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New metabolic and genetic factors associated with *Arabidopsis thaliana* response to ammonium stress

Doctoral thesis

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ABBREVIATIONS

ABA: Abscisic acid

Ami-RNA: artificial microRNA

BLUP: Best linear unbiased prediction

CRK: Cysteine-rich receptor-like kinase

EMMAx: Efficient mixed-model association expedited

ICDH: Isocitrate dehydrogenase

IPCC: Itergovernmental Panel on Climate Change

GDH: Glutamate dehydrogenase

GOGAT: Glutamate synthase

GS: Glutamine synthetase

GWA: Genome-wide association

NAD-ME: NAD-dependent malic enzyme

NADP-ME: NADP-dependent malic enzyme

NR: Nitrate reductase

NUE: Nitrogen use efficiency

MARF: Minor allele relative frequency

MDH: Malate dehydrogenase

LD: Linkage disequilibrium

PEPC: Phosphoenolpyruvate carboxylase

QTL: Quantitative trait locus

ROS: Reactive oxygen species

SB: Shoot biomass

TCA: Tricarboxilic acid

RESUMEN DE LA TESIS DOCTORAL

Nuevos factores genéticos y metabólicos asociados con la respuesta al amonio de *Arabisdopsis thaliana*

Asier Sarasqueta

Contexto general

Las plantas necesitan nitrógeno para su crecimiento y desarrollo, siendo la concentración de este elemento el mayor factor limitante en el suelo para la producción vegetal. Los suelos de uso agrícola continuo suelen carecer de las concentraciones adecuadas de este elemento, y los agricultores se ven obligados a introducirlo mediante el uso de fertilizantes. Los fertilizantes se enriquecen principalmente con nitrato (NO_3^-) y amonio (NH_4^+). Desgraciadamente, el uso excesivo de fertilizantes tiene efectos medioambientales nocivos como la eutrofización de acuíferos derivada de la lixiviación del nitrato (su carga negativa facilita su pérdida ya que los propios suelos están negativamente cardados y lo repelen) y de la emisión de compuestos gaseosos como los óxidos de nitrógeno. Entre ellos el N_2O, que se producen desde el NO_3^- por desnitrificacion en condiciones anaerobias y desde el NH_4^+ por nitrificación (proceso mediante el cual el NH_4^+ es oxidado por los microorganismos del suelo hasta NO_3^-), tiene un gran poder efecto invernadero, entre 265 y 298 veces superior al del CO_2, contribuyendo de manera importante al calentamiento global. Por otra parte, la pérdida de una parte del nitrógeno aplicado al medio ambiente se traduce en una disminución del uso eficiente de nitrógeno por parte de las plantas, una de las razones por las cuales es necesario su continuo aporte. Los fertilizantes con una fuente de nitrógeno basado en el amonio y combinado con inhibidores de la nitrificación se usan de manera común para mantener el nitrógeno en el suelo por periodos más largos de tiempo. Se ha visto que el uso de este tipo de combinaciones (amonio junto con inhibidores de la nitrificación) es capaz de reducir significativamente los efectos medioambientales negativos causados por los fertilizantes comunes con alto contenido en nitrato.

En teoría, debido al menor estado de reducción en el que se encuentra el amonio respecto del nitrato su asimilación requiere de menor gasto energético que el necesario para la asimilación

del nitrato. Sin embargo, , muchas plantas muestran síntomas de estrés cuando crecen con una fuente de nitrógeno basada exclusivamente en el amonio. Estos síntomas, entre otros, incluyen clorosis foliar, desbalance iónico, desregulación hormonal, desordenes en la regulación del pH interno y cambios en el metabolismo. Cuando analizamos la planta en su conjunto, es evidente que la reducción del crecimiento de la planta es un efecto común de la nutrición amoniacal, la cual es considerada como situación de estrés común en el reino vegetal ya que afecta a prácticamente todas las especies vegetales. Con todo, el grado de estrés amoniacal puede ser diferente y se observa variabilidad en su tolerancia tanto entre especies como entre genotipos de la misma especie. A pesar de que los mecanismos mediante los cuales las plantas responden al estrés amoniacal se han estudiado durante décadas, a día de hoy, aun no se conocen en su totalidad y solo se han identificado algunos componentes genéticos y moleculares asociados a la nutrición amoniacal.

Objetivos

El objetivo general del presente estudio es llegar a un mejor entendimiento de los mecanismos metabolicos y genéticos asociados a la tolerancia al amonio utilizando *Arabidopsis thaliana* como especie modelo.

Este objetivo general está dividido en tres objetivos específicos, cada uno correspondiente a un capítulo diferente:

1. Explorar la variabilidad intraespecífica en la tolerancia del amonio de *Arabidopsis thaliana* centrándonos en especial en los mecanismos de asimilación del N.

2. Identificar genes relacionados con la variabilidad natural en la tolerancia del amonio en *Arabidopsis thaliana* mediante un estudio de asociación de genoma completo.

3. Entender el ajuste metabólico de *Arabidopsis thaliana* al estrés amoniacal en función del pH del medio externo.

1. Explorando la tolerancia al amonio en un colección de accesiones naturales de **Arabidopsis thaliana**

Metodologia

Para determinar la existencia de la variabilidad natural en la tolerancia al amonio en *A. thaliana*, en primer lugar llevamos a cabo un análisis de 47 accesiones naturales de Arabidopsis crecidas con una fuente de N exclusivamente amoniacal o nítrica.. El rendimiento de cada una de las accesiones naturales utilizadas se determinó en función de la biomasa de la roseta. Por otro lado, el grado de tolerancia al amonio de cada accesión, lo determinamos basándonos en el ratio de la biomasa de la roseta de las plantas crecidas con amonio entre lla de las plantas crecidas con nitrato. Además, también analizamos diversas características relacionadas con el metabolismo del nitrógeno, como el contenido total de amonio y amino ácidos y las actividades enzimáticas nitrato reductasa (NR), , glutamina sintetasa (GS) y , glutamato deshidrogenasa (GDH) en sentido aminante y desaminante.

Resultados y conclusiones

En este estudio, quedó de manifiesto que *Arabidopsis thaliana* muestra una gran variabilidad intraespecífica a la tolerancia al amonio. Entre los parámetros determinados, la acumulación de amonio en los tejidos resulto determinante para la biomasa de las rosetas, independientemente de la fuente de nitrógeno con la que se desarrollaban las plantas. Por otro lado, se observó que las actividades enzimáticas no tienen ningún efecto sobre la variación de la biomasa, a pesar de que su actividad era modificada por la fuente de nitrógeno. En este sentido, la actividad en sentido aminante de la glutamato deshidrogenasa era mayor cuando la fuente de nitrógeno con las que crecían las plantas era el amonio, mientras que su actividad desaminante era mayor cuando la fuente de nitrógeno era el nitrato. En general, parece que la acumulación del NH_4^+ juega un papel importante en la variabilidad en la tolerancia al amonio de *Arabidopsis thaliana*, siendo este factor más relevante que la capacidad que tienen sus células para su asimilación.

2. Un estudio de asociación de genoma completo revela un nuevo locus en *Arabidopsis thaliana* relacionado con la variabilidad natural del uso eficiente del amonio.

Metodología

Para estudiar las bases genéticas que explican la variabilidad natural en la tolerancia al amonio de *Arabidopsis thaliana*, crecimos en las condiciones anteriormente descritas, de un colección de 337 accesiones naturales y llevamos a cabo un estudio de asociación de genoma completo utilizando la biomasa obtenida de las rosetas en función de la fuente de nitrógeno como carácter cuantificable para así identificar regiones del genoma relacionadas con la variabilidad en la tolerancia al amonio. Con los datos de biomasa obtenidos llevamos a cabo diferentes estudios de asociación de genoma completo independientes, separando las accesiones en función de su procedencia geográfica para así tener en cuenta la posible variabilidad adaptativa a la nutrición amonical en función de la escala geográfica.

Resultados y conclusiones

Al igual que en el estudio previo, observamos una gran variabilidad intraespecífica en todas las escalas geográficas estudiadas, desde una escala local hasta la global. Además, cuando analizamos los resultados obtenidos del estudio en la escala geográfica de Francia, identificamos un pico de asociación significativo relacionado con la biomasa de las rosetas obtenidas bajo un régimen nutricional amoniacal, que no aparecía en el análisis de esa misma escala geográfica realizado con plantas crecidas con nitrato como fuente de nitrógeno. Este pico de asociación, corresponde a una región del genoma en la que se encuentran situados en tándem un grupo de 19 genes, correspondientes a la familia de las “Cysteine-rich receptor-like kinases” (CRKs). Las CRKs son una familia compuesta por 44 miembros, distribuidos por en todo el genoma, relacionados con la respuesta de las plantas a estreses bióticos y abióticos. Para validar la implicación potencial de las CRKs en la tolerancia al amonio, utilizamos líneas mutantes de tipo T-DNA para cada uno de los genes CRKs situados en la región de interés que crecimos en condiciones nutricionales iguales a las usadas para la obtención de los valores de biomasa de las rosetas utilizados para el análisis de asociación de genoma completo. En este estudio, no observamos ningún fenotipo en las líneas mutantes que se diferenciase al de Col-0, el genotipo salvaje del que derivan. También realizamos un análisis de la expresión génica de las diferentes *CRKs* en Col-0 crecidas bajo nutrición nítrica o amonical. Así, observamos que

algunos de los genes se inducían en las plantas crecidas en condiciones amoniacales, lo que vuelve a sugerir la potencial participación de estos genes en la respuesta al amonio de *A. thaliana*. Sin embargo, la probable redundancia funcional de las CRK impide confirmar su implicación real en la respuesta de las plantas ante el estrés amoniacal.

3. La interacción de la fuente de nitrógeno y del pH del medio externo afecta de manera diferente al metabolismo o de Arabidopsis en parte aérea y raíz.

Metodología

Para el estudio de la adaptación de *Arabidopsis thaliana* al estrés amoniacal en función del pH del medio externo, crecimos plantas de Arabidopsis variando la fuente de nitrógeno (NH_4^+ o NO_3^-), su concentración (2 mM o 10 mM) y el pH (5.7 o 6.7) del medio nutricional de las plantas. Analizamos en parte aérea y raíz, parámetros relacionados con el estrés amoniacal como el crecimiento, el contenido de algunos metabolitos, la acividad de enzimas relacionadas con la asimilación del nitrógeno así como el de encimas envueltas en el metabolismo del carbono.

Resultados y conclusiones

Los resultados obtenidos revelaron que el grado de estrés amoniacal que sufren las plantas, depende delrégimen nutricional. La maquinaria encargada de la asimilación del amonio, así como de la actividad de las enzimas anapleróticas asociadas al ciclo de los ácidos tricarboxílicos (TCA) según el tejido analizado (parte aérea o raíz) y del régimen nutricional respondían a los cambios rpovocados en el medio externo. El mayor grado de estrés amoniacal fue detectado en las condiciones nutricionales de pH 5.7, las cuales estaban asociadas con una mayor acumulación de NH_4^+. Este estrés no pudo evitarse a pesar del incremento de la actividad de la maquinaria de asimilación del amonio (GS, GDH) y de las enzimas anapleróticas asociadas al TCA. Además, los resultados obtenidos, sugieren roles específicos para las distintas isoformas de GS y GDH en función del régimen nutricional. En general hemos visto que la acumulación de amonio en los tejidos vegetales es la causante del estrés amoniacal y que esta acumulación no parece estar relacionada con el desajuste de la asimilación del nitrógeno.

ABSTRACT

Plants are exogenous nitrogen (N) dependent. To provide plants with the necessary N, fertilizers are enriched mostly with nitrate (NO_3^-) and (NH_4^+). However, the excessive supply of N in soils has negative environmental effects as water eutrophication due to nitrate leaching and greenhouse gas emissions which contribute to global warming. Ammonium-based fertilizers formulated with nitrification inhibitors are commonly used to maintain the N available in the soil for longer periods and their use is known to greatly mitigate the environmental effects associated to nitric fertilization. Paradoxically, although NH_4^+ assimilation requires less energy than that of NO_3^-, many plants display symptoms of stress when grown with NH_4^+ as the sole N source. These symptoms include, among others, leaf chlorosis, ion imbalance, hormone deregulation, disorder in pH regulation, and changes in metabolite levels. At the whole-plant level, a reduction in plant growth is a common effect of ammonium nutrition. Indeed, ammonium nutrition is considered as a universal stressful situation, virtually affecting every plant species. However, the degree of stress it generates is variable and intra- and inter-specific variability towards ammonium nutrition has been reported. Although the mechanisms through plants respond to ammonium stress have been studied for decades, they are not well understood yet and very few molecular components associated with ammonium nutrition have been identified. In this context, the present pretends to go deeper unlocking the metabolic and genetic mechanisms associated to ammonium sensitivity/tolerance using *Arabidopsis thaliana* as a model. To fulfill this purpose we carried out two different approaches. Firstly, we aimed exploiting the potential natural intraspecific variability of Arabidopsis in ammonium tolerance. Secondly, since ammonium stress is related with pH deregulation we aimed understanding the metabolic adaptation of Arabidopsis to ammonium stress in function of the external medium pH.

Regarding natural variability, we first analyzed a panel of 47 natural accessions of Arabidopsis and observed large intraspecific variation in ammonium tolerance using rosette biomass as a marker of plant performance and the ratio between rosette biomass under NH_4^+ and NO_3^- conditions to estimate ammonium tolerance. We also determined several traits related to N metabolism: NH_4^+ and total amino acid content, and nitrate reductase (NR), glutamine synthetase (GS) and glutamate dehydrogenase (GDH) enzyme activities. Among the determined parameters, tissue NH_4^+ accumulation resulted determinant for shoot biomass independently of the N source provided. Enzymes activities did not have an effect on biomass variation but their activities were modified by the N source. In this sense GDH aminating

activity was enhanced under ammonium nutrition and GDH deaminating activity was higher under nitrate nutrition. Overall, NH_4^+ accumulation seems to be an important player in Arabidopsis natural variability in ammonium tolerance rather than the cell NH_4^+ assimilation capacity.

To further understand the genetic basis of the observed natural variability of *Arabidopsis thaliana* in ammonium tolerance. We enlarged the analysis, using this time a panel of 337 natural accessions with the aim to carry out a genome wide association study (GWAS) that could help us to identify genomic regions associated with ammonium tolerance. To do so, we took into account the potential spatial scale of adaptive variation by performing independent GWAS in function of the geographical origin of the accessions studied. Overall, we observed great intraspecific variability at every scale studied, from local scale to a world scale. When performing the GWAS, at French geographical scale we identified a significant peak of association in relation with shoot biomass under ammonium nutrition that was absent in the analysis performed with shoot biomass under nitrate nutrition. This association peak corresponds to a genomic region that encompasses a tandem array of nineteen genes encoding Cysteine-rich receptor-like kinases (CRKs). CRKs are a family of 44 members that have been suggested important in plant response to biotic and abiotic stress. To validate the potential implication of these CRK genes we analyzed a complete panel of T-DNA mutant lines covering the mentioned region. We did not observe any phenotype regarding ammonium tolerance of this lines respect to Col-0 wild type plants. Nevertheless, we also analyzed their gene expression and observed that some of these genes were induced upon ammonium nutrition, suggesting again their potential role during ammonium nutrition. Overall, the probable redundancy in their function did not allow confirming the true implication of any of the CRK members in *Arabidopsis thaliana* ammonium tolerance.

Regarding the metabolic adaptation to ammonium stress in function of the external medium pH we grew Arabidopsis plants under different nutritional regimes by varying the nitrogen source (NO_3^- and NH_4^+), its concentration (2 and 10mM) and the pH of the external medium (5.7 and 6.7). The results obtained revealed changes in ammonium assimilation machinery and in the activity of the anaplerotic enzymes associated to tricarboxylic acids (TCA) cycle depending of the analyzed organ and in function of the nutritional regime that provoked a different degree of ammonium stress. A greater stress severity at pH 5.7 was associated with NH_4^+ accumulation and could not be circumvented in spite of the stimulation of GS, GDH and TCA cycle anaplerotic enzymes. Furthermore, this work suggests specific roles for different GS

and GDH isoforms in function of the nutritional regime. Overall, NH_4^+ accumulation triggering ammonium stress appears to bear no relation to nitrogen assimilation impairment.

GENERAL INTRODUCTION

INTRODUCTION

1. PLANT NUTRITION AND THE IMPORTANCE OF NITROGEN

Plants growth is controlled by different factors as light, CO_2, water and mineral nutrients availability, which in turn determine the yield of a cropland. When some of the cited factors availability is modified it usually has an effect on growth, e.g. with nutrients supply (Figure I).

Plants take up the nutrients they need from the environment. Mineral nutrients and water are incorporated from the root medium to the plant, although they can be incorporated also through leaves, and carbon (C) is obtained from the atmosphere (Table I). Once the nutrients are inside the plant, they can be stored, metabolized or transported to different cells, tissues or organs. A great number of studies have determined the positive relation between the concentration of available nutrients in the root medium and growth. However, an excessive nutrient supply may cause the contrary effect, provoking an inversion point as it is shown in Figure 1 for micronutrients. Inversion points can be caused by different factors, as the toxicity caused by a nutrient *per se* or the induction of a deficiency of another nutrient among others (Engels et al., 2011).

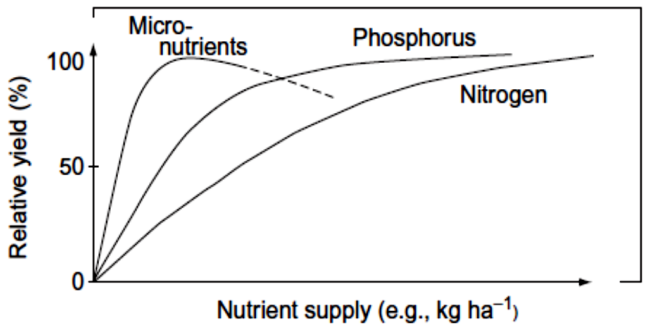


Figure I. Representation of yield response curves for N, P and micronutrients. Adapted from Engels et al., 2011)

Nutrient level maintaining in agricultural field is fundamental to obtain an optimum production and this is achieved by an appropriate fertilization. In general fertilizers could be divided in two categories: organic and inorganic (or synthetic). Organic fertilizers are composed by animal

and vegetable wastes and also from the synthesis of different organic compounds as amino acids. Inorganic fertilizers are made with mineral compounds extracted from rocks among others. The exception of this general classification is urea ($CO(NH_2)_2$), which is one of the most used N source and is classified into inorganic fertilizers although it has animal origin (Rach et al., 2016).

Among the nutrients absorbed by roots, N is the major growth limiting factor. The importance of N resides in the fact that it is the central component of different structural biomolecules such as nucleic acids and amino acids. Therefore, to maintain the productivity agriculture has the need to replace the lost nutrients, especially N, the most abundant component of fertilizers, which is lost by different chemical and biological pathways.

Table I. Classification of plant nutrients. Modified from Kirkby, (2011).

Classification of plant nutrients		
Nutrient	**Uptake**	**Biochemmical function**
Group 1		
C, H, O, N, S	As CO_2, HCO_3^-, H_2O, O_2, NO_3^-, NH_4^+, N_2, SO_4^{2-}, SO_2 ions from the soil solution, gases from the atmosphere	Major constituents of organic material. Essential elements of atomic groups involved in enzymatic processes. Assimilation by oxidation-reduction reactions.
Group 2		
P, B, Si	As phosphates, boric acid or borate, silic acid from the soil solution	Esterification with alcohol groups. Phosphate esters involved in energy transfer reactions.
Group 3		
K, Na, Ca, Mg, Mn, Cl	As ions from the soil solution	Non-specific functions establishing osmotic potential. More specific functions for optimal confirmation of enzymes (enzyme activation). Bridging of reaction partners. Balancing anions. Controlling membrane permeability and electrochemical potentials.
Group 4		
Fe, Cu, Zn, Mo	As ions or chelates from the soil solution	In chelated form in prosthetic groups of enzymes. Enable electron transport by valency change.

2. NITROGEN CYCLE

N is one of the most abundant elements in nature and it can be found in different reservoirs as in the atmosphere and in terrestrial and marine ecosystems. The biogeochemical reactions that allow the N interchange among the different N reservoirs form the N cycle. The largest N pool in the biosphere is the atmosphere where it is found in form of gas nitrogen (N_2) and represents 78 % of the dry air composition. N_2 is formed by a triple bound that binds two N

atoms. This triple bound makes it a very stable molecule, and because of that, most organisms are unable to metabolize it. However, N_2 is breakable by a few natural processes as lightings and more commonly by N_2-fixing microbes (Figure II), and by non natural processes as Haber-Bosch ammonium synthesis process (Robertson and Groffman, 2015; Robertson and Vitousek, 2009). The greatest difference of N respect to other macro-elements as K, P, and Ca and other elements required in lesser amounts such as Mg and B, is that there is not a mineral bound in the soil to replace the loss of the N. N is lost by the harvesting of vegetables, which do not return to the soil, by the transformation of N into volatile N forms, lost in the atmosphere, or into NO_3^- which is leached. In consequence, the N has to be replaced from outside of the soil-plant system (Robertson and Vitousek, 2009). Different N sources can enrich the soil from outside. In general, most of the N proceeds from the atmospheric N fixed by N_2-fixing microbes and from fertilizers added by agriculturists, although, in very polluted regions, N from the rainwater and from dry depositions onto leaf and soil surfaces can be important (Robertson and Vitousek, 2009; Xu et al., 2012; Hoffman et al., 2014; Robertson and Groffman, 2015) .

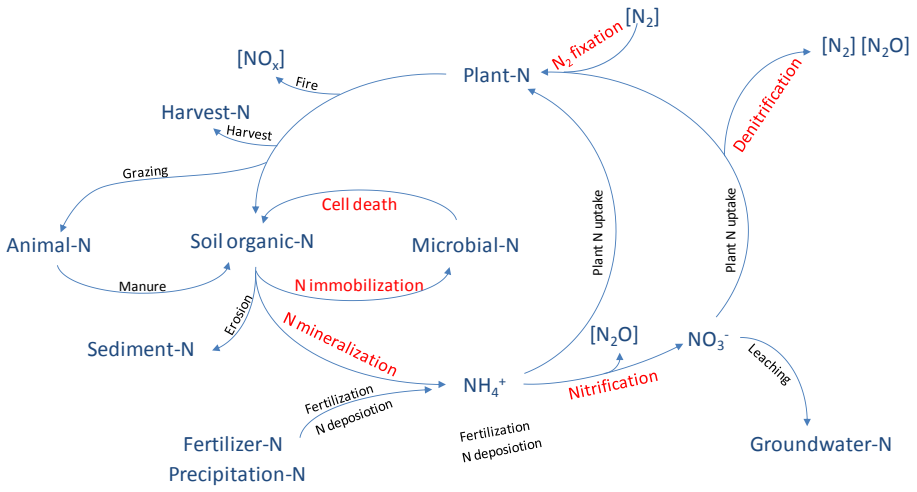


Figure II. Schematic representation of the major elements of the terrestrial nitrogen cycle. Those processes mediated by soil microbes appear in red. Gases appear in brackets (Modified diagram from Robertson and Groffman, (2015).

2.1. Nitrogen fixation

Biological N fixation

Biological N fixation occurs thanks to the activity of the nitrogenase, an enzyme complex synthesized by some microorganisms that catalyzes the conversion of atmospheric N_2 to NH_4^+ (Hoffman et al., 2014; McGlynn et al., 2012). N fixing microorganisms are included into the group of diazotrophs, composed by some bacteria and archaea. This group is divided into two different subgroups depending on whether they are free-living diazotrophs or they are forming symbiotic associations with other organisms. The first subgroup is formed by organisms as some bacterial species of *Clostridium, Klebsiella, Azotobacter* or *Nostoc* genera among others (Cooper and Scherer, 2012). The second one is formed by bacteria as *Rhizobium*, *Frankia* and *Burkholderia* genera that form symbiotic interaction with different types of plants. In agricultural systems, the most relevant N fixation comes from legume plants (Paramasivan et al., 2016; Walker et al., 2015).

Industrial N fixation

Industrial N fixation comes from the Haber-Bosch process, in which atmospheric nitrogen is reduced to produce NH_3, which then, can be oxidized to form NO_3^- by Ostwald process (Kandemir et al., 2013; Offermans et al., 2006). This industrial NH_3 allows to replace the removed N during crops harvest easily and more economically than with more traditional methods such as adding organic manures and wastes which are only profitable in places where there is cattle industry and the transport of the organic fertilizers is viable (Robertson and Vitousek, 2009).

2.2. N losses from soil are promoted by different processes

Soil microorganisms are able to metabolize different N sources. Through the mineralization of organic N compounds, it is produced NH_4^+, which nitrifiers can oxidize up to NO_3^-. Denitrifiers can use NO_3^- as electron acceptor in respiration processes producing more reduced N forms as N_2, and furthermore, anammox bacteria carry out the NH_4^+ anaerobic oxidation producing N_2 (Britto and Kronzucker, 2002; Hayatsu et al., 2008; Xu et al., 2012).

NO_3^- and NH_4^+ concentrations in the soil are extremely variable. The NH_4^+/ NO_3^- ratio depends on a variety of edaphoclimatic factors, as oxygenation state pH or temperature, that affect the microbial community living in the soil (Sanz-Cobena et al., 2017). In soils containing a low rate of oxygen, as in waterlogged soils, nitrification is inhibited and denitrification is promoted and NH_4^+ can be accumulated. In contrast, in well aerated soils, the nitrification process is rapid, and NH_4^+ is fast transformed into NO_3^- (Robertson and Groffman, 2015; Sanz-Cobena et al., 2017). These processes are important due to they determine the NH_3/NH_4^+ and NO_3^- concentrations in the soil, which are the main N sources that are lost from soil to the environment, through processes in which are implicated soil microbes, ion leaching and volatilization.

NH_3 losses from soil to atmosphere

NH_4^+can be deprotonated to NH_3 which can be lost in the atmosphere. NH_3 volatilization is a relevant process due to in addition of its effect on climate change (see page 20, section "Environmental problems related with agriculture"), it can be a great source of N loss in agriculture. Most important factors affecting NH_3 volatilization process are the type and amount of the applied fertilizer, soil pH and temperature (Chen et al., 2015).

Conversion of urea to biologically available N

When N is in form of urea, it can be rapidly converted to biologically available N. Urea is hydrolyzed, in a reaction catalyzed in the soil by urease enzyme, to form NH_3 and CO_2 (Reaction 1) (Robertson and Vitousek, 2009).

Reaction 1. $(NH_2)_2CO + H_2O \xrightarrow{\text{urease}} 2NH_3 + CO_2$

The generated NH_3 can be lost in the atmosphere or can be rapidly transformed to NH_4^+ in non alkaline soils, which is soluble and well retained, but, it can suffer transformations as nitrification and anammox oxidation provoking N loss and detrimental effects in the environment (explained below) (Hayatsu et al., 2008; Robertson and Groffman, 2015; Robertson and Vitousek, 2009).

Nitrification

The nitrification process is based in the transformation of NH_4^+ into more oxidized N forms, principally nitrite (NO_2^-) and NO_3^-. In soils the process is carried out by different microbes from archaeal, bacterial and fungal live kingdoms (Robertson and Groffman, 2015). During the process, previously deprotonated NH_4^+ is oxidized forming hydroxylamine (NH_2OH) by

ammonia mono-oxygenase enzyme (AMO). Then, NH_2OH is oxidized to NO_2^- by hydroxylamine-oxidoreductase (HAO), and finally the NO_2^- is oxidized to NO_3^- by nitrite oxidoreductase (NOR). During the process, volatile N forms, as nitrogen oxides (NO_x) and nitrous oxide (N_2O), may be produced (Figure III) (Robertson and Groffman, 2015). Although there are autotrophic and heteretrophic nitrifiers, autotrophic nitrification is the dominant form in most soils (Robertson and Groffman, 2015).

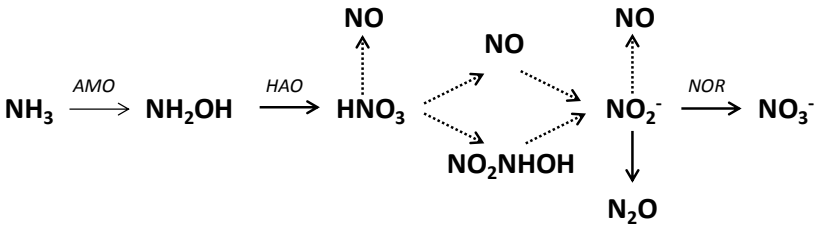


Figure III. Autotrophic nitrification pathways. Dashed lines indicate unconfirmed reactions (diagram modified from Robertson and Groffman, 2015)

Anammox (anaerobic ammonium oxidation)

It is the biological process in which NH_4^+ is oxidized anaerobically. This oxidation pathway is carried out by some microorganisms known as Anammox bacteria. Anammox bacteria known species are divided into five genera belonging in the order *Brocadiales*, which is constituting a monophyletic clade, inside the *Planctomicetes* phylum (Jetten et al., 2001; Kartal et al., 2012). The reaction is given by the combination of NH_4^+ and NO_2^- which under anaerobic conditions form N_2 (Hayatsu et al., 2008).

The process is driven by hydrazine hydrolase (HH), hydrazine oxidizing enzyme (HZO) and nitrite reducing enzymes (NR) in the bacterial organelle known as anammoxsome (Figure IV).

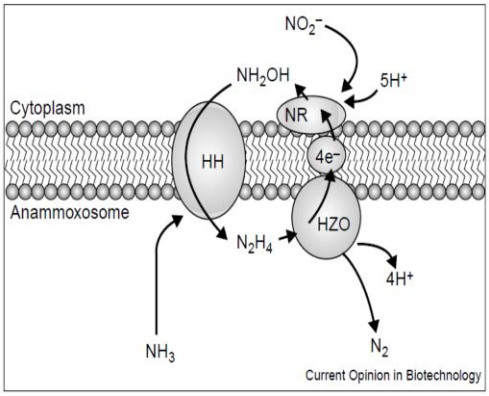


Figure IV. Mechanism of anaerobic ammonium oxidation. NR is a nitrite-reducing enzyme (NH_2OH is the assumed product); HH(hydrazine hydrolase) condenses hydrazine out of ammonia and hydroxylamine; HZO is a hydrazine-oxidizing enzyme (which might be equivalent to hydroxylamine oxidoreductase). Diagram retrieved from Jetten et al., (2001).

Denitrification

Denitrification is the process in which NO_3^- is transformed to more reduced N forms (Figure V). The organisms able to carry out this process are globally known as denitrifiers and include facultative anaerobes, principally *Pseudomonas* and *Alcaligenes*, and to a lesser extent, *Bacillus*, *Agribacterium*, and *Flavibacterium*. Denitrification is given by reduction of NO_3^- to NO_2^- that is further reduced to NON_2O and finally to N_2 (Figure V). The denitrification takes place in low O_2 level environments when oxidized forms of N are used as electron acceptor in the respiration process to the obtaining of ATP.

Figure V. Bacterial denitrification pathway. Each step is enacted by individual enzyme. Small arrows show steps in which intermediates products are released to the atmosphere.

Each step is driven by an enzyme: the first step by nitrate reductase (NAR), the second by nitrite reductase (NIR), then acts the nitric oxide reductase (NOR) and finally the nitrous oxide reductase (NOS). Until the formation of N_2, intermediates as nitric oxide (NO) or/and N_2O can also be exchanged to the atmosphere (Jackson et al., 2008; Robertson and Groffman, 2015; Robertson and Vitousek, 2009).

2.3. Environmental problems related with agriculture

Agriculture has to face the challenge of increasing the global food production to be able to feed the increasing world population (Alexandratos and Bruinsma, 2012). Unfortunately, intensive agricultural practices can have consequences in human health and a great detrimental impact in the environment. The most evident environmental impact comes from the conversion of a natural ecosystem to an anthropic one. Besides, there are other environmental costs as the pollution caused from the excessive use of agricultural fertilizers or from pesticides. Fertilizers can provoke acidification of croplands and the entry of nutrients to natural ecosystems provoking detrimental effects. Regarding to N, agriculture systems are highly inefficient in N conservation in the field and great quantities of this nutrient are lost in the environment during crops growth because of gas emissions and leaching. These N losses provoke environmental damages which comes from the biological activity of N compounds in soils and its reactivity in the atmosphere (Robertson and Vitousek, 2009; Stocker et al., 2013).

Soil acidification derived from excessive N fertilization

The excessive use of N fertilizers can decrease soil pH. On the one hand, during the nitrification of NH_4^+ to NO_3^-, $2H^+$ are formed (Robertson and Groffman, 2015), and furthermore, the NH_4^+ uptake can also acidify soils due to when it is taken up, it generates a charge unbalance that is compensated stoichiometrically with H^+ exchanged from plants to soil (Britto and Kronzucker, 2002). In addition, the volatile NO produced through N transformations could form nitric acid in the atmosphere, which can be deposited on soils decreasing the pH (Jackson et al., 2008; Robertson and Vitousek, 2009).

Soil acidification has multiple effects on ecosystem health, including the mobilization of toxic metals as aluminum ions and the loss of important cations for plants. Also acidic soils are related with P deficiency and reduced biodiversity and productivity (Blake et al., 1994).

Nitrate accumulation in vegetables

High concentration of NO_3^- in the soil can promote a great absorption of NO_3^- by roots and its accumulation in the plants tissues. When plants NO_3^- content exceed a threshold, the vegetable consumption could be harmful to human. Due to this toxic aspect, the NO_3^- content in human foodstuff is rigorously regulated (Bryan and Loscalzo, 2011).

When NO_3^- arrives to human organism, it is metabolized to nitrosamine. Nitrosamine formation process can be carcinogenic and could result in methemoglobinemia or blue baby syndrome, a condition found especially in infants, which is characterized by the reduced ability of blood cells to carry oxygen to the different tissues (Bryan and Loscalzo, 2011; Yusof et al., 2016).

Gas emissions derived from soil N and their environmental effects

Croplands with an excessive N supply can generate great N lost to the atmosphere in form of NH_3 or nitrogen oxides, as NO_x and N_2O (equation 4 and Figure III) generated by soil microbial activity (Robertson and Groffman, 2015; Robertson and Vitousek, 2009; Torralbo et al., 2017).

In alkaline pH soils, the NH_4^+/NH_3 ratio is lower and consequently the volatilization of NH_3 increases and it can be transferred as reactive N to downwind ecosystems (Robertson and Vitousek, 2009). Regarding to nitrogen oxides, on the one hand, the excessive release of NO_x to the atmosphere can cause the photochemical production of O_3. O_3 is both, a greenhouse gas and an oxidant that harms human health and plant growth at concentrations that are reached frequently in high NO_x regions (Robertson and Groffman, 2015; Robertson and Vitousek,

2009). On the other hand, N_2O is a greenhouse effect gas with a warming potential 265–298 fold higher than CO_2. Additionally, N_2O is the most important O_3 destroyer in the atmosphere and has negative impact in the ozone layer. Currently the great amount of the anthropogenic emissions of N_2O comes from agriculture (70-80%) (Stocker et al., 2013).

Aquatic systems pollution derived from N compounds

NH_4^+ and NO_3 are soluble, in the case of NO_3^-, because of its anionic properties, its retention on the normally negatively charged soil is much lower compared to NH_4^+ (Robertson and Groffman, 2015). Excess of NO_3^- is commonly lost through leaching. When NO_3^- is leached, or NH_4^+ in lower concentration, it arrives to aquatic systems and raises the total available nutrient concentration in the media causing eutrophization (Robertson and Groffman, 2015; Robertson and Vitousek, 2009; Xu et al., 2012). This increasing of nutrients allows algae to present higher growth rates than naturally can occur. This alga can precipitate to the bottom of the aquatic system. Where they precipitated algae are decomposed by bacteria in an aerobic process, in which the dissolved oxygen is consumed leading to hypoxia and the reduction of the deeper water organisms that require oxygen (Robertson and Vitousek, 2009). Moreover, in the case of NO_3^-, it could reach water sources for human consumption. This aspect of the N movement can be relevant due to high levels of NO_3^- can adversely affect human health, causing methemoglobinemia or blue baby syndrome (Bryan and Loscalzo, 2011).

3. STRATEGIES TO REDUCE N IMPACT IN AGRICULTURE

Key aspects towards a more environmental friendly agriculture are, among others, to obtain higher yields per cropland, to reduce the conversion of natural ecosystems into agricultural systems and to improve the nutrient use efficiency of plants reducing the nutrient inputs by fertilizers. Those strategies can match perfectly to the approaches focused on the problems derived from the excessive use of N in the agriculture, which can be faced by the developing of crops with better nitrogen use efficiency (NUE) by plants or the use of fertilization methods that would help to improve this plant trait.

NUE refers to the total biomass or grain yield produced per unit of applied N fertilizer (Xu et al., 2012). N loss is in part responsible of the low NUE in agriculture. This loss has economic and environmental impact, in the first case because of the high amount of resources needed to the production of N fertilizers, and in the second, due to the described detrimental effects that

it can provoke in the environment. For these reasons to achieve a better NUE in plants is a key aspect to obtain a more friendly agriculture with the environment.

Although there are multiple ways to achieve better NUE, the approaches to improve the plant NUE can be classified into the following four main options: i) a proper selection of the crop varieties with high NUE and its rotations could improve the uptake of the available N. To do so, it is fundamental to understand the genetic and molecular mechanisms involved in NUE and its components characteristic. ii) to apply the correct amounts of N, adjusting the fertilization to the crop requirements, and to avoid the fertilization below of the root zone, iii) a good programming of the timing and placement and iv) the appropriate manage of watersheds to decrease N losses downstream from cropland (Cherry et al., 2008; Robertson and Vitousek, 2009; Xu et al., 2012).

The use of urease and nitrification inhibitors

Nitrification and denitrification are the main sources of N volatile forms to the atmosphere and thus, main N loss factors. Nitrification can be inhibited by natural or human made nitrification inhibitors which can be useful in mitigation the N compound emissions (Torralbo et al., 2017). The great amount of the available commercial nitrification inhibitors are derived from pyridines, pyrimidines, amino-triazoles and sulfur compounds (Coskun et al., 2017). The most regularly used and best understood are 2-chloro-6-(trichloromethyl)-pyridine (nitrapyrin), dicyandiamide (DCD), and 3,4-dimethylpyrazole phosphate (DMPP). The inhibition process has a big agricultural potential value due to it has the ability to maintain the NH_4^+ for longer time in the soil avoiding some of the negative effects caused by the lost of N, notably as NO_3^- leached and as N_2O emission. (Torralbo et al., 2017).

Urease inhibitors inhibit the transformation of urea into NH_3/ NH_4^+. There are various types of urease hydrolase inhibitors, with a validated effectiveness avoiding the N lost to the environment (Abalos et al., 2012; Sanz-Cobena et al., 2012). The most common commercial urease inhibitor is N-(n-butyl) thiophosphorictriamide (NBPT). As in the case of nitrification inhibitors, urease inhibitors also have a great agricultural value due to they can maintain this N source for longer time in the soil enhancing the NUE of plants. Furthermore, the reduction of urease enzyme activity also mitigate NH_3 volatilization and the generation of NO_3^- leaching and denitrification processes (Sanz-Cobena et al., 2017).

4. NITROGEN METABOLISM

4.1. Plants can use different N forms

Plants can use a wide array of N forms as N source, ranging from simple inorganic N compounds as NH_4^+ and NO_3^-, to organic N compounds such as amino acids, peptides and also proteins (Paungfoo-Lonhienne et al., 2008).

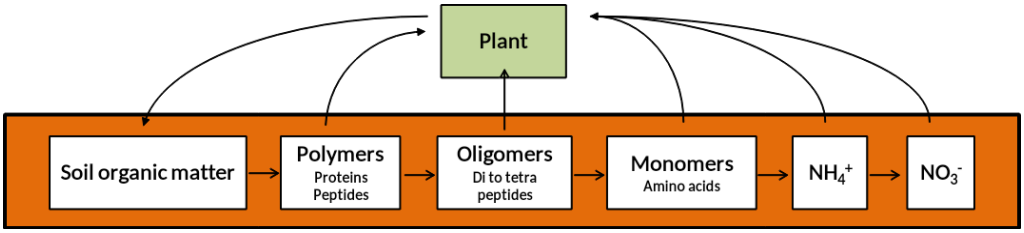


Figure VI. Plant N sources in soil. Root-derived enzymes contribute to organic matter depolymerization. The generated composes could be taken up as organic polymers and oligomers, which are directly incorporated into roots, or can be totally mineralized until NH_4^+ and NO_3^-. Figure modified from Paugfoo-Lonhienne et al. (2012).

Proteins as N source are taken up by different ways. One way is based on the exudation of proteolytic enzymes from root cells to the root medium to digest soil proteins into smaller peptides or amino acids, which then are taken up. This process takes place in the root surface and possibly in the apoplast of the root cortex. The second way is based on protein endocytosis by root cells, which then are catabolised. The endocytic uptake pathway has been observed in different plant species such as *Arabidopsis thaliana* or *Hakea actites* (Paungfoo-Lonhienne et al., 2008). Other N sources, as short peptides and amino acids, are taken up via membrane transporters into root cells (Näsholm et al., 2009; Paungfoo-Lonhienne et al., 2012). As example of peptide uptake, Komarova et al., (2008) have described in Arabidopsis the function of *Arabidopsis thaliana* peptide transporter 1 (AtPTR1*)* protein, a peptide transporter localized in the plasma membrane of root cells, able to take up peptides from the root media. Similarly, amino acids also can be taken up by membrane transporters in roots. There are several transporters already identified in the absorption of amino acids from the root media as lysine histidine transporter 1 (LHT1) or amino acid permease 1 (AAP1) among others (Kant et al., 2011). Once the amino acids are inside of the plant cells, they can be directly incorporated into proteins, or metabolized for the later synthesis of other biomolecules (Näsholm, 2009).

At present, modern agriculture has a great dependence on the use of inorganic N fertilizers (Graham and Miller, 2006; Matson et al., 1997) and because of that research on plant N nutrition has had a strong focus on inorganic N forms. This focus is motivated by the prominent role of inorganic N in agriculture soils and the dependence of many crops on this N source (Nasholm 2009). In this context, increasing the knowledge of the mechanisms through which plants acquire and assimilate inorganic N is fundamental.

4.2. Nitrate and ammonium acquisition and assimilation

Nitrate (NO_3^-) and ammonium (NH_4^+) comprise the main inorganic forms of N absorbed by plants. The preference for either NO_3^- or NH_4^+ as the N source is an important ecological determinant which affects plant diversity; while this aspect has not yet been precisely defined, it is however known to depend on the one hand, on the genetic background, and on the other hand on environmental features, such as soil properties (including pH) and plant physiology (van den Berg *et al.*, 2005). N transfer from soil to plant is carried out by plasma membrane-localized transport proteins. Taken up N will be stored or assimilated covering plants N demands during their development. For that purpose, plants have the availability of a great set of N transporters, which allows plant´s root adjusting N transport to soil environmental fluctuations, including N composition and concentration or pH fluctuations. In this sense it is important to highlight that pH fluctuates widely between natural and agricultural soils and represents an important feature that may limit N availability and the plant´s capacity to absorb essential nutrients (Hawkesford et al., 2011).

Today, it is thought that different N transporters and downstream N assimilatory systems could have in common some regulatory mechanisms, which need to be understood because they may be essential to improve the ability of plants to use soil N (Plett et al., 2017; Tegeder and Masclaux-Daubresse, 2018).

NO_3^- transport:

In Arabidopsis, nitrate transporter genes are encoded, at least, by four gene families, *nitrate transporter 1 (NRT1)* or *peptide transporter (PTR)* gene, *nitrate transporter 2 (NRT2)*, chloride channel (CLC), and slow anion channels and their homologs (SLAC1, SLAH) (Fan et al., 2017). However, nitrate is mainly taken up by transporters of the NRT1 and NRT2 families, which are low affinity nitrate transporters (LATS) and high affinity nitrate transporters (HATS), respectively (Fan et al., 2017). Most nitrate transporters are proton coupled importers, but

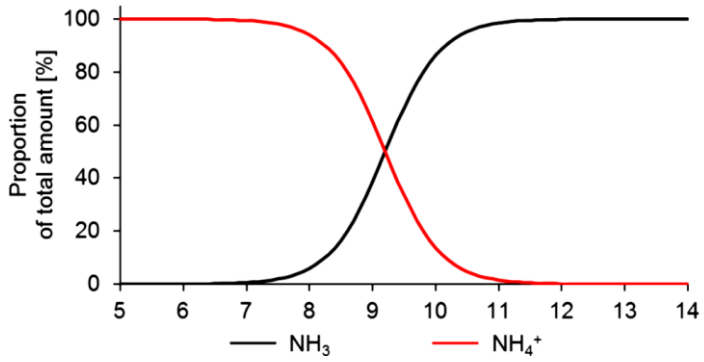
there are some exceptions as, the NPF7.3/NRT1.5 transporter, which participates in NO_3^- loading into the xylem and that is bidirectional (Lin et al., 2008), the NPF2.7/Nitrate Excretion Transporter1 (NAXT1), which mediates nitrate efflux (Segonzac et al., 2007), or the transporter of nitrate found in Chloride Channel (CLC) family, which works as anion channel or anion-proton exchangers (De Angeli et al., 2006).

LATS are characterized due to their ability to take up NO_3^- when high soil NO_3^- concentrations are available, normally higher than 250 µM (Plett et al., 2017). However, there are exceptions in some plants in which a member of NRT1 family function as high and low affinity transporter simultaneously (*e.g.* NPF6.3/NRT1.1 in Arabidopsis and NRT1.1B in rice (*Oryza sativa*)) (Huang et al., 1999; Liu et al., 1999; Wang et al., 1998). On the other hand, NRT2 are HATS and carry out their function under low NO_3^- soil concentration, below 250 µM (Plett et al., 2017). In *A. thaliana* six NRT transporters have been linked to HATS and LATS NO_3^- transporter systems. Under high soil NO_3^- concentration, NPF6.3/NRT1.1 (also called Chlorate-resistance Protein 1 (CHL1)) and NPF4.6/NRT1.2 are the responsible of NO_3^- acquisition, while under low NO_3^- concentration, the transporters identified in NO_3^- uptake are NRT2.1, NRT2.2, NRT2.4 and NRT2.5 (Plett et al., 2017). LATS take up around 95% of the total N, furthermore among the LATS transporters, NRT2.1 and NRT2.2 are the mayor contributors of the NO_3^- uptake (Lezhneva et al., 2014).

NO_3^- uptake by transporters is regulated, at least, at transcriptional and post-translational level. At transcriptional level, it has been observed that changing NO_3^- availability, from starvation status to optimum NO_3^- supply and *vice versa*, genes as *NRT2.1* and *NRT2.2* are differentially transcribed, decreasing their transcription level in high N availability conditions and increasing under NO_3^- starvation conditions (Plett et al., 2017; Ruffel et al., 2014). NO_3^- itself, through NRT1.1/NPF6, which besides transporting NO_3^- also functions as NO_3^- sensor, is able to trigger a signaling cascade that controls the *NRT2.1* gene expression (Plett et al., 2017; Ruffel et al., 2014). Post translational regulation is also an important regulatory mechanism in the NO_3^- uptake control. As example and as previously mentioned, AtNRT1.1 (CHL1/NPF6.3) has been described as a dual affinity transporter under post-translational control. Phosphorylated AtNRT1.1 functions as high affinity NO_3^- transporter, but when it is dephosphorylated, it functions as low affinity transporter (Plett et al., 2017).

NH_4^+ transport:

Ammonium is taken up by plants in two forms, the protonated form NH_4^+ and the electro-neutral form NH_3. The form in which the ammonium is presented in the root medium depends of the pH of the soil; but due to its pKa is 9.25 (Figure VII), in most soils, the predominant form is NH_4^+ (Straub, 2016; Van Den Berg et al., 2005).



Figrure VII. Schematic model of ammonium (NH_4^+) and ammonia (NH_3) proportions dependent on pH. In acidic solutions and also the neutral cell cytosol, the ammonium ion is the most abundant species. Neutral ammonia could be dominant in highly alkaline soils (Diagram retrieved from Straub, 2016).

As NO_3^-, NH_4^+ can be taken up directly from soil by high affinity transporters system (HATS), which are able to transport NH_4^+ in the range of µM and by low affinity transporters (LATS) responsible of the NH_4^+ transport in the mM range (Wang et al., 1993).

In the µM range, the responsible of NH_4^+ absorption are members of the Ammonium transporter/methylamine permease/rhesus (AMT/Mep/Rh) superfamily (HATS system). This family is composed by proteins of the clades AMT, MEP and Rh (McDonald et al., 2016). HATS is a saturable system, that catalyses the thermodynamically secondary active uptake of ammonium against a chemical gradient when the external concentration is low (Wang et al., 1993). Thanks to the crystal structures obtained of AMTB ammonium transporters from *Aquifex aeolicus* and *Escherichia coli* (Andrade et al., 2005; Khademi et al., 2004; Zheng et al., 2004), it is known that these proteins are homotrimeric structures and that each homomer contains an hydrophobic channel composed by trasmembrane domains which are proposed as the responsible of the NH_3 transport. It is though that the transporters are able to deprotonate

the NH_4^+ into NH_3 to then, facilitate the transport of NH_3 (Khademi et al., 2004; Zheng et al., 2004). To enhance the plant environmental adaptability, plants codify different activity HATS transporters, with different characteristics (McDonald et al., 2016). Among these transporters, *Mep* genes which encodes for AMT2 proteins, are electroneutral ammonium carriers, and drive ammonium in favor of its chemical gradient (Neuhäuser et al., 2009). *AMT* genes encodes for AMT1 proteins. These kind of transporters are electrogenic ammonium transporters, and function thanks to the negative cell membrane potential (Mayer et al., 2006). *A. thaliana* genomes encode 6 AMT proteins divided into five AMT1 members and a single AMT2 transporter. It is important to mention that AMT2 transporters do not contribute to NH_4^+ absorption from soil. It is thought that its function consists in the transport of ammonium among the apoplast and simplast (Sohlenkamp et al., 2002). Transcriptional control of AMTs in response to N and C nutritional status is considered their major regulatory mechanism in plants (Gazzarrini et al., 1999; Lejay et al., 2003; Loqué et al., 2006). For example, under N starvation, increases of the transcript levels of *AMT1*;*1*, *AMT1*;2 and *AMT1;3* have been detected (Lanquar et al., 2009). In addition, It has been shown that the overexpression of *AtAMT1;1* in transgenic tobacco can be used to increase ammonium uptake capacity (Yuan et al., 2006). Ammonium HATS can be also regulated post-translationally. It is known that AMT transporters are regulated by phosphorylation, in this sense, it is mentionable the function of CIPK23 (CBL-interacting protein kinase 23), which is able to phosphorylate AMT1;1 and AMT1;2 regulating their NH_4^+ flux (Straub et al., 2017).

Regarding LATS, they operate next to mM range (Nacry et al., 2013) a non saturable system that exhibits linear increase of its activity as ammonium concentration increases (Wang et al., 1993). It is important to highlight that the genetic identity of transport proteins responsible for the LATS pathway is not sufficiently understood, but it is known that among the responsible to acquire NH_4^+ at high concentrations are some aquaporins (AQP) (Kirscht et al., 2016), some K^+ channels (Hoopen et al., 2010) and some non-selective cation channels (NSCCs) (Balkos et al., 2010). AQP family members are highly expressed in roots and it is thought that the ability of this kind of transporters to transport NH_4^+ comes from the structural similarity between NH_3 and H_2O (Maurel et al., 2015). As example of NH_4^+ LATS, the AQP TIP2 (Tonoplast Intrinsic Protein 2) is a known NH_3 permeation facilitator who is involved, at least, in sequestering ammonium into cell vacuoles to control ammonium homeostasis in the cytosol and whose expression is up-regulated under N starvation conditions (Kirscht et al., 2016).

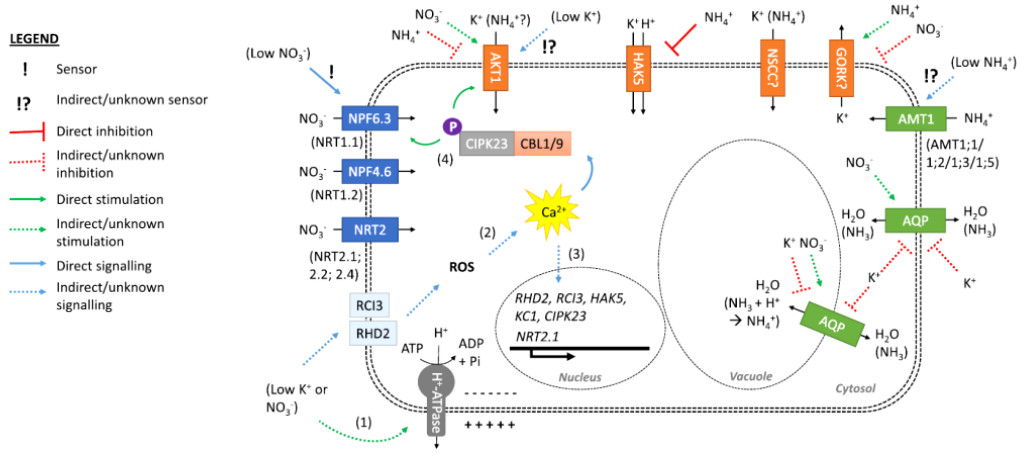


Figure VIII. Schematic representation of plant NO_3^-, NH_4^+ and K^+ transporters and their principal regulatory elements (figure retrieved from Coskun et al., 2017)

4.3. N assimilation

When N is taken up as NO_3^- , it is reduced to NH_4^+ by *nitrate reductase* (NR), which catalyses the transference of two electrons producing NO_2^- and by *nitrite reductase* (NiR), which transforms NO_2^- to NH_4^+ in a six electron transfer process (Figure VIII). NO_3^- reduction into NO_2^- happens by the NR in the cytosol together with three co-factors, flavine adenine dinucleotide (FAD), a heme (bound to a domain which resembles a family of cytochromes) and molybdopterin (a molybdenum containing co-factor) which participate in the electron transfer from NADH/NAD(P)H to nitrate (Figure IX). Then, the NO_2^- generated, is transported to plastids where it is reduced into NH_4^+ by NiR, expressed only in plastids. The electron donor in this case is the ferredoxin (Hawkesford et al., 2011). NR activity can be regulated both transcriptionally and post-translationally. It has been shown that expression of the nitrate reductase genes are induced by nitrate, generating active protein rapidly after addition of NO_3^- (Patterson et al., 2016). Post-transcriptional regulation is given by the phosphorylation and dephosphorylation of nitrate reductase depending of the presence of light among others (Lea et al., 2006).

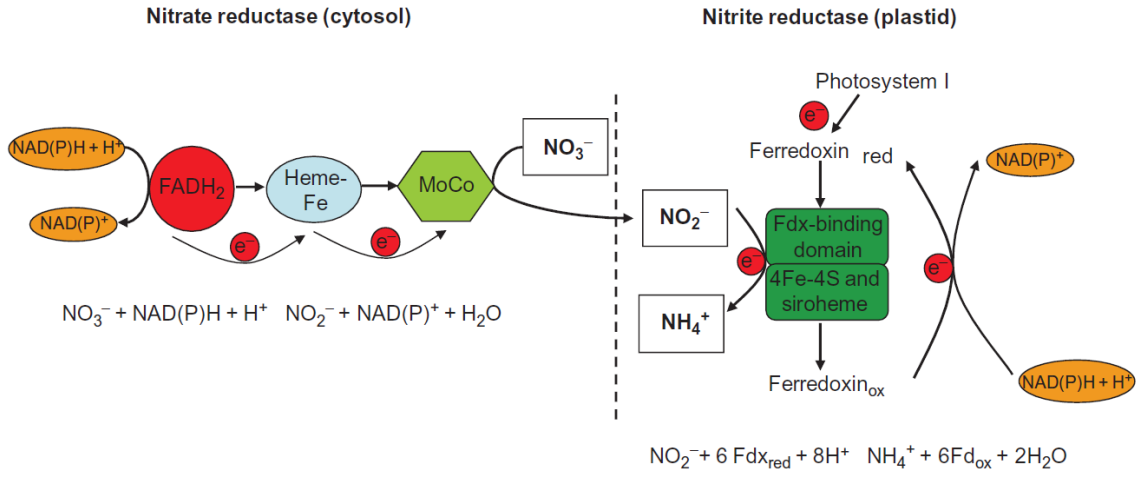


Figure IX. Schematic representation of the sequence of nitrate reduction process (Diagram retrieved from Hawkesford et al., 2011).

Inorganic nitrogen is only incorporated into biomolecules as NH_4^+. Ammonium can be directly taken up from the soil, it can be produced from the reduction of NO_3^- or generated through deamination steps in different metabolic pathways. Independently of its origin, NH_4^+ follows the same assimilation pathway and is mainly incorporated into amino acids via the glutamine synthetase/ glutamate synthase (GS/GOGAT) cycle (figure X) (Masclaux-Daubresse et al., 2010). Although it is still under debate, asparagine synthetase (AS) and glutamate dehydrogenase (GDH) have also been described as enzymes with the capacity to directly assimilate ammonium (Figure X)(Ferraro et al., 2015; Skopelitis et al., 2007).

GS/GOGAT cycle

The assimilation process begins with the condensation of NH_4^+ with glutamate (Glu) into glutamine (Gln) in ATP-dependent reaction catalyzed by glutamine synthase (GS) enzyme. Then, the produced Gln is used as amide source to transfer it to 2-oxoglutarate (2-OG), a reaction in which two Glu are generated (Forde and Lea, 2007; Lea and Miflin, 2010). The generated products are used by plants as precursor of other N biomolecules (Figure X). There are two GS enzyme isoforms, GS1 and GS2. GS1 is expressed in cytosol, and it is responsible for the primary NH_4^+ assimilation in roots and also for the reassimilation of the NH_4^+ generated in the cytosol during amino acids catabolism (Guan et al., 2016; Lothier et al., 2011). GS2 is expressed in plastids and it is responsible of the assimilation of the NH_4^+ produced from the

reduction of NO_3^-, which as described above finalizes inside the plastids. Furthermore, GS2 also has the functions of assimilating the NH_4^+ produced during photorespiration (Bernard and Habash, 2009).

GS1 is encoded by a multi gene family composed by a variable number of genes depending of the plant species. For example, in *Arabidopsis thaliana* genome, there are five GS1 genes, from *GLN1:1* to *GLN1:5*, and a single gene encoding GS2 enzyme (Ishiyama et al., 2004a). The regulation of the expression of the different GS genes is different depending of the analyzed cell, tissue, the physiological stage of the plant and the circumstances in which the plant is growing (Bernard and Habash, 2009; Lothier et al., 2011; Orsel et al., 2014). GS function is regulated at the transcriptional, translational and post-translational level by different factors including external and internal N availability, light and abiotic and biotic stresses. For example, it has been shown that GS1 isoenzymes expression and activity are dependent on nitrogen availability in *A. thaliana* (Ishiyama et al., 2004b). On the other hand the addition of sucrose to the growth medium causes the induction of *GLN1;1, GLN1*;2, and *GLN1;3* expression. This induction was attenuated by the external supply of amino acids, suggesting that a metabolic regulation of GS1 is associated with the relative abundance of carbon skeleton versus amino acids content in the root tissue (Ishiyama et al., 2004b). Glutamine 2-oxoglutarate aminotransferase (GOGAT) is the other fundamental piece of the ammonium assimilation pathway. It is presented in two forms, the ferredoxin dependent GOGAT (Fd-GOGAT), encoded by GLU1 and GLU2 genes and the NADH dependent GOGAT, which is codified by GLT gene (Potel et al., 2009). Fd-GOGAT is the predominant isoform in plastids of photosinthetically active tissues and its main function is to reassimilate the NH_4^+ generated during the photorespiration coupled with GS2 (Forde and Lea, 2007). The NADH-GOGAT is expressed in photosinthetically non active tissues, and besides, it is important in the NH_4^+ assimilation when this N source is predominant in soil (Konishi et al., 2014). GOGAT enzyme is induced in response to ammonium supply, condition in which the transcription of NADH-GOGAT is induced (Konishi et al., 2014).

NH_4^+ assimilatory alternative pathways

Asparagine synthetase (AS) is an enzyme that catalyzes the transference of the amide group from Gln to aspartate (Asp) to produce asparagine (Asn) (Figure X). AS is a cytosolic enzyme and in *A. thaliana*, it is codified by three genes, *ASN1*, *ASN2* and *ASN3* (Gaufichon et al., 2016).

It has been suggested that under special circumstances, as under an exclusive NH_4^+ based nutrition, it could be involved in the direct assimilation of NH_4^+ (Gaufichon et al., 2016). AS activity is dependent on its substrate availability and therefore AS needs a proper Asp supply. In this sense, Asp synthesis, driven by Aspartate amino transferase (AAT), is essential and thus, the coordinated action between AAT, GS and AS is the final responsible of Asn synthesis (Leasure and He, 2015). In addition, AS activity is also known to be regulated at transcriptional level, for example; it has shown that the transcription of *ASN2* is induced by low content of sucrose at the end of the night, whereas sucrose accumulation in the light repressed *ASN2* expression (Gaufichon et al., 2013).

Glutamate dehydrogenase (GDH) is also an enzyme implicated in N metabolism that catalyzes the reversible deamination of the glutamate (Glu) to NH_4^+ and 2-Oxoglutarate (2-OG). There is controversy about its role in plants, but it is accepted that GDH activity *in vivo* is primarily directed towards 2-OG production (Fontaine et al., 2012; Labboun et al., 2009). In this line, it is thought that its main function is to support plant metabolism under C- limitation circumstances providing 2-OG (Fontaine et al., 2012). Although controversial, it is thought that, under some circumstances NADH-GDH might also be collaborating in the direct amination of 2-OG to form glutamate, such as during fruit ripening (Ferraro et al., 2015) or ammonium stress (Skopelitis et al., 2006) (Figure X). NAD(H)-GDH, in Arabidopsis, is encoded by three genes (*GDH1* to *GDH3*), which are expressed in mitochondria, and by a fourth gene encoding an NADP(H)-dependent GDH isoform, expressed in plastids, although it is an inactive isoform (Fontaine et al., 2012). In Arabidopsis, GDH is a hexameric protein composed by combination of α, β and γ subunits leading to different 28 possible isoenzymes by their combination (Fontaine et al., 2013). GDH expression can be regulated by external conditions; for example, under stress conditions it is induced. Besides, GDH isoforms are differentially expressed depending of N availability, for instance, when plants are grown exclusively with an ammoniacal N source, its expression and activity is greatly induced (Qiu et al., 2009; Tercé-Laforgue et al., 2015).

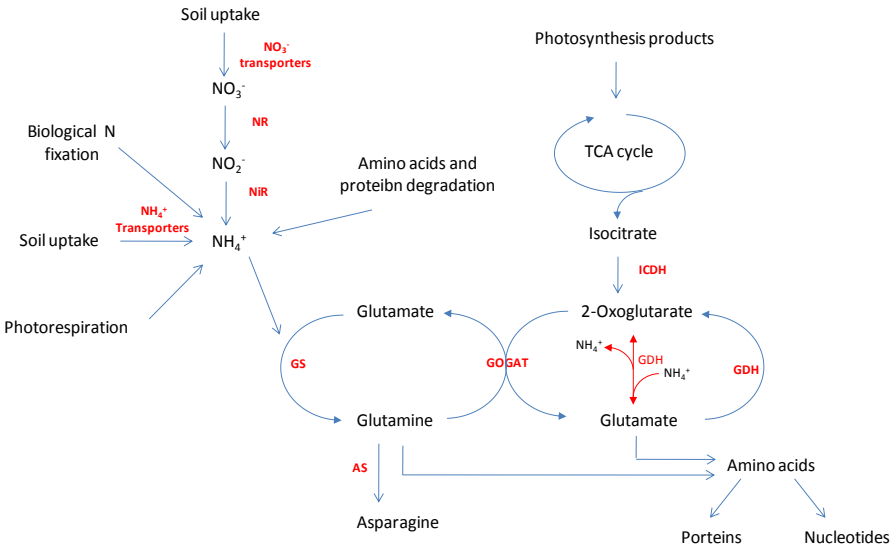


Figure X. Schematic representation of different N sources uptake and their metabolizing processes. Red arrows show the hypothetical GDH dependent assimilatory pathway.

4.4. Interaction between C and N metabolisms

N assimilation depends on many factors, obviously in N availability, but also in the access to C-skeletons, energy and appropriate amount of reducing power essential to assimilate inorganic nitrogen into a wide array of organic nitrogenous compounds (Nunes-Nesi et al., 2010). Indeed, several studies have highlighted the importance of a suitable C supply to alleviate NH_4^+ toxicity by controlling environmental conditions in order to favor C assimilation (Roosta and Schjoerring, 2008; Setién et al., 2013; Vega-Mas et al., 2015). In this sense, it is known that the TCA cycle and its associated anaplerotic enzymes play a central role (re)generating 2-OG for NH_4^+ assimilation through GS/GOGAT cycle (Figure XI). TCA cycle is a fundamental process for all aerobic organisms. It takes place in the mitochondria, where organic compounds are oxidized to generate reducing equivalents (NADH and $FADH_2$) that could be used to ATP production through the oxidative phosphorylation (Sweetlove et al., 2010). Furthermore, TCA cycle provides other metabolic pathways with substrates, as in the case of the N assimilatory pathway that needs C-skeletons, mainly 2-OG and oxaloacetate (OAA) (Figures IX and X).

Anaplerotic enzymes are key actors in maintaining TCA with intermediates. On the one hand, phosphoenolpyruvate carboxylase (PEPC) is an anaplerotic enzyme that catalyzes the carboxilation from phosphoenol pyruvate (PEP) to OAA. On the other hand, NAD(H) dependent malate dehydrogenase (MDH) anaplerotic enzyme, reduces malate into OAA in a reversible

reaction of the TCA cycle. Finally, closing the circle among PEP, OAA and malate, it is the malic enzyme (ME), which can decarboxylate the malate into pyruvate by the NADP-dependent ME (NADP-ME), expressed in plastids and in cytosol, or by NAD-depending ME (NAD-ME) in mitochondria (Drincovich et al., 2001) (Figure XI).

TCA enzymes, function as crossroad between N and C metabolism and their regulation depends on the availability of N. Different authors have shown how PEPC is induced under NH_4^+ supply (Ariz et al., 2013; Setién et al., 2014). PEPC activity is linked to GS activity due to its function depends on the Gln/Glu ratio, which in turns depends on the activity of the GS (Britto and Kronzucker, 2005). As expected, TCA cycle enzymes regulation is coordinated. For example, it is described that the overexpression of the PEPC induces the expression of enzymes as NADP-ME, NAD-ME and also NADP-dependent isocitrate dehydrogenase (ICDH) or that they respond coordinately to stress conditions (Doubnerová and Ryšlavá, 2011; Häusler et al., 2001). Furthermore, it has been observed that ICDH and MDH enzymes are induced under NH_4^+ supply, contributing to its assimilation in cases in which PEPC is not induced (Vega-Mas et al., 2015).

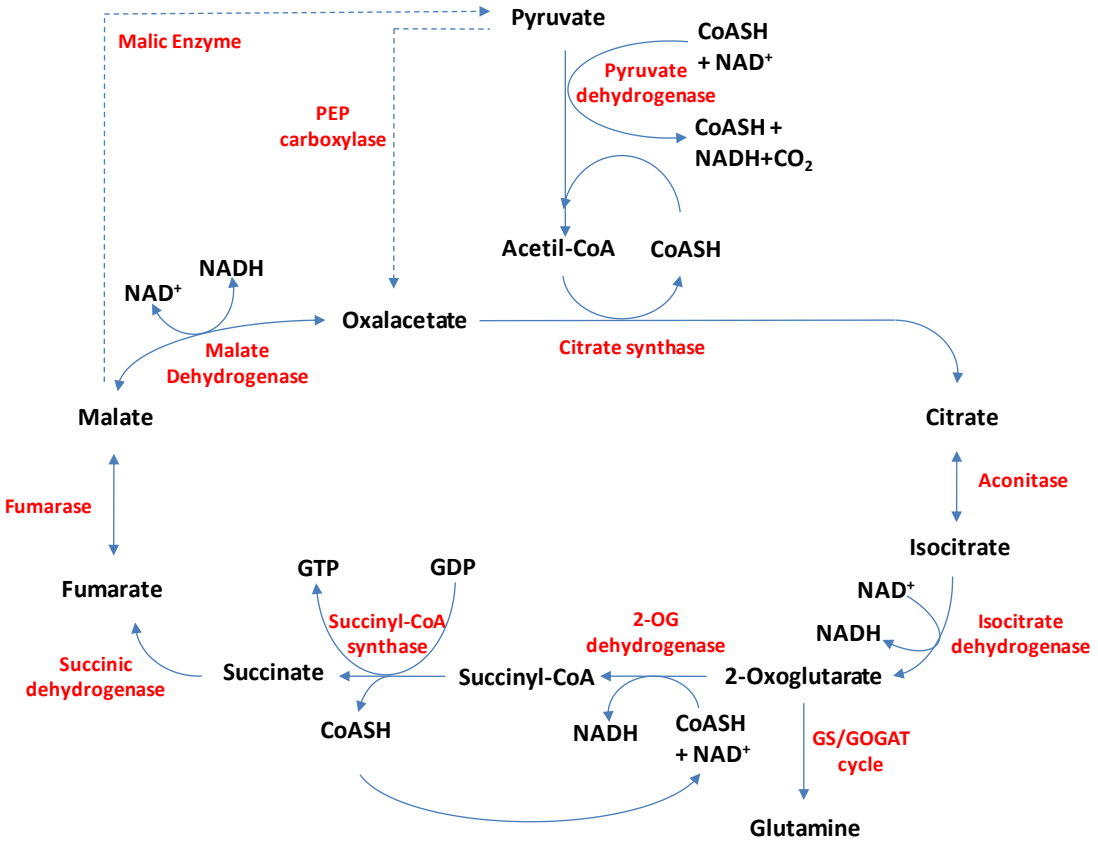


Figure XI.TCA cycle and anaplerotic routes: The diagram shows TCA intermediates and substrates (black) and the enzymes responsible of the conversion from one intermediate to other one (red). Discontinuous and continuous arrows, in the first case anaplerotic route and in the second classical route, show the direction of the reactions.

5. AMMONIUM TOXICITY

When inorganic N is taken up from the root medium, before of being assimilated, it is necessary to convert it into NH_4^+. NH_4^+ is in lower redox state than NO_3^- and because of that plants have not the need to reduce it in an energetically expensive process. The energy required to reduce one molecule of NO_3^- into NH_4^+ is of 15 ATP equivalents (Salsac et al., 1987). Even though N is only incorporated into biomolecules as NH_4^+, paradoxically, an elevated abundance of this cation is toxic for plants, especially when it is supplied as the sole N source, although the toxicity threshold greatly depends on NH_4^+ concentration (Britto and Kronzucker, 2002; Li et al., 2014). In spite of, a robust classification of plants species adapted to NO_3^- or NH_4^+ does not exist, it appears that most non-bred plants preferentially take up NH_4^+ (Bloom et al., 1993; Kronzucker et al., 2001).

5.1. Ammonium nutrition effects on plants

Ammonium toxicity syndrome in plants include several symptoms and physiological effect as, leaf chlorosis, ion imbalance, hormones deregulation, disorders in pH regulation, decrease in net photosynthesis and changes in metabolite levels and oxidative stress among others. These alterations together, provoke a reduction in plant growth. Indeed, growth inhibition with increasing external NH_4^+ concentrations, as compared with NO_3^- nutrition, is probably the best indicator of NH_4^+ stress as it is a comprehensive measure of the physiology of the plant as a whole (Ariz et al., 2011b; Cruz et al., 2006; Domínguez-Valdivia et al., 2008). Importantly, NH_4^+ specially affects plant at root level, where root elongation, lateral branches production, root gravitropism, and root hair development are inhibited (Li et al., 2014).

One of the consequences of NH_4^+ nutrition is the cationic imbalance caused in plants. Nutrients content, as K^+, Mg^{2+} and Ca^{2+}, decreased in the plant, among others, because of the competition that these cations have with NH_4^+ to enter into the cell, due to, they share some ion transporters (Szczerba et al., 2008). In this sense, it is known that under K^+ limiting conditions increasing the K^+ concentration of the nutritional solution could reduce the NH_4^+ toxicity (Balkos et al., 2010).

Plants grown in NH_4^+ stress conditions also show altered hormone homeostasis. Different studies have reported that cytokinins, auxins, ethylene and ABA levels change in response to this nutrition (Li et al., 2011a, 2012, 2013; Liu et al., 2013; Rahayu et al., 2005). The importance of these changes resides in the functions that hormones perform in plants development; for example, it is well established that auxins and ethylene play a fundamental role in the correct development of the roots, whose growth, as previously mentioned, is inhibited during NH_4^+ stress (Li et al., 2011a, 2013). Furthermore, it is thought that under NH_4^+ nutrition, the alleviation of the toxicity mediated by adding NO_3^- in the root media, could be due to the positive effect NO_3^- has on cytokinin signaling (Hachiya et al., 2012; Roosta and Schjoerring, 2008).

Under NH_4^+ nutrition plants produce more reactive oxygen species (ROS) than under NO_3^- or mixed nutrition. It is thought that due to NH_4^+ is already reduced, the reductive power that is destined to reduce NO_3^- into NH_4^+ is over-accumulated, and thus, the oxidized forms of these molecules are not available in electron chain reactions to be reduced provoking the production

of ROS molecules with the damages linked to the excessive presence of these molecules (Podgórska et al., 2013).

5.2. Plant strategies to face ammonium stress

Several studies have compared the growth of different plant species under NH_4^+ and NO_3^- nutrition, showing that most of them grow better under NO_3^- or mixed nutrition than under exclusively NH_4^+ nutrition. Under NH_4^+ nutrition plants accumulate more NH_4^+ in their tissues (Britto et al., 2001), in this way, it is interesting to highlight that plants shown a correlation between the NH_4^+ accumulated in their tissues and the growth reduction (Lasa et al., 2001, 2002). In general NH_4^+ is assimilated in the root to avoid the detrimental effects that it causes in photosynthetic active tissues. In this sense, roots may act as a kind of barrier. As other strategies to control NH_4^+ levels, the isolation of NH_4^+ inside the vacuole or NH_4^+ efflux to the rhizosphere are alternative strategies to control cytosolic NH_4^+ levels. In general, an enhanced activity of these processes contributes to improve the plant NH_4^+ tolerance. However, these strategies are linked to biomass reduction because of carbohydrate limitation for growth due to excessive sugars consumption during NH_4^+ assimilation and to the great energy cost of NH_4^+ influx/efflux, known as futile transmembrane NH_4^+ cycle (Coskun et al., 2013). The futile NH_4^+ cycle is a plasma membrane cycle of NH_4^+ uptake and efflux through cell roots. In a context of NH_4^+ toxicity, in which NH_4^+ would be the principal N source, NH_4^+ futile cycle is a continuous process to avoid the NH_4^+ tissue accumulation and its detrimental effects. It is energetically expensive process, and it is said that because of that, plants has to employ resources in the NH_4+ detoxification instead of in other processes causing growth inhibition (Britto et al., 2001). Afterwards, the same research group suggested that the futile cycling was probably in form of NH_3 and thus, without requiring the energy consumption of active NH_4^+ transport (Coskun *et al*., 2013).

The increase of the assimilation of NH_4^+ is another detoxification strategy in plants. It is known that under NH_4^+ nutrition enzymes involved in the N assimilation processes, as GS1, are induced, mainly in roots where most of the N assimilation happens (Guan et al., 2016; Lothier et al., 2011). However, GS activity can be overfilled in excessive NH_4^+ concentration since it has been observed that when amino acids level increases assimilation capacity of the GS1 is saturated (Prinsi and Espen, 2015). Besides, NH_4^+ assimilatory capacity is highly dependent on C-skeletons supply, due to that, as already mentioned, TCA anaplerotic enzymes are related to plant tolerance capacity providing 2-OG and OAA when they are needed (Setién et al., 2014).

Plants can isolate toxic compounds into vacuoles. Due to vacuole pH is *ca.* two units more acidic than the cytosol pH, it can contain 100 fold higher NH_4^+ concentration. The transport of NH_4^+ from the cytosol to the lumen of the vacuole is mediated by passive transport and by membrane bound ATP-driven pumps. In the case of the passive transport, this is mediated by aquaporins, which transport NH_4^+ only as NH_3. Once in the vacuole, the NH_3 is protonated to NH_4^+, and consequently it is trapped inside (Bittsánszky et al., 2015).

5.3. pH control: fundamental factor in ammonium stress response

Soil pH fluctuates widely between natural and agricultural soils and represents an important feature that may limit N availability and the plant´s capacity to absorb essential nutrients (Marschner and Rengel, 2011). Moreover, pH alterations may have an influence on cellular expansion (Cosgrove, 1999) and water conductance in roots besides other effects (Kamaluddin and Zwiazek, 2004). Furthermore, H^+s also play a role as second messengers in cell signaling cascades and so internal pH control is essential for the fine tuning of cells functioning (Felle, 2001). High NH_4^+ content is common in acidic soils and the connection between NH_4^+ stress and pH alteration has been known from a long time (Chaillou et al., 1991; Gerendas and Ratcliffe, 2000). It is known that ammonium-tolerant plants commonly tolerate acidic conditions, and that by controlling external medium pH, it is possible to mitigate NH_4^+ toxicity (Li et al., 2014). NH_4^+ uptake is known to induce acidification of the rhizosphere and apoplast through proton extrusion coupled to the NH_4^+ transport into the cells (Kronzucker et al., 2001). On the other hand, NO_3^- uptake promotes external alkalinization. Further to this, it has been suggested that NH_4^+ uptake causes cytosolic alkalinization, while NO_3^- uptake provokes cytosolic acidification (Hawkesford et al., 2011). However, this potential cytosolic alteration associated to N uptake is transient, because of when the uptake and assimilation are considered as a whole process, both NO_3^- and NH_4^+ nutritions tend to alkalinize cell cytosol (Britto and Kronzucker, 2005). Indeed, although intracellular pH is sensitive to external pH, cytosolic pH is extremely stable thanks to the fine tuning of cell metabolism. This is evidenced by several studies, which observed that external pH changes over a range of pH 4-10 had very little impact on internal cytosolic pH (Gerendás and Ratcliffe, 2013; Hartung and Ratcliffe, 2002). A further example is the work of Hachiya et al., (2012), who by the use of *A. thaliana* plants expressing a cytosolic fluorescent pH sensor, observed that although apoplast pH decreased upon NH_4^+ stress, cytosolic pH remained stable. Indeed, cell metabolic adjustment

in response to changes in soil medium parameters, such as N source and availability, is crucial for plants in order to maintain their growth rates and fitness.

5.4. Genes identified in the response to ammonium stress

Recent genetic approaches have been useful to identify new molecular players involved in the signaling pathways that lead to NH_4^+ sensitivity or tolerance. However, still few genetic components of the ammonium perception and signaling have been identified.

Genes encoding NH_4^+ transports and regulators of these transporters are, as expected, closely related with cellular NH_4^+ homeostasis. In this sense through the analysis of Arabidopsis mutants for AMT transporters, it has been observed that AMT1;3 has a major role in the plant NH_4^+ tolerance response. Under a exclusive NH_4^+ N source, plants with a mutation in AMT1;3 are smaller than WT plants, and furthermore, *amt1:3* mutants present improper root development under NH_4^+ nutrition (Lima et al., 2010). AMT transporters are regulated by phosphorylation (Lanquar et al., 2009). Straub et al. (2017), reported that, CIPK23 kinase is able to phosphorylate, among others, AMT1;1 and AMT1;2 transporters, decreasing their activity when phosphorylated. Accordingly, NH_4^+ uptake was induced in *cipk23* mutants and therefore, these mutants show more sensitive phenotype under NH_4^+ nutrition (Straub *et al*., 2013). In this context, also observed that mutants of CBL1 (calcineurin B-like binding protein 1), protein required for the CIPK23-dependent phosphorylation of the potassium transporter AKT1, also displayed increased sensitivity towards ammonium nutrition (Straub et al., 2017).

In addition to NH_4^+ transporters, NO_3^- transporters have also a role in the plant response to NH_4^+ stress. For example, *NRT1.1* gene that encodes the most studied nitrate transporter has been shown to be related with low pH tolerance in plants because of NO_3^- uptake is coupled to H^+ uptake, which promotes the increasing of the rhizosphere pH (Fang et al., 2016). Interestingly, *nrt1.1* mutant is more tolerant to NH_4^+ stress in a pH-dependent manner (Hachiya et al., 2011). Other NO_3^- transporters also implicated in the alleviation of the NH_4^+ toxicity are NRT2.1, NRT1.5 or SLAH3, which are NO_3^- efflux channels. The plant mutants to these genes displayed a growth decrease under mixed or exclusive NH_4^+ nutrition (Krouk et al., 2006; Meng et al., 2016; Zheng et al., 2015).

As previously mentioned, subcellular NH_4^+ compartmentalization is another strategy to avoid NH_4^+ toxicity. In this context, it has been shown that the $[Ca^{2+}]_{cyt}$-associated receptor protein kinase (CAP1) is located the tonoplast and that is involved in the ammonium homeostasis in

the cytosol facilitating the NH_4^+ isolation inside de vacuole through the regulation of NH_4^+ transporters in the tonoplast (Bai et al., 2014).

N-molecules in general, and NH_4^+ in particular, besides of being essential nutrients, are important signals in plants and regulates a great number of functions in relation with nutrient uptake, metabolic balance and plant development, among others. As previously said, there are several mechanisms under the influence of the presence of NH_4^+, for example the AMT family proteins functioning, which are phosphorylated responding to external NH_4^+ concentration (Lanquar et al., 2009). Two different models have been proposed to explain NH_4^+ sensing and signaling: the transceptor model and the receptor kinase model (Figure XII). In the transceptor model, which is based on protein that possesses both transporter and receptor functions at the same time, when there is a high external NH_4^+ concentration, AMT1 proteins are phosphorylated and shut down NH_4^+ transport. In this sense, it is known CIPK23 directly phosphorylates AMT1;1 and AMT1;2 in a complex with CBL1 (calcineurin B-like protein) regulating the transport activity (Straub, 2016). In the receptor kinase model, AMT1 only functions as a transporter when a receptor kinase senses high external NH_4^+ concentration and phosphorylates the transporter interrupting the NH_4^+ flux (Lanquar et al., 2009).

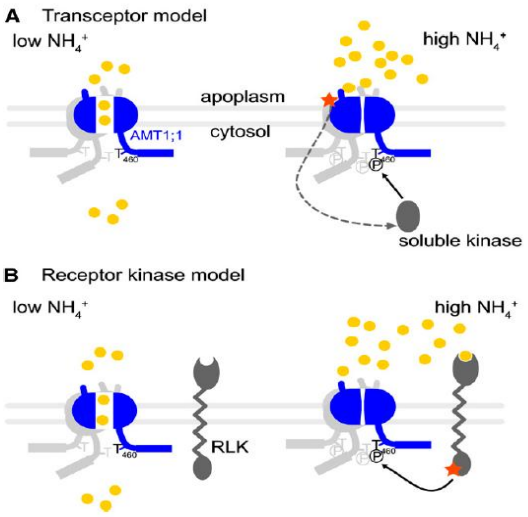


Figure XII. A) Transceptor model: At low external ammonium concentration, the transporter senses the extracellular NH_4^+ status and T460 is not phosphorylated. NH_4^+ can enter the cell through the transporter. When ammonium levels increase, the transporter senses the high NH_4^+ external status. A kinase is recruited and phosphorylation shuts down NH_4^+ import via the allosteric trans-regulatory mechanism. (B) Receptor kinase model: At low ammonium concentration, a receptor kinase senses the extracellular NH_4^+ status and T460 is not phosphorylated. When ammonium increases, the receptor kinase senses the high NH_4^+ external status and triggers phosphorylation of T460. The transporter is converted to the shut conformation. Retrieved from Lanquar et al., (2009).

The correct coordination between hormones and nutrient signaling is essential for proper plant development. Hormonal status is influenced by N-availability but N-sensing, uptake and assimilation is under hormones control (Krouk et al., 2011). NH_4^+ stress promotes hormonal imbalances that may cause roots and shoots growth inhibition in many plant species including *Arabidopsis thaliana.* In this context, auxin is the most studied hormone and there are several studies showing the interaction between NH_4^+ and this hormone. For example Yang et al., (2015), showed how auxin response decreases under $(NH_4)_2SO_4$ nutrition provoking impaired root growth, which was rescued by external auxin supply. In this sense, the same study reveals that the Arabidopsis knock-out mutants for auxin resistant 1 (*AUX1)* and pin formed 2 (*PIN2)* genes, which encode for auxin transporters, have an altered root growth under NH_4^+ nutrition compared to wild type (WT), which is rescued when are supplied with external auxins. Other hormone influenced by NH_4^+ nutrition is abscisic acid (ABA). Indeed, an Arabidopsis mutant for the plastid metalloprotease (Ethylene-dependent Gravitropism-deficient and Yellow-green-like Protein 1), displayed hypersensitivity to NH_4^+ stress in relation with impaired ABA signaling (Li et al., 2012).

NH_4^+ assimilation is a known plant strategy to reduce the detrimental effects of NH_4^+ nutrition. In this sense the correct functioning of N metabolism related enzymes is essential to maintain cellular NH_4^+ homeostasis. For example, *gln1;2* mutants show higher NH_4^+ accumulation than WT plants and are more to NH_4^+ nutrition, (Lothier et al., 2011). NADH-GOGAT is also fundamental in NH_4^+ stress response. For instance, Konishi et al., 2014 reported that Arabidopsis *nadh-gogat-2* mutant developed smaller rosettes than the WT plants when grown under NH_4^+ nutrition.

GDP-mannose participates in both L-ascorbic acid (AsA) and in N-glycoprotein synthesis. It has been shown that Arabidopsis *vtc1* mutant, which does not express GDP-mannose pyrophosphorylase enzyme (GMPase), is hypersensitive to NH_4^+ stress (Qin et al., 2008). Thus suggest that GMPase plays a role in NH_4^+ tolerance process. Barth et al., (2010) showed that effective protein glycosylation in the roots, rather than decreased AsA content, was linked to the hypersensitivity of *vtc1* to NH_4^+.

The toxicity of NH_4^+ has been related with changes in the cellular redox state. The cellular oxidant/antioxidant balance, among others, is under the control of electron transport chains. Podgórska et al., (2015) have studied how in *A. thaliana fro1* plants, which have a defective mitochondrial respiratory chain complex I and consequently accumulate more ROS, are able to grow better under NH_4^+ nutrition than WT plants, probably because of compensation in

energetic metabolism. In rice, another gene related with NH_4^+ tolerance is OsSE5 (PHOTOPERIOD SENSITIVITY 5) which encodes a Heme-oxygenase 1 (HO1) protein. HO1 is involved in rice antioxidant defense, and *OsSE5* RNAi-transgenic plants revealed NH_4Cl^- hypersensitive phenotype with impaired antioxidant defense. In agreement, the overexpression of OsSE5 in *A. thaliana* conferred enhanced NH_4^+ tolerance linked to a higher antioxidant capacity (Xie et al., 2015).

GENERAL OBJECTIVES

OBJECTIVES

The increase of the world population supposes a great challenge for agriculture, which, among others, is forced to increase the agricultural soil and to improve the fertilizer management in order to cover the food demand augmentation. The intensive use of fertilizers is associated to environmental pollution, which is mostly linked to nitrogen compounds. The reduction of the pollution caused by nitrogen compounds in agriculture is a key aspect that humanity has to face at present. In this line, the use of NH_4^+ as N source, in combination with nitrification inhibitors, has been shown more efficient to preserve the environment compared to the more traditional use of NO_3^- as N source. However, ammonium nutrition represents a stressful situation and provokes a number of problems for plants health.

In this context, the **general objective** of the current study is **to better understand plant metabolic and genetic mechanisms associated to ammonium tolerance using *Arabidopsis thaliana* as a model.**

This general objective is divided in three specific objectives, each one corresponding to a different chapter:

4. To explore *Arabidopsis thaliana* intraspecific natural variability in ammonium tolerance with a special focus on N assimilation mechanisms.

5. To identify genes related to *Arabidopsis thaliana* natural variability in ammonium tolerance through genome wide association analysis.

6. To understand *Arabidopsis thaliana* metabolic adjustment to ammonium stress in function of the external medium pH.

CHAPTER 1.

Exploring ammonium tolerance in a large panel of *Arabidopsis thaliana* natural accessions

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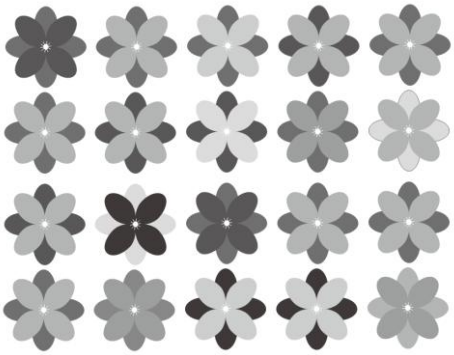


1. ABSTRACT

Plants are dependent on exogenous nitrogen (N) supply. Ammonium (NH_4^+), together with nitrate (NO_3^-), is one of the main nitrogenous compounds available in the soil. Paradoxically, although NH_4^+ assimilation requires less energy than NO_3^-, many plants display toxicity symptoms when grown with NH_4^+ as sole N source. However, besides the species-specific ammonium toxicity, intra-specific variability has also been shown. Thus, the aim of this work was to study the intra-specific ammonium tolerance in a large panel of *Arabidopsis thaliana* natural accessions. We grew plants either with 1 mM NO_3^- or NH_4^+ as N source, and we determined several parameters related to ammonium tolerance and assimilation. Overall, high variability was observed in *A. thaliana* shoot growth under both N nutritions. From the parameters determined tissue ammonium content was the one with the highest impact on shoot biomass, and interestingly this was also the case when N was supplied as NO_3^-. Enzymes of nitrogen assimilation did not have an impact on *A. thaliana* biomass variation, but the N source affected their activity. GDH aminating activity was, in general, higher in NH_4^+-fed plants. In contrast, GDH deaminating activity was higher in its deaminating direction in NO_3^--fed plants, suggesting a differential role of this enzyme in function of the N form supplied.

2. INTRODUCTION

Plants have a fundamental dependence on inorganic nitrogen (N) and intensive agriculture requires the use of N compounds to supplement the natural supply from the soil. Indeed, more than 100 million metric tonnes of nitrogenous fertilizers are added to the soil worldwide annually (Good and Beatty, 2011). In part because of the intense use of fertilizers, agriculture is now a dominant force behind many environmental threats, including climate change and degradation of land and freshwater (Foley et al., 2011; Tilman et al., 2011)). Moreover, recent studies suggest that agricultural output would need to roughly double to meet the expected demand associated with world population increase (FAO, 2009).

Nitrate (NO_3^-) and ammonium (NH_4^+) are the main forms of N available for plants. There is serious concern regarding NO_3^- loss in the field, giving rise to soil and water pollution. Moreover, incomplete capture and poor conversion of nitrogen fertilizer also causes global warming through emissions of nitrous oxide. Due to these detrimental effects of adding high

NO_3^- concentrations to ecosystems (Gruber and Galloway, 2008) the potential of NH_4^+ as N source for agriculture has been reconsidered alongside the search to improve N use efficiency while mitigating agriculture impact (IPCC 2007). Similarly, lowering fertilizer input and breeding plants with better nitrogen use efficiency without affecting yield is a main goal for research in plant nutrition (Xu et al., 2012).

Plants have differential N-source preference, but it depends not only of their genetic background but also on a wide and dynamic range of environmental variables including soil pH, temperature, etc. Thus, a robust classification of plants species adapted to NO_3^- or NH_4^+ does not exist. However, it appears that most non-bred plants preferentially take up NH_4^+ (Bloom et al., 1993; Kronzucker et al., 2001). Moreover, crop species have traditionally been bred under nitric or combined nitrogen nutrition, provoking a negative selection pressure towards NH_4^+ assimilation and this is surely one of the reasons they prefer NO_3^-, although NO_3^- must be taken against electrochemical gradient and then be reduced to NH_4^+ with the consequent energy cost (Kronzucker et al., 2001). In this sense, NH_4^+ nutrition has been generally considered as toxic for plants, particularly when NH_4^+ is supplied as the sole N source. Indeed, NH_4^+ is also toxic to animals and fungi when present in excess amounts (Britto and Kronzucker, 2002).

Ammonium toxicity syndrome in plants include several symptoms, among others leaf chlorosis, ion imbalance, hormones deregulation, disorder in pH regulation, decrease in net photosynthesis and changes in metabolites levels including amino acids, organic acids and carbohydrates. At whole-plant level, a reduction in plant growth with increasing external NH_4^+ concentrations, as compared with NO_3^- nutrition, is a common effect of NH_4^+ nutrition (Cruz et al., 2006). Biomass reduction has been associated with carbohydrate limitation for growth due to excessive sugars consumption for NH_4^+ assimilation and to the energy costs of futile transmembrane NH_3/ NH_4^+ cycling in root cells (Coskun et al., 2013). Indeed, plant growth is probably the best indicator of NH_4^+ stress as it is a comprehensive measure of the physiology of the plant as a whole (Ariz et al., 2011a; Cruz et al., 2006; Domínguez-Valdivia et al., 2008).

Substantial variations in NH_4^+ tolerance have been observed amongst closely related species (Monselise and Kost, 1993) and even within species (Cruz et al., 2011; Li et al., 2011b), suggesting the evolution of highly distinct mechanisms to cope with this stress. The strategies plants deploy to avoid NH_4^+ toxicity include enhancing NH_4^+ assimilation and increasing the efflux outside the cell or into the vacuole. Nevertheless, at present there is no consensus on which traits confers NH_4^+ tolerance or sensitivity to plants. Ammonium assimilation mainly

occurs via glutamine synthetase / glutamate synthase cycle (GS/GOGAT). However, it seems that other alternative pathways could be involved in ammonium assimilation when NH_4^+ is supplied as the sole source of N. Although controversial, under these conditions, glutamate dehydrogenase (GDH), that catalyzes the reversible deamination of glutamate (Glu) to 2-oxoglutarate, might be collaborating in NH_4^+ assimilation (Setién et al., 2013; Skopelitis et al., 2006).

Arabidopsis thaliana and the *Brassicaceae* family are considered to be a species, and a family, sensitive to NH_4^+. Most of the works focused on NH_4^+ toxicity in *Arabidopsis* have compared plants fed with NO_3^- versus plants fed with a combined nutrition of NO_3^- supplemented with increasing concentrations of NH_4^+. The works that have grown *Arabidopsis* under long-term ammonium supply as sole N-source are rare and have shown how NH_4^+ causes a retardation of seedlings growth or a dramatic reduction in plant biomass (Helali et al., 2010; Hoffmann et al., 2007). Besides, recent genetic approaches have been useful to identify new molecular actors involved in the signalling pathways that lead to NH_4^+ sensitivity, for example a GDP-mannosepyrophosphorylase enzyme (Qin et al., 2008) or the ammonium transporter AMT1:3 (Lima et al., 2010).

Overall, the evolutionary trade-off between high productivity, adaptation to low-nutrient environments and the use of ammonium as fertilizer is a challenge to most plant cultivars that have been selected under non-limiting NO_3^- or combined NH_4^+ / NO_3^- fertilization (Presterl et al., 2003; Xu et al., 2012). In this sense, approaches based in intra-specific natural variation have become an important mean to study plants adaptation to the environment. Thus, the present work evaluates the natural variability of *A. thaliana* grown under a low NO_3^- or NH_4^+ supply focusing on the importance of N assimilation mechanisms in relation to the differential N-source provided.

3. MATERIALS AND METHODS

Experimental procedures and growth conditions

Forty seven *Arabidopsis thaliana* world natural accessions lines (http://public-lines.versailles.inra.fr/naturalAccession/index) were used throughout the study. Seeds were directly sown in 37 cm^3 pots containing a perlite:vermiculite substrate mixture (1:1, v:v).
Seeds were cold-treated during 4 days in the dark at 4ºC and then transferred into a controlled conditions phytotron : 14 h day, 200 $\mu mol.m^{-2}.s^{-1}$ light intensity, 60% RH and 22ºC day conditions and 70% RH and 18ºC night conditions. Pots were initially misted with a modified MS solution containing 0.5 mM of NH_4NO_3. Nine days after transfer into the growth chamber a single seedling was retained per pot and treatment was initiated. Plants were irrigated three times a week with a modified MS solution (3 mM $CaCl_2$, 1.25 mM KH_2PO_4, 1.5 mM $MgSO_4$, 5 mM KCl, 0.085 mM Na_2EDTA, 0.5 mM MES, 5µM KI, 0.1 µM $CuSO_4$, 100 µM $MnSO_4$, 100 µM H_3BO_3, 0.1 µM $CoCl_2$, 100 µM $FeSO_4$, 30 µM $ZnSO_4$ and 0.1 µM Na_2MoO_4) with 0.5 mM $Ca(NO_3)_2$ or 0.5 mM $(NH_4)_2SO_4$ as N source. NH_4^+-fed plants were supplemented with 1 mM $CaSO_4$ to compensate the Ca^{2+} supplied together with the NO_3^-.

Thirty days after transfer into the growth chamber, rosette biomass was recorded and leaves were immediately frozen in liquid nitrogen and stored at -80ºC.

Determination of ammonium and total amino acids content

Aliquots of ca. 25 mg of frozen material were ground to powder with liquid nitrogen and homogenised with 800 µL of ultrapure water. Samples were then incubated at 80ºC during 5 minutes and centrifuged 20 min at 4000 *g* and 4ºC and supernatants were recovered.

Total free amino acids were determined by the ninhydrin method (Yemm et al., 1955). Ammonium content was determined by using the colorimetric method based in the phenol hypochlorite assay (Berthelot reaction).

Protein extraction

Proteins were extracted as described in Gibon, (2004). Briefly, leaves (about 40 mg\sample) were homogenised in a mortar and pestle with 0.8 mL of extraction buffer (10 mM $MgCl_2$, 1

mM EDTA, 1 mM EGTA, 10 mM DTT, 0.1% Triton X-100, 10% Glycerol, 0.05% BSA, 0.5% PVPP, 50 mM HEPES pH 7.5) in presence of a cocktail of proteases inhibitors (1 mM PMSF, 1 mM ε-aminocaproic acid, 10 µM leupeptin, 1 mM benzamidin). Samples where then centrifuged at 4000 g for 30 min at 4ºC and the supernatants recovered. Protein content of the supernatants was quantified by the Bradford assay (Bradford, 1976).

Enzyme activities

GS reaction was measured at 30ºC in a reaction buffer containing: 50 mM Tris-HCl (pH 7,6), 20 mM $MgSO_4$, 8 mM sodium glutamate, 6 mM hydroxylamine, 4 mM Na_2-EDTA and 8 mM ATP. The reaction was stopped by adding 0.12 M $FeCl_3$, 0.5 M TCA and 2 N HCl. Samples were centrifuged at 13,200 g for 5 min, and the absorbance of γ-glutamyl monohydroxamate (γ-GHM) was measured at 540 nm.

GDH activity was carried out in the aminating sense in a reaction buffer containing 100 mM Tris-HCl (pH 8), 1 mM $CaCl_2$, 13 mM 2-oxoglutarate, 50 mM $(NH_4)_2SO_4$ and 0.25 mM NADH, and in the deaminating sense in 100 mM Tris-HCl (pH 9), 1 mM $CaCl_2$, 30 mM glutamic acid and 0.25 mM NAD. Both kinetic activities were monitored spectrophotometrically at 30ºC by consumption of NADH (aminating sense) or appearance of NADH (deaminating sense) at 340 nm.

NR activity was measured at 30ºC. The reaction medium consisted of 50 mM HEPES-KOH, pH 7.6, 5 mM KNO_3, 0.2 mM NADH, 10 µM flavin adeninedinucleotide phosphate, 1 mM DTT, 20 mM EDTA. The reaction was started by adding 50 µl of protein extract to 250 µL of reaction medium and stopped by adding 32 µL of 50 mM zinc acetate. Then, samples were centrifuged, 100 µL of supernatant was recovered, 8 µL of phenacin metosulfate 50 mM added and the samples incubated during 20 min at room temperature. Finally, 80 µL of sulfanilamide 1% in 3 M HCl and 80 µL of 0.02% N-(1-naftyl) ethylenediamine dihydrochloride were added and the absorbance determined at 546 nm.

Western blotting

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed in a 1.5-mm thick 10% (w/v) resolving gel and a 4.6% acrylamide (w/v) stacking gel in a vertical electrophoresis cell (Mini- Protean III; Bio-Rad) at 150 V during 150 min. Gels were electroblotted onto nitrocellulose membrane during 75 min at 100 V in a Mini Trans-Blot

Electrophoretic Transfer Cell (Bio-Rad). Blots were blocked in 5% (w/v) skim milk in 20 mM Tris buffered saline at 4°C during 1 hour. We used α-GDH (1:5000), α-GS (1:2000) and α-NR (1:1000; Agrisera, Sweeden) as primary antibodies. The secondary antibody was goat anti-rabbit horseradish peroxidase conjugate (1:50000, Sigma-Aldrich). Immunoreactive bands were visualized with a highly sensitive chemiluminescent substrate for peroxidase detection (GE Healthcare Europe GmbH, Freiburg, Germany).

Data analysis

Data analyses were carried out using the SPSS 17.0 (Chicago, IL). Statistical differences between nitrate and ammonium nutrition for each accession and variable were assessed comparing the mean values by paired t-test. To test the connectivity between variable, Pearson's correlation coefficient was calculated for $P \leq 0.05$. Multiple regressions provided a view of the relationship between a trait and shoot biomass independent of other correlated traits. Multiple regression estimations can suffer from multicollinearity wherein highly correlated traits might act redundantly. Thus, to help our interpretation we also used Akaike´s information criterion (AIC) to determine the "best" model by rewarding added explanatory power but penalizing the inclusion of additional terms. This provides the simplest model with the least collinearity and thus, supposedly, the best estimation of selection (Shaw and Geyer, 2010).

4. RESULTS

To evaluate plants nitrogen use efficiency with ammonium as sole N-source we compared *Arabidopsis* rosette biomass after three weeks of growth under 1 mM NH_4^+ (0.5 mM $(NH_4)_2SO_4$) or 1 mM NO_3^- (0.5 mM $Ca(NO_3)_2$) and we used the ratio between shoot biomass under NH_4^+ and NO_3^- conditions (SB NH_4^+/ NO_3^-) to estimate ammonium tolerance as it has been previously used in other works (Ariz et al., 2011a; Cruz et al., 2006). In general, *Arabidopsis* is a species sensitive to NH_4^+ and nearly every ecotype analysed showed shoot biomass inhibition in response to NH_4^+. Twenty four out of the forty seven accessions analysed experienced a significant growth inhibition upon NH_4^+ nutrition (Fig. 1.1A). The accession Te-0 was the one showing the lowest SB NH_4^+/ NO_3^-ratio (< 0.4), which was significantly lower than the next most sensitive accession to NH_4^+ (Rubenzhnoe-1; SB NH_4^+/ NO_3^- 0.56). Only three accessions had a SB NH_4^+/ NO_3^- ratio higher than one but without significant differences between both nutritions (Akita, Enkheim-T and Gre-0; Fig. 1.1B). Overall, intra-specific shoot growth

variability under contrasted N-source is evident by the use of this accessions collection (Fig. 1.1B).

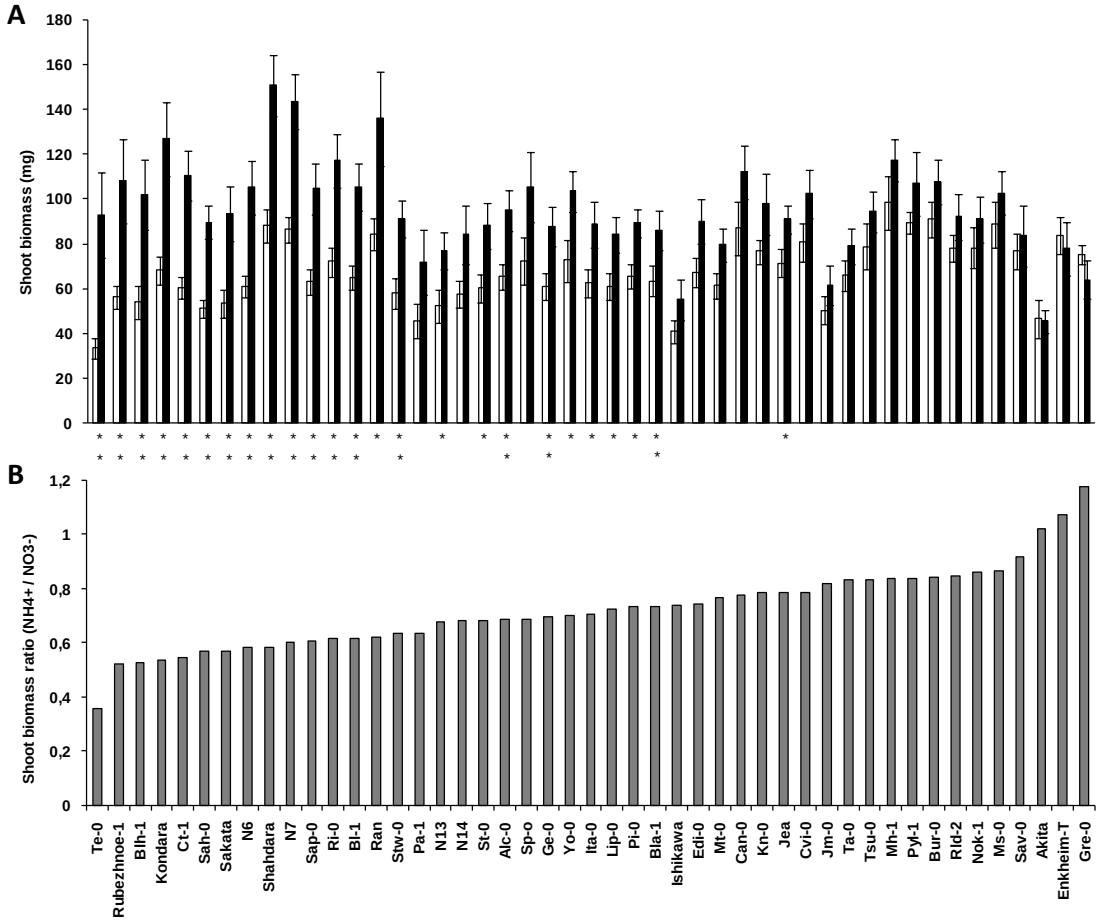


Fig. 1.1. Natural variation of *Arabidopsis thaliana* growth under nitrate or ammonium as N source. (A) Shoot biomass. (B) Ratio between shoot biomass under NH_4^+ and NO_3^- nutrition. Means and standard errors were calculated from 8–12 plants. Significant differences between shoot biomass under ammonium compared with nitrate nutrition are indicated for each accession (**P*<0.05; ***P*>0.01)

The content of ammonium and free amino acids (Supp. Table 1.1) as well as NR, GS and GDH enzyme activities (Supp. Table 1.2A-Supp. Table 2D) were determined. GDH activity was measured both in the aminating (GDHam) and deaminating (GDHdeam) directions). Regarding NH_4^+ content, overall, plants under NH_4^+ nutrition significantly contained more NH_4^+ compared to plants fed with NO_3^-. Eight accessions (Enkheim-T, Gre-0, Ishikawa, Jea, Ms-0, Ran, Ta-0 and Tsu-0) did not show significant differences among both treatments (Supp. Table 1). Amino acids content followed a similar trend as NH_4^+ content (Supp. Fig. 1.1) and every accession under NH_4^+ nutrition significantly contained more amino acids compared with NO_3^- nutrition (Supp. Table 1; Supp. Fig. 1.1). Concerning the enzyme activities, as expected, every accession

under NO_3^- nutrition had a higher NR activity (Supp Table1, Fig. 1.2B). GS activity was similar for every accession under both nutritions, except for Mt-0 and Ct-1 that showed a slightly higher GS activity under NO_3^- nutrition and for Rld-2, N7 and N14 that experienced a small increase under NH_4^+ nutrition (Supp. Table 1, Fig. 1.2A). GDH activity was higher in its aminating sense under NH_4^+-nutrition in 35 out of the 47 accessions. On the contrary, the activity in its deaminating sense was higher under NO_3^- nutrition in every accession except for Akita, Ishikawa, Rld-2, Pa-1 and Sah-0, which did not show significant differences between both forms of nutritions (Supp. Table 1; Fig. 1.2C and D).

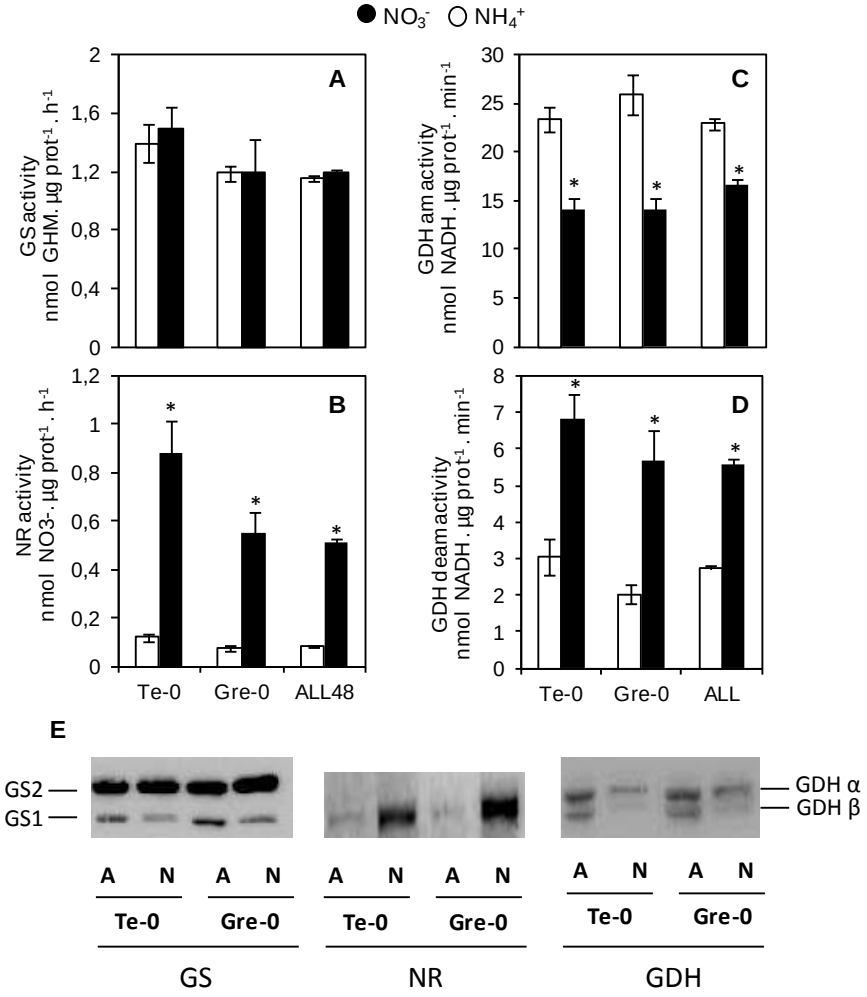


Fig. 1.2. Enzyme activities of Te-0 and Gre-0 accessions and the mean of every accession (ALL) for (A) GS, (B) NR, (C) GDHam, and (D) GDH deam, and (E) western blot of GS, GDH, and NR for Te-0 and Gre-0 accessions grown under ammonium or nitrate nutrition. An asterisk indicates a significant difference for $P<0.05$ ($n=6$).

Table 1.1. Pearson correlations between the determined parameters in *Arabidopsis thaliana* plants under NH_4^+ nutrition. SB means shoot biomass and SB NH_4^+ / NO_3^- denotes de shoot biomass ratio between NH_4^+ - and NO_3^- -fed plants.

		SB NH_4^+/NO_3^-	SB	NH_4^+	Amino acids	NR activity	GS activity	GDHam activity	GDHdeam activity
SB NH_4^+ / NO_3^-	**r2**	1							
	P								
SB	**r2**	**-0.524****	1						
	P	**0.000**							
NH_4^+ content	**r2**	**0.547****	**-0.566****	1					
	P	**0.000**	**0.000**						
Amino acids content	**r2**	**0.478****	**-0.544****	**0.496****	1				
	P	**0.001**	**0.000**	**0.000**					
NR activity	**r2**	0.124	-0.138	**0.340***	0.085	1			
	P	0.403	0.349	**0.018**	0.567				
GS activity	**r2**	0.029	-0.105	0.139	-0.014	**0.335***	1		
	P	0.845	0.478	0.346	0.927	**0.020**			
GDHam activity	**r2**	**0.438****	-0.238	**0.389***	**0.326***	0.162	0.066	1	
	P	**0.002**	0.103	**0.006**	**0.024**	0.271	0.655		
GDHdeam activity	**r2**	0.048	0.146	0.078	-0.187	0.220	**0.489****	0.156	1
	P	0.744	0.321	0.596	0.204	0.133	**0.000**	0.288	

To investigate the connectivity between the different parameters we performed a Pearson correlation analysis for each parameter pair. Values are given for the correlation coefficient (r^2) and the significance (*P*). The results are presented separately for the plants grown under NH_4^+ (Table 1) and NO_3^- nutrition (Table 2). Shoot biomass both under ammonium or nitrate nutrition was negatively correlated with NH_4^+ and free amino acids content (Tables 1, 2; Fig. 1.3A), which is reasonable because it could mean that the absorbed N is not been used for growth, and ammonium accumulation inside plant tissues is known to be deleterious for plant performance (Britto and Kronzucker, 2002; Ludewig et al., 2007). None of the parameters determined in NH_4^+-fed plants showed any correlation with the SB NH_4^+/ NO_3^- ratio (Table 1). In contrast, in NO_3^--fed plants, NH_4^+ and amino acids content, together with GDHam activity, were positively correlated with SB NH_4^+/ NO_3^- ratio (Table 2).

Table 1.2. Pearson correlations between the determined parameters in *Arabidopsis thaliana* plants under NO_3^- nutrition. SB means shoot biomass and SB NH_4^+ / NO_3^- denotes de shoot biomass ratio between NH_4^+ - and NO_3^- -fed plants.

		SB NH_4^+/NO_3^-	SB	NH_4^+	Amino acids	NR activity	GS activity	GDHam activity	GDHdeam activity
SB NH_4^+ / NO_3^-	r^2	1							
	P								
SB	r^2	**0.43****	1						
	P	**0.00**							
NH_4^+	r^2	-0.14	**-0.45****	1					
	P	0.33	**0.00**						
Amino acids	r^2	-0.00	**-0.41****	**0.55****	1				
	P	0.99	**0.00**	**0.00**					
NR activity	r^2	-0.01	-0.19	0.10	0.05	1			
	P	0.93	0.19	0.51	0.74				
GS activity	r^2	0.11	-0.02	-0.12	0.00	0.25	1		
	P	0.48	0.91	0.43	0.99	0.09			
GDHam	r^2	0.21	0.01	**0.33***	**0.32***	0.06	0.05	1	
activity	***P***	0.15	0.94	**0.02**	**0.07**	0.70	0.72		
GDHdeam activity	r^2	0.14	-0.05	0.27	**0.31***	0.14	0.01	**0.69****	1
	P	0.36	0.72	0.07	**0.04**	0.35	0.95	**0.000**	

Regarding the enzyme activities, in NH_4^+-fed plants, neither GS nor NR activity showed any correlation with none of the parameters determined (Table 1.1). GDH activities in both directions were positively correlated among each other, suggesting that when a genotype shows high GDH activity, it occurs in both aminating and deaminating directions. Both, GDHam and GDHdeam activities were positively correlated with amino acids content; but only GDHam was positively correlated with NH_4^+ content (Table 1.1). In NO_3^--fed plants, NR activity was positively correlated with NH_4^+ content and with GS activity (Table 1.2). Besides, GS activity was also correlated with GDH activity in both the aminating and deaminating directions. Interestingly, and similarly to NH_4^+-fed plants, in NO_3^--fed plants GDHam activity was also correlated with ammonium and amino acids content (Table 1.2).

In order to better understand the relationships between the ratio SB NH_4^+/ NO_3^- and the different determined parameters, we applied a multiple regression full model and Akaikes´s information best model (AIC-selected). The full model only indicated a significant selection for the ammonium content in NO_3^--fed plants (Table 1.3) and explained the 23% of the variance in SB NH_4^+/ NO_3^-. In the best model the percentage of the variance in SB NH_4^+/ NO_3^- explained increased up to 38%. From the four traits retained in the best model (ammonium content both in NH_4^+- and NO_3^--fed plants; amino acids content in NO_3^--fed plants and NR activity under NH_4^+ nutrition) NH_4^+ and amino acids accumulated in NO_3^--fed plants were significantly retained. Interestingly, NH_4^+ content explained the 53% of the best model.

We performed the same analysis for the shoot biomass under both nutritions. For NH_4^+ -fed plants the models only indicated selection for ammonium content, and both the full and best models only explained the 19% of the variance in shoot biomass (Supp. Table 1.3). For NO_3^--fed plants both the full and the best model explained 39 % of the variance in shoot biomass. The full model indicated selection for ammonium and amino acids content (Supp. Table 1.2) and both models retained significant the ammonium and amino acids content (Supp. Table 1.2).

According to the importance given both by Pearson correlations and the multiple regression models, we represented the correlation of ammonium content both with shoot biomass and with SB NH_4^+/ NO_3^- was illustrated (Fig. 1.3). As shown by Pearson analysis (Tables 1.1, 1.2) ammonium content was negatively correlated with shoot biomass both under NH_4^+- or NO_3^- -nutritions (Fig. 1.3A). Interestingly, and as suggested by the multiple regression model, only ammonium content in NO_3^--fed plants was correlated with SB NH_4^+/ NO_3^- ratio (Fig. 1.3B).

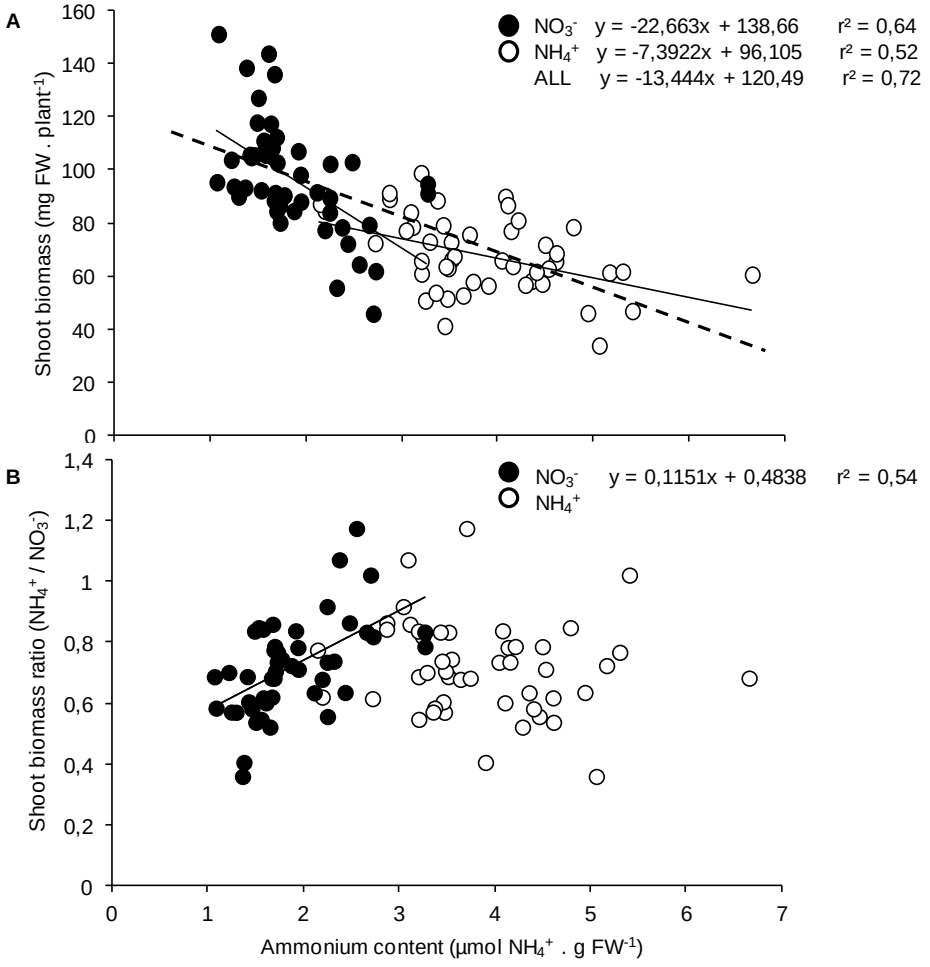


Fig.1.3. Scatter plots of ammonium content (horizontal axis) versus (A) shoot biomass and (B) the ratio between shoot biomass under NH_4^+ and NO_3^-. Linear regression and Pearson r^2 are given only if *P* was <0.05.

Table 1.3. Full and Akaikes information criterion (AIC)-selected best multiple regression models of *Arabidopsis thaliana* ammonium tolerance based in the ratio of the rosette biomass between plants grown under NH_4^+ or NO_3^- nutritions. Selection gradients (β) and standard errors (SE) are presented along with P-values. Significant selection gradients are presented in bold. A means ammonium-fed plants and N means nitrate-fed plants.

		SB NH_4^+ / NO_3^-			
		Full model		AIC-selected best model	
Trait	**Treatment**	β ± SE	P value	β ± SE	P value
NH_4^+	**A**	-0.037 ± 0.029	0.214	-0.033 ± 0.020	0.108
NH_4^+	**N**	***0.155 ± 0.045***	**0.002**	**0.106 ± 0.041**	**0.002**
NO_3^-	**A**	-0.001 ± 0.003	0.848	-	-
NO_3^-	**N**	-0.001 ± 0.002	0.651	-	-
Amino acids	**A**	0.001 ± 0.002	0.630	-	-
Amino acids	**N**	0.010 ± 0.005	0.081	**0.008 ± 0.004**	**0.040**
NR activity	**A**	-0.882 ± 1.064	0.412	-1.266 ± 0.795	0.119
NR activity	**N**	-0.031 ± 0.183	0.867	-	-
GS activity	**A**	-0.053 ± 0.172	0.760	-	-
GS activity	**N**	-0.042 ± 0.143	0.773	-	-
GDHam activity	**A**	-0.005 ± 0.009	0.594	-	-
GDHam activity	**N**	0.010 ± 0.008	0.215	-	-
GDHdeam activity	**A**	0.021 ± 0.048	0.669	-	-
GDHdeam activity	**N**	-0.004 ± .0.022	0.852	-	-
		r^2 0.23		r^2 0.38	

To understand further the behaviour of the N-assimilating enzymes determined, we represented the enzyme activities and we performed a western blotting analysis for the accessions Te-0 and Gre-0, the most sensitive and tolerant accessions to ammonium, respectively (Fig. 1.3). This analysis did not show any difference for any of the three enzymes under both nutritions. However, it was useful to ascertain that although there were not significant differences in GS activity, the GS1 isoform content was clearly accumulated upon ammonium nutrition (Fig. 1.3E). NR protein content, in agreement with NR activity, was dramatically induced in NO_3^--fed Te-0 and Gre-0 plants. Finally, both GDH α and β isoforms content increased in NH_4^+-fed plants, according to GDHam activity increase (Fig. 1.3C). In contrast, although both isoforms were induced upon ammonium nutrition, as described above GDHdeam activity increased in NO_3^--fed plants (Fig. 1.3D). However, it must be noted that under NH_4^+ nutrition the average of GDHam activity was around eight times higher than GDHdeam activity, whilst under NO_3^- nutrition GDHam was about three times higher than GDHdeam activity.

5. DISCUSSION

Plant response to N availability depends on the genotype, the N source and N fertilization level and the limiting steps in N metabolism are different at low and high N supply (Chardon et al., 2012; Xu et al., 2012). Overall, NUE is higher when N supply is limiting. In general, adaptation to low-nitrogen environments is challenging to most cultivars, because they have been selected under high-nutrient environments but plants in natural field conditions are faced to environmental changes where N availability varies and the better NUE under low N conditions is a competitive advantage (Kant et al., 2011). Moreover, reducing N fertilizers input in the soil while maintaining productivity is an unavoidable strategy to reduce agriculture impact in the environment. Thus, in this work, a low-N dose (1 mM) was used throughout the study. Moreover, since *Arabidopsis*, and *Brassicaceae* family, has been described as a very susceptible species to ammonium nutrition. Indeed, because of this high sensitivity, most of the studies about ammonium toxicity in *Arabidopsis* have been performed with a mixed nutrition and thus, long-term ammonium-based nutrition studies involve the use of a low ammonium concentration.

Approaches based in intra-specific natural variation have become an important mean to study plants adaptation. Regarding nitrate nutrition, studies based in natural variation have already been used in several species including maize (Coque and Gallais, 2007) and rice (Namai et al., 2009). *Arabidopsis* natural variation has also been studied for example under nitrate supply under limiting and ample N supply (Chardon et al., 2010; North et al., 2009) and to evaluate the capacity of different genotypes for nitrogen remobilization during seed filling (Masclaux-Daubresse and Chardon, 2011). In contrast, the studies focused in intra-specific variation of N use with ammonium as sole N-source are more scarce although there exist examples studying four maize cultivars (Schortemeyer, 1997), a collection of rice inbred lines (Obara et al., 2010) or four pea cultivars (Cruz et al., 2011). In this work, we have collected data from 47 natural accessions of *Arabidopsis* and we have measured several traits related to N-metabolism to determine the natural variation of *Arabidopsis* growth and N metabolism (ammonium and amino acids content and NR, GS, and GDH activities) under two different N sources (nitrate or ammonium). Biomass is considered as the best indicator of plant performance because it integrates every aspect of the plant metabolism, nutrient uptake to its assimilation and we considered the ratio of the shoot biomass under ammonium versus nitrate nutrition as indicator of the plant tolerance/sensitivity to ammonium as it has been previously used in other works (Ariz et al., 2011a; Cruz et al., 2006). *Arabidopsis* N1438 accession grown under

2.5 mM NH_4^+ during 21 days showed three times less biomass compared with plants grown under NO_3^- and the authors suggested ionic imbalance as a major cause of this toxicity (Helali et al., 2010). Similarly Hoffmann et al., (2007) reported a retardation of *Arabidopsis* Col-0 seedlings growth in NH_4^+- nutrition compared to NO_3^- nutrition. The present study confirms an overall sensitivity or *Arabidopsis* to ammonium, since out of the 47 genotypes, 44 had a ratio below one (23 accessions showing significant differences in shoot biomass between both nutritions). However, this study highlights great intra-specific variation of ammonium tolerance expressed as SB NH_4^+/ NO_3^-, which varied between 0.36 and 1.18. These values are in harmony with the values registered by Ariz et al., (2011) working with 7 different species and ammonium concentrations. Thus, the present study, working with a low ammonium concentration, reveals a similar degree of intra-specific *Arabidopsis* variability in ammonium tolerance than the inter-specific degree of ammonium tolerance variability. This underscores the high variability within a single species and the powerfulness of natural variation approaches for plants adaptation studies.

Ammonium accumulation affects plant growth

Ammonium "excessive" accumulation is toxic to cells. However, the concept of "excessive" is extremely variable depending on the plant species and on soil NH_4^+ concentration. In fact, ammonium toxicity is considered to be "universal" even in species labeled as "NH_4^+ specialists" (Li et al., 2014). Excess ammonium unbalances among others pH homeostasis, ionic equilibrium and primary metabolism (Britto and Kronzucker, 2002). Ammonium accumulation might come from its direct uptake but also from amino acids deamination, protein degradation and photorespiration. To prevent cells cytosol from ammonium overload plants deploy different strategies including AMT-type ammonium transporters regulation (Lanquar et al., 2009) or increasing ammonium assimilation (Setién et al., 2013). In our work, as expected, NH_4^+-fed plants accumulated more NH_4^+ and amino acids than NO_3^--fed plants and this NH_4^+ accumulation was negatively correlated with *Arabidopsis* rosette biomass (Fig. 1.2A). Interestingly, this correlation was found both plants under NH_4^+ nutrition and under NO_3^- nutrition, suggesting that ammonium accumulation negatively influences plant growth even under nitric nutrition. NH_4^+ accumulation under low-N supply might be due to a lack of proper carbohydrate supply for ammonium assimilation or by the toxicity provoked by the excess of NH_4^+ as stated above. Anyway, to our knowledge, this is the first time that a correlation between plant shoot growth under NO_3^- as sole N source and the accumulation of NH_4^+ in leaves has been reported, which evidences the extreme sensitivity of *Arabidopsis* to ammonium.

Regarding SB NH_4^+/NO_3^- ratio, out of the parameters determined only ammonium, amino acids content and GDHam from NO_3^--fed plants showed a significant correlation (Table 2). Multiple regressions full and best models retained ammonium and amino acids content, which both show a strong correlation (Sup. Fig. 1.1), as significant factors explaining the variation in SB NH_4^+/NO_3^- ratio (Table 1.3). Interestingly, NH_4^+ content of NH_4^+-fed plants did not show any significant correlation with SB NH_4^+/NO_3^- ratio. Thus, the fact that NO_3^--fed plants that accumulate more NH_4^+ present a smaller rosette biomass (Fig. 1.2A) could explain the relationship between ammonium content of NO_3^--fed plants with SB NH_4^+/NO_3^- ratio (Fig. 1.2B). Alternatively, it can be speculated that evolutionarily a plant that under NO_3^- nutrition is able to accumulate more ammonium could be genetically better adapted to an ammonium based nutrition.

NR, GS and GDH role in Arabidopsis response to ammonium

After NO_3^- and NH_4^+ uptake, N can be reduced to ammonia or directly assimilated for plant growth. As expected, NR activity was induced upon NO_3^- exposure but it was not related to differential plant growth. Indeed, NR or nitrite overexpression in tobacco, potato or *Arabidopsis* did not increase plant biomass, thus nitrate reduction does not seem to be a limiting step for plant growth (Masclaux-Daubresse et al., 2010; Pathak et al., 2008). Ammonium assimilation in normal conditions is mainly assimilated in plants via de GS/GOGAT cycle. There are two different GS isoforms. GS1 is encoded by five genes in *Arabidopsis* and functions primarily in assimilating ammonia during nitrogen remobilization. GS2 is encoded by a single gene in *Arabidopsis* and has been involved in assimilating the ammonia coming from nitrate reduction or photorespiration (Xu et al., 2012). In general, plants with higher GS activities are considered more tolerant to ammonium and Cruz et al., (2006) showed a relationship between GS activity in the dark and ammonium tolerance. In this work, we found no difference in GS activity in almost every accession between NH_4^+- and NO_3^--fed plants (Supp. Table 1.2, Fig. 1.3A) and there was no correlation between GS activity and shoot biomass in plants under both nutritions (Table 1.1, 1.2). We performed a western blot analysis in two accessions with contrasted ammonium tolerance (Te-0 and Gre-0) and in both cases there was a clear accumulation of GS1 isoform in response to ammonium nutrition. Overall, total GS activity does not seem to be crucial for ammonium tolerance in *Arabidopsis*; however GS1 could have an important role when ammonium is supplied as N-source. Moreover, out of the five genes encoding for GS1 in *Arabidopsis* GS1,2 is the most highly expressed in leaves and it is induced by ammonium (Lothier et al., 2011). Indeed, an Arabidopsis mutant lacking GS1,2 expression exhibited reduced growth under a seven-day ammonium treatment compared to

the wild type (Lothier et al., 2011). Similarly, a rice mutant in *GS1;1* gene was also more sensitive upon ammonium nutrition (Kusano et al., 2011). Thus, it remains to be determined whether GS1,2 and the rest of GS isozymes, are related to *Arabidopsis* variability under ammonium nutrition. Besides, very recently NADH-GOGAT has been suggested to play an important role in ammonium assimilation under ammonium nutrition (Konishi et al., 2014).

GDH enzyme is able to catalyze the *in vitro* reversible amination of 2-oxoglutarate to glutamate. *In vivo,* the existence of the GDH N assimilating capacity is controversial and in the last years several proofs are accumulating in favor of the major role of GDH deamination, among others, by the use of ^{15}N-NMR-labeling studies showing that there was no direct incorporation of ammonia into Glu when GS was inhibited (Labboun et al., 2009; Tercé-Laforgue et al., 2013). However, although in unstressed plants GDH ammonia assimilating capacity seems to be negligible, it appears that under stress conditions and under ammonium nutrition, GDH could be incorporating NH_4^+ (Setién et al., 2013; Skopelitis et al., 2006). In this work, we found a contrasted behavior of GDH activity. GDHam was generally induced upon NH_4^+ exposure and GDHdeam was repressed (Supp. Table 1.2, Fig. 1.3D). Moreover, both in NH_4^+ and NO_3^--fed plants ammonium and amino acids content was positively correlated with GDHam activity, and not with GDHdeam (Tables 1.1, 1.2). Thus, our data suggest that NH_4^+ accumulation might be stimulating GDH ammonium incorporating capacity rather than being a consequence of NH_4^+ release associated to GDH Glu deamination. Anyway, experiments designed to ascertain the actual GDH aminating activity in conditions of plant growth under an exclusive ammoniacal nutrition such as ^{15}N-NMR-labeling are necessary.

GDH is traditionally accepted to form seven isoenzymes composed of α and β homo- or heterodimers. In our work, after SDS-PAGE we were able to differentiate α and β subunits and both subunits were accumulated under ammonium nutrition (Fig. 1.3E). Recently, it has been shown the existence in *Arabidopsis* of a third gene encoding for a γ subunit (Fontaine et al., 2012). However, the activity of this γ isoenzyme was exclusive from root (Fontaine et al., 2012), which is in line with the hypothesis that each of the GDH subunits may have specific biological functions (Purnell et al., 2005; Tercé-Laforgue et al., 2013). An accumulation of GDH polypepetides has already been reported in several species including wheat (Setién et al., 2013), pea (Ariz et al., 2013) and tomato (Setién et al., 2014). The overall data indicate a key role for GDH in *Arabidopsis* under NH_4^+ nutrition.

Concluding remarks and future prospects

Overall, the results obtained in this work reveal that there exists high natural variation in *A.*

thaliana growth in function of the N source. This variation was partially due to the differential tissue NH_4^+ and amino acids accumulation both in NO_3^-fed and NH_4^+-fed plants. Similarly, significant natural variability was detected in NH_4^+ tolerance expressed as SB NH_4^+/NO_3^- ratio, and interestingly NH_4^+ accumulation in NO_3^-fed was the parameter showing the highest relevance. Although plant NH_4^+ assimilation capacity is known to be a key aspect for ammonium tolerance, GS and GDH enzymes does not seem to be responsible of the variability shown in *A. thaliana*. However, we clearly observed the modulation of GDH activity in function of the supplied N source which suggests an important role of this enzyme in NH_4^+ assimilation. Similarly the observed GS1 isoform higher content in NH_4^+-fed plants could be also contributing to NH_4^+ assimilation. Besides, the quality of the root system has also been suggested to partly explain the differences in nitrogen uptake and use efficiency (Loudet et al., 2005). Furthermore, several works have highlighted the importance of the root in NH_4^+ tolerance (Sasaki and Kojima, 2018; Setién et al., 2013, 2014). Thus, future works leading with root metabolism will be useful to ascertain whether N assimilation in this organ could be related to *A. thaliana* natural variability in NH_4^+ tolerance. Besides, approaches leading with bigger *A. thaliana* natural populations in combination with genome wide association studies (Atwell et al., 2010) will surely be very helpful to elucidate the genetic basis underlying the *Arabidopsis* intra-specific variability in ammonium tolerance.

6. SUPPLEMENTAL INFORMATION

Supplemental Table 1.1.A. Ammonium content. Attached file.

Supplemental Table 1.1.B. Amino- acid content. Attached file.

Supplemental Table 1.2.A. NR activity. Attached file.

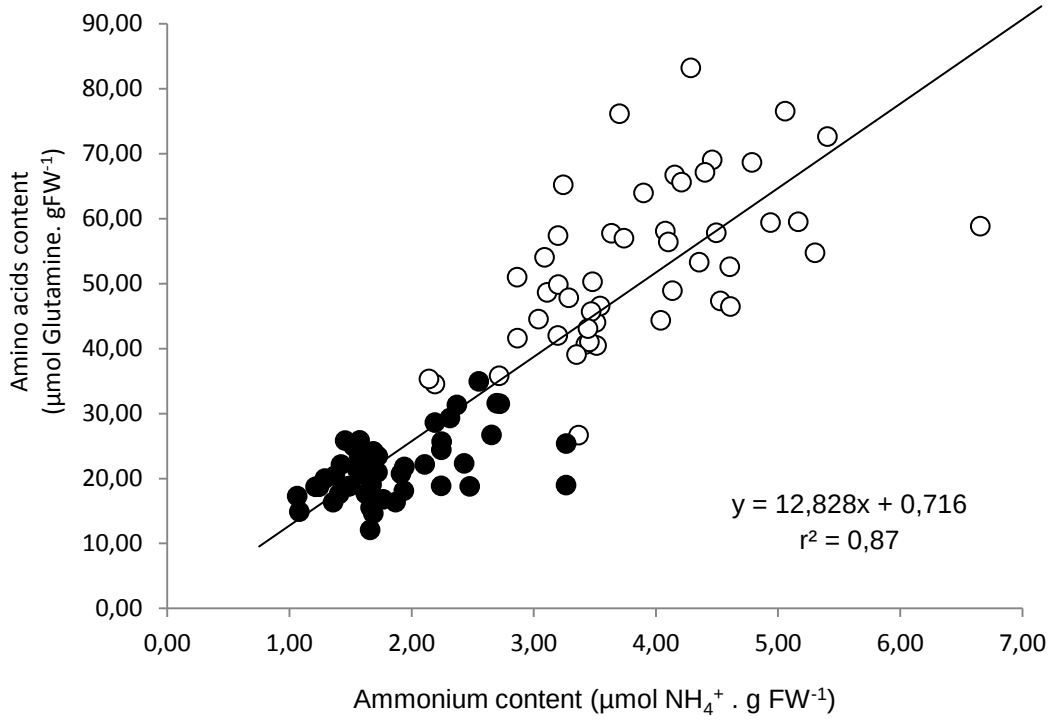
Supplemental Table 1.2.B. GS activity. Attached file.

Supplemental Table 1.2.C. GDH aminating activity. Attached file.

Supplemental Table 1.2.D. GDH deaminating activity. Attached file.

Supplemental Table 1.3. Full and Akaikes information criterion (AIC)-selected best multiple regression models of *Arabidopsis thaliana* rosette biomass under NH_4^+ or NO_3^- nutritions. Selection gradients (β) and standard errors (SE) are presented along with P-values. Significant selection gradients are presented in bold.

	Biomass (NH_4^+-fed)				**Biomass (NO_3^--fed)**			
	Full model		AIC-selected best model		Full model		AIC-selected best model	
	β ± SE	P value	β ± SE	P value	β ±SE	P value	β ± SE	P value
NH_4^+	**-6,011± 2,717**	**0,033**	**-7,382 ± 2,185**	**0,002**	**-12,27 ± 5,64**	**0,006**	**-14,307 ± 5,161**	**0,008**
Amino acids	-0,284 ± 0,183	0,128	-	-	**-1,246 ± 0,561**	**0,032**	**-1,474 ± 0,528**	**0,008**
NR activity	-94,95 ± 86,21	0,337	-	-	13,24 ± 20,19	0,316	-	-
GS activity	-3,081 ± 15,16	0,586	-	-	-22,84 ± 15,88	0,158	-	-
GDHam activity	0,792 ± 0,713	0,274	-	-	0,094 ± 0,757	0,902	-	-
GDHdeam activity	0,013 ± 4,160	0,824	-	-	4,523 ± 2,569	0,086	-	-
	r^2 0.19		r^2 0.19		r^2 0.39		r^2 0.39	



Supplemental Figure 1.1. Scatter plots of amino acids versus ammonium content of leaves of *Arabidopsis thaliana* grown under NH_4^+ and NO_3^-. Linear regression equations and person r^2 are given.

CHAPTER 2.

Genome-wide association study reveals a new locus involved in *Arabidopsis thaliana* natural variation in ammonium use efficiency

1. ABSTRACT

The use of ammonium as nitrogen source together with nitrification inhibitors has been evidenced as good alternative to reduce some of the negative effects of nitric fertilizers. However, ammonium nutrition often entails a stressful situation. Although ammonium stress is considered as universal, different species show contrasting ammonium tolerance. Moreover, variability has also been observed among genotypes of the same species. In this work, we took advantage of the natural variability of *Arabidopsis thaliana* natural populations and studied a panel of 337 natural accessions to identify genomic regions associated with ammonium tolerance following a genome wide association (GWA) approach. To do so, we took into account the potential spatial scale of adaptive variation. Overall, we observed great intraspecific variability at every geographical scale studied. At French geographical scale we identified a significant peak of association in chromosome IV related with shoot biomass under ammonium nutrition that was absent in the analysis performed with shoot biomass under nitrate nutrition. This association peak corresponds to a genomic region that encompasses a tandem array of 19 genes encoding for Cysteine-rich receptor-like kinases (CRKs). CRKs are a family of 44 members that have been suggested important in plant response to biotic and abiotic stress. To validate the potential implication of these CRK members we analyzed a complete panel of T-DNA mutant lines covering the mentioned region and in two microRNA lines targeting five of the members located in the identified region. No differences in ammonium tolerance were observed in any of these lines respect to Col-0 wild type plants. We also analyzed their gene expression in wild type plants and observed that some of these genes were induced upon ammonium nutrition, suggesting again their potential role during ammonium nutrition. Overall, the probable redundancy in CRKs function did not allow confirming the true implication of any of the CRK members in *Arabidopsis thaliana* ammonium tolerance.

2. INTRODUCTION

In agricultural soils nitrogen (N) is a major growth limiting factor for plants and thus, there is a constant need of supplying soils with N fertilizers to sustain crops yield. Importantly, a great part of the applied N fertilizers is lost to the environment and causes important environmental problems including water eutrophication and greenhouse gases emissions. Plants take up N mostly in inorganic form, essentially nitrate (NO_3^-) or ammonium (NH_3/NH_4^+). NO_3^-, being negatively charged, is prone to be leached while NH_4^+, with positive charge, tends to be

retained in soil clays (Lin et al., 2001). Besides, microbial NO_3^- denitrification and NH_4^+ nitrification processes provoke the emission of nitrogenous gases including nitrous oxide (N_2O), a greenhouse gas with a global warming potential, 265-298 times higher than CO_2 for a 100-year timescale (IPCC 2014). The use of NH_4^+ based fertilizers formulated together with nitrification inhibitors, which maintain NH_4^+ stable in the soil for long periods, have been proven efficient to reduce both NO_3^- leaching and N_2O emission; therefore, improving plants N use efficiency (Ruser and Schulz, 2015).

When the plant takes up N as NO_3^-, in order to be incorporated into amino acids it needs to be reduced to NH_4^+ in an energetically expensive manner. NH_4^+ is then assimilated via the glutamine synthetase/glutamate synthase (GS/GOGAT) cycle, which is intertwined with the respiratory metabolism. In this sense, tricarboxilic Acids (TCA) cycle and its associated anaplerotic enzymes, provide the needed carbon skeletons for NH_4^+ assimilation. In theory, the direct uptake of NH_4^+ by the root, thus bypassing the mandatory nitrate reduction, is advantageous for plant performance. However, when NH_4^+ is present in the soil, or root medium, at high concentrations plants commonly suffer the so-called ammonium stress. The symptoms associated to this stress are, among others, deficiency of competing cationic nutrients, deregulation in hormone homeostasis, changes in amino acids, organic acids and carbohydrates levels, disorders in pH regulation, or decrease in net photosynthesis. The most evident consequence of these symptoms, comparing to plants grown under nitrate nutrition, is the reduction of the plant growth and even the appearance of leaf chlorosis (Britto and Kronzucker, 2002). Although ammonium sensitivity is considered as universal, virtually affecting every species, great variation in ammonium tolerance or sensitivity has been reported between closely related species (Monselise and Kost, 1993) and also within the same species (Cruz *et al*., 2011; Di *et al*., 2018). In *Arabidopsis thaliana* several authors have reported the existence of intraspecific variability upon ammonium nutrition (Li et al., 2011b; Menz et al., 2018; Rauh et al., 2002; Sarasketa et al., 2014 corresponding to Chapter 1). Interestingly, in Chapter 1 (Sarasketa et al., 2014) studying a panel of 47 natural accession of *A. thaliana*, we observed that the accumulation of free NH_4^+ in the leaves was correlated with ammonium tolerance. In fact, the excessive accumulation of NH_4^+ in cells´ cytosol has been put forward as the most probable cause of the biomass reduction typically observed in plants subjected to ammonium stress. In fact, growth reduction would be a trade-off of the energy consumed by the cell to deal with free NH_4^+, through its assimilation into organic molecules, its efflux outside the cell or its confinement inside the vacuole (Coskun et al., 2013; Kirscht et al., 2016).

Physiological effects of ammonium stress have been greatly studied; nevertheless, the genetic basic underlying ammonium stress or sensitivities remain barely explored (Li et al., 2014). The relationship between Arabidopsis genotype and its phenotype upon exposure to high concentrations of ammonium has been studied for instance with quantitative trait loci (QTL) mapping using RIL population to identify chromosomal regions associated for instance to root development (Rauh et al., 2002; Sasaki and Kojima, 2018). In addition, the use of mutant screenings appeared efficient strategy to identify molecular actors governing Arabidopsis response to ammonium stress such as GDP-mannose pyrophosphorylase (Qin et al., 2008) or the metalloprotease AMOS1/EGY1 (Li et al., 2012). In the present work, as a mean to further dig into plants ammonium tolerance, we took advantage of naturally occurring variability to try identifying new molecular players potentially involved in the plants response to ammonium stress by means of Genome-Wide Association (GWA) mapping. The great genetic diversity of *Arabidopsis thaliana* and its relative rapid decay of linkage disequilibrium (LD; ~10 kb) together with the available genotypic data based on 250K SNP array with a marker density higher than supports a mapping resolution close to the gene level made Arabidopsis to emerge as an excellent species for GWA studies (Bergelson and Roux, 2010; Brachi et al., 2013; Horton et al., 2012). Multiple studies have revealed the power of GWA to study the phenotypic variation observed at broad geographical scale for instance in relation with plant development, plant response to pathogens and also in relation with plant nutrition and metabolism. For instance, Rosas et al., (2013) found that Pi exporter PHOSPHATE 1 was associated to Arabidopsis root system plasticity in response to nitrate and Fusari et al., (2017) using GWA studies described genetic associations with enzyme activities and primary metabolites levels.

Plant biomass is considered as the best marker of ammonium stress (Britto and Kronzucker, 2002; Cruz et al., 2006; Sarasketa et al., 2014). Thus, we performed a GWA study analyzing rosette biomass of 349 diverse Arabidopsis accessions grown under the exclusive supply of 1 mM NH_4^+ or NO_3^- as N source. The study revealed a genomic region in chromosome IV significantly associated with shoot biomass when plants were grown under ammonium nutrition that was absent when grown under nitrate nutrition. Intriguingly, this region encompasses a tandem array of twenty genes encoding Cysteine-rich receptor-like kinases (CRKs). CRKs have been suggested important in plant response to biotic and abiotic stress (Bourdais et al., 2015) and thus, we assessed the potential implication of these CRK genes through gene expression and T-DNA mutant analysis.

3. MATERIALS AND METHODS

Plant material

We analyzed a set of 337 *Arabidopsis thaliana* accessions of the French RegMap panel (Horton et al., 2012). According to the geographical scales described in Brachi et al., (2013) we distributed these 337 accession in the following sets: WORLD (n = 145), EUROPE (n = 124), FRANCE (n = 180), BURGUNDY (regional scale, n = 110), MIB and TOU (two local populations in Burgundy; n = 48 and n = 62, respectively).

Regarding *A. thaliana* mutants, all the lines studied were in Col-0 background. The mutants *crk5*, *crk6*, *crk7-1*, *crk8*, *crk10-2*, *crk11*, *crk12*, *crk13*, *crk16*, *crk17*, *crk20*, *crk21-1*, *crk22*, *crk23-2*, *crk24* were previously described (Bourdais et al., 2015). The mutants *crk14-2* (SALK_005139), *crk14-3* (SALK_144908), *crk15-2* (GK-008C04), *crk19-3* (SALK_004196), *crk19-4* (SALK_105919), were obtained from the Nottingham Arabidopsis Stock Center (NASC) and characterized (Fig. S2.1) with the primers described in Table S2.1. *crk6/7/8/10/15 -1* and *crk6/7/8/10/15 -2* ami-RNA lines were described in (Idänheimo et al., 2014).

Growth conditions and phenotyping

For accessions phenotyping, plants were sown on previously autoclaved 1:1 perlite:vermiculite mixture in trays of 104 individual cells (37 cm^3/cell) using a split-plot design arranged as a randomized complete block design. Twelve rounds of phenotyping were performed each one with one individual per accession and per treatment. To control micro-environmental variations within blocks (trays), 96 different accessions were randomly sown per tray and in the resting eight cells four individuals of Einkheim-T and Oy-0 were grown. Seeds were cold-stratified for 4 d at 4 °C and then transferred to a controlled conditions growth chamber (14 h day, 200 $\mu mol.m^{-2}.s^{-1}$ light intensity, 60% RH and 22°C day conditions and 70% RH and 18°C night conditions). The substrate mixture was initially misted with a modified MS solution (Sarasketa et al., 2014) containing 0.5 mM NH_4NO_3. Nine days after transfer, seedlings were thinned to one per port and the nitrogen nutrition treatment was initiated. To do so, trays were bottom-watered three times per week with modified MS-solution containing as exclusive nitrogen source 0.5 mM $Ca(NO_3)_2$ or 0.5 mM $(NH_4)_2SO_4$ for nitrate-fed and ammonium-fed plants, respectively. NH_4^+-fed plants were supplemented with 0.5 mM $CaSO_4$ to compensate

the Ca^{2+} supplied together with the NO_3^-. 21-days after the onset of the treatment rosette biomass was recorded.

Gene expression analysis

RNA was extracted with the Nucleospin RNA plant kit (Marcharey-Nagel), out of 25 mg of frozen Arabidopsis Col-0 leaf or root tissue. After checking RNA quality, 1 µg of RNA was retrotranscribed to cDNA (PrimeScript™ RT; Takara Bio Inc.). Gene expression was analyzed by quantitative PCR using SYBR Premix ExTaq™ (Takara Bio Inc.) in a Step One Plus Real Time PCR System (Applied Biosystems).The PCR program was 95 °C for 5 min followed by 40 cycles (94 °C for 15 s and 60 °C for 1 min) and a melting curve (40–95 °C with one fluorescence read every 0.3 °C). Relative expression was calculated using *SAND family* (At2g28390) and β-*tubulin 4* (At4g44340) as housekeeping genes. The primers used are described in Table S2.1).

Data analysis

All accessions have been previously genotyped using a custom Affymetrix SNP tiling array (AtSNPtile1), which surveys 248,544 SNPs that provides a marker density is on average 1 SNP every 500 bp (Horton et al., 2012; http://bergelson.uchicago.edu/regmap-data/). GWA analyses were performed using a mixed-model (Efficient Mixed-Model Association eXpedited; EMMAX) (Kang et al., 2010). This model efficiently controls population structure and relatedness by including an identity-by-state kinship matrix among the accessions. To minimize bias due to rare alleles, we only considered SNPS with minor allele frequency (MARF) bigger that 10%. Analyses were based on best linear unbiased predictions (BLUPs) obtained by the statistical model described in the following equation using the PROC MIXED procedure in SAS (SAS Institute Inc., Cary, NC, USA).

$$Y_{igc} = \mu_{biomass} + block_i + genotype_g + covMicro_c + \varepsilon_{igc}$$

Where “Y” in the trait studied, "µ" is the overall mean, block accounts for differences among the trays, “covMicro” is a covariate accounting for the microenvironmental effects within blocks (Einkheim-T and Oy-0 biomass were used as a covariate) and “ε” is the residual term.

For gene expression analysis and mutants phenotyping, statistical analysis of normality and homogeneity of variance were analyzed by Kolmogorov-Smirnov and Levene tests and the significance of the results was assessed using independent samples t-test.

4. RESULTS AN DISCUSSION

A. thaliana accessions display extensive natural variation in their response to growing with ammonium or nitrate as N source independently of their geographical origin

Previous research with *Arabidopsis thaliana* accessions reported the existence of natural variability in Arabidopsis NUE (Rauh et al., 2002; Chardon et al., 2010; Masclaux-Daubresse and Chardon, 2011). In Chapter 1 (Sarasketa et al., 2014) we also confirmed that variability, under both ammonium and nitrate as N source. Regarding the ability to grow with ammonium as N source Rauh et al., (2002) and Sasaki and Kojima, (2018) identified, respectively, 5 and 6 QTLs analyzing a RIL population of the cross Columbia-4 × Landsberg erecta (Ler). However, in both cases no positional cloning or candidate gene analysis was performed. Menz et al., (2018) compared the nitrogen source preference of Col-0, considered an accession with low NUE, vs. Tsu-0, considered of higher NUE. Although, Tsu-0 showed superior shoot growth stimulation by nitrate than Col-0 Menz et al., (2018) concluded that the differences the observed in NUE were independent of the reported N-source preference. Overall, the genes underlying Arabidopsis preference towards ammonium or nitrate is still far to be understood.

An increasingly number of works extensively demonstrated the convenience of GWA analysis to finely map and identify the genetic basis underlying natural variation respect to different traits (*e.g.* Atwell et al., 2010; Brachi et al., 2013; Fusari et al., 2017; Rosas et al., 2013). Therefore, to advance in the molecular players associated to ammonium tolerance, we carried out a GWA study using as quantitative trait/phenotype the biomass of the rosette of *A. thaliana* plants grown under exclusive ammonium or nitrate nutrition. We phenotyped 337 natural accessions (Table S2.2) and observed significant genetic variation in rosette biomass (Table S2.3, S2.4) with high variability under both ammonium (Fig 2.1A) and nitrate nutrition (Fig 2.1B). In agreement with the widely reported high sensitivity of *A. thaliana* towards ammonium nutrition almost every accession displayed reduced rosette biomass accumulation when grown with ammonium nutrition but with a differential degree in their sensitivity (Fig.

2.1C). Indeed, the interaction between the 337 accessions and the received treatment (nitrate vs. ammonium nutrition) was highly significant (Table S2.3).

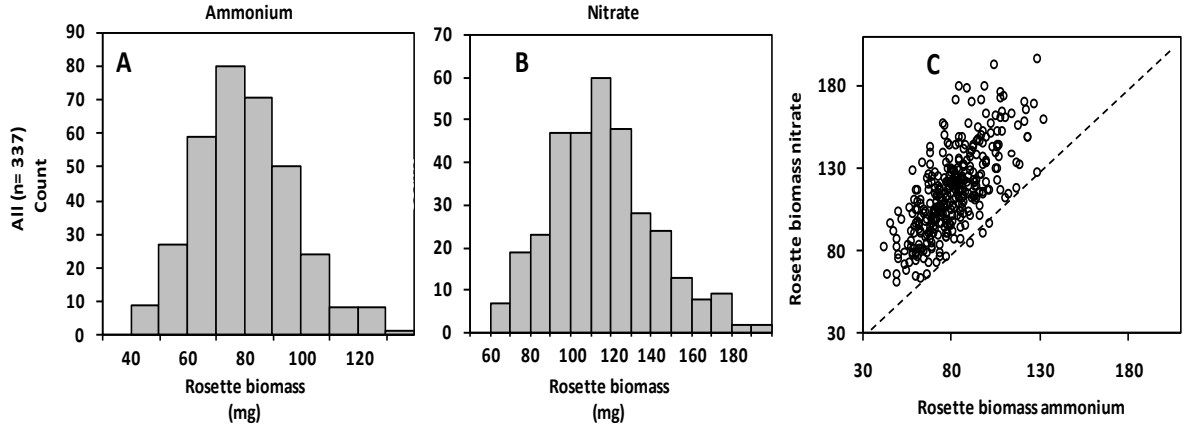


Figure 2.1. Distribution of rosette biomass based on BLUPs calculated for each one of the 337 accessions grown under ammonium (A) or nitrate nutrition (B). (C) Plot representing the relation between the two nutritions. Count refers to the number of accessions.

Multiple studies have shown the power of GWA studies to identify the genetics of phenotypic variation at broad geographical scale (Atwell et al., 2010; Chao et al., 2012). In this line, we firstly performed a GWA mapping considering all the phenotyped accessions but we did not detect any significant genetic association between rosette biomass either when plants were grown under ammonium or nitrate nutrition (Fig S2.2). Secondly and based on the assumption that populations may be adapted to local environmental conditions and thus, that a certain phenotype may depend upon the geographical scale considered because of different selection pressure acting at each scale (Brachi et al., 2013), we shorted the phenotyped accessions into different subpopulations depending on the geographical scale (World, Europe, France, Burgundy, MIB and TOU). Interestingly, we also observed strong variability in rosette biomass at every scale, from worldwide scale to local scale (Fig. 2.2 and Fig S2.3). Therefore, suggesting that natural selection related to growth with ammonium or nitrate as N source acted at spatial scale. Genetic mapping at different scale may reduce some of the limitations of GWA mapping such as detecting rare alleles, potentially acting at smaller geographical scale, which may be difficult to detect at larger and worldwide scale (Atwell et al., 2010) or reducing the confounding by population structure that may introduce false negatives and positives (Brachi et al., 2010).

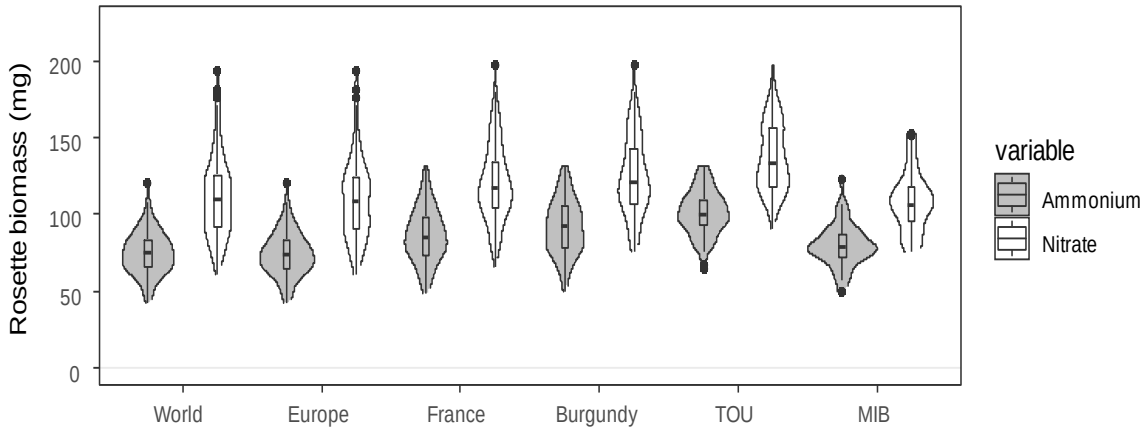


Figure 2.2. Natural variation in function of the geographical scale for rosette biomass based on BLUPs calculated for each accession grown under ammonium or nitrate nutrition. World includes 145 accessions, France 180, Burgundy 110, TOU 62 and MIB 48.

GWA mapping at different geographical scale reveals a genetic association between ammonium nutrition and members of the CRK family

Accordingly, we also performed GWA mapping at the different geographical scales (Fig.S2.4). We only detected significant associations in France population where we identified 34 significant SNPs associated with rosette biomass growth under ammonium nutrition (Table S2.5). Out of them, 18 were located within a genomic region of 22,718 bps of chromosome IV (4_12138079 to 4_12160796 bp) being the most highly associated SNP located in the position 4_12155356 (p-value $2.29.10^{-6}$). Importantly, this association peak was absent when the GWA mapping was performed in France population grown under nitrate nutrition (Fig. 2.3); therefore, suggesting the specific relationship of this genomic region with the use of ammonium as N source.

When studying ammonium tolerance the biomass ratio between plants grown under ammonium compared when grown under nitrate nutrition has been sometimes used as a proxy to somehow estimate the ammonium tolerance degree of a certain genotype (Ariz et al., 2011a; Esteban et al., 2016). We calculated the rosette biomass ratio for every genotype (Table S2.2) and used it as a phenotypical trait for GWA mapping (Fig. S2.5). This time, we identified 12 significant SNPs in France population and 6 in MIB population (Table S2.6). Importantly, 3 of the 6 SNPs identified in MIB population were also located within the region identified using rosette biomass under ammonium nutrition (4_12142581, 4_12143177, 4_12143292). This result, reinforces the potential role of a gene/genes located in this genomic region as important for *A. thaliana* growth upon ammonium nutrition. The preference for an N

source and in particular ammonium tolerance has an important contribution for ecosystems functioning and the structure of communities (Boudsoq et al., 2012; Dias et al., 2014; Van Den Berg et al., 2005). Indeed, a number of environmental attributes such as light incidence or soil N pools (organic, NH_4^+, NO_3^-) and pH (Britto and Kronzucker, 2013) have been associated with plants ammonium tolerance. For instance, at soil pH below 6 nitrogen can remain as ammonium, becoming the predominant form (Gödde and Conrad, 2000) and the local adaptation of a certain species or genotype to soil acidity has been show of relevance to determine its ammonium tolerance capacity (Van Den Berg et al., 2005; Wang et al., 2016). Thus, the finding of different genetic association in function of the geographical scale studied may be related to plan adaptation to specific edaphoclimatic conditions.

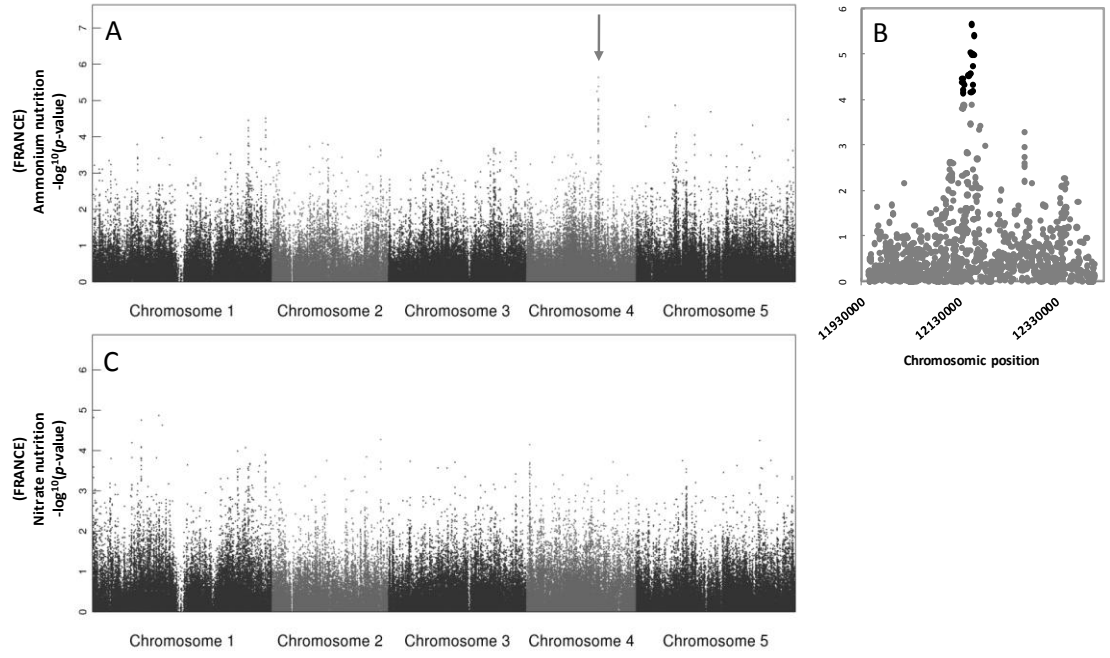


Figure 2.3.Manhattan plots of *Arabidopsis thaliana* rosette biomass based on BLUPs calculated for each accession grown under ammonium (A and B) or nitrate nutrition (C) at France geographical scale. The peak representing the genomic region associated with NH_4^+ tolerance is highlighted by a grey arrow. B plot is a detailed plot of the genomic region associated with NH_4^+ tolerance with the significant SNPs marked in black. The *x*-axis indicates the position along each chromosome. The five chromosomes are presented in a row along the *x*-axis in different degrees of grey. The *y*-axis indicates the $-\log^{10}$ p-values using the EMMAX method. Minor allele relative frequency (MARF) >10%.

The association peak identified in France population is encompassed within a genomic region that harbors a gene cluster almost exclusively formed by genes of the Cysteine-rich receptor-like kinase (CRK) family, which occupy a chromosomic region of 74.2 kb. (Chr4: 12117491 to 12191702) (Fig.2.4). Arabidopsis genome contains 44 CRK genes (plus the truncated *CRK9* and

the pseudogene *CRK35*) (Bourdais et al., 2015; Chen, 2001). Out of them, 38 are located in different clusters in Chromosome IV (Bourdais et al., 2015) being the cluster identified in this study the largest one and contains 19 CRK members, from CRK5 to CRK24 (CRK9 is truncated and excluded), in a tandem array (Fig 2.4). CRK family is a large subfamily of Arabidopsis RLKs, and as most RLKs, they possess an extracellular domain, a transmembrane domain and an intracellular Ser/Thr protein kinase domainch (Chen, 2001). In Arabidopsis, tandem duplication represents one of the major mechanisms of RLK expansion and 210 genes of Arabidopsis RLKs are found in tandem repeats representing 33.6% of all RLKs (Shiu and Bleecker, 2003). CRKs extracellular domain is characterized because it has two copies of domain of unknown function 26 (DUF26, recently renamed to stress-antifung PF01657). This domain has a conserved C-X8-C-X2-C motif with three Cys that have been predicted to potentially act as sensor of apoplastic redox modifications (Bourdais et al., 2015; Wrzaczek et al., 2010).

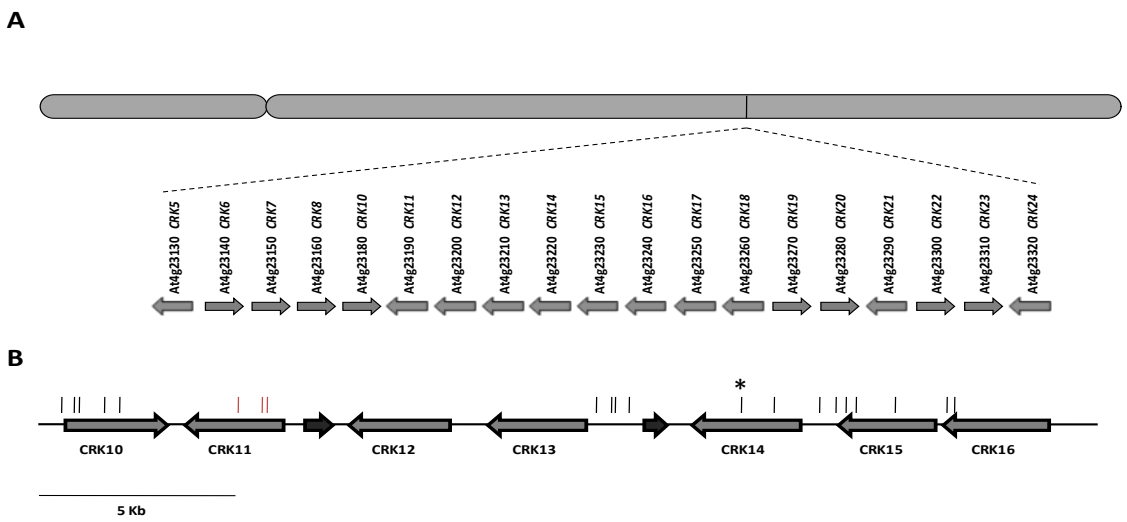


Figure 2.4. Location of *CRK* genes and the SNPs significantly related with NH_4^+ tolerance in *Arabidopsis thaliana*. A. *CRK* gene positioning in the largest arm of the chromosome IV forming a 19 genes cluster arranged in a tandem array. B. Details of the genomic region where the SNPs associated with *CRK* genes are located, from the GWA studies from rosette values under ammonium nutrition in French population (black vertical lines) and from ratio values in MIB subpopulation (red vertical lines). Top SNP is indicated by asterisk (*).

The induction of CRK family members in response to pathogen attack, salicylic acid or to abiotic stresses, including ozone and UV light has been previously reported (Bourdais et al., 2015; Chen et al., 2004; Wrzaczek et al., 2010), suggesting their potential role in stress sensing and/or signal transduction. Although, in view of their similarity, high redundancy among CRK members is expected, knocked-out mutants of individual genes have already displayed phenotypes related to different plant functions revealing that some CRKs have specific and

even are antagonistic. Notably Bourdais et al., (2015) analyzed the phenotype of a panel of T-DNA mutants, covering almost the whole CRK family, upon exposure to different stresses and observed that some individual mutants displayed altered response for instance to pathogen infection or salt stress, again, in agreement with the idea that CRKs may be key actors in stress adaptation and in extracellular stimuli perception.

In this line, and to check the hypothesis of any of these 19 genes that compose the *CRK5-CRK24* cluster could be causal for the observed variability in ammonium tolerance, we analyzed their gene expression (Fig. 2.5) and obtained individual mutants for every CRK members localized in our cluster except for CRK18 since we did not find any T-DNA mutant displaying altered expression for this gene (Bourdais et al., 2015 and Fig. S2.1). Regarding gene expression, we observed significant differences in the transcript levels of 8 out of the 19 genes located in the association peak. The expression of *CRK5*, *CRK6*, *CRK10*, *CRK17*, *CRK20*, *CRK22* and *CRK23* was enhanced under ammonium nutrition while the expression of *CRK13* was reduced (Fig. 2.5).

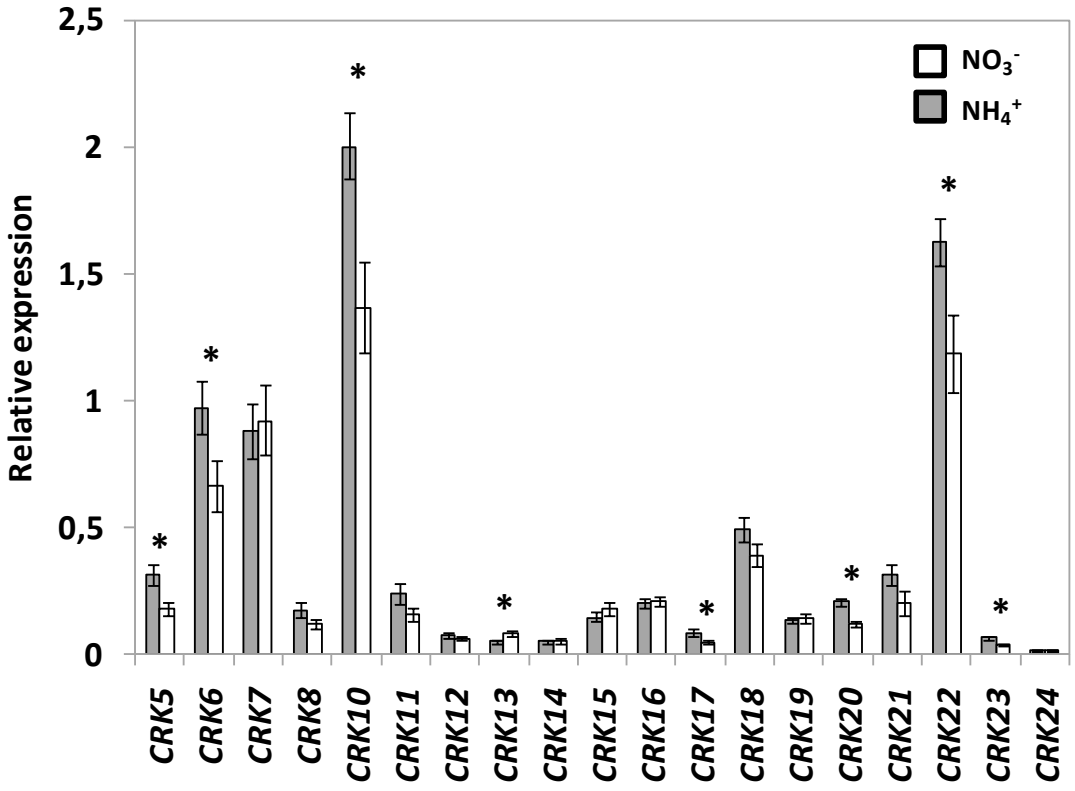


Figure 2.5. *CRK* gene expression profile of Arabidopsis Col-0 wild type grown under NH_4^+ (grey bars) or NO_3^- (white bars) nutritional regimes. Asterisks (*) indicates significant differences between both nutrition. Data were analyzed by t-test (*p<0.05*). Columns represent the average of the expression values of ± SE (*n=7-9*).

Regarding mutant analysis, we recorded the biomass of the aerial part of every mutant when grown, in the same conditions used for the accessions phenotyping, under the exclusive access to ammonium as N source and compared it to Col-0 wild type plants. Significant differences were only obtained for *crk5* mutant, whose biomass was notably reduced compared to Col-0 (Fig. 2.6). Moreover, its expression in Col-0 was increased ca. 40 % in ammonium respect to nitrate nutrition (Fig. 2.5). Previous studies already reported that *crk5* displays a developmental phenotype showing decreased growth rate respect to Col-0 (Bourdais et al., 2015; Burdiak et al., 2015). Moreover, *crk5* showed accelerated senescence in response to darkness and UV radiation (Bourdais et al., 2015; Burdiak et al., 2015). Besides, CRK5 function has been associated with stress adaptation among others in relation with abscisic acid signaling for example participating in stomatal closure control (Bourdais et al., 2015; Burdiak et al., 2015; Lu et al., 2016). To check the potential specificity of *crk5* mutant phenotype grown under ammonium nutrition, we grew *crk5* under nitrate conditions and observed a similar phenotype as under ammonium conditions, thus potentially discarding its development phenotype with plant N source preference (Fig. S2.6).

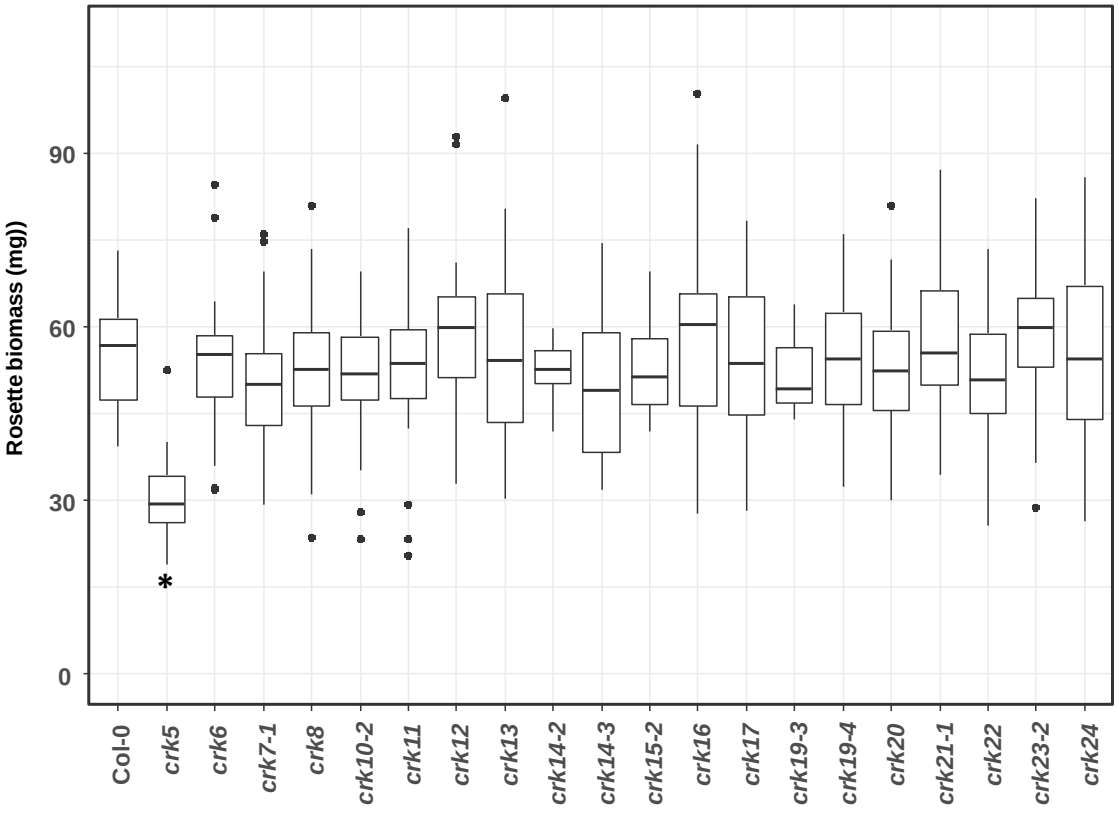


Figure 2.6. Box plots of the rosette biomass of different *crk* mutant lines grown under NH_4^+ nutritional regime. t-test (*p<0.05*) revealed differences among mutants and Col-0. Asterisks (*) indicates significant difference in each mutant rosette biomass respect to Col-0. Data were analyzed by t-test (*p<0.05*). Boxes represent the rosette biomass distribution among 11-40 individual.

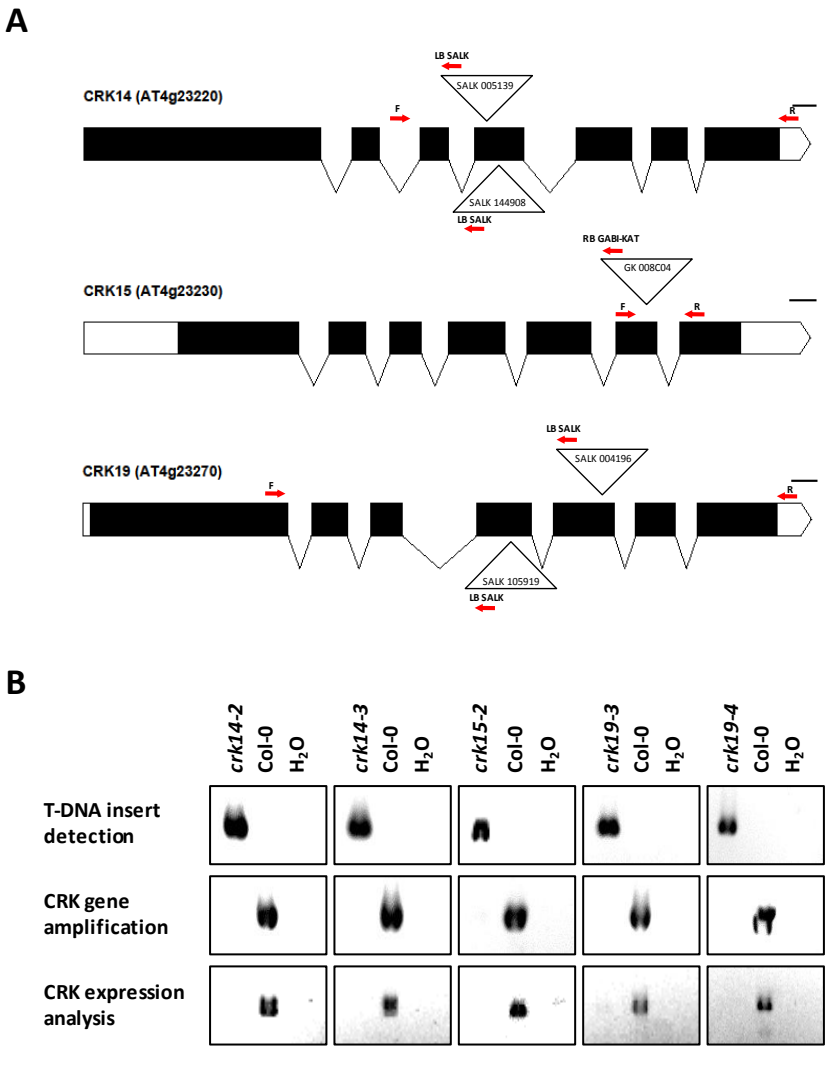
Phylogeny of the CRK family based on the whole protein sequence or in the intracellular kinase or extracellular region differentiates five groups (I-V) (Bourdais et al., 2015). Out of the 7 CRK genes induced five of them (*CRK5*, *CRK6*, *CRK10*, *CRK20* and *CRK23)* are closely related and located within the Group V as established by Bourdais et al., (2015). This could suggest potential functional redundancy between CRKs members of the Group V. Indeed, among these genes, *CRK5* has been suggested to work redundantly with its closest homologues *CRK4* and *CRK19* in ABA signaling (Lu et al., 2016). Besides, the transcript abundance of *CRK6* an *CRK10*, which show 79,6 % of protein similarity, was also higher under ammonium nutrition. CRK6 and CRK10 are within a phylogenetic subgroup with *CRK7*, *CRK8* and *CRK15* (Bourdais et al., 2015). This group of CRKs has been suggested to be involved in the coordination of Arabidopsis response to stress-induced alterations in extracellular reactive oxygen species (ROS) (Idänheimo et al., 2014). Interestingly, ammonium stress is commonly associated with ROS overproduction and thus, the stimulation of cell antioxidant machinery (Podgórska and Szal, 2015).Taking advantage of two ami-RNA lines (*crk6/7/8/10/15-1* and *crk6/7/8/10/15-2*) tackling members of this CRK group (Idänheimo et al., 2014) we tried to deal with the potential redundancy among these genes . Idänheimo et al., (2014) described a variable efficiency of these two lines to reduce CRK expression. For examples *crk6/7/8/10/15-1* displayed a reduction of *ca*. 90 % in the expression of *CRK6* and *CRK8* but an insignificant reduction in *CRK10* and *CRK15* expression (Idänheimo et al., 2014). We assessed the growth of these two lines upon ammonium nutrition but as with individual mutants, we did not observe any significant difference respect to Col-0 wild type plants.

The large number of CRKs together with their redundancy and overlapping functions among CKRs is likely a result of evolutionary pressure to guarantee plant flexibility to adapt to changing environmental conditions. Although, some works have assigned specific functions for individual CRKs (Bourdais et al., 2015; Hunter et al., 2018; Lu et al., 2016), different studies have tha this redundancy between CRK family members would be the main reason for the sometimes lack of phenotypes in single CRK loss-of- function mutants (Acharya et al., 2012; Chen et al., 2004; Yeh et al., 2015).

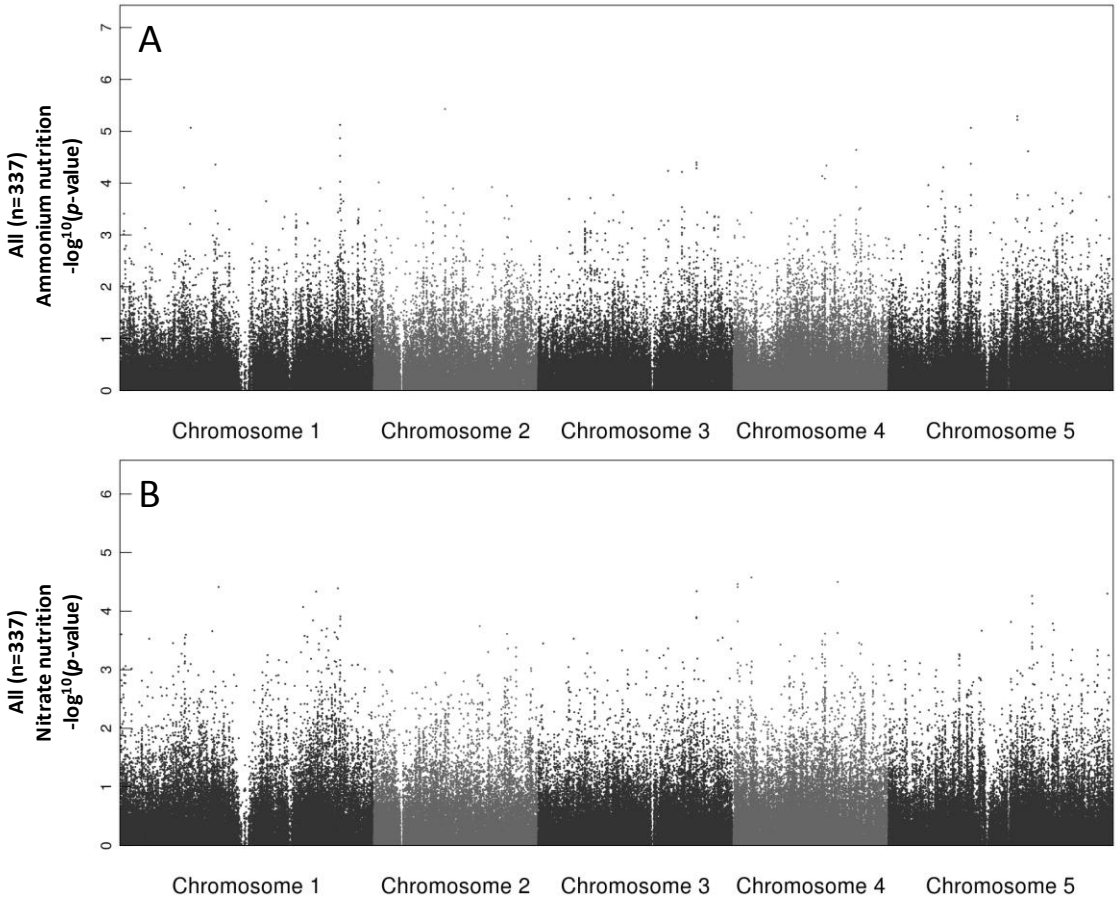
Overall, in this work we observed high natural variability in Arabidopsis ammonium tolerance at every geographical scale that together with GWA mapping suggests that the selective agents shaping individuals ammonium tolerance could be particular to every geographical scale. The association peak identified in France population together with gene expression results supports the potential involvement of CRKs in plant response to ammonium. However, further

experiments are required to confirm the true involvement of members of the CRK family in Arabidopsis. Among others, the study of over-expression lines, the generation of knocked-out mutants for several genes with the use for instance of CRISPR/Cas technology or studying CRKs gene-function in a genetic background other than Col-0 would be surely helpful to confirm and understand the involvement of CRKs in plants ammonium tolerance.

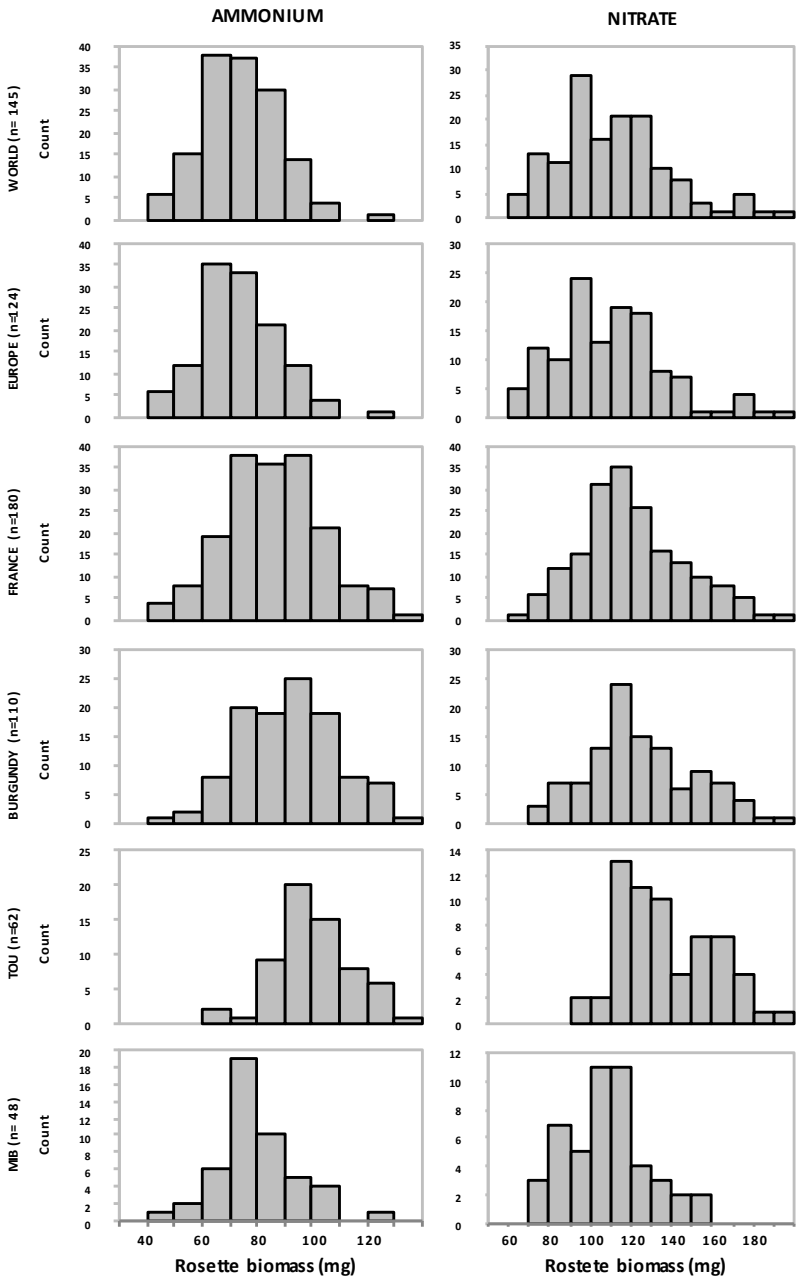
5. SUPPLEMENTARY INFORMATION



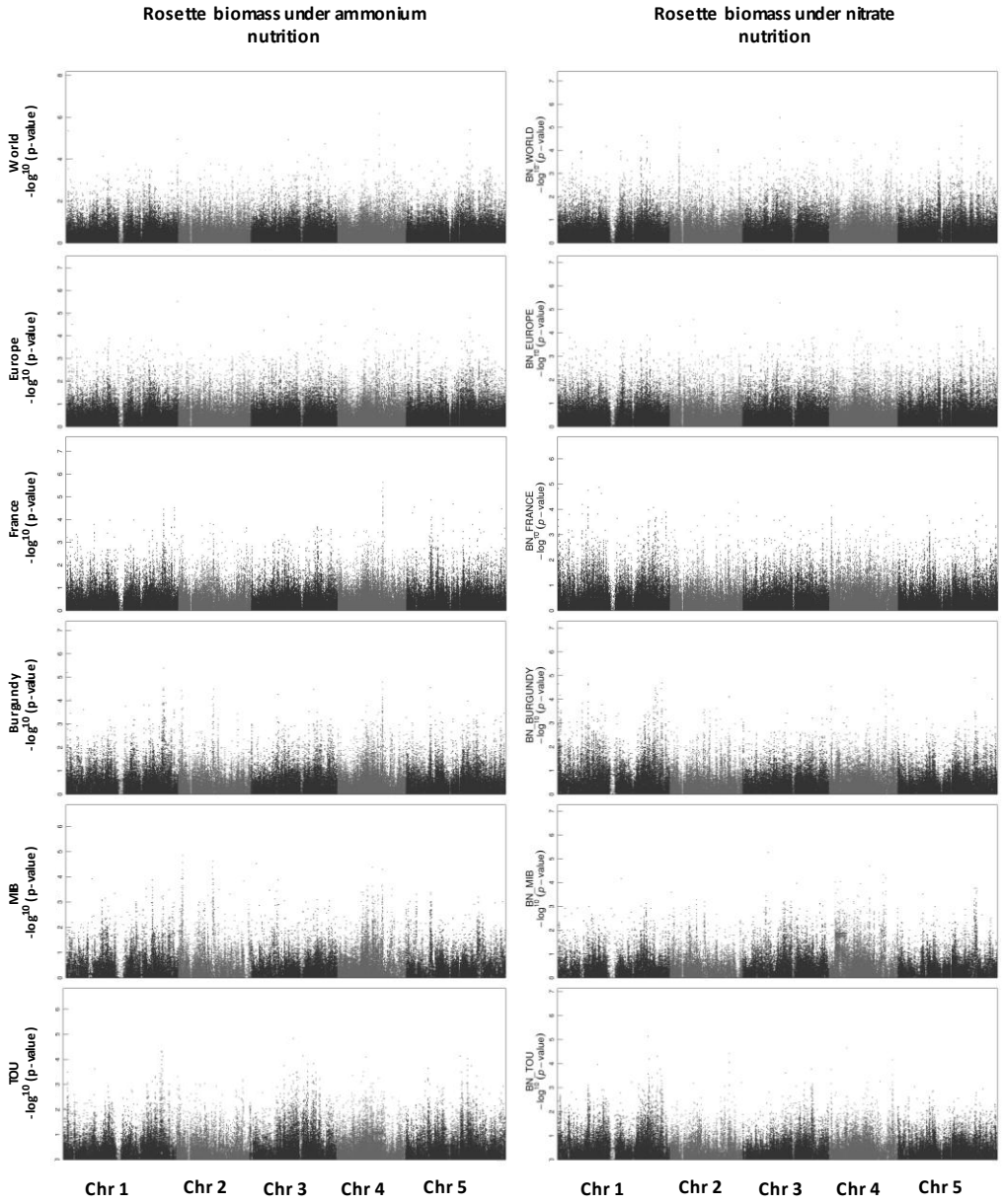
Supplementary figure 2.1. *crk14-2* (SALK_005139), *crk14-3* (SALK_144908) *crk15-2* (GK-008C04) *crk19-3* (SALK_004196) and *crk19-4* (SALK_105919) mutant lines characterization. (A) Gene structures indicating with a triangle the position of the T-DNA insertions. Arrows indicate the position and orientation of the primers used for mutant genotyping, F means forward and R reverse. (B) *crk* mutants characterization. Upper panel shows T-DNA insert detection, middle panel shows the absence of genomic amplification confirming the homozygousness of the mutant lines and low panel shows the absence of transcript detection of the corresponding genes.



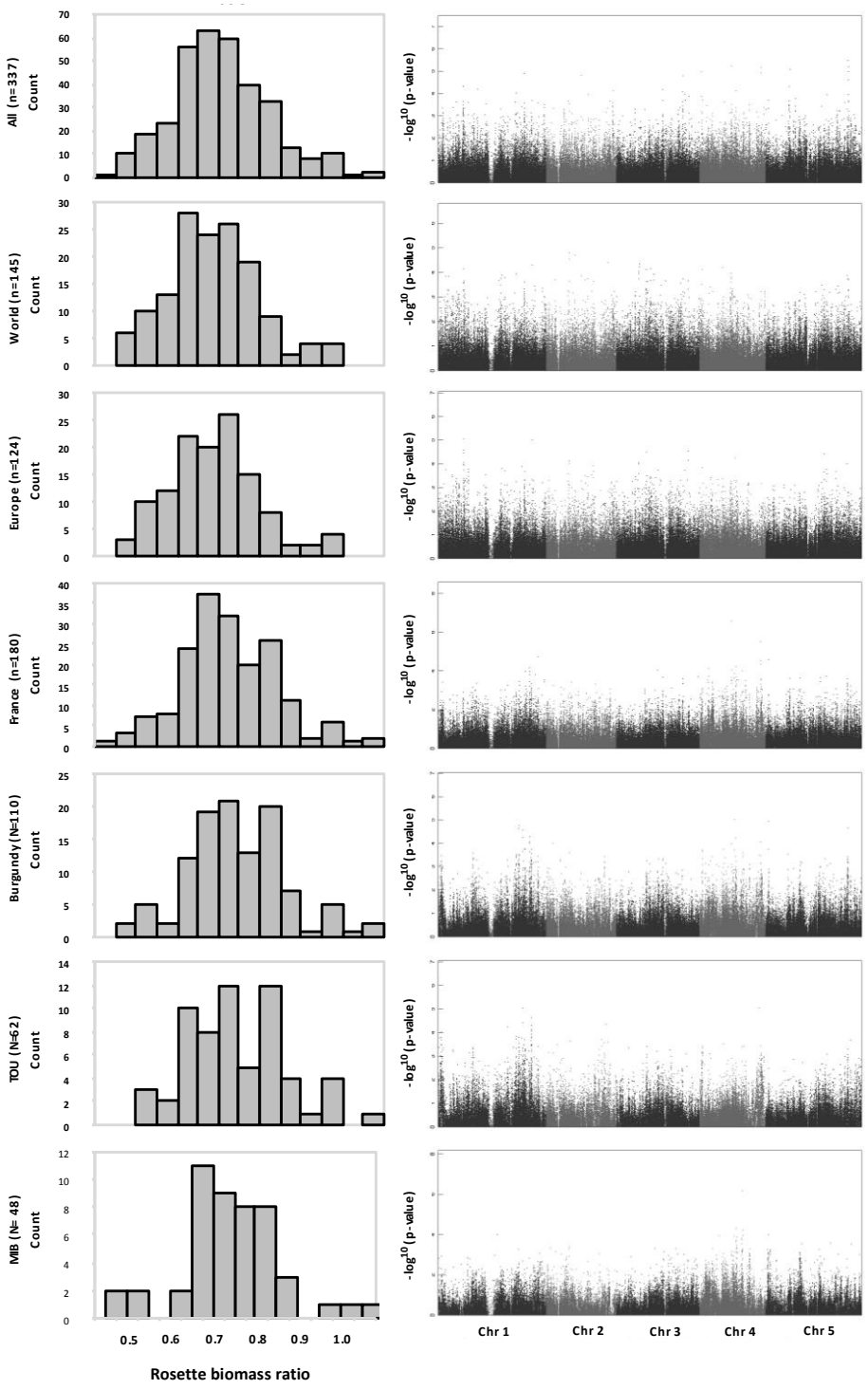
Supplementary figure 2.2. Manhattan plots of *Arabidopsis thaliana* rosette biomass based on BLUPs calculated for each accession grown under ammonium (A) or nitrate nutrition (B) in all the phenotyped natural accessions. The *x*-axis indicates the position along each chromosome. The five chromosomes are presented in a row along the *x*-axis in different degrees of grey. The *y*-axis indicates the $-\log_{10}$ p-value using the EMMAX method. Minor allele relative frequency (MARF) >10%.



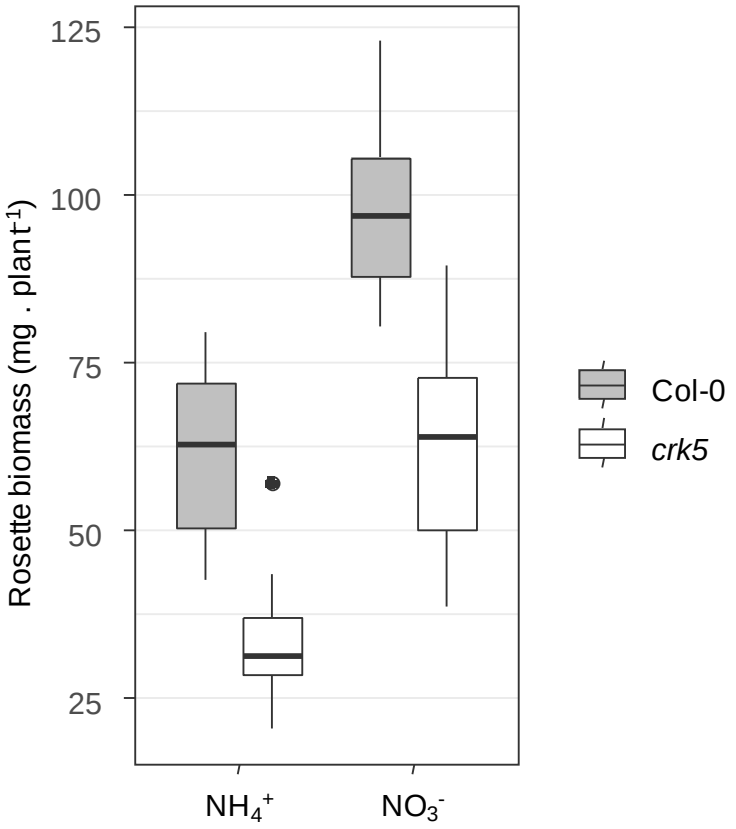
Supplementary figure 2.3. Distribution of rosette biomass based on BLUPs calculated for each one accessions at the studied geographical scales grown under ammonium (left) or nitrate (right) nutrition. Count refers to the number of accessions.



Supplementary figure 2.4. Manhattan plots of Arabidopsis rosette biomass based on BLUPs, under ammonium (left) and nitrate nutrition (right) in function of the different geographical scales. The *x*-axis indicates the position along each chromosome. The five chromosomes are presented in a row along the *x*-axis in different degrees of grey. The *y*-axis indicates the $-\log_{10}$ p-value using the EMMAX method. Minor allele relative frequency (MARF) >10%.



Supplementary figure 2.5. Natural variability of the ratio between the rosette biomass calculated from BLUPS for each accession grown under ammonium vs nitrate nutrition at different geographical scales (left histograms). Right plots are the manhattan plots generated from the GWA mapping of the ratio as trait of study. The *x*-axis of the manhattan plots indicates the position along each chromosome. The five chromosomes are presented in a row along the *x*-axis in different shades of grey. The *y*-axis indicates the $-\log_{10}$ *p*-values using the EMMAX method. MARF >10%.



Supplementary figure 2.6. Rosette biomass based on BLUPs of *crk5* mutant line (white boxes) and Col-0 ecotype (grey boxes) under ammonium or nitrate nutrition. Boxes represent the rosette biomass distribution among 19-20 individuals.

Supplementary table 2.1. Primer list

Gene name	Primer name	Sequence	Efficiency
RT-PCR			
SAND	qPCR-HK-F	AACTCTATGCAGCATTTGATCCACT	2
At2g28390	qPCR-HK-R	TGATTGCATATCTTTATCGCCATC	
Beta-Tub 4	qPCR-HK-F	GAGGGAGCCATTGACAACATCTT	2
At4g44340	qPCR-HK-R	GCGAACAGTTCACAGCTATGTTCA	
CRK5	qPCR-*CRK5*-F	TTGTTGTGCCAGTCGCTATCTCAGT	1.98
At4g23130	qPCR-*CRK5*-R	ACCCTGCAGTTGTGATGTCATCCTC	
CRK6	qPCR-*CRK6*-F	AGTAACATTCTCCTAGATGCGGATATAAA	2
At4g23140	qPCR-*CRK6*-R	TATTCTGCTTGTGTTATCTTGGG	
CRK7	qPCR-*CRK7*-F	GCTTTATCGAGACAGATGACGCACA	2
At4g23150	qPCR-*CRK7*-R	TGGCTGGACGTTTTACAGGATCTTC	
CRK8	qPCR-*CRK8*-F	GCGCACCAAACACATCGAAAGT	2
At4g23160	qPCR-*CRK8*-R	CCCCCTCAAGATGGGAGAAAGATAC	
CRK10	qPCR-*CRK10*-F	ATCTACGCGTTTTACACCGAAT	2
At4g23180	qPCR-*CRK10*-R	TTTGAATTCCCATCTTTTCCA	
CRK11	qPCR-*CRK11*-F	ATGCGATGCATGGTCAATACTCCA	2
At4g23190	qPCR-*CRK11*-R	CTCCAAAGCCTCGAAGCATAAGTGA	
CRK12	qPCR-*CRK12*-F	GCTTCTTGGGTACTGTCTGGA	2
At4g23200	qPCR-*CRK12*-R	GCCCTTGCTTTGTAGGATCA	
CRK13	qPCR-*CRK13*-F	CCGGTTTGCTCGGAAGGAAAAA	1.97
At4g23210	qPCR-*CRK13*-R	CAGGCAACCTTCCCTTGAAAACA	
CRK14	qPCR-*CRK14*-F	GTGGGAGCTTTTTCCCTTCTCTGA	2
At4g2322099	qPCR-*CRK14*-R	ATGATTGCCCAGACAATTCCTATCG	
CRK15	qPCR-*CRK15*-F	TGGAATGGACCAAACCCAGGAAAAC	1.86
At4g23230	qPCR-*CRK15*-R	TGCGCCGTCTGTCTCGTAAAAG	
CRK16	qPCR-*CRK16*-F	CCTCCCGAATATGTGGCGAACG	1.68
At4g23240	qPCR-*CRK16*-R	GCCGGGTCTACAAGTTCCAAGAATG	
CRK17	qPCR-*CRK17*-F	AGCCCGAGCTGTTATATGCGATG	1.92
At4g23250	qPCR-*CRK17*-R	CAAGAGCAACAGTGATTCCGAGGAT	
CRK18	qPCR-*CRK18*-F	GGCGTTATGTCAAGGCCAAACTG	1.72
At4g23260	qPCR-*CRK18*-R	TTGCTGCGATTTTTCCTCCTGAT	
CRK19	qPCR-*CRK19*-F	AACGAATCTAATGTTGGAACACC	2
AT4G23270	qPCR-*CRK19*-R	GAGGAGTTTCCACCTTTTCCA	
CRK20	qPCR-*CRK20*-F	ACCCTACGATGCAAGGGCAGCTAGACT	1.67
At4g23280	qPCR-*CRK20*-R	TCGTATTGGCTTCTGTTTGGTCCATC	
CRK21	qPCR-*CRK21*-F	GTCGTCGTGAGCACTGTACTGCTTG	2
At4g23290	qPCR-*CRK21*-R	AGCGAACCTGAGGACGCTGTAAGAT	
CRK22	qPCR-*CRK22*-F	TGCTGGCAACTTGGTTACCTATGC	2
At4g23300	qPCR-*CRK22*-R	TGGTTGACAACTTTGGACGGTCTT	
CRK23	qPCR-*CRK23*-F	TTGATTACATGTTTAAGCGC	2
At4g23310	qPCR-*CRK23*-R	GCAATAATTATGACAGAGGA	
CRK24	qPCR-*CRK24*-F	GGATCGGGGAACATCCAAACAGA	1.96
At4g23320	qPCR-*CRK24*-R	CGGCAACATGACCTATACTCAACCA	
Genotyping/Expression analysis			
CRK14	*crk14* genotyping-R	GAACAATGTCTCACTGGAATAGC	
At4g2322099	*crk14* genotyping-F	TGCACAATCCAAAGGCTGTC	
CRK15	*crk15* genotyping-R	ACAGACTTGGTGGTCGTTGG	
At4g23230	*crk15* genotyping-F	TGGAATGGACCAAACCCAGGAAAAC	
CRK19	*crk19* genotyping-R	GCATGAAAAATCAGCGTGAG	
AT4G23270	*crk19* genotyping-F	AACGAATCTAATGTTGGAACACC	
Salk T-DNA	*Salk lines genotyping-LB*	CCCTTTAGGGTTCCGATTTAGTGCT	
Gabi-Kat T-DNA	*GK-lines genotyping-RB*	GTGGATTGATGTGATATCTCC	
CRK14	*CRK14*-semiquantitative-R	GAACAATGTCTCACTGGAATAGC	
expression	*CRK14*-semiquantitative-F	GTGGGAGCTTTTTCCCTTCTCTGA	
CRK15	*CRK15*-semiquantitative-R	ACAGACTTGGTGGTCGTTGG	
expression	*CRK15*-semiquantitative-F	TGGAATGGACCAAACCCAGGAAAAC	
CRK19	*CRK19*-semiquantitative-R	GGTTGGACGATCTTCAGCATCTTCCT	
expression	*CRK19*-semiquantitative-F	AACGAATCTAATGTTGGAACACC	

Supplementary Table 2.2. List of the natural accessions, indicating their geographical origin, used providing the phenotypic values obtained for each accession (rosette biomass under ammonium or nitrate nutrition and the ratio between both nutrition). Available as attached .xls file**.**

Supplementary Table 2.3. Biomass variation among 337 natural accessions. Model random terms were tested with likelihood ratio tests of models with and without these effects. Random effects are in italic.

Model terms	*F* or LRT	*P*
Block	5.19	0.005
Treatment	44.17	< 0.0001
Accession	245.00	< 0.0001
Treatment*Accession	36.90	< 0.0001
Control Enkheim-T	82.56	< 0.0001
Control Oy-0	41.49	< 0.0001

Supplementary Table 2.4. Biomass variation among accessions within each treatment (ammonium and nitrate). Model random terms were tested with likelihood ratio tests of models with and without these effects. Random effects are in italic.

Model terms	AMMONIUM		NITRATE	
	***F* or LRT**	***P***	***F* or LRT**	***P***
Block	175.00	< 0.0001	161.50	< 0.0001
Accession	620.20	< 0.0001	686.60	< 0.0001
Control Enkheim-T	83.06	< 0.0001	89.69	< 0.0001
Control Oy-0	60.18	< 0.0001	37.08	< 0.0001

Supplementary table 2.5. Significant SNPs associated with rosette biomass under ammonium nutrition at France geographical scale. The SNPs corresponding to the identified CRK genomic region in Chromosome IV Are highlighted in bold

Geographical scale	Analyzed trait	SNP_id	p-value	MARF
France	Ammonium	1_26403649	5,69E-05	0,4611
France	Ammonium	1_26404546	6,79E-05	0,4111
France	Ammonium	1_26404879	3,51E-05	0,4222
France	Ammonium	1_26413906	9,93E-05	0,2833
France	Ammonium	1_29342131	8,99E-05	0,3278
France	Ammonium	1_29343282	3,08E-05	0,3111
France	Ammonium	1_29344040	3,90E-05	0,3056
France	Ammonium	1_29346750	6,51E-05	0,3056
France	Ammonium	4_11939116	5,54E-06	0,3333
France	**Ammonium**	**4_12138079**	**7,31E-05**	**0,4278**
France	**Ammonium**	**4_12138380**	**5,83E-05**	**0,4333**
France	**Ammonium**	**4_12138515**	**3,27E-05**	**0,4389**
France	**Ammonium**	**4_12139160**	**4,10E-05**	**0,4278**
France	**Ammonium**	**4_12139564**	**4,48E-05**	**0,4333**
France	**Ammonium**	**4_12151678**	**3,01E-05**	**0,2444**
France	**Ammonium**	**4_12152075**	**8,91E-06**	**0,2389**
France	**Ammonium**	**4_12152155**	**2,62E-05**	**0,2556**
France	**Ammonium**	**4_12152513**	**2,62E-05**	**0,2556**
France	**Ammonium**	**4_12155356**	**2,29E-06**	**0,2222**
France	**Ammonium**	**4_12156188**	**6,57E-05**	**0,1722**
France	**Ammonium**	**4_12157351**	**1,76E-05**	**0,1944**
France	**Ammonium**	**4_12157779**	**6,27E-05**	**0,2611**
France	**Ammonium**	**4_12158031**	**9,88E-06**	**0,2333**
France	**Ammonium**	**4_12158261**	**4,58E-05**	**0,25**
France	**Ammonium**	**4_12159123**	**1,03E-05**	**0,2389**
France	**Ammonium**	**4_12160582**	**9,88E-06**	**0,2333**
France	**Ammonium**	**4_12160796**	**4,07E-06**	**0,2389**
France	Ammonium	5_12620456	2,03E-05	0,1944
France	Ammonium	5_1607457	5,14E-05	0,2278
France	Ammonium	5_19706020	4,80E-05	0,4889
France	Ammonium	5_25739673	3,33E-05	0,3778
France	Ammonium	5_6587431	1,35E-05	0,3222
France	Ammonium	5_6809568	7,97E-05	0,4944
France	Ammonium	5_9896114	8,98E-05	0,3167

Supplementary table 2.6. Significant SNPs associated with the ratio values rosette biomass under ammonium and nitrate nutrition at France geographical scale and with the MIB subpopulation

Geographycal scale	Analyzed trait	SNP_id	p-value	MARF
France	Ratio	1_25637119	6,88E-05	0,3167
France	Ratio	1_28110050	1,94E-05	0,15
France	Ratio	3_11611598	9,11E-05	0,1056
France	Ratio	4_10242761	6,03E-05	0,4833
France	Ratio	4_17147820	2,93E-05	0,1889
France	Ratio	4_17148493	4,59E-05	0,1833
France	Ratio	4_17148748	3,18E-06	0,2333
France	Ratio	4_2495314	7,41E-05	0,4389
France	Ratio	4_9007859	2,65E-07	0,2222
France	Ratio	4_9036259	9,08E-05	0,4778
France	Ratio	5_21075331	3,96E-05	0,04444
France	Ratio	5_814779	2,61E-05	0,3111
MIB	Ratio	1_16669946	9,97E-05	0,125
MIB	Ratio	4_10288102	4,91E-05	0,3333
MIB	Ratio	4_11946439	6,63E-07	0,4583
MIB	**Ratio**	**4_12142581**	**6,11E-05**	**0,25**
MIB	**Ratio**	**4_12143177**	**6,11E-05**	**0,25**
MIB	**Ratio**	**4_12143292**	**6,11E-05**	**0,25**

CHAPTER 3.

Nitrogen sources and external medium pH modulates *Arabidopsis thaliana* ammonium tolerance and metabolic adaptation

Sarasketa A, González-Moro MB, González-Murua C and Marino D (2016)
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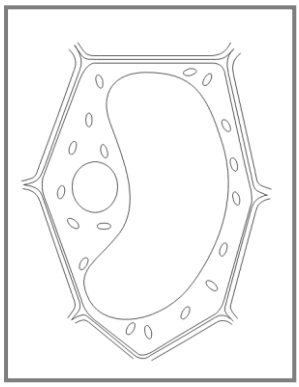


1. ABSTRACT

Ammonium nutrition often represents an important growth-limiting stress in plants. Some of the symptoms that plants present under ammonium nutrition have been associated with pH deregulation, in fact external medium pH control is known to improve plants ammonium tolerance. However, the way plant cell metabolism adjusts to these changes is not completely understood. Thus, in this work we focused on how *Arabidopsis thaliana* shoot and root respond to different nutritional regimes by varying the nitrogen source (NO_3^- and NH_4^+), concentration (2 and 10 mM) and pH of the external medium (5.7 and 6.7) to gain a deeper understanding of cell metabolic adaptation upon altering these environmental factors. The results obtained evidence changes in the response of ammonium assimilation machinery and of the anaplerotic enzymes associated to Tricarboxylic Acids (TCA) cycle in function of the plant organ, the nitrogen source and the degree of ammonium stress. A greater stress severity at pH 5.7 was related to NH_4^+ accumulation; this could not be circumvented in spite of the stimulation of glutamine synthetase, glutamate dehydrogenase and TCA cycle anaplerotic enzymes. Moreover, this study suggests specific functions for different *gln* and *gdh* isoforms based on the nutritional regime. Overall, NH_4^+ accumulation triggering ammonium stress appears to bear no relation to nitrogen assimilation impairment. Finally, this work also highlights the importance of taking external medium pH into account when optimizing ammonium nutrition.

2. INTRODUCTION

Nitrate (NO_3^-) and ammonium (NH_4^+) comprise the main inorganic forms of nitrogen (N) absorbed by plants. The preference for either NO_3^- or NH_4^+ as the N source is an important ecological determinant which affects plant diversity; while this aspect has not yet been precisely defined, it is however known to depend on environmental features such as soil properties (including pH), plant physiology and genetic background (Van Den Berg et al., 2005). Regardless of the N source, nitrogen is only incorporated into biomolecules as NH_4^+; however, paradoxically, an elevated abundance of this cation is toxic for plants, although the toxicity threshold greatly depends on ammonium concentration (Li et al., 2014). Symptoms experienced by plants when facing ammonium stress include chlorosis, ionic imbalance,

reduced photosynthetic activity, changes in NH_4^+, amino acids, organic acids and carbohydrates pool or pH deregulation.

Soil pH fluctuates widely between natural and agricultural soils and represents an important feature that may limit N availability and the plant´s capacity to absorb essential nutrients (Marschner, 2012). Moreover, pH alterations may have an influence on cellular expansion (Cosgrove, 1999) and water conductance in roots (Kamaluddin and Zwiazek, 2004), besides other phenomena. Furthermore, H^+s also play a role as second messengers in cell signaling cascades and so internal pH control is essential for the fine tuning of cells functioning (Felle, 2001). High ammonium content is common in acidic soils and the connection between ammonium stress and pH alteration has been known from a long time (Chaillou et al., 1991; Gerendas and Ratcliffe, 2000). Indeed ammonium-tolerant plants can sometimes also tolerate acidic conditions and controlling external medium pH has been shown to mitigate ammonium toxicity (Li et al., 2014).

NH_4^+ uptake is known to induce acidification of the rhizosphere/apoplast, whereas NO_3^- uptake promotes external alkalinization. Further to this it has been suggested that NH_4^+ uptake causes cytosolic alkalinization, while NO_3^- uptake provokes cytosolic acidification (Marschner, 2012). However, this potential cytosolic alteration associated to N uptake is transient because when uptake and assimilation are considered as a whole process both nitrate and ammonium nutritions tend to alkalinize cell cytosol (Britto and Kronzucker, 2005). Indeed, although intracellular pH values are sensitive to external pH values, cytosolic pH is extremely stable thanks to the fine tuning of cell metabolism. This is evidenced by several studies which observed that external pH changes over a range of pH 4-10 had very little impact on internal cytoplasmic pH (Gerendás and Ratcliffe, 2013; Hartung and Ratcliffe, 2002). A further example is the work of Hachiya et al., (2012) who, by the use of *A. thaliana* plants expressing a cytosolic fluorescent pH sensor, observed that although apoplast pH decreased upon ammonium stress, cytosolic pH remained stable. Indeed, cell metabolic adjustment in response to changes in soil medium parameters, such as N source and availability, is crucial for plants in order to maintain their growth rates and fitness.

NO_3^- is reduced to NH_4^+ by nitrate and nitrite reductases; subsequently ammonium is mainly incorporated into amino acids via the glutamine synthetase/glutamate synthase (GS/GOGAT) cycle in which both nutrition pathways (NO_3^- and NH_4^+) converge. Nevertheless, it has been proposed that under some circumstances NADH-glutamate dehydrogenase (GDH), enzyme

that catalyzes the reversible deamination of glutamate to 2-oxoglutarate (2-OG) could also collaborate in NH_4^+ assimilation (Ferraro et al., 2015). Nitrogen assimilation is intertwined with the respiratory metabolism; and it is known that the Tricarboxilic Acids (TCA) cycle and its associated anaplerotic enzymes play a central role (re)generating 2-OG for NH_4^+assimilation. Indeed, several studies have highlighted the importance of a suitable carbon supply to alleviate NH_4^+ toxicity by controlling/modulating environmental conditions in order to favor carbon assimilation (Roosta and Schjoerring, 2008; Setién et al., 2013; Vega-Mas et al., 2015).

To develop a deeper understanding of how plants respond to ammonium nutrition in relation to external medium pH changes and to study how cell carbon and nitrogen metabolism adapts to these changes, we have grown *Arabidopsis thaliana* Col-0 plants in liquid *in vitro* conditions providing ammonium or nitrate as N source at concentrations of 2 or 10 mM and external medium pHs of 5.7 or 6.7. Moreover, in this work we studied not only shoot but also root metabolism, an organ not often considered in Arabidopsis studies concerned with metabolic adaptation to ammonium stress. The overall results reveal that pH determines the degree of ammonium stress with respect to NH_4^+ tissue accumulation and how TCA anaplerotic and ammonium assimilating enzymes adjust to these changes.

3. MATERIALS AND METHODS

Experimental procedure and growth conditions

Arabidopsis thaliana Col-0 seeds were surface sterilized and sown in 0.6% agar Petri dishes with a modified MS solution (2.25 mM $CaCl_2$, 1.25 mM KH_2PO_4, 0.75 mM $MgSO_4$, 5 mM KCl, 0.085 mM Na_2EDTA, 5 µM KI, 0.1 µM $CuSO_4$, 100 µM $MnSO_4$, 100 µM H_3BO_3, 0.1 µM $CoCl_2$, 100 µM $FeSO_4$, 30 µM $ZnSO_4$ and 0.1 µM Na_2MoO_4; 20.5 mM MES, pH 5.9) containing 1 mM of NH_4NO_3 and 0.5% sucrose. Plates were kept during 4 days in the dark at 4 ºC and then moved into a controlled conditions phytotron: 14 h, 200 µmol m^{-2} s^{-1} light intensity, 60% RH and 22 ºC day conditions and 10 h, 70% RH and 18 ºC night conditions.

Nine day-old seedlings were transferred to 24-well plates containing 1 ml of nutrient solution (1 plant/well). Eight different treatments were assayed, all of them with the same MS-solution used for germination but varying pH (5.7 or 6.7), N-source (NH_4^+ or NO_3^-) and N concentration (2 and 10 mM). NH_4^+ was provided as $(NH_4)_2SO_4$ and nitrate as $Ca(NO_3)_2$. To

properly compare different N nutritions, NH_4^+-fed plants were supplemented with 1 or 5 mM $CaSO_4$ to compensate the Ca^{2+} supplied together with the NO_3^-.

Plates were kept under continuous shaking (120 rpm) during 12 days. The nutrient solution was renewed in days 5 and 9 and the evolution of the pH of the external medium monitored (Figure S1) Sterility was maintained until harvest. Six independent experiments were performed. In each experiment six 24-well plates were analyzed, each plate containing 3 plants per treatment. When harvesting, shoots and roots were dried with paper towels, biomass recorded and immediately frozen in liquid nitrogen and stored at -80ºC. Biomass was determined as the mean value of three plants grown in the same plate as one biological replicate.

Ammonium and total amino acids determination

Tissue accumulation of ammonium and total amino acid content were determined as described in Sarasketa et al., (2014) following ninhydrin method for free amino acids determination and phenol hypochlorite assay for ammonium quantification. Glutamine was used as standard for the calibration curve for total amino acid content determination.

Protein extraction and quantification

Leaves and roots were homogenized using a mortar and pestle with 20 µL of extraction buffer per mg of FW [10 mM $MgCl_2$, 1 mM EDTA,1 mM EGTA, 10 mM dithiothreitol (DTT), 0.1% Triton X-100, 10% glycerol, 0.05% bovine serum albumin (BSA), 0.5% polyvinylpolypyrrolidone (PVPP), 50 mM HEPES pH 7.5] in the presence of a cocktail of proteases inhibitors [1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM ε-aminocaproic acid, 10 µM leupeptin]. Homogenates were then centrifuged at 4,000 *g* for 30 min at 4 ºC and the supernatants recovered. Soluble protein content was determined by a dye binding protein assay (Bio-Rad Bradford Protein assay) with BSA as standard for the calibration curve.

Enzyme activities

For all the enzymes determined, except for glutamine synthetase (GS), 20 µL of protein extraction supernatants were incubated with 280 µL of reaction buffer in 96-well microplates and the evolution of NAD(P)H was spectrophotometically monitored at 340 nm during 20 min at 30 ºC. The reaction buffers were for NAD(H)-GDH: 100 mM Tris-HCl (pH 8), 1 mM $CaCl_2$, 13

mM 2-oxoglutarate, 50 mM $(NH_4)_2SO_4$ and 0.25 mM NADH; for NADH-dependent glutamate synthase (GOGAT): 100 mM Tricine-KOH (pH 8.6), 0.2 mM NADH, 10 mM DTT, 1 mM 2-oxoglutarate, 3 mM glutamine; for phosphoenolpyruvate carboxylase (PEPC): 100 mM Tricine-KOH (pH 8), 5 mM $MgCl_2$, 5 mM NaF, 0.25 mM NADH, 6.4 U/mL MDH, 2 mM $NaHCO_3$ and 3 mM phosphoenolpyruvate; for MDH: 100 mM HEPES-KOH pH (7.5), 5 mM $MgSO_4$, 0.2 mM NADH, 2 mM oxaloacetate; for NAD-dependent malic enzyme (NAD-ME): 50 mM HEPES-KOH (pH 8), 0.2 mM EDTA-Na_2, 5 mM DTT, 2 mM NAD, 5 mM malate, 25 µM NADH, 0.1 mM acetyl Coenzyme A, 4 mM $MnCl_2$; for NADP-dependent malic enzyme (NADP-ME): 100 mM Tris-HCl (pH 7), 10 mM $MgCl_2$, 0.5 mM NADP and 10 mM malate; for NADP-dependent isocitrate dehydrogenase (ICDH): 100 mM Tricine-KOH (pH 8), 0.25 mM NADP, 5 mM $MgCl_2$ and 5 mM isocitrate. In the case of malate dehydrogenase (MDH) due to its high activity supernatants were diluted 30 times.

For GS, 50 µL of sample supernatants were incubated during 30 min at 30ºC with 100 µL of reaction buffer [50 mM Tris-HCl (pH 7.6), 20 mM $MgSO_4$, 80 mM sodium glutamate, 6 mM hydroxilamine, 4 mM Na_2-EDTA and 8 mM ATP] and the reaction stopped with 150 µL of acid ferric mixture [0.5 M TCA, 2 N HCl, 120 mM $FeCl_3$]. Samples were centrifuged at 2,128 *g* for 5 min, and γ-glutamylmonohydroxamate (γ-GHM) colorimetrically quantified in the supernatants at 540 nm.

Gel blots

Sodium dodecyl sulphate/polyacrylamide gel electrophoresis (SDS/PAGE) was performed with a 12% acrylamide resolving gel and a 4.6% (w/v) stacking gel in a vertical electrophoresis cell (Mini-Protean III;Bio-Rad). Equal amounts of proteins were loaded in each well and separated at 150 V for 150 min. Proteins were then transferred into nitrocellulose membranes by wet electroblotting (Bio-Rad). Antibodies used were anti-GS (1:2,000) and anti-GDH (1:5,000) and goat anti-rabbit IgG-HRP as secondary antibody (1:20,000). Proteins were visualized using the Pierce ECL Western Blotting substrate (Thermo Scientific). Two bands were detected with anti-GS corresponding to GS1 and GS2. With anti-GDH we only detected a single band. The densitometry of the bands was calculated using the Image J software. The relative quantification was done respect to the most intense band of each blot (value “1”).

RNA Extraction and Q-RT-PCR Analysis

Leaves and roots were homogenized in liquid nitrogen and total RNA was isolated using the Nucleospin RNA plant kit (Macherey-Nagel) according to the manufacturer's recommendations. RNA quality was checked and 1 µg of RNA retrotranscribed into cDNA using the PrimeScript™ RT reagent Kit (Takara Bio Inc.). Gene expression was measured by quantitative PCR in a 15 µL reaction using the SYBR Premix ExTaq™ Takara Bio Inc.) in a Step One Plus Real Time PCR System (Applied Biosystems) and 2 µL of cDNA diluted 1:10. The primers used for *gln* and *gdh* expression are described in Lothier et al., (2011a) and Fontaine et al., (2012a), respectively. The PCR program used was as follows: polymerase activation (95 °C for 5 min), amplification and quantification cycles repeated 40 times (94 °C for 15 s, 60 °C for 1 min), and melting curve (40–95 °C with one fluorescence read every 0.3 °C). Relative expression was calculated as the ΔCp between each gene and the average of the housekeeping genes *SAND family* (At2g28390) and *β-tubulin 4* (At5g44340) with the primers described in (Marino et al., 2013). Average ΔCp was calculated from 3 samples (each one representing a pool of three plantlets).

Statistical analysis

Data were analysed using SPSS 17.0 (Chicago, IL, USA). Statistical analysis of normality and homogeneity of variance were analysed by Kolmogorov-Smirnov and Levene tests. Analysis of significant differences within each nitrogen dose included one-way ANOVA and comparison of means (Duncan´s test). Nitrogen dose effect was carried out by t-student statistical analysis. Relationships between variables were tested by Pearson's correlation. Additional details about statistical analyses and significance levels are presented in figure legends.

4. RESULTS

Arabidopsis thaliana Col-0 plants were grown for 12 days under ammonium nutrition in axenic hydroponic conditions to avoid the possibility of nitrification. Nitric nutrition was used as a reference for comparison. It should be noted that due to *Arabidopsis thaliana´s* sensitivity to ammonium nutrition most of the studies published in relation to ammonium

stress applied a mixed nutrition of nitrate with increasing concentrations of ammonium; however, as stated earlier, in this work ammonium was applied as the sole N-source.

Biomass accumulation is surely the most comprehensive parameter used to evaluate plants performance in response to a long-term stressful situation. As expected, *Arabidopsis thaliana* shoot biomass was overall reduced in ammonium-fed plants compared to equivalent nitrate-fed plants (Figure 3.1). This inhibition depended on the pH, since biomass accumulation was lower at pH 5.7, particularly at 10 mM dose. With respect to NO_3^--fed plants, at 2 mM they grew at an equal rate in a pH independent manner whereas with 10 mM supply shoots biomass only presented a significant increase under pH 5.7 (Figure 3.1A). Root biomass and length responded to the different nutritional regimes in a similar manner as the shoots; however, these parameters were lesser at 10 mM according to the reduced need of surface exploration to acquire nutrients (Figure 3.1B; Figure S3.2).

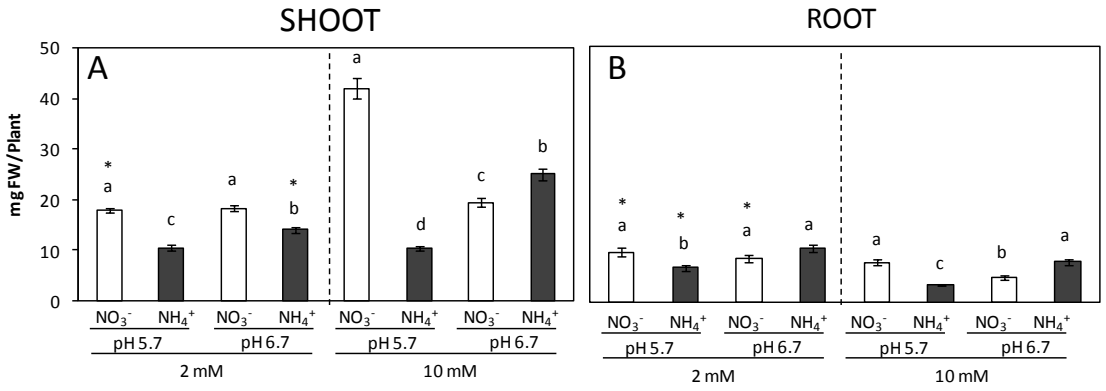


Figure 3.1. Shoot (A) and root (B) biomass of plants grown under different conditions of pH (5.7 or 6.7), N source (NO_3^- or NH_4^+) and dose (2 or 10 mM). Letters represent significant differences between treatments within the same N dose ($p < 0.05$). Asterisk represents the effect of N-dose between plants grown under the same pH and N source ($p < 0.05$). Columns represent mean ± se (n=25-35).

NH_4^+ content in both shoots and roots increased in NH_4^+-fed plants mainly under 10 mM dose. Interestingly, the degree of ammonium stress, estimated from the biomass, correlated to NH_4^+ accumulation; under pH 5.7 ammonium accumulation was around six and five times higher in shoots and roots, respectively, in comparison with plants grown at pH 6.7 (Figure 3.2A, 3.2B). An increase in the total free amino acid content is a typical response to ammonium nutrition (Britto and Kronzucker, 2002; Sarasketa et al., 2014). When the supplied nitrogen dose was 10 mM the increase in amino acid content under ammonium nutrition compared to nitrate nutrition was evident (Figure 3.2C, 3.2D). However, no differences were detected when comparing the effects of pH. Besides, amino acid content was always higher in roots compared

to shoots (Figure 3.2C, 3.2D). Protein accumulation did not show any clear trends in function of the different nutritional conditions (Figure 3.2E, 3.2F); nevertheless, protein accumulation was notably greater in some ammonium treatments compared to nitrate counterparts, such as in shoots grown at 2 mM pH 5.7 and at 10 mM pH 6.7 (Figure 3.2E). Interestingly, the roots revealed a capacity to accumulate high levels of amino acids, while leaves preferentially accumulated NH_4^+ in the form of soluble proteins (Figure 3.2).

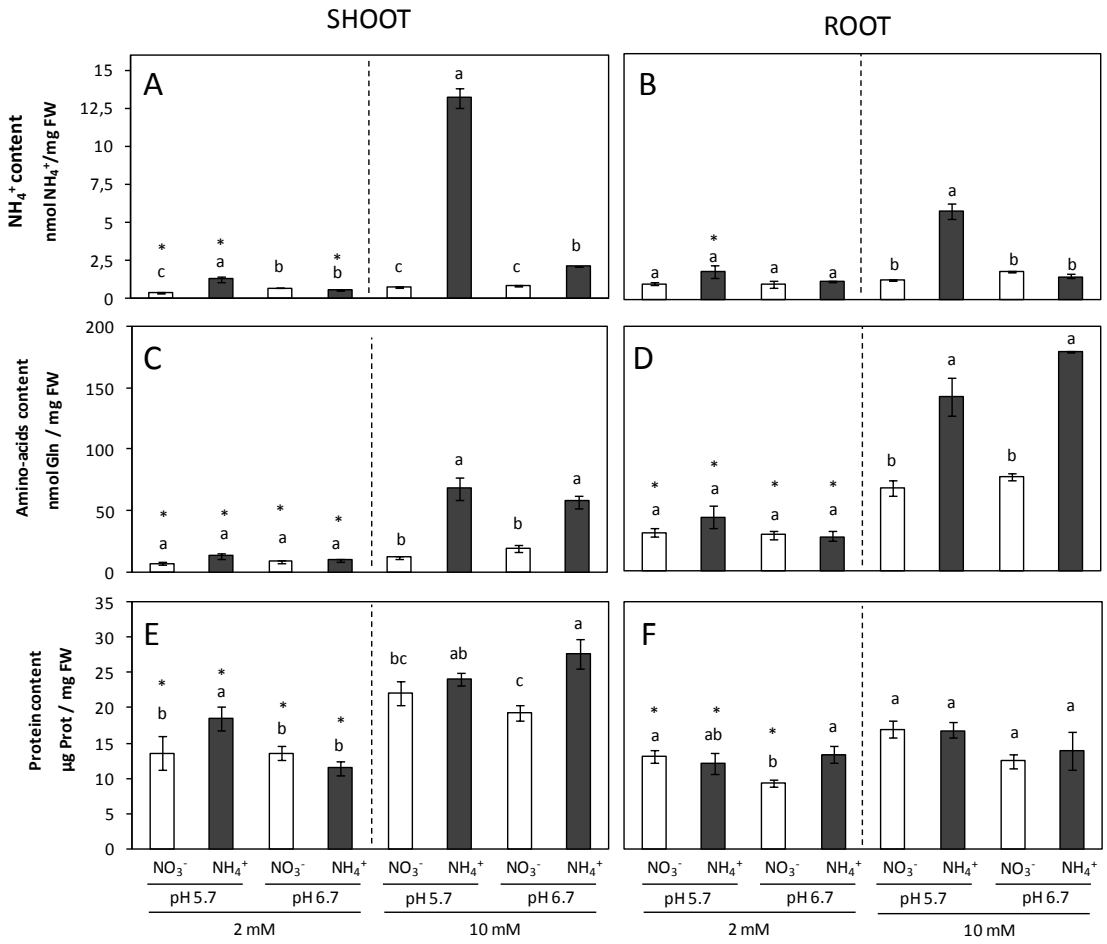


Figure 3.2. Ammonium content (A, B), amino acids content (C, D) and protein content (E, F), of of shoot (A,C,E) and root (B,D,F) of plants grown under different conditions of pH (5.7 or 6.7), N source (NO_3^- or NH_4^+) and concentration (2 or 10 mM). Letters represent significant differences between treatments within the same N dose ($p < 0.05$). Asterisk represents the effect of N-dose between plants grown under the same pH and N source ($p < 0.05$). Columns represent mean ± se (n=3). Each sample is a pool of three plants.

The GS/GOGAT cycle is the main ammonium assimilation pathway. GS activity in shoots did not vary in response to the N source, dose or pH (Figure 3.3A). Contrastingly, in roots at 10 mM dose, GS activity was greater- under pH 6.7 compared to pH 5.7, regardless of the N source (Figure 3.3B). Control over the cycle has mainly been attributed to GS but a recent paper reported that the NADH-GOGAT enzyme in roots could be involved in ammonium tolerance

(Konishi et al., 2014) and so we also included this enzyme in our study. NADH-GOGAT activity increased in both roots and shoots in response to N dose independent of the N source and pH (Figure 3.3C, 3.3D). On the other hand, GDH enzyme activity was clearly induced under ammonium nutrition compared with nitrate nutrition (Figure 3.3E, 3.3F). Overall, this induction was consistently more marked at pH 5.7 than pH 6.7. For instance, at 2 mM regime at pH 5.7 GDH activity in both shoots and roots of ammonium-fed plants was twice that of their nitrate counterpart (Figure 3.3E, 3.3F). Similarly, at 10 mM dose and pH 5.7 GDH activity in NH_4^+-fed shoots was nearly eight times higher than in those under nitrate nutrition, whereas at pH 6.7 the activity was only three times higher (Figure 3.3E). In roots, at high NH_4^+-dose GDH activity doubled that of those cultured with nitrate regardless of the external medium pH (Figure 3.3F). Interestingly, GDH activity in shoots was correlated with tissue NH_4^+ accumulation highlighting the tight relationship between these two parameters (Figure S3.3). GDH activity determined in its deaminating sense showed a similar trend as the one observed in its aminating sense (Figure S3.4).

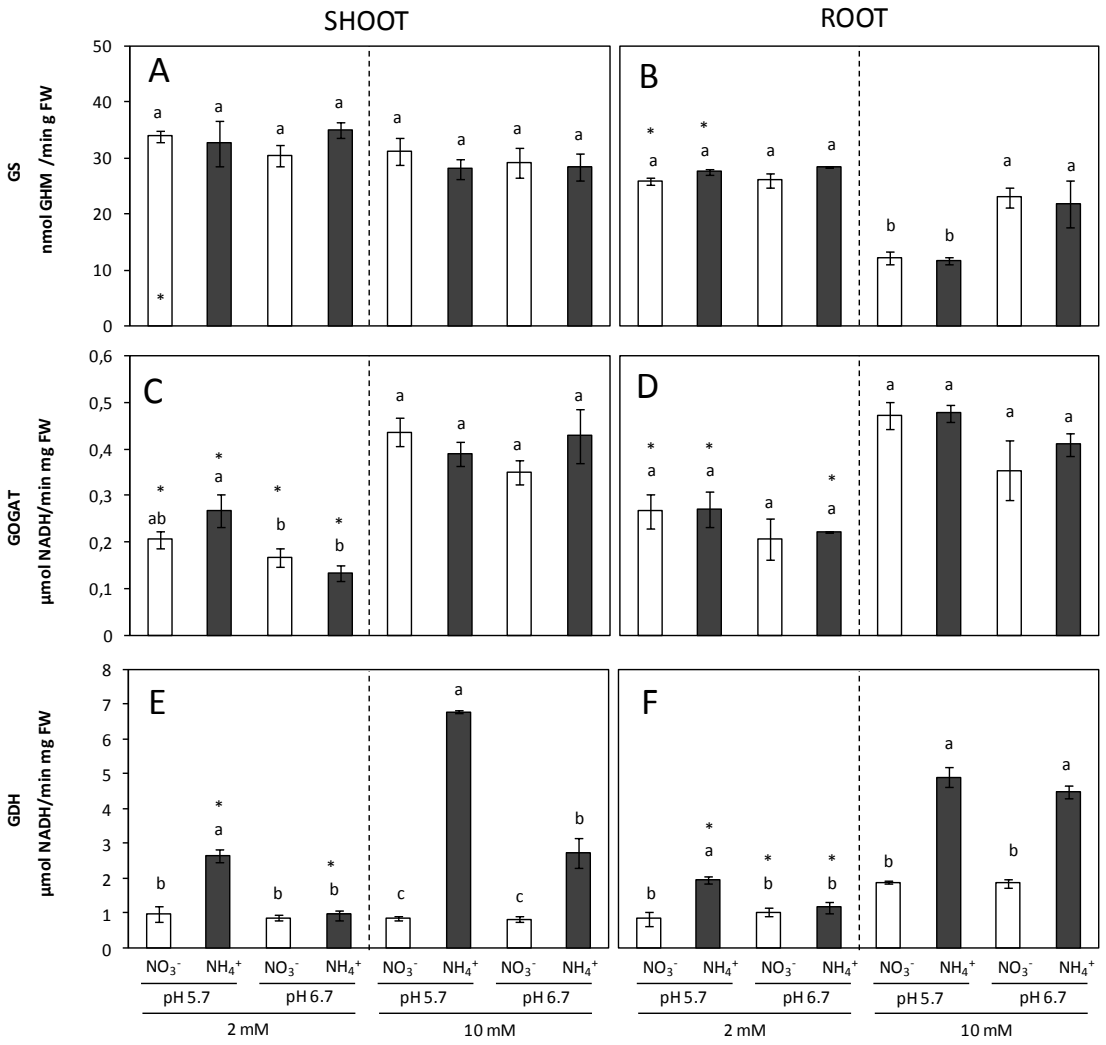


Figure 3.3. GDH (A,B), GOGAT (C,D) and GS (E,F) enzyme activities of shoot (A,C,E) and root (B,D,F) of plants grown under different conditions of pH (5.7 or 6.7), N source (NO_3^- or NH_4^+) and dose (2 or 10 mM). Letters represent significant differences between treatments within the same N dose ($p < 0.05$). Asterisk represents the effect of N-dose between plants grown under the same pH and N source ($p < 0.05$). Columns represent mean ± se (n=3-6). Each sample is a pool of three plants.

To further analyze how pH and N-source affected GS and GDH and how their activity relates to the different isoforms we determined their protein content and gene expression when grown under 10 mM nitrogen concentration. We chose this condition because at this N dose the effect of external medium pH on plants response under ammonium stress was more evident. In *A. thaliana* the cytosolic GS1 isoform is encoded by five genes (*gln1;1* to *gln1;5*). In shoots *gln1;1* and *gln1;2* were the genes that showed higher expression levels, while in roots *gln1;3* expression was also remarkable (Figure 3.4A, 3.4B). Ammonium nutrition provoked *gln1;2* induction in both shoots and roots under both pH regimes (Figure 3.4A). In addition, *gln1;3* was also induced by ammonium nutrition in shoots; however, in roots grown at pH 5.7 the expression was higher under nitrate nutrition (Figure 3.4A, 3.4B). According to *gln1* genes expression, GS1 protein content accumulated in both tissues when cultured under ammonium

nutrition particularly when the external medium pH was pH 5.7 (Figures 3.4C, 3.4D). Nitrate nutrition induced the expression of plastidic GS2 in both shoots and roots (Figure 3.4A, 3.4B). However the content of GS2, as detected by western blotting, was only higher in the shoots of NO_3^--fed plants (Figure 3.4). As expected, the most abundant GS isoform in shoots was GS2, while in roots it was GS1; however, due to the induction of *gln1* genes, GS1 and GS2 were present at similar levels in shoots of ammonium-fed plants at pH 5.7 (Figures 3.4C, 3.4D).

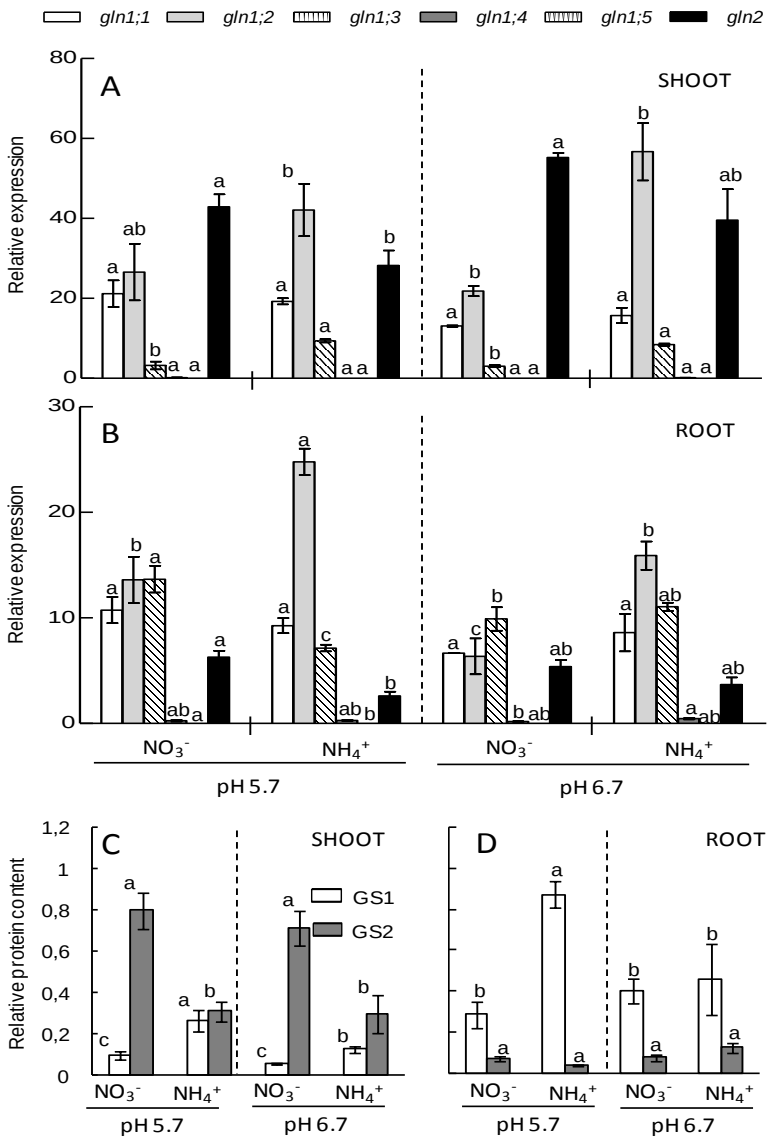


Figure 3.4. *GLN* gene expression patterns of shoot (A) and root (B) and GS enzyme content of shoot (C) and root (D) of plants grown under different conditions of pH (5.7 or 6.7), N source (NO_3^- or NH_4^+) and 10 mM N dose. Letters represent significant differences between treatments within the same N dose ($p < 0.05$). Asterisk represents the effect of N-dose between plants grown under the same pH and N source ($p < 0.05$). Columns represent mean ± se (n=3). Each sample is a pool of three plants. In Supplementary Figure 6 a zoom of gln 4;4 and gln 1;5 genes expression is available.

NAD(H)-GDH in Arabidopsis is encoded by three genes (*gdh1* to *gdh3*). A fourth gene encoding an NADP(H) dependent GDH isoform has been described but this isoform seems to be inactive (Fontaine et al., 2012). In this work, *gdh2* was the most expressed gene in both shoots and roots while ammonium nutrition further induced its expression in both tissues (Figures 3.5A, 3.5B). Again, this induction was more pronounced at pH 5.7, the conditions under which biomass was more affected by ammonium stress. Moreover, *gdh1* expression was also induced in ammonium-fed plants but only at pH 5.7. Interestingly, the *gdh3* gene, whose expression was much lower than that of *gdh1* and *gdh2*, was induced in both shoots and roots under nitrate nutrition (Figures 3.5A, 3.5B). According to the increased expression of genes, GDH protein content was also greater under ammonium nutrition in both shoots and roots, with the highest induction observed in shoots at pH 5.7 (Figures 3.5C, 3.5D).

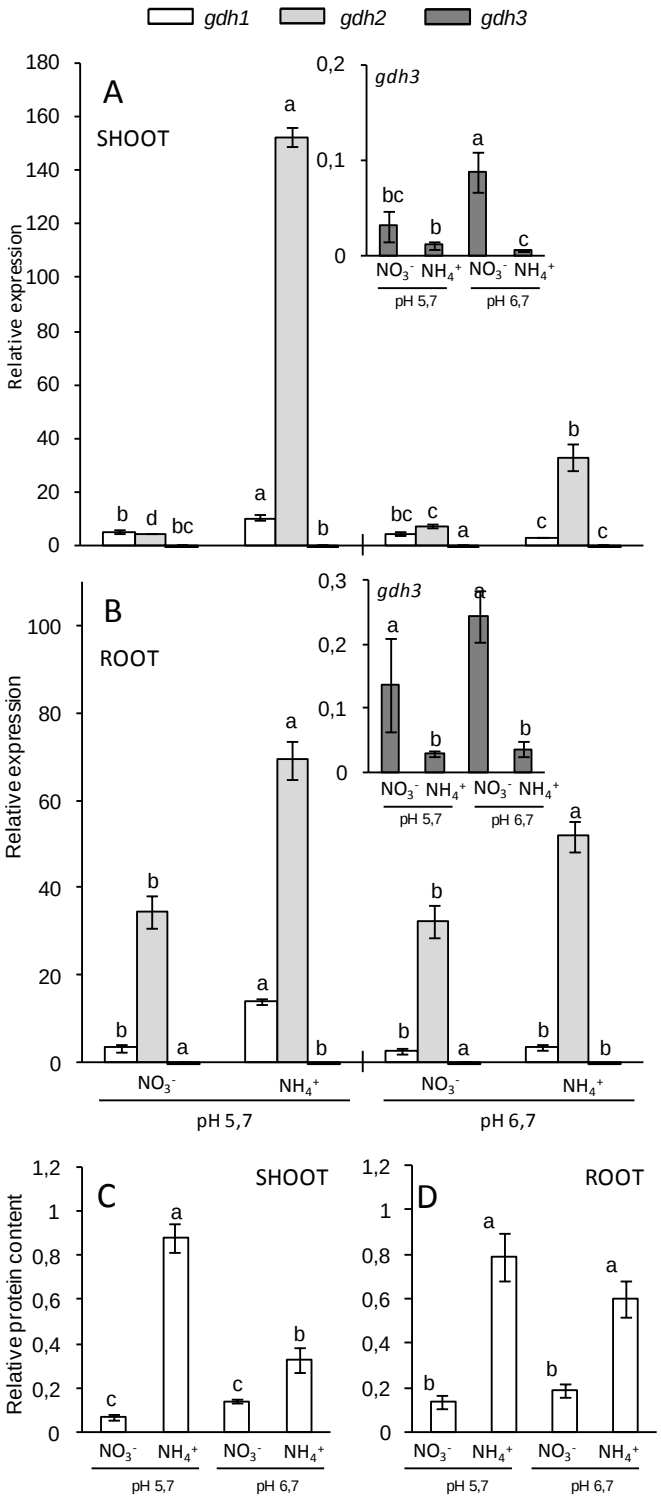


Figure 3.5. *GDH* gene expression patterns of shoot (A) and root (B) and GDH enzyme content of shoot (C) and root (D) of plants grown under different conditions of pH (5.7 or 6.7), N source (NO_3^- or NH_4^+) and 10 mM N dose. Letters represent significant differences between treatments within the same N dose ($p < 0.05$). Asterisk represents the effect of N-dose between plants grown under the same pH and N source ($p < 0.05$). Columns represent mean ± se (n=3). Each sample is a pool of three plants.

TCA cycle anaplerotic enzymes presented a differential behavior depending on the organ. ICDH, MDH, NAD-ME and NADP-ME activities were all induced in the shoots of plants grown under ammonium nutrition regardless of the external medium pH (Figure 3.6). This induction was generally greater under regimes which involved a higher degree of ammonium stress. For example, at 2 mM dose, ICDH and MDH induction was significant at pH 5.7, while at pH 6.7 it remained at the same level as that of nitrate-fed plants (Figures 3.6C, 3.6I). On the other hand, the effect of a higher ammonium concentration on the induction of TCA enzymes was evident; for example, it can be observed that NAD-ME activity remained stable at 2 mM dose while it was clearly induced under 10 mM ammonium dose (Figure 3.6E). Conversely, PEPC activity was greater in shoots of NO_3^--fed plants, particularly when cultured at 10 mM concentration (Figure 3.6A). In roots, NADP-ME and ICDH activities responded in a similar manner as to the behavior observed in shoots, with the highest level of induction reported at 10 mM NH_4^+ and a pH of 5.7 (Figures 3.6B, 3.6F). Interestingly, the behavior of NAD-ME and PEPC changed significantly when comparing shoots against roots. NAD-ME activity was induced in shoots under ammonium nutrition, while in roots it was higher under nitrate nutrition (Figures 3.6E, 3.6F); and PEPC activity, which was greater in nitrate-fed shoots, was induced in ammonium-fed roots at 10 mM dose and pH 5.7 (Figure 3.6A, 3.6B).

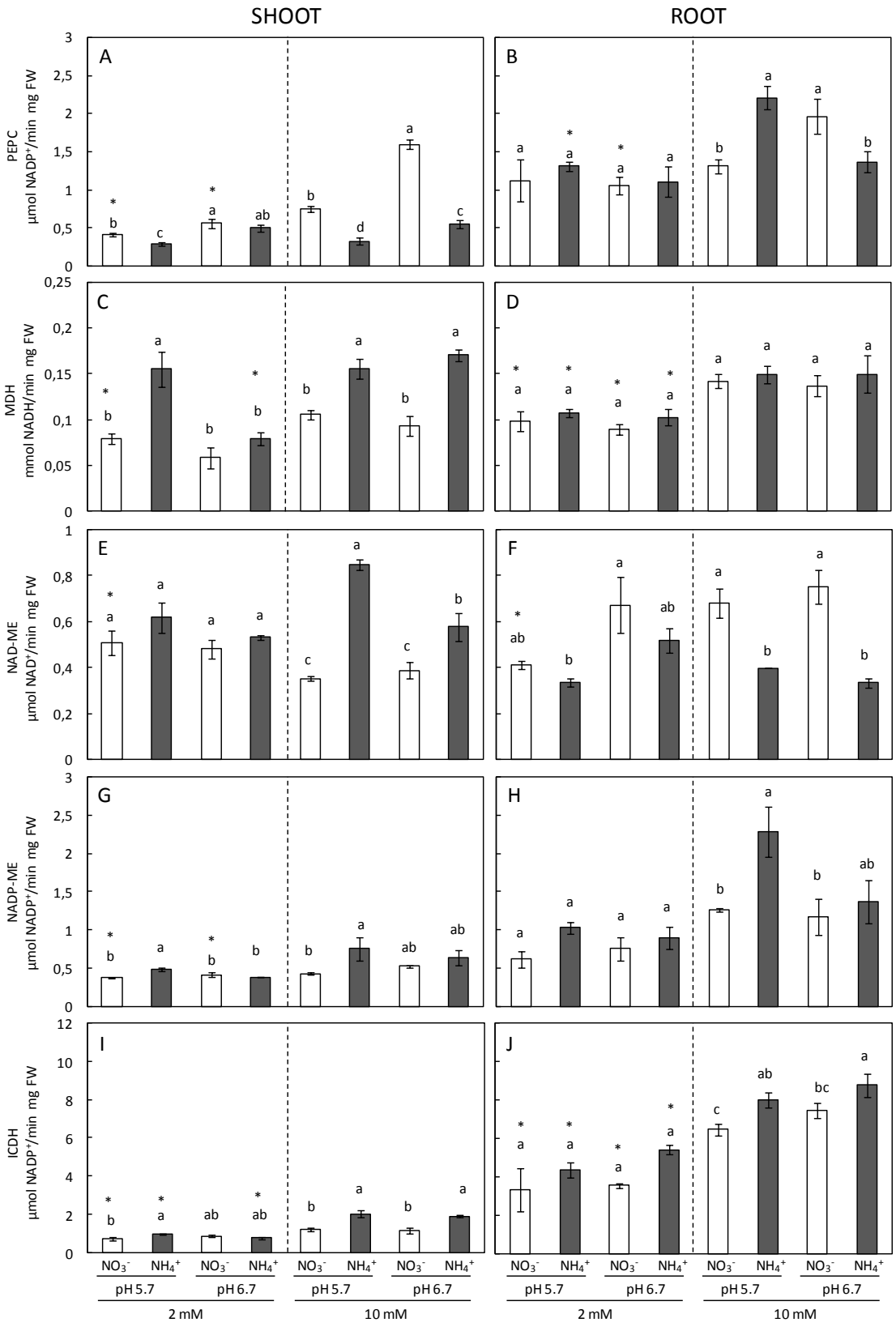


Figure 3.6. PEPC (A,B), ICDH (C,D), MDH (E,F), NAD^+-ME and $NADP^+$-ME enzyme activities of shoot (A,C,E, I) and root (B,D,F, J) of plants grown under different conditions of pH (5.7 or 6.7), N source (NO_3^- or NH_4^+) and dose (2 or 10 mM). Letters represent significant differences between treatments within the same N dose ($p < 0.05$). Asterisk represents the effect of N-dose between plants grown under the same pH and N source ($p < 0.05$). Columns represent mean ± se (n=3-6). Each sample is a pool of three plants.

5. DISCUSSION

The control of external medium pH has been shown to improve Arabidopsis tolerance to ammonium-induced stress (Britto and Kronzucker, 2002; Hachiya et al., 2012; Zheng et al., 2015). As expected, in our work we also found that the growth of ammonium-fed plants was improved when cultured at pH 6.7 compared to pH 5.7 (Figure 3.1). Indeed, the importance of pH regarding ammonium stress has also been highlighted by the use of Arabidopsis mutants with an altered ammonium tolerance. For instance, *vtc1,* mutant deficient in GDP-mannose pyrophosphorylase (Kempinski et al., 2011); *frostbite1,* mutant of mitochondrial respiratory chain Complex I (Podgórska et al., 2015); or *slah3,* mutant of the anion channel SLAC1 Homologue 3 (Zheng et al., 2015), all showed phenotypes under ammonium nutrition that were at least partially related to the control of external medium pH . Besides, Arabidopsis is a very sensitive to ammonium nutrition and ammonium stress has commonly been induced by applying increasing concentrations of ammonium concomitantly with nitrate usually in proportions of between 4:1 and 12:1 (ammonium:nitrate). The reasons behind the nitrate-dependent alleviation of ammonium stress are not yet fully understood, but it has been suggested that it could be related to pH regulation (Hachiya et al., 2012). In addition, plasma membrane H^+-ATPases activity is closely related to ion uptake compensating charge movements and the energy needed to feed H^+-ATPases has been associated with poor root growth in a species-dependent manner at acidic pH values of around 3.5 (Yan et al., 1992, 1998). Indeed, one reported response of ammonium nutrition is to increase H^+-ATPase activity (Yamashita et al., 1995; Zhu et al., 2009) and thus the energy consumed to maintain H^+-ATPase could be involved in the higher stress degree commonly observed at acidic pHs. Therefore, all these data underline the importance of studying the relation between external medium pH and ammonium nutrition. In the present study, we focused, mainly by examining NH_4^+ assimilation and TCA cycle anaplerotic enzymes, on how the metabolism of Arabidopsis plants, adapts to different degrees of ammonium stress.

At pH 6.7 the ammonium stress was alleviated and so at this pH Arabidopsis plants responded positively to an increase in external ammonium concentration whereas at pH 5.7 plants yielded a reduced biomass (Figure 3.1). At pH 6.7 nitrate-fed plants did not respond to an increase in N-dose and were therefore significantly smaller than those grown at the same concentration of nitrate but at pH 5.7 (Figure 3.1). Previous studies have also observed impaired growth of nitrate-fed plants in response to medium alkalinization across similar pH ranges, for example, in maize (Schortemeyer et al., 1993), *Typha latifolia* (Brix et al., 2002) or

tomato (Zhao and Ling, 2007). Thus, it seems that the availability of essential nutrients could be responsible for this pH-dependent growth effect in plants fed with 10 mM nitrate. In our study, we did not observe any significant alterations in the metabolic parameters analyzed that could explain such growth differences (Figures 3.2-3.5). Future work will help to elucidate the negative effect that certain plant species experience in relation to nitrate nutrition and external medium alkalinization.

In several works, acidic pHs have been shown to induce ammonium uptake or accumulation in tissues (Chaillou et al., 1991; Coskun et al., 2013; Ortiz-Ramirez et al., 2011; Søgaard et al., 2009). In our study, the degree of ammonium stress was correlated with NH_4^+ tissue accumulation since both roots and shoots accumulated much more ammonium at pH 5.7 compared to pH 6.7 (Figure 3.2). And so NH_4^+ accumulation could be due to ammonium transport rather than a result of impairing the metabolic pathways involved in its assimilation, as the contents of both amino acids and proteins were at similar levels in ammonium-fed plants regardless of the external medium pH (Figure 3.2).

It is known that ammonium assimilation is mainly driven by the GS/GOGAT cycle. Concerning GDH, there is still controversy about its role in plants but it is now accepted that GDH activity *in vivo* is primarily directed towards 2-oxoglutarate production (Fontaine et al., 2012; Labboun et al., 2009). However, under some circumstances it seems that GDH might also be collaborating in the direct amination of 2-OG to form glutamate, such as during fruit ripening (Ferraro et al., 2015) or ammonium stress (Skopelitis et al., 2006). It is apparent that an increased capacity to assimilate ammonium would help to prevent NH_4^+ content rising to toxic levels while simultaneously increasing plant growth potential. Indeed, GS1 overexpression in tobacco plants accumulated less NH_4^+ than wild-type plants under nitrate-based nutrition (Oliveira et al., 2002). Similarly, it has been proposed that plants which are capable of maintaining high levels of GS activity in the dark present an enhanced tolerance to ammonium stress (Cruz et al., 2006). In the present work, neither GS nor NADH-GOGAT activities presented any response to a different N-source. Contrastingly, GDH clearly showed an overall induction under ammonium nutrition. This induction was greatest in shoots at pH 5.7, where ammonium accumulation was higher; suggesting that GDH induction in the shoot depends on stress severity (Figure 3.3).

Different functions have been proposed for different GS and GDH isoforms (Guan et al., 2015; Lothier et al., 2011; Marchi et al., 2013). The main function of GS2 has been associated to the

reassimilation of photorespiratory ammonium in photosynthetic tissues (Pérez-Delgado et al., 2015) and primary nitrogen assimilation in green tissues (Xu et al., 2012). Considering that NO_2^- is reduced to NH_4^+ in the chloroplasts, we expected to encounter higher GS2 levels in nitrate-fed plants compared to ammonium-fed plants (Figure 3.4) was as this has previously been observed in other plants including Arabidopsis (Sarasketa et al., 2014) or maize (Prinsi and Espen, 2015). GS1 content was only higher under the more toxic ammonium treatment in relation with increased *gln1;2* and *gln1;3* gene expression. Interestingly, Arabidopsis *gln1;2* mutants grown *in vitro* were about 20% smaller than wild-type plants grown under ammonium nutrition (Lothier et al., 2011). Similarly, rice mutants lacking OsGS1:1 experienced growth retardation under ammonium nutrition (Kusano et al., 2011). Overall, GS1 is essential under ammonium nutrition and different isoforms present non-overlapping functions. However, GS activity is subjected to tight post-transcriptional and post-translational regulation by, among others, phosphorylation (Prinsi and Espen, 2015) or nitration (Melo et al., 2011); and these regulatory mechanisms could explain the observation that GS activity did not vary in function of the N-source (Figure 3.2), contrary to its genes expression levels or protein content (Figure 3.4).

Ammonium has been known to induce GDH activity for decades, while heavier hexamers (enriched in α subunits) are often induced by ammonium (Cammaerts and Jacobs, 1985; Skopelitis et al., 2006). In our work, *gdh1* and *gdh2* were induced in response to ammonium nutrition, but interestingly *gdh1* was only induced at pH 5.7, and *gdh2* induction in shoots was greater at pH 5.7 than pH 6.7. This suggests that that the observed increase in GDH protein content and activity was due to the induction of both genes (Figures 3.3 and 3.5). Interestingly, expression of the until recently unstudied *gdh3* gene was higher in nitrate-fed plants, thus revealing a differential behavior for this isoform. Whether GDH3 could be playing a specific role under nitrate nutrition is still unknown. However, Marchi et al., (2013) proposed a role for GDH3 in nutrient remobilization during the Arabidopsis reproductive phase; furthermore, they showed *gdh3* induction by cytokinins, hormones known to regulate plant growth in response to nitrate (Krouk et al., 2011). Thus, our data suggest specific functions for the different GDH isoforms depending on both the type of N source and the degree of ammonium stress. Future research is still required to decipher the importance of GDH with regards to ammonium nutrition and to reveal the functional specificity of each isoform in plant metabolism. Overall, GS1, *gdh1* (encoding GDHβ) and *gdh2* (encoding GDHα) seem to be responding to the level of ammonium stress, which occurs to a higher extent at pH 5.7, and collectively suggest an important role of increased nitrogen assimilation capacity during ammonium nutrition.

However, the content of total protein and amino acids did not accumulate at pH 5.7 compared to pH 6.7 suggesting that induction of N assimilation enzymes was not sufficient to scavenge the excess of ammonium into biomolecules. On the other hand, ammonium-fed plants may suffer from carbon limitation for NH_4^+ assimilation (Ariz et al., 2011a; Setién et al., 2013; Vega-Mas et al., 2015) and it has been shown that the main function of GDH activity is to provide 2-oxoglutarate when C becomes limiting (Fontaine et al., 2012). In the present work, when correlating GDH activity with NH_4^+ accumulation (Supplementary Figure S3.3), we found a negative correlation in shoots for nitrate nutrition ($r = -0.994$, $p= 0.006$), while under ammonium nutrition this correlation was positive ($r = 0.969$, $p= 0.031$), which could be a sign that the role of GDH induction is directed towards 2-OG production to meet GS/GOGAT demand. However, the induction of GDH in response to NH_4^+ accumulation to collaborate in its assimilation cannot be discarded and future work using isotopic labeling together with mutant analysis under ammonium stress will surely help to shed more light on GDH function.

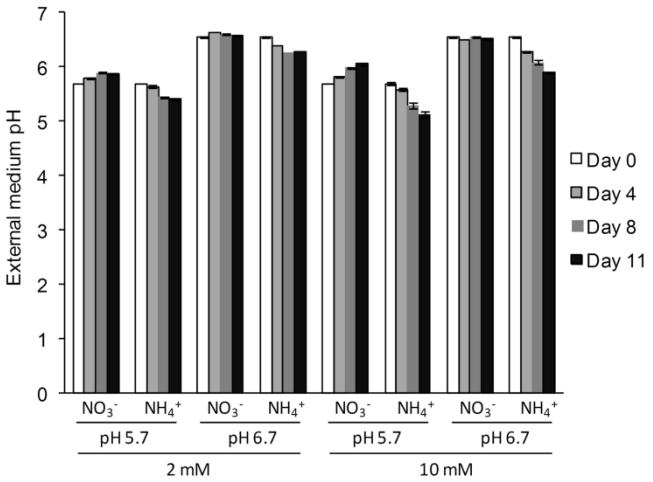
TCA cycle anaplerotic enzymes induction has been revealed important in order to counteract the depletion of TCA intermediates diverted to NH_4^+ assimilation; thus, they are crucial upon ammonium nutrition. Indeed, organic acids and malate pools decline in correlation with an increase in amino acid content has often been observed under ammonium nutrition (Britto and Kronzucker, 2005; Setién et al., 2013). In the present work, MDH, NAD-ME and NADP-ME were induced in shoots and could play a role in organic acids consumption (Figure 3.5). Furthermore, shoot NAD-ME and root NADP-ME induction was greater under a harsher degree of ammonium stress. Interestingly, NAD-ME levels in the roots were induced by nitrate nutrition and the plastidic and mitochondrial localization of this enzyme (Maier et al., 2011) may suggest a differential localization or function of malate pool in function of the N source. ICDH is a key enzyme in the provision of 2-OG, in the present study it was also induced in response to ammonium nutrition (Figure 3.5), as it has been observed in other plants such as pea (Ariz et al., 2013). In line with ICDH´s key role in 2-OG production, the amino acids content in shoots was observed to correlate with ICDH activity (Supplementary Figure S3.5). The importance of this enzyme was evident in plants lacking total or partial ICDH expression, since they presented reduced pools of 2-OG under carbon limitation (Boex-Fontvieille et al., 2013). On the other hand, ammonium nutrition is known to provoke redox alterations (Podgórska et al., 2013) and ICDH function supplying NADPH has also been related to redox homeostasis control (Marino et al., 2007; Mhamdi et al., 2010), thus the possibility that ICDH induction could also be related to cell redox control cannot be ruled out. With regards to PEPC, it has recently been shown that ammonium assimilation was impaired in the Arabidopsis *PEPC*

double mutant *ppc1/ppc2* grown in standard 1/2 MS medium (Shi et al., 2015) and, although disparate results have been found in different species, PEPC is known to be induced under ammonium stress, mainly in roots (Ariz et al., 2013; Britto and Kronzucker, 2005; Lasa et al., 2002). In the present study, higher PEPC activity in shoots of nitrate fed-plants will corroborate the need to replenish carbon intermediates in shoots when nitrate is the N source, whereas under ammonium stress, NH_4^+ assimilation would preferentially occur in the roots. Thus, fine regulation of TCA anaplerotic enzymes appears to be a key aspect when trying to improve plants NH_4^+ assimilation capacity under ammonium stress.

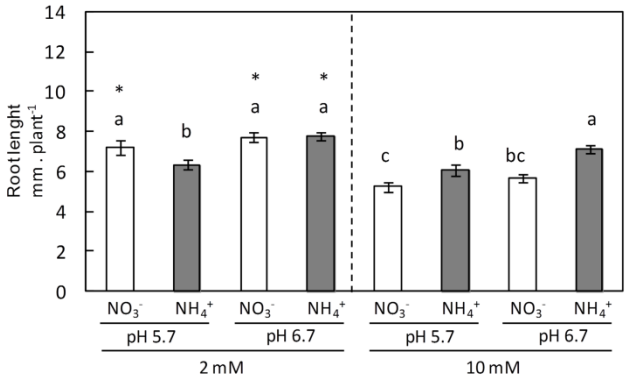
Final conclusions

Variations in the pH of the external medium are known to affect plants N nutrition. Regarding ammonium nutrition, pH control appears to play a key role in determining plant ammonium tolerance or sensitivity. In Arabidopsis, external medium buffering or medium alkalinization has been shown to mitigate some of the detrimental effects associated with ammonium stress, but how plant cell metabolism adapts to those changes has barely been studied, especially in the roots. In the present work, the higher degree of ammonium stress was related to NH_4^+ accumulation at pH 5.7 which could not be circumvented by the induction of ammonium assimilation machinery, including TCA cycle anaplerotic enzymes. Moreover, this study suggests specific roles for different GS and GDH isoforms in function of the nutritional regime. Similarly, anaplerotic enzymes seem to play an important role at the interface between carbon and nitrogen metabolism and future studies into ammonium nutrition with the use of knockout mutants in the different TCA cycle anaplerotic enzymes will be extremely helpful in gaining a better understanding of their role in ammonium stress. Finally, fluxomic analysis, paying special attention to metabolites subcellular localization, will elucidate the changes occurring in plant cell metabolism under ammonium-based nutrition.

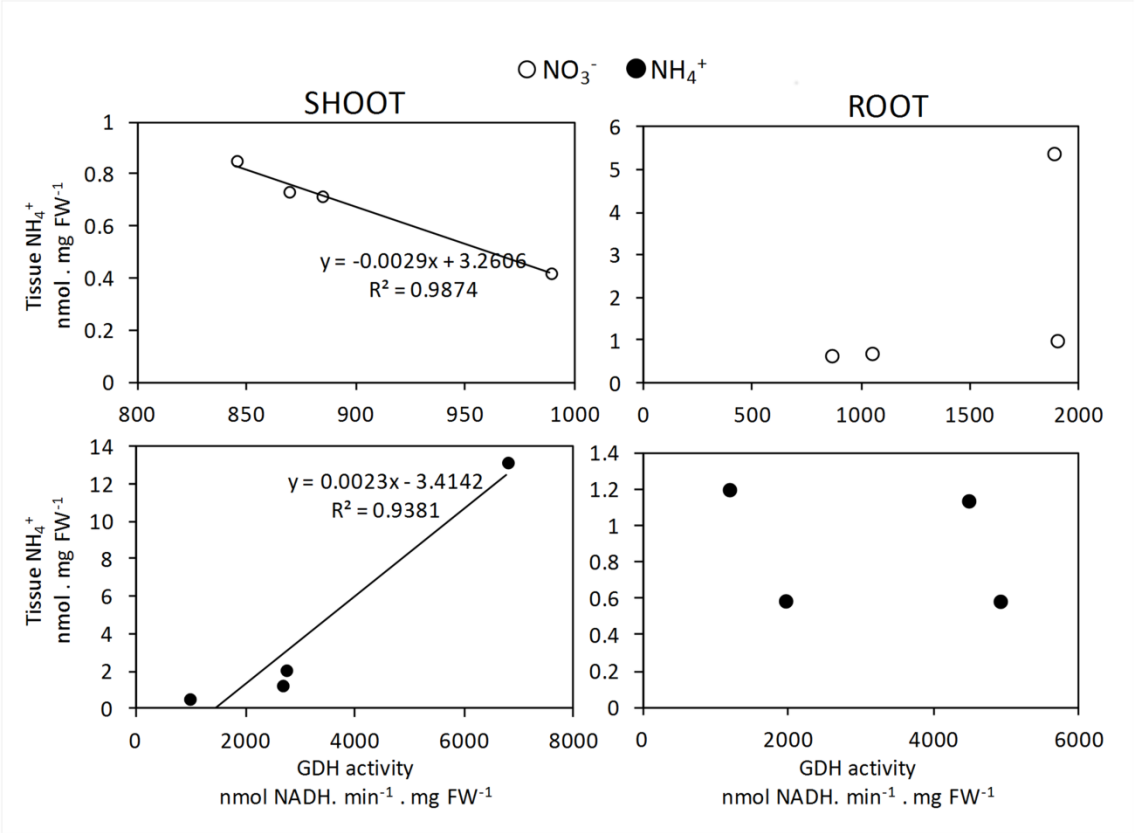
6. SUPPLEMENTARY INFORMATION



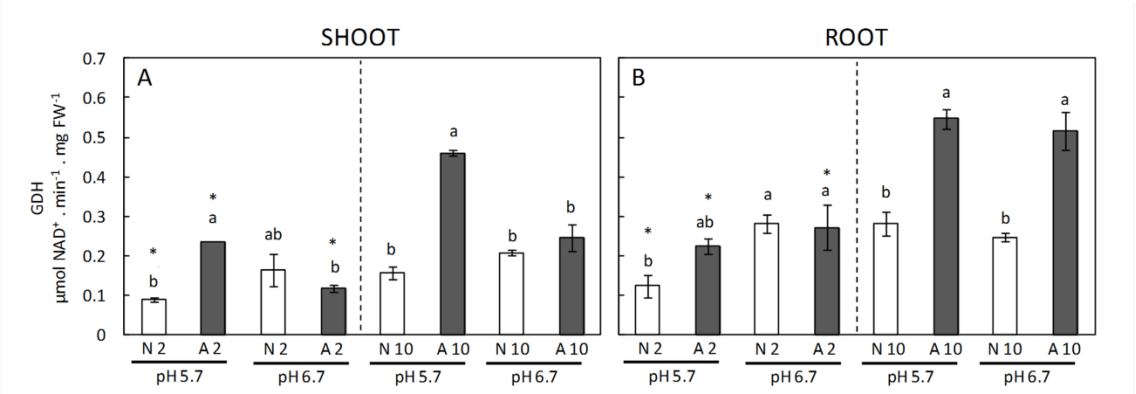
Supplementary Figure 3.1. External medium pH monitoring during Arabidopsis plants growth under different conditions of pH (5.7 or 6.7), N source (NO_3^- or NH_4^+) and concentration (2 or 10mM).



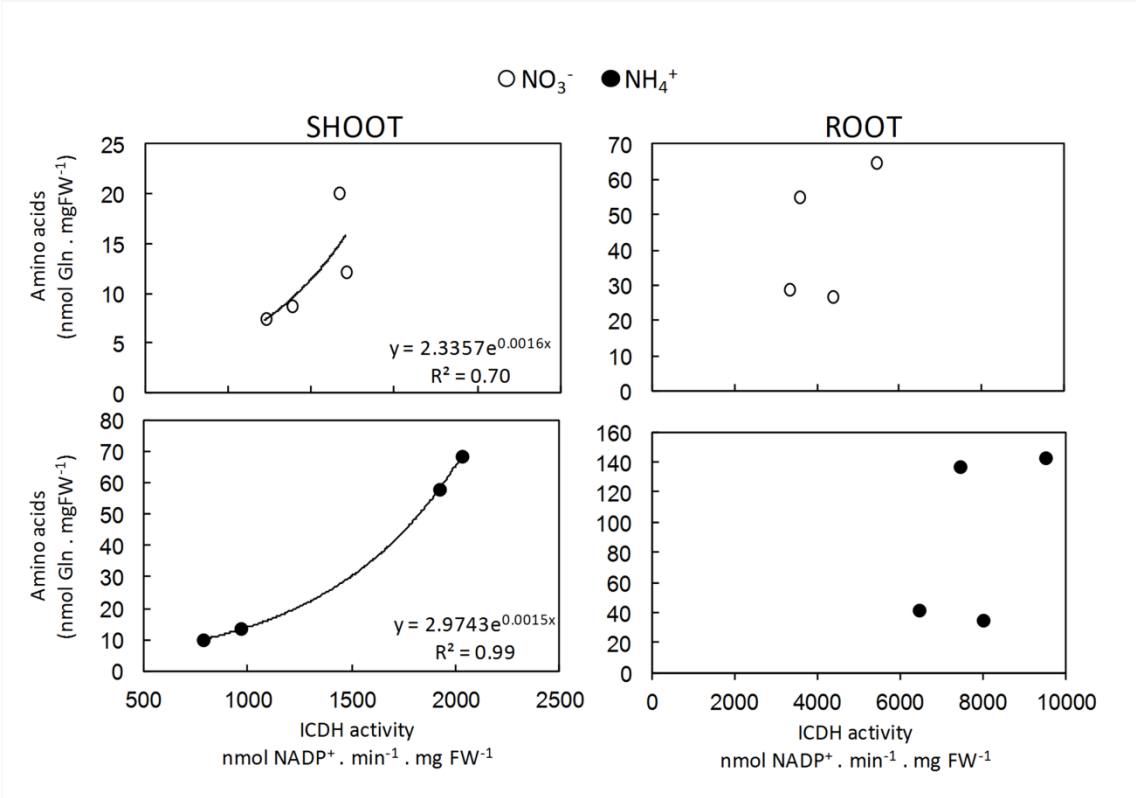
Supplementary Figure 3.2. Root length of plants grown under different conditions of pH (5.7or6.7), N source (NO−3 or NH+4), and concentration (2 or10mM). Statistical analysis was described in Figure 1. Columns represent mean ± se (*n* = 25–35).



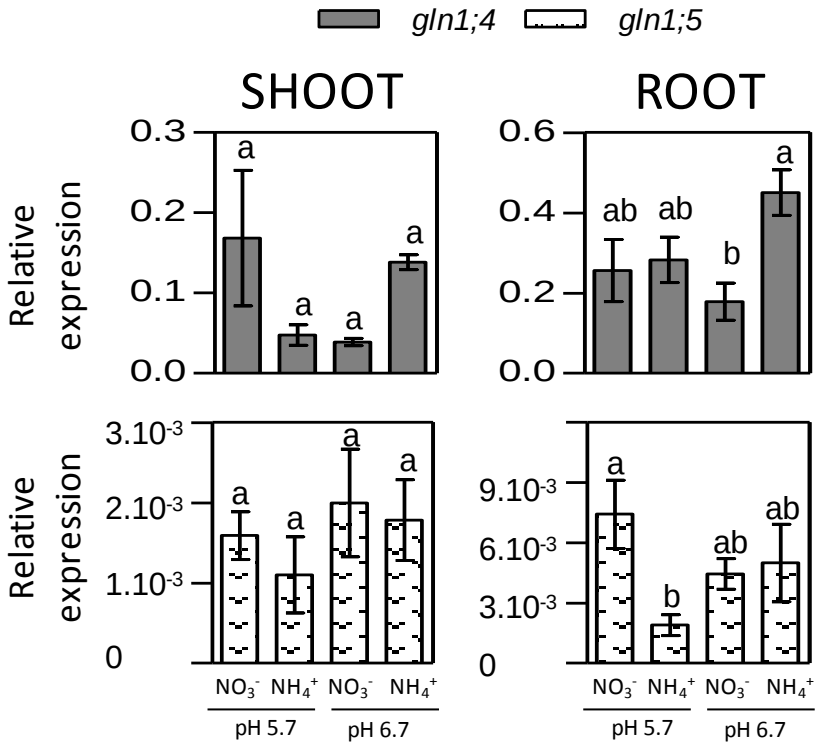
Supplementary Figure 3.3. Pearson correlations between GDH activity and tissue NH_4^+ content in roots and leaves of plants grown under nitrate or ammonium as nitrogen source. Correlationlinesarepresentedonlyif $p < 0.05$.



Supplementary Figure 3.4. GDH enzyme activity measured on its deaminating sense from shoots (A) and roots (B) of plants grown under different conditions of pH (5.7 or 6.7), N source (NO_3^- or NH_4^+), and concentration (2 or 10mM). Statistical analysis was described in Figure 1. Columns represent mean ± se ($n = 3$). Each sample is a pool of three plants.



Supplementary Figure 3.5. Pearson correlations between ICDH activity and amino acid content in roots and leaves of plants grown under nitrate or ammonium as nitrogen source. Correlation lines are presented only if $p < 0.05$.

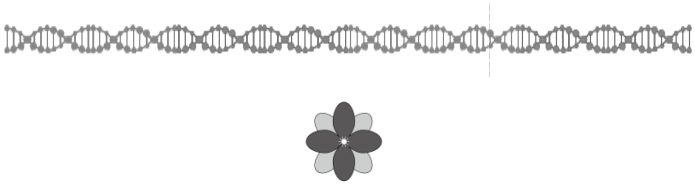


Supplementary Figure 3.6. Zoom of *gln1-4* and *gn1-5* genes expression shown in Figure 4.

Supplementary Table 3.1. Primer list

Primer List					
Primer Function	**Primer name**	**Sequence**	**Lenght**	**TAIR gene Code**	**Efficiency**
***β-tubulin 4* quantification**	qPCR-*β-TUB*-F	GAGGGAGCCATTGACAACATCTT	23	At5g44340	2
	qPCR-*β-TUB*-R	GCGAACAGTTCACAGCTATGTTCA	24		
***SAND* quantification**	qPCR-*SAND*-F	AACTCTATGCAGCATTTGATCCACT	25	At2g28390	2
	qPCR-*SAND*-R	TGATTGCATATCTTTATCGCCATC	24		
***GDH1* quantification**	qPCR-*GDH1*-F	TGGCTCAAGCTACCATTCTCAGA	23	At5g18170	Not calculated
	qPCR-*GDH1*-R	CCTCTAAGCCAAAGTAGGAGGATAAA	26		
***GDH2* quantification**	qPCR-*GDH2*-F	GTGGTTGGGAAGCTTAATTCAGTT	24	*At5g07440*	Not calculated
	qPCR-*GDH2*-R	CCATTTCGGAAAGCTCAATGAT	22		
***GDH3* quantification**	qPCR-*GDH3*-F	TCGCTCAAGCTACCACTATCAG	22	*At3g03910*	Not calculated
	qPCR-*GDH3*-R	CTGCAATTAGCACATAGTTTTTATTACTC	29		
***GLN1.1* quantification**	qPCR-*GLN1.1*-F	CAACCTTAACCTCTCAGACTCCACT	25	AT5G37600	Not calculated
	qPCR-*GLN1.1*-R	CAGCTGCAACATCAGGGTTGCTA	23		
***GLN1.2* quantification**	qPCR-*GLN1.2*-F	TAACCTTGACATCTCAGACAACAGT	25	At1g66200	Not calculated
	qPCR-*GLN1.2*-R	TCAGCAATAACATCAGGGTTAGC	23		
***GLN1.3* quantification**	qPCR-*GLN1.3*-F	TAACCTCAACCTCACCGATGCCACC	25	AT3G17820	Not calculated
	qPCR-*GLN1.3*-R	CTTGGCAACGTCGGGGTGGCTG	22		
***GLN1.4* quantification**	qPCR-*GLN1.4*-F	CAATCTCGATCTCTCCGATTCCACT	25	AT5G16570	Not calculated
	qPCR-*GLN1.4*-R	GGCGACAACACTAGGGTCTTCA	22		
***GLN1.5* quantification**	qPCR-*GLN1.5*-F	CCTAAACCTTGATCTATCAGACACC	25	AT1G48470.1	Not calculated
	qPCR-*GLN1.5*-R	GCCTTCACATTGGGATGATCG	21		
***GLN2* quantification**	qPCR-*GLN2*-F	CCAACATGTCAGATGAGAGTGCC	23	AT5G35630	Not calculated
	qPCR-*GLN2*-R	CCAGGTGCTTGACCGGTACTC	21		

GENERAL CONCLUSIONS.



GENERAL CONCLUSIONS

1. *Arabidopsis thaliana* shows great intraspecific variability in ammonium tolerance using rosette biomass as a marker of plant performance.

2. Intraspecific variability was observed at different geographical scales, from worldwide scale to local scale, suggesting that adaptative variation towards ammonium tolerance acted at every spatial scale.

3. NH_4^+ accumulation seems to be an important player in Arabidopsis natural variability in ammonium tolerance rather than the cell NH_4^+ assimilation capacity.

4. NH_4^+ accumulation was negatively associated with shoot growth independently of the N source provided.

5. Genome wide association mapping at French geographical scale identified a significant peak of association in relation with shoot biomass under ammonium nutrition that was absent in the analysis performed with shoot biomass under nitrate nutrition. This association peak corresponds to a genomic region that encompasses a tandem array of nineteen genes encoding Cysteine-rich receptor-like kinases (CRKs).

6. The study of *crk* T-DNA mutant lines covering the identified genomic region did not provide any clue regarding CRK implication on ammonium tolerance. However, *CRK* gene expression analysis on Col-0 shows that the expression of some *CRK* members was induced upon ammonium nutrition. Thus, suggesting again their potential role during ammonium nutrition. Overall, the probable redundancy in their function did not allow confirming the true implication of any of the CRK members in Arabidopsis ammonium tolerance.

7. External medium pH has a key impact on Arabidopsis response to ammonium nutrition.

8. Arabidopsis shoot and root metabolism differentially adapts to the nutritional regime in function of the external medium pH, N source and concentration, adjusting the NH_4^+ assimilation machinery and carbon metabolism in function of ammonium stress degree.

9. The greatest stress severity at pH 5.7 was associated with NH_4^+ accumulation and could not be circumvented in spite of the stimulation of GS, GDH and TCA cycle anaplerotic enzymes. Thus, NH_4^+ accumulation triggering ammonium stress is not due to N assimilation impairment.

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