform optimally under a range of conditions as broad as possible. A complete understanding is only obtained by the integration of distinct levels of research from ecology, over physiology to the molecular biology and functionality of individual genes (Malcolm, 2001)

In addition, the analysis of natural variation in wild species has begun to elucidate the molecular bases of phenotypic differences related to plant adaptation in distinct natural environments and to determine the ecological and evolutionary processes that maintain this variation (Mitchell-Olds *et al.,* 2007).

Studies on local adaptation in plants are of great value to conservation biologists and climate change researchers (Leimu and Fischer, 2008), and such studies are beneficial in examining how gene flow and other drivers of evolution impact natural selection (Kawecki and Ebert, 2004). In the planning of restoration projects research on local adaptation provides valuable information. Plants used in restoration projects can be more carefully selected if it is known how introduced plants will adapt to a new location. Plants most suitable for restoration are usually collected locally or from areas of similar habitats (McKay *et al.,* 2005).

Some experts believe that adaptation does not always have to be reciprocal; fitness reaction norms do not always have to cross to demonstrate local adaptation (Wright and Station, 2011). Most studies on local adaptation in plants typically use reciprocal transplant experiments in the field and test fitness traits of two or more plant groups transplanted into their home and away sites. Fitness can be estimated with floral, vegetative, and survival measurements.

Flowering time is an important measure because differences in the maturation of reproductive structures can lead to changes in pollination, herbivory, and reproductive success (Levin, 2006). The experiment of Yang *et al.,* 2010 demonstrated that two accessions that differ in

their flowering time surprisingly respond to Fe deficiency in a similar way. In turn, both biotic and abiotic stress may influence the flowering time.

Both drought and salinity are well-known to affect flowering time in Arabidopsis. Drought is an abiotic stress factor that affects many regions of the world. Drought causes an early arrest of floral development and leads to sterility (Su *et al.,* 2013). To ensure survival during drought stress, plants often accelerate the flowering process, and this response is known as 'drought escape' (Sherrard and Maherali, 2006; Franks *et al.,* 2007; Bernal *et al.* 2011; Franks, 2011). The related concept 'drought avoidance' refers to the condition where the plant reduces water loss to prevent dehydration (Kooyers, 2015).

Salinity substantially delays flowering time in Arabidopsis (Kim *et al.,* 2007), and several flowering regulators that mediate this response have been identified. Salt delays flowering in a process dependent on DELLA proteins acting as negative regulators of GA signalling and the plant hormone ethylene (Achard *et al.,* 2006). Salt stress suppresses the expression of *CO* and *FT*, contributing to the delay in flowering (Kim *et al.,* 2007; Li *et al*., 2007). In addition, the salinity-induced delay in flowering time appears to be dependent on the floral repressor BROTHER OF FT AND TFL1 (BFT), as the delay observed in wild-type plants was not evident in *bft* mutants (Ryu *et al.,* 2011).

Biotic stress factors such as attack by pests and pathogens can have a significant effect on plant development, including flowering. In Arabidopsis, pathogen infection alters flowering time in response to infection with the vascular wilt fungal pathogen *Fusarium oxysporum* (Lyons *et al.,* 2015), and the bacterial pathogen *Pseudomonas syringae* (Korves and Bergelson, 2003).

Wallace (1858) recognized that plant adaptation to different soil types is evidence of the strong natural selection imposed by ecological discontinuities. Plants need elements present in the soil. However, both deficiency or toxicity of some of these elements represent a stress for the plant. Most nutrients that plants require for growth and development are supplied as mineral ions to the roots, and they are classified as macronutrients (Ca, K, Mg, N, P, and S) or micronutrients (B, Cl, Fe, Mn, Co, Cu, Mo, Ni, and Zn) depending on the necessary quantities. The composition of mineral nutrients and trace elements (i.e., the inorganic component of an organism) is now referred to as the ionome (Salt *et al.,* 2008). There is substantial natural variation for mineral use efficiency, root uptake, translocation from roots to shoots, and accumulation in the seed as storage and supply for the germinating seedling. This variation has been reported in many species, leading to breeding programs such as those aiming to improve zinc and iron status of cereal grains or tuber crops (www.harvestplus.org).

Detailed analyses of the ionome in *A. thaliana* have shown considerable variation for leaf mineral concentrations under various mineral/metal supply conditions (Salt *et al.,* 2008). QTLs

have been identified for accumulation of different elements (Ca, Cu, Fe, K, Mg, Mn, Na, P, S, and Zn) in seeds, siliques, leaves, and roots under different growth conditions (Vreugdenhil *et al.,* 2004; Waters and Grusak, 2008; Ghandilyan *et al.,* 2009)

In addition, *A. thaliana* QTL analyses have been focused on accumulation of specific minerals, including N (as nitrate; Loudet *et al.,* 2003; Harada *et al.,* 2004), K (Harada and Leigh, 2006), Cu (Kobayashi *et al.,* 2008), Mo (Baxter *et al.,* 2008), and Na (Rus *et al.,* 2006). These studies have led to the isolation of three genes underlying large-effect QTLs, which also encode different mineral transport components. A root copper tolerance locus corresponds to the *HMA5* gene encoding a Cu-transporting ATPase. Several natural alleles differing in missense mutations in conserved motifs show lower activity and Cu translocation to the shoot (Kobayashi *et al.,* 2008). A mitochondrial molybdenum transporter encoded by the nuclear *MOT1* gene underlies shoot Mo concentration QTL. The absence of the *MOT1* promoter region has been associated with low gene expression and low shoot Mo concentration, suggesting that this regulatory mutation is the causal nucleotide polymorphism (Baxter *et al.,* 2008). Finally, *HKT1* encodes a Na^+ transporter for which two loss-of function alleles associated with promoter deletions produce lower root expression and enhanced shoot Na^+ levels in two coastal accessions (Rus *et al.,* 2006; Busoms *et al.,* 2015).

Importantly, the molecular analysis of natural genetic variation has not only led to the correlation of allelic variation of known genes with phenotypic variation, but also to the discovery of novel genes. This identification of genes that account for natural phenotypic variation is and will remain one of the principal goals in this field. However, beyond this goal, the analysis of natural genetic variation also offers an excellent opportunity to overcome the often-perceived dichotomy between molecular and organismal biology (Shindo *et al.,* 2007).

Gene functions involved in plant survival and adaptation can partially be identified by induced mutant analyses of different wild genotypes, where mutants with reduced fitness are easily selected. However, current mutant collections have been obtained using a limited number of laboratory strains, which harbour only a small portion of *A. thaliana* natural variation. Interestingly, Clark *et al.,* (2007) showed that 9.4% of *A. thaliana* protein-coding genes are naturally absent or knocked out in wild accessions, limiting the mutant spectra that can be obtained from each accession. Therefore, natural variation provides a relevant complementary resource to discover novel gene functions, as well as those allelic variants that specifically interact with the genetic background and/or the environment or alleles showing small effects on phenotype, particularly for traits related to plant adaptation (Benfey and Mitchell-Olds, 2008)

Genetic analyses of natural variation in plants are mainly performed by QTL mapping, often called linkage mapping, in which phenotypic variation is associated with allelic variation at molecular markers segregating in experimental mapping populations derived from directed

crosses (Doerge, 2002). Thus, genomic regions accounting for trait variation are located in large physical intervals containing the causal QTLs. Further analyses of these regions, with a combination of functional strategies, allows the final identification of QTGs and nucleotide polymorphisms altering the function of those genes (reviewed in Koornneef *et al.,* 2004; Alonso-Blanco *et al.,* 2005; Weigel and Nordborg, 2005; González-Martínez *et al.,* 2006). Association mapping, which involves looking for phenotype-genotype associations in a general population of individuals whose degree of relatedness or pedigree is unknown, is also becoming more popular and useful in plant systems. Improvements in statistical and analytical tools and in gene sequencing technology are being crucial for progress in this field (see Myles *et al.,* 2009).

The wide geographical and environmental distribution of *A. thaliana*, combined with its small genome and the availability of unprecedented genetic and genome resources, have strongly facilitated the molecular analysis of this species in the last decade.

Arabidopsis thaliana (L.) Heyhn commonly known as wild thale or mouse ear cress, belongs to the mustard family (Brassicaceae, formerly Cruciferae). The genus Arabidopsis comprises nine species and eight subspecies (Al-Shehbaz and O'Kane, 2002). Among them, A. thaliana can be distinguished by morphological characteristics such as fruit and seed shape. The nine species of the genus Arabidopsis are mainly found in Europe. Two species are also found in Asia and North America, but only A. thaliana has a worldwide distribution. In fact, A. thaliana can be found in diverse habitats, for instance in open or disturbed habitats, on sandy soils or on river banks, at sea level or at high altitude (Al-Shehbaz and O'Kane, 2002). The rapid expansion of habitat colonization by A. thaliana implies that this species has a huge capacity to adapt to a wide range of ecological niches.

The high capacity of Arabidopsis to colonize a broad geographical spectrum is probably connected to its life cycle strategy, especially the timing of seed germination and flowering initiation. In Europe, Arabidopsis accessions generally flower in spring and early summer, and the mature seeds are available from May to July, occasionally also in late summer up to early autumn (Lawrence, 1976; Koornneef et al., 2004).

In general, accessions from Southern Europe are either winter- or summer-annual types, whereas most Northern European accessions are typically winter-annual.

It is commonly assumed that Arabidopsis is a completely, or nearly completely, self-fertilizing species, owing to its characteristic flowering morphology, which is typical for inbreeding plants: the flowers are small, lack strong scent and the anthers are positioned close to the stigmata (Charlesworth and Vekemans, 2005). Indeed, the selfing rate in natural environments has been estimated in some studies to be greater than 95 % (Abbott and Gomes, 1989; Charlesworth and Vekemans, 2005; Stenøien et al., 2005). Thus, local Arabidopsis populations are generally regarded to consist of a single inbred sibship. However, despite inbreeding, an

unexpected amount of genetic variation has been found within local populations (Nordborg et al., 2005; Bakker et al., 2006b), suggesting gene flow between populations, which might be facilitated through exchange of pollen rather than by seed dispersal (Bakker et al., 2006b).

Arabidopsis accessions show an extraordinarily wide phenotypic variation. Thus far, significant natural variation has been reported for every phenotypic trait investigated (Koonneef et al., 2004). Some developmental traits, such as flowering time or seed dormancy, have drawn special attention, partly because they are of applied interest to crop breeding, and partly because they are easy to investigate. In addition to visually obvious phenotypes, natural variation has also been observed in genetic mechanisms such as cytosine methylation (Riddle and Richards, 2002). Moreover, assays of metabolite profiles by large-scale unbiased metabolomic methods have uncovered natural variation at the level of small molecules, suggesting that they reflect physiological phenotypes that could be selected in nature (Keurentjes et al., 2006).

Finally, the natural variation resources of Arabidopsis are complemented by the annotated genome sequence, which enables high-density genotyping, and by collections of knockout mutants, which provide a powerful tool to verify the prospective roles of genes involved in natural trait variation by independent means.

Thus, A. thaliana has provided the largest number of genes and nucleotide polymorphisms underlying natural variation of any plant species (Alonso-Blanco et al., 2005). However, the specific ecological niche and life history of A. thaliana limits the plant traits and processes that can be approached in a single species. Therefore, new plant models phylogenetically related to A. thaliana (e.g., Arabidopsis lyrata; Clauss and Koch, 2006) as well as unrelated species, e.g., of the genera Aquilegia (Kramer, 2009), Mimulus (Wu et al., 2007), Ipomoea (Clegg and Durbin, 2003), and Helianthus (Rieseberg et al., 2003), are beginning to be used in studies of natural variation and speciation.

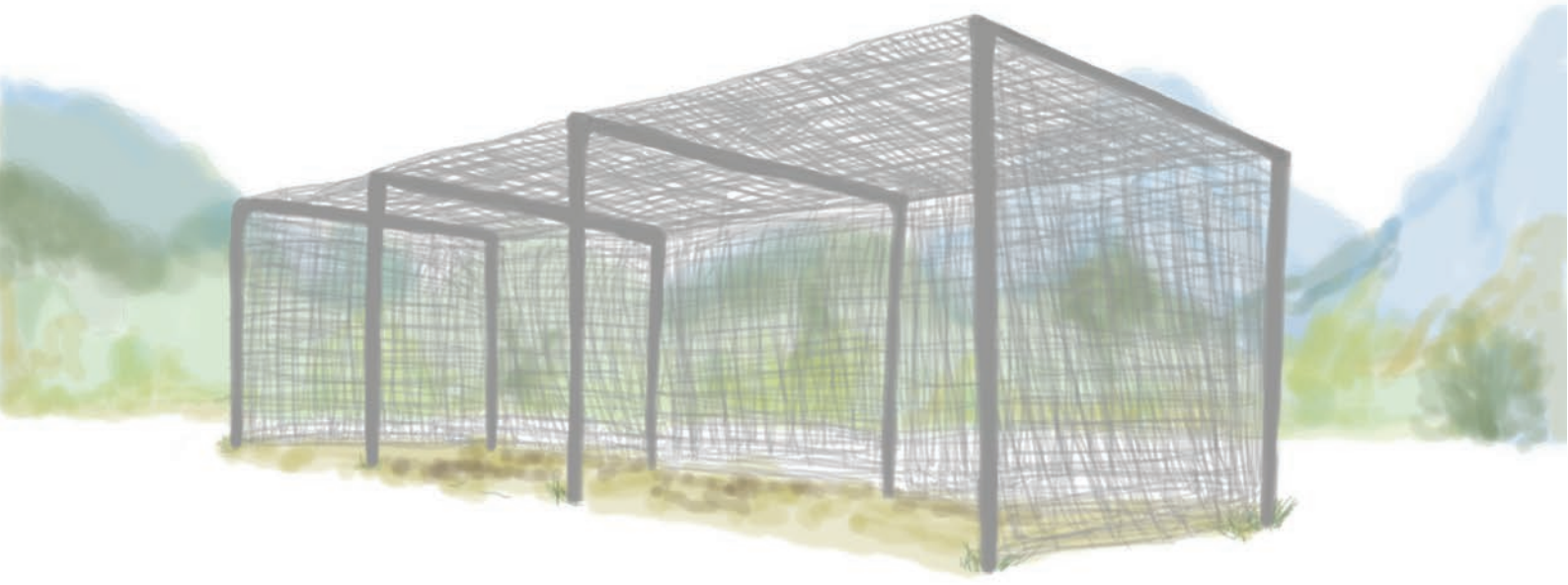
Document presentation

To structure the presentation of this doctoral work and related results, this thesis has been divided into four chapters not completely independent but with a clear common target and trajectory. The first chapter is devoted to present the field experiments with natural populations of *A. thaliana*. The plant localization, its behaviour, characterization and classification as well as the used methods and materials and related results are addressed and discussed. Next two chapters (II and III) are more focused on laboratory activities and protocols, being the central topics of study the physiological and genetic traits for two demes previously selected due to its extreme behaviour in front of carbonated soils. To corroborate the observed phenotypic variations among two demes from Catalonia we carried out a

genomic analysis using the Genome Wide Association Analysis (GWA) tools and this topic plus related results are reported in chapter IV.

All PhD work has been done under the support, facilities and resources of Unitat Fisiologia Vegetal - Universitat Autònoma de Barcelona. Ionomic and genetic analyses were made in collaboration with Prof. David Salt in the University of Aberdeen (https://www.abdn.ac.uk/) facilities during several stages. Finally, the phenolic analyses were made at Estación Experimental Aula Dei, Zaragoza (http://www.eead.csic.es/web/guest/home) in cooperation with Prof. Ana Álvarez-Fernández. The statistical analyses presented in this work were performed using the JMP software (https://www.jmp.com/en_us/home.html).

Chapter I
Field experiments



Introduction

Calcareous soils

Calcareous soils occur naturally in arid and semi-arid regions because of relatively little leaching (Brady and Weil, 1999). They also can develop in humid and semiarid zones under certain conditions: parent material rich in $CaCO_3$, (e.g. limestone, shells or calcareous glacial tills), that is relatively young and has undergone little weathering.

Calcareous soils often contain more than 15% $CaCO_3$ that may occur in various forms (powdery, nodules, crusts etc.). Soils with high $CaCO_3$ belong to the calcisols and related calcic subgroups of other soils. They are relatively widespread in the drier areas of the earth (Figure I-1).

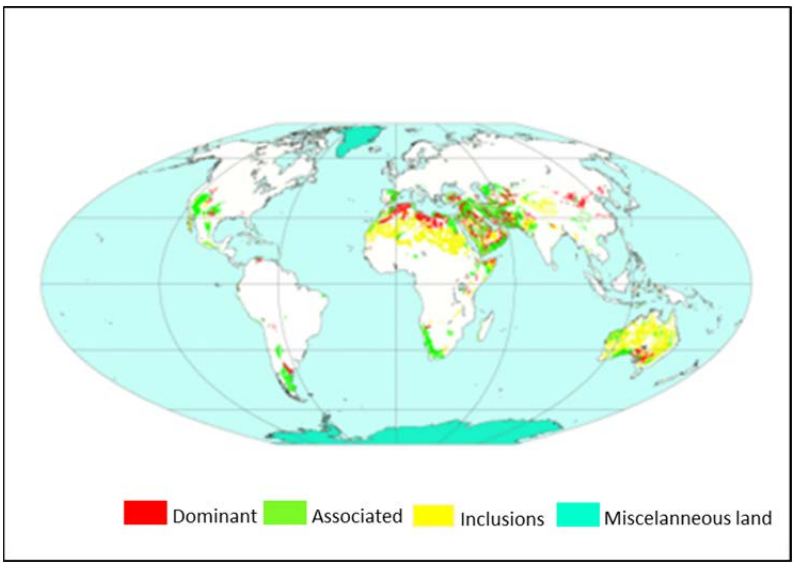


Figure I-1: Map of Calcisol distribution in the world from FAO

Some soils originated from calcareous parent materials can be calcareous throughout their profile. This will generally occur in the arid regions where precipitation is scarce. In other soils, $CaCO_3$ has been leached from the upper horizons, and accumulated in B or C horizons. These lower $CaCO_3$ layers can be brought to the surface after deep soil cultivation (Brady and Weil, 1999).

In some soils, the $CaCO_3$ deposits are concentrated into layers that may be very hard and impermeable to water. These *caliche* layers are formed by rainfall leaching the salts to a depth in the soil at which water content is so low that carbonates precipitate (Jackson and Erie, 1973).

Soils can also become calcareous through long periods of irrigation with water containing dissolved $CaCO_3$ (Hagin and Tucker, 1982).

Calcareous soils are alkaline because of the presence of $CaCO_3$, which dominates their chemistry. The carbonates are characterized by a relatively high solubility, reactivity, and

alkaline nature; their dissolution results in a high solution bicarbonate (HCO_3^-) concentration which buffers the soil in the pH range of 7.5 to 8.5:

$$CaCO_3 + H_2O \rightarrow Ca^{2+} + HCO_3^- + OH^-$$

Usually, the pH does not exceed 8.5 regardless of the $CaCO_3$ concentration, unless a significant quantity of sodium is present (Lindsay, 1979). Calcareous soils have 100% base saturation and calcium is the dominant cation in the exchange complex and in the soil solution (Loeppert and Suarez, 1996).

Problems associated with carbonated soils

Calcareous soils cover more than 30% of the earth's land surface (Chen and Barak; 1982) and their $CaCO_3$ content varies from a few percent to 95% (Marschner, 1995).

The carbonate minerals, due to their relatively high solubility, reactivity, and alkaline character, act as pH buffers; the pH values of most calcareous soils are within the range of 7.5 to 8.5. It is because of these properties that carbonates play an important role in pedogenic, chemical and rhizosphere processes in the soil (Loeppert and Suarez, 1996).

Pedogenetic processes can be characterized by the dynamics of the most soluble elements on the specific alteration conditions of the site. In areas of low rainfall, calcium carbonate is the characteristic mineral and its dynamics describe the pedogenesis. Moreover, the existence of alkaline carbonate controlling the chemical environment of the soil through its buffering action determines the availability of many nutrients, the humidification, the flocculation of colloids and, in general, restrains other chemical evolutions of the soil (Vallejo, 1986).

Calcareous soils in the Mediterranean area are expected to favour metal immobilization because of the presence of $CaCO_3$, a high pH, and the climatic conditions (low rainfall, high evapotranspiration), which favour the accumulation of metals in the soil's surface layer. In such conditions, metal bioavailability patterns are difficult to predict, being modulated by the action of carbonates and interactions with other reactive soil phases such as organic matter or oxides. Recent work on microbial properties in metal-contaminated calcareous agricultural soils has reported such complex interactions (Calvarro *et al.,* 2014).

Mediterranean soils often show relatively high values of pH and carbonate content which confers a high metal sorption capacity and therefore a low risk of metal toxicity. Nevertheless, previous works have shown that there is a significant fraction of potentially available metals in calcareous Mediterranean soils (Santiago-Martin *et al.,* 2013)

According to Frische *et al.,* (2003), bioavailability can be defined as a complex process of mass transfer and uptake of contaminants into soil-living organisms, depending on substance properties, soil properties, the biology of the organisms, and climatic influences. The dynamics of bioavailability comprise 2 phases: a physico-chemically driven desorption process and a physiologically driven uptake process by a specific biotic organism (Peijnenbur, 2003)

Metal availability patterns in calcareous soils cannot be explained by neither the pH value nor the carbonate content alone, but must be viewed in combination with the content and composition of organic matter (OM) and the fine mineral fraction, thus highlighting the need for further study of these soil fractions and their role in the metal (bio) availability (Santiago-Martin *et al.,* 2013)

Reported symptoms of impaired nutrition in calcareous soils are chlorosis and stunted growth. This is attributed to the high pH and reduced nutrient availability, as direct toxicity of bicarbonate ions (HCO_3^-) to physiological and biochemical systems are much less likely (Pearce *et al.,* 1999). Nonetheless, excess HCO_3^- can be harmful for crop growth due to the inhibition of protein synthesis and respiration and decreased nutrient absorption (Alhendawi, 1997).

The presence of $CaCO_3$ directly or indirectly affects the chemistry and availability of nitrogen, phosphorus, magnesium, potassium, manganese, zinc, copper and iron (Marschner, 1995; Obreza *et al.,* 1993).

Zinc (Zn) is an essential microelement for plant growth in all kinds of soils. It influences many biological processes, including carbohydrate metabolism, cell proliferation and phosphorus-Zn interactions (Rengel, 2015; Rehman *et al.,* 2012). Excess HCO_3^- or Zn deficiency inhibits photosynthesis and PS II, which influences photosynthetic and chlorophyll fluorescence parameters (Mohsenian, 2015). HCO3-, which is considered the key factor that influences Fe deficiency chlorosis and Zn deficiency in many plant species (McCray, 1992) is the major anion found in calcareous soils in karst regions.

Iron chlorosis is frequent in dicot (Strategy I) plants, mainly in calcareous soils (Römeld, 1986a). In these soils, Fe bioavailability can be severely limited because of the low solubility of iron oxides and hydroxides at high pH (Hell, 2003). Moreover, the elevated bicarbonate concentration of these soils, besides its effect on pH, can inhibit the Fe uptake mechanisms (Lucena, 2007). The high demand for Fe in the soil together with its low availability in soils leads to a competition between plant and other living organisms, being particularly strong in alkaline soils (Colombo, 2014)

Nitrogen fertilizers should be incorporated into calcareous soils to prevent ammonium-N volatilization. The availability of phosphorus and molybdenum is reduced by the high levels of calcium and magnesium that are associated with carbonates. In addition, iron, boron, zinc, and manganese deficiencies are common in soils that have a high $CaCO_3$ due to reduced solubility at alkaline pH values (Marschner, 1995; Brady and Weil, 1999).

Calcium carbonate provides a reactive surface for adsorption and precipitation reactions, for example, of phosphate, trace metals and organic acids (Talibudeen and Arambarri, 1964; Amer *et al.,* 1985). Carbonate reactivity influences the rate of volatilization of ammonia (Ryan *et al.,* 1981). Carbonate affects also rhizosphere processes, especially those processes in

which acidification is an important factor. For example, the Fe-deficiency response of dicotyledons involves the exudation of protons and acidification of the rhizosphere. The effectiveness of Fe-deficiency stress response is therefore negatively influenced by the neutralization of plant-produced acidity, which is influenced by the reactivity of the carbonate phase (Loeppert *et al.,* 1988; Morris *et al.,* 1990).

Calcicole and Calcifuge plants

Soils differ greatly in their pH and Ca concentration, particularly in non-cultivated soils. During evolution, plant species have adapted to these variations of pH and Ca conditions. For this reason, there are large differences between plant species and even between varieties of a single species in terms of tolerance. Plant species are therefore divided into calcicoles and calcifuges, depending on their level of tolerance (Lee *et al*., 1998)

The calcifuge plants are unable to develop efficient responses mechanisms to the deficiency of Fe, P, Zn and other micronutrients. Calcifuges struggle to solubilize these elements from the sparely soluble sources in calcareous soils and to keep them metabolically active in sufficient quantities in their tissues when growing on calcareous soils. In contrast, calcicoles have developed numerous mechanisms for mobilizing nutrients from different forms. Especially relevant is the ability to make iron available, since in the limestone soils the exchangeable and soluble Fe concentrations are much lower than those required for adequate plant growth (Lindsay, 1984).

There are clear differences in the metabolism of Ca^{2+} between the two groups of plants. Many calcareous species contain high levels of intracellular Ca^{2+} and high concentrations of malate. Contrastingly, calcifuges are usually poor in soluble $Ca^{2+}$and precipitate Ca in the form of calcium oxalate. Species and even cultivars can considerably differ in this capacity to form crystals of calcium oxalate or other crystals that contain calcium (Bangerth, 1979). To satisfy their demand for Fe, plants adapted to high-pH soils exude mainly compounds that help to increase iron availability, such as citric acid; this forms a soluble Fe-citrate chelate which is more available for uptake. Grasses (Poaceae) have the additional power of solubilizing Fe by exuding phytosiderophors (Marschner and Kissel, 1986), and this exudation may be related to calcicole/calcifuge behaviour (Gries and Runge, 1992, 1995).

However, a main and most puzzling problem of species richness in relation to calcicole/calcifuge behaviour is the question why the calcifuge plants have "lost" their ability to develop in calcareous soil and to compete successfully for survival on slightly acid soils. It seems like the development of tolerance to strongly acid conditions and the ability to detoxify or avoid uptake of Al ions is in some way related to their loss of ability to process the critical nutrients available in high pH soils. Some calcifuges may respond to a "signal'' of Al at their root surfaces by exuding compounds that may be similar or identical to compounds exuded by calcicoles at nutrient deficiency; e.g. organic acids like citrate, malate or oxalate (Jones, 1998; Schötteldreier *et al.,* 2001). This would mean that calcifuges and calcicoles respond to different

types of "signals" with a similar reaction, which forms the real basis of calcicole and calcifuge behaviour. Detailed studies comparing organic acid exudation under Al toxicity and P deficiency revealed differences in both time frame and sites of exudation patterns (Kochian *et al.,* 2004).

Germund Tyler (2003) propose that the response to the "deficiency signal" is a primary reaction of plants, whereas the response to an "ion toxicity signal" would be a secondary evolutionary property. This secondary property may take evolutionary time to develop, which would be an additional explanation of the fact that we have fewer calcifuge plants. It is not easy to prove such a hypothesis experimentally.

Natural populations of *Arabidopsis thaliana*

The analysis of natural variation in wild species has begun to elucidate the molecular bases of phenotypic differences related to plant adaptation to distinct natural environments and to determine the ecological and evolutionary processes that maintain this variation (Mitchell-Olds *et al.,* 2007). The model plant *A. thaliana* shows a wide range of genetic and trait variation among wild-type lines collected in the field. In addition, because of the unparalleled availability of genomic resources, the potential of *A. thaliana* for studies of natural genetic variation is increasingly recognized (Shindo *et al.,* 2006).

When different *Arabidopsis* accessions are grown together and compared under similar environmental conditions, genetic variation can be observed in many traits. Phenotypic variation in morphological and physiological traits is abundant and enables almost every *A. thaliana* accession to be distinguished from other accessions collected at different locations.

This variation is of interest from two general points of view. First, analysing this natural variation makes it possible to identify the function of individual genes. Second, analysis of natural variation has an increasing interest from an ecological and evolutionary perspective (Kliebenstein *et al.,* 2001; Remington & Purugganan, 2003). Thus, the patterns of phenotypic and molecular variation observed are analysed with the aim of inferring the mechanisms generating and maintaining this variation, and to identify which allelic variants are adaptive under specific environmental conditions (Koornneef *et al.,* 2004).

A large-scale sampling by SDM (Species Distribution Model) throughout Catalonia located new wild populations of *A. thaliana thaliana* (Busoms *et al.,* 2015a). The systems that characterize adjacent inland areas where *A. thaliana* grows in Catalonia are the Catalan Costal depression and Catalan Pre-Coastal Range. In the northern half of Catalan Coast *A. thaliana* can be found only in two specific areas: (1) Cap de Creus and Golf de Roses and (2) Serra Litoral (Busoms, 2015a) (Figure I-2)

Geology of Catalonia

Catalonia has a Mediterranean climate, except for the Val d'Aran, which has an oceanic climate; this valley is orientated and open to the Cantabrian Sea (www.meteo.cat). The Mediterranean climate is characterized by mild winters and short, hot, dry summers; precipitation is rare and seasonal (spring and autumn). However, there is some variation in the interior climate zones, which can experience more extreme temperatures, lower humidity and less rainfall. This is due to the distance from the sea; on the coast and areas near the coast, the sea has a more moderating effect on the climate.

The Catalan region consists of three areas or units: Pyrenees, located north of Catalonia, is a mountainous formation that connects the Iberian Peninsula with the European mainland; the Central Depression, a structural unit forming the eastern sector of the Ebro Valley; and the Catalan Mediterranean system, also called Catalan Coastal Ranges, alternating hills and plains parallel to the coast.

The siliceous substrates (eruptive granite and other rocks, Palaeozoic schists, etc.) predominate in the Pyrenean Axial Zone of the Aran Valley and Alta Ribagorça at the Canigó, the Albera and the Cap de Creus. These silicate substrates generate non-carbonate soils that, under favourable weather conditions, often can be oligotrophic and acidic. The second important rock surface of this type occupies the northern part of the Catalanic territory between Gironès and Baix Empordà on one site and the Baix Llobregat on the other. Smaller spots of siliceous material can be found in the Olositanic territory (rather eutrophic, volcanic rocks, etc.) and in the central part of the Catalanic territory (at Anoia, in the mountains of Prades, in the Priorat, and the Baix Camp).

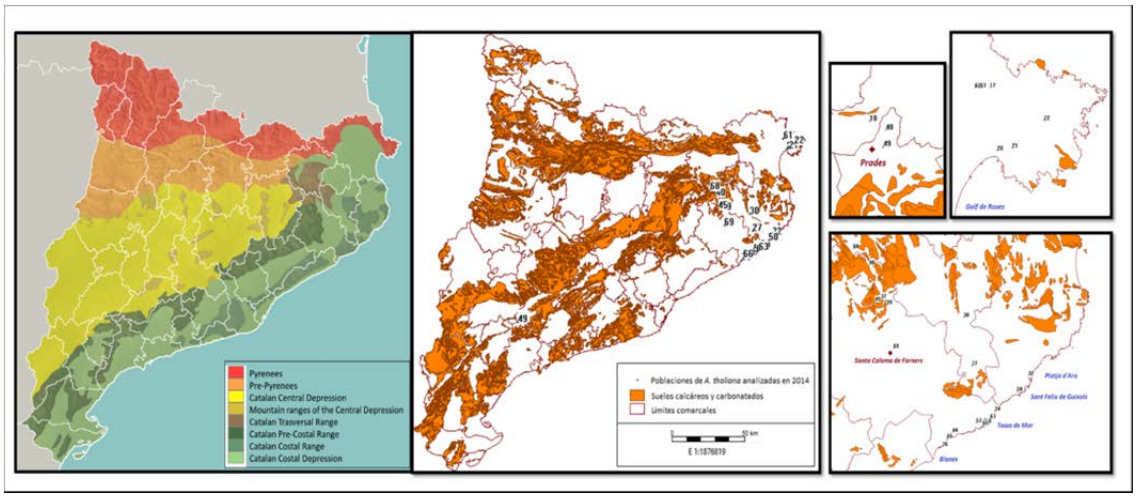


Figure I-2: (A) Geomorphological map of Catalunya (B): location of natural populations of *A. thaliana*

Carbonate-rich limestone is much more abundant in Catalonia than siliceous substrates. Most of the Pre-Pyrenean mountains are formed by compact, fissured calcareous rocks with a tendency to permeability (Karst formation). Compact limestone is also found in the Northern Catalanic Mountains (Bertí, Serra Superior del Valles) and especially in the southern central

part of the range, where the karst and limestone massifs predominate, from Garraf to Penyagolosa (Bolòs *et al.,*1993).

Specific Objectives

- Characterization of soils able to support natural populations of *A. thaliana* in Catalonia
- Evaluation of soil proprieties to see whether there are limiting factors related to plant tolerance to carbonate soils.
- Classification of natural accessions into different groups according to their tolerance to carbonate soils.

Materials and methods

Soil analysis

For three years, soil was collected from the original sites of natural populations of *A. thaliana* in Catalonia. Rhizosphere soils were sampled to analyse their physical and chemical properties.

Three independent soil analyses per site were performed: pH, water-holding capacity, and texture were measured using fresh soil following the methods described by Carter & Gregorich (2006). Organic matter and carbonate content were analysed following the procedures described by Black *et al.,* (1965) and Loeppert *et al.,* (1996). Sulphate concentrations were determined per Rehm & Caldwell (1968) and chloride concentrations were measured with a chloride ion-selective Electrode (Crison Instruments, Barcelona).

Ionomic analysis of soils were made in collaboration with Aberdeen University. To characterize the elemental composition of the soils, analyses were performed on the 2-mm fraction samples. Soil samples (5g) were dried for 42h at 60°C in 50-mL Falcon tubes. The extraction method, adapted from Soltanpour and Schwab (1977) consisted of a digestion with 20 mL of 1 M NH_4HCO_3, 0,005 M diaminetriaminepentaacetic acid, and 5 mL of pure water during 1h of shaking on a rotary shaker at low speed. Each sample was gravity filtered through qualitative filter papers until obtaining approximately 5 mL of filtrate, which was transferred into Pyrex tubes; 0.7 mM trace grade HNO_3 was added and digested at 115°C for 4,5 h. Each sample was diluted to 6.0 mL with 18 MV water and analysed for As, B, Ca, Cd, Co, Cu, Fe, K, Li, Mg, Mn, Mo, Na, Ni, P, Rb, S, Se, Sr and Zn content (ppb) on an Elan DRCe ICP-MS (PerkinElmer Sciex). National Institute of Standards and Technology traceable calibration standards (ULTRAScientific, North Kingstown RI, USA) were used for the calibration.

Plants analysis

Ionomic analysis of leaf tissue were made in collaboration with Aberdeen University.

Plants from the common garden experiments 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 42h at 60 ºC, and weighed before open-air digestion in Pyrex tubes using 0,7 mL concentrated HNO3 (Mallinckrodt AR select grade) at 110 ºC for 5 h. Each sample was diluted to 6.0 mL with 18 MΩ water and analysed for As, B, Ca, Cd, Co, Cu, Fe, K, Li, Mg, Mn, Mo, Na, Ni, P, Rb, S, Se, Sr and Zn content (ppm) on an Elan DRCe ICP-MS (PerkinElmer Sciex). NIST traceable calibration standards (ULTRAScientific, North Kingstown RI) were used for the calibration.

Garden experiments

To detect local adaptation to carbonate soil common garden experiments were conducted at two field sites with contrasting soil carbonate levels (Table I-1): Les Planes (42° 03' 45.1"N; 2° 32' 46.6"E) a representative for carbonate-rich soil and Santa Coloma de Farners (41° 50' 41.04"N;2 40' 36.13"E) a representative for low-carbonate soil.

Site	Geology (*)	Texture (*)	O.M.(%)	pH	%$CaCO_3$
LP	Limestones (7)	Clay –loam (5)	4.73	7.86	33,25
SCF	Granitoids (1)	Loamy sand (2)	2.83	7.11	4,81

Table I-1: Physical and chemical properties of soils from rhizosphere of selected soils from field experiment localizations. (*) Texture: numbers from more to less sandy. Geology: numbers from more to less silicon.

Same common garden design was reproduced at both sites. The common garden occupied a surface of 2x6 metres in the native soil at each site, and each garden was covered with a shading mesh that reduced 70% light on sunny days and 50 % on cloudy days (Figure I-3-A).

In March 2013, 2014 and 2015, 100 seeds (10 in each square) of 9 demes (Figure I-3-B) were sown at both sites with individual genotypes planted into 30 x 30 cm squares (Figure I-3-C) obtaining 10 plots of 90 x 90 cm with 10 demes distributed randomly (in each replicated plot each deme had a different position). Two weeks after germination, 2 plants were left in each square. We studied the fitness of 10 plants for each deme at each site and the other 10 plants of each deme per site were harvested in April 2013 and 2014 to analyse their leaf ionome. Rosette diameter was measured every week for 2 months and the number of siliques was counted at maturity as a proxy for fitness. During the 3 months of the field experiments, minimum and maximum temperatures, precipitation and soil composition were monitored.

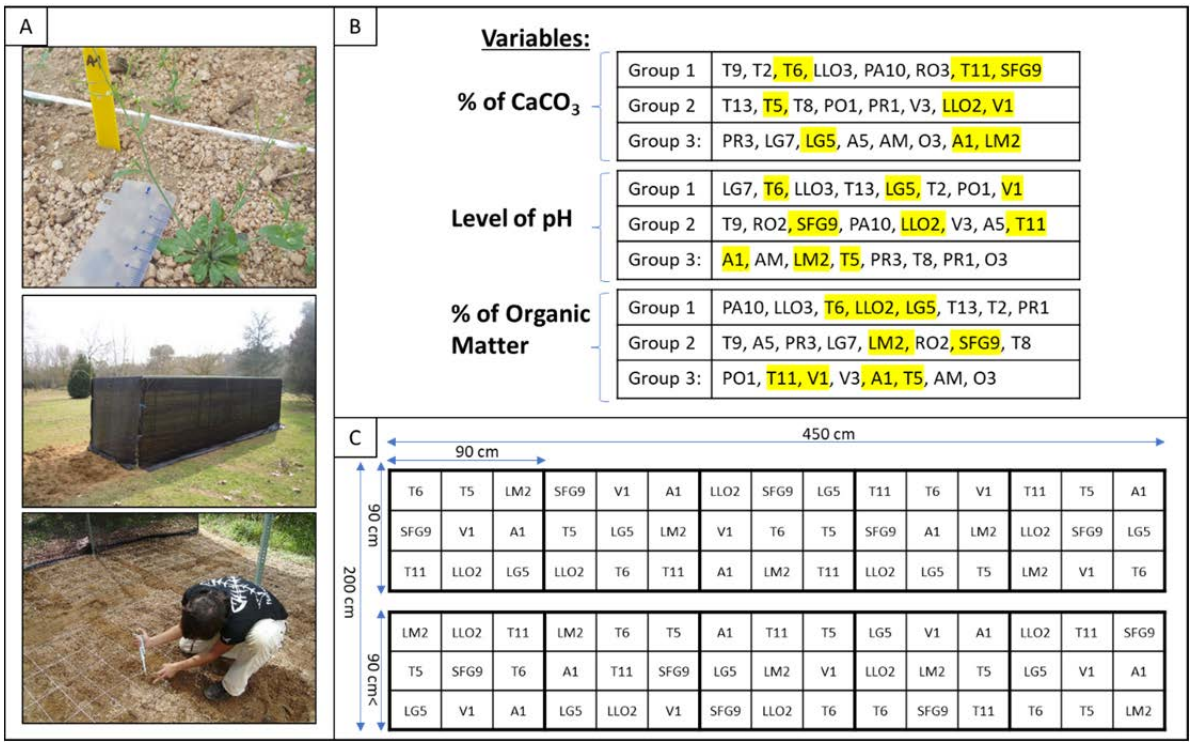


Figure I-3: (A) Pictures of common garden plots used in a transplant experiment in LP and SCF. (B) Demes associates to classifications: %CaCo3, pH level, %O.M, in yellow demes selected to do the transplant experiments. (C) Schema-Representation of each plant distribution inside plots.

Results & Discussion

Characterization of the driving factor for enhanced tolerance to carbonate soils

To test which the most relevant agent is driving the divergent selection of differential tolerance to carbonate soils in natural populations of *A. thaliana,* the demes were ordered according to the increasing values of the following soil parameters found in their original habitat: concentration of carbonates, soil pH, and soil organic matter. The resulting series from lowest to highest value were divided into 3 groups, each containing the same number of demes (Figure I-4).

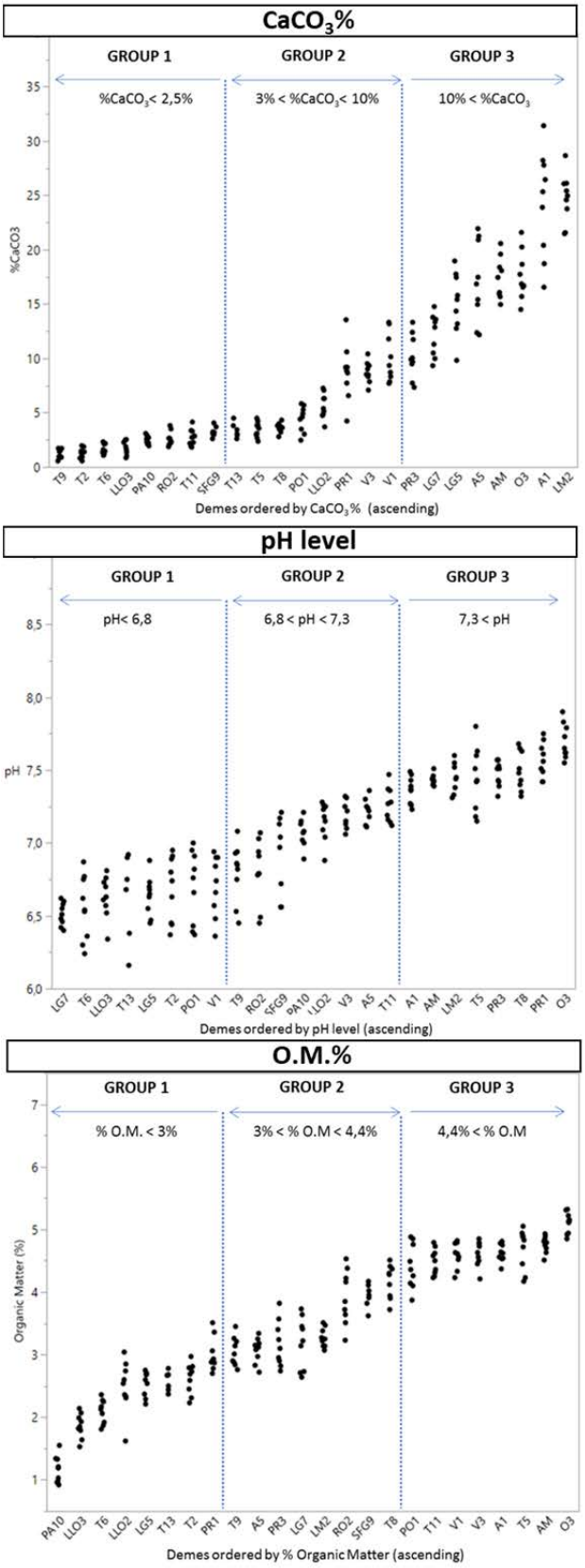


Figure I-4: Classification of 24 demes in relation to (A) CaCO3%, (B) pH level, (C) Organic Matter% in the original soils for 3 years.

Reciprocal transplant experiment

Fitness analysis

For assaying carbonate tolerance in soil, a total of 9 demes were selected, with representations of demes from each group, and sown in both common gardens.

Fitness based on siliqua production was a more reliable parameter than rosette diameter. The number of siliqua is a parameter directly related to the adaptability and survival of a plant, while its vegetative size can be variable and a non-indicative trait of carbonate tolerance. Therefore, siliqua number per plant, not rosette diameter, was used for a proxy of fitness.

After three years of measurements it was found that plants from all groups had higher fitness on low carbonate soil (SCF) than in carbonate-rich substrate (LP).

In Figure I-5 it can be observed that plants from all groups (regardless the variables used for classification) had similar fitness on low carbonate soil (SCF). In SCF there were no intergroup differences in any of the analysed variables (Figure I-5 A-1, 2 and 3). Also no correlation was found between fitness of the demes and any of the considered parameters (R^2 <0,005) (Figure I-5 B-1, 2 and 3).

Contrastingly, on the carbonate-rich substrate there was a strong variation in fitness.

Considering the percentage of soil organic matter as the grouping variable, all groups growing at LP had lower fitness than those grown in SCF, but there were no intergroup differences (Figure I-5 A.3). Moreover, no correlation with the fitness of the demes was observed (R^2 <0,005) (Figure I-5 B.3). Therefore, the percentage of organic matter in the original soils is not a factor that is directly related with the plants' tolerance to carbonated soils.

Intergroup differences were observed for both: $CaCO_3$% and pH level. The results for both variables agree that group number 3 is the one with highest fitness (Figure I-5 A.1-2). The composition of demes of group number 3 for pH level and $CaCO_3$ only differ in one deme: LG5. Curiously, this deme is from group 1 in relation to pH level groups (Figure I-5 B-2); this differential behaviour explains the high variability in group 1 regarding the pH level.

The correlation of groups and fitness in LP exists only for the variable: $CaCO_3$ % (R^2 >0,3) (Figure I-5 B.1) while none is found for groups classified by pH level (R^2 <0,05) (Figure I-5 B.2).

For this reason, from now on, we assign the % of carbonate in the original soil as the leading factor driving the difference in carbonate tolerance in the natural population of *A. thaliana thaliana*. Consequently, all further analysis and graphs are done grouping the demes by the $CaCO_3$ % in their native soil.

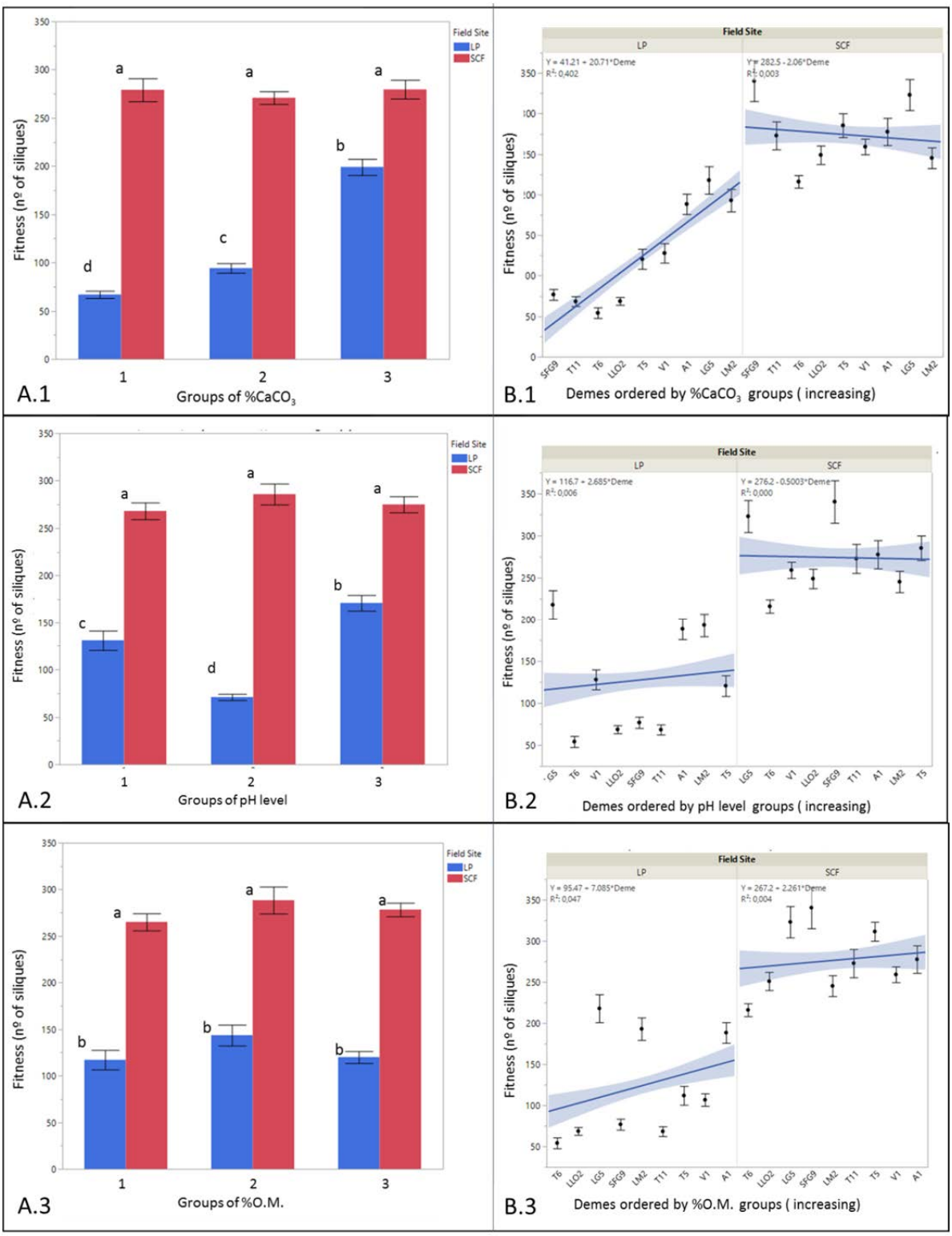


Figure I-5: (A) Representation of mean and standard error of fitness (number of siliques) in both sites SCF (red) and LP (blue) during three years; Group classifications by %CaCO3 (A.1), pH level (A.2) and %Organic Matter (A.3). (B) Correlation between fitness (number of siliques) and order of demes according to increase of %CaCO3 at both sites: LP and SCF (B.1), pH level (B.2) and %Organic Matter (B.3)

Ionomic analysis

Sodium: At SCF Na concentrations were similar for all groups, while at LP intergroup differences were observed: plants from group 1 had distinctively higher Na leaf concentrations than those from group number 3 (Figure I-6)

Potassium: Soil concentrations of potassium at SCF were much lower than at LP. Leaf K concentrations were not differing between groups in neither soil (Figure I-6).

Calcium: Soil Ca concentrations were similar at both experimental sites. However, plants from all groups grown in LP had higher leaf Ca concentrations than those in SCF (Figure I-6). There are statistically significant differences ($p \leq 0{,}005$) between group 1 and group 3 grown in LP, (group 1 accumulate more calcium than group 3).

Magnesium: soil concentration of Mg was higher in SCF than in LP, Mg concentration in leaf was also higher for all groups grown in SCF, and no differences between groups were observed neither in SCF, nor LP (Figure I-6).

Phosphorus: Soil concentration was lower in SCF than in LP, and the same relation is observed for leaf concentrations in all groups (Figure I-7). While in SCF there were no differences among groups, in LP group 3 showed higher concentrations than group 2 ($p \leq 0{,}005$).

Sulphur: Soil concentration of S was lower in SCF. Leaf concentrations of sulphur were similar for plants grown in SCF, but in LP there was a positive relationship between leaf sulphur concentrations and increasing group number.

Soil Fe concentrations tended to be higher in SCF than in LP (Figure I-7). Plants grown at LP showed intergroup differences for iron accumulation in the leaves. Highest leaf Fe concentrations were observed in group 3 with values between 100 and 120 mg kg^{-1}, while in group 1 leaf Fe concentrations of around 55 mg kg^{-1} indicate iron deficiency. Intermediate concentrations around 70 mg kg^{-1} were observed for group 2. Leaf Fe accumulation in SCF do not show differences for groups.

Zinc: Soil at LP had the highest Zn concentration but leaf Zn accumulation do not show differences for groups at neither site.

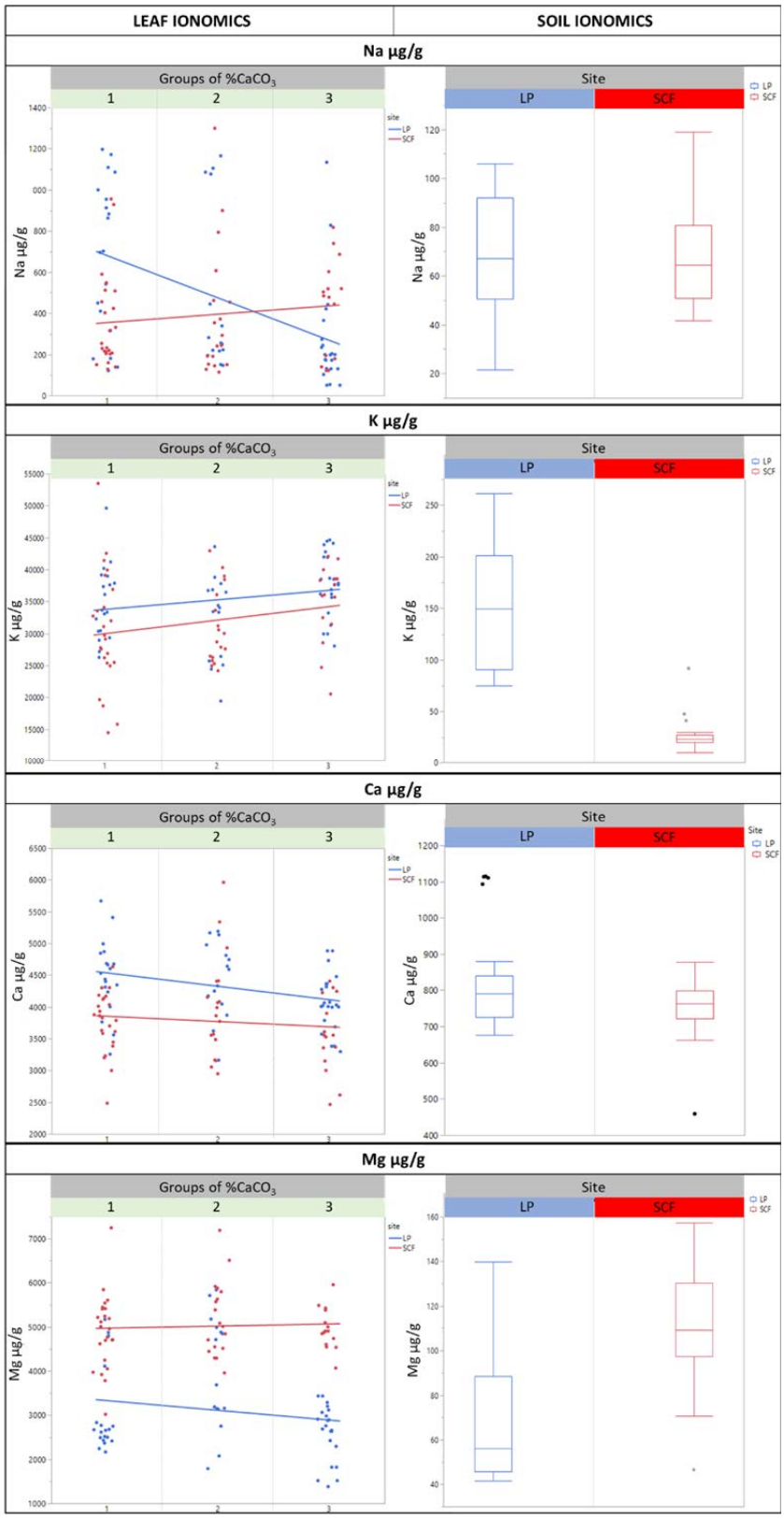


Figure I-6: (Leaf-left graphic): Analysis of mineral nutrient in leaves of three $CaCO_3$ groups: Na^+, K^+, Ca^+, Mg^+ (µg/g) grown in LP & SCF. (Soil-right graphic): same mineral nutrients analysed in soils of LP & SCF

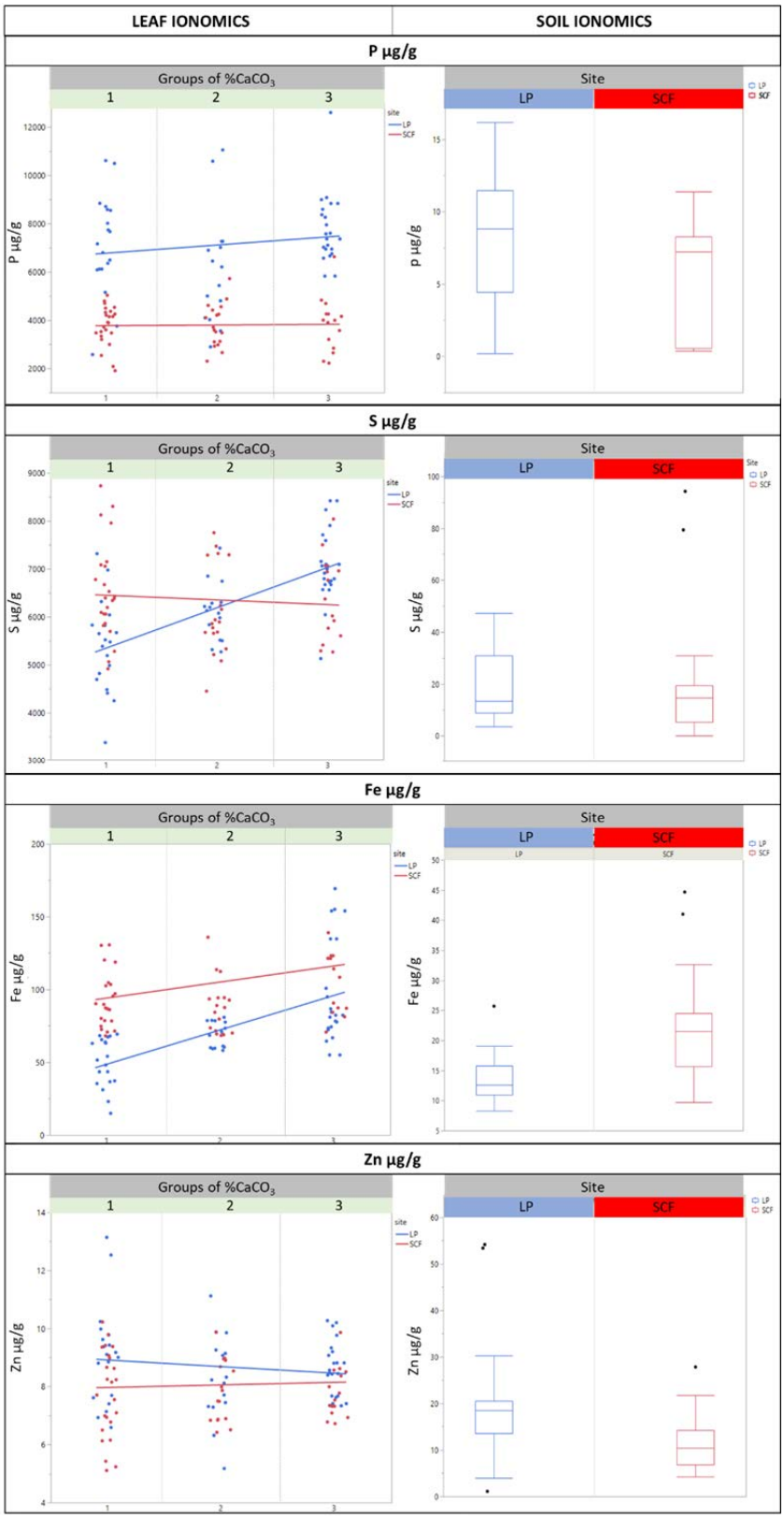


Figure I-7: (Leaf-left graphic): Analysis of mineral nutrient in leaves of three $CaCO_3$ groups: P, S, Fe, Zn (µg/g) grown in LP and SCF. (Soil-right graphic): same mineral nutrients analysed in soils of LP and SCF

Natural habitat

All soils of our study region were located on gravel, granodiorite or granitic rocks, and originated from similar geological bases. Nonetheless, the samples collected from 24 sites of *A. thaliana* demes during three years reveal some differences in soil properties.

Soils underlying group 3 plants (Figure I-8) have a high water holding capacity and the sulphate and chloride concentrations were lower in comparison to group 1 and group 2, with lower $CaCO_3$%.

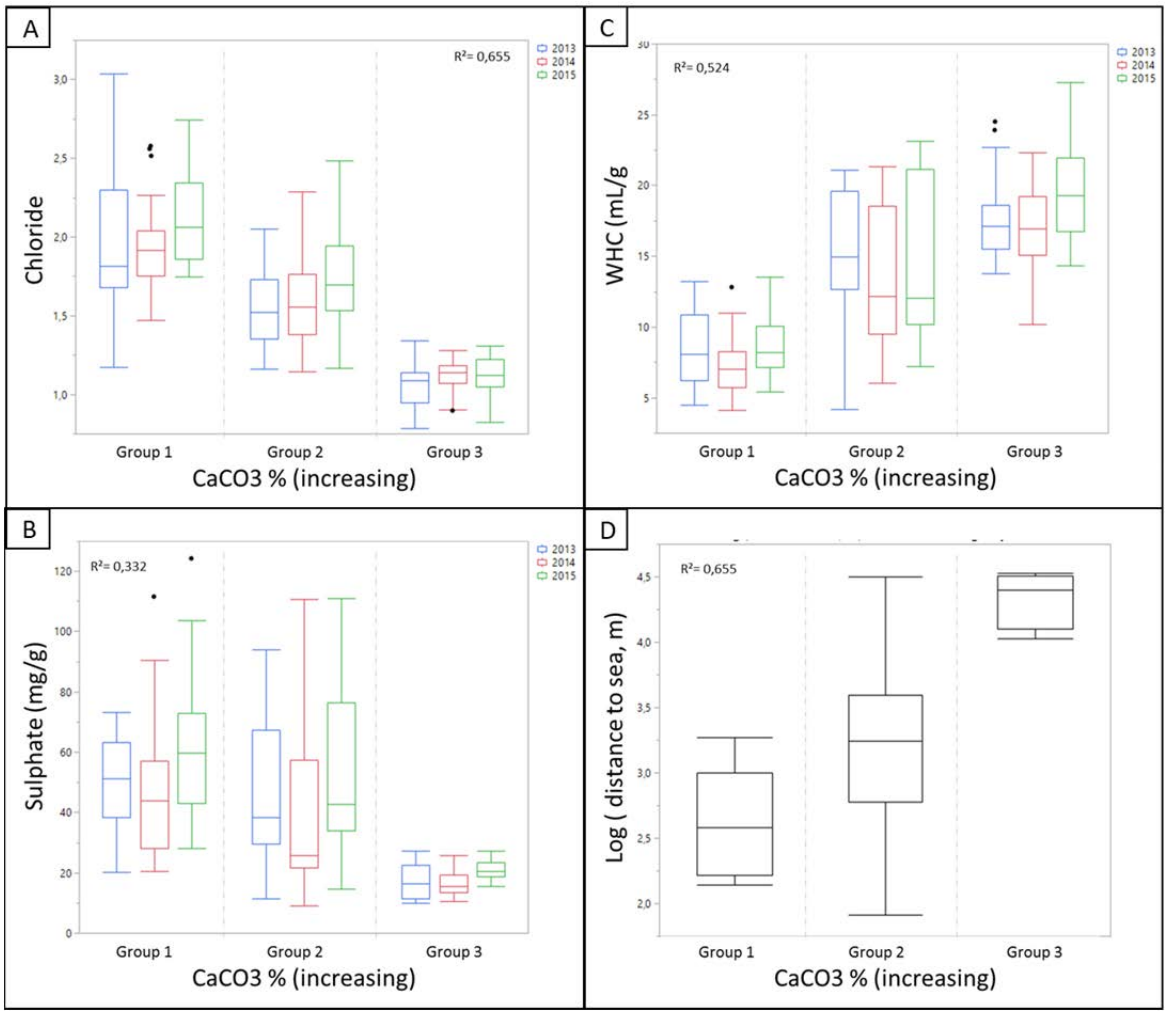


Figure I-8: Means and standard deviation of (A)chloride (mg/g); (B) sulphates (mg/g); C Water Holding Capacity (WHC, mL/g); (D) Distance to the sea (Logarithm of meters to the sea) from soil samples of natural *A. thaliana* populations collected in 2013, 2014, 2015, and their relationship with CaCO3% groups. Data include X sample of soils per site and year (ANEX)

Analysis of mineral nutrients (Figure I-9) show that plants from group 1 and 2 inhabited in soils with a high concentration of sodium, which can be explained by the proximity to the sea of these two groups. A negative correlation exists with magnesium (high in group 1, medium in group 2 and low in group 3). In contrast, for potassium there is a positive relation with level of carbonates in the soil. As expected the level of calcium is high correlated with level of carbonates of the soil.

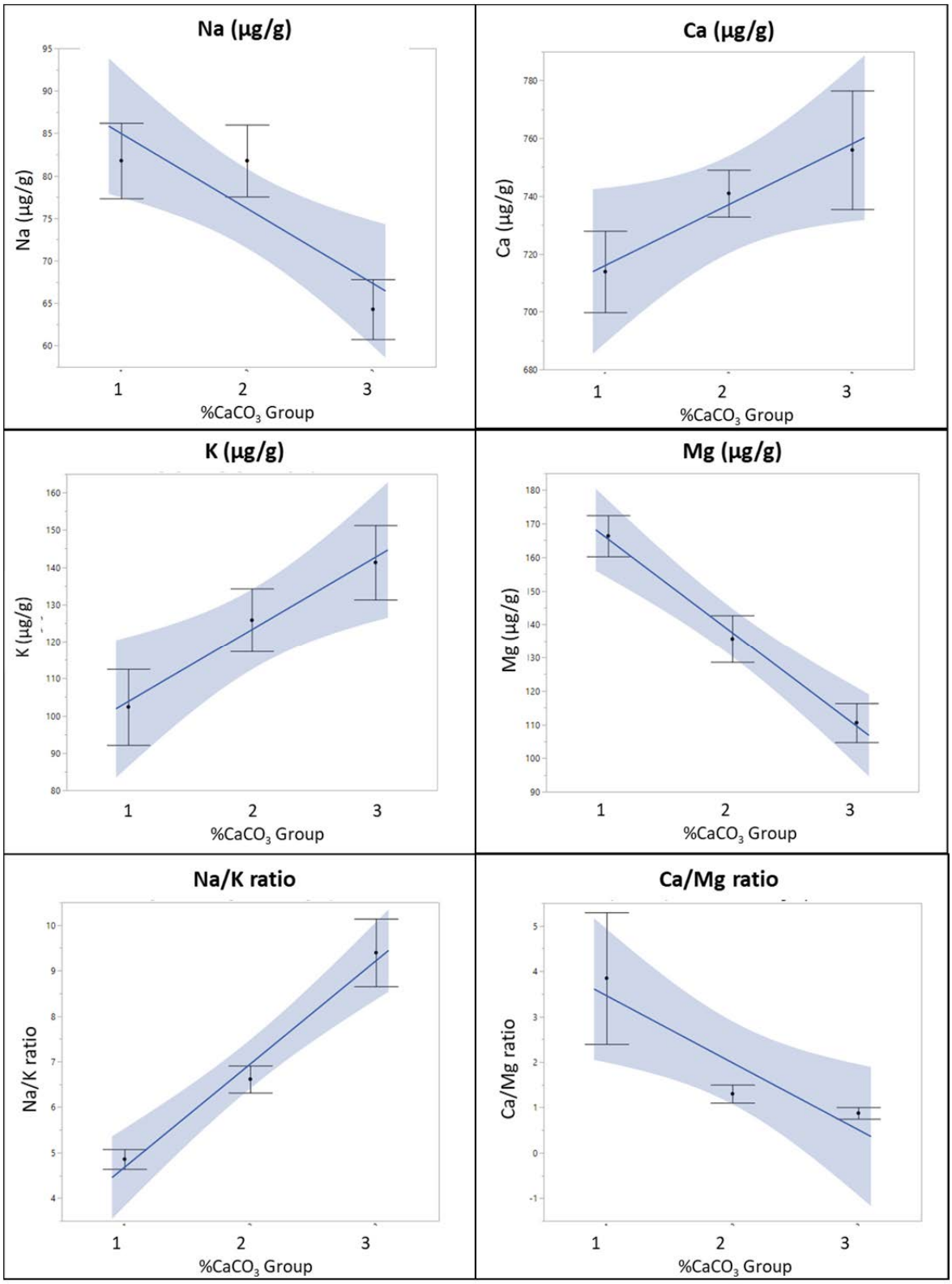


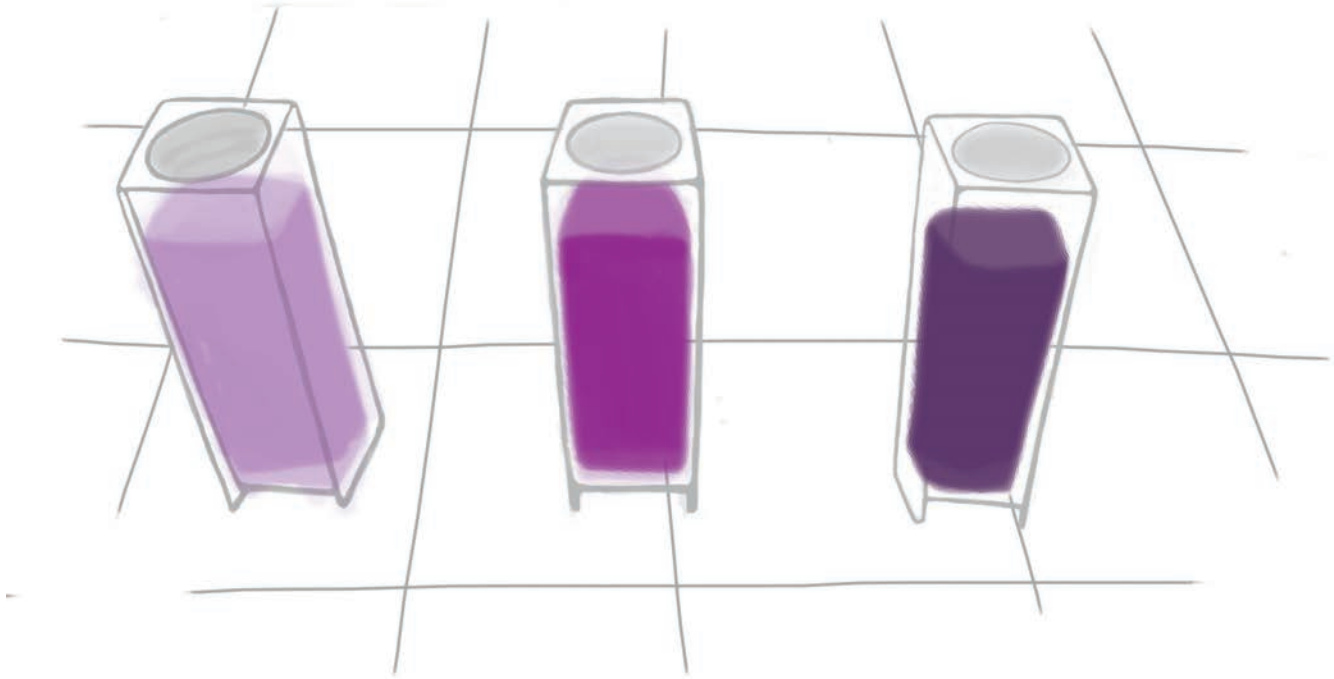
Figure I-9: Selected mineral nutrients in soils from natural habitats of A thaliana demes: (A) Na^+, (B) K^+, (D) Ca^+, (E) Mg^+ (mg/kg DW) and (C) Na^+/K^+ and (D) Ca^+/mg^+ ratios and their relationship with %$CaCO_3$.

Conclusions

- The carbonate level in the original soil is the leading factor driving the difference in carbonate tolerance of natural population of *A. thaliana*.
- Transplanted to carbonate rich soils, those populations originating from sites near the carbonate-rich area with moderate carbonate soil concentrations produced more siliques than individuals collected from sites distant to carbonate-rich soils where soils have no or low carbonate.
- *A. thaliana* plants collected from sites near carbonate-rich soils are locally adapted to soil carbonate, potentially through mechanisms that decrease calcium uptake and improve iron and phosphorus efficiency.

Note: Different statistical data related to this Chapter I can be found in 0 to Annex 4.

Chapter II
Physiological traits



Introduction

Iron in plants

Iron (Fe), mainly in the form of FeIII, is very abundant in most soils, but its availability to plants is low, especially in high pH and calcareous soils (Römheld and Marschner, 1986b). On the other hand, excessive iron accumulation by plants may lead to toxic effects (Romera *et al.,* 2014; Brumbarova *et al.,* 2015). Therefore, plants Fe acquisition is highly regulated.

Among the essential micronutrients in plants, iron is required in highest amounts. The nutrient is required in various key processes, including photosynthesis, respiration, and chlorophyll biosynthesis. Furthermore, the element is a component in heme proteins, the Fe-sulfur cluster, and other Fe-binding sites. The chemical properties of Fe that make it suitable for redox reactions also prone it to the generation of reactive oxygen species when it exists in a free ionic state and in large quantities (Marschner,1995). Despite its abundance in the soil, Fe is only slightly soluble under aerobic conditions, especially in high-pH and calcareous soils.

Based on the mechanisms developed to facilitate mobilization and uptake of Fe, plants are classified into Strategy I species and Strategy II species (Figure II-1). Strategy I species include all higher plants excluding most of the Poaceae, while Strategy II species are characteristically found in the Poaceae (Römheld and Marschner, 1986a; Ivanov *et al.,* 2012; Kobayashi and Nishizawa, 2012).

The first step to iron up take of Strategy I consist in rhizosphere acidification to liberate FeIII ions, by proton pumps and phenolic acid exdudation The main characteristic of Strategy I species is the necessity for reduction of FeIII to FeII, by the enzyme ferric-chelate reductase, located in the plasma membrane of root epidermal cells. The responsible gene has been cloned in *A. thaliana* (AtFRO2, Robinson *et al.,* 1999) and in other species, such as cucumber (CsFRO1, Waters *et al.,* 2007) and tomato (SLFRO1, Li *et al.,* 2004). Once iron has been reduced, it is transported into the cell through a transporter located in the plasma membrane of root epidermal cells, whose gene has also been cloned in *A. thaliana* (AtIRT1, Eide *et al.,* 1996), cucumber (CsIRT1; Waters *et al.,* 2007), and tomato (SlIRT1, Eckhardt *et al.,* 2001). These iron-acquisition genes are regulated at the transcriptional level by FIT (a transcription factor bHLH type in *A. thaliana*, whose homologue in tomato is FER, that acts together with two other transcription factors, bHLHH38 and bHLH39 (Colangelo and Guerinot, 2004; Jakob *et al.,* 2004; Yuan *et al.,* 2008; Bauer *et al.,* 2007). IRON REGULATED1/Ferroportin 1 (IREG1/FPN1) and IREG2/FPN2. were reported to be expressed in the roots of iron-deficient plants (Colangelo and Guerinot, 2004).

To obtain Fe from the soil, Strategy II species release PS (PhytoSiderophores) from their roots, which form stable FeIII-chelates. These FeIII-chelates (FeIII-PS) are then taken up by specific epidermal root cell plasma membrane transporters.

Both the strategy I and II plants distribute iron intra- and extracellularly with the help of nicotianamine which forms stable complexes with FeII and protects cells from oxidative damage (Kaway, 2001).

The ferrous ion is transported through the root cortex via symplast by the plasmodesms, Apparently in the form of FeII-nicotianamine (Stephan, 2002). Then, and still in the symplast of the root system, FeII is oxidized to the FeIII form and is transported to the aerial parts of plant via xylem, in the form of a soluble complex of dicitrate (Stephan and Sholz, 1993).

In the strategy I plant pea, an inward Fe^{2+} transport across the internal membrane of the chloroplast was described (Shingles *et al.,* 2001, 2002). This Fe transport was inhibited by Zn^{2+}, Cu^{2+}, and Mn^{2+} in a competitive manner, and was activated by protons, similar to the reduction based iron (Fe^{2+}) acquisition mechanism in roots. More recently, Fe-up take experiments using bathophenantroline disulfonate (BPDS) on isolated sugar beet (*Beta vulgaris*) chloroplasts described that ferric (Fe[III]) citrate was preferred over ferrous (Fe[II]) citrate as an iron source (Solti *et al.,* 2012). This Fe uptake was strongly connected to the photosynthetic performance of the chloroplast and subjected to negative feedback regulation. There are evidences of a reduction based mechanism for chloroplast Fe-acquisition in strategy I and II plants, since the existence of a chloroplast ferric chelate oxidoreductase (FRO) has been demonstrated at the enzymatic activity level (Mikami *et al.,* 2011;).

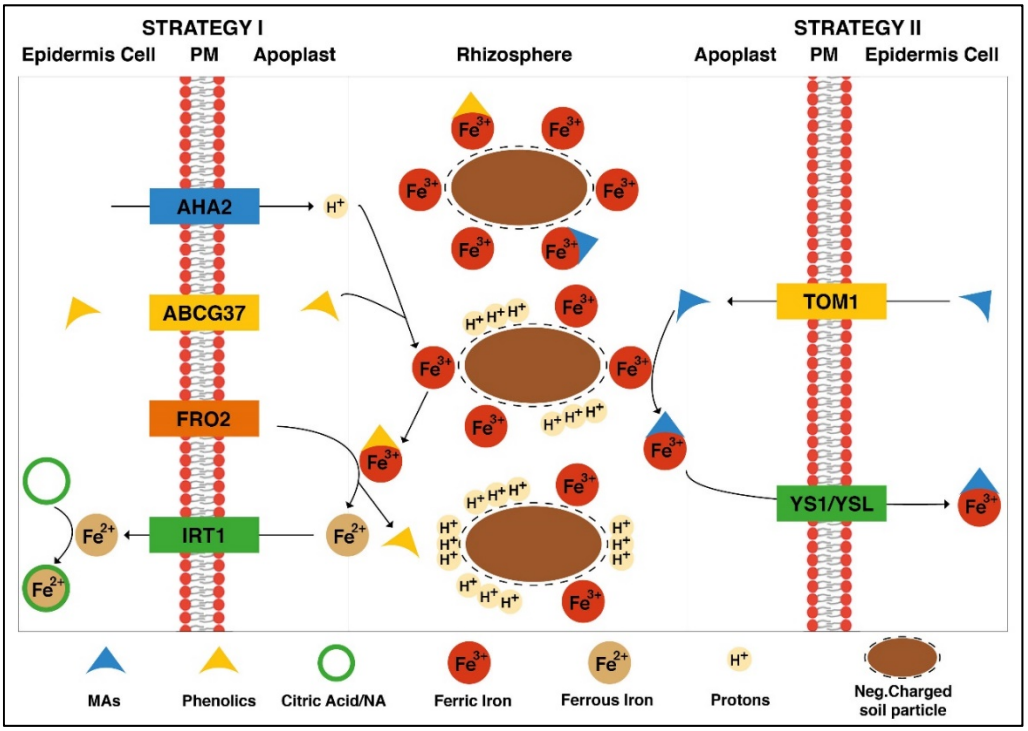


Figure II-1: Strategy I and Strategy II iron acquisition in plants. (Mod. from: Naranjo-Arcos & Bauer, 2016).

Iron deficiency

Iron deficiency occurs in a variety of soils, affected soils usually have a pH higher than 6 (Brown, 1971). Iron stress (deficiency or toxicity) in crop plant often represents a serious constraint for stabilizing and/or increasing crop yields. Any factor that decreases the availability of Fe in a soil or competes in a plant absorption process contributes to Fe-deficiency.

When grown under Fe deficiency, Strategy I plants induce several morphological and physiological responses in their roots, aimed to facilitate Fe mobilization and uptake. Some of these responses include development of subapical swelling with abundant root hairs, development of transfer cells, enhancement of ferric reductase activity (due to enhanced expression of *AtFRO2*-like genes), enhancement of Fe^{2+} uptake capacity (due to enhanced expression of *AtIRT1*-like genes), acidification of the extracellular medium (due to enhanced expression of H^+- ATPase genes), and release of flavins and phenolics (Römheld and Marschner 1986b; Hell and Stephan 2003). The *A. thaliana* basic helix-loop-helix (bHLH) transcription factor bHLH29/FRU, also known as FIT (for Fe deficiency-induced transcription factor), controls some of the root responses upon Fe limitation at different levels (for review, see Guerinot, 2000; Hindt and Guerinot, 2012; Ivanov *et al.,* 2012).

Under Fe-deficient conditions, Strategy II species greatly increase the production and release of *PS*, the number of FeIII-PS transporters and develop other physiological and regulatory responses (Kobayashi and Nishizawa, 2012)

Once adequate Fe amounts have been absorbed, Fe deficiency responses need to be down regulated to avoid toxicity and to conserve energy. The regulation of these responses is not fully understood but several hormones and signalling substances have been proposed to participate in the activation, like auxin (Landsberg, 1984), ethylene (Romera and Alcántara, 1994), and *NO* (Graziano and Lamattina, 2007), as well as in their suppression, like cytokinins (Séguéla *et al.,* 2008), jasmonic acid (Maurer *et al.,* 2011), and brassinosteroids (Wang *et al.,* 2012). These hypotheses have been mainly focused on Strategy I species, while the role of hormones and signalling substances on the regulation of Fe deficiency responses in Strategy II species has been less studied.

It is well documented that Fe deficiency in field crops primarily occurs in high pH, alkaline soils (calcareous conditions) (Hansen *et al.,* 2003). The solubility of Fe minerals decreases exponentially for each unit increase of pH, within the common pH range for soils (Lindsay and Schwab, 1982). Elevated levels of bicarbonates (HCO_3^-) in the soil solution will further aggravate Fe deficiency stress (Coulombe *et al.,* 1984).

High concentrations of bicarbonate appear to disturb plant metabolic processes which ultimately affect growth and the nutrient uptake (Marschner, 1995; Mengel *et al.*, 2001a,b). In calcareous soils, bicarbonate concentrations can reach values up to 9–15 mmol kg^{-1} (Boxma 1972; Zuo *et al.,* 2007), implying higher values when expressed on a molar base (mmol per L of

soil solution). When working with nutrient solutions, researchers frequently use bicarbonate concentrations ranging from 5 to 35mmol L−1 (Wadleigh and Brown 1952; Porter and Thorne 1955; Coulombe *et al.,* 1984).

Factors that increase bicarbonate concentration in soils, like high moisture and compaction, can also induce Fe chlorosis (Boxma 1972; Mengel *et al.,* 1984; Bloom and Inskeep, 1986). However, the bicarbonate mode of action is not well understood. Due to its pH buffering capacity, bicarbonate can maintain a high pH (7.5–8.0) in the medium, which can diminish both Fe solubility and root ferric reductase activity, since the latter has an optimum pH around 5.0 (Römheld *et al.,* 1983; Romera *et al.,* 1994; Moog and Brüggemann 1994; Kosegarten *et al.,* 2004). Besides these pH-mediated effects, bicarbonate can inhibit the induction of enhanced ferric reductase activity in Fe-deficient cucumber, sunflower (*Helianthus annuus* L.) and peach (*Prunus persica* L.) plants (Romera *et al.,* 1994; Alcántara *et al.,* 2000; Bohórquez *et al.,* 2001).

This is of special relevance to the micronutrients, iron in particular, in relation to high pH in calcareous soils, which are renowned for so called lime-induced chlorosis (Marschner, 1995; Alhendawi *et al.,* 2011).

Within the roots, bicarbonate promotes dark fixation of CO_2. This process may have important consequences for the plants mineral nutrition since the primary products of dark fixation in the roots are malate and other organic acids (Rhoads and Wallace, 1960; Lee and Woolhouse, 1969). The mode of action of bicarbonate; however, is not yet fully understood. It is still not clear whether the effects of bicarbonate result from the bicarbonate ion itself or from the high pH that induce the rhizosphere or a combination of both.

Iron containing primary minerals are specifically dissolved by bacteria (e.g., Thiobacillium and Metallogenium sp.), a weathering factor. These processes are strictly bacteria dependent and known as “sorption, solubilization (chelation), accumulation, transformation and precipitation”. Within the rhizosphere, these mechanisms are even more complex because of the presence of plants roots. In fact, plants can affect microbes (abundance, diversity, and activity), Fe availability, and the interactions between Fe minerals and microbes, as a consequence of root activity (exudation and nutrient uptake) to satisfy their need of this essential micronutrient. Therefore, the low supply of FeIII to the soil solution and the high Fe demand of plants and microorganisms (for their intense growth) could induce a considerable level of competition for Fe in the rhizosphere (Loper and Buyer 1991; Guerinot and Yi 1994).

Oxidative stress and defence

Adverse environmental conditions are reported to induce oxidative stress in plants because the production of reactive oxygen species (ROS) (Foyer *et al.,* 1997).

The term ROS includes any derivative of molecular oxygen (O_2) that is considered more reactive than O_2 itself. Thus, ROS refers to free radicals such as superoxide ($\cdot O_2^-$) and the hydroxyl radical (·OH), but also to non-radicals like singlet oxygen ($^1O_2$) and peroxide hydrogen

(H_2O_2). Because of its relative stability, H_2O_2 has received attention as a signal molecule involved in the regulation of specific biological processes such as plant–pathogen interactions. H_2O_2 is generated by a two-electron reduction of O_2, catalysed by certain oxidases or indirectly via reduction or dismutation of O_2^- which is formed by oxidases, peroxidases, or by photosynthetic and respiratory electron transport chains (Foyer and Noctor, 2005; Mittler *et al.,* 2004; Bindschedler *et al.,* 2006; Sagi and Fluhr, 2006). Most of the cellular compartments (chloroplast, mitochondria, peroxisome, and cytoplasm) in higher plants participate in the generation of ROS inside the cell (Figure II-2)

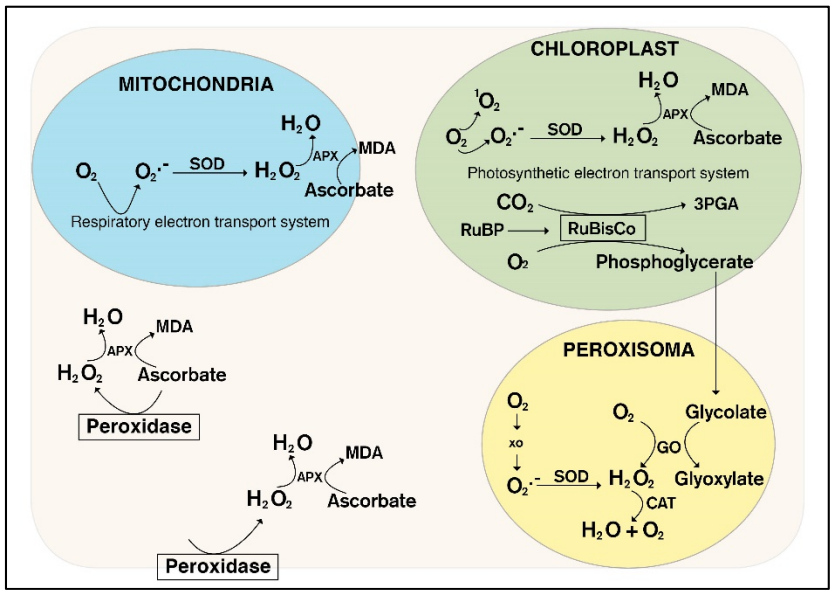


Figure II-2: Oxidative environment and redox homeostasis in plants: dissecting out significant contribution of major cellular organelles. GO, glycolate oxidase; 3PGA, 3-phosphoglycerate; RuBisCo, ribulose 1,5-bisphosphate carboxylase/oxygenase; RuBP, ribulose 1,5-bisphosphate; SOD, superoxide dismutase; XO, xanthine oxidase; CAT, catalase; APX, ascorbate peroxidase. Mod. From Das *et al.,* 2015

Oxidative-stress-response-related proteins were among the most affected protein categories under Fe deficiency conditions. These changes included increases in superoxide dismutases (CuZnSOD and MnSOD), monodehydroascorbate reductase (MDAR1), peroxidase 12 (PER12) and a decrease in catalase (CAT-2) (López-Millán, 2013). These observations point to the strong impact of Fe deficiency on redox homeostasis, not only because free Fe ions induce reactive oxygen species (ROS) formation via Fenton reactions, acting as a prooxidant, (Halliwell and Gutteridge 1984), but also because many proteins involved in oxidative stress, such as peroxidases and catalase, are Fe-containing proteins.

Iron is a constituent of several components of the electron transport chain in mitochondria and chloroplasts, and thus iron deficiency disrupts normal electron transfer resulting in the overproduction of ROS. Under these conditions, the high levels of ROS generated exceed the possibility of being controlled by the antioxidant system, causing cell oxidative damage (Allen, 1997)

In its role as an enzyme constituent, Fe is part of catalase (CAT, EC 1.11.1.6), non-specific peroxidases (POD, EC 1.11.1.7), ascorbate peroxidase (APX, EC 1.11.1.11) and Fe superoxide dismutase (Fe-SOD, EC 1.15.1.1).

The labile iron pool is composed of weakly chelated iron low-molecular-weight compounds. Most of this iron is Fe^{II} or Fe^{III} associated with ligands with a low affinity for iron. Protein synthesis during plant growth processes generates a constant flow of iron from the extracellular environment to the cytoplasm (Kruszewski, 2003). The cytoplasmic labile iron pool supplies iron for the synthesis of heme or [Fe-S] clusters, and is therefore essential in controlling numerous metabolic reactions.

Superoxide Dismutase

Superoxide radical ($\cdot O_2^-$) is produced at any location where an electron transfer is present and thus in every compartment of the cell. Superoxide dismutase, converting $\cdot O_2^-$ to H_2O_2, constitutes the first line of defence against ROS in different plant species under several stress conditions (Elsten, 1991; Alscher *et al.,* 2002; Blokhina *et al.,* 2003).

The importance of SOD has been demonstrated by analysis of mutants in microbes and animals. SOD mutants in *Escherichia coli*, *Saccharomyces cerevisiae*, *Neurospora crassa*, and *Drosophila melanogaster* exhibit increased sensitivity to methyl viologen (paraquat), a redox-active compound that enhances the production of O_2^- (Carlioz and Touati, 1986; Phillips *et al.,* 1989; Gralla *et al*., 1991; Chary *et al.,* 1994). SOD is also essential for DNA integrity and normal life span: The *E. coli* and *N. crassa* mutations cause an increased spontaneous mutation rate, whereas the *D. melanogaster* mutant has a significantly shorter life span then the wild type (Carlioz and Touati, 1986; Phillips *et al.,* 1989; Chary *et al.,* 1994). Mutations in human and mouse Cu/ZnSOD have been linked to the disease familiar amyotrophic lateral sclerosis, which is characterized by premature neuron death (Rosen *et al.,* 1993). Taken together, this evidence indicates a vital role for SOD in preventing ROS generated cell damage and death in aerobically growing organisms. SOD is also thought to be important in converting O_2^- to H_2O_2 during the pathogen-induced oxidative burst in animal phagocytic immune cells and in plant cells (Desikan *et al.,* 1996; Babior *et al.,* 1997).

Based on metal co-factor used by the enzyme, SODs are classified into three groups: iron SOD (Fe SOD), manganese SOD (Mn SOD) and cooper-zinc SOD (Cu/Zn SOD), and these SODs are in different compartments of the cell (Figure II-3). Fe SODs are located in the chloroplast, Mn SODs in the mitochondrion and the peroxisome and Cu-Zn SODs in the chloroplast, the cytosol and possibly in the extracellular space.

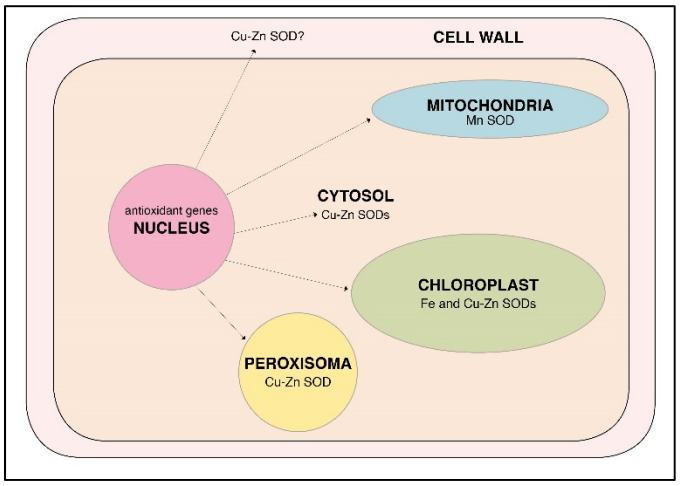


Figure II-3: Role of superoxide dismutase (SODs) in controlling oxidative stress in plants. (Mod from: Alscher *et al.,* 2002).

Catalase

Catalase action in plant and animal tissues was first observed in 1818 by Thenard, who noted that such tissues readily degraded hydrogen peroxide, a substance he had also discovered some years earlier

Catalase (H2O2:H2O2 oxidoreductase, EC 1.11.1.6; CAT) is a tetrameric heme containing enzyme that is found in all aerobic organisms and serves to rapidly degrade H_2O_2.

Catalase is one of the most active catalysts produced by nature. It decomposes H_2O_2 at an extremely rapid rate. Depending on the concentration of H_2O_2, it exerts a dual function (Deisseroth and Dounce 1970). At low concentrations (<10-6 M) of H_2O_2, it acts like a peroxidant where a variety of hydrogen donors (e.g., ethanol, ascorbic acid) can be oxidized in the following manner.

$$RH_2 + H_2O_2 \rightarrow R + 2H_2O$$

At high concentrations of the substrate, catalase decomposes toxic H_2O_2 at an extremely rapid rate using the catalytic reaction in which H_2O_2 acts as both acceptor and donor of hydrogen molecules.

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

Spectrophotometric and kinetic evidence suggests that catalase uses a two-step mechanism in both the peroxidising and catalytic reactions (Deisseroth and Dounce 1970; Dounce 1983). In the first step, the heme iron of catalase interacts with H_2O_2 to form high valent iron complex containing an oxoferryl porphyrin cation radical (Alfonso-Prieto *et al.,* 2009).

$$\text{Enz (Por-FeIII)} + H2O2 \rightarrow \text{Cpd I (Por·+-FeIV = O)} + H2O$$

Compound I then react with a second H_2O_2 molecule forming water and oxygen:

$$\text{Cpd I (Por·+-FeIV} = O) + H_2O_2 \rightarrow \text{Enz (Por-FeIII)} + H_2O + O_2$$

This intermediate compound I, which can be detected *in vitro* and *in vivo*, because it alters the spectrophotometric properties of the catalase heme. In fact, because of special kinetic properties of catalase, compound I is utilized as an indicator of H_2O_2 concentrations *in vivo* (Oshino *et al.,* 1975).

Phenolic compounds in root exudates

Iron deficient Strategy I plant species have long been known to increase efflux of root exudates (Cesco *et al.,* 2010). Some species, such as *A. thaliana thaliana*, produce phenolic compounds (Fourcroy *et al.,* 2014; Schmid *et al.,* 2014) while other species, including sugar beet, cucumber and melon, produce flavin compounds (Susin *et al.,* 1994; Welkie, 2000; Rodríguez-Celma *et al.,* 2011). Although the function of flavin compounds in plant Fe deficiency is not well defined, they may function in reduction or complexation of extracellular Fe to facilitate Fe acquisition (Cesco *et al.,* 2010; Sisó-Terraza *et al.,* 2016b). Proteins involved in riboflavin synthesis increased in abundance in response to Fe deficiency or Fe deficiency in alkaline conditions (Rellán-Álvarez *et al.,* 2010; Rodríguez-Celma *et al.,* 2011) and genes involved in riboflavin biosynthesis were up-regulated in iron-deficient roots in alkaline conditions (Rellán-Álvarez *et al.,* 2010; Rodríguez-Celma *et al.,* 2013).

Root secretion of coumarin-type phenolic compounds has been recently shown to be related to *A. thaliana* tolerance to Fe deficiency at high pH. Previous studies revealed the identity of a few simple coumarins occurring in roots and exudates of Fe-deficient *A. thaliana* plants, and left open the possible existence of other unknown phenolics (Sisó-Terraza, *et al.,* 2016a).

Objectives

- Characterization of Fe deficiency responses in contrasting two demes of *A. thaliana* originally developing on soils with different carbonate contents
- Evaluation of physiological parameters potentially associates with tolerance to carbonate soils
- Characterization of physiological traits in extreme behaviours

Materials and methods

Plant Culture and Experimental Design

Hydroponics

Seeds from natural habitat of two natural demes of *A. thaliana*, A1 and T6, were germinated, pre-grown and grown as indicated in Fourcroy *et al.,* (2014) with several modifications. Seeds were sown in 0.2 ml tubes containing 0.6 % agar prepared in nutrient solution 1/4 Hoagland, pH 5.5. Iron was added as 45 µM Fe(III)-EDTA. After 10 days in the growth chamber, the

bottom of the tubes containing seedlings was cut off and the tubes were placed in opaque 300 ml plastic boxes (pipette tip racks; Starlab, Hamburg, Germany), containing aerated nutrient solution 1/2 Hoagland, pH 5.5, supplemented with 20 µM Fe(III)- EDTA. Plants were grown for 11 d and nutrient solutions were renewed weekly. After that plants (12 plants per rack) were grown for 14 days in different treatments:

- Nutrient solution 1/2 Hoagland with 0 (control) or 20 µM Fe(III)-ethylendiaminedi (o-hydroxyphenylacetate) (treatment) (Fe(III)-EDDHA); Sequestrene, Syngenta, Madrid, Spain). Solutions were buffered at pH 5.5 (with 20 mM MES) or at 7.5 (with 5 mM HEPES) to maintain a stable pH during the whole treatment period. Nutrient solutions were renewed weekly. Two batches of plants were grown and analysed. Pots without plants, containing only aerated nutrient solution (with and without Fe) were also placed in the growth chamber and the nutrient solutions sampled as in pots containing plants; these samples were later used as blanks for root exudate analyses.
- in nutrient solution 1/2 Hoagland with 5 or 20 µM Fe(III)-ethylendiaminedi (o-hydroxyphenylacetate) (Fe(III)-EDDHA; Sequestrene, Syngenta, Madrid, Spain). Solutions were started at pH.7.5 without buffered, to analyse changes in pH solution. Nutrient solutions were renewed weekly. Enzymatic activities were analysed.

Cultivation in soil

Same demes, A1 and T6 were also used for soil experiments. Two different soil cultivation experiments were performed

- Plants from both natural populations of *A. thaliana* were grown from seeds in potted soils from Santa Coloma de Farmers (SCF) representative of no-carbonate soil and soil from Les Planes (LP), representative of carbonate soil (used for field experiment in chapter I). Plants were watered two times per week with distilled water. Measures of growth (dimeter of rosette) were taken weekly during a month, and samples for genotyping and ICP were taken.
- Plants from both A1 and T6 were grown on universal substrate (Compo Sana Semilleros) watered two times per week. After 21 days from sowing the plants were watered twice a week with 20mM NaHCO. Rosette diameter was measured every week.

Germination and plant growth (both in soil and hydroponic experiments) took place in a controlled-environment chamber (Conviron CMP5090, Canada), at 21ºC, 70% relative humidity and a photosynthetic photon flux density of 220 µmol m^{-2} s^{-1} photosynthetic active radiation with a photoperiod of 8 h light/16 h dark.

Ferric-reductase

Ferric-reducing capacity was measured before harvest according to Romera *et al.,* (1999) (Figure II-4. Intact plants (40 days old) were pre-treated for 30 min in 1 mL of solution A with the following composition in mM: 2 $Ca(NO_3)_2$; 0,75 K_2SO_4; 0,65 $MgSO_4$; 0,5 KH_2PO_4. Then transferred for 1 h to a similar solution that also contained 100 µM Fe $^{3+}$ EDTA and 300 µM ferrozine, pH 5,0 (assay solution). The ferric-reducing capacity was determined by measuring the concentration of Fe^{2+}-ferrozine complex formed, via absorbance measurements at 562 nm in a (Shimadzo UV-2450). Reduction rates were calculated using an extinction coefficient of 29 800 M^{-1} cm^{-1}. Finally, the fresh weight of roots was measured.

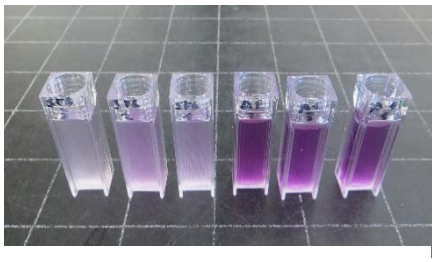


Figure II-4: Image of Ferric-chelate-reductase assay

To calculate reduction capacity (enzyme activity) we used the following formula:

$$\textbf{RC}\ (\text{nmol } Fe^{2+} \cdot g^{-1} \text{ root f. Wt } h^{-1}) = \frac{V(L) \times OD_{562nm}(cm^{-1})}{\varepsilon.Coef \times time(h) \times P_{root.F.Wt}}$$

Enzyme extract preparation

For preparation of crude enzyme extracts, a 0.05 g sample of fresh leaves was ground in 2 mL of 0.1 M cool phosphate buffer (pH 6.8) on ice bath (Kar and Mishra, 1976). The crude extract was centrifuged at 15,000 x g for 15 min at 4°C. The supernatant was used for catalase and SOD activity assays. The protein concentration of the supernatant was measured according by NANODROP-2000. Catalase and peroxidase activities were measured according to Erdei *et al.,* (2002).

Catalase assay

Catalase activity was measured according to Erdei *et al.,* (2002), the assay mixture (3 mL) contained 15 mM H_2O_2, 50mM phosphate buffer (pH 6.8) and 100µL enzymes extract. The decline of absorbance at 240 nm was scanned automatically with a spectrophotometer (Shimadzo UV-2450) in kinetic mode. Molar extension coefficient of catalase is 43.6 L $mM^{-1}cm^{-1}$. Activity was expressed as a function of total protein.

SOD-assay

The superoxide anion scavenging activity of plant extracts was determined with the WST (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) reduction method, using the Superoxide dismutase (SOD) assay Kit-WST (Dojindo Lab, Kumamoto, Japan). In this method, O2 reduces WST- 1 to produce the yellow formazan, which is measured spectrophotometrically at 450 nm. Antioxidants can inhibit yellow WST formation.

All measurements were done in triplicate. The percentage of inhibition of superoxide radicals was calculated using the above formula.

Chlorophyll concentration

Measures of chlorophyll concentration were taken with a SPAD device (CCM300, Opti-Sciences, Hudson, USA) after 14 days of treatment. For each plant three measures on leaves of different age (old/medium/young) were taken in order to have more representative results.

Genotyping (PCR) AtFPN2

An SSR marker was developed based on this insertion in Ts-1 plants (CS1552) with forward primer 5'ACATTTGCAGCTTGGGCTAC-3' and reverse primer 5'- CTCCGGTTCTGAGAGGTGAG-3', according to Morrissey *et al.,* 2009

DNA was extracted using 50 mM TRIS (pH 9) and 5 mM EDTA (pH 8). After heating at 95°C for 5 min, 4µl of extract was directly used as a template for PCR. 10 µl PCR reactions contained 2µl 5X Green GoTaq® reaction buffer (Promega), 0.8 µl 25mM MgCl2, 0.8 µl 2.5 mM dNTPs, 0.4 µl 10 mM forward and reverse primer and 0.3 µl homemade Taq polymerase. A total 45 cycles PCR was performed with 30 secs at 94°C, 15 sec annealing at 60°C followed by 30 sec extension at 72°C. PCR product was then digested with enzyme XhoI overnight and separated on 3% agarose gel.

Soil analysis

To characterize the elemental composition of the soils, analyses were performed on the 2-mm fraction samples. Soil samples (5g) were dried for 42 h at 60°C in 50-mL Falcon tubes. The extraction method, adapted from Soltanpour & Schwab, 1977, consisted of a digestion with 20 mL of 1 M NH_4HCO_3, 0,005 M diaminetriaminepentaacetic acid, and 5 mL of pure water during 1 h of shaking on a rotary shaker at low speed. Each sample was gravity filtered through qualitative filter papers until obtaining approximately 5 mL of filtrate, which was transferred into Pyrex tubes; 0.7 mM trace grade c. HNO_3 was added and digested at 115°C for 4,5 h. Each sample was diluted to 6.0 mL with 18 MV of water and analysed for Cd, Co, Mn and Ni content (ppb) on an Elan DRCe ICP-MS (PerkinElmer Sciex). National Institute of Standards and Technology traceable calibration standards (ULTRAScientific) were used for the calibration.

Phenolic Compounds

Extraction and analysis of phenolics compounds were made in collaboration with Estación Experimental Aula Dei

Extraction of phenolic compound of nutrient solutions and roots

Nutrient solutions were changed weekly and sampled at 14 days after the onset of Fe deficiency treatment, and immediately stored at -20 ºC until extraction of phenolic compounds.

Phenolic compounds in the nutrient solutions (100 ml of solution used for the growth of 4 plants) were retained in a SepPack C18 Cartridge (Waters), eluted from the cartridge with 2 ml of 100% LC-MS grade methanol, and the eluates stored at -80°C. Samples were thawed and a 300 µl aliquot was dried under vacuum (SpeedVac) alone or supplemented with 10 µl of a IS solution (80 µM Artemicapin C and 150 µM Matairesinol). Dried samples were dissolved in 15% methanol and 0.1% formic acid to a final volume of 100 µl, and then analyzed by HPLC-MS.

Frozen roots (100 mg) were ground in liquid N2 using a Retsch M301 ball mill (Restch, Düsseldorf, Germany) for 3 min and then phenolic compounds were extracted with 1 ml of 100% LC-MS grade methanol, either alone or supplemented with 20 µl of a IS solution (37.5 µM Artemicapin C, 50 µM Esculin and 37.5 µM Matairesinol) by homogenization in the same mill for 5 min. The supernatant was recovered by centrifugation (12,000 g at 4°C and 5 min), and stored at -20°C. The pellet was re-suspended in 1 ml of 100% methanol, homogenized again for 5 min and the supernatant recovered. The two supernatant fractions were pooled, vacuum dried in a SpeedVac (SPD111V, Thermo-Savant, Thermo Fisher Scientific, Waltham, Massachusetts, MA, USA) and dissolved with 250 µl of a solution containing 15% methanol and 0.1% formic acid. Extracts were filtered through poly-vinylidene fluoride (PVDF) 0.45 µm ultrafree-MC centrifugal filter devices (Millipore) and stored at -80°C until analysis.

Phenolic compounds were extracted from roots and nutrient solutions as described in Fourcroy *et al.,* (2014), with some modifications described previously. First, extraction was carried out without adding internal standards (IS) to identify relevant compounds. This extract was also used to check for the presence of the compounds used as IS and other endogenous isobaric compounds that may co-elute with them, since in both cases there will be analytical interferences in the quantification process. The extraction was then carried out adding the following three IS compounds (Figure II-5): Artemicapin C, a methylenedioxy-coumarin, for quantification of the coumarins scopoletin, fraxetin, isofraxidin and fraxinol; Esculin, the glucoside form of the coumarin esculetin, for quantification of coumarin glycosides; and the lignan Matairesinol, for quantification of coumarinolignans.

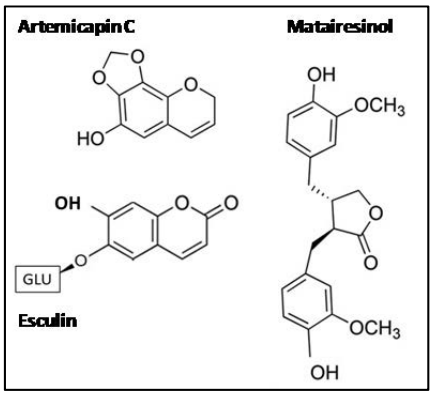


Figure II-5: Internal Standards used for phenolic compound quantification: Artemicapin C, Esculin and Matairesinol

Analysis HPLC-UV/VIS/ESI-MS(TOF)

HPLC-UV/VIS/ESI-MS(TOF) analysis was carried out with an Alliance 2795 HPLC system (Waters) coupled to a UV/VIS (Waters PDA 2996) detector and a time-of-flight mass spectrometer [MS(TOF); MicrOTOF, Bruker Daltonics, Bremen, Germany] equipped with an electrospray (ESI) source. The ESI-MS(TOF) operating conditions and software used were as described in Fourcroy *et al.,* (2014). Mass spectra were acquired in positive ion mode in the range of 50–1000 mass-to-charge ratio (m/z) units. The mass axis was calibrated externally and internally using Li-formate adducts [10 mM LiOH, 0.2% (v/v) formic acid and 50% (v/v) 2-propanol]. The internal mass axis calibration was carried out by introducing the calibration solution with a divert valve at the first and last 3 min of each HPLC run. Molecular formulae were assigned based on exact molecular mass with errors <5 ppm (Bristow, 2006).

Concentrations of phenolic compounds were quantified using external calibration with internal standardization except for Cleomiscosins because no reference product was available. The levels of the Cleomiscosins are expressed in peak area ratio, relative to the lignan Matairesinol used as IS. For quantification, analytes and IS peak areas were obtained from chromatograms extracted at the m/z (±0.05) ratios corresponding to $[M^+H]^+$ ions, with the exception of glycosides, where the m/z ratios corresponding to [M-hexose+H]+ ions were used.

Results and Discussion

Results

Hydroponic Experiments

When grown in hydroponics without Fe supply, plants from both demes exhibited chlorosis (Figure II-6 A). Analysis of chlorophyll concentrations on 20 plants from each deme revealed statistically significant differences (P< 0.05) in leaf chlorophyll concentration between both demes. A1 show higher levels of chlorophyll than T6 (Figure II-6 B).

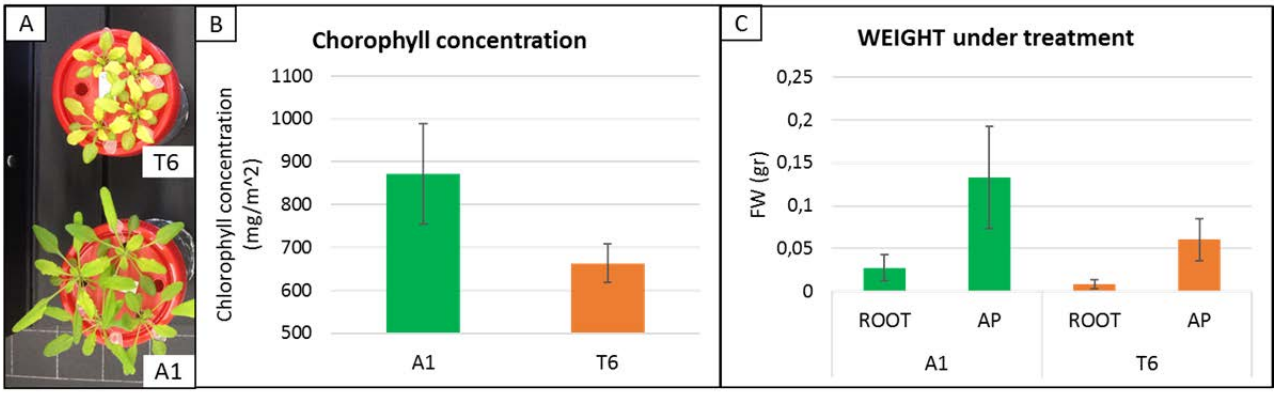


Figure II-6 (A): photo of hydroponics, up to down T6 and A1 under iron deficiency treatment. (B): Chlorophyll concentration (mg/m^2) of A1 and T6 under treatment (T) and control (C). (C): Fresh Weight of aerial part (AP) and roots (ROOT) of A1 and T6 under treatment

To further characterize the response in both demes, the activity of different Fe-deficiency marker enzymes was analysed in the roots of hydroponically grown plants comparing low/high pH (5.5/7.5) treatments. Activities of SOD, CAT and FCR were considered (Figure II-7.A,B,C).

Catalase activity was very low in both demes and no differences due to solution pH were observed (Figure II-7.B). Under the slightly acidic control conditions (pH 5.5) both demes displayed the same relatively low SOD activity. At pH7.5, SOD activity was substantially increased in both demes, but with significantly higher values for A1 originally coming from a soil with enhanced carbonate levels, than for T6 coming from siliceous soil (Figure II-7.A).

In growth medium with pH 5.5, both demes did not differ in ferric reductase activity (FCR), which was relatively low. Contrastingly, T6 displayed considerably higher FCR activity than A1 when grown under slightly alkaline conditions (pH 7.5) (Figure II-7.C).

Monitoring plant-induced pH changes in the nutrient solution during this experiment revealed that both demes tended to decrease the solution pH when grown under slightly alkaline conditions. Plants from deme A1 decreased the pH from the initial value of 7.5 to 5.9 (±0.3), while T6 was less efficient lowering decrease the pH level from 7.5 to 6.9 (± 0.2) (Figure II-7.D).

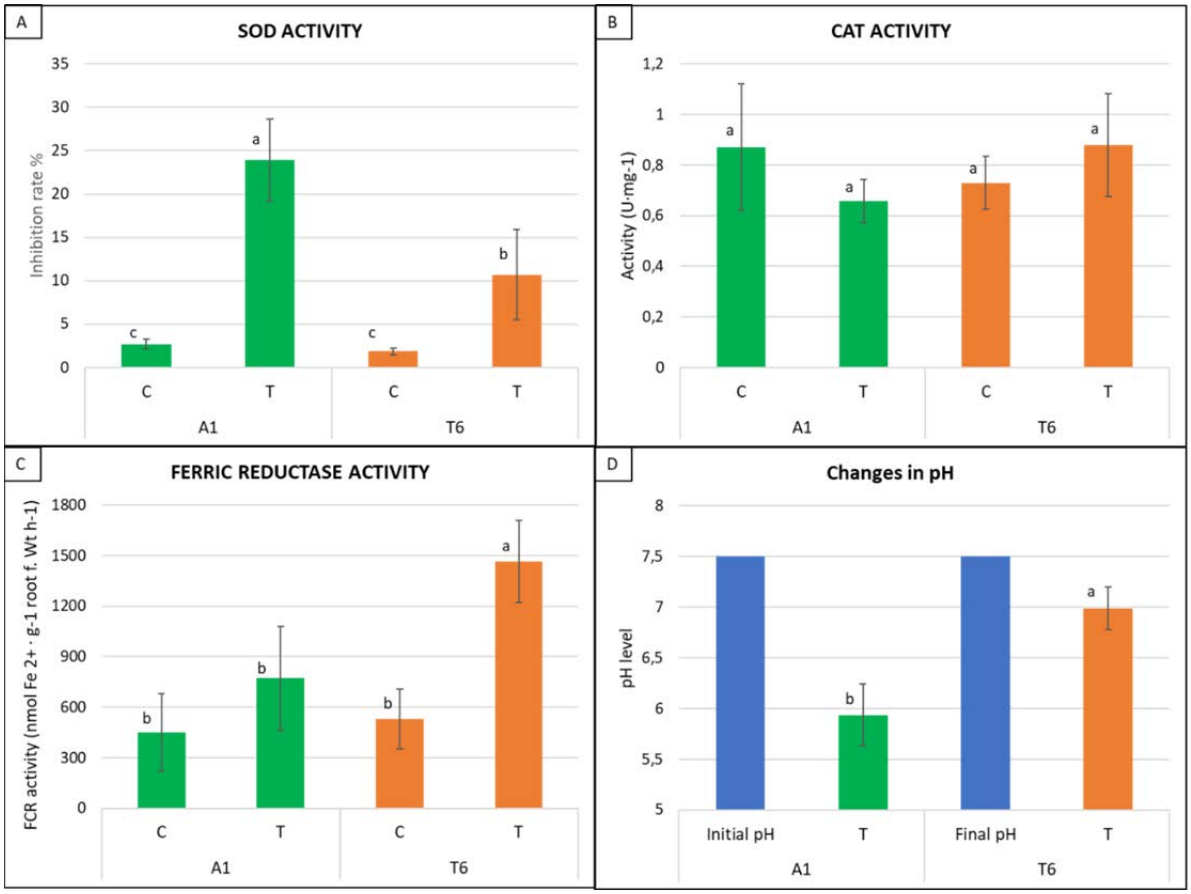


Figure II-7: (A) SOD activity (inhibition rate%) for demes T6 and A1 under treatment (T) and control (C) conditions; (B): CAT activity (U·mg-1) for demes: A1 and T6 under treat. (T) and cont. (C); (C): Ferric Chelate Reductase activity for demes T6 and A1 under treat. (T) and cont. (C); (D): level of pH after a week of treat., initial pH: 7,5. Treat. (T): pH 7.5, Cont. (C): pH 5.5. Mean and Standard deviation is presented in all graphics

Soil experiments

Demes A1 and T6 were grown in the same soils that had already been used for the reciprocal transplant experiment described in chapter I; control soil from Santa Coloma de Farners (SCF) and carbonate soil from Les Planes (LP) (Table 1- Chapter I). Plant growth rates are displayed in Figure II-8-A, as increase of rosette diameter. Both demes grew better in SCF than in LP soil. The growth of A1 plants was the same in both soils, while rosette diameter of T6 was markedly decreased in carbonate soil (Figure II-8.B).

Further experiments with plants from both demes grown on potting mix either irrigated with distilled water or 20mM of bicarbonate also revealed clear growth differences between the demes (Figure II-8-C,D). Both suffered growth inhibition when irrigated with bicarbonate solution. However, in plants from A1 the decrease was not as pronounced as in T6 and only in T6 the differences in rosette diameters between the treatments were statistically significant.

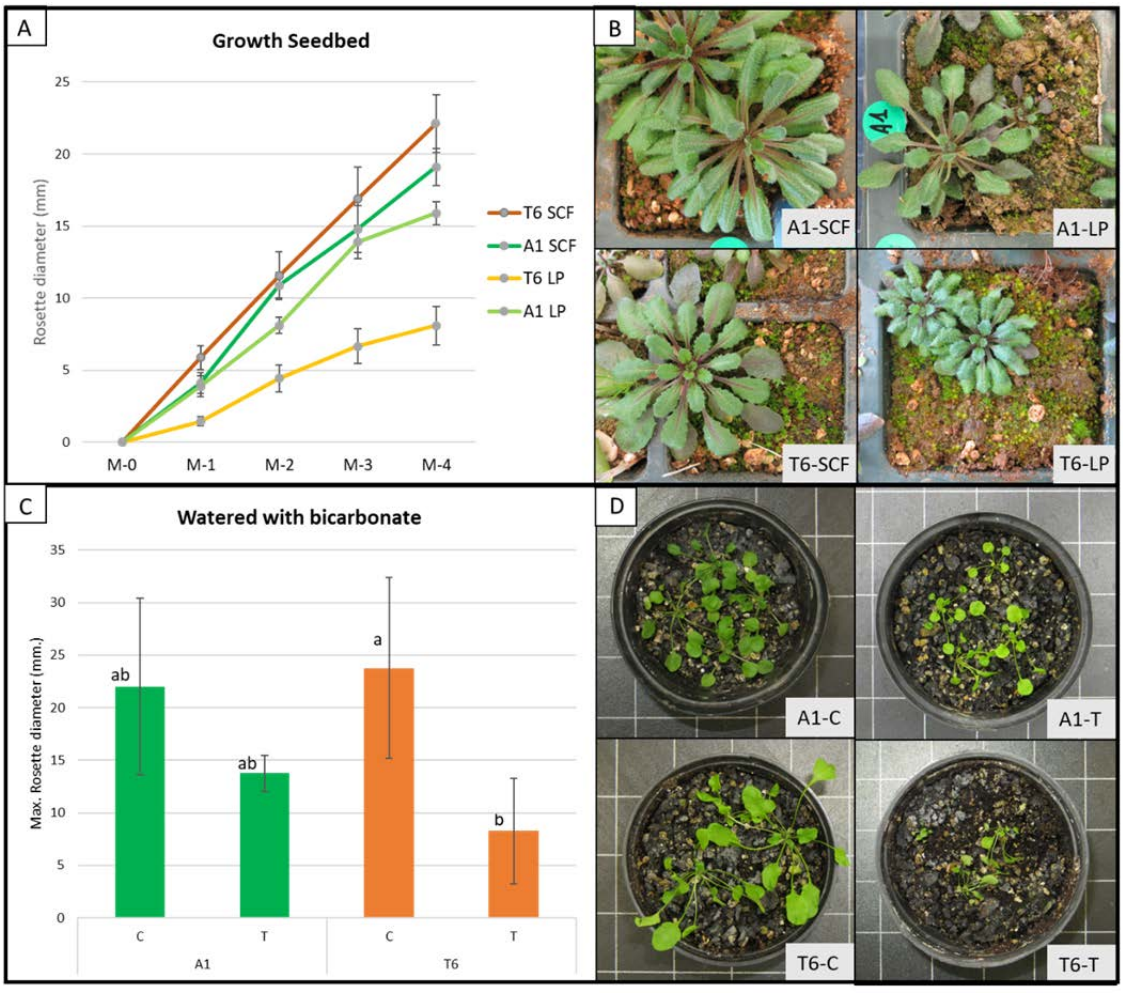


Figure II-8: (A): Increment of rosette diameter of A1 and T6 grown in carbonate soil (LP)and control soil (SCF) during one month; (B) Photographs of A1 and T6 in and control soil (SCF) and carbonate soils (LP); (C) maximum rosette diameter after watering A1 and T6 during one month with either 20mM $NaHCO_3$ (treatment, T) or distilled water (Control, C); (D): Photographs of A1 and T6 under treatment (T) and control (C) conditions.

FPN2 genotype

Previous investigations on natural population in the region reported the occurrence of genetic variability concerning the DNA sequence of the ferro metal efflux protein AtFPN2 (Busoms, 2015 a). To see whether such differences could be related to the differential behaviour of the demes studied here, the *AtFPN2* gene was genotyped in A1 and T6 demes.

The results show that the DNA sequence for *AtFPN2* differed between both demes: A1 has the *AtFPN2* allele like Col.0, while T6 has the *AtFPN2* allele like Ts-1. (Figure II-9.A,B).

To further visualize functional consequences of this genetic difference, analysis of leaf concentration of different divalent metals was done in A1 and T6 gown in SCF and LP soil.

For cobalt (Co) both demes showed an increase of shoot metal concentrations when growing in LP soil, but A1 had lower Co levels than T6 ($p < 0{,}005$). On SCF soil Co shoot levels were lower, but also statistically differences between A1 and T6 were found (Figure II-10.A).

Nickel concentrations followed the same pattern as cobalt; for both demes concentrations were low on SCF soil without statistically differences between demes ($p > 0{,}005$), while concentrations in the shoot increased when plants grew on LP. Again, concentrations in T6 were higher than in A1 ($p < 0{,}005$).

Regardless the soil type, manganese shoot concentrations were lower in A1 than in T6 ($p < 0{,}005$). Contrastingly, the soil type had a strong influence on shoot Fe concentrations, which were higher in plants on SCF soil than on LP soil. For T6 the difference was statistically significant, but this was not the case for A1 (Figure II-10.E).

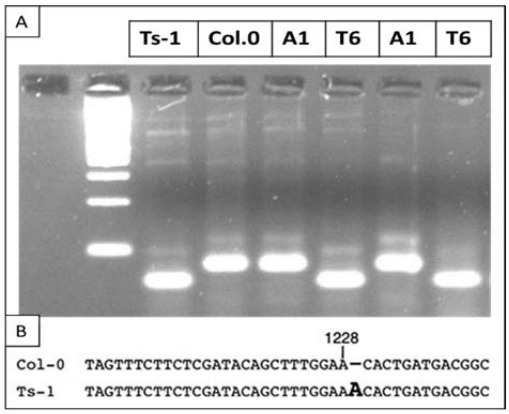


Figure II-9: (A) Picture of PCR gels from A1 and T6 demes in comparison to Ts-1 and Col.0. (B): Alignment of FPN2 showing adenine inserted after position 1228 of the Ts-1 genomic sequence (mod. From Morrissey *et al.*, 2009).

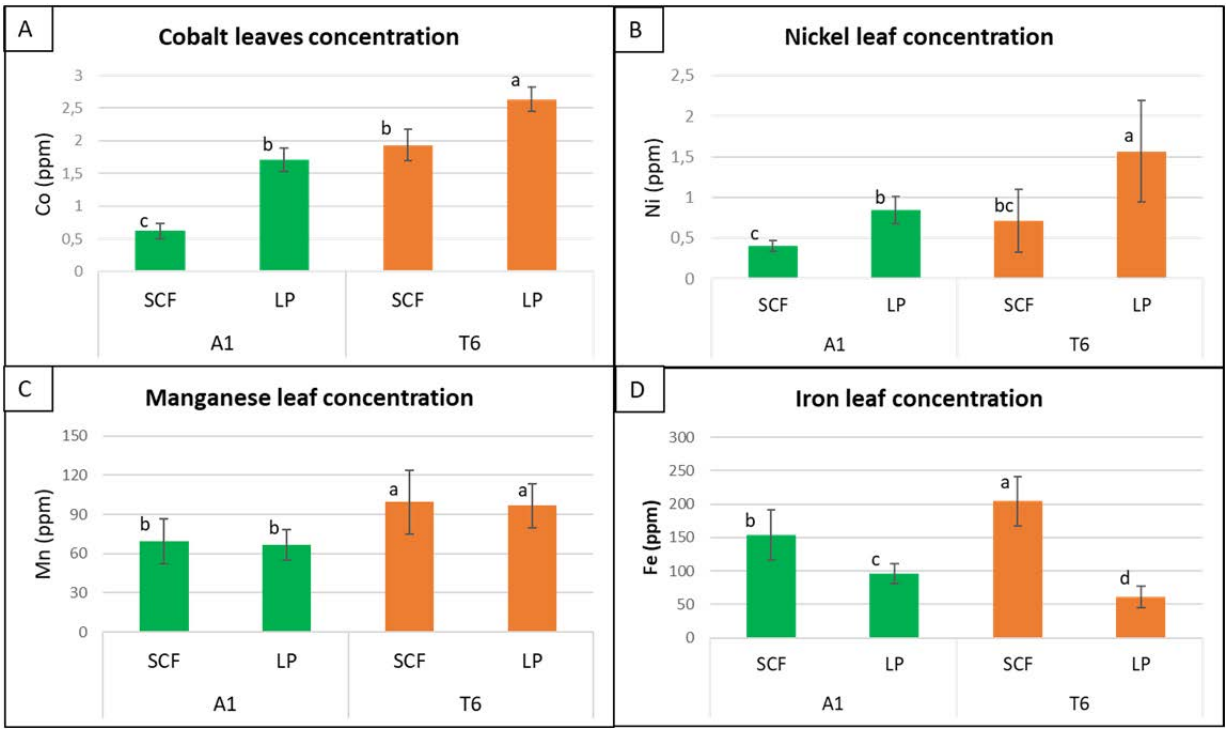


Figure II-10: Leaf concentrations (ppm) mean ± standard deviations of selected micronutrients of A1 and T6 grown in SCF and LP soils; (A): Cobalt (Co), (B): Nickel (Ni); (C) Manganese (Mn); (D) Iron (Fe).

Phenolic compounds in roots and root exudates

Structural formula of the identified phenolic compounds is shown in **¡Error! No se encuentra el origen de la referencia.**. Differences in hydroxyl groups and methoxylation are related to the tendency of Fe complex formation. Structural differences are also related to antioxidant activity which may be relevant for maintenance of Fe in the reduced state.

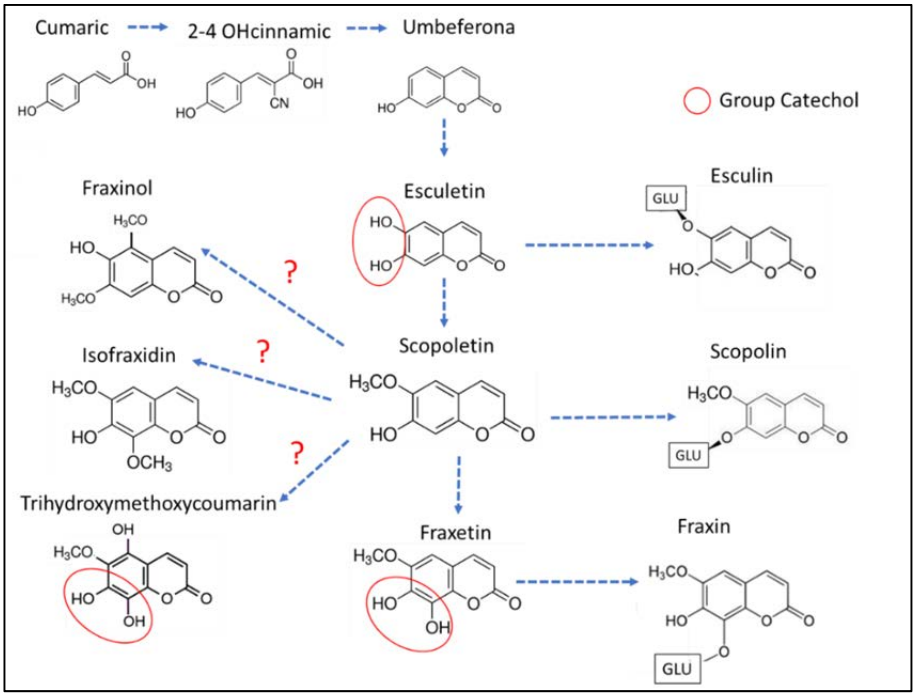


Figure II-11: Scheme of biosynthesis pathway of coumarins. Red circles show the group catechol present in esculetin, thrihydroxy-methoxycomuarin and fraxetin

Phenolic patterns of root extracts of both demes were quite similar. The major compound was scopolin (86-87%), followed by fraxetin (5-8%), scopoletin (4-6%) and with minimum concentrations of trihydroxymethoxycoumarin (1%) and Fraxinol (1%) (Figure II-12.A).

Differences between both demes were found in nutrient solutions. Quantitatively, plants from A1 secreted 2.5 times more total phenolics than T6 (Figure II-12.B.1). Qualitatively, plants had a different profile of the studied compounds (Figure II-12.A).

Scopoletin was the main component of exudates in T6 (86%), while in A1 fraxinol (50%) followed by scopoletin were the most representative fractions (Figure II-12.A). No differences in the percent distribution for fraxetin (10%) and esculetin (2-4%) were observed between the deme exudates, while in A1, but not in T6, isofraxidin (6%) was detected.

Esculetin, trihydropxymethoxycoumarin, and fraxetin bear catechol groups (Figure II-12.B.2 Both demes present a similar catechol/no catechol compounds ratio in root exudates.

Deme T6 exudate higher cleomiscocines concentrations. Contrastingly, A1 had higher cleomiscocines concentrations inside root tissues.

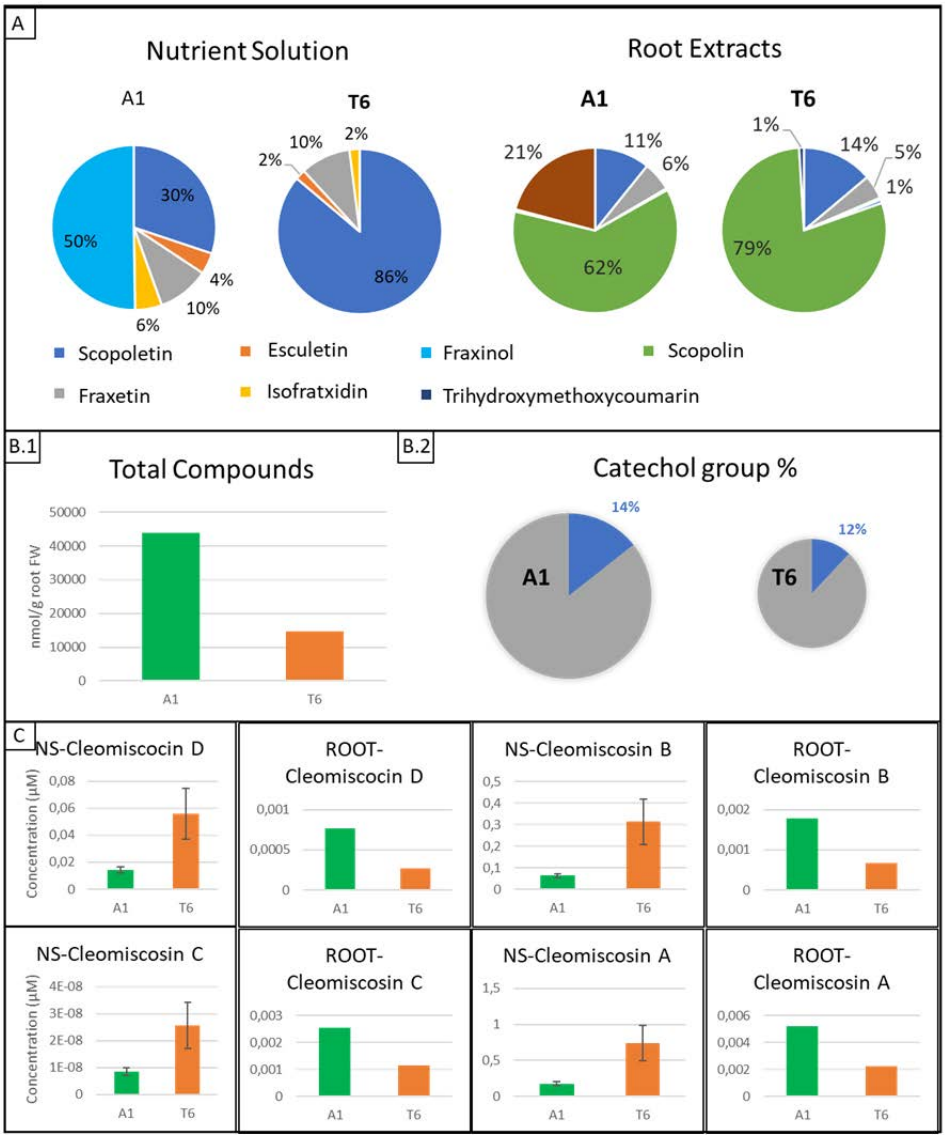


Figure II-12: (A): Proportion of different compounds in Nutrient solution and root extracts. (B)-1: Total compounds (nmol/g root FW) of A1 and T6 demes. -2: Proportion of catechol group% in relation of total compounds per deme (Diameter of circle of A1 2.5 time big than t6). (C): Cleomiscocin (D, C, B, A) concentrations (µM).

Discussion

Based on our field survey reported in chapter 1, two demes with contrasting behaviour were chosen for further analysis of physiological traits related to carbonate tolerance. In this chapter 2, deme A1 (from group nº3 of %$CaCO_3$, Chapter I) as a representative of "moderate-tolerant to carbonate soils" and deme T6 (from group nº1 of % $CaCO_3$, Chapter I) as a representative of "sensitive to carbonate soils".

After two weeks of treatment clear differences between Fe sufficient and Fe deficient plants were visible (Figure II-6-A). A decrease of leaf chlorophyll content was observed in both demes (Figure II-6-B). Under control conditions, deme T6 shows a higher initial chlorophyll concentration than A1. However, under iron deficiency, a considerable decrease of chlorophyll

concentrations was observed in T6, while in A1 no statistical differences between chlorophyll levels of control and iron deficient plants were found. Higher chlorophyll leaf concentrations in A1 under Fe deficiency cannot be attributed to a concentration effect caused by growth inhibition. On the contrary, A1 plants had higher root and shoot fresh weights than plants from T6 (Figure II-6-C). As all plants were pre-cultured the first two weeks with a Fe-containing control solution, this result could indicate higher efficiency in the use and mobilization of the previously accumulated iron in A1 than in T6.

To increase Fe availability in the rhizosphere under Fe-deficient conditions, dicots and non-gramineous monocots increase their ferric reduction capacity at the root surface, enhance proton excretion in the rhizosphere, and release reductants and chelators (Römheld and Marschner, 1983). Reduction by ferric chelate reductase is thought to be the rate-limiting step in Fe uptake (Grusak *et al.,* 1990). The expression of the gene responsible for ferric reduction in *A. thaliana*, *FR02* (Robinson *et al.,* 1999) involves posttranscriptional regulation, as shown for iron regulated transporter 1, IRT1 (Connolly *et al.,* 2002, 2003). Overexpression of the *FR02* gene leads to improved growth in low-Fe conditions at pH 6.0. After FeIII reduction, iron is transported into the epidermal cells by the divalent metal transporter IRT1 (Vert *et al.,* 2002), that also transports zinc, manganese, cadmium, cobalt (Korshunova *et al.,* 1999), and nickel (Schaaf *et al.,* 2006). Iron likely moves symplastically to the pericycle, where it then needs to be exported into the xylem to move to the shoot. FPN2 has previously been reported to be expressed in the roots of iron-deficient plants (Colangelo and Guerinot, 2004) and to localize to the vacuolar membrane (Schaaf *et al.,* 2006). Although upregulated in response to iron deficiency, FPN2 also functions in nickel sequestration (Schaaf *et al.,* 2006).

The reduction of Fe III to Fe II is generally localized on the surface of the subapical parts of the root, as well as in the root hairs (Moog and Brüggemann, 1994). Reduction of iron occurs in the plasma membrane through a specific enzyme, ferric-chelate reductase, capable of reducing chelated-Fe III.

The process requires the generation of a coordination vacancy in the FeIII L6 complex I (Figure II-13) to get the most stable species in solution at pH<7 to achieve the octahedral environment around the metal by incorporation of a single water molecule from proton excretion (Escudero *et al.,* 2012). The optimum pH for the reduction of Fe III in intact roots, in vivo, is around 5.5. For this reason, most experiments to analyses ferric reductase activity are performed in this pH range (Romera *et al.,* 1999). The reduction of Fe varies with the chelate concentration, following Michaelis-Menten kinetics (Bienfait *et al.,* 1983). The presence of heavy metals such as Ni and Cu negatively affects the reduction (Romera *et al.,* 1998, Schmidt, 1999).

Susin *et al.,* (1996) reported that the FCR activity at pH 6.5 was much lower than at pH 6.0. Further decreases occurred at pH higher than 6.5. In the Fe-sufficient plants, the FCR activity did not change from pH 3.0 to pH 6.5 but decreased at higher pH values.

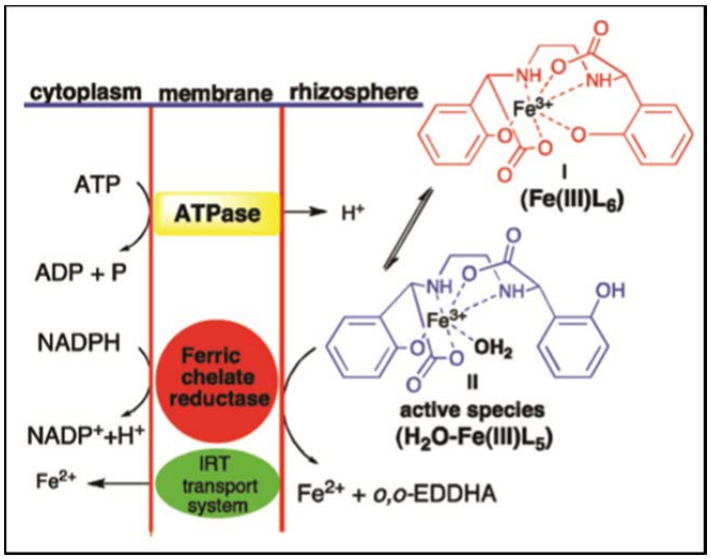


Figure II-13: Scheme of Fe reduction. Mod from Escudero *et al*., 2012

Here we compared FCR activity between both contrasting *A. thaliana* demes growing in solution with an initial pH of 7.5 or 5.5. In our study plants treated with pH 7.5 showed higher FCR activity than controls, in both demes. These results demonstrate that FCR enzyme works well in both demes at pH 5.5. Plants that had been cultivated for 2 weeks at pH7.5 may have lower tissue Fe than the controls (pH 5.5). This may explain the higher FCR activity in the plants from the basic nutrient solution treatment.

For studies in carbonated soils, the pH ranges are high, so the reduction of FeIII to FeII is a limiting factor closely related with high pH in the medium. Here we found that T6 demes have a high ferric-reductase activity, in comparison to demes A1 (Figure II-6.D). On a first glance, this seems incongruous with the studies that show that in plants adapted to iron deficit, the activity of ferric-chelate reductase increases when iron is deficient (Chaney, *et al.,* 1972; Bienfait, 1985). In our study leaves of T6 demes were more chlorotic and levels of chlorophyll were lower than A1. So, the enhanced ferric –reductase activity in T6 was rather a sign of Fe deficiency than an efficient mechanism for Fe acquisition.

The relationship between proton excretion and Fe reductase is not yet clear. Bienfait (1985) proposed a route to connect both phenomena: the proton extrusion would be coupled to the accumulation of citrate; this citrate would be isomerized to isocitrate that would lead to reduction of the $NADP^+$ and the formation of α-oxoglutarate. The obtained $NADPH^+$ would directly or indirectly donate electrons to the FCR of the plasma membrane. However, when simultaneous measurements of both phenomena have been carried out in fruit tree seedlings, only a significant pH decrease in the nutrient solution in plum varieties has been found, where the maximum FCR activity is reached when acidification is highest (Romera *et al.,* 1999).

In Figure II-6.C we can see that A1 is able to decrease the pH to values around 6, while T6 maintains the pH of the rhizosphere at values close to 7. Considering the optimum pH values for the ferric reductase activity discussed above, it is clear that A1 presents the ideal scenario

for developing ferric reductase activity, while T6 is outside the ideal pH required by the proper activation of the ferric reductase.

It is evident that the limiting factor for the absorption of iron in T6 is not the state of the enzyme, because when its activity at pH 5.5 is evaluated it shows high values, but apparently, it is its inability to decrease the pH of the medium, which causes that ferric reductase cannot develop this function correctly.

A. thaliana thaliana plants produce and secrete an array of phenolics in response to Fe deficiency when the pH of the nutrient solution is high. Phenolics found in this study include previously reported coumarins (scopoletin, fraxetin, isofraxidin and fraxinol) and several coumarinolignans recently reported *in A. thaliana* (cleomiscosins A, B, C, and D) (Sisó-Terraza *et al.,* 2016a).

The secretion of coumarins by Fe-deficient roots involves an ABC (ATP-binding cassette) transporter, ABCG37/PDR9, which is strongly over-expressed in plants grown in media deprived of Fe (Yang *et al.,* 2013; Fourcroy *et al.,* 2014, 2016) or containing insoluble Fe(III) at high pH (Rodríguez-Celma *et al.,* 2013). The export of scopoletin, fraxetin, isofraxidin, and an isofraxidin isomer was greatly impaired in the mutant abcg37 (Fourcroy *et al.,* 2014), which, as it occurs with F6'H1, is inefficient in taking up Fe from insoluble Fe(III) at pH 7.0 (Rodríguez-Celma *et al.,* 2013). It was found a significant level of secreted coumarins for both demes that suggest no obstacle with ABC transporter, ABCG37/PDR9.

Catechol groups promotes Fe-complex formation that increase the Fe-III mobilization (Schmid *et al.,* 2014). Three coumarins containing the catechol group (trihydroxymethoxycoumarin, esculetin and fraxetin) were found in root exudates of A1 and T6. Even the percentage of coumarins with catechol groups was similar for both demes (14%-12% respectively), while the biggest differences were found in quantity of total compounds. In other words, both demes shown the same proportion of catechol-coumarins, but A1 produce 2.5 times more catechol-coumarins than T6. This trait could explain the phenotypical chlorosis differences observed in A1 and T6.

Fe deficiency present a strong impact on redox homeostasis, not only because free Fe ions induce ROS formation via Fenton reactions, but also because many proteins involved in oxidative stress such as peroxidases and catalase are Fe-containing proteins.

These changes included increases in superoxide dismutases (SOD), monodehydroascorbate reductase (MDAR1), peroxidase 12 (PER12) and a decrease in catalase (CAT-2).

Differences in SOD activity were observed between both demes and treatments. Increases of SOD-activity, as a result of the compensatory mechanism of the SOD isoenzymes, have already been reported for in *Pyrus dulcis × P. persica, Medicago truncatula,* and *A. thaliana* (López-Millán *et al.,* 2013). However, it is interesting to analyse the differences in this increase between two demes with contrasting behaviour: for A1 the increase of SOD activity was 2.5

times greater than for T6. This indiactes a better ROS defence mechanism in A1 than in T6 (Figure II-7.A).

The decrease of catalase activity is likely associated with the decrease in Fe availability and it is observed in *S. lycopersicum and P. dulcis × P. persica*, (López-Millán *et al.,* 2013), but in our experiment, we did no appreciate significant differences neither intra treatments, nor between demes (Figure II-7.B); in general CAT-activity was extremely low.

In some species, the treatment of plants with bicarbonate improves the FeIII reduction capacity of the roots; examples are pear trees (Donnini *et al.,* 2009), vinyard (Ksouri *et al.,* 2007), peanut (Zuo *et al.,* 2007), *A. thaliana* (Msilini *et al.,* 2009) and pea plants (Jelali *et al.,* 2010). In roots of these species a bicarbonate induced increase of the FCR activity was observed. However, the values did not reach the high levels detected in plants cultivated without Fe. However, other studies show opposite results, in cucumber, pea, tomato and *A. thaliana* (Waters *et al.,* 2007) and in citrus and peach trees with a high supply of HCO_3^- . Bicarbonate supplied in high concentrations causes a reduction of FCR activity (Chouliaras *et al.,* 2004; Molassiotis *et al.,* 2006).

Bicarbonate in the soil solution is a strong pH buffer, mainly in the presence of calcium carbonate. Since bicarbonate is quite mobile, and CO_2 diffusion is a slow process, the pH decrease in such soils after proton release by plants is small (Lucena, 2000). Also, the ferric reductase activity of plant roots declines sharply at high pH values. The chemical FeIII reduction depends on the pH; so, the lower the pH, the more favoured is the formation of Fe(II) from the Fe(III) in the rhizosphere. Although FCR activity was not determined in this experiment, chlorosis observed in leaves of treated plant (Figure II-8.B,D), especial for T6, could be a consequence of that phenomenon.

Plants watered with 20 mM of bicarbonate showed a decrease of growth for both demes, but this inhibitory effect of growth was much stronger in T6 than A1 (Figure II-8.C,D). This relation between growth inhibition and bicarbonate in the medium has previously been reported for cucumber plants after 10 days of treatment with 10mM bicarbonate (Garcia *et al.,* 2014), and also for pea, with a 15mM bicarbonate treatment (Barhoumi *et al.,* 2007). No differences were observed for tomato productivity (Kg/plant) under treatments with 2.5-5 mM of bicarbonate (Parr-Terraza *et al.,* 2012); however, this study was performed with solutions adjusted to pH: 5.5±0.1 (with HCl 1N or NaOH 1N).

These results carry on evidencing that one of the most limiting factors to growth in carbonate soils is the pH level. A1 and T6 demes show a decrease of growth both in carbonated soils and watered with bicarbonate solution, but T6 present a higher difference between control treatments, suggesting a clear pattern on of sensitivity in comparison to A1, which exhibits a tolerant behaviour.

FPN2

Under iron deficiency, IRT1 transports iron and other metals from the rhizosphere to the root cytoplasm. As under iron deficiency stress this transporter is upregulated not only the uptake of Fe, but also that of other metal ions can be increased. This may lead to an excessive accumulation of these metals. FPN2 may transport part of these excess metals to the root vacuole so avoiding its transport to the xylem via FPN1 and preventing the translocation of potential toxic metal to the aerial part.

Results of Ni, Co, Mn concentrations in leaves (Figure II-10.C,B,D) show that A1 presents lower concentrations than T6. The concentrations of Ni, Co, Mn in aerial part of plants grown in carbonate soils, suggest that A1 is more capable that T6 to avoid the metal translocation to the xylem. These accessions present a different allele for FPN2, A1 like col.0 and T6 like Ts-1. The difference is a frame shift in Ts-1, which produces a stop codon 117 amino acids earlier than in Col-0.

These results are supported by experiments of Morrysey (2009), who found that without FPN2 cobalt is not sequestered in the root vacuoles; instead, it is able to move to the shoot via FPN1, resulting in an increase in shoot cobalt and cobalt sensitivity. In a similar way, Schaaf (2006) demonstrated that wild type plants in comparison to mutants for FPN2 accumulate less nickel in roots and show increased nickel sensitivity under iron deficiency.

We propose that this modification in FPN2 results in a decreased activity of metal transport into the root vacuole. On the one hand, this induces the high accumulation of toxic metal in aerial parts and, on the other hand, increases the iron deficiency through the competition with other metals to be transported by FPN1.

In Figure II-14 is proposed a model of action of A1 and T6 under iron deficiency.

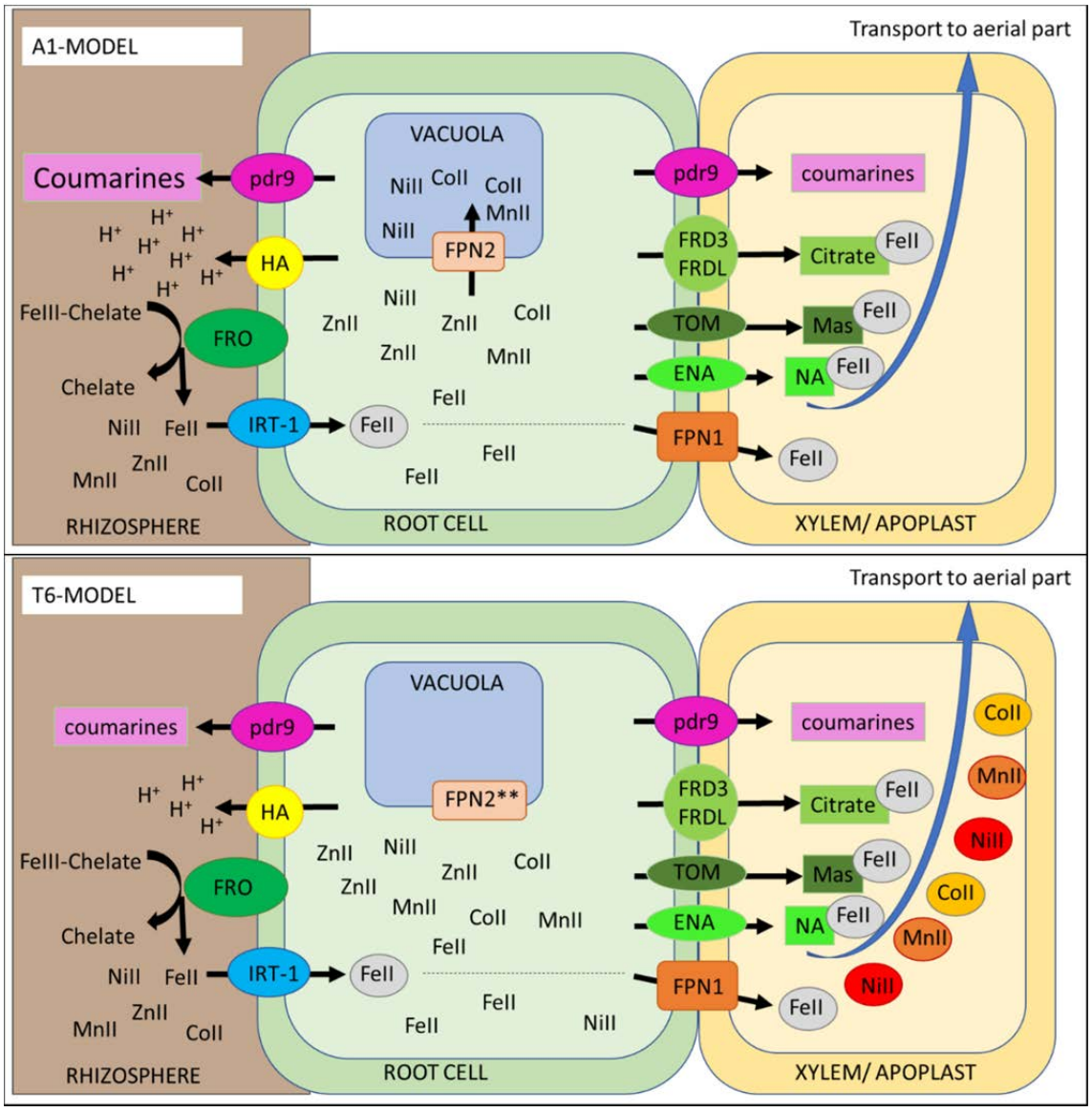
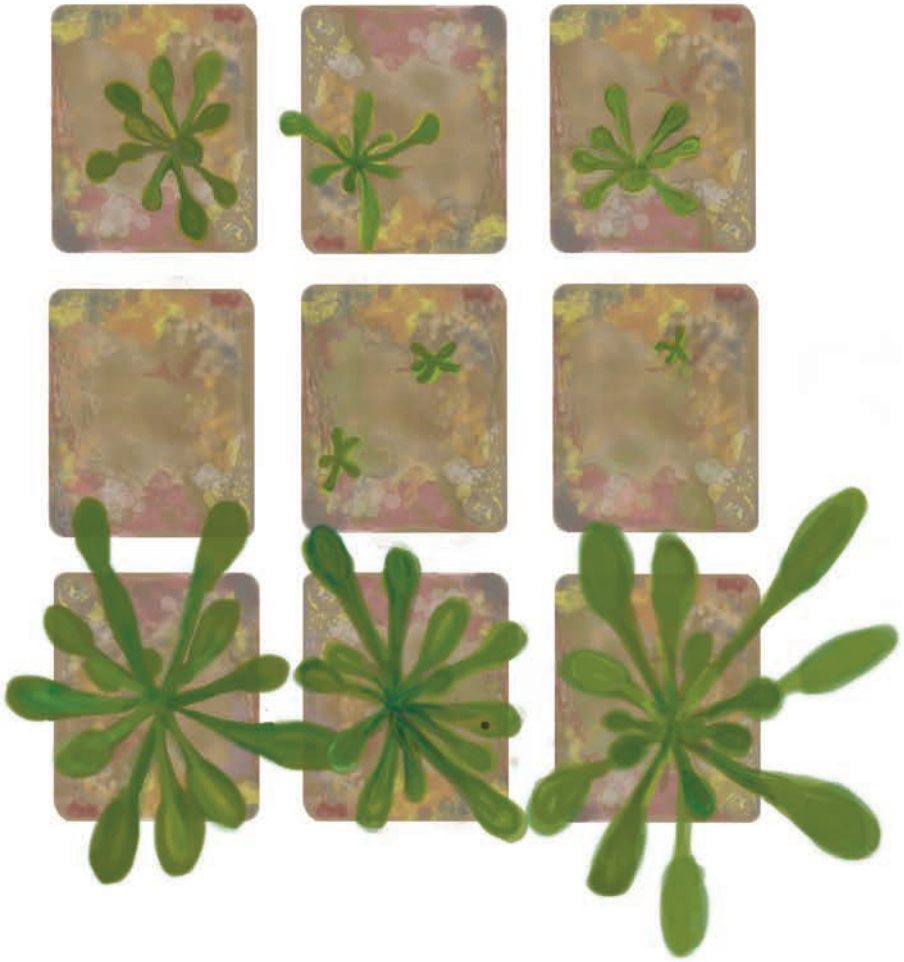


Figure II-14: Scheme of mode of action of A1 (upper image) and T6 (lower image) under iron deficiency

Conclusions

- Two demes of *A.thaliana* originally occurring on soils differing in carbonate content display clear differences in tolerance to iron deficiency and/or carbonate
- Under Fe deficiency, A1, deme from soil with low carbonate content was able to maintain higher chlorophyll concentrations than T6, originally growing on siliceous soil without carbonate.
- On carbonate soil, fitness, in terms of siliques production, was considerably more affected in T6 than in A1.
- The adaptive responses of A1 versus iron deficiency, bicarbonate in the medium, and high pH, were maintenance of chlorophyll concentration, ability to diminish pH of rhizosphere, higher quantity of exudates, and early activation of SOD activity, an efficient mechanism for controlling ROS.
- Moreover, in contrast to T6, the ability of A1 to lower the rhizosphere pH provides ideal conditions for FCR activity.
- The dimorphism of FPN2 leading to lower metal ion vacuolar storage in T6 may be a main reason for the higher shoot translocation of Co, Ni and Mn in this deme, which may interfere with efficient Fe translocation leading to higher sensitivity to carbonate and/or Fe deficiency.

Chapter III
Genetic traits



Introduction

A. thaliana thaliana shows a wide range of genetic and trait variations among wild collected accessions (Shindo *et al.,* 2007). The use of natural genetic variation in *A. thaliana* has already proven to be a very powerful approach for the discovery of novel genes and alleles (reviewed by Alonso-Blanco *et al.,* 2009).

To understand the significance of natural genetic variation in functional terms, it is necessary to identify the traits of ecological relevance and to determine their genetic basis. To achieve this, it is critical to first identify adapted populations in a plant species amenable to the rapid molecular genetic dissection of the phenotype.

A. thaliana is a tempting species for such studies due to its a small genome size, its excellent genomic tools, and extensive collections of native populations, along with the general availability of high-throughput whole-genome resequencing, (Bergelson & Roux, 2010). Different from most of its congeners, *A. thaliana* is self-compatible, and its life cycle can be as short as 6 weeks; both properties greatly facilitate genetic studies. Its native range is continental Eurasia and North Africa (Al-Shehbaz and O'Kane, 2002), but it has been introduced throughout much of the rest of the world, especially around the northern hemisphere.

The analysis of natural variation in wild species has begun to elucidate the molecular bases of phenotypic differences related to plant adaptation to distinct natural environments and to determine the ecological and evolutionary processes that maintain this variation (Mitchell-Olds *et al.,* 2007). The model plant *A. thaliana* shows a wide range of genetic and trait variation among accessions collected in the field. In addition, because of the unparalleled availability of genomic resources, the potential of *A. thaliana* for studies of natural genetic variation is increasingly recognized (Buescher *et al.,* 2010; Weigel, 2012).

Correlations of life history traits with edaphic conditions and interspecific competition (Brachi *et al.,* 2013) in natural populations of *A. thaliana* suggest that these are strong selective agents driving adaption in local populations.

However, the final proof of the adaptive role of a given allelic variant will require testing the fitness effects of alternative alleles of the gene in the same genetic background in the field under the contrasting environmental conditions to which the alleles are assumed to be adaptive.

As a first step towards such proof, reciprocal transplant experiments are usually performed to test if the populations containing the contrasting alleles are locally adapted (Blanquart *et al.,* 2013). Conventionally, local adaptation is considered to exist when demes (local populations or small stands of plants) have higher fitness in their own habitat compared to demes from any other habitat, and this has been termed the 'local vs. foreign' criterion (Kawecki & Ebert, 2004). Ideally, to establish such local adaptation experimentally requires reciprocal transplant

experiments in the field in which the fitness of genotypes from different demes are all directly compared by growing them together in each of the demes local habitats.

When different *A. thaliana* accessions are grown together and compared under similar environmental conditions, genetic variation can be observed for many traits. This kind of experiment has been done in chapter II, to analyze the local adaptation of natural populations of *A. thaliana* to carbonated soils.

To understand the significance of natural genetic variation in functional terms it is necessary to identify the traits of ecological relevance and determine the genetic basis of these traits. Furthermore, such an understanding would provide significant benefits to efforts directed to developing crop varieties that can maintain yields against a backdrop of changing global temperature and precipitation patterns (for review see Friesen & Wettberg, 2010).

Plant breeding is the art and science of changing the traits of plants in order to produce desired characteristics (Poehlman & Sleper, 1995). That activity started with sedentary agriculture and particularly the domestication of the first agricultural plants, a practice which is estimated to date back 9,000 to 11,000 years.

Initially, early farmers simply selected food plants with particular, desirable characteristics and employed these as progenitors for subsequent generations. In consequence, they unconsciously selected for the accumulation of favorable traits over time. Despite the poor understanding of the process, plant breeding was a popular activity. Gregor Mendel himself, the father of genetics, was a plant breeder, as were some of the leading botanists of his time. Mendel's 1865 paper explaining how dominant and recessive alleles could produce the traits we see and could be passed to offspring was the first major insight into the science behind the art. The paper was largely ignored until 1900, when three scientists working on breeding problems rediscovered it and published Mendel's findings.

Mendel established the basis of genetics. The most relevant findings include the Mendelian genetic theory of inheritance, dominance and recessivity of traits, segregation of character recombination or re-assortment of characters, the distinction between germ and soma and between genotype and phenotype, and the finding that chromosomes are the vehicles of the units of heredity

Molecular techniques, particularly large-scale DNA sequencing and expression microarrays, have heralded a new era of research on the evolution and diversity of domesticated plants (Doebley *et al.,* 2006; Burke *et al.,* 2007)

The fusion between the chromosomal and the Mendel theories had many remarkable effects. If Mendelian factors or genes were part of chromosomes, then it was easy to understand why two copies of every gene exist in all cells of a diploid organism. This provided a mechanistic foundation of Mendel’s first law, according to which a zygote receives only one version of a given gene from each parent (law of segregation). But the chromosomal theory also explained

why Mendel's second law (law of reassortment of genes) has many exceptions, since this law does not apply when two gens located in the same chromosome segregate together.

Objectives

In this study, the traits looked for are those that enable the plants to survive in carbonate soil. The first step to achieve this was to characterize the deme already adapted to this kind of soils, as it was done in chapter I and II. The following step will be to select those genes that confers the ability to grow on carbonate soils. This requires the following actions:

- Elaboration of different crossings between contrasting accessions to characterize the heritability of "tolerance to carbonate soil".
- Selection of the crossing showing a clear heredity (F1 like tolerant or sensitive parental) for getting next generations.
- Selection in F2 generation of the crossings showing tolerance to carbonate soil as a dominant trait for getting F3 generations.
- Getting a F3 generation for ensuring a greater genetic variability by gene segregation
- Grouping a pool of most tolerant families to be sequenced (and compare with sensitive demes) by BSA-seq.
- Getting a list of different genes between parental lines (A1 and T6)

Materials and methods

Plant culture

Experiments were performed in the green house of Aberdeen University during the months May to July in 2014, 2015, and 2016.

Soil used in this experiment was from Les Planes d'Hostoles (used in Chapter I as a carbonate soil). Soil was passed through a 2-mm sieve and mixed with perlite (Soil:Perlite, 3:1) for pot cultivation.

The following representative demes from the three groups of carbonate classes defied in chapter 1 were used for the crossings:

- Group 1 of $CaCO_3$%: T6 and T11
- Group 2 of $CaCO_3$%: V1 and LLO2
- Group 3 of $CaCO_3$%: A1 and LM2

The steps taken in each generation are summarized in Figure III-1.

For getting the first generation, F1, a total of 16 crossings were made (8 pairs of crossings) using the same deme as a male and female (e.g. T6 x LLM2 and LLM2 x T6).

Intermediated-crossing: demes from group 1 (T6 and T11) were crossed with a deme from group 2 (V1); demes from group 3 (A1 and LM2) were crossed with a deme from group 2 (T11).

Extreme-crossings: demes from group 1 (T6 and T11) were crossed with demes of group 3 (A1 and LM2).

Measures of dimeter were taken weekly.

In the second generation, F2, experiments were continued with the decency of 2 crosses, A1-T6 and LM2-T6.

Measures of rosette diameter, number of siliques, % of flowering were taken.

In the third generation, F3, A1 and T6 was the chosen crossing. A total of 20 seeds decency of each individual of F2 were planted in carbonate soil.

Measures of rosette diameter, number of siliques, % of flowering were taken in order to select ten families representative of “tolerant-traits”

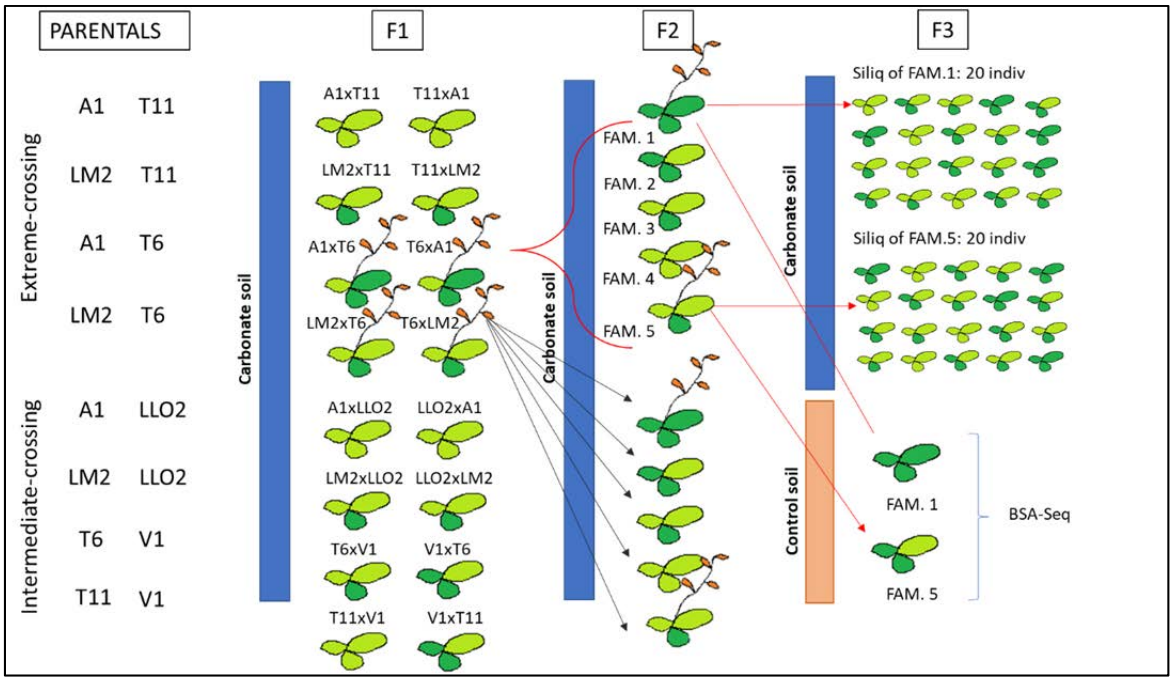


Figure III-1: Scheme of steps to selection of the three generations

Sequencing of parental lines: A1 and T6

Sequencing and data analysis were made in collaboration with Purdue University and John Innes Center.

DNA extraction and sequence processing:

Leaf material from 4 individuals of A1 and 4 individuals of T6 were used for DNA extraction. DNA libraries were prepared using TruSeq DNA PCR-Free Sample Preparation Kit. Whole-genome sequencing was performed on Illumina HiSeq 2000 at 15x coverage.

Raw sequence data were processed as follows: (1) removing duplicate reads using Picard (MarkDuplicates); (2) applying a 'namefix' to the bam files using Picard (AddOrReplaceReadGroups) and (3) realigning Indels using the GATK 'GenomeAnalysis' Toolkit. Bi-allelic SNPs were identified using 'HaplotypeCaller' and genotyped using 'GenotypeGVCF' (both tools in GATK). Data were filtered using GATK SelectVariants using these parameters: QD < 2.0 || MQ < 40.00 || FS > 60.0 || SOR > 4.0 || MQRankSum < -8.0 || ReadPosRankSum < -8.0 and a minimum coverage of 10 per sample.

Data analysis:

To obtain a consensus sequence for each deme, private SNPs of T6 and A1 (shared for the 4 samples) were selected using GATK (selecting AF<0,1 and AF >0.9) and Col-0 TAIR10 sequence as a reference.

Example (Table III-1): For T6, in position X_0 all samples share the same nucleotide with Col-0 so AF will be 1 (AF > 0.9) and in position X_4 all samples differ from Col-0 so the AF of this position will be 0 (AF < 0.1). For positions X0 and X4 in deme A1 the opposite happens. For position X1, X2, and X3 AF will be <0.9 and > 0.1, so those positions will not consider for the consensus list.

	T6				**AF**	**Col-0**	A1				**AF**
Position	T6-1	T6-2	T6-3	T6-4			A1-1	A1-2	A1-3	A1-4	
X0	A	A	A	A	**1**	**A**	C	C	C	C	**0**
X1	A	A	A	C	0.75	**A**	A	C	C	C	0.25
X2	A	A	C	C	0.5	**A**	A	A	C	C	0.5
X3	A	C	C	C	0.25	**A**	A	A	A	C	0.75
X4	C	C	C	C	**0**	**A**	A	A	A	A	**1**

Table III-1: Example to create a consensus sequence.

Once we obtained the consensus sequence, we selected the sites that differ between demes using GATK 'concordance' command obtaining one vcf file with T16 AF<0.1 and A1 AF > 0.9 and a second vcf file with T16 AF>0.9 and A1 AF< 0.1. We merged the files using VCFtools and the amino acid changes between A1 and T6 were obtained and quantified using SNPeff.

From the final list, all genes with less than 3 variant-modifiers were eliminated; also, those with more than 10 variants modifier to avoid the selection of "Highly variables genes".

Results

F1 selection

For intermediated crossings (Figure III-2) all parental and crosses grown at SCF had a similar increase of rosette diameter. When growing on carbonate–rich soil at Les Planes, crosses between A1 deme from group 3 of $CaCO_3$% with group 2 deme LLO2 (Figure III-2.1) showed less increment of rosette diameter that either parental. This was observed for both ways of

crossings (A1xLLO2 and LLO2xA1). However, when LM2 was used as the group 3 parental (Figure III-2.2) a different pattern was observed. Cross LLO2xLM2 showed better growth than parental LLO2, while cross LM2xLLO2 showed the lowest growth. In the crossings with demes from group 1 and 2 of $CaCO_3$% we can observe that cross V1xT11 (where deme from group 2 act as a female) grew better than cross T11xV1 where the group 2 deme acted as a male. The same pattern was observed in T6-V1 crossings (Figure III-2.4).

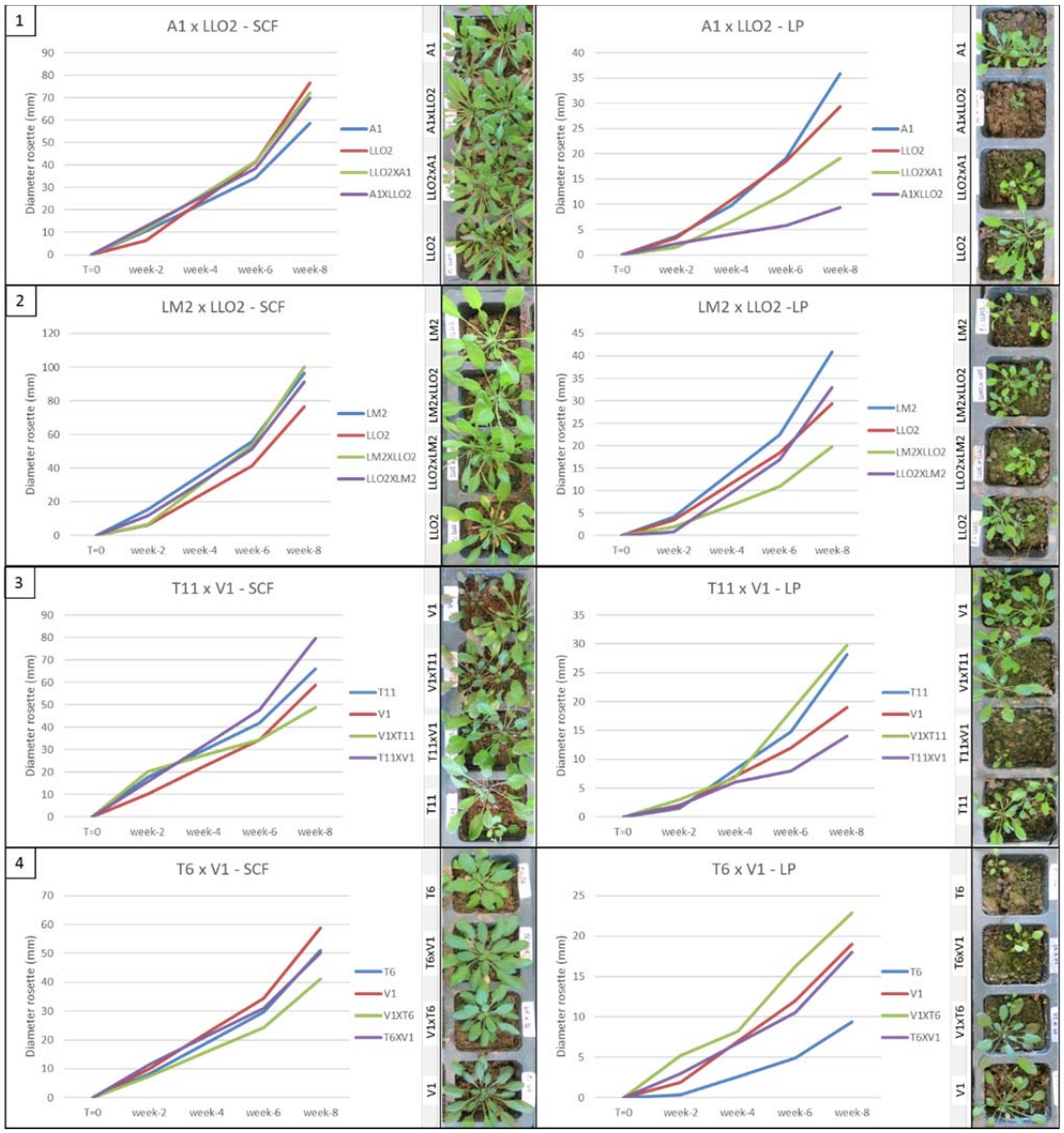


Figure III-2: F1 results of increase dimeter rosette (mm) of crossing and parental demes grown in SCF (left-graphs and photos) and LP (right graphs and photos).

For extreme crossings (Figure III-3) most plants grew better in SCF than in LP except for the crosses T6xLM2 and LM2xT6, which grew slowly (Figure III-3.5). These same crossing grown in LP present high differences with parental LM2; the behaviour of both crosses was like the sensitive parental T6. The opposite pattern was found in A1-T6 crosses (Figure III-3.7); no growth differences among parentals and crosses were observed at SCF. Contrastingly, on the

carbonate- rich soil at LP all crosses, A1xT6 and T6xA1, behaved like the tolerant parent A1; the sensitive parent T6 showed the lowest values.

When LM2 was crossed with T11 (Figure III-3.6), the crossing also showed a behaviour like T11, but the differences were not so marked. When A1 is crossed with T11 the behaviour of crossings is like A1, but differences are not so pronounced (Figure III-3.8).

Considering the small number of the seeds obtained in some of the crosses, we decided to continue to F2 with the crosses that displayed the most pronounced differences: A1-T6 and LM2-T6.

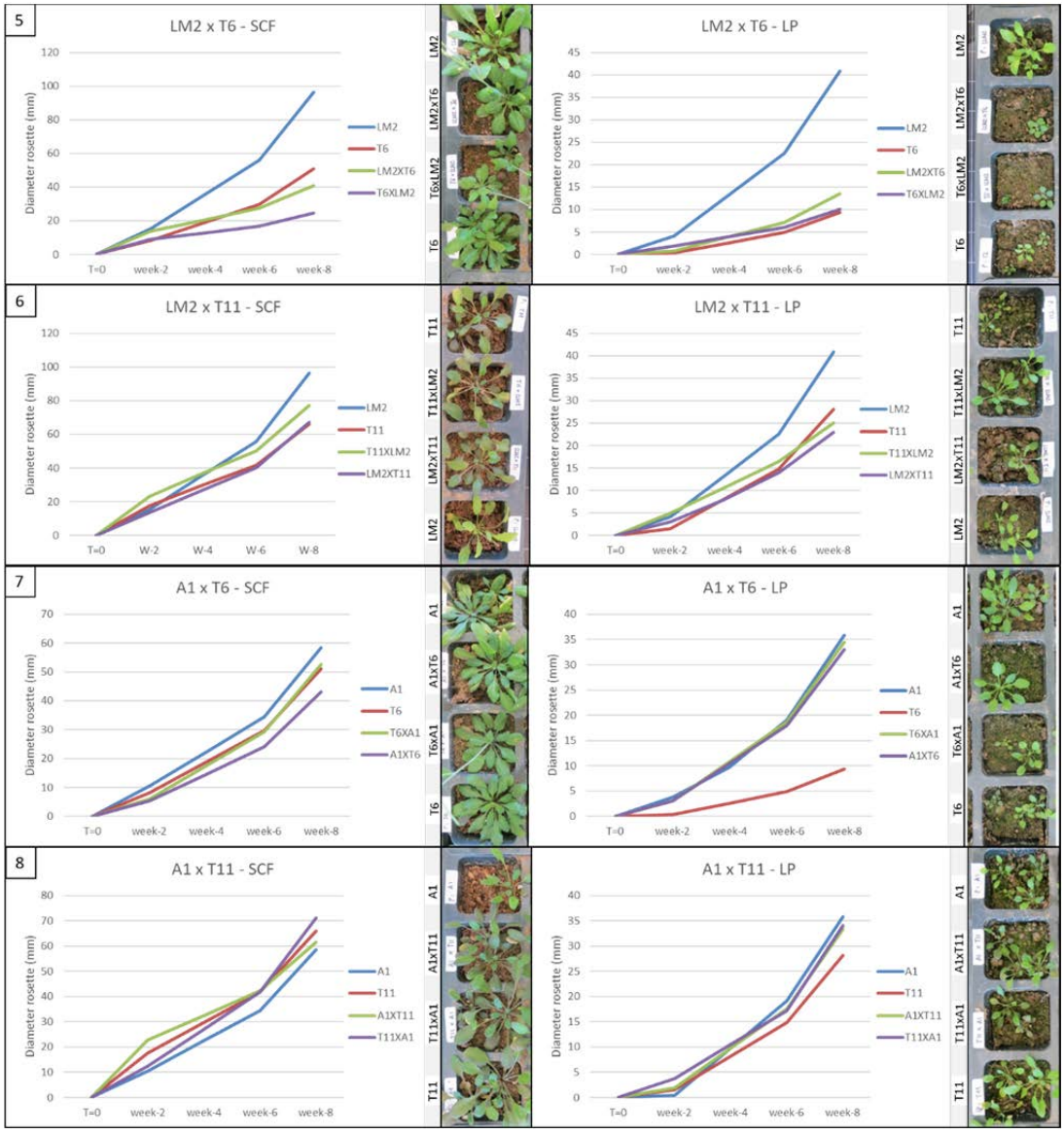


Figure III-3: F1 results of increase diameter rosette (mm.) of crossing and parental demes grown in SCF (left-graphs and photos) and LP (right graphs and photos). (5-6) Deme from group 3 of $CaCO_3$% (LM2) crossed with demes from group 1 (T6 and T11). (7-8) Deme from group 3 of $CaCO_3$% (A1) crossed with demes from group 1 (T6 and T11).

F2 selection

For F2 generation the mean of siliques number and percentage of flowering plants is represented in Figure III-4.

In the case of cross A1-T6, there were no significant differences in silique numbers between the A1 parental and the crossings (A1xT6 and T6XA1), while the sensitive parental T6 had a low number of siliques.

The percentages of flowering plants for both crossings (A1xT6 and T6xA1) was generally low compared to parental A1, but higher than parental T6.

For the cross LM2-T6, there were no differences of siliques number neither between crossings and the tolerant parental deme. The percentage of flowering plants was low for T6 and both crossings in comparison to the tolerant parental, LM2.

Considering the small number of seeds and low percentage of flowering plants it was decided to continue the F3 experiments with A1-T6.

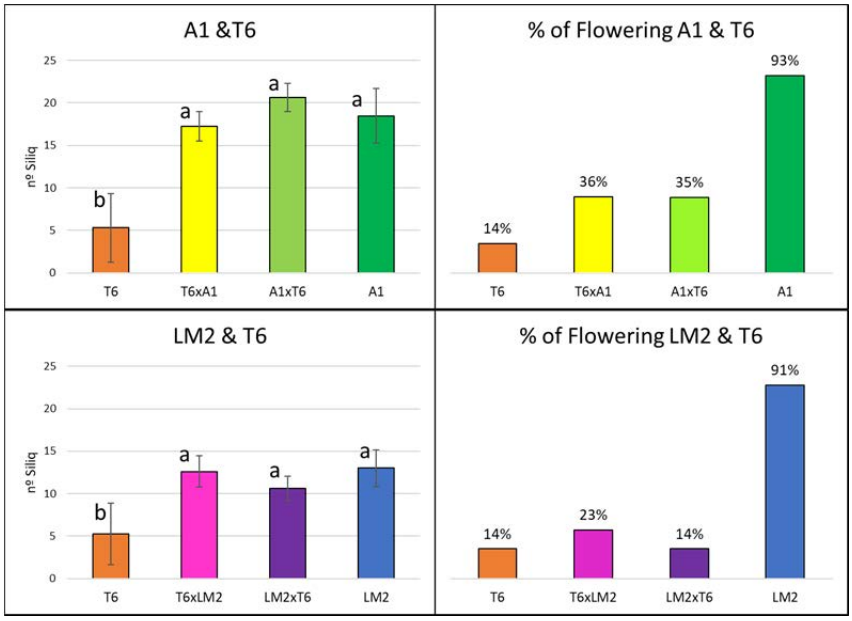


Figure III-4: F2 generation results: left site shows the Mean ± Std Dev of number of siliques of (top box) A1 & T6 and (bottom box) LM2 & T6. In right site, the percentage of flowered plants in each deme is shown.

F2 generation from crossing A1 & T6 was analysed (Figure III-5). All plants with seeds were taken into account and divided in two categories: less than 10 siliques (that represent a maxim number found in T6 parental) and more than 10 siliques (that represent a behaviour like A1 parental). The results revealed that all crossings have a heredity around 25:75 (sensible:tolerant), T6xA1 was 31:69 and A1xT6 was 18:82. Therefore we decided to carry on with the two ways of crossing (A1xT6 and T6xA1) for the generation of F3, in order to have more genes variability represented.

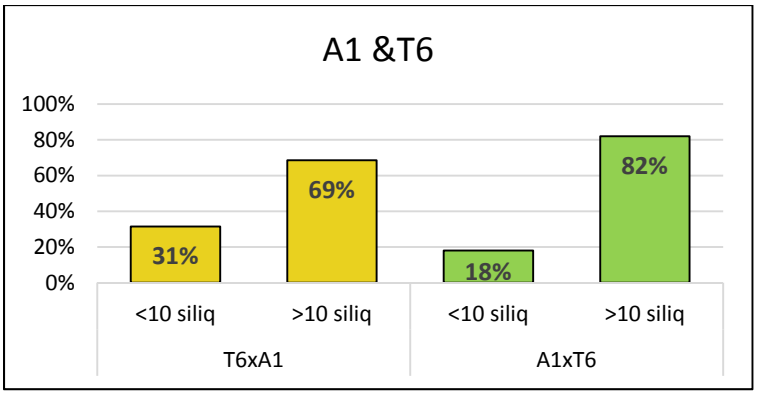


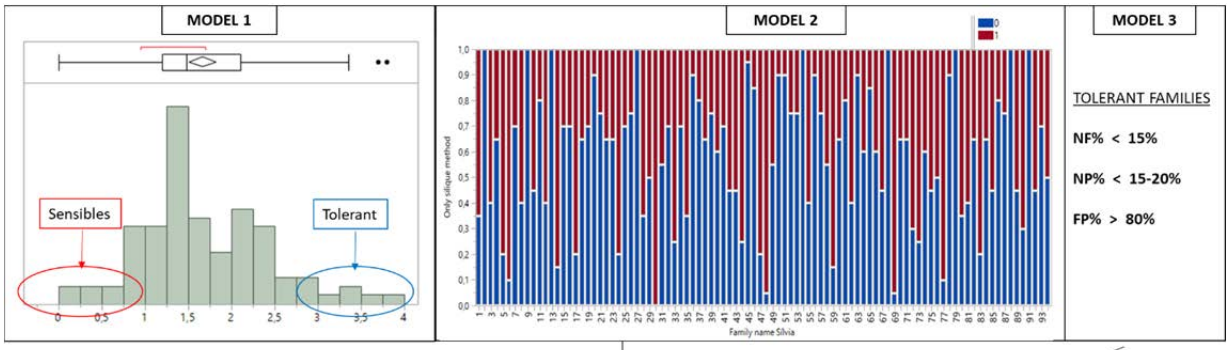
Figure III-5: Percentage of number of plants that have less than 10 siliques (siliq) and more than 10 siliques (siliq) for both crossings (T6xA1 and A1xT6)

F3 selection

Three different approaches were assayed to select the tolerant families of F3 (Figure III-6):

- Model 1: results of fitness (mean of number of siliques for family) were used to organize all families following these categories: 0 = NP (no plant); 1= NF (no flower), 2= 1-10 siliques, 3= 11-20 siliques, 4= 21-30 siliques. 10 families from category 4 where selected as representation of the most tolerant families.
- Model 2: results of fitness from each individual plant in F3; results were represented giving the following values to each single individual out of 20 individuals for family: 0 if there was no plant or plant without flower and 1 if there was a plant with flower and/or siliques. The mean of 0 and 1 values represent the number of family. Families selected as tolerant were those with a number close to 1.
- Model 3: Characters chosen for tolerant families were: less than 15% of no flowering plants (NF), less than 15-20% of no plants (NP) and more than 80% of individuals with flower (FP). Families that present these characters are chosen as representatives of tolerant families.

The table below in Figure III-6 shows the selected tolerant families for each model. Families that are repeated in two of three models are shown in green; families that appear in all models are marked in orange colour. As any family appeared just one time, we decided to take all families to do the BSA-seq, which is under progress.



TOLERANT FAMILIES		
MODEL-1	MODEL-2	MODEL-3
6	6	17
14	14	14
17	30	24
24	33	33
47	47	47
48	48	48
59	59	59
69	69	69
5	5	30
77	70	70
83	77	83

Figure III-6: Three different approaches were assayed to select the tolerant families of F3. Down table show the selected tolerant families for each model, in green are represented families that are repeated in two of three models and in orange are marked the families that appear in all models

Parental Sequencing

A1, the deme tolerant to moderate carbonate levels and T6, the sensitive deme, were sequenced in order to find genes differing between these two demes. A list of 968 genes with 3-10 nucleotides variants was found (see annex XX).

Discussion

In F1 we observed that when demes of group 1 of $CaCO_3$% (T11 and T6) are crossed with a deme of group 2 of $CaCO_3$% (V1), the direction of the cross was important. When deme from group 2 acted as a female the increment of diameter was higher (sometimes higher than parental) than when the cross was done using demes of group 1 of $CaCO_3$% as a female. In that case these individuals showed the lowest increment of diameter rosette. This could suggest that genes related with increase of diameter could be associated with maternal effect of heredity.

In crossings T6-A1 it was observed that F1 generation looks like A1 (tolerant parental). This suggests that “traits of tolerance to carbonated soils” are dominant in this crossing. The opposite happens with the other extreme crossing (T6-LM2), where F1 looks like the sensitive

parental, indicating that “tolerance to carbonated soils” is a recessive trait recessive in that crossing.

The different heredity of crosses A1-T6 and LM2-T6 where it is observed that “tolerance to carbonated soils” seems a character dominant for A1-T6 but recessive for LM2-T6 may imply that different traits are responsible for adaptation to carbonate in A1 and LM2. In chapter-II only physiological trait of A1 and T6 were examined, but not for LM2.

In chapter-II the adaptive responses of A1 versus iron deficiency, bicarbonate in the medium, and high pH, were maintenance of chlorophyll concentration, ability to diminish pH of rhizosphere, higher quantity of exudates, and early activation of SOD activity. But these parameters were not checked for LM2.

LM2 could present other mechanism that make those demes suitable to be adapted to carbonated soils (other genetic modification, activities of another enzymes…). But the results of heredity of LM2 would suggest that traits that made LM2 tolerant to carbonates soils are less dominant than the traits of “tolerance to carbonate soils” present in A1; because when LM2 is crossed with T6 the F1 heredity present a sensitive phenotype, so characters of LM2 behave as a recessives in front of T6.

These results suggest that tolerance to carbonate soil it is a “trait” related with a multigroup of genes, and different combinations of these genes could offer similar phenotype of tolerance with multiple genotype variations.

For ensuring a greater variability by gene segregation, the F2 and F3 generations were done for the cross A1-T6. The pool of tolerant families selected for BSA-seq present part of the genome of T6.

With BSA-seq we will find common genes between pool and T6; These genes came from “original genotype of T6” and are not related with tolerance to carbonated soils. Only those genes that appear in genomes of pool of tolerant families and in sequence of parental A1 will describe the genetic traits that confers tolerance to carbonate soil to A1 (**¡Error! No se encuentra el origen de la referencia.**).

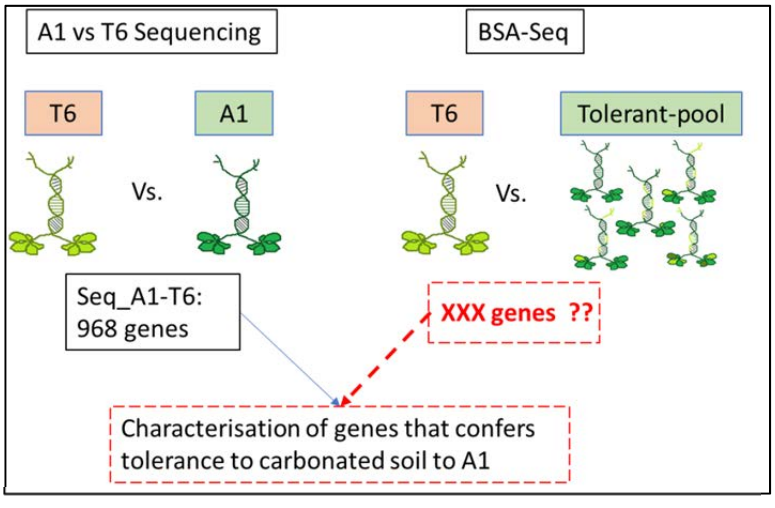
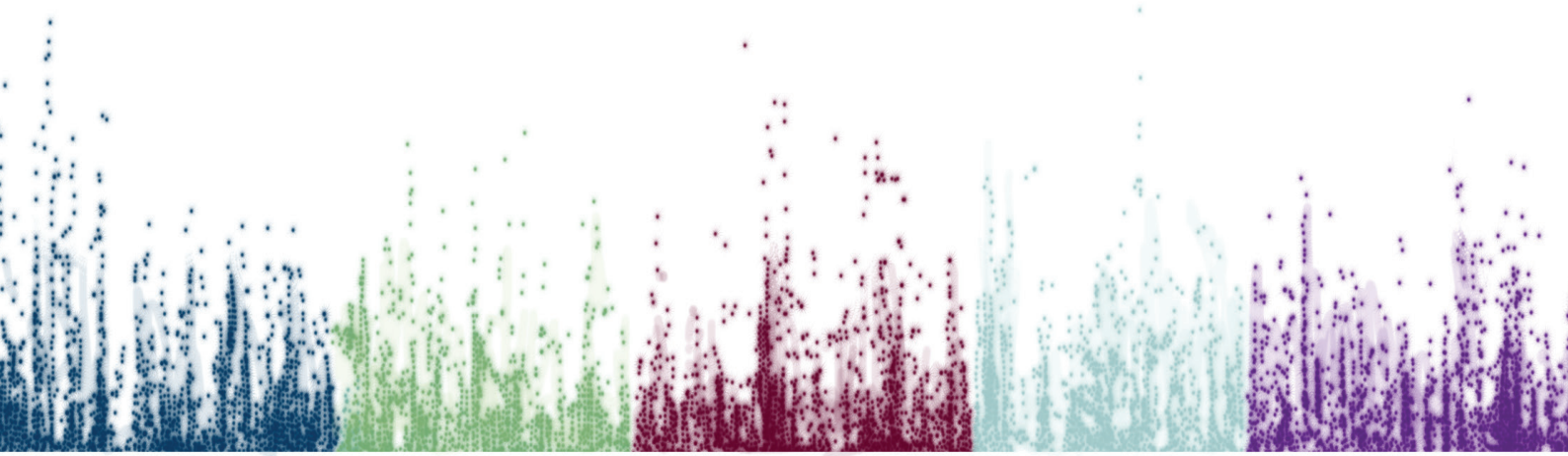


Figure III-7: Comparison of results from A1-T6 sequencing with future results of BSA-seq from tolerant pool families and T6.

Conclusions

- Multiple gene combinations confer the capacity to be tolerant to moderate levels of soil carbonate in *A. thaliana*
- Genetic traits that confers tolerance to deme A1 (tolerant) show a dominant heredity versus T6 (sensitive)
- The difference between A1 (carbonate tolerant) and T6 (carbonate sensitive) is based on differences in 968 genes.
- Bulk sequencing of the pool of tolerant F3 families obtained after A1-T6 crossings is expected to reduce this list of genes potentially involved in carbonate tolerance.

Chapter IV
GWAs



Introduction

Once observed the existence of phenotypic variations in tolerance to moderate soil carbonate levels in natural populations from Catalonia, we decided to test a larger set of accessions under more intense carbonate stress to see if more distinctive features can be observed.

For this purpose, a genome wide association analysis with the Hapmap collection of 338 natural accessions was performed at the GWA portal (https://gwas.gmi.oeaw.ac.at/).

GWAS

Genome wide association (GWA) technique was originally developed for human genetics where it is impossible to obtaining synthetic mapping populations (Hirschhorn *et al.,* 2005).

The genetic sources of phenotypic variation have been a major focus of both plant and animal studies aimed to identify causes of disease, to improve agriculture, and to understand adaptive processes. In plants, quantitative trait loci (QTL) were originally mapped in biparental crosses, but they were restricted in allelic diversity and in having limited genomic resolution (Borevitz, 2003).

GWA mapping has some important advantages over traditional linkage mapping using synthetic mapping populations. Firstly, natural accessions have experienced more recombination events than mapping populations such as RIL. That fact allows mapping with greater precision. Secondly, GWA mapping takes advantage of a much larger range of genetic variation. This approach however brings some disadvantages as well. Not all variants are equally likely to be discovered in GWAS. Rare alleles on the scale of the used population are less likely to be discovered comparing to frequent ones due to smaller statistical power (Asimit & Zeggini 2010; Gibson, 2011). Such interesting rare alleles could be discovered in QTL studies as frequency of each variant in mapping population is equal. Another problem of GWA mapping is the heterogeneity that takes place when certain alleles of different genes produce the same phenotype. Allelic heterogeneity is observed when different alleles occurring in one gene lead to the same phenotype. It makes the associations of each of these genetic factors weaker. Population structure can cause false positive as well false negative results (Zhao *et al.,* 2007). Correction for effects of population structure using statistical approaches can cause on the other hand false negative results (Korte & Farlow, 2013).

Using linkage disequilibrium (LD), that is based on non-random association between alleles and phenotypes, GWA mapping identifies important polymorphisms and evaluates the statistical significance of associations between differences in a quantitative phenotype and genetic polymorphisms tested across many genetically different individuals.

GWA mapping was successfully used in several economically important species apart from *A. thaliana*, predominantly in maize (*Zea mays*) (e.g. Hao *et al.,* 2011; Tian *et al.,* 2011; Yang *et*

al., 2013), rice (*Oryza sativa*), and wheat (*Triticum vulgare*) (Cockram *et al.,* 2010; Long *et al.,* 2013).

The first extensive study that used the GWA approach was performed by Atwell (2010) in *A. thaliana*. This study investigated the genetic architecture of 107 different traits related to flowering time, development, resistance to pathogens and element composition. From 76 to 194 accessions were phenotyped for different traits and genetic information was obtained using microarray platform that contained almost 250 000 genetic markers - single nucleotide polymorphisms (SNPs). The GWA approach was validated through finding several loci for traits that already had been confirmed as highly relevant.

Combining several techniques has advantages and offer different possibilities that could be used to find and confirm genetic factors taking part in important biological processes and responsible for traits of interest. GWA approaches in *A. thaliana* for example can be joined with traditional linkage mapping (Zhao *et al.,* 2007; Brachi *et al.,* 2010) or with BSA (Chao *et al.,* 2012; Chao *et al.,* 2014) to identify false negative and false positive results. Finding causal genetic factors and confirmation of results of genetic mapping can be achieved using various molecular biology techniques and available resources.

Objectives

- Test if there is a genetic variation related with ionomic analysis with a HapMap collection grown in carbonate soils
- Test if there is a genetic variation related with rosette diameter with a HapMap collection grown in carbonate soils

Materials and methods

Plant Culture and Experimental Design

For assessing natural variation in response to carbonated soils we used 338 natural accessions of *A. thaliana* from the HapMap collection (Baxter *et al.,* 2010) representing the within-species genetic variation (Supplementary Table 1). We obtained the accessions from the Nottingham *A. thaliana* Stock Centre (NASC, Nottingham, UK).

A pair of seeds of each accession was sown in two soils from Mallorca with highly contrasting carbonate contents, but similar in other physical and chemical properties (see table XX). Irrigation was done twice a week using distilled water.

The rosette diameter of the plants was measured two weeks after sowing. Further 3 measurements were performed in intervals of 10 days. After the last measurement, the plants were collected and analyzed for shoot ionome.

Plant analysis

Chlorophyll

Measure of chlorophyll concentration were taken using a SPAD (Opti-sciencies CCM300) after 14 days of treatment. For each plant three measurements in leaves of different stages (old/medium/young) were taken in order to get more representative results.

ICP

Plants 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 hr at 60 ºC, and weighed before open-air digestion in Pyrex tubes using 0,7 mL concentrated HNO3 (Mallinckrodt AR select grade) at 110 ºC for 5 h. Each sample was diluted to 6.0 mL with 18 MΩ water and analysed for As, B, Ca, Cd, Co, Cu, Fe, K, Li, Mg, Mn, Mo, Na, Ni, P, Rb, S, Se, Sr and Zn content (ppm) on an Elan DRCe ICP-MS (PerkinElmer Sciex). NIST traceable calibration standards (ULTRAScientific, North Kingstown RI) were used for the calibration.

Soils

Soils used in this experiment came from Mallorca. There are two different soils: Haplic Calcisol (carbonated soil) (X: 505185Y: 4390717) and Chromic Endoleptic Luvisol (X: 516699, Y: 4392835).

Soil characteristics were done at the Universitat de les Illes Balears (UIB); the following analyses were performed: Trace Elements, by microwave digestion with *aqua regia* described by Marin *et al.,* (2008) and quantification 5300DV Optima ICP-OES (Perkin-Elmer, Massachusetts), total organic carbon described by Nelson and Sommers (1982), calcic carbonated using a Bernard calcimeter (Porta *et al.,* 1986), cation exchange capacity: using ammonic acetate (Rhoades, 1982), texture and particle size by Porta *et al.,* (1986).

Genome-wide association (GWA) analysis

GWAPP consists of a Web front end with a graphical user interface, and a back end that handles the data and performs the mapping.

Once a phenotype file has been uploaded, the results are viewed via the *Plots* tab, an interactive Manhattan plot (a scatterplot with the negative logarithm P values for the SNP association plotted against the SNP positions) for all five chromosomes is shown.

The Benjamini-Hochberg-Yekutieli multiple testing procedure (Benjamini and Yekutieli, 2001) was used to control the false discovery rate. Assuming arbitrary dependence between SNPS, the 5% false discovery rate (FDR) threshold is plotted as a dashed horizontal line. Only the SNPs with higher value of 5 were taken in account.

Linkage disequilibrium (LD) structure can be also detected with GWAAP, that will calculate genome-wide r^2 values between the selected SNP and all other displayed SNPs and colour code them in the Manhattan plot

We used two types of phenotype traits for performing the association with the genotype: Ionomic analysis (Li, B, Na, Mg, P, S, K, Ca, Mn, Fe, Co, Ni, Cu, Zn, As, Se, Rb, Sr, Mo and Cd) and growth, as an increase of rosette diameter.

For analysis of variable *ionomics* two scenarios were analysed: leaf concentration of plants grown in carbonate soils (C) and carbonate and no carbonate leaf concentration difference (CND), as the relative leaf concentration between plants grown in carbonate and non-carbonate soils per unit of leaf concentration in non-carbonate soil. Calculated using follow equation:
(C)-(N)/(N)*100, where (C) is leaf concentration in carbonate soil and (N) is the concentration in no carbonate soil.

For analysis of the variable *diameter* the data used were: "differential growth rate" (GRD). The GRD is obtained by calculating Growth rate from measure 1 until measure 4 between in plants grown in carbonate soil per unit of non-carbonate soil. Calculated using follow equation: GR_14_C/GRD_14_N.

Results & Discussion

Soil analysis

Table IV-1 displays the multiple variables analysed in the two soils used for the GWAS experiment. Significant differences in the concentration of CaCO3 (663.5 g / kg carbonated soil vs. 1.5 g / kg control soil) active lime (136 9 g / kg ground vs. carbonated 0 g / kg ground control), the electrical conductivity (EC) (116 μS / cm soil carbonated vs. 33 μS / cm soil control), pH (8.5 vs 7.1 in soil carbonate ground control) and presence of coarse elements (29.5 g / kg ground control ground carbonated vs. 2.5). By contrast there are no significant differences regarding the percentage of sand, silt, clay or measures cationic exchange capacity (CEC), field capacity and permanent wilting point.

Soil ionomic analysis revealed that concentrations of most elements tend to be higher in the control soil than in the carbonate soil (Fe, Zn, Mn, Cu, Mg, Na and Mo), but only for Ca and Mn the differences are statistically significant. Concentrations of Ni and Co are very similar in both soils. Although one of the main differences between soils analysis was Ca content, no genetic variations were associated to leaf Ca content (Figure IV-1).

	Carbonated Soil	Control Soil		Carbonated Soil	Control Soil
WRB (2014)	Haplic Calcisol	Chromic Endoleptic Luvisol	**C/N**	8.8	3.8
Soil Taxonomy (2014)	Chromic Luvisol	Typic Haploxeralf	**P Olsen (mg/kg)**	29,4	13.4
Color Munsell wet	2.5Y 5/4	5YR 4/3	**CaCO3 (g/kg)**	664	1.5
Colour Munsell Dry	2.5Y 7/3.5	5YR 5/6	**Active Lime (g/kg)**	259	0.0
Depth (cm)	0-30	0-30	**CEC (mmol/kg)**	167,0	170.5
Sands (g/kg)	168	127	**EC 1:5 (uS/cm)**	116,0	33.0
Slime (g/kg)	565	527	**pH H_2O**	8,5	7.1
Clays (g/kg)	267	346	**pH KCl**	7,4	5.3
Organic C (g/kg)	9.45	6.44	**Field capacity (g/kg)**	227,3	204.9
Organic Matter (g/kg)	16.28	11.10	**Permanent wilting point (g/kg)**	97,5	113.6
N (g/kg)	1.07	1.70			

Table IV-1: Physical and Chemical proprieties of two different Mallorca soils

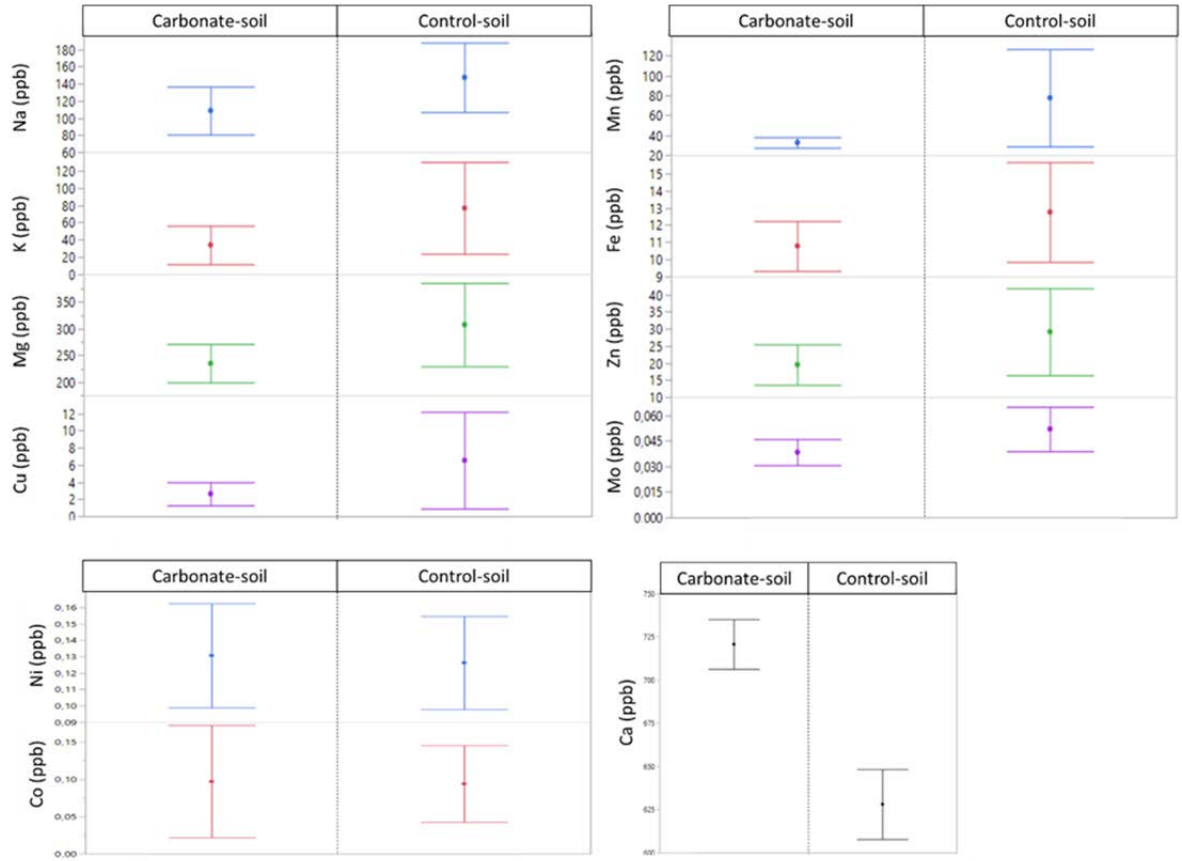


Figure IV-1: Mean and standard error of ionomic analysis of sodium (Na), potassium (K), magnesium (Mg), copper (Cu), manganese (Mn), iron (Fe), zinc (ZN), Molybdenum (Mo), nickel (Ni), cobalt (Co) and calcium (Ca) from carbonate soil and control soil from Mallorca.

Extreme Selection

During the experiment, 30 ecotypes were selected for extreme behaviour according to the following parameters: better growth in control soil and worse growth in carbonated soil (Figure IV-2-A) and better growth in carbonated soil and weak growth in control soil (Figure IV-2-B).

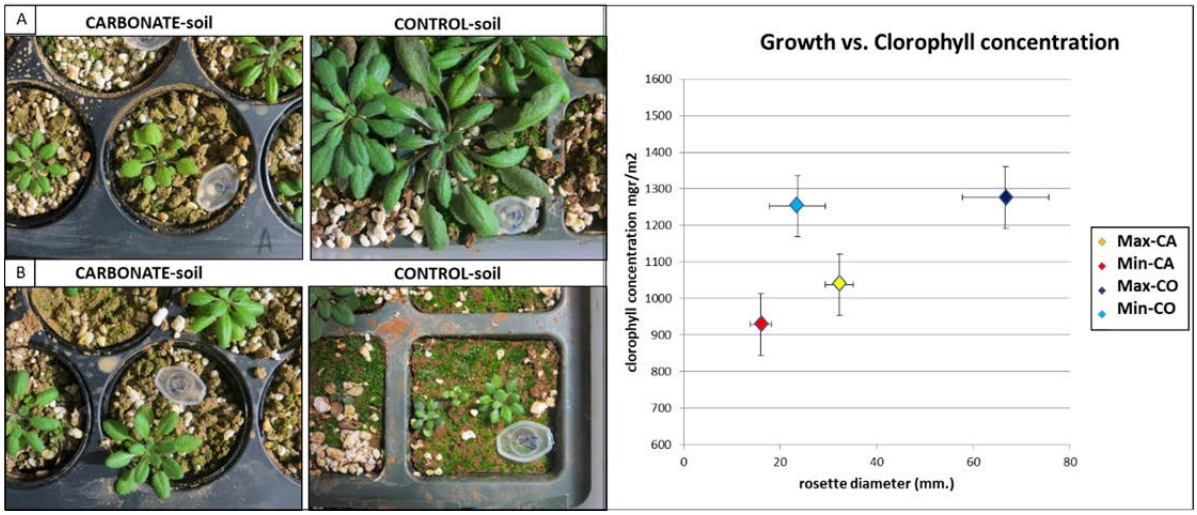


Figure IV-2: (A): Example of extremes behaviours selected up to down: better growth in control soil; better growth in carbonated soil. (B): Measures of diameter and chlorophyll concentration of extremes behaviours in carbonated soil (CA) and control soils (CO)

Growth measurements revealed that the maximum diameter of plants grown in control soil was twice the maximum diameter of plants grown in carbonated soil. Furthermore, the minimum diameters of plants form control soil were not statistically different from the maximum diameters of plants from carbonate soil (Figure IV-2).

All plants grown in control soil had higher chlorophyll concentrations than plants from carbonate soils. Although all plants grown on carbonate soils had lower concentrations of chlorophyll, there was a positive correlation between the rosette diameter and the level of chlorophyll in the plants.

In general, plants grown on carbonate soil presented a lower maximum diameter in comparison to plants grown in control soil These data allow us to verify that the presence of carbonates in soil have a negative effect on plant growth. Plants on carbonated soil had lower chlorophyll concentrations in comparison to plants grown in control soil, chlorophyll concentration is an important variable associated with tolerance carbonate soils.

GWAS

Nutritional Analysis

For ionomic analysis 2 parameters of each element were analysed by GWAS (Table IV-2):

- Concentration of element in carbonate soil (C). Significant SNPs were found for shoot concentrations of the following elements: B, Cd, Co, Cu, Fe, Mg, Ni and Zn.
- Analysing the differences of shoot element concentrations (CND) between plants grown on non-carbonate soil (N) and carbonate soil (C), significant SNPs were found for the following elements: B, Co, Mn, Mo, Na, Ni, P and Zn.

Carbonate soil									
Elements	B	Cd	Co	Cu	Fe	Mg	Ni	Zn	
SNPs	-	-	1	3	1	-	4	2	
LD	1	1	1	5	2	1	4	7	Total gens:
Nº gens	1	31	15	53	19	2	61	67	247
Difference Control-carbonate soil									
Elements	B	Co	Mn	Mo	Na	Ni	P	Zn	
SNPs	-	-	-	-	9	-	-	1	
LD	1	1	1	2	5	2	1	2	Total gens:
Nº gens	11	0	10	7	91	24	5	35	182

Table IV-2: nº of genes associated to SNPs, or locus disequilibrium (LD) of elements in carbonate soil analysis and difference of control and carbonate soil element concentration.

Diameter analysis

Results of GWAs show important genes present in LD and SNPs regions. According to TAIR the corresponding gene description is as shown in Table IV-3:

Gene	Description
AT2G27010.1	Member of CYP705A
AT2G27020.1	Encodes 20S proteasome alpha 7 subunit PAG1.
AT5G02910.1	F-box/RNI-like superfamily protein
AT5G02920.1	F-box/RNI-like superfamily protein
AT5G02930.1	F-box/RNI-like superfamily protein
AT5G02940.1	Ion channel POLLUX-like protein, putative (DUF1012)
AT5G02950.1	Tudor/PWWP/MBT superfamily protein
AT5G02960.1	Ribosomal protein S12/S23 family protein
AT5G02970.1	Alpha/beta-Hydrolases superfamily protein
AT5G02980.1	Galactose oxidase/kelch repeat superfamily protein

Table IV-3: List of genes and descriptions resulting from GWAs analysis using diameter as a variable.

Both ionomics and rosette diameter data provided a list of genes potentially implicated in differences for these variables in plants grown on carbonate soil. Taking into account the existent description of part of these genes (proteins), some genes can be related to already described functions relevant for variance in these variables (ionomics and rosette diameter). For example, for Zn shoot concentrations in plants grown on carbonate soil *AT1G10480* was identified. This gene encodes a Zn finger binding protein (ZFP5), that acts as a positive regulator of root hair development in *A. thaliana* (An *et al.,* 2012). However, paying attention

only to genes that have a known description that could explain the differences observed in ionomics or rosette diameter could be misleading, because not all genes and gene functions have already been described. Moreover, not all genes and their functions have been tested in carbonate soil.

To evaluate whether these genes indicated by the GWAS analysis can also be relevant for differences in the behaviour of our natural populations the list of gene differences between parental A1 (tolerant) and T6 (sensitive) (chapter-3) was compared to the GWAS results.

The list of differential genes comparing A1 and T6 (SeqA1-T6) contained 14 genes also present in the list of GWAS ionomics. For rosette growth, only one gene from the GWAS list was also among the genes that differ between A1 and T6.

Table IV-4 shows the list of these 14 matching genes and the corresponding element associated in GWAs ICP analysis. Moreover, the gen matched for GWAs-diameter analysis and it description is shown.

Matchings SeqA1-T6 & GWAS-ICP		
Element	**Gene**	**Description**
Zn_H	AT1G10490.1	GNAT acetyltransferase (DUF699)
Zn_H	AT1G10500.1	Involved in chloroplast Fe-S cluster assembly. Located in the chloroplast stroma..
Zn_H	AT1G10510.1	RNI-like superfamily protein
Zn_H	AT1G10520.1	Encodes a homolog of the mammalian DNA polymerase lambda. Involved in the repair of UV-B induced DNA damage.
Zn_H	AT1G10522.1	Encodes PRIN2 (plastid redox insensitive 2).
Zn_H	AT1G16150.1	Encodes a WAK-like receptor-like kinase with a cytoplasmic Ser/Thr protein kinase domain and an extracellular domain with EGF-like repeats. Involved in Arabidopsis root mineral responses to Zn2+, Cu2+, K+, Na+ and Ni+. The mRNA is cell-to-cell mobile.
Zn_H	AT1G16160.1	WAK-like kinase The mRNA is cell-to-cell mobile.
Zn_H	AT1G16170.1	ephrin-A3 protein
Zn_H	AT1G16180.1	Serinc-domain containing serine and sphingolipid biosynthesis protein
Zn_H	AT1G16190.1	Encodes a member of the RADIATION SENSITIVE23 (RAD23) family: proteins play an essential role in the cell cycle, morphology, and fertility of plants through their delivery of UPS substrates to the 26S proteasome.
Cu_H	AT2G42840.1	Encodes a putative extracellular proline-rich protein is exclusively expressed in the L1 layer of vegetative, inflorescence and floral meristems and the protoderm of organ primordia.
Cu_H	AT2G42860.1	hypothetical protein
Cu_H	AT2G42870.1	Encodes PHYTOCHROME RAPIDLY REGULATED1 (PAR1), an atypical basic helix-loop-helix (bHLP) protein. Up regulated after simulated shade perception. Acts in the nucleus to control plant development and as a negative regulator of shade avoidance response. Functions as transcriptional repressor of auxin-responsive genes SAUR15 (AT4G38850) and SAUR68 (AT1G29510).
Ni_H	AT5G20580.1	TMEM192 family protein
Matching SeqA1-T6 & GWAS-diameter		
GRD14	**Gene**	**Description**
	AT5G02980.1	Galactose oxidase/kelch repeat superfamily protein

Table IV-4: List of gens matching when comparing the Seq A1-T6 with the list of GWAs-ICP genes. Analyzed element where those genes were detected in the GWAS-ICP analysis and description of Tair page are indicated

The gene matching with diameter analysis was AT5G02980.1 that encodes for a galactose oxidase/kelch repeat superfamily protein according Tair descripction.

This superfamily refers to a very large group of proteins that contains a kelch repeat in their amino acid sequence. Galactose oxidase (GO, EC 1.1.3.9) is a monomèric 6- kDa enzyme that contains a single copper ion and an amino acid-derived cofactor that selectively oxidizes primary alcohols to aldehydes.

Among the 14 matching genes (from ionomic variable), 10 where found using Zn shoot concentrations in plants grown in carbonate soil (H) as a variable in GWAS. That fact definitively suggest that Zn plays a significant role in adaptation to carbonate soils.

Zn is an essential trace element (micronutrient) required in small but critical amounts by both plants and animals (including humans). It is required for the structure and function of a wide range of macromolecules including hundreds of enzymes. Zn is the only metal to be involved in all six classes of enzymes: oxido-reductases, transferases, hydrolases, lyases, isomerases and ligases (Barak and Helmke 1993). Zn ions exist primarily as complexes with proteins and nucleic acids and participate in all aspects of intermediary metabolism (Tapeiro and Tew, 2003; Alloway, 2009).

The Zn which is available to plants is that present in the soil solution, or is adsorbed in a labile (easily desorbed) form. The soil factors affecting the availability of Zn to plants are those which control the amount of Zn in the soil solution and its sorption-desorption from/into the soil solution. These factors include: the total Zn content, pH, organic matter content, clay content, calcium carbonate content, redox conditions, microbial activity in the rhizosphere, soil moisture status, concentrations of other trace elements, concentrations of macro-nutrients, especially phosphorus and climate (Alloway, 2004).

The relationship of Zn and carbonated soils has been the subject of study for several decades (Yoshida, 1969; Udo, 1970; Harter, 1983). Kiekens (1980) also studied the adsorption of Zn on a calcareous soil and found that the reaction was not reversible due to some of the Zn being irreversibly fixed by the soil. These findings on the fixation/sorption of Zn on calcium carbonate have some important implications for the behaviour of Zn in calcareous soils. Some of the worst Zn deficiency problems in crops occur on calcareous soils in arid and semi-arid regions of the world. Uygur and Rimmer (2000) have pointed out that calcareous soils tend to have pH values of 8 or above and that under these pH conditions, iron oxides readily precipitate out and form coatings on the carbonate minerals. They showed that an increase in pH from 8 to 8.3 can double the strength of bonding of Zn to calcite but with 0.05% of iron oxide on the calcite the bonding increases 7-fold between pH 8 and 8.3. They found that with a coating of iron oxide on the calcite, the sorption of Zn was greater than it is with pure calcite and the extent to which Zn is immobilized is greater and it is less readily desorbed than it is from pure calcite. Therefore, the occurrence in calcareous soils in semi-arid and arid regions of calcite with thin coatings of iron oxide results in Zn being even less available to plants than with pure calcite, and a higher risk of Zn deficiency in crops.

Copper is another one of eight essential micronutrients for all higher plants. In soil, Cu is restricted mainly in the top layer because of its ability to tightly bind with carbonates, clay minerals, hydrous oxides of Al, Fe and Mn and organic matter (Mengel and Kirkby 2001). Copper mobility along the soil profile, bioavailability for root uptake and consequently phytotoxicity threshold for crops depend on soil pH (Chaignon *et al.,* 2003), cation exchange capacity (CEC), quality of organic matter, texture etc. (Parat *et al.,* 2002).

Copper occurs in the soil almost exclusively in divalent form. The largest fraction of Cu is usually present in the crystal lattices of primary and secondary minerals. In addition, a high proportion of Cu is bound by the soil organic matter. The Cu ion is adsorbed to inorganic and organic negatively charged groups and is dissolved in the soil solution as Cu^{2+} and organic Cu complexes. Copper is specifically adsorbed to carbonates, soil organic matter, phyllo silicates, and hydrous oxides of AI, Fe, and Mn (Reed & Martens 1996).

A particularly important and widely used feature of Cu(I) is its ability to bind small molecules such as oxygen donor ligands (Krämer et al., 2005). This explains why Cu is a co-factor of a large number of oxidases and why Cu-dependent oxidases are the principal catalysts of terminal oxidation reactions in cells, for example tyrosinase, lacase, phenolases, ascorbic acid oxidase and cytochrome c oxidase (Barceló, 2001)

Cu it is also involved in plant growth, there are apparently also multi-copper oxidase-like proteins such as SKU5, which are involved in cell wall formation yet lack any detectable oxidase activity (Sedbrook *et al.,* 2002), Copper also is required for lignin synthesis which is needed for cell wall strength and prevention of wilting (Ranocha *et al.,* 2002).

Some smaller proteins with one mononuclear blue copper (type I) centre do not function as oxidases, but as electron carriers. The best-known and quantitatively most important example in plants is plastocyanin, which accounts for about 50% of the plastidic Cu (Marschner 1995). This protein mediates the electron transfer from the cytochrome b6f complex to PS-I.

Cu availability decrease with high pH (Huff *et al.,* 1970) so that means that all cited “vital functions” could be threatened when plants grown in carbonated soils.

Nickel (Ni) occurs abundantly in igneous rocks as a free metal or as a complex with iron. It stands at twenty-second position amongst most abundant elements in the earth crust (Sunderman and Oskarsson 1991)

The uptake of Ni in plants is mainly carried out through the root system via passive diffusion and active transport (Seregin and Kozhevnikova 2006). The ratio of uptake between active and passive transport varies with the species, form of Ni and concentration in the soil or nutrient solution (Vogel-Mikus *et al.,* 2005). The overall uptake of Ni by plants depends on the concentration of Ni^{2+}, plant metabolism, the acidity of soil or solution, the presence of other metals and organic matter composition (Chen *et al.,* 2009). However, uptake of Ni usually

declines at higher pH values of the soil solution due to the formation of less soluble complexes (Temp, 1991).

Moreover, Ni^{2+} ion may also compete with other essential metal ions when it is absorbed by roots. The uptake of heavy metals from the soil solution is strongly affected by calcium ion. Ca^{2+} lowered the absorption of Ni^{2+} in *A. thaliana bertolonii*, (Gabrielli and Pandolfini 1984).

Besides this, Ni an important component of many enzymes, where it coordinates either with S-ligands and O-ligands (e.g. Urea), S-ligands (cystein residue e.g. hydrogenase) or ligands of tetrapyrol structure (Marschner 2002). However, urease is the only enzyme in higher plants that has been reported to possess Ni as an integral component, in stoichiometric quantities (Dixon *et al.,* 1980). Moreover, embryonic root was poorly developed or even failed to develop; in addition to this several other anomalies were also reported in the development of endosperm together with decreased activity of dehydrogenase. In some legumes, small amount of Ni is essential for root nodule growth and hydrogenase activation. The efficiency of nitrogen fixation depends on largely hydrogenase activity because the oxidation of hydrogen provides ATP required for N reduction to ammonia (Yusuf *et al.,* 2011).

The pH, cation exchange capacity and CaCO3 content of the soils are important characteristics which affect the adsorption and subsequent plant uptake of Ni from soil (Ramachandran et al., 2013). So, in the same way of Cu and Zn reported above, taking into account that Ni availability decrease in carbonated soils, potential tolerant plants (to carbonate soils) should be provided by mechanisms that permits uptake enough Ni from the carbonate soil.

Conclusions

- The presence of carbonates in the soil reduced the growth of all accessions of the Hapmap collection in comparison to the non-carbonate soil. This indicates that in this large, world-wide collection there is no genotype with preference to carbonate over siliceous substrate.
- Differences in rosette diameter increase of plants growing on carbonate soil versus siliceous soil reveals differences in adaptation to soil carbonate among the Hapmap accessions
- As expected, low chlorophyll content is an important variable indicating sensitivity to soil carbonate. The positive correlation between rosette diameter and chlorophyll concentrations guarantee that the higher chlorophyll concentration in certain accessions is not the consequence of reduced leaf expansion growth.
- Genetic variations were found by GWAs analysis in the Hap Map collection in relation to ionomics and rosette diameter.

- Differences in rosette ionomics was associated with 432 genes that potentially influence the nutrient leaf concentrations of plants growing on carbonated soils.
- The relative increase of rosette diameter of plants growing on carbonate soils is related to only 10 genes that potentially determine the differences in growth rate under carbonate stress.
- Comparison of potentially important genes for carbonate tolerance got from GWAs and those differing between genes the natural accessions A1 (carbonate tolerant) and T6 (carbonate sensitive) identifies only 15 matching genes (14 genes from ionomic analysis and 1 from rosette diameter analysis)
- From ionomic analysis 10 out of 14 matching genes are associated to zinc leaf content. That fact definitively suggests that Zn plays a significant role in adaptation to carbonate soils. Further 3 genes are related to rosette Cu concentrations.
- From rosette dimeter analysis only one gene matches: *At5G02980.1*, probably coding for a Cu-containing galactose oxidase.

Note: Different statistical data related to this Chapter IV can be found in 0Annex 5 to Annex 7.

Concluding Remarks

Arabidopsis thaliana can be considered a calcifuge species. According to both the distribution data of 24 demes in Catalonia and growth results on carbonate versus siliceous soil of 361 accessions originating from all over the world this species prefers siliceous over carbonate substrates. However, the species has evolved local adaptation to moderate carbonate levels with an apparent upper limit in the Catalonian area of 30 % soil carbonate. Differential responses were shown by common garden experiments. Observed differences in fitness of *A. thaliana* on high carbonate soil was strictly dependent on the carbonate level in the soil of origin

Accessions with extreme behaviour, A1 moderately tolerant to carbonate and T6 sensitive, clearly differed in several physiological markers for carbonate tolerance:

- In hydroponics under iron deficiency treatment plants from T6, but not A1 present an extreme chlorosis. FCR activity was different between demes. However, in hydroponics with initial nutrient solution at pH 7.5, A1 plants decrease pH level to 5.9 (±0.3) while those of T6 only achieve pH 6.9 (± 0.2). This result suggests that although the FCR enzyme has a normal activity in both demes, only A1 was able to adjust the pH to the optimal range of FCR activity.
- As pH and iron have a strong impact on redox homeostasis, enzymes related to oxidative stress were tested. Catalase activity was not different between demes but SOD activity in A1 under treatment exhibits a higher inhibition rate than in T6. These results suggest that A1 demes produces higher levels of antioxidant defences under these conditions.
- Analysis on phenolic compound in root exudates also reveals differences between demes. A1 produces 2.5 times more quantity of total exudates with a higher percentage of catechol groups than T6. Phenolic compounds may function in reduction or complexation of Fe. So different quantities of these compounds suggest that A1 has more facilities to mobilize iron under high pH and iron deficiency conditions.
- Genetic variability in the DNA sequence of *AtFPN2* were found for demes A1 and T6. The A1 *AtFPN2* allele is like that of Col.0 and the T6 allele is like that of TS-1. FPN2 is a metal transporter protein located in the tonoplast. Concentrations of Ni, Co and Mn in aerial parts were higher for T6 than for A1, suggesting that the modification in *AtFPN2* could affect the metal transport to the vacuole. It is proposed that modification of *AtFPN2* in T6 decreases the mobility of excess metals in the root vacuole, so the proportion of

cytoplasmic Fe became lower and some of these metals (instead of iron) could be transported to the aerial parts, reducing iron availability for the leaves and incrementing metal toxicity.

Carry on with genetic traits we have crossed our demes. When the tolerant deme LM2 was crossed with the sensitive T6 the F1 phenotype was like T6. Contrastingly, when the tolerant deme A1 was crossed with T6 the F1 phenotype was like the tolerant parental A1. This result suggests that multiple genetic combinations can confer a phenotype with tolerance to carbonate soils and some of these characters act as dominant and some as recessive traits.

The complete genome was obtained for A1 and T6 and the comparison of sequences provided a list of 968 genes. Although, most probably only a fraction of these genes is directly involved in the differential response to soil carbonate, this list is a first approach to identify the genetic trait that confers tolerance to carbonate soil to A1 accession.

For exploring the response to carbonate soil in of a wider source of *A. thaliana* germplasm the Hapmap collection of 361 accessions was grown on carbonate and siliceous soils. Rosette diameter increase and rosette ionome data were used for GWAS analysis. 442 potential genes related to carbonate response were detected.

A comparison of the GWAs results with the list of genes differing between A1 and T6 reveals 14 matching genes for ionomic parameters and 1 gene for the rosette growth parameter. Genes matching for the ionomic parameters were found for Zn, Cu and Ni rosette concentrations.

The results from the BSA-seq obtained with the pool of tolerant families will be compared with the list of genes differing between the parentals, A1 and T6. The expected results will be very helpful to finally define the genes that are directly related to the tolerance to carbonates soil of the A1 deme. Moreover, the comparison of results of BSA-seq with the GWAs results will allow us to see the implication of these genes in the tolerance to carbonated soils for a large range of *A. thaliana* accessions.

BIBLIOGRAPHY

References

Abbott, R. J., & Gomes, M. F. (1989). Population genetic structure and outcrossing rate of Arabidopsis thaliana. Heredity 62:411-418.

Achard, P., Cheng, H., De Grauwe, L., Decat, J., Schoutteten, H., Moritz, T., ... & Harberd, N. P. (2006). Integration of plant responses to environmentally activated phytohormonal signals. Science, 311:91-94.

Alfonso-Prieto, M., Biarnés, X., Vidossich, P., & Rovira, C. (2009). The molecular mechanism of the catalase reaction. Journal of the American Chemical Society, 131:11751-11761.

Alhendawi, R. A. (2011). Comparisons between Effects of Bicarbonate and High pH on Iron Uptake, Fem Reducing Capacity of the Roots, PEP Carboxylase Activity, Organic Acid Composition and Cation-anion Balance of the Xylem Sap of Maize Seedlings. American Journal of Plant Nutrition and Fertilization Technology, 201:1.

Alhendawi, R. A., Kirkby, E. A., Pilbeam, D. J., & Römheld, V. (2008). Effect of iron seed dressing and form of nitrogen-supply on growth and micronutrient concentration in shoots of sorghum grown in a calcareous sand culture. Journal of Plant Nutrition, 31:1855-1865.

Alhendawi, R. A., Römheld, V., Kirkby, E. A., & Marschner, H. (1997). Influence of increasing bicarbonate concentrations on plant growth, organic acid accumulation in roots and iron uptake by barley, sorghum, and maize. Journal of Plant Nutrition, 20:1731-1753.

Alloway, B. J. (2004). Zinc in soils and crop nutrition. IZA Publications. International Zinc Association, Brussels, pp 1–116

Alloway, B. J. (2009). Soil factors associated with Zn deficiency in crops and humans. Environmental Geochemistry and Health, 31:537-548.

Alonso-Blanco, C., Aarts, M. G., Bentsink, L., Keurentjes, J. J., Reymond, M., Vreugdenhil, D., & Koornneef, M. (2009). What has natural variation taught us about plant development, physiology, and adaptation? The Plant Cell, 21:1877-1896.

Alonso-Blanco, C., Gomez-Mena, C., Llorente, F., Koornneef, M., Salinas, J., & Martínez-Zapater, J. M. (2005). Genetic and molecular analyses of natural variation indicate CBF2 as a candidate gene for underlying a freezing tolerance quantitative trait locus in Arabidopsis. Plant Physiology, 139:1304-1312.

Alscher, R.G., N. Erturk and L.S. Hearth. (2002). Role of superoxide dismutases (SODs) in controlling oxidative stress in plants. Journal of Experimental Botany, 53:1331–1341

Al-Shehbaz, I. A., & O'Kane Jr, S. L. (2002). Taxonomy and phylogeny of Arabidopsis (Brassicaceae). The Arabidopsis Book, e0001.

Amer, E.A., A. Mahmoud and V. Sabel. (1985) Zeta potential and surface area of calcium carbonate as related to phosphate sorption. Soil Science Society of America Journal, 49:1137-1142.

An, L., Zhou, Z., Sun, L., Yan, A., Xi, W., Yu, N. & Gan, Y. (2012). A zinc finger protein gene ZFP5 integrates phytohormone signaling to control root hair development in Arabidopsis. The Plant Journal, 72:474-490.

Asimit, J., & Zeggini, E. (2010). Rare variant association analysis methods for complex traits. Annual Review of Genetics, 44, 293-308.

Atwell, S., Huang, Y.S., Vilhjalmsson, B.J., Willems, G., Horton, M., … & Nordborg, M. (2010) Genome-wide association study of 107 phenotypes in Arabidopsis thaliana inbred lines. Nature. 465:627–631

Babior, B. M. (1997). Superoxide: a two-edged sword. Brazilian Journal of Medical and Biological Research, 30:141-155.

Bakker, E. G., Toomajian, C., Kreitman, M., & Bergelson, J. (2006). A genome-wide survey of R gene polymorphisms in Arabidopsis. The Plant Cell, 18:1803-1818.

Bangerth, F. (1979). Calcium-related physiological disorders of plants. Annual Review of Phytopathology, 17:97-122.

Barak, P., & Helmke, P. A. (1993). The chemistry of zinc. In Zinc in soils and plants (pp. 1-13). Springer Netherlands.

Barceló, J., Nicolás, G., Sabater, B., & Sánchez, R. (2001). Fisiología vegetal. Ediciones Pirámide, Madrid, 275-306.

Barhoumi, Z., Rabhi, M., & Gharsalli, M. (2007). Effect of two nitrogen forms on the growth and iron nutrition of pea cultivated in presence of bicarbonate. Journal of Plant Nutrition, 30:1953-1965

Bauer P, Ling H-Q, Guerinot ML (2007) FIT, the FER-LIKE iron deficiency induced transcription factor in Arabidopsis. Plant Physiolgy and Biochemestry 45:260-61.

Baxter, I., Brazelton, J. N., Yu, D., Huang, Y. S., Lahner, B., Yakubova, E., ... & Vitek, O. (2010). A coastal cline in sodium accumulation in Arabidopsis thaliana is driven by natural variation of the sodium transporter AtHKT1. PLoS Genetics, 6,

Baxter, I., Muthukumar, B., Park, H. C., Buchner, P., Lahner, B., Danku, J., ... & Salt, D. E. (2008). Variation in molybdenum content across broadly distributed populations of

Arabidopsis thaliana is controlled by a mitochondrial molybdenum transporter (MOT1). PLoS Genetics, 4,

Benfey, P. N., & Mitchell-Olds, T. (2008). From genotype to phenotype: systems biology meets natural variation. Science, 320:495-497.

Benjamini, Y., & Yekutieli, D. (2001). The control of the false discovery rate in multiple testing under dependency. Annals of Statistics, 57:1165-1188.

Bergelson J, Roux F (2010) Towards identifying genes underlying ecologically relevant traits in Arabidopsis thaliana. Natural Reviews Genetics 11:867-879

Bienfait, H. F. (1985). Regulated redox processes at the plasmalemma of plant root cells and their function in iron uptake. Journal of Bioenergetics and Biomembranes, 17:73-83.

Bienfait, H. F., Bino, R. J., Bliek, A. V. D., Duivenvoorden, J. F., & Fontaine, J. M. (1983). Characterization of ferric reducing activity in roots of Fe-deficient Phaseolus vulgaris. Physiologia Plantarum, 59:196-202.

Bindschedler LV, Dewdney J, Blee KA, (2006). Peroxidase dependent apoplastic oxidative burst in Arabidopsis required for pathogen resistance. The Plant Journal, 47:851–863.

Black CA (1965) Particle fraction and particle-size analysis. Methods of Soil Analysis: Physical and Mineralogical Properties Including Statistics of Measurement and sampling, 1:550-551

Blanquart F, Kaltz O, Nuismer SL, Gandon S (2013) A practical guide to measuring local adaptation. Ecology Letters 16:1195-1205

Blokhina, O., E. Virolainen and K.V. Fagerstedt. (2003). Antioxidants, oxidative damage and oxygen deprivation stress. Annals of Botanny, 91:179–194.

Bloom P. R and Inskeep W. P. (1986) Kinetics of Calcite Precipitation in the Presence of Water-soluble Organic Ligands. Soil Science 50:1167-1172

Bohórquez, J. M., Romera, F. J., & Alcántara, E. (2001). Effect of Fe^{3+}, Zn^{2+} and Mn^{2+} on ferric reducing capacity and regreening process of the peach rootstock Nemaguard (Prunus persica (L.) Batsch). Plant and Soil, 237:157-163.

Bolòs, O., & Vigo, J. (2015). Flora dels països catalans, vol I-IV.

Borevitz, J. O., & Nordborg, M. (2003). The impact of genomics on the study of natural variation in Arabidopsis. Plant Physiology, 132:718-725

Boxma .R (1972) Bicarbonate as the most important soil factor in lime-induced chlorosis in the Netherlands. Plant and Soil, 37:233-243

Brachi B, Villoutreix R, Faure N, Hautekèete N, Piquot Y, Pauwels M, Roby D, Cuguen J, Berge lson J, Roux F (2013) Investigation of the geographical scale of adaptive phenological variation and its underlying genetics in Arabidopsis thaliana. Molecular Ecology 22:4222–4240

Brachi, B., Faure, N., Horton, M., Flahauw, E., Vazquez, A., Nordborg, M., Bergelson, J., Cuguen, J., Roux, F. (2010) Linkage and association mapping of Arabidopsis thaliana flowering time in nature. PLoS Genetics. 6

Brady, N.C. and R.R. Weil (1999) The Nature and Properties of Soils. 12th Edition. Prentice Hall, Upper Saddle River, New Jersey.

Bristow, A. W. T. (2006). Accurate mass measurement for the determination of elemental formula–A tutorial. Mass Spectrometry Reviews, 25:99-111.

Brown, J. C., Chaney, R. L., and Ambler, J. E. (1971). A new tomato mutant inefficient in the transport of iron. Plant Physiology, 25:48–53.

Brumbarova, T., Bauer, P., and Ivanov, R. (2015). Molecular mechanisms governing Arabidopsis iron uptake. Trends in Plant Science. 20:124–133.

Buescher, E., Achberger, T., Amusan, I., Giannini, A., Ochsenfeld, C., Rus, A., Lahner, B., Hoekenga, O., Yakubova, E. & Guerinot, M. L. (2010). Natural genetic variation in selected populations of Arabidopsis thaliana is associated with ionomic differences. PLoS One, 5

Burke, J. M., Burger, J. C., & Chapman, M. A. (2007). Crop evolution: from genetics to genomics. Current Opinion in Genetics & Development, 17:525-532.

Busoms, S. (2015) Local adaptation of wild populations of Arabidopsis thaliana to coastal and inland habitats in Catalonia (Thesis) Universitat Autònoma de Barcelona and University of Aberdeen.

Busoms, S., Terés, J., Huang, X., Bomblies, K., Danku, J., Douglas, A., Weigel, D., Poschenrieder, C. & Salt, D. (2015) Salinity is an agent of divergent selection driving local adaptation of Arabidopsis to coastal habitats. Plant Physiology, 168:915-929.

Calvarro, L. M., de Santiago-Martín, A., Gómez, J. Q., González-Huecas, C., Quintana, J. R., Vázquez, A., ... & Vera, R. R. (2014). Biological activity in metal-contaminated calcareous agricultural soils: the role of the organic matter composition and the particle size distribution. Environmental Science and Pollution Research, 21:6176-6187.

Carlioz, A., & Touati, D. (1986). Isolation of superoxide dismutase mutants in Escherichia coli: is superoxide dismutase necessary for aerobic life? The EMBO Journal, 5:623.

Carter MR, Gregorich EG (2006) Soil Sampling and Methods of Analysis, 2nd edn. Taylor and Francis Group, CRC Press, Boca Raton, FL, pp 1223.

Cesco, S., Neumann, G., Tomasi, N., Pinton, R., & Weisskopf, L. (2010). Release of plant-borne flavonoids into the rhizosphere and their role in plant nutrition. Plant and Soil, 329:1-25.

Chaignon, V., & Hinsinger, P. (2003). A biotest for evaluating copper bioavailability to plants in a contaminated soil. Journal of Environmental Quality, 32:824-833.

Chaney, R. L., Brown, J. C., & Tiffin, L. O. (1972). Obligatory reduction of ferric chelates in iron uptake by soybeans. Plant Physiology, 50:208-213.

Chao, D.-Y., Chen, Y., Chen, J., Shi, S., Chen, Z., Wang, C., Danku, J.M., Zhao, F.-J, Salt, D.E. (2014) Genome-wide association mapping identifies a new arsenate reductase enzyme critical for limiting arsenic accumulation in plants. PloS Biology. 12

Chao, D.-Y., Silva, A., Baxter, I., Huang, Y.S. Nordborg, M., Danku, J., Lahner, B., Yakubova, E., Salt, D.E. (2012) Genome-wide association studies identify heavy metal ATPase3 as the primary determinant of natural variation in leaf cadmium in Arabidopsis thaliana. PLoS Genetics 8

Charlesworth, D., & Vekemans, X. (2005). How and when did Arabidopsis thaliana become highly self-fertilising. Bioessays, 27:472-476.

Chary, P., Dillon, D., Schroeder, A. L., & Natvig, D. O. (1994). Superoxide dismutase (sod-1) null mutants of Neurospora crassa: oxidative stress sensitivity, spontaneous mutation rate and response to mutagens. Genetics, 137:723-730.

Chen C, Huang D, Liu J (2009) Functions and toxicity of nickel in plants: recent advances and future prospects. Clean 37:304–313

Chouliaras, V., Therios, I., Molassiotis, A., & Diamantidis, G. (2004). Iron chlorosis in grafted sweet orange (Citrus sinensis L.) plants: physiological and biochemical responses. Biologia plantarum, 48:141-144.

Clark, R. M., Schweikert, G., Toomajian, C., Ossowski, S., Zeller, G., Shinn, P., ... & Chen, H. (2007). Common sequence polymorphisms shaping genetic diversity in Arabidopsis thaliana. Science, 317:338-342.

Clauss, M. J., & Koch, M. A. (2006). Poorly known relatives of Arabidopsis thaliana. Trends in Plant Science, 11:449-459.

Clegg, M. T., & Durbin, M. L. (2003). Tracing floral adaptations from ecology to molecules. Nature Reviews. Genetics, 4:206.

Cockram, J., White, J., Zuluaga, D.L., Smith, D., Comadran, J., … & O'Sullivan, D.M. (2010) Genome-wide association mapping to candidate polymorphism resolution in the unsequenced barley genome. Proceedings of the National Academy of Sciences of the United States of America, 107:21611-21616

Colangelo EP y Guerinot ML (2004) The essensial basic helix-loop-helix protein FIT1 is required for the iron deficiency response. Plant Cell, 16:3400- 3412

Colombo, C., Palumbo, G., He, J. Z., Pinton, R., & Cesco, S. (2014). Review on iron availability in soil: interaction of Fe minerals, plants, and microbes. Journal of Soils and Sediments, 14:538-548.

Connolly, E. L., Campbell, N. H., Grotz, N., Prichard, C. L., & Guerinot, M. L. (2003). Overexpression of the FRO2 ferric chelate reductase confers tolerance to growth on low iron and uncovers posttranscriptional control. Plant Physiology, 133:1102-1110.

Connolly, E. L., Fett, J. P., & Guerinot, M. L. (2002). Expression of the IRT1 metal transporter is controlled by metals at the levels of transcript and protein accumulation. The Plant Cell, 14:1347-1357.

Coulombe, B.A., Chaney R.L., Weibold W.J. (1984). Use of bicarbonate in screening soybeans for resistance to iron chlorosis. Plant Nature, 7:411-425

Darwin, C., & Wallace, A. (1858). On the tendency of species to form varieties; and on the perpetuation of varieties and species by natural means of selection. Zoological Journal of the Linnean Society, 3:45-62.

Das, P., Nutan, K. K., Singla-Pareek, S. L., & Pareek, A. (2015). Oxidative environment and redox homeostasis in plants: dissecting out significant contribution of major cellular organelles. Frontiers in Environmental Science, 2:70.

Deisseroth, A. and A. Dounce. (1970). Catalase: Physical and chemical properties, mechanism of catalysis and physiological role. Physiological Reviews, 50:319-375.

Desikan, R., Hancock, J. T., Coffey, M. J., & Neill, S. J. (1996). Generation of active oxygen in elicited cells of Arabidopsis thaliana is mediated by a NADPH oxidase-like enzyme. FEBS letters, 382:213-217.

Dixon NE, Hinds JA, Fihelly AK, Gozala C, Winzor DJ, Blakeley RL, Zerner B (1980) Jack bean urease (EC 3.5.1.5). IV. The molecular size and mechanism of inhibition by hydroxamic acids. Spectrophotometric fixation of enzymes with reversible inhibitors. Canadian Journal of Biochemestry, 58:1323-1334

Doebley, J. F., Gaut, B. S., & Smith, B. D. (2006). The molecular genetics of crop domestication. Cell, 127:1309-1321.

Doerge, R. W. (2002). Mapping and analysis of quantitative trait loci in experimental populations. Nature Reviews. Genetics, 3: 43.

Donnini, S., Castagna, A., Ranieri, A., & Zocchi, G. (2009). Differential responses in pear and quince genotypes induced by Fe deficiency and bicarbonate. Journal of Plant Physiology, 166:1181-1193.

Dounce, A.L. (1983). A proposed mechanism for the catalatic action of catalase. Journal of Theoretical Biology, 105:553-567.

Eckhardt U, Marques AM, Buckout TJ (2001) Two iron-regulated cation transporters from tomato complement metal uptake-deficient yeast mutants. Plant Molecular Biology 45:437-448.

Eide D, Broderius M, Fett J, Guerinot ML (1996) A novel iron-regulated metal transporter from plants identified by functional expression in yeast. Proceedings of Natural Academy of Science 93:5624-5628.

Elstner, E. F. (1991). Mechanisms of oxygen activation in different compartments of plant cells. Current. Active Oxygen/Oxidative Stress and Plant Metabolism, 6:13-26.

Erdei, S., A. Hegedûs, G. Hauptmann, J. Szalai and G. Horváth (2002) Heavy metal induced physiological changes in antioxidative response system. Plant Physiology, 46:89-90

Escudero, R., Gómez-Gallego, M., Romano, S., Fernández, I., Gutiérrez-Alonso, Á., Sierra, M. A., … & P., Lucena, J. J. (2012). Biological activity of Fe (III) aquo-complexes towards ferric chelate reductase (FCR). Organic & Biomolecular Chemistry, 10:2272-2281.

Fourcroy, P., Sisó-Terraza, P., Sudre, D., Savirón, M., Reyt, G., Gaymard, F., et al., (2014). 951 Involvement of the ABCG37 transporter in secretion of scopoletin and derivatives by 952 Arabidopsis roots in response to iron deficiency. New Phytology, 201:155-167

Fourcroy, P., Sisó-Terraza, P., Sudre, D., Savirón, M., Reyt, G., Gaymard, F., ... & Briat, J. F. (2014). Involvement of the ABCG37 transporter in secretion of scopoletin and derivatives by Arabidopsis roots in response to iron deficiency. New Phytologist, 201:155-167.

Foyer, C. H., and Noctor, G. (2005). Redox homeostasis and antioxidant signaling: a metabolic interface between stress perception and physiological responses. Plant Cell. 17:1866–1875

Foyer, C. H., Lopez-Delgado, H., Dat, J. F., & Scott, I. M. (1997). Hydrogen peroxide-and glutathione-associated mechanisms of acclimatory stress tolerance and signalling. Physiologia Plantarum, 100:241-254.

Friesen, M. L., & von Wettberg, E. J. (2010). Adapting genomics to study the evolution and ecology of agricultural systems. Current Opinion in Plant Biology, 13:119-125.

Frische T, Mebes KH, Filser J. (2003) Assessing the bioavailability of contaminants in soils: A review of recent contents. Research Report. Federal Environmental Agency (Umweltbundesamt), Berlin, Germany

Gabrielli R, Pandolfini T (1984) Effect of Mg^{2+} and Ca^{2+} on the response to nickel toxicity in a serpentine endemic and nickelaccumulating species.Physiologia Plantarum, 62:540–544

García, M. J., García-Mateo, M. J., Lucena, C., Romera, F. J., Rojas, C. L., Alcántara, E., & Pérez-Vicente, R. (2014). Hypoxia and bicarbonate could limit the expression of iron acquisition genes in Strategy I plants by affecting ethylene synthesis and signaling in different ways. Physiologia Plantarum, 150:95-106.

Ghandilyan, A., Barboza, L., Tisné, S., Granier, C., Reymond, M., Koornneef, M., Schat, H. and Aarts, M. G. (2009). Genetic analysis identifies quantitative trait loci controlling rosette mineral concentrations in Arabidopsis thaliana under drought. New Phytologist, 184:180-192

Gibson, G. (2011) Rare and common variants: twenty arguments. Nature Reviews Genetics. 13:135-145

González-Martínez, S. C., Krutovsky, K. V., & Neale, D. B. (2006). Forest-tree population genomics and adaptive evolution. New Phytologist, 170:227-238.

Gralla, E. B., Thiele, D. J., Silar, P., & Valentine, J. S. (1991). ACE1, a copper-dependent transcription factor, activates expression of the yeast copper, zinc superoxide dismutase gene. Proceedings of the National Academy of Sciences, 88:8558-8562.

Graziano, M., and Lamattina, L. (2007). Nitric oxide accumulation is required for molecular and physiological responses to iron deficiency in tomato roots. Plant Journal, 52:949–960.

Gries, D., & Runge, M. (1992). The ecological significance of iron mobilization in wild grasses. Journal of Plant Nutrition, 15:1727-1737.

Gries, D., & Runge, M. (1995). Responses of calcicole and calcifuge poaceae species to iron-limiting conditions. Plant Biology, 108:482-489.

Grusak, M. A., Welch, R. M., & Kochian, L. V. (1990). Physiological characterization of a single-gene mutant of Pisum sativum exhibiting excess iron accumulation I. Root iron reduction and iron uptake. Plant Physiology, 93:976-981.

Guerinot ML, Yi Y (1994) Iron: nutritious, noxious, and not readily available. Plant Physiology 104:815-820

Guerinot, M. L. (2000). The ZIP family of metal transporters. Biochimica et Biophysica Acta (BBA)-Biomembranes, 1465:190-198.

Hagin, J. and B. Tucker. (1982). Fertilization of Dryland and Irrigated Soils. Springer Verlag, New York.

Halliwell, B., & Gutteridge, J. M. (1984). Role of iron in oxygen radical reactions. Methods in Enzymology, 105:47-56.

Hansen, N.C., Schmitt M.A., Anderson J.E., Strock J.S. (2003). Iron deficiency of soybean in the Upper Midwest and associated soil properties. Agronomy Journal, 95:1595-1601.

Hao, Z., Li, X., Xie, C., Weng, J., Li, M., Zhang, D., … & Zhang, S. (2011) Identification of functional genetic variations underlying drought tolerance in maize using SNP markers. Journal of Integrative Plant Biology, 53:641-52

Harada, H., & Leigh, R. A. (2006). Genetic mapping of natural variation in potassium concentrations in shoots of Arabidopsis thaliana. Journal of Experimental Botany, 57: 953-960.

Harter, R. D. (1983). Effect of soil pH on adsorption of lead, copper, zinc, and nickel. Soil Science Society of America Journal, 47:47-51.

Hell R, Stephan UW. (2003) Iron uptake, trafficking and homeostasis in plants. Planta. 216:541-51

Helmke, R.H. Loeppert, P.N. Soltanpour, M.A. Tabatabai, C.T. Johnson and M.E. Sumner (eds.). Methods of Soil Analysis Part 3 Chemical Methods. SSSA Special Publication No. 5. Madison, WI., pp. 437-474.

Hindt, M. N., & Guerinot, M. L. (2012). Getting a sense for signals: regulation of the plant iron deficiency response. Biochimica et Biophysica Acta (BBA)-Molecular Cell Research, 1823:1521-1530.

Hirschhorn, J. N., & Daly, M. J. (2005). Genome-wide association studies for common diseases and complex traits. Nature Reviews Genetics, 6:95-108.

Huff, L. C., Marranzino, A. P., & Nakagawa, H. M. (1970). A geochemical study of alluvium-covered copper deposits in Pima County, Arizona (No. 1312-C). US Govt. Print. Off.

Ivanov, R., Brumbarova, T., and Bauer, P. (2012). Fitting into the harsh reality: regulation of iron-deficiency responses in dicotyledonous plants. Molecular Plant 5:27-42.

Jackson, R.D. and L.J. Erie. (1973). Soil and water management practices for calcareous soils. In: FAO Soils Bulletin 21 - Calcareous Soils. Food and Agriculture Organization of the United Nations, Rome

Jakoby M, Wang HY, Reidt W, Eisshaar B, Bauer P (2004) FRU (BHLH029) is required for induction of iron mobilization genes in Arabidopsis thaliana. FEBS Letters, 577:528-534.

Jelali, N., Dell'orto, M., Abdelly, C., Gharsalli, M., & Zocchi, G. (2010). Changes of metabolic responses to direct and induced Fe deficiency of two Pisum sativum cultivars. Environmental and Experimental Botany 68:238-246.

Jones, D. L. (1998). Organic acids in the rhizosphere–a critical review. Plant and Soil, 205: 25-44.

Kawai, S., Kamei, S., Matsuda, Y., Ando, R., Kondo, S., Ishizawa, A., & Alam, S. (2001). Concentrations of iron and phytosiderophores in xylem sap of iron-deficient barley plants. Soil Science and Plant Nutrition, 47:265-272.

Kawecki, T. J., & Ebert, D. (2004). Conceptual issues in local adaptation. Ecology letters, 7:1225-1241.

Keurentjes, J. J., Fu, J., De Vos, C. R., Lommen, A., Hall, R. D., Bino, R. J., ... & Koornneef, M. (2006). The genetics of plant metabolism. Nature genetics, 38:842.

Kiekens, L. (1980). Heavy metal sorption in soil. Doctoral Thesis, Gent

Kim, B. G., Waadt, R., Cheong, Y. H., Pandey, G. K., Dominguez-Solis, J. R., Schültke, S., ... & Luan, S. (2007). The calcium sensor CBL10 mediates salt tolerance by regulating ion homeostasis in Arabidopsis. The Plant Journal, 52: 473-484.

Kliebenstein, D. J., Kroymann, J., Brown, P., Figuth, A., Pedersen, D., Gershenzon, J., & Mitchell-Olds, T. (2001). Genetic control of natural variation in Arabidopsis glucosinolate

accumulation. Plant physiology, 126(2), 811-825.Kobayashi, T., & Nishizawa, N. K. (2012). Iron uptake, translocation, and regulation in higher plants. Annual Review of Plant Biology, 63:131-152.

Kobayashi, T., and Nishizawa, N. K. (2012). Iron uptake, translocation, and regulation in higher plants. Annual Review of Plant Biology, 63:131-152.

Kobayashi, Y., Kuroda, K., Kimura, K., Southron-Francis, J. L., Furuzawa, A., Kimura, K., ... & Koyama, H. (2008). Amino acid polymorphisms in strictly conserved domains of a P-type ATPase HMA5 are involved in the mechanism of copper tolerance variation in Arabidopsis. Plant Physiology, 148:969-980.

Kochian, L. V., Hoekenga, O. A., & Piñeros, M. A. (2004). How do crop plants tolerate acid soils? Mechanisms of aluminum tolerance and phosphorous efficiency. Annual Review of Plant Biology, 55:459-493.

Koornneef, M., Alonso-Blanco, C., & Vreugdenhil, D. (2004). Naturally occurring genetic variation in Arabidopsis thaliana. Annual Review of Plant Biology,55:141-172.

Kooyers, N. J. (2015). The evolution of drought escape and avoidance in natural herbaceous populations. Plant Science, 234:155-162.

Korshunova, Y. O., Eide, D., Clark, W. G., Guerinot, M. L., & Pakrasi, H. B. (1999). The IRT1 protein from Arabidopsis thaliana is a metal transporter with a broad substrate range. Plant Molecular Biology, 40:37-44.

Korte, A., Farlow, A. (2013) The advantages and limitations of trait analysis with GWAS: a review. Plant Methods, 9:29

Korves, T. M., & Bergelson, J. (2003). A developmental response to pathogen infection in Arabidopsis. Plant Physiology, 133:339-347.

Kosegarten H., Hoffmann B., Rroco E., Grolig F., Glüsenkamp K., Mengel K. (2004) Apoplastic pH and Fe^{III} reduction in young sunflower (Helianthus annuus) roots. Physiologia Plantarum: 95-106

Krämer, U., & Clemens, S. (2005). Functions and homeostasis of zinc, copper, and nickel in plants. In Molecular biology of metal homeostasis and detoxification (pp. 215-271). Springer Berlin Heidelberg

Kramer, E. M. (2009). Aquilegia: a new model for plant development, ecology, and evolution. Annual Review of Plant Biology, 60:261-277.

Kruszewski, M. (2003). Labile iron pool: the main determinant of cellular response to oxidative stress. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 531:81-92.

Ksouri, R., Megdiche, W., Debez, A., Falleh, H., Grignon, C., & Abdelly, C. (2007). Salinity effects on polyphenol content and antioxidant activities in leaves of the halophyte Cakile maritima. Plant Physiology and Biochemistry, 45:244-249.

Landsberg, E.C. (1984). Regulation of iron stress response by whole plant activity. Journal of Plant Nutrition 7:609-621.

Lawrence, M. J. (1976). Variations in natural populations of Arabidopsis thaliana (L.) Heynh. the Biology and Chemistry of the Cruciferae. JG Vaughan, AJ Macleod & BMG Jones, eds.

Lee, J. A. (1998). The calcicole—calcifuge problem revisited. Advances in Botanical Research, 29:1-30.

Lee, J. A., & Woolhouse, H. W. (1969). Root growth and dark fixation of carbon dioxide in calcicoles and calcifuges. New Phytologist, 68:247-255.

Levin, D. A. (2006). Flowering phenology in relation to adaptive radiation. Systematic Botany, 31:239-246.

Li L, Cheng X, Ling HQ (2004) Isolation and characterization of Fe (III) chelate reductase gene LeFRO1 in tomato. Plant Molecular Biology 54:125-136.

Lindsay W.L., Schwab A.P. (1982). The chemistry of iron soils and its availability to plants. Journal of Plant Nutrition, 5:821-840

Lindsay, W. L. (1979). Chemical Equilibria in soil. John Wiley & Sons, New York

Lindsay, W. L. (1984). Soil and plant relationships associated with iron deficiency with emphasis on nutrient interactions. Journal of Plant Nutrition, 7:489-500.

Loeppert, R.H. and D.L. Suarez. (1996). Carbonate and gypsum, In: D.L. Sparks, A.L. Page, P.A.

Long, N. V., Dolstra, O., Malosetti, M., Kilian, B., Graner, A., Visser, R. G. F., van der Linden, C. G. (2013). Association mapping of salt tolerance in barley (hordeum vulgare L.). Theoretical and Applied Genetics. 126:2335-2351

Loper, J. E., & Buyer, J. S. (1991). Siderophores in microbial interactions on plant surfaces. Molecular Plant-Microbe Interact, 4:5-13.

López-Millán, A. F., Grusak, M. A., Abadía, A., & Abadía, J. (2013). Iron deficiency in plants: an insight from proteomic approaches. Frontiers in Plant Science, 4:254.

Loudet, O., Chaillou, S., Merigout, P., Talbotec, J., & Daniel-Vedele, F. (2003). Quantitative trait loci analysis of nitrogen use efficiency in Arabidopsis. Plant Physiology, 131:345-358.

Lowenstam HA (1981) Minerals formed by organisms. Science 211:1126-1131

Lower SK, Hochella MF, Beveridge TJ (2001) Bacterial recognition of mineral surfaces: nanoscale interactions between Shewanella and α- FeOOH. Science 292:1360-1363

Lucena, C., Romera, F. J., Rojas, C. L., García, M. J., Alcántara, E., & Pérez-Vicente, R. (2007). Bicarbonate blocks the expression of several genes involved in the physiological responses to Fe deficiency of Strategy I plants. Functional Plant Biology, 34: 1002-1009.

Lucena, J. J. (2000). Effects of bicarbonate, nitrate and other environmental factors on iron deficiency chlorosis. A review. Journal of Plant Nutrition, 23, 1591-1606

Lyons, R., Stiller, J., Powell, J., Rusu, A., Manners, J. M., & Kazan, K. (2015). Fusarium oxysporum triggers tissue-specific transcriptional reprogramming in Arabidopsis thaliana. PLoS One, 10, e0121902.

Malcolm, J. (2001) Molecular Analysis of Plant Adaptation to the Environment, Dordrecht, The Netherlands, Kluwer academic publishers

Marin, B., Chopin, E. I. B., Jupinet, B., & Gauthier, D. (2008). Comparison of microwave-assisted digestion procedures for total trace element content determination in calcareous soils. Talanta, 77:282-288.

Marschner, H., Römheld, V., & Kissel, M. (1986). Different strategies in higher plants in mobilization and uptake of iron. Journal of Plant Nutrition, 9:695-713.

Marschner H. (1995) Mineral Nutrition of Higher Plants. London: Academic. 2nd ed.

Marschner H (2002) Mineral nutrition of higher plants, 3rd edn. Academic Press, London, 364-369

Maurer, F., Müller, S., and Bauer, P. (2011). Suppression of Fe deficiency gene expression by jasmonate. Plant Physiology and Biochemistry, 49:530–536.

McCray JM, Matocha JE. (1992) Effects of soil water levels on solution bicarbonate, chlorosis and growth of sorghum. Journal of Plant Nutrition, 15:1877–1890.

McKay, J. K., Christian, C. E., Harrison, S., & Rice, K. J. (2005). “How local is local?”—a review of practical and conceptual issues in the genetics of restoration. Restoration Ecology, 13:432-440.

Mendel, G. (1996). Experiments in plant hybridization (1865). Verhandlungen des naturforschenden Vereins Brünn. Available online: www.mendelweb.org/Mend

Mengel K., Breininger M. Th., Bübl W.(1984) Bicarbonate, the most important factor inducing Iron chlorosis in vine grapes on calcareous soil. Plant and Soil, 81.333-334.

Mengel, K., Kirkby, E. A., Kosegarten, H., & Appel, T. (2001). Soil Copper. In Principles of plant nutrition (pp. 599-611). Springer Netherlands.

Mikami, Y., Saito, A., Miwa, E., & Higuchi, K. (2011). Allocation of Fe and ferric chelate reductase activities in mesophyll cells of barley and sorghum under Fe-deficient conditions. Plant Physiology and Biochemistry, 49:513-519.

Mitchell-Olds, T., Willis, J. H., & Goldstein, D. B. (2007). Which evolutionary processes influence natural genetic variation for phenotypic traits? Nature Reviews Genetics, 8:845-856

Mittler R, Vanderauwera S, Gollery M, Van Breusegem F. (2004). The reactive oxygen gene network in plants. Trends in Plant Science 9:490–498.

Mohsenian, Y., & Roosta, H. R. (2015). Effects of grafting on alkali stress in tomato plants: datura rootstock improve alkalinity tolerance of tomato plants. Journal of Plant Nutrition, 38:51-72.

Molassiotis, A., Sotiropoulos, T., Tanou, G., Diamantidis, G., & Therios, I. (2006). Boron-induced oxidative damage and antioxidant and nucleolytic responses in shoot tips culture of the apple rootstock EM 9 (Malus domestica Borkh). Environmental and Experimental Botany, 56:54-62.

Moog P.R., Brüggemann W., Schiefelbein J.W., Kuiper J.C. (1994) Responses to iron deficiency in Arabidopsis thaliana: The Turbo iron reductase does not depend on the formation of root hairs and transfer cells. Planta, 195:505-513

Morris, D.R., R.H. Loeppert and T.J. Moore. (1990). Indigenous soil factors influencing Fe chlorosis of soybean in calcareous soils. Soil Science of Society America Journal, 15: 1329-1336.

Morrissey, J., Baxter, I. R., Lee, J., Li, L., Lahner, B., Grotz, N., ... & Guerinot, M. L. (2009). The ferroportin metal efflux proteins function in iron and cobalt homeostasis in Arabidopsis. The Plant Cell, 21:3326-3338.

Msilini, N., Attia, H., Bouraoui, N., M'rah, S., Ksouri, R., Lachaâl, M., & Ouerghi, Z. (2009). Responses of Arabidopsis thaliana to bicarbonate-induced iron deficiency. Acta Physiologiae Plantarum, 31:849-853.

Myles, S., Peiffer, J., Brown, P. J., Ersoz, E. S., Zhang, Z., Costich, D. E., & Buckler, E. S. (2009). Association mapping: critical considerations shift from genotyping to experimental design. The Plant Cell, 21:2194-2202.

Naranjo-Arcos, M. A., & Bauer, P. (2016). Nutritional Deficiency. Chapter 4: Iron Nutrition, Oxidative Stress, and Pathogen Defense.

Nelson DW, Sommers LE. (1982). Total carbon, organic carbon and organic matter. A: Page AL, Miller RH, Keeney DR (Eds) Methods of soil analysis. Part 2. Chemical and microbiological properties. American Society of Agronomy and Soil Science Society of America, Madison, 539-579.

Nordborg, M., Hu, T. T., Ishino, Y., Jhaveri, J., Toomajian, C., Zheng, H., ... & Jakobsson, M. (2005). The pattern of polymorphism in Arabidopsis thaliana. PLoS biology, 3.

Obreza, T.A., A. K. Alva and D.V. Calvert. (1993) Citrus fertilizer management on calcareous soils. Circular 1127, Soil and Water Science Department, Florida Cooperative Extension Service, Institute of Food and Agricultural Sciences, University of Florida.

Oshino, N., D. Jamieson, T. Sugano, and B. Chance. (1975). Optical measurement of the catalase-hydrogen peroxide intermediate compound I in the level of anaesthetized rats and its implication to H202 production in viva. Biochemistry Journal, 146:67-77.

Parat, C., Chaussod, R., Lévêque, J., Dousset, S., & Andreux, F. (2002). The relationship between copper accumulated in vineyard calcareous soils and soil organic matter and iron. European Journal of Soil Science, 53:663-670.

Parra Terraza, S., Lara Murrieta, P., Villarreal Romero, M., & Hernandez Verdugo, S. (2012). Plant growth and tomato yield at several nitrate/ammonium ratios and bicarbonate concentrations. Revista Fitotecnia Mexicana, 35:143-153.

Pearce, R.C., Y. Li and L.P. Bush (1999) Calcium and bicarbonate effects on the growth and nutrient uptake of burley tobacco seedlings: hydroponic culture. Journal of Plant Nutrition, 22:1069-1078.

Peijnenburg WJGM, Jager T. (2003). Monitoring approaches to assess bioaccessibility and bioavailability of metals: Matrix issues. Ecotoxicological and Environmental Safety 56:63-77.

Phillips, J. P., Campbell, S. D., Michaud, D., Charbonneau, M., & Hilliker, A. J. (1989). Null mutation of copper/zinc superoxide dismutase in Drosophila confers hypersensitivity to paraquat and reduced longevity. Proceedings of the National Academy of Sciences, 86:2761-2765.

Poehlman, J. M., & Sleper, D. A. (1995). Methods in Plant Breeding. Breeding Field Crops, 172-174.

Porta, J., Lopez-Acevedo, M., Rodriguez, R. (1986). Técnicas y experimentos en edafología. Col·legi Oficial d'Enginyers Agrònoms de Catalunya, Barcelona.

Porter L., Thorne, D. W. (1955) Interrelation of Carbon Dioxide and Bicarbonate Ions in Causing Plant Chlorosis. Soil Science, 79:373-382

Ramachandran, V., & D'Souza, S. F. (2013). Adsorption of nickel by Indian soils. Journal of Soil Science and Plant Nutrition, 13:165-173

Ranocha, P., Chabannes, M., Chamayou, S., Danoun, S., Jauneau, A., Boudet, A. M., & Goffner, D. (2002). Laccase down-regulation causes alterations in phenolic metabolism and cell wall structure in poplar. Plant Physiology, 129:145-155.

Reed, S. T., & Martens, D. C. (1996). Copper and zinc. Methods of Soil Analysis Part 3—Chemical Methods, 703-722.

Rehm GW, Caldwell AC (1968) Sulfur supplying capacity of soils and the relationship to soil type. Soil Science 105:355-361

Rehman, H. U., Aziz, T., Farooq, M., Wakeel, A., & Rengel, Z. (2012). Zinc nutrition in rice production systems: a review. Plant and soil, 361:203-226.

Rellán-Álvarez, R., Andaluz, S., Rodríguez-Celma, J., Wohlgemuth, G., Zocchi, G., Álvarez-Fernández, A., ... & Abadía, J. (2010). Changes in the proteomic and metabolic profiles of Beta vulgaris root tips in response to iron deficiency and resupply. BMC Plant Biology, 10:120.

Remington, D. L., & Purugganan, M. D. (2003). Candidate genes, quantitative trait loci, and functional trait evolution in plants. International Journal of Plant Sciences, 164:7-20.

Rengel, Z. (2015). Availability of Mn, Zn and Fe in the rhizosphere. Journal of Soil Science and Plant Nutrition, 15:397-409.

Rhoades, JD. (1982). Cation exchange capacity. A: Page AL, Miller RH, Keeney DR (Eds) Methods of soil analysis. Part 2. Chemical and microbiological properties. American Society of Agronomy and Soil Science Society of America, Madison, 149-152.

Rhoads, W. A.; Wallace, A. (1960) Possible involvement of dark fixation of coz in lime-induced chlorosis. Soil Science 89:248-256

Robinson NJ, Procter CM, Connolly EL, Guerinot ML (1999) A ferric chelate reductase for iron uptake from soils. Nature 397:694-697.

Rodríguez-Celma, J., & Schmidt, W. (2013). Reduction-based iron uptake revisited: on the role of secreted iron-binding compounds. Plant Signaling & Behavior, 8:1473-1485.

Rodríguez-Celma, J., Lattanzio, G., Grusak, M. A., Abadia, A., Abadia, J., & López-Millán, A. F. (2011). Root responses of Medicago truncatula plants grown in two different iron deficiency conditions: changes in root protein profile and riboflavin biosynthesis. Journal of Proteome Research, 10:2590-2601.

Rodríguez-Celma, J., Lin, W. D., Fu, G. M., Abadía, J., López-Millán, A. F., & Schmidt, W. (2013). Mutually exclusive alterations in secondary metabolism are critical for the uptake of insoluble iron compounds by Arabidopsis and Medicago truncatula. Plant Physiology, 162:1473-1485.

Romera, F. J., Alcántara, E., & De la Guardia, M. D. (1998). The induction of the "turbo reductase" is inhibited by cycloheximide, cordycepin and ethylene inhibitors in Fe-deficient cucumber (Cucumis sativus L.) plants. Protoplasma, 205:156-162.

Romera, F. J., Alcantara, E., and De La Guardia, M. D. (1999). Ethylene production by Fe-deficient roots and its involvement in the regulation of Fe-deficiency stress responses by strategy I plants. Annals of Botany, 83:51-55.

Romera, F. J., and Alcántara, E. (1994). Iron-deficiency stress responses in cucumber (Cucumis sativus L.) roots (A possible role for ethylene?) Plant Physiology, 105:1133–1138.

Romera, F.J., Lucena, C. García, M.J., Alcántara, E., and Pérez-Vicente, R. (2014). Regulation of Fe deficiency responses in wt pea and some of its mutants (brz and dgl), in Pisum sativum: Cultivation, Functional Properties and Health Benefits. Ed. S. Becket: 1–20.

Römheld V, Marschner H. (1986a) Evidence for a specific uptake system for iron phytosiderophore in roots of grasses. Plant Physiology, 80:175–180

Römheld, V., & Marschner, H. (1983). Mechanism of iron uptake by peanut plants I. FeIII reduction, chelate splitting, and release of phenolics. Plant Physiology, 71:949-954.

Romheld, V., & Marschner, H. (1986a). Mobilization of iron in the rhizosphere of different plant species. Advances in plant nutrition (USA).

Rosen, D. R., Siddiquef, T., Patterson, D., Figlewicz, D. A., Sapp, P., Hentatif, A and Gaston, S. M. (1993). Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic. Nature, 362:4.

Rus, A., Baxter, I., Muthukumar, B., Gustin, J., Lahner, B., Yakubova, E., & Salt, D. E. (2006). Natural variants of AtHKT1 enhance Na+ accumulation in two wild populations of Arabidopsis. PLoS genetics, 2:210.

Ryan, J., D. Curtin and I. Safi (1981) Ammonia volatilization as influenced by calcium carbonate particle size and iron oxides. Soil Science of Society America Journal, 45:338-341.

Ryu, J. Y., Park, C. M., & Seo, P. J. (2011). The floral repressor BROTHER OF FT AND TFL1 (BFT) modulates flowering initiation under high salinity in Arabidopsis. Molecules and cells, 32:295-303.

Sagi, M., & Fluhr, R. (2006). Production of reactive oxygen species by plant NADPH oxidases. Plant physiology, 141:336-340.

Salt, D. E., Baxter, I., & Lahner, B. (2008). Ionomics and the study of the plant ionome. Annual Reviews of Plant Biology, 59:709-733.

Santiago-Martín A, Valverde-Asenjo I, Quintana JR, GonzálezHuecas C, Lafuente AL (2013b) Soil properties affecting metal extractability patterns in periurban calcareous agricultural soils in the Mediterranean area. International Journal of Environment Research, 7:831–840.

Schaaf, G., Honsbein, A., Meda, A. R., Kirchner, S., Wipf, D., & von Wirén, N. (2006). AtIREG2 encodes a tonoplast transport protein involved in iron-dependent nickel detoxification in Arabidopsis thaliana roots. Journal of Biological Chemistry, 281:25532-25540.

Schmidt, H., Gunther, C., Weber, M., Sporlein, C., Loscher, S., Bottcher, C., et al.,. (2014). Metabolome analysis of Arabidopsis thaliana roots identifies a key metabolic pathway for iron acquisition. PLoS ONE 9

Schmidt, W. (1999). Mechanisms and regulation of reduction-based iron uptake in plants. New Phytologist, 141:1-26.

Schöttelndreier, M., Norddahl, M. M., Ström, L., & Falkengren-Grerup, U. (2001). Organic acid exudation by wild herbs in response to elevated Al concentrations. Annals of Botany, 87:769-775.

Sedbrook, J. C., Carroll, K. L., Hung, K. F., Masson, P. H., & Somerville, C. R. (2002). The Arabidopsis SKU5 gene encodes an extracellular glycosyl phosphatidylinositol–anchored glycoprotein involved in directional root growth. The Plant Cell, 14:1635-1648.

Séguéla, M., Briat, J. F., Vert, G., and Curie, C. (2008). Cytokinins negatively regulate the root iron uptake machinery in Arabidopsis through a growth-dependent pathway. Plant Journal, 55:289–300

Seregin IV, Kozhevnikova AD (2006) Physiological role of nickel and its toxic effects on higher plants. Russian Journal of Plant Physiology 53:257-277

Sherrard, M. E., & Maherali, H. (2006). The adaptive significance of drought escape in Avena barbata, an annual grass. Evolution, 60:2478-2489.

Shindo, C., Bernasconi, G., & Hardtke, C. S. (2007). Natural genetic variation in Arabidopsis: tools, traits and prospects for evolutionary ecology. Annals of Botany, 99:1043-1054.

Shindo, C., Lister, C., Crevillen, P., Nordborg, M., & Dean, C. (2006). Variation in the epigenetic silencing of FLC contributes to natural variation in Arabidopsis vernalization response. Genes & Development, 20:3079-3083.

Shingles, R., North, M., & McCarty, R. E. (2001). Direct measurement of ferrous ion transport across membranes using a sensitive fluorometric assay. Analytical Biochemistry, 296:106-113.

Shingles, R., North, M., & McCarty, R. E. (2002). Ferrous ion transport across chloroplast inner envelope membranes. Plant Physiology, 128:1022-1030.

Sisó-Terraza, P., Luis-Villarroya, A., Fourcroy, P., Briat, J. F., Abadía, A., Gaymard, Fourcroy, P., …, & Álvarez-Fernández, A. (2016). Accumulation and Secretion of Coumarinolignans and other Coumarins in Arabidopsis thaliana Roots in Response to Iron Deficiency at High pH. Frontiers in Plant Science, 7.

Sisó-Terraza, P., Rios, J. J., Abadía, J., Abadía, A., & Álvarez-Fernández, A. (2016). Flavins secreted by roots of iron-deficient Beta vulgaris enable mining of ferric oxide via reductive mechanisms. New Phytologist, 209:733-745.

Soltanpour PA, Schwab AP (1977) A new soil test for simultaneous extraction of macro-and micro-nutrients in alkaline soils. Communications in Soil Science and Plant Analysis 8:195-207

Solti, Á., Kovács, K., Basa, B., Vértes, A., Sárvári, É., & Fodor, F. (2012). Uptake and incorporation of iron in sugar beet chloroplasts. Plant Physiology and Biochemistry, 52:1-97.

Stenøien, H. K., Fenster, C. B., Tonteri, A., & Savolainen, O. (2005). Genetic variability in natural populations of Arabidopsis thaliana in northern Europe. Molecular Ecology, 14:137-148.

Stephan, U. W. & Sholz, G. (1993). Nicotianamine: mediator of transport of iron and heavy metals in the phloem? Plant Physiology, 88:522-529

Stephan, U. W. (2002). Intra- and intercellular iron trafficking and subcellular compartmentation within roots. Plant and Soil 241:19-25

Su, Z., Ma, X., Guo, H., Sukiran, N. L., Guo, B., Assmann, S. M., & Ma, H. (2013). Flower development under drought stress: morphological and transcriptomic analyses reveal acute responses and long-term acclimation in Arabidopsis. The Plant Cell, 25:3785-3807.

Sunderman FW, Oskarsson A (1991) Metals and their compounds in the environment. In: Merian E, Weinheim VCH (eds), 1101-1126.

Susin, S., Abadia, A., González-Reyes, J. A., Lucena, J. J., & Abadia, J. (1996). The pH requirement for in vivo activity of the iron-deficiency-induced" Turbo" ferric chelate reductase (a comparison of the iron-deficiency-induced iron reductase activities of intact plants and isolated plasma membrane fractions in sugar beet). Plant Physiology, 110:111-123.

Susín, S., Abián, J., Peleato, M. L., Sánchez-Baeza, F., Abadía, A., Gelpi, E., & Abadía, J. (1994). Flavin excretion from roots of iron-deficient sugar beet (Beta vulgaris L.). Planta, 193:514-519.

Talibudeen, O. and P. Arambarri (1964) The influence of the amount and the origin of calcium carbonates on the isotopically-exchangeable phosphate in calcareous soils. Journal of Agricultural Science, 62:93-97.

Tapeiro, H., & Tew, K. D. (2003). Trace elements in human physiology and pathology: Zinc and metallothioneins. Biomedical Pharmacotherapy, 57:399–411

Temp, GA (1991) Nickel in plants and its toxicity: Ustoichivost'k tyazhelym metallam dikorastushchikh vidov (Resistance of wild species to heavy metals). In: Alekseeva-Popova NV (ed). Lenuprizdat, Leningrad,139–146

Tian, F., Bradbury, P.J., Brown, P.J., Hung, H., Sun, Q., Flint-Garcia, S., Rocheford, T.R., McMullen, M.D., Holland, J.B., Buckler, E.S. (2011) Genome-wide association study of leaf architecture in the maize nested association mapping population. Nature Genetics, 43:159-62

Tyler, G. (2003). Some ecophysiological and historical approaches to species richness and calcicole/calcifuge behaviour—contribution to a debate. Folia Geobotanica, 38:419-428.

Udo, E. J., Bohn, H. L., & Tucker, T. C. (1970). Zinc adsorption by calcareous soils. Soil Science Society of America Journal, 34:405-407.

Uygur V., & Rimmer, D. L. (2000). Reactions of zinc with iron coated calcite surfaces at alkaline pH. European Journal of Soil Science, 51:511-516

Vallejo, V. R. (1986) La distribució dels carbonats en els sòls de la Depressió Central Catalana. Institut Català de Historia Natural, 53:69-79.

Vert, G., Grotz, N., Dédaldéchamp, F., Gaymard, F., Guerinot, M. L., Briat, J. F., & Curie, C. (2002). IRT1, an Arabidopsis transporter essential for iron uptake from the soil and for plant growth. The Plant Cell, 14:1223-1233.

Vogel-Mikus K, Drobne D, Regvar M (2005) Zn, Cd and Pb accumulation and arbuscular mycorrhizal colonization of pennycress Thlaspi praecox Wulf. (Brassicaceae) from the vicinity of a lead mine and smelter in Slovenia. Environnemental Pollution 133:233-242

Vreugdenhil, D., Aarts, M. G. M., Koornneef, M., Nelissen, H., & Ernst, W. H. O. (2004). Natural variation and QTL analysis for cationic mineral content in seeds of Arabidopsis thaliana. Plant, Cell & Environment, 27:828-839.

Wadleigh, C. H., & Brown, J. W. (1952). The chemical status of bean plants afflicted with bicarbonate-induced chlorosis. Botanical Gazette, 373-392.

Wang, B., Li, Y., and Zhang, W. H. (2012). Brassinosteroids are involved in response of cucumber (Cucumis sativus) to iron deficiency. Annals of Botany, 110: 681-688

Waters, B. M., & Grusak, M. A. (2008). Whole-plant mineral partitioning throughout the life cycle in Arabidopsis thaliana ecotypes Columbia, Landsberg erecta, Cape Verde Islands, and the mutant line ysl1ysl3. New Phytologist, 177:389-405.

Waters, B. M., Lucena, C., Romera, F. J., Jester, G. G., Wynn, A. N., Rojas, C. L., Alcantara, E., Pérez-Vicente, R. (2007). Ethylene involvement in the regulation of the H+-ATPase CsHA1 gene and of the new isolated ferric reductase CsFRO1 and iron transporter CsIRT1 genes in cucumber plants. Plant Physiology and Biochemistry, 45:293-301.

Weigel, D., & Nordborg, M. (2005). Natural variation in Arabidopsis. How do we find the causal genes?. Plant Physiology, 138:567-568.

Weigel, D (2012) Natural Variation in Arabidopsis: From Molecular Genetics to Ecological Genomics. Plant Physiology 158:2-22

Welkie, G. W. (2000). Taxonomic distribution of dicotyledonous species capable of root excretion of riboflavin under iron deficiency. Journal of Plant nutrition, 23, 1819-1831.

Woolhouse H. W., Lee J. A. (1969) Root growth and dark fixation of carbon dioxide in calcicoles and calcifuges. New Phytologist, 68:247–255

Wright, S. I., & Gaut, B. S. (2004). Molecular population genetics and the search for adaptive evolution in plants. Molecular Biology and Evolution, 22:506-519.

Wu, C. A., Lowry, D. B., Cooley, A. M., Wright, K. M., Lee, Y. W., & Willis, J. H. (2008). Mimulus is an emerging model system for the integration of ecological and genomic studies. Heredity, 100:220.

Yang, Q., Li, Z., Li, W., Ku, L., Wang, C., Ye, J., …, & Xu, M. (2013) CACTA-like transposable element in ZmCCT attenuated photoperiod sensitivity and accelerated the postdomestication spread of maize. Proceedings of the National Academy of Sciences of the United States of America. 110:16969-16974

Yoshida, S., & Tanaka, A. (1969). Zinc deficiency of the rice plant in calcareous soils. Soil Science and Plant Nutrition, 15:75-80.

Yuan Y, Wu H, Wang N, Li J, Zhao W, Du J, Wang D, Ling H-Q (2008) FIT interacts with AtbHLH38 and AtbHLH39 in regulating iron uptake gene expression for iron homeostasis in Arabidopsis. Cell Research, 18:385-397.

Yusuf, M., Fariduddin, Q., Hayat, S., & Ahmad, A. (2011). Nickel: an overview of uptake, essentiality and toxicity in plants. Bulletin of Environmental Contamination and Toxicology, 86:1-17.

Zhao K, Aranzana MJ, Kim S, Lister C, Shindo C, Tang C, Toomajian C, Zheng H, Dean C, Marjoram P, Nordborg M. (2007) An Arabidopsis example of association mapping in structured samples. PLoS Genetics. 3:e4

Zuo, Y., Ren, L., Zhang, F., & Jiang, R. F. (2007). Bicarbonate concentration as affected by soil water content controls iron nutrition of peanut plants in a calcareous soil. Plant Physiology and Biochemistry, 45:357-364.

Web Pages & Programmes

Bruker Compas Data Analysis: DataAnalysis version 4.2

GATK: **McKenna, A., Hanna, M., Banks, E., Sivachenko, A., Cibulskis, K., Kernytsky, A., Garimella, K., Altshuler, D., Gabriel, S., Daly, M. & DePristo, M. A.** (2010). The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. Genome Research, 20:1297-1303.

GWAPP: https://gwas.gmi.oeaw.ac.at

JMP: Version 12 (2013-2016) & Version 13 (2016-2017) SAS Institute Inc, Cary, NC

Meteocat: Servei Meteorològic de Catalunya. «Trets generals». Web. [Consulta: 18 de febrer del 2017]. CC-BY-SA-3.0. http://meteo.cat

PICARD: http://broadinstitute.github.io/picard

SnpEff: **Cingolani, P., Platts, A., Wang, L. L., Coon, M., Nguyen, T., Wang, L., Land, S.J., Lu, X. & Ruden, D. M.** (2012). A program for annotating and predicting the effects of single nucleotide polymorphisms, SnpEff: SNPs in the genome of Drosophila melanogaster strain w1118; iso-2; iso-3. Fly, 6:80-92.

VCFtools: **Danecek, P., Auton, A., Abecasis, G., Albers, C. A., Banks, E., DePristo, M. A., Handsaker, R. E., Lunter, G., Marth G. T., Sherry, S.T. & McVean, G.** (2011). The variant call format and VCFtools. Bioinformatics, 27:2156-2158.

ANNEXES

Annex 1. Siliques statistics for years 2013-2015

Annex Chapter I Table 1: Mean ± Standard Deviation of number of siliques of years: 2013, 2014 and 2015 grown in two soils LP and SCF. ANOVA between group variable (%$CaCo_3$, pH level, %O.M.) in both common garden sites.

Variable	year	Site	Group nº	N	mean	Std Dev	DF	F ratio	Prob > F
$CaCO_3$%	2013	LP	1	30	86.7	31.1	2	44.0	<.0001
			2	30	97.0	41.8			
			3	30	256.2	92.1			
		SCF	1	30	338.3	99.6	2	1.5	0.2247
			2	30	309.1	76.3			
			3	30	355.7	110.5			
	2014	LP	1	30	73.5	30.6	2	82.7	<.0001
			2	30	125.3	48.0			
			3	30	221.3	53.9			
		SCF	1	30	245.6	55.3	2	3.8	0.0261
			2	30	278.1	58.9			
			3	30	279.2	46.2			
	2015	LP	1	30	53.6	34.5	2	52.9	<.0001
			2	30	71.2	27.9			
			3	30	141.0	52.7			
		SCF	1	30	277.6	137.6	2	2.9	0.0595
			2	30	239.2	41.3			
			3	30	228.6	78.6			
O.M.%	2013	LP	1	30	221.7	136.5	2	3.4	0.0385
			2	20	177.4	102.6			
			3	40	137.4	81.9			
		SCF	1	30	364.3	94.7	2	2.7	0.0727
			2	20	293.5	93.6			
			3	40	342.6	95.9			
	2014	LP	1	30	122.9	71.4	2	1.3	0.2734
			2	20	143.7	102.1			
			3	40	153.1	63.7			
		SCF	1	30	239.4	41.0	2	10.0	0.0001
			2	20	259.2	47.1			
			3	40	292.7	58.0			

Variable	year	Site	Group nº	N	mean	Std Dev	DF	F ratio	Prob > F
	2015	LP	1	30	73.6	56.3	2	5.7	0.0043
			2	20	119.6	49.6			
			3	40	87.2	51.7			
		SCF	1	30	242.1	76.4	2	7.0	0.0014
			2	20	308.2	161.2			
			3	40	225.2	49.0			
pH level	2013	LP	1	30	257.6	106.1	2	19.2	<.0001
			2	30	83.4	29.3			
			3	30	189.2	99.7			
		SCF	1	30	342.4	104.1	2	0.1	0.904
			2	30	334.9	92.8			
			3	30	328.8	100.6			
	2014	LP	1	30	138.7	63.3	2	54.8	<.0001
			2	30	74.1	32.0			
			3	30	216.3	53.5			
		SCF	1	30	255.6	47.0	2	9.6	0.0002
			2	30	246.7	55.4			
			3	30	299.9	49.6			
	2015	LP	1	30	80.6	56.9	2	17.9	<.0001
			2	30	61.9	30.6			
			3	30	126.2	53.3			
		SCF	1	30	244.0	75.0	2	5.0	0.0087
			2	30	283.3	133.6			
			3	30	216.4	49.9			

Annex 2. Ionomic analysis of soil from common garden experiment. Statistics for years 2013-2015

Annex Chapter-I Table 2: Mean ± Standard Deviation (µg/g) from common garden experiment. Soil from carbonated site (LP) and control soil (SCF), collected in April of 2013, 2014, 2015. Anova between common garden sites of 5 samples from each site and year.

Element	Year	Site	N	Mean (µg/g)	Std Dev (µg/g)	DF	F ratio	Prob>F
Na	2013	LP	5	34	17	1	3.13	0.1514
		SCF	5	49	4.5			
	2014	LP	5	54	7.6	1	3.49	0.0862
		SCF	5	47	4.0			
	2015	LP	5	84	17	1	0.68	0.4161
		SCF	5	79	18			
K	2013	LP	5	205	39	1	25.27	0.0073
		SCF	5	32	39			
	2014	LP	5	91	4.2	1	1043.70	<.0001
		SCF	5	27	1.2			
	2015	LP	5	180	54	1	74.26	<.0001
		SCF	5	24	8.2			
Ca	2013	LP	5	488	91	1	2.14	0.2176
		SCF	5	619	107			
	2014	LP	5	741	27	1	4.01	0.0684
		SCF	5	775	35			
	2015	LP	5	869	153	1	4.74	0.0371
		SCF	5	784	48			
Mg	2013	LP	5	50	8.6	1	2.24	0.2088
		SCF	5	66	14			
	2014	LP	5	45	2.1	1	865.79	<.0001
		SCF	5	101	5.1			
	2015	LP	5	82	26	1	24.64	<.0001
		SCF	5	122	21			
P	2013	LP	5	7.4	8.7	1	0.03	0.8725
		SCF	5	8.1	0.8			

Element	Year	Site	N	Mean (µg/g)	Std Dev (µg/g)	DF	F ratio	Prob>F
	2014	LP	5	7.5	3.4	1	0.43	0.5248
		SCF	5	8.6	1.7			
	2015	LP	5	8.7	4.7	1	11.40	0.002
		SCF	5	3.5	4.1			
S	2013	LP	5	12	3.4	1	1.00	0.4226
		SCF	5	24	10			
	2014	LP	5	32	26	1	4.63	0.0525
		SCF	5	6.7	7.4			
	2015	LP	5	14	9.8	1	0.66	0.4244
		SCF	5	21	27			
Fe	2013	LP	5	15	6.7	1	6.63	0.0617
		SCF	5	37	9.9			
	2014	LP	5	16	0.7	1	53.08	<.0001
		SCF	5	25	3.9			
	2015	LP	5	11	2.2	1	36.37	<.0001
		SCF	5	17	4.2			
Zn	2013	LP	5	11	6.7	1	13.36	0.0217
		SCF	5	4.4	0.1			
	2014	LP	5	21	3.9	1	136.01	0.0545
		SCF	5	10	7.1			
	2015	LP	5	21	16	1	80.92	<.0001
		SCF	5	14	3.3			

Annex 3. Ionomic analysis in leaf of demes grown in LP and SCF. Statistics for years 2013-2015

Annex Chapter-I Table 3: Mean ± Standard Deviation of ionomic analysis (µg/g) in leaf from *A.thaliana* plants ordered in relation to groups of $CaCo_3$% growing in carbonated soil (LP) and control soils (SCF) common gardens. ANOVA between $CaCO_3$% groups in each site for three years: 2013, 2014, 2015.

Element	Year	Site	Group %CaCO3	N	Mean (µg/g)	Std Dev (µg/g)	DF	F ratio	Prob >F
Na	2013	LP	1	21	644	125	2	1.8963	0.1762
			2	21	387	144			
			3	21	322	118			
		SCF	1	21	262	111	2	1.2903	0.3008
			2	21	549	140			
			3	21	364	118			
	2014	LP	1	21	615	132	2	3.4403	0.0632
			2	21	539	186			
			3	21	167	122			
		SCF	1	21	324	79	2	0.9364	0.4125
			2	21	349	91			
			3	21	492	100			
	2015	LP	1	21	815	171	2	2.4039	0.1266
			2	21	537	156			
			3	21	308	156			
		SCF	1	21	480	82	2	1.1471	0.3439
			2	21	322	88			
			3	21	517	134			
K	2013	LP	1	21	35051	2505	2	1.5821	0.2302
			2	21	32099	2892			
			3	21	38638	2362			
		SCF	1	21	32051	2365	2	0.4049	0.6733
			2	21	31468	2992			
			3	21	34608	2528			
	2014	LP	1	21	34344	2050	2	4.4179	0.0344

Element	Year	Site	Group %CaCO3	N	Mean (µg/g)	Std Dev (µg/g)	DF	F ratio	Prob >F
			2	21	28748	2900			
			3	21	38857	1898			
		SCF	1	21	28915	2710	2	0.6869	0.5174
			2	21	33037	3130			
			3	21	33152	3428			
	2015	LP	1	21	35317	2008	2	0.0341	0.9666
			2	21	34619	1833			
			3	21	35044	1833			
		SCF	1	21	30106	3147	2	1.1471	0.3439
			2	21	29100	3364			
			3	21	38074	5139			
Ca	2013	LP	1	21	4292	188	2	1.4423	0.2599
			2	21	4658	217			
			3	21	4194	177			
		SCF	1	21	3714	150	2	0.3157	0.7334
			2	21	3875	190			
			3	21	3690	160			
	2014	LP	1	21	4646	243	2	1.7094	0.2192
			2	21	4115	344			
			3	21	4064	225			
		SCF	1	21	3971	180	2	0.5898	0.566
			2	21	3676	208			
			3	21	3801	228			
	2015	LP	1	21	4610	232	2	2.7669	0.0971
			2	21	4404	212			
			3	21	3903	212			
		SCF	1	21	3622	287	2	4.0273	0.0398
			2	21	4354	307			
			3	21	2815	468			
Mg	2013	LP	1	21	3393	367	2	1.0979	0.3529
			2	21	3309	423			
			3	21	2704	346			
		SCF	1	21	4617	234	2	0.7104	0.5054
			2	21	5040	296			
			3	21	4906	250			
	2014	LP	1	21	2927	341	2	6.7109	0.01
			2	21	4802	483			
			3	21	2779	316			
		SCF	1	21	5036	228	2	0.1513	0.8608
			2	21	5007	263			

Element	Year	Site	Group %CaCO3	N	Mean (µg/g)	Std Dev (µg/g)	DF	F ratio	Prob >F
			3	21	5206	288			
	2015	LP	1	21	2473	365	2	9.3444	0.0026
			2	21	4140	333			
			3	21	2262	333			
		SCF	1	21	5064	358	2	0.7345	0.4962
			2	21	5479	382			
			3	21	4671	584			
P	2013	LP	1	21	7422	645	2	0.9475	0.4044
			2	21	6120	745			
			3	21	7165	608			
		SCF	1	21	3975	288	2	0.2091	0.8133
			2	21	3691	365			
			3	21	3783	308			
	2014	LP	1	21	7094	655	2	4.662	0.0298
			2	21	4453	926			
			3	21	7810	606			
		SCF	1	21	4015	273	2	0.9384	0.4118
			2	21	3942	316			
			3	21	3436	346			
	2015	LP	1	21	6773	1022	2	1.116	0.3551
			2	21	7076	933			
			3	21	8652	933			
		SCF	1	21	3339	410	2	1.3126	0.2983
			2	21	3719	438			
			3	21	4610	669			
S	2013	LP	1	21	4967	237	2	19.2813	<.0001
			2	21	6147	274			
			3	21	6987	224			
		SCF	1	21	6930	281	2	2.8085	0.0883
			2	21	6912	356			
			3	21	6040	301			
	2014	LP	1	21	5661	391	2	3.2951	0.0696
			2	21	6359	553			
			3	21	7030	362			
		SCF	1	21	6092	230	2	0.3495	0.7103
			2	21	5948	266			
			3	21	6277	291			
	2015	LP	1	21	5701	373	2	5.3099	0.0192
			2	21	5948	340			
			3	21	7199	340			

Element	Year	Site	Group %CaCO3	N	Mean (µg/g)	Std Dev (µg/g)	DF	F ratio	Prob >F
		SCF	1	21	6597	393	2	2.8614	0.0886
			2	21	5688	420			
			3	21	7434	641			
Fe	2013	LP	1	21	55	10	2	5.4122	0.0132
			2	21	63	12			
			3	21	99	10			
		SCF	1	21	79	10	2	5.108	0.0183
			2	21	82	13			
			3	21	123	11			
	2014	LP	1	21	45	10	2	4.3822	0.0351
			2	21	66	15			
			3	21	87	10			
		SCF	1	21	97	7	2	0.9128	0.4213
			2	21	85	8			
			3	21	99	8			
	2015	LP	1	21	47	9	2	11.7062	0.001
			2	21	76	8			
			3	21	105	8			
		SCF	1	21	118	20	2	0.716	0.5047
			2	21	114	21			
			3	21	158	32			
Zn	2013	LP	1	21	89	5	2	0.6108	0.5527
			2	21	80	6			
			3	21	87	5			
		SCF	1	21	77	5	2	0.1199	0.8877
			2	21	80	6			
			3	21	77	5			
	2014	LP	1	21	90	6	2	0.2902	0.7528
			2	21	81	9			
			3	21	86	6			
		SCF	1	21	75	8	2	0.9785	0.3973
			2	21	91	10			
			3	21	75	10			
	2015	LP	1	21	95	6	2	0.8442	0.4506
			2	21	86	6			
			3	21	85	6			
		SCF	1	21	81	7	2	0.227	0.7996
			2	21	87	7			
			3	21	85	11			

Annex 4. Ionomic analysis in soils from natural habitat. Statistics for years 2013-2015

Annex Chapter-I Table 4: Mean ± Standard Deviation of mineral nutrients (µg/g) in soils from Natural habitat collected in April 2013, 2014 and 2015. ANOVA between %$CaCO_3$ groups of 21-24 samples from each year.

Element	Year	%CaCO3 group	N	Mean (µg/g)	Std Dev (µg/g)	DF	F ratio	Prob>F
Na	2013	1	24	104	5.5	2	36.98	<.0001
		2	24	56.6	5.5			
		3	21	37.7	5.9			
	2014	1	24	96.0	4.9	2	21.74	<.0001
		2	24	62.6	4.9			
		3	21	50.8	5.3			
	2015	1	24	118	5.3	2	31.31	<.0001
		2	24	91.9	5.3			
		3	21	56.5	5.7			
K	2013	1	24	105	12	2	42.45	<.0001
		2	24	130	12			
		3	21	257	13			
	2014	1	24	105	14	2	31.27	<.0001
		2	24	143	14			
		3	21	267	15			
	2015	1	24	119	17	2	21.54	<.0001
		2	24	155	17			
		3	21	278	18			
Ca	2013	1	24	664	20	2	1.28	0.2849
		2	24	709	20			
		3	21	687	21			
	2014	1	24	692	19	2	0.35	0.7088
		2	24	715	19			
		3	21	705	21			
	2015	1	24	760	24	2	1.68	0.1946
		2	24	796	24			
		3	21	731	26			

Element	Year	%CaCO3 group	N	Mean (µg/g)	Std Dev (µg/g)	DF	F ratio	Prob>F
Mg	2013	1	24	171	10	2	15.48	<.0001
		2	24	131	10			
		3	21	91	10			
	2014	1	24	172	10	2	11.20	<.0001
		2	24	126	10			
		3	21	102	11			
	2015	1	24	179	8	2	20.92	<.0001
		2	24	129	8			
		3	21	100	9			

Annex 5. Ionomic analysis in Mallorca soils

Annex Chapter-IV Table 1 Mean ± Standard Deviation of elements (µg/g) in soils from Mallorca. ANOVA between carbonate soil and no carbonate soil (control) of 6 samples.

Element	Soil	N	Mean	Std Dev	F ratio	Prob > F
Na	Carbonate	6	108	28.1	3.7	0,0833
	Control	6	147	40.5		
Mg	Carbonate	6	235	36.1	4.2	0,0666
	Control	6	307	78.4		
K	Carbonate	6	34.1	22.4	3.3	0,0992
	Control	6	76.8	53.1		
Ca	Carbonate	6	720	35.4	14.5	0,0041
	Control	6	628	45.3		
Mn	Carbonate	6	32.8	5.2	4.9	0,0495
	Control	6	77.7	49		
Fe	Carbonate	6	10.8	1.5	2.2	0,1682
	Control	6	12.8	2.9		
Co	Carbonate	6	0.10	0.08	0.007	0,9352
	Control	6	0.09	0.05		
Ni	Carbonate	6	0.13	0.03	0.07	0,8047
	Control	6	0.13	0.03		
Cu	Carbonate	6	2.65	1.36	2.7	0,1308
	Control	6	6.55	5.64		
Zn	Carbonate	6	19.5	5.9	2.8	0,1242
	Control	6	29.2	12.8		
Mo	Carbonate	6	0.04	0.01	4.8	0,0515
	Control	6	0.05	0.01		

Annex 6. List of genes differing from A1 and T6 demes

Annex Chapter-IV Table 3: List of genes differing from A1 and T6 demes

AT1G01120; AT1G01940; AT1G01950; AT1G01960; AT1G02190; AT1G02270; AT1G02280; AT1G02305; AT1G02310; AT1G02390; AT1G04280; AT1G04290; AT1G04295; AT1G05710; AT1G05720; AT1G05730; AT1G05740; AT1G05760; AT1G05770; AT1G05780; AT1G06890; AT1G06900; AT1G06910; AT1G07702; AT1G07705; AT1G08150; AT1G08160; AT1G08165; AT1G08180; AT1G08190; AT1G08360; AT1G08370; AT1G08380; AT1G08410; AT1G08430; AT1G08450; AT1G08810; AT1G08830; AT1G09000; AT1G09020; AT1G09160; AT1G09180; AT1G09260; AT1G09270; AT1G09280; AT1G09290; AT1G09300; AT1G09340; AT1G09580; AT1G09590; AT1G09610; AT1G09620; AT1G09640; AT1G09660; AT1G09700; AT1G09710; AT1G09720; AT1G09730; AT1G09850; AT1G09860; AT1G09870; AT1G09880; AT1G09890; AT1G09900; AT1G10030; AT1G10040; AT1G10060; AT1G10070; AT1G10160; AT1G10180; AT1G10430; AT1G10455; AT1G10490; AT1G10500; AT1G10510; AT1G10520; AT1G10522; AT1G11280; AT1G11300; AT1G14000; AT1G14040; AT1G14048; AT1G14060; AT1G14071; AT1G14080; AT1G14090; AT1G14110; AT1G14130; AT1G14140; AT1G14150; AT1G14160; AT1G14180; AT1G14182; AT1G15620; AT1G15630; AT1G15650; AT1G15660; AT1G16010; AT1G16060; AT1G16140; AT1G16150; AT1G16160; AT1G16170; AT1G16180; AT1G16190; AT1G16210; AT1G16220; AT1G16230; AT1G16240; AT1G16290; AT1G16300; AT1G16310; AT1G16320; AT1G16340; AT1G16360; AT1G16370; AT1G16390; AT1G16410; AT1G16440; AT1G16445; AT1G16450; AT1G16460; AT1G16470; AT1G16480; AT1G16489; AT1G16490; AT1G16620; AT1G16635; AT1G16640; AT1G16730; AT1G16740; AT1G16760; AT1G16770; AT1G16780; AT1G16820; AT1G16825; AT1G16840; AT1G16850; AT1G16900; AT1G16910; AT1G16916; AT1G16920; AT1G17040; AT1G17130; AT1G17200; AT1G17220; AT1G17275; AT1G17277; AT1G20490; AT1G23170; AT1G23250; AT1G23260; AT1G23290; AT1G23300; AT1G23400; AT1G23670; AT1G23680; AT1G23700; AT1G26610; AT1G27390; AT1G27400; AT1G27420; AT1G27430; AT1G27450; AT1G27461; AT1G27470; AT1G27490; AT1G27500; AT1G27510; AT1G27520; AT1G29179; AT1G29190; AT1G29195; AT1G29750; AT1G29760; AT1G29780; AT1G29785; AT1G29790; AT1G30450; AT1G30460; AT1G31480; AT1G31485; AT1G31500; AT1G31510; AT1G32120; AT1G32130; AT1G32150; AT1G32160; AT1G32510; AT1G32520; AT1G32860; AT1G34418; AT1G34430; AT1G36180; AT1G44125; AT1G44130; AT1G45474; AT1G47380; AT1G47389; AT1G47395; AT1G47890; AT1G48050; AT1G48060; AT1G48080; AT1G48090; AT1G48500; AT1G48520; AT1G48530; AT1G48550; AT1G49160; AT1G49630; AT1G49640; AT1G49660; AT1G49670; AT1G49680; AT1G49690; AT1G49700; AT1G49715; AT1G49990; AT1G52770; AT1G52790; AT1G52800; AT1G55630; AT1G55640; AT1G55660; AT1G60270; AT1G60290; AT1G60300; AT1G60310; AT1G60610; AT1G60625; AT1G60630; AT1G60640; AT1G60650; AT1G60670; AT1G60680; AT1G60700; AT1G61040;

AT1G61050; AT1G61060; AT1G61065; AT1G61070; AT1G61320; AT1G61460; AT1G61480; AT1G61490; AT1G61510; AT1G61520; AT1G61540; AT1G61560; AT1G61665; AT1G61685; AT1G61940; AT1G61950; AT1G61980; AT1G62020; AT1G62095; AT1G62110; AT1G62130; AT1G62670; AT1G62695; AT1G63450; AT1G63460; AT1G63470; AT1G63580; AT1G63610; AT1G66430; AT1G66440; AT1G66450; AT1G66460; AT1G66630; AT1G66640; AT1G66820; AT1G67170; AT1G67220; AT1G69450; AT1G69650; AT1G69660; AT1G69680; AT1G72000; AT1G72110; AT1G72120; AT1G72130; AT1G72270; AT1G74170; AT1G74190; AT1G75540; AT1G76450; AT1G76620; AT1G76630; AT1G76640; AT1G76660; AT1G76728; AT1G76730; AT1G76750; AT1G76770; AT1G76790; AT1G77110; AT1G77120; AT1G77130; AT1G77131; AT1G77370; AT1G77490; AT1G77510; AT1G77525; AT1G77530; AT1G77540; AT1G77580; AT1G77610; AT1G77630; AT1G77640; AT1G77655; AT1G77660; AT1G77765; AT1G77770; AT1G77780; AT1G77800; AT1G77810; AT1G78630; AT1G78640; AT1G78660; AT1G78740; AT1G78750; AT1G78770; AT1G78830; AT2G02950; AT2G02960; AT2G02970; AT2G02980; AT2G03430; AT2G03460; AT2G03740; AT2G03760; AT2G03780; AT2G10980; AT2G16250; AT2G16260; AT2G16280; AT2G16365; AT2G16367; AT2G16380; AT2G16485; AT2G16490; AT2G16500; AT2G19120; AT2G19140; AT2G19910; AT2G19930; AT2G20310; AT2G21520; AT2G21530; AT2G21550; AT2G21570; AT2G21930; AT2G21940; AT2G21950; AT2G21960; AT2G21970; AT2G21990; AT2G22000; AT2G22080; AT2G22150; AT2G22155; AT2G22160; AT2G22180; AT2G22450; AT2G22840; AT2G22860; AT2G22870; AT2G22880; AT2G22900; AT2G22940; AT2G22950; AT2G22970; AT2G23020; AT2G23040; AT2G23060; AT2G23093; AT2G23096; AT2G23100; AT2G23110; AT2G24430; AT2G24460; AT2G24470; AT2G25160; AT2G25170; AT2G25430; AT2G25460; AT2G25480; AT2G26360; AT2G26370; AT2G26390; AT2G26400; AT2G26420; AT2G26830; AT2G26860; AT2G26940; AT2G29910; AT2G29920; AT2G29940; AT2G32150; AT2G32160; AT2G32170; AT2G32179; AT2G32180; AT2G34900; AT2G35630; AT2G35637; AT2G35658; AT2G35660; AT2G35680; AT2G35742; AT2G35743; AT2G35744; AT2G35747; AT2G35750; AT2G35760; AT2G35765; AT2G35770; AT2G35780; AT2G35850; AT2G35859; AT2G38140; AT2G38150; AT2G38160; AT2G39970; AT2G41060; AT2G41460; AT2G41510; AT2G41530; AT2G41905; AT2G41930; AT2G42840; AT2G42860; AT2G42870; AT2G43950; AT2G43980; AT2G44680; AT2G44850; AT2G44860; AT2G44890; AT2G44920; AT2G44925; AT2G46360; AT2G46470; AT2G46480; AT2G46490; AT2G46493; AT2G46494; AT2G46495; AT2G46570; AT2G46572; AT2G47250; AT3G01220; AT3G01230; AT3G01250; AT3G01260; AT3G01270; AT3G01280; AT3G02020; AT3G02040; AT3G02050; AT3G02065; AT3G02320; AT3G02330; AT3G02335; AT3G02360; AT3G10490; AT3G10520; AT3G11200; AT3G11370; AT3G13720; AT3G13724; AT3G13740; AT3G17230; AT3G17240; AT3G17250; AT3G17310; AT3G17320; AT3G17340; AT3G17350; AT3G17365; AT3G19770; AT3G19790; AT3G20010; AT3G20020; AT3G20040; AT3G20100; AT3G20155; AT3G20160; AT3G20240; AT3G20270; AT3G20290; AT3G20330; AT3G20340; AT3G20362; AT3G20680; AT3G21650; AT3G21755; AT3G22700; AT3G22710; AT3G22720; AT3G22723; AT3G22730; AT3G22740; AT3G23350; AT3G23360; AT3G23370; AT3G23410; AT3G23430; AT3G23470; AT3G23490; AT3G23940; AT3G23960; AT3G24700; AT3G24710; AT3G24730; AT3G24740; AT3G25520; AT3G25530; AT3G26000; AT3G26020; AT3G26030; AT3G26830; AT3G26855; AT3G26860; AT3G26890; AT3G26900; AT3G26910; AT3G26922; AT3G26930; AT3G26932; AT3G27540; AT3G27550; AT3G27555; AT3G27560; AT3G28730; AT3G42721; AT3G42722; AT3G42724; AT3G42725; AT3G42783; AT3G44070; AT3G45390; AT3G45400; AT3G45420; AT3G45430; AT3G45870; AT3G45890; AT3G45900; AT3G45910; AT3G46658; AT3G46668;

AT3G46670; AT3G46930; AT3G47430; AT3G47440; AT3G47460; AT3G47750; AT3G47770; AT3G48670; AT3G48675; AT3G48680; AT3G48690; AT3G48710; AT3G48860; AT3G48880; AT3G48890; AT3G49060; AT3G49080; AT3G49100; AT3G49180; AT3G49200; AT3G49990; AT3G50000; AT3G50020; AT3G50376; AT3G50930; AT3G50940; AT3G50950; AT3G50960; AT3G50970; AT3G51180; AT3G51470; AT3G51478; AT3G51490; AT3G51950; AT3G52605; AT3G52620; AT3G52630; AT3G52900; AT3G52905; AT3G53310; AT3G53330; AT3G53340; AT3G53650; AT3G53670; AT3G53680; AT3G53690; AT3G53700; AT3G54010; AT3G54100; AT3G54110; AT3G54130; AT3G54140; AT3G56880; AT3G56890; AT3G56891; AT3G56900; AT3G56910; AT3G59950; AT3G59960; AT3G60060; AT3G60070; AT3G62620; AT3G62630; AT3G62735; AT3G62740; AT3G62760; AT3G62770; AT3G62880; AT3G62890; AT4G00695; AT4G00910; AT4G01026; AT4G01030; AT4G02060; AT4G02075; AT4G04350; AT4G04360; AT4G04375; AT4G04380; AT4G04460; AT4G09200; AT4G09730; AT4G09731; AT4G09745; AT4G09750; AT4G09760; AT4G11120; AT4G11945; AT4G12650; AT4G12680; AT4G12700; AT4G13760; AT4G13970; AT4G13985; AT4G13990; AT4G13996; AT4G14147; AT4G14149; AT4G14270; AT4G14272; AT4G14276; AT4G14290; AT4G14368; AT4G14830; AT4G14840; AT4G14860; AT4G14870; AT4G14880; AT4G15242; AT4G15248; AT4G15258; AT4G15260; AT4G15320; AT4G15340; AT4G16070; AT4G16095; AT4G16100; AT4G16380; AT4G16400; AT4G16410; AT4G16420; AT4G19160; AT4G19180; AT4G19350; AT4G19370; AT4G19380; AT4G19540; AT4G19550; AT4G19570; AT4G19580; AT4G19960; AT4G20070; AT4G20090; AT4G20150; AT4G21150; AT4G23850; AT4G23870; AT4G23880; AT4G23890; AT4G23895; AT4G24440; AT4G24470; AT4G24480; AT4G24520; AT4G24530; AT4G25730; AT4G25740; AT4G25910; AT4G25940; AT4G28680; AT4G28700; AT5G01040; AT5G01130; AT5G01140; AT5G01240; AT5G01260; AT5G01270; AT5G01290; AT5G01300; AT5G01320; AT5G01330; AT5G01420; AT5G01430; AT5G01445; AT5G01450; AT5G01460; AT5G01470; AT5G01490; AT5G01910; AT5G01920; AT5G01930; AT5G01950; AT5G01960; AT5G02980; AT5G02990; AT5G03010; AT5G03030; AT5G03040; AT5G03610; AT5G03630; AT5G03790; AT5G03795; AT5G03810; AT5G06560; AT5G06650; AT5G06660; AT5G06700; AT5G07380; AT5G07440; AT5G08010; AT5G08020; AT5G08040; AT5G08050; AT5G08055; AT5G08060; AT5G08720; AT5G08740; AT5G08750; AT5G08760; AT5G08780; AT5G08790; AT5G09220; AT5G09225; AT5G09240; AT5G09250; AT5G09960; AT5G10220; AT5G10240; AT5G10260; AT5G10270; AT5G10900; AT5G11600; AT5G11610; AT5G11630; AT5G13205; AT5G13220; AT5G13340; AT5G13360; AT5G13500; AT5G13520; AT5G13530; AT5G13590; AT5G13610; AT5G13620; AT5G13900; AT5G13910; AT5G13930; AT5G13940; AT5G15880; AT5G15890; AT5G15900; AT5G15910; AT5G15920; AT5G16050; AT5G16060; AT5G16250; AT5G16260; AT5G16280; AT5G16390; AT5G16420; AT5G16715; AT5G16840; AT5G16880; AT5G16890; AT5G16910; AT5G17130; AT5G17140; AT5G17165; AT5G19940; AT5G19950; AT5G20130; AT5G20140; AT5G20160; AT5G20165; AT5G20170; AT5G20200; AT5G20240; AT5G20580; AT5G20590; AT5G20600; AT5G20610; AT5G20620; AT5G20635; AT5G20650; AT5G22460; AT5G22510; AT5G22520; AT5G22530; AT5G22545; AT5G22550; AT5G22600; AT5G23700; AT5G23710; AT5G23720; AT5G23730; AT5G23880; AT5G23900; AT5G23903; AT5G23908; AT5G23920; AT5G23955; AT5G23970; AT5G23980; AT5G37020; AT5G37030; AT5G37050; AT5G37055; AT5G37060; AT5G40080; AT5G40316; AT5G40370; AT5G40382; AT5G40440; AT5G41170; AT5G41190; AT5G41770; AT5G41790; AT5G42146; AT5G42150; AT5G42180; AT5G42190; AT5G42470; AT5G45780; AT5G45790; AT5G45800; AT5G46250; AT5G46260; AT5G46270; AT5G47230; AT5G47240; AT5G47260; AT5G47400; AT5G47420; AT5G47930; AT5G48070;

AT5G48090; AT5G48100; AT5G48330; AT5G48335; AT5G48350; AT5G48360; AT5G48657; AT5G48660; AT5G48675; AT5G48920; AT5G48930; AT5G48990; AT5G49590; AT5G49600; AT5G49615; AT5G49740; AT5G49760; AT5G50210; AT5G50230; AT5G50240; AT5G50260; AT5G51650; AT5G51740; AT5G51845; AT5G51860; AT5G52010; AT5G53910; AT5G54440; AT5G54460; AT5G54910; AT5G55140; AT5G55150; AT5G55630; AT5G55640; AT5G55650; AT5G55660; AT5G55670; AT5G55680; AT5G55700; AT5G55710; AT5G55720; AT5G55730; AT5G55750; AT5G55855; AT5G56150; AT5G56170; AT5G56180; AT5G56320; AT5G56330; AT5G56380; AT5G56430; AT5G56440; AT5G56450; AT5G56452; AT5G56460; AT5G56520; AT5G56680; AT5G56690; AT5G56710; AT5G56720; AT5G56730; AT5G57360; AT5G58840; AT5G59930; AT5G59945; AT5G59950; AT5G59960; AT5G60080; AT5G60090; AT5G61980; AT5G63760; AT5G63810; AT5G63820; AT5G63840; AT5G63870; AT5G63880; AT5G63890; AT5G63905; AT5G63910; AT5G63920; AT5G63930; AT5G63940; AT5G63941; AT5G63950; AT5G64410; AT5G65610; AT5G65615; AT5G66820; AT5G66840; AT5G66850; AT5G66890; AT5G66900; AT5G66910; AT5G66920; AT5G67000; AT5G67010; AT5G67030; AT5G67100; AT5G67265; AT5G67270;

Annex 7. GWAs results

Annex Chapter-IV Table 3: List of genes related to significant SNPs with p-value and chromosome (Chr) where located. For variable ionomics genes are grouped by the elements and the scenario (C or CND). For variable rosette diameter scenario GRD_14 is shown.

Noted that when SNP is found associated to a LD, the gene region data are the positions (bases) that limit the LD, while for the SNPs not associated to a LD the region take into account was the SNP position ±10Kb

Ionomic Results

Element	Data	Chr	p-valor	Region	Gene Model Name
B	C	5	7.92	612824-612844	AT5G02710.1
	CND	5	6.81	13781553-1386391	AT5G35603.2; AT5G35604.1; AT5G35610.1; AT5G35620.1; AT5G35630.1; AT5G35640.1; AT5G35660.1; AT5G35670.1; AT5G35680.3; AT5G35688.1; AT5G35690.1
Cd	C	4	10.24	14646525-14802361	AT4G29940.1; AT4G29950.1; AT4G29960.1; AT4G29970.1; AT4G29990.1; AT4G30000.2; AT4G30010.1; AT4G30020.1; AT4G30030.1; AT4G30040.1; AT4G30050.1; AT4G30060.1; AT4G30064.1; AT4G30067.1; AT4G30070.1; AT4G30074.1; AT4G30080.1; AT4G30090.1; AT4G30097.1; AT4G30100.1; AT4G30110.1; AT4G30120.1; AT4G30130.1; AT4G30140.1; AT4G30150.1; AT4G30160.2; AT4G30170.1; AT4G30180.1; AT4G30190.2; AT4G30200.2; AT4G30210.1
Co	C	1	6.68	24329888±10kb	AT1G65470; AT1G65480
		3	6.63	2057938-2108596	AT3G06600; AT3G06610; AT3G06620; AT3G06630; AT3G06640; AT3G06650; AT3G06660; AT3G06670; AT3G36659; AT3G66652; AT3G66654; AT3G66656; AT3G66658
	CND	4	7.16	5077876-5086000	No cod
Cu	C	1	6.77	16480134-16529395	AT1G43690.1; AT1G43700.1; AT1G43710.1; AT1G43720.1; AT1G43722.1; AT1G43730.1
		2	7.56	17804160-17870970	AT2G42790.1; AT2G42800.1; AT2G42810.2; AT2G42820.1; AT2G42830.2; AT2G42840.1; AT2G42850.1; AT2G42860.1; AT2G42870.1; AT2G42880.1; AT2G42885.1; AT2G42890.1; AT2G42900.1; AT2G42910.1; AT2G42920.1;

Ionomic Results

Element	Data	Chr	p-valor	Region	Gene Model Name
					AT2G42930.1; AT2G42940.1; AT2G42950.1; AT2G42955.1; AT2G42960.1
		3	6.46	940937-948608	AT3G03750.2; AT3G03760.1; AT3G03770.1
		4	6.35	2694152-2696156	AT4G05260.1; AT4G05270.1
			6.54	7969817-7971311	AT4G13730.1
			6.15	8419223±10kbKb	AT4G14660.1; AT4G14670.1; AT4G14680.1; AT4G14690.1; AT4G14700.1; AT4G14710.5; AT4G14713.1
			7.3	15268789±10kb	AT4G31460.1; AT4G31470.1; AT4G31480.1; AT4G31490.1; AT4G31500.1; AT4G31510.1; AT4G31520.1
			6.32	17516327±10kb	AT4G37190.1; AT4G37200.1; AT4G37210.1; AT4G37220.1; AT4G37230.1; AT4G37235.1; AT4G37240.1
Fe	C	1	7.56	11219975±10kb	AT1G31320.1; AT1G31330.1; AT1G31335.1; AT1G31340.1
			6.34	29888436-29938104	AT1G31350.1; AT1G79450.1; AT1G79460.1; AT1G79470.1; AT1G79480.1; AT1G79490.1; AT1G79510.1; AT1G79520.2; AT1G79530.1; AT1G79540.1; AT1G79550.1; AT1G79560.1; AT1G79570.1
		5	7.43	13782455-13799218	AT5G35603.2; AT5G35604.1
Mg	C	3	8.7	3438537-3442703	AT3G10980.1; AT3G10985.1
Mn	CND	1	6.32	26389344-26392275	AT1G70060.1; AT1G70070.1
		5	6.39	8490721-8525213	AT5G24750.1; AT5G24760.1; AT5G24770.1; AT5G24780.1; AT5G24790.1; AT5G24800.1; AT5G24810.2; AT5G24820.1
Mo	CND	4	6.52	16400142-16418933	AT4G34260.1; AT4G34265.1; AT4G34270.1; AT4G34280.1; AT4G34290.1; AT4G34400.1; AT4G34410.1
Na	CND	1	6.76	3979166±10kb	AT1G11750; AT1G11760; AT1G11765; AT1G11770; AT1G11780; AT1G11785; AT1G11790; AT1G11800; AT1G11810
			8.11	4008593-4041017	AT1G11880.1; AT1G11890.1; AT1G11900.1; AT1G11905.1; AT1G11910.1
		2	6.88	16788878	AT2G40160; AT2G40170; AT2G40180; AT2G40190; AT2G40200; AT2G40205; AT2G40210; AT2G40220
		3	8.65	10284807-10311811	AT3G27750.1; AT3G27770.1; AT3G27785.1; AT3G27809.1; AT3G27810.1
			6.51	1439513	No COD

Ionomic Results

Element	Data	Chr	p-valor	Region	Gene Model Name
		4	6.05	710256±10kb	AT4G01630; AT4G01640; AT4G01650; AT4G01660; AT4G01670; AT4G01671; AT4G01680
			6.71	736303±10kb	AT4G01690; AT4G01700; AT4G01703; AT4G01710; AT4G01720
			6.2	2433157-2452828	AT4G04790.1; AT4G04800.1; AT4G04810.1; AT4G04830.1; AT4G04840.1; AT4G04850.2
			6	8009942±10kb	AT4G13790; AT4G13800; AT4G13810; AT4G13820; AT4G13830; AT4G13840
			6.25	9335683±10kb	AT4G16560; AT4G16563; AT4G16566; AT4G16570; AT4G16580; AT4G16590
			8.2	13425225-13504372	AT4G26610.1; AT4G26620.1; AT4G26630.1; AT4G26640.2; AT4G26650.1; AT4G26660.1; AT4G26670.1; AT4G26680.1; AT4G26690.1; AT4G26700.1; AT4G26701.1; AT4G26710.1; AT4G26720.1; AT4G26730.1; AT4G26740.1; AT4G26750.1; AT4G26760.1; AT4G26770.1; AT4G26780.1; AT4G26790.1; AT4G26800.1; AT4G26810.1; AT4G26820.1; AT4G26830.1; AT4G26840.1; AT4G26850.1; AT4G26860.2
		5	6.84	15190986-15191867	AT5G38070.1; AT5G38080.1
			6.8	16269901±10kb	AT5G40610.1; AT5G40620.1; AT5G40630.1; AT5G40640.1
Ni	C	1	6.2	9926937-9929381	no cod
		3	7.09	9588977-9675806	AT3G26200.1; AT3G26210.1; AT3G26220.1; AT3G26230.1; AT3G26235.1; AT3G26240.1; AT3G26250.1; AT3G26280.1; AT3G26290.1; AT3G26300.1; AT3G26310.1; AT3G26320.1; AT3G26330.1; AT3G26340.1; AT3G26350.1; AT3G26360.1; AT3G26370.1; AT3G26380.1; AT3G26390.1; AT3G26400.1; AT3G26410.1
		3	7.09	9588977-9675806	AT3G26420.1
		5	7.61	6520538-6556355	AT5G19350.1; AT5G19360.1; AT5G19370.1; AT5G19380.2; AT5G19390.1; AT5G19400.1; AT5G19410.1; AT5G19420.2; AT5G19430.1; AT5G19440.1
			6.93	6938501±10kb	AT5G20490.1; AT5G20500.1; AT5G20510.1; AT5G20520.1; AT5G20540.1
			7.51	6950055±10kb	AT5G20510.1; AT5G20520.1; AT5G20540.1; AT5G20550.1; AT5G20560.1; AT5G20570.2; AT5G20580.1
			6.61	7092292±10kb	AT5G20860.1; AT5G20870.1; AT5G20885.1; AT5G20890.1; AT5G20900.1; AT5G20910.1; AT5G20920.1; AT5G20930.1

Ionomic Results

Element	Data	Chr	p-valor	Region	Gene Model Name
			6.03	17196637±10kb	AT5G42870.1; AT5G42880.1; AT5G42890.1; AT5G42895.1; AT5G42900.1; AT5G42905.1; AT5G42910.1; AT5G42920.2
	CND	3	6.79	9588977-9675806	AT3G26200.1; AT3G26210.1; AT3G26220.1; AT3G26230.1; AT3G26235.1; AT3G26240.1; AT3G26250.1; AT3G26270.1; AT3G26280.1; AT3G26290.1; AT3G26300.1; AT3G26310.1; AT3G26320.1; AT3G26330.1; AT3G26340.1; AT3G26350.1; AT3G26360.1; AT3G26370.1; AT3G26380.1; AT3G26390.1; AT3G26400.1; AT3G26410.1
		3	6.79	9588977-9675806	AT3G26420.1
P	CND	3	7.27	5240515-5265661	AT4G08290.1; AT4G08300.1; AT4G08310.1; AT4G08320.2; AT4G08330.1
Zn	C	1	6.51	644814-678264	AT1G02890.1; AT1G02900.1; AT1G02910.1; AT1G02920.1; AT1G02930.1; AT1G02940.1; AT1G02950.2; AT1G02960.2; AT1G02965.1; AT1G02970.1; AT1G02980.1
			8.19	3446115-3551054	AT1G10480.1; AT1G10490.1; AT1G10500.1; AT1G10510.1; AT1G10520.1; AT1G10522.1; AT1G10530.1; AT1G10540.1; AT1G10550.1; AT1G10560.1; AT1G10570.1; AT1G10580.1; AT1G10585.1; AT1G10586.1; AT1G10588.1; AT1G10590.3; AT1G10600.1; AT1G10610.1; AT1G10620.1; AT1G10630.1; AT1G10640.1; AT1G10650.1; AT1G10657.1; AT1G10660.2; AT1G10670.3; AT1G10680.1; AT1G10690.1
			8.32	5520641-5546768	AT1G16110.1; AT1G16120.1; AT1G16130.1; AT1G16150.1; AT1G16160.1; AT1G16170.1; AT1G16180.1; AT1G16190.1
		4	6.4	6657678±10kb	AT4G10820.1; AT4G10840.1
		5	6.13	2067554-2071431	AT5G06710.1
			6.33	4001555±10kb	AT5G12340.1; AT5G12350.1; AT5G12360.1; AT5G12370.1; AT5G12380.1; AT5G12390.1
			6.14	18046772-18061286	AT5G44730.2; AT5G44740.2; AT5G44750.2; AT5G44760.1
			6.18	18975826-18992062	AT5G46760.1; AT5G46770.1; AT5G46780.1; AT5G46790.1; AT5G46795.1; AT5G46800.1; AT5G46810.1
			9.04	19291111-19292148	No cod
	CND	1	7.03	3446115-3551054	AT1G10480.1; AT1G10490.1; AT1G10500.1; AT1G10510.1; AT1G10520.1; AT1G10522.1; AT1G10530.1; AT1G10540.1; AT1G10550.1; AT1G10560.1; AT1G10570.1; AT1G10580.1;

Ionomic Results

Element	Data	Chr	p-valor	Region	Gene Model Name
					AT1G10585.1; AT1G10586.1; AT1G10588.1; AT1G10590.3; AT1G10600.1; AT1G10610.1; AT1G10620.1; AT1G10630.1; AT1G10640.1; AT1G10650.1; AT1G10657.1; AT1G10660.2; AT1G10670.3; AT1G10680.1; AT1G10690.1
		2	6.21	19044542±10kb	AT2G46370.4; AT2G46375.1; AT2G46380.1; AT2G46390.1; AT2G46400.1; AT2G46410.1; AT2G46420.1
		3	6.6	6797800-6800113	AT3G19570.2

Diameter results

Data	Chr	p-valor	Region	Gene Name
GRD14	2	7.23	11526938-11543244	AT2G27010.1; AT2G27020.1
	5	10.19	678730-700016	AT5G02910.1; AT5G02920.1; AT5G02930.1; AT5G02940.1; AT5G02950.1; AT5G02960.1; AT5G02970.1; AT5G02980.1