Analysis of the biochemical and genetic properties of alanine aminotransferase enzymes

by

Chandra H. McAllister

A thesis submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

in

Plant Biology

Department of Biological Sciences
University of Alberta

Abstract

Quantitatively, nitrogen (N) has shown to be one of the most important nutrients for proper plant growth and development, and a key limiting factor in crop productivity. In the last 40 years, there has been a significant increase in the production of synthetic N-fertilizers, as well as an increase in the overall application of these N-fertilizers to crops and subsequently considerable negative impacts on the environment. In order to mitigate N compounds released into the environment while maintaining crop yields to feed a growing world population, there is a need for plants that more effectively uptake, assimilate, and mobilize applied N. While advances in plant breeding practices and agricultural technologies have increased greatly due to Norman Borlaug's "Green Revolution" in the 1960's, the selection of traits associated with efficiency of nutrient uptake and usage was greatly ignored because of excessive fertilizer applications during this time. Consequently, there is a need for crop plants with increased nutrient efficiency, specifically nitrogen use efficiency (NUE).

Alanine aminotransferase (AlaAT), is a pyridoxal-5'-phosphate-dependent (PLP) enzyme that catalyzes the reversible transfer of an amino group from alanine to 2-oxoglutarate to produce glutamate and pyruvate, or vice versa. It has been well documented in both greenhouse and field studies that tissue-specific over-expression of AlaAT from barley (*Hordeum vulgare*, *HvAlaAT*) results in a significant increase in plant NUE in both canola and rice. While the physical phenotypes associated with over-expression of *HvAlaAT* have been well characterized, specifically in regards to NUE, the role this enzyme plays *in vivo* to create a more N efficient plant remains unknown. Furthermore, the importance of HvAlaAT and not other AlaAT homologues in creating this phenotype has not yet been explored.

To address the role of HvAlaAT in NUE, the *in vitro* K_M values of a selection of AlaAT variants was analyzed. Based on this kinetic analysis, several of these enzymes were chosen to be expressed, both tissue specifically and constitutively, in *Arabidopsis thaliana* Col-0 background and alaat1;2 (alaat1-1;alaat2-1) knockout background. The analysis and comparison here of both the physical and physiological properties of AlaAT transgenic plants revealed significant differences between plants expressing different AlaAT enzymes in regard to externally applied factors as well as differences in internal C and N concentrations. The analysis reported herein indicates that, the over-expression of AlaAT variants other than *HvAlaAT* in crop plants could further increase the NUE phenotype(s) previously observed.

Preface

Chapter 2 of this thesis has been published as McAllister, C. H., Facette, M., Holt, A. and Good, A. G. (2013) Analysis of the enzymatic properties of a broad family of alanine aminotransferases. *PloS One*, *8*(2), e55032. I was responsible for the experiment conceptualization, data collection and analysis as well as the manuscript composition. M. Facette assisted with the experiment conceptualization and data collection. A. Holt assisted with experimental design and manuscript edits. A. G. Good was the supervisory author and was involved with concept formation and manuscript composition.

Acknowledgements

I would first and foremost like to thank my supervisor, Dr. Allen Good, for allowing me the opportunity to carry out this research in his lab. His guidance and support over the last several years, both during my undergraduate degree and during my graduate studies, has been invaluable. I would also like to extend thanks to my advisory committee, Dr. Martin Srayko and Dr. Enrico Scarpella for their comments and guidance.

I would like to further thank Dr. Andrew Holt for his assistance in both understanding and designing the kinetics assays as well as Dr. Michelle Facette for her assistance with these assays. Thanks also to Dr. Tracy Ravio, for providing the TR61 *E. coli* line for expression studies as well as all those who made available the various AlaAT cDNAs: Dr. Anis Limami (Université d'Angers), Dr. Laura Olsen (University of Michigan) and Dr. Rong ze Yang (University of Maryland).

Thank-you to the Department of Biological Sciences and the Faculty of Graduate Studies and Research at the U of A for their financial support during my graduate program, as well the Natural Sciences and Engineering Research Council of Canada (NSERC) and the Alberta Crop Industry Development Fund for funding of this project.

Finally, I would like to say a very big thank-you to my parents, Analee and Glenn McAllister, for always supporting and encouraging me. And finally, thank-you to Anthony Oko for his unwavering support and love; thank-you for making me smile.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

A	Absorbance
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BLAST; BLASTn	Basic local alignment search tool; nucleotide BLAST
bp	Base pairs
btg26	Brassica turgor gene-26 promoter
C-myc-tag	C-myc epitope tag, human transcription factor
°C	Degrees Celsius
cDNA	Complementary DNA
cm; nm	Centimeter; nanometer
Col-0	Columbia ecotype
C_t; $\Delta\Delta C_t$	Cycle number that passes threshold; comparative C_T
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraaceitc acid
$FADH_2$	Flavin nicotinamide dinucleotide
g; rcf	Relative centrifugal force
GUS	ß-Glucuronidase
His-tag	Histidine tag
hr	Hours
Hyg	Hygromycin
k_{cat}	Turnover number, overall catalytic rate of an enzyme
kD	Kilodalton
kg; g; mg; µg	Kilograms; grams; milligrams; micrograms

K_M	Michaelis constant
Kan	Kanamycin
L; ml; µl	Liter; milliliter; microliter
lac gene	ß-galactosidase gene
M; mM; µM	Molar; millimolar; micromolar
Min	Minute
mol	Mole
mRNA	messenger RNA
MES	4-Morpholineethanesulfonic acid monohydrate
MS	Murashige and Skoog medium
µCi	Microcurie
µE	Microeinstein
n	Number of replicates
NAD(H)	Nicotinamide adenine dinucleotide
NADP(H)	Nicotinamide adenine dinucleotide phosphate
NCBI	National Center for Biotechnology Information
OD	Optic density
Oligo-(dT)s	Short sequence of deoxy-thymine nucleotides
OsAnt1	*Oryza sativa* antiquitin gene promoter
PCR	Polymerase chain reaction
PMSF	Phenylmethasesulphonylfluoride
PVPP	Polyvinylpolypyrrolidone
qRT-PCR	Quantitative reverse transcriptase-PCR
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulphate

sec	Second
SEM	Standard error of the mean
T-DNA	Transfer-DNA
TILLING	Targeting induced local lesions in genomes
Tris	Tris (hydroxymethyl) aminomethane
U	Units
V_{max}	Maximum velocity of reaction
w/v	Weight per volume

1. General Introduction

Quantitatively, nitrogen (N) is the most important factor limiting plant growth and development (Vitousek and Howarth, 1991). In agricultural systems, increased crop yields rely heavily on the application of nitrogenous fertilizers (N-fertilizers) because biologically active N rarely meets anthropogenic demands. N-fertilizers have shown to be harmful to the environment (Vitousek *et al*., 2009; Wuebbles, 2009). The negative impacts of N fertilizers are one reason that we are seeing a shift in agriculture, from a focus on food production to that of increased agricultural sustainability (de Bossoreille de Ribou *et al*., 2013). For these reasons, plants, specifically cereal crops, with increased nitrogen use efficiency (NUE) are of great importance.

There are numerous ways to increase NUE in plants. This includes altering the process of nutrient management and application. In terms of altering the plant itself, selective breeding practices and genetic modification have been attempted. While both selective breeding and nutrient management have been used successfully in the past to improve NUE in plants, these methods appear to have reached a plateau. Even with the surge of investment in agricultural research and development since the 1960's Green Revolution, many of the main food crops, including rice, wheat and maize, have shown decreases in their relative rates of yield (Hawkesford & Barraclough, 2011; Grassini *et al*., 2013). Recently, it has been shown that globally there is a significant deceleration in the relative rate of increase of average crop yields, and evidence of yield plateaus or even yield drops in some cases (Grassini *et al*., 2013).

Recent advances in both plant genetics and physiology have allowed for selective breeding and genetic modification of plants in order to produce crops that have desired traits. While the genetic engineering of plants to produce desirable traits has been fairly simple in some cases, for example, the introduction of pesticide resistance (*Bacillus thuringiensis* (*Bt*)) in cotton, other more complex traits have been much harder to genetically engineer, as in the case of yield. (Kraakman *et al*., 2004; Tester and Langridge, 2010). For instance, in barley 18-20 genetic markers are associated with 40-58% of the yield (Kraakman *et al*., 2004). NUE, like yield,

NOTE: *A version of this chapter has been published.* McAllister, C. H., Beatty, P. H. and Good, A. G. (2012) Engineering nitrogen use efficient crop plants: the current status. *Plant Biotech. J.* **10:** 1011–1025.

is a genetically complex trait with multiple genes contributing to the phenotype; this is also compounded by environmental factors. This makes selection difficult using traditional breeding and selection methods (Cattivelli *et al*., 2008; Hawkesford & Barraclough, 2011). And, while selective breeding practices have previously selected for NUE as an advantageous trait associated with low-nutrient conditions, much more effort is needed to deduce N-specific traits.

In this thesis I approach the problem of increasing plant NUE through the transgenic expression of a number of variants of the enzyme alanine aminotransferase (AlaAT) [E.C.2.6.1.2]. Previous work has shown that tissue-specific over-expression of this enzyme in both canola and rice increases NUE in these plants, however, little is known about the reasons for this increase or how alterations in AlaAT enzyme expression, structure or activity could modify or affect an NUE phenotype. While previous work has focused on how tissue and/or temporal expression of this enzyme affects the observed NUE phenotype, this thesis focuses on the enzymatic properties of various AlaAT enzymes, including the kinetic differences of AlaAT homologues. Specifically, I looked at how differences in the enzymatic properties of different AlaAT isoforms could potentially affect the NUE of plants using the model plant *Arabidopsis thaliana*. Any of the isoforms affecting the phenotype of Arabidopsis can then be tested in crop plants (such as rice) to provide a better understanding of the physiological basis of the phenotype.

1.1. Nitrogen and the environment

1.1.1. The nitrogen cycle

The natural cycling of N compounds consists of five major N transformations: nitrogen fixation, nitrification, denitrification, anammox (volatilization) and ammonification (Figure 1.1), which occur in both terrestrial and aquatic ecosystems. In order to convert N_2 into biologically active N, including nitrate (NO_3^-), ammonium (NH_4) and N-oxides, the triple bond between N atoms must be broken; this process is known as nitrogen fixation and requires a large amount of energy (Bowden *et al*., 2000; Bouwman *et al*., 2013). Due to the high energy input required for this process, nitrogen is biotically fixed by only a small handful of prokaryotes, and abiotically fixed by both lightning and industrial processes (Bernhard, 2012). N_2 is generally converted to the gas NH_3, which then goes through a series of oxidations. Under anaerobic conditions,

volatization can occur, resulting in the reformation of N_2 from both NO_2^- and NH_3. In aerobic conditions, NH_3 is converted to a variety of compounds (NO_x), including amidogen (NH_2), NH_4^+ and NO_3^-, through a process known as nitrification, which is carried out by prokaryotes in two main steps. First, NH_3 is converted to nitrite (NO_2^-), followed by the conversion of NO_2^- to the stable N compound NO_3^- (Bernhard, 2012). Both NO_2^- and NO_3^- products from bacterial nitrification are subject to denitrification by bacteria, whereby NO_x compounds are converted back to N_2 gas. The greenhouse gas nitrous oxide (N_2O) is a by-product of denitrification. NO_3^- and NH_4^+ that are not denitrified are available to be taken up and used by plants for growth and development, ultimately being converted into organic N compounds, including amino acids and nucleic acids, RNA and DNA. To complete the cycle, plant and animal wastes, as well as decomposing organic N compounds, can then be converted back to ammonia by bacteria in a process known as ammonification.

1.1.2. Biological nitrogen fixation

The N compounds necessary for anthropogenic purposes, both agricultural and industrial, are produced via the N cycle. The anthropogenic N cycle is driven by the need to fix inert atmospheric nitrogen (N_2) into biologically available forms of N for industrial purposes and crop production. However, the amount of reactive N (N_r: all N species except N_2) produced through natural means (biological nitrogen fixation; BNF) does not meet the demands for fixed nitrogen required by industries such as energy, transportation, military and agriculture (Compton *et al*., 2011; Galloway *et al*., 2013). The global natural sources of fixed N species (both terrestrial and marine) approximated 203 teragrams (Tg) N yr^{-1} before the industrial revolution, one third of which was derived from terrestrial systems (Fowler *et al*., 2013). Since then, it is estimated that anthropogenic practices contribute another 210 Tg N yr^{-1} above the estimates of natural N_r production, and that this amount increases annually (Figure 1.2; Fowler *et al*., 2013).

The need for increased N_r is a direct result of increasing energy and food production needs. Global cereal production alone rose 20% (from 1897 to 2270 million tonnes) from 1995 to 2005, and meat production increased 26% (from 207 to 260 million tonnes) in the same time frame (Galloway *et al*., 2008). The production of N_r to meet the demands for an ever increasing global population is accomplished via the Haber-Bosch method, which is an anthropogenic

means of fixing N. Named after Fritz Haber who created the method in 1909, and Carl Bosch who increased the efficiency of the method for industrial production in 1913, this method synthesizes ammonia from N_2 and H_2 in the presence of high heat and pressure (Galloway *et al*., 2013). Initially, the process of anthropogenic N fixation was developed because of two key needs: 1) the increasing demand for crops and therefore fertilizers, and 2) the requirement of base materials involved in making explosives and weapons (Erisman *et al*., 2008). Therefore, it is of no surprise that the Haber-Bosch process has had a direct impact on the increases in global agriculture since its development. In fact, estimates indicate that half of the total food consumed by the world's population is grown using N-fertilizers created using the Haber-Bosch process (Galloway and Cowling 2002).

In 2005, approximately 100 Tg of N produced by the Haber-Bosch process was used in global agriculture, (about 80% of the total N produced by this process; Erisman *et al*., 2008). However, there is a large discrepancy between the amount of N used by agriculture, and the total amount of N that is eventually consumed by humans; estimates indicate that of the 100 Tg N produced for agriculture and applied to the environment in 2005, only about 17 Tg N was available for human consumption from crop, dairy or meat products (Erisman *et al*., 2008). This in turn leads to an interesting philosophical consideration: how much N_r is truly needed versus how much do we use. Depending on the population (animal and human), population density (rural or urban) or topography (including plant species and variety) the question of, and discrepancy between amounts needed versus amounts made could vary greatly. Although here I address the issues surrounding excessive environmental N_r in terms of decreasing N-fertilizer amounts, there are other ways in which this problem may be addressed. Clearly, humans make conscience decisions about how much N we "need" based on factors including the amount of and type of meat in our diet. While this thesis will not focus on these other ways in which humans can consciously decrease N_r amounts, it is a possible solution to excessive N_r in the environment, and one that should not be overlooked.

This difference in the N_r made and released into the environment and the portion utilized has resulted in serious environmental problems, including eutrification of water systems, biodiversity loss, greenhouse gas emissions and ozone depletion (Erisman *et al*., 2013). If human populations reach expected numbers in 2050 of ~8.9 billion people, N_r production is also

expected to increase, reaching a staggering ~900 Tg N yr^{-1} (Galloway and Cowling, 2002). Given the recent precautions taken to reduce N emissions and production however, it is hoped that such increases in global N_r are not necessary. A summary of human produced N_r and the effects these compounds have on the environment is depicted in Figure 1.3 and summarized in the next section.

1.1.3. Negative impacts of N_r on the environment

Once N_r has been created, either naturally or anthropogenically, it can then be used for a variety of purposes, including fertilizer production (Galloway and Cowling 2002). In general, fertilizer production and usage is the largest contributor of "new" N to the environment (Compton *et al*., 2011; Thomson *et al*., 2012) and has been charged with numerous negative impacts on the ecosystems where it is applied, including: depletion of soil minerals, soil acidification, eutrophication of water systems and changes in community structure and biodiversity (Liu and Greaver, 2009; Skiba *et al*., 2011; Vitousek *et al*., 1997).

Between 1960 and 1990, rising N-fertilizer inputs were necessary to deliver the gains in yield associated with the Green Revolution, where selective breeding strategies along with alterations in fertilization, irrigation and pesticides resulted in a doubling of global cereal production (Tilman *et al*., 2002; Davies, 2003; Pingali 2012). Unfortunately, while the benefits of the Green Revolution include increases in carbon sequestration and improved crop yields (by improved partitioning of photosynthetic products in cereal crops) these have now been off-set by the widespread use of pesticides, water and agricultural land shortages, land-use changes and increases in world population size. Increases in N-fertilizer application and the resulting increased release of N_r products also contribute to global warming and ozone depletion (Tilman *et al*., 2002; Davies 2003; Hawkesford and Barraclough 2011).

One of the byproducts of denitrification and fossil-fuel combustion is N_r in the form of nitrous oxides (N_2O and NO_x), which can then be converted to nitric oxide (NO), a substance that reacts directly with, and depletes, ozone (O_3) (McElroy *et al*.,1986; Solomon, 1999; Hofstra and Bouwman, 2005; Pilegaard, 2013). Currently, N_2O has the highest global warming potential (Ravishankara *et al*., 2009; Wuebbles, 2009; Galloway *et al*., 2013). Along with its negative impacts on ozone, N_2O is one of four greenhouse gases (GHGs) (not including water vapor)

contributing to increases in global warming, the other three being carbon dioxide (CO_2), methane (CH_4) and fluorinated gases (PFCs (perfluorocarbons), HFCs (hydrofluorocarbons) and SF6 (sulfur hexafluoride)) (van Aardenne *et al*., 2004; Bouwman *et al*., 2013). GHGs are compounds that trap heat in the Earth's atmosphere, not allowing energy to return to space. This trapped energy, depending on the compound, will have a radiative force or global warming potential (GWP) based on the lifetime of that molecule (Johnson *et al*., 2007). While N_2O is only the third most abundant GHG (behind CO_2 and CH_4 respectively) (Montzka *et al*., 2011) it is estimated to be ~300X more potent than CO_2 (with CH_4 being ~20X more potent a GHG than CO_2) (Johnson *et al*., 2007; Thomson *et al*., 2012). Increases in GHG emissions and thus global warming are suggested to have impacts on both terrestrial and marine ecosystems, with overall impacts resulting in decreased carbon sequestration (Montzka *et al*., 2011). Interestingly, N_2O does not only have a warming effect, as it traps heat and degrades O_3 in the troposphere as a GHG, but also a cooling effect as it decreases O_3 in the stratosphere (Solomon *et al*., 2007; Galloway *et al*., 2008; Sutton *et al*., 2008; Bouwman *et al*., 2013). These opposing effects have created much debate about the overall effects of increased N_r in the Earth's atmosphere.

Once in the atmosphere, N_r compounds can also interact with water molecules resulting in nitric acid (NO) in precipitation (Galloway *et al*., 2013). While not the primary cause of acid rain (which is sulfuric acid), NO in rainwater can react with NH_3 and NH_4^+ in the soil resulting in soil acidification as well as contributing to the acidification of waterways (Galloway *et al*., 2013). N_r increases in marine systems can have a number of detrimental effects, including algal blooms and eutrophication, leading to loss of marine life and biodiversity (Vitousek *et al*., 1997, 2009; McAllister *et al*., 2012; Galloway *et al*., 2013). N_r compounds in rain water also contribute to soil N deposition, and have shown to contribute about 20-45 kg N ha^{-1} yr^{-1} in high density metropolitan areas (Fenn *et al*., 1996; Lohse *et al*., 2008)

1.1.4. Positive impacts of N_r on the environment

Increases in N-fertilizers can also have positive impacts on ecosystems, including increases in carbon sequestration, resulting in increases in cereal crop yields and food production (Liu and Greaver, 2009). Increases in N availability to crops have also increased the nutritional value of these plants, increasing the overall nutrition that humans gain by eating such crops (Galloway and Cowling, 2002). Furthermore, as atmospheric CO_2 concentrations increase (ie:

greenhouse effect) so does the value of increased C sequestration by plants (Langley and Megonigal, 2010). The ability for different plant species to utilize increases in N or C would therefore have an impact on the diversity within a given ecosystem, and could result in plant community shifts, specifically to ecosystems dominated by C_4 plants from systems previously dominated by C_3 plants (Langley and Megonigal, 2010). These alterations could be advantageous or disadvantageous depending on the circumstances. Even in more natural terrestrial ecosystems, increases in N input have shown to increase CO_2-stimluated plant activity, resulting in increased C sequestration (Langley and Megonigal, 2010). Naturally, if growers are obtaining increased yields and the demand for these products is also increasing, then there should also be an increase in wealth and standard of living in these areas; this is often the trend in areas that have had access to N-fertilizers (Galloway and Cowling, 2002).

It is widely agreed that at lower N concentrations, the positive outcomes of applying N-fertilizers, discussed above, outweigh the negative. However, unlike the negative impacts of N_r in the environment, the positive benefits become saturated at higher levels in both the agricultural and industrial sectors, with the negative impacts surmounting the positive ones (Vitousek *et al*., 1997; Townsend *et al*., 2003). Therefore, it is important to decrease the amount of N_r made and used anthropogenically to levels below that advised by the World Health Organization (WHO) (10 parts per million in ground water), and Health Canada and the United States Environmental Protection Agency (10 mg/ml nitrate-nitrogen in drinking water), so that the benefits of N_r can once again outweigh the disadvantages (Health Canada, 1992; Townsend et al., 2003; United States Environmental Protection Agency, 2009).

1.1.5. Indirect impacts of N on the environment

As well as directly affecting ecosystems and the environment, N_r compounds have many indirect effects due to the impact these molecules have on both plant and microbial activity, and the influence N_2O and NO_x molecules have on the flux of other GHGs (Bowden *et al*., 2000; Butterbach-Bahl *et al*., 2002; Mosier *et al*., 2003, 2006; Liu and Greaver, 2009; Pilegaard, 2013). In both agricultural and natural ecosystems, the addition of N compounds to plant systems has a large impact on growth and development, often increasing plant biomass and yield in the case of cereal crops. It has been estimated that the addition of N to forest ecosystems increases the carbon content by 6%, and to agricultural systems by 2% (Liu and Greaver, 2009).

Unfortunately, these effects are often undermined by not only the increase in N_2O emissions, but also by the increase in CH_4 emissions (97%) and reduced uptake of CH_4 (38%), caused by the addition of N in these ecosystems (Liu and Greaver, 2009).

1.1.6. Recent changes in the management of land and N_r usage

Interestingly, recent changes in land-usage to mitigate the use of fossil fuels, and to decrease GHG emissions, have actually resulted in increased loss of N_2O into the environment and decreased the total amount of carbon captured (Melillo *et al*., 2009). There are two main reasons for these alterations in agricultural land usage: 1) the conversion of traditional agricultural land to that used for biofuel production and, 2) the conversion of forests and other habitats into agricultural land, often in an effort to increase land for crops after losing this land to the production of biofuels (Searchinger *et al*., 2008; Melillo *et al*., 2009). Due to the GHG emissions caused by the burning of fossil fuels, the use of biofuels (most commonly corn grain (*Zea mays* ssp.) ethanol and soybean (*Glycine max*) biodiesel) is increasing in popularity as alternative transportation fuels (Hill *et al*., 2006). In order for these biofuels to be considered viable alternate fuels they must fulfill several criteria, which include providing a net energy gain while maintaining environmental benefits and being economically competitive (Hill *et al*., 2006). Initially, the changes in land-usage mentioned above were encouraged, and it was commonly accepted that such changes would increase carbon sequestration however, this is not always the case. Soil disturbances resulting in increased carbon loss, fertilizer usage and the economic and environmental cost of transporting biofuels are all disadvantages (Hill *et al*., 2006; Dunn *et al*., 2013). Growth of crops for food-based biofuels and feedstocks, such as corn, ultimately decreases the amount of land devoted to food production, however, there are a number of strategies being considered to increase carbon sequestration and decrease GHG emissions, as seen in Figure 1.4. For example, changing biofuel crops from corn and soy to switchgrass would have a positive impact on not only carbon sequestration above and below ground, but also fertilizer inputs (Dunn *et al*., 2013). Inevitably, if biofuels are to become economically and environmentally sound fuel alternatives, the above challenges need to be addressed.

Ultimately, many different factors contribute to the release of N_r, both natural and anthropogenic. However, increases in the anthropogenic production and usage of N_r species has had devastating impacts on both land and marine environments, and will continue to have

detrimental effects unless the production and usage of these compounds is lessened (Liu and Greaver, 2009; Pingali, 2012; Thomson *et al*., 2012). In spite of this knowledge, the use of N-fertilizers and the release of N_r compounds have not decreased, despite our recognition of the negative environmental impacts. Many different proposals to decrease the effects of N_r and other greenhouse gases have been applied in the last several years, including the Kyoto Protocol in 1997, the implementation of carbon credits in many countries and numerous adaptive and mitigative carbon sequestration strategies (Figure 1.4) (McCarty and Ritchie, 2002; Lal, 2004; Thomson *et al*., 2012). In Canada specifically, several different programs are being conducted, such as the National Agri-Environmental Health Analysis and Reporting Program (NAHARP) and the National Carbon and Greenhouse gas Accounting and Verification System (NCGAVS) (Huffman *et al*., 2008). Although these changes have led to decreases in total emissions in certain sectors, ultimately more must be done in order to maintain globally sustainable crop yields while also ensuring the sustainability and productivity of the environment (Pingali, 2012; Thomson *et al*., 2012)

1.2. Nitrogen as a plant nutrient

1.2.1. Plant Nutrition

As sessile organisms, plants are dependent on their environment to obtain and take up nutrients (Fageria *et al*., 2008). There are 14 mineral elements obtained from the soil that are required for plant growth and development (White and Brown, 2010). These mineral elements include macronutrients: nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg) and sulphur (S), as well as micronutrients: chlorine (Cl), boron (B), iron (Fe), manganese (Mn), copper (Cu), zinc (Zn), nickel (Ni) and molybdenum (Mo) (White and Brown, 2010). Along with these mineral nutrients found in the soil, water (oxygen (O) and hydrogen (H)) and carbon (C) are essential for plant growth and development (Jones and Jacobson, 2001; Maathuis, 2009).

It is common for soils to be deficient in mineral compounds, with specific chemical deficiencies depending on the soil type, the amount of organic matter present, as well as other environmental conditions such as weather (ie: temperature) and human or animal activity (Fernández and Hoeft, 2009). There are a number of ways that soils can obtain or lose nutrients. Inputs include: mineral fertilizer, organic fertilizer, deposition, N-fixation and sedimentation,

while nutrient losses include: crop products, crop residues, leaching, gaseous loss and soil erosion (Tan *et al*., 2005).

In Canadian prairie soils, N and P are most commonly depleted, with micronutrient deficiencies occurring less frequently (R. McKenzie, 1998, 2003). In 2010, Agriculture Canada reported that N-fertilizers were the largest nutrient utilized for agricultural production, making up 72% of the annual fertilizer usage, equaling approximately 4.2 million tonnes (mt). Of the nitrogenous fertilizers utilized, urea based mixtures were most popular (Agriculture and Agri-Food Canada, 2012; Temple Scott Associates Incorporated, 2013). According to the Food and Agriculture Organization of the United Nations (FAO), the demand for fertilizers in North America alone is expected to increase by 0.8% from 2011-2015, and global demand is expected to increase by 1.7% in the same time frame (FAO, 2011).

Globally, the expected supply and demand for N-fertilizers world-wide in 2011-2012 was approximately 154 000 kilotonnes (kt) and 139 000 kt, respectively, leaving a surplus of ~15 000 kt (FAO, 2008). However, N-fertilizer demand is expected to continue to increases between 1-3% worldwide each year, as it has previously. Over-production of N-fertilizers does not appear to be as worrisome a prospect as under-production which would impact the economic and social sustainability of agriculture and food production globally (FAO, 2008; Goulding *et al*., 2008; Agriculture and Agri-Food Canada, 2012). Application of fertilizers alone has shown to increase average yield of crops 40-60% in long-term studies in the United States and England, with even higher percentage increases in the tropics (Stewart *et al*., 2005). Thus, although the excessive addition of nutrients to crop plants is harmful to the environment and economically demanding, because yield and crop sustainability is dependent on these additions, fertilizer usage will continue, although it is hoped that crop plants can be developed that maintain yields with fewer nutrients supplied.

In general, low levels of a particular soil nutrient will result in reduction of plant growth, however, the addition of some mineral compounds in large amounts can also inhibit plant growth, such as sodium (Na), aluminum (Al), Cl, Mn, Fe and B (White and Brown, 2010). Also, anthropogenic activity has resulted in increased amounts of chemicals that are toxic for plants in certain environments, including the addition of cadmium (Cd), Cu, Zn, lead (Pb) and mercury (Hg) (White and Brown, 2010). While generalities about “macro” and “micro” nutrients can be

made, there are significant differences in nutrient needs and acquisition abilities between different plant species and in different environments (Macnicol and Beckett, 1985; Brown *et al*., 1987; White *et al*., 2004; Stewart *et al*., 2005; Pilon-Smits *et al*., 2009; White and Brown, 2010). Therefore, achieving ideal nutrient conditions for a plant is not simple, but involves the addition and possibly the elimination of chemical components from soils, as well as an understanding of that particular species' nutritional needs.

In many plant species, alterations in nutrient availability have been studied extensively, and deficiencies are highly visible as changes in yield, root system architecture, colouration and biomass of both above and below ground tissues (Clarkson, 1985; Goulding *et al*., 2008; Gruber *et al*., 2013). It is also a common misconception that application of nutrients to soils results in the availability of that nutrient to the plant, as soils will often differ in their ability to bind a specific nutrient and make it available for plant growth and development. Returning to the concept of nutrient use efficiency, as of 2005, it was estimated that the global nutrient use efficiency of N was 50%, P was 40% and K was 75% (Tan *et al*., 2005). Because plants obtain the majority of their nutrients through their roots, inhibition of root growth has a large impact on the uptake of nutrients. Another contributor to mineral uptake is pH, which effects mineral solubility and therefore root uptake ability, particularly of the nutrients N, P, K and S (Murphy *et al*., 2000; McKenzie, 2003) . Temperature, and the presence of other minerals also influence the ability of plants to obtain nutrients (McKenzie, 2003; Fernández and Hoeft, 2009).

1.2.2. Nitrogen in the soil

As noted, quantitatively N is the most important plant nutrient (Kraiser *et al*., 2011; McAllister *et al*., 2012). It is used as a signaling molecule as well as a component of proteins and nucleic acids (Miller *et al*., 2007, 2008). Imbalances of N in the environment lead to numerous effects on the plant, including sub-optimal yields in the case of cereal crops (Miller *et al*., 2007). Since N availability has such great effects on the growth and development of the plant, the ability to sense, assimilate and utilize this nutrient must be tightly regulated, with a highly integrated system of both positive and negative feedback loops (Forde 2000, 2002; Miller *et al*., 2008). This is particularly true for those plants that have symbiotic relationships with diazotrophic bacteria (Frugier *et al*., 2008).

N is most often taken up by plants as water soluble nitrate (NO_3^-; usually the most abundant), ammonium (NH_4^+), and to a lesser extent, as proteins, peptides or amino acids (Figure 1.5) (Good *et al*., 2004; Miller *et al*., 2007; Rentsch *et al*., 2007; Tsay *et al*., 2007; Näsholm *et al*., 2009). Plants have evolved a number of nitrogen transport systems that discriminate between these forms of N, as well as sense both the external and internal concentrations of specific N molecules (Gojon *et al*., 2011). The AMT1 (ammonium transporter 1) family of unitransporters mediates transfer of ammonium into plants using an electrochemical gradient (Ludewig *et al*., 2007; Kraiser *et al*., 2011), while external proteins, peptides and amino acids are transported across the cell membrane by a variety of transporters and transporter families present in the roots (Rentsch *et al*., 2007; Miller *et al*., 2008; Näsholm *et al*., 2009). Amino acids taken up by plants include both D and L-enantiomers, however, in most cases plants have a very low capacity to metabolize D-amino acids, and increased abundance of internal D-amino acids results in plant toxicity (Svennerstam *et al*., 2007; Näsholm *et al*., 2009). There are two families of transporters that take up NO_3^- from the environment: NRT1 and NRT2. In general NRT1 mediates low-affinity transport of NO_3^- (Tsay *et al*., 2007), while NRT2 proteins mediate high-affinity transport (Williams and Miller, 2001). Both of these NO_3^- transporter families use pH gradients to move N across the cell membrane (Miller *et al*., 2007, 2008). In *Arabidopsis thaliana*, NRT1.1 (also known as CH1) acts as a dual-affinity transporter (Liu *et al*., 1999; Ho *et al*., 2009; Masclaux-Daubresse *et al*., 2010).

NRT1.1 has also been classified as a NO_3^- transceptor, indicating that it not only transports but also senses NO_3^- concentrations and alters N uptake, root growth and transcription of downstream genes accordingly (Gojon *et al*., 2011). It has been known for some time that yeast and animals have transceptors, but not until recently has it become widely accepted that such a system exists in plants (Krouk *et al*., 2006; Gojon *et al*., 2011). The AMT family of ammonium transporters is also thought to potentially have transceptor-like activities, with AMT1;1 (Loqué *et al*., 2007; Lanquar *et al*., 2009) and AMT1;3 (Lima *et al*., 2010) involved in sensing/signaling decreasing ammonium transport and altering root growth respectively (De Michele *et al*., 2013). Recent studies also support the existence of a nitrite-specific transporter in *Arabidopsis thaliana* (Kotur *et al*., 2013), which previous research in both wheat (Jackson *et al*., 1974) and barley (Ibarlucea *et al*., 1983) had supported, but the evidence has remained inconclusive. Such systems allow for plants to effectively and rapidly alter uptake of N as well

as the use of other plant nutrients, including C, H and O, in response to changes in the environment. While NRT1.1 is currently classified as a transceptor in only *Arabidopsis thaliana*, such findings pave the way for research in other plants on previously identified transporter proteins.

N conditions in the soil also regulate root architecture, which is influenced by temperature, circadian rhythm, water availability, soil type and the C/N soil ratio (Kant *et al*., 2011). N availability can also have a direct influence on these other root architecture-regulating changes including plant diurnal rhythms, photosynthesis, internal C concentrations and plant developmental stage (Stitt *et al*., 2002; Gutiérrez *et al*., 2008).

Under N-limiting conditions, there is an inhibition of lateral root growth. The result of these changes is that the C to N ratio in the plant is increased, and there is a decrease in photosynthetic activity as well as early entry into leaf senescence (including discolouration) and flower induction (Paul and Driscoll, 1997; Malamy and Ryan, 2001; Martin *et al*., 2002; Malamy, 2005; Wingler *et al*., 2006; Zhang, 2007; Kant *et al*., 2011; Fischer *et al*., 2013; Gruber *et al*., 2013). In non-limiting N conditions, that are uniform throughout the environment, lateral root emergence is shown to be inhibited. This is contrary to what occurs in pockets of high N in the soil, in which plants have been shown to increase lateral root emergence (Malamy and Ryan, 2001; Forde, 2002; Kant *et al*., 2011). In conditions where N is not limiting, it is the increase in plant internal N concentrations that is believed to decrease lateral root initiation (Zhang and Forde, 1998, 2000). In both N-limiting and non-limiting conditions there are immediate alterations in N uptake and reduction, as well as organic acid and carbon metabolism in congruence with the alterations seen in root architecture (Scheible *et al*., 2004).

In order to fully understand the alterations in plant growth and development that occur both directly and indirectly with changes in environmental N, we must not only understand external sources of N, but also the fate of N molecules that enter the plant. N metabolism in plants has been studied in depth by various researchers, and is relatively well understood (Näsholm *et al*., 2009; Masclaux-Daubresse *et al*., 2010; Kant *et al*., 2011; McAllister *et al*., 2012). It is the secondary and indirect outcomes of primary N uptake and metabolism that are

less well understood and continue to challenge researchers looking to increase not only nitrogen uptake and utilization in plants, but nutrient use efficiency in general.

1.2.3. Primary nitrogen metabolism

N use by plants involves two main steps: uptake and utilization. Utilization can be further compartmentalized into assimilation and translocation/remobilization (Masclaux-Daubresse *et al*., 2010). While plants can sense external N in all forms mentioned above, the presence of both external and internal nitrate is known to affect plant metabolism and alter the expression of specific plant genes, influencing root and shoot morphology, time to flowering and relief of seed dormancy (Bernier *et al*., 1993; Alboresi *et al*., 2005; Zhang *et al*., 2007; Dechorgnat *et al*., 2011).

NO_3^- is taken up from the environment by two main families of transporters, NRT1 and NRT2 (Miller *et al*., 2007). As depicted in Figure 1.5, NO_3^- is converted to NH_4^+ and amino acids for transport within the plant. The initial reduction of NO_3^- to nitrite (NO_2^-) occurs in the cytoplasm and is carried out by nitrate reductase (NR). Further reduction of NO_2^- occurs in the plastid/chloroplast by nitrite reductase, which converts NO_2^- to NH_4^+ (Masclaux-Daubresse *et al*., 2010). Assimilation of NH_4^+ into glutamine and glutamate also takes place in the plastid/chloroplast through the glutamine synthetase/glutamate synthase (GS/GOGAT) system of reactions, allowing plants to assimilate N in both photosynthetic (ferredoxin (Fd)-GOGAT) and non-photosynthetic (NADH-GOGAT) tissues (Suzuki and Knaff, 2005; Masclaux-Daubresse *et al*., 2010; Foyer *et al*., 2011). Once N is taken up by the root, pathways are required to not only assimilate this compound but also transport the various assimilated forms to various plant tissues. Release of ammonium in leaf tissues due to remobilization of nutrients during senescence, as well as photorespiration in C_3 plants, requires that these tissues also have the ability to return N to the amino acid pool to be distributed as the plant requires (Liepman and Olsen, 2003). The carbon skeletons utilized by these reactions are obtained from the tricarboxylic acid (TCA) cycle as seen in Figure 1.6, making these reactions not only essential for N metabolism within the plant, but also of importance to the cycling of C (Lawlor, 2002).

Once N has been taken up and assimilated, it is transported throughout the plant predominantly as glutamine, asparagine, glutamate and aspartate for utilization and storage

(Figure 1.5) (Okumoto and Pilot, 2011); NO_3^-, as well as NH_4^+, can also move throughout the plant, however this is usually at much lower concentrations (Schjoerring *et al*., 2002). The conversion of glutamine to asparagine and glutamate to aspartate requires two cytoplasmic aminotransferase enzymes, asparagine synthetase (ASN) and aspartate aminotransferase (AspAT), respectively (Hodges, 2002; Masclaux-Daubresse *et al*., 2010). These amino acids (and NH_4^+) are transported via the xylem and most often distributed to mesophyll cells where they are either stored or utilized for carbon assimilation (Tegeder and Rentsch, 2010). Chloroplastic proteins are known to make up approximately 80% of the stored N in leaf tissues. Phosphoenolpyruvate carboxylase (PEPc) and GS are abundant plant proteins that are often used for N storage (Beatty and Good, 2011), along with ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco, a carbon fixation enzyme) which has been shown to account for up to 50% of the stored N in C_3 plants, and approximately 20% of stored N in C_4 plants (Mae *et al*., 1993; Beatty and Good, 2011; Kant *et al.*, 2011).

Once N reaches sink tissues, numerous enzymes are involved, either directly or indirectly, in the assimilation of N, including GS1 and glutamate dehydrogenase (GDH) which are involved in assimilation of glutamine and remobilization of N during senescence and grain filling. Both of the enzymes, Fd-GOGAT and GS2 have been shown to be involved in re-assimilation of N during photorespiration (Tobin and Yamaya, 2001; Masclaux-Daubresse *et al*., 2005, 2010; Tabuchi *et al*., 2007). The ability of plants to effectively remobilize N into the maturing fruits or grains is of critical importance to overall NUE, especially in cereal crops where it is the grain that is of economic importance. All of the above mentioned enzymes, and pathways are controlled by many factors including but not limited to, soil N availability, plant N status, external and internal C status as well as changes in plant hormones.

In the last several years is has become apparent that a number of small, non-coding microRNAs (miRNAs) also play key roles in the regulation of N, including the uptake, assimilation and partitioning (Gifford *et al*., 2008; Pant *et al*., 2009; Valdés-López *et al*., 2010; Xu *et al*., 2011; Zhao *et al*., 2011; Cai *et al*., 2012; Liang *et al*., 2012; Trevisan *et al.*, 2012; Zhao et al., 2012; Fischer *et al*., 2013). Comparisons between studies in maize (Xu *et al.*, 2011; Trevisan *et al*., 2012; Zhao *et al*., 2012), bean (Valdés-López *et al*., 2010), rice (Jeong *et al*., 2011; Nischal *et al*., 2012) and Arabidopsis (Gifford *et al*., 2008; Pant *et al*., 2009; Zhao *et al*.,

2011; Liang *et al*., 2012) indicate that there are several conserved miRNAs involved in the regulation of N metabolism. miR156 shows up-regulation in rice, maize and Arabidopsis in response to N starvation, while miR160 and miR447 show up-regulation in just Arabidopsis and rice in response to N starvation (Fischer *et al*., 2013). Those miRNAs showing down-regulation in response to N limitation in all four of the above mentioned species (bean, rice, maize and Arabidopsis) include miR169, miR397, miR399 and miR408, while other miRNAs share down-regulating properties in only two or three of these plant types (Fischer *et al*., 2013). Currently, our understanding of how these non-coding RNA molecules affect the overall physiological response of plants to any change in the environment is minimal. It is clear however, that changes in miRNAs in response to N status of the plant result in multiple physiological changes, including flower maturation, anthocyanin production and root morphology among others. miRNAs are also an example of post-transcriptional controls utilized by plants, which allow for rapid alterations in gene and therefore protein expression, and which could ultimately also be utilized by researchers to alter plant/crop properties in a similar fashion (Trevisan *et al*., 2012). Relating miRNA expression to the already established N uptake, assimilation and mobilization pathways is therefore important and will be fundamental for a full understanding of N metabolism.

1.3. The carbon:nitrogen balance in plants

It has been shown that N levels can significantly affect C fixation and distribution (Makino *et al.,* 1997; Reich *et al*., 2006). N is stored in large quantities in photosynthetic proteins, such as Rubisco and PEPc as mentioned above, two proteins involved in photosynthesis (Nunes-Nesi *et al.*, 2010). Decreases in N assimilation and storage have shown to decrease the overall amount of carbon fixed by the plant (Nunes-Nesi *et al*., 2010). Limitations in soil N when C is abundant have been demonstrated to affect lateral root growth and distribution; high C/low N in control plants tends to initiate lateral root growth (Little *et al*., 2005; Zheng, 2009).

Also crucial to plant C:N ratios are the products of the GS-GOGAT pathway. Glutamate regulates C and N metabolism in both C_3 and C_4 plants. Glutamate acts as a signaling and N transport molecule, and is a substrate in the production of other amino acids and keto-acids, which are fundamental components of C metabolism (ie: pyruvate and 2-oxoglutarate) (Foyer *et al*., 2003; Nunes-Nesi *et al*., 2010). Of key importance to the maintenance and overall

preservation of N metabolism in the plant is glycolysis, which produces substrates that are both directly and indirectly involved in the production of amino acids and therefore plant proteins (Zheng, 2009). PEPc is a key enzyme in the glycolytic pathway, and is involved in production of the keto-acid, 2-oxoglutarate. In high NO_3^- conditions, C_3 plants increase expression of PEPc. The changes that occur in source or sink concentrations, of either C or N, must be coordinated with changing environmental inputs, such as water and nutrient availability and light conditions (Coruzzi and Zhou, 2001).

1.3.1. Pyruvate and 2-oxoglutarate

The availability of glycolytic and TCA cycle intermediates is critical for the C/N balance in plants. As depicted in Figure 1.5, some of these intermediates can be used directly to produce amino acids or indirectly to produce amino acid precursor molecules. Some of these compounds also feed into N pathways for nucleic acid synthesis and hormone biosynthesis (Rocha *et al*., 2010). ATP and reducing compounds NADH and $FADH_2$ are also produced through C metabolism, both of which are involved in N metabolism as well as numerous other cellular functions. TCA cycle intermediates have also shown to be an important part of oxidative stress defense, not only in plants but living organisms in general (Mailloux *et al*., 2007).

Two very important compounds linking C and N metabolism in plants are 2-oxoglutarate (also known as α-ketoglutarate) and pyruvate. These two compounds readily accept amine groups from a variety of amino acid donors, and both compounds also act as the C-backbone for numerous cellular reactions in both the cytoplasm, mitochondria and plastid(s) (Hodges, 2002; Bunik and Fernie, 2009; Nunes-Nesi *et al*., 2010), including the initial assimilation of N in the root plastid, which requires 2-oxoglutarate. 2-oxoglutarate is considered by many to be the major organic acid at the interface of N and C metabolism, not only in plants but also in other organisms (Lancien *et al*., 2000). The ratio of glutamine to 2-oxoglutarate has shown to be a controller of GS activity, and in some cases, specifically in cyanobacteria but also in plants, this keto-acid is a good candidate for a metabolic signal affecting nitrogen efficiency (Hodges, 2002; Lancien *et al*., 2000; Herrero *et al*., 2001; Radchenko *et al*., 2013).

Pyruvate, on the other hand, is a critical precursor for the production of acetyl-coenzyme A (acetyl-CoA), which is the mandatory precursor for the production of fatty acids (Ke *et al*.,

2000); the production of this compound is also required in the glycolytic process regardless of normoxic, hypoxic or anoxic conditions (Good and Muench, 1993; Rocha *et al*., 2010). Pyruvate is also the starting compound for gluconeogenesis, and recently it has been shown that in light, pyruvate can serve to restore the glycolytic intermediate phosphoenolpyruvate (PEP), which is the starting point for the shikimate pathway (Tcherkez *et al*., 2011).

The importance of both pyruvate and 2-oxoglutarate can be observed not only in plants, but numerous other organisms, including bacteria. The significance of these two compounds in relation to amino acid synthesis and overall nitrogen metabolism will be discussed in more detail in a later section.

1.4. Amino acid metabolism

Once external N has been taken up by the plant and assimilated, several different parallel systems exist for the biosynthesis of amino acids (Miller *et al*., 2007). These pathways are regulated by numerous factors, including developmental stage, as well as physiological, environmental, metabolic and stress-derived signals (Galili *et al*., 2008); the biosynthesis of amino acids is most often regulated by end-product feedback inhibition loops (Less and Galili, 2008). A family of enzymes known as transaminases contributes greatly to the levels of amino acids in plants as well as the turnover of amino acids to keto-acids and other non-amino acid products as part of these feedback systems (Mehta *et al*., 1993).

Amino acids serve as N carriers for long distance transport in plants as discussed previously, however, they have numerous other functions within individual cells and the plant as a whole (Okumoto and Pilot, 2011). Serving as the building blocks of proteins, amino acids are essential for proper cell function. They also serve in antioxidant defense, heavy metal detoxification and as signaling molecules within cells, specifically in the case of the amino acids proline, histidine and glutamate (Sharma and Dietz, 2006), and are implicated in feedback inhibition, specifically in the case of glutamine in the uptake of N by roots (Davenport, 2002).

In the case of signaling, ionotropic glutamate receptors (iGluRs), homologous to those that mediate the nervous system in mammals, have been found in plants. These receptors have previously shown to be ligand-selective for the amino acids glutamate, glycine, asparagine and serine, and most recently, a family of iGluRs has been discovered that accepts an even broader

range of amino acid ligands, mostly hydrophobic amino acids, a characteristic unlike the mammalian homologues (Lam *et al*., 1998; Davenport, 2002; Tapken *et al*., 2013). In plants, these receptors have been implicated in numerous functions including: Ca^{2+} allocation, light response, hormone biosynthesis, water balance, coordination of mitotic development, response to aluminum toxicity, control of root activity and sensing of C/N status (Tapken *et al*., 2013).

Unlike most animals, plants, like bacteria, can synthesize all 20 essential amino acids in the L-enantiomer form. (Plants have also shown to utilize amino acids in their D-enantiomeric forms (ie: D-alanine) for N uptake and assimilation (Hill *et al*., 2011).) Along with the 20 essential amino acids synthesized by plants, several non-protein amino acids are also made, many of which are involved in defense (Huang *et al*., 2011). The synthesis of the L-enantiomers of all amino acids in plants can be broken down into roughly six major families: aspartate, serine, aromatic, histidine, pyruvate (including branched chain amino acids), and glutamate. These six families of amino acids will be briefly outlined below.

Aspartate can be derived directly from the amino acid asparagine, or from oxaloacetate, a component of the TCA cycle. From aspartate, a large number of essential amino acids can be manufactured, including: threonine, lysine, methionine and isoleucine as well as the intermediate amino acid homoserine (Figure 1.6) (Jander and Joshi, 2010; Kirma *et al*., 2012). Because this essential amino acid pathway does not exist in animals, it has been the subject of much research, showing a strong link between lysine and cellular energy metabolism (Kirma *et al*., 2012). Methionine is a direct product of the aspartate-derived amino acids however, its synthesis is highly controlled by a separate pathway/cycle of reactions. It plays an important role in the production of S-adenosylmethionine (SAM), which is a substrate for pathways that produce the plant signaling compound ethylene, as well as the nicotianamine and polyamine biosynthesis pathways and the sulphur assimilation pathway (also known as the methionine salvage cycle or Yang cycle) (Sauter *et al*., 2013). Research surrounding this pathway has increased in the last several years as lysine and methionine are the most limiting essential amino acids in both cereal and legume crops. For this reason, increasing the concentration of these compounds in both human foods as well as livestock feeds is of great interest (Kirma *et al*., 2012).

The biosynthesis of the amino acid serine proceeds through two main pathways: the glycolate pathway involved in cellular photorespiration and the 3-phosphoglycerate pathway

(Figure 1.6), which takes place in the plasmid and is involved in non-photosynthetic processes, specifically environmental stresses (Ho and Saito, 2001). Serine can be used to generate both glycine and cysteine; glycine being a key component of photorespiration and cysteine being an important component of sulphur metabolism (Ho and Saito, 2001; Bonner *et al*., 2005; Timm *et al*., 2013). Importantly, in the glycolate pathway, research indicates that serine, not glycine, acts as a metabolic signal, controlling transcription of other genes involved in photorespiration (Timm *et al*., 2013).

The aromatic amino acids, tryptophan, phenylalanine and tyrosine are produced in bacteria, fungi and plants via the shikimate pathway (Figure 1.6). These amino acids play roles in plant defense, development, reproduction and environmental response; tryptophan is a precursor molecule for the plant hormone auxin (Maeda and Dudareva, 2012). It is estimated that >30% of the carbon fixed by the plant is shunted into this pathway, indicating that this pathway is highly coordinated with both C and N metabolism (Maeda and Dudareva, 2012).

The production of the aromatic amino acid tryptophan can also be integrated with that of histidine; histidine biosynthesis can be linked to the production of purines, pyrimidines, pyridine nucleotides, and folates, linking this amino acid tightly with DNA synthesis and repair (Stepansky and Leustek, 2006). In many proteins, histidine can be found as a part of the active site of the enzyme, due to the ability of this amino acid to exist in both protonated and unprotonated forms (Petersen *et al*., 2010). These alternate forms of histidine also make this amino acid ideal for binding of metals, specifically the chelating of Ni^{2+} ions in plant cells (Stepansky and Leustek, 2006; Petersen *et al*., 2010). Histidine is synthesized indirectly from glycolytic products, linking the biosynthesis and regulation of this amino acid tightly with C metabolism.

As a product of glycolysis and a precursor of the TCA cycle, pyruvate is a key component of C metabolism. Pyruvate can also be utilized in the synthesis of amino acids, including alanine, and the branched chain amino acids (BCAAs), valine, leucine and isoleucine (Binder *et al*., 2007). Alanine has a number of functions in plants, which includes being a sink for N under hypoxic conditions (Miyashita *et al*., 2007). BCAAs on the other hand, are often constituents of transmembrane regions of membrane proteins as they have high hydrophobicity (Binder *et al*., 2007).

Glutamate synthesis is also highly linked to C metabolism, as 2-oxoglutarate, a component of the TCA cycle can be used as a direct substrate to generate this amino acid. As well as glutamate, 2-oxoglutarate can be converted to lysine in some organisms including plants. Glutamate can also be used to produce several other amino acids, including proline, arginine, γ-aminobutyric acid (GABA) and glutamine; glutamine being a product of the GS-GOGAT series of reactions discussed previously in primary N assimilation (Forde and Lea, 2007). Ornithine, a metabolic intermediate and non-protein amino acid, is also produced from glutamate, and can be used in combination with arginine to produce proline. Proline is an important component of plant stress response, specifically as an osmoregulator in drought and saline soils (Delauney and Verma, 1993; Hare and Cress, 1997; Szabados and Savouré, 2010). Proline synthesis and accumulation is a direct effect of plant stress, much like that of alanine, and is the result of changes in N metabolism under adverse environmental conditions (Hare and Cress, 1997). It is clear that amino acid metabolism is highly linked to that of carbon, and that amino acids serve more than just protein building blocks. Because amino acid synthesis and therefore N metabolism is dependent on glycolytic products or C metabolism, and vice versa, it is not surprising that in DNA microarray studies over one half of the transcriptome of plants has shown to regulate N, C or both N and C (Zheng, 2009). Understanding the roles of individual components of these systems is critical to understanding the link between these two processes and the overall growth and development of plants.

1.4.1. Glutamate metabolism

Glutamate is a key component of the GS-GOGAT series of reactions as discussed previously, combining with NH_4^+ in the presence of ATP to produce glutamine early on in primary N assimilation (specifically NADH-GOGAT) (Finnemann and Schjoerring, 1998; Schjoerring *et al*., 2002; Suzuki and Knaff, 2005; Masclaux-Daubresse *et al*., 2010). Glutamate can also act in the photorespiratory pathway with NH_4^+ and ATP to produce glutamine in green tissues (Fd-GOGAT) (Suzuki and Knaff, 2005; Zheng, 2009; Masclaux-Daubresse *et al*., 2010). Beyond N assimilation, glutamate also plays other key roles within the plant cell and the plant as a whole.

As discussed above, glutamate is essential for the production of other amino acids, including glutamine, arginine, proline and GABA. GABA, a non-protein amino acid, has shown to be critical in plant defense and signaling, specifically in hypoxic conditions, where increased production of GABA allows plants to survive acidic cellular conditions (Miyashita and Good, 2008). The production of GABA is also imperative for proper seed development, where this compound helps balance C/N ratios (Fait *et al*., 2011) along with the enzymes glutamate synthetase (GS1) and glutamate dehydrogenase (GDH) (Grabowska *et al*., 2012). Production of glutamate for this purpose does not usually come from GS-GOGAT; 2-oxoglutarate from the TCA cycle is the key contributor of the C backbone for the production of glutamate, along with other keto-acids (ie: oxaloacetate) and a variety of amine donors and acceptors including alanine, glutamine, aspartate, NAD^+ and NH_4^+.

Enzymes involved in the catabolism or anabolism of glutamate include, but are not limited to, glutamate synthase (GLT1), glutamate dehydrogenase (GDH), glutamate decarboxylate (GAD), glutamine synthetase (GS), aspartate aminotransferase (AspAT) and alanine aminotransferase (AlaAT). These enzymes allow for glutamate, or glutamate derived products to be shunted into pathways involved in pyrimidine metabolism, purine metabolism, aminosugar metabolism, the TCA cycle, amino acid metabolism and nitrogen metabolism. In addition, TCA cycle intermediates can be limiting (ie: 2-oxoglutarate), and these products are relied on heavily by numerous other pathways for C skeletons, making glutamate concentration important in the maintenance and replenishing of compounds in both the C and N cycles and therefore the junction between N assimilation and energy production (Miflin and Habash 2002; Bunik and Fernie, 2009).

Along with the conversion of glutamate to essential plant products, it can also act as a signaling molecule. Initially it was known that glutamate acted as an homeostatic intracellular signal, to indicate the level of N nutrition (Forde and Lea, 2007; Price *et al*., 2012; Tobin and Yamaya, 2001). Now it is thought that glutamate also acts intercellularly as a response to light, Ca^{2+} channel gating, sensing of C/N, hormone biosynthesis, water balance, stage of development, and resistance to infection and toxicity, through a family of glutamate receptors (iGluRs) (Miller *et al*., 2008; Price *et al*., 2012; Tapken *et al*., 2013). Taken together, glutamate concentration, both at a cellular and an organismal level is critical for proper plant development.

1.4.2. Alanine metabolism

The importance of alanine in plant stress response and N metabolism has been known for some time. Along with the production of GABA, increases in alanine, specifically under hypoxic conditions, contribute greatly to the post-stress recovery of plants (Wallace *et al*., 1984; Good and Muench, 1992; Miyashita *et al*., 2007). In times of hypoxia, plants alter metabolism, lowering the rate of glycolysis and beginning fermentation in order to produce ATP with reduced oxygen (Good and Muench, 1993). Levels of pyruvate, lactate and ethanol accumulate quickly under hypoxic conditions; pyruvate is a primary substrate for the fermentative pathways in plants that produce both lactate and ethanol (Good and Crosby, 1989; Rocha *et al*., 2010). However, production of these fermentative products results in N lost to the plant as well as the creation of toxic internal pH levels. Alanine also accumulates under hypoxic conditions, specifically in plant root tissues using pyruvate as a primary substrate however, increases in this product do not result in either N loss or alterations in internal pH. Evidence indicates that increases in this amino acid allow for conservation of the plant N pool in times of stress, which can then be utilized effectively once aerobic conditions are returned to, unlike the products of fermentation (Miyashita *et al*., 2007).

Since alanine production utilizes pyruvate directly, this amino acid also links C and N metabolism (Figure 1.6) (Rocha *et al*., 2010; McAllister *et al*., 2013). Besides being linked to the TCA cycle, alanine is also involved in photorespiration and C_4 photosynthesis. During photorespiration, alanine acts as a main amino donor in green tissues (Joy, 1988; Liepman and Olsen, 2001, 2003; Bauwe *et al*., 2010), and in C_4 photosynthesis, alanine acts as a four-C carrier molecule along with malate and aspartate, diffusing from mesophyll to bundle sheath cells (Ghannoum, 2009). Alanine, in addition to leucine and glutamine, is also a C and N storage molecule, and can be found in high concentrations in plant vacuoles when amino acid synthesis exceeds cellular demand for such products (Dietz *et al*., 1990).

In general, alanine production and usage contributes to overall plant health and growth, specifically in times of stress. For these reasons, alterations in alanine levels, or those of glutamate as discussed previously, could have a significant impact on the distribution of plant compounds and the overall status of C and N metabolism in plants.

1.5. Nitrogen use efficiency in plants

The definition of plant nutrient use efficiency has varied over time. In some cases, plants are nutrient use efficient if they have the ability to realize a yield above average under conditions of suboptimal nutrient supply (Sattelmacher *et al*., 1994), while in other instances plants are considered nutrient efficient if they take up a certain amount of nutrient relative to that applied to the soil or if they are able to partition or store nutrients in a particular manner (Appalachian Soil and Water Conservation Research Laboratory, 1990). Regardless of the definition, increasing plant nutrient use efficiency has played a major role in increasing overall crop yields that has been required for a growing world population (Fageria *et al*., 2008).

1.5.1. Defining plant nitrogen use efficiency

Traditional plant breeding practices have increased crop yields significantly, and, concomitantly increased the NUE of the crops as well (Hawkesford and Barraclough, 2011). However, there is still a gap between actual yields and attainable yields and between attainable yields and potential yields for particular crops. Actual yields are the currently obtained yields and are defined as the farm yield obtained for a given crop in a given area (Fischer *et al*., 2009). However, actual yields, in most cases, do not accurately reflect the attainable yield of that farmland and that crop, which is the estimated yield when the best technology is utilized along with sensible economics (Fischer *et al*., 2009). The potential yield is the theoretical yield of a crop which is when the best variety is grown with the best agronomy without harmful biotic or abiotic stresses (Singh *et al*., 2001; Fischer *et al*., 2009). The differences in these yields are the yield gap, and the goal of current agricultural practices and technology is to decrease this gap. Estimates of yield gaps in developed countries are often smaller than those in developed countries partly due to the inability to afford high cost N fertilizers, especially in Sub-Saharan Africa where soil nutrient depletion is an issue (Sanchez, 2002). The plateau seen in yield improvement through traditional breeding strategies and nutrient management practices indicates that new solutions are needed in order to decrease yield gaps and to obtain yields closer to the estimated attainable and potential yields (Hawkesford and Barraclough, 2011).

When referring to crop management and nutrient usage, several different definitions and measurements have emerged in order to evaluate overall nutrient use efficiency of crops (Figure

1.7). Generally speaking, nutrient efficiency is based on the amount of applied or available nutrient and two main resulting measurements: 1) the total plant biomass or 2) the total grain weight. These two measurements are often obtained along with other physiological nutrient measurements including uptake of nutrients from the soil and nutrient utilization, which includes mobilization of nutrients, often from root to shoot, and remobilization of nutrients, from a storage tissue or organ to the grain.

The nitrogen use efficiency of plants comprises both uptake and utilization efficiencies, and can be calculated as UpE x UtE = Nt/Ns x Gw/Nt = Gw/Ns; total grain weight divided by the total N (Nt) supplied to the plant. Another approach is to measure the above ground biomass NUE, measured as Sw/Ns, where the dry shoot weight is divided by the N supplied (Good *et al*., 2004; Pathak *et al*., 2011). There are also other NUE calculations that take into account the available soil N prior to fertilizer application (Figure 1.7. gives an overview of NUE calculations).

1.5.2. Constructing nitrogen use efficient plants

Three main ideas have emerged to deal with the problem of reducing N fertilizer application without reducing yield: improved agronomy using best nutrient management practices (BNMP) that are tailored to the crop, climate and location (Goulding *et al*., 2008), engineering of nitrogen fixing cereals (Charpentier and Oldroyd, 2010; Good and Beatty, 2011), and the breeding or engineering of either traditional or transgenic crop plants with increased NUE (Good *et al*., 2007; Shrawat *et al*., 2008).

The most straightforward approach to reducing N fertilizer inputs is to use less N fertilizer on crops. There are various management practices associated with decreased input of N fertilizers with the goal of sustaining yields, however all take into account the four R's of Nutrient Stewardship: right source, right rate, right time and right place (International Plant Nutrition Institute, 2009). Agricultural practices to decrease N losses vary with soil type, as well as crop type. The management practices used are aimed at not only decreasing the soil N lost to leaching and volatization, but also to increasing N taken up by the crops (NUE) (Goulding, 2000). Application of enhanced efficiency fertilizers, such as controlled release fertilizers (CRF) or slow release fertilizers (SRF) has been shown in some cases to decrease N losses to the

environment (Motavalli *et al*., 2008). Timing of fertilizer application, including weather and season have also shown to contribute to fertilizer management practices; N_2O emissions have shown to be affected by freeze-thaw cycles, mostly due to microbial activities (Johnson *et al*., 2007). Using conservation tillage or no-tillage practices has also shown to decrease N leaching while maintaining, and in some cases increasing, crop yields. This is also true for the use of cover crops and crop rotation, using primarily legumes, which allow for decreases in applied fertilizers (Sainju *et al*., 2003; Johnson *et al*., 2007).

Legumes, and other nodulating plants, form a symbiotic relationship with nitrogen fixing soil-borne bacteria (Kraiser *et al*., 2011). These bacteria, which belong to various genera, are broadly referred to as diazotrophic bacteria due to their ability to convert N_2 in the surrounding environment to NH_4^+ through the activity of the enzyme complex nitrogenase, which is composed of both a dinitrogenase and a dinitrogenase reductase (Charpentier and Oldroyd, 2010; Kraiser *et al*., 2011). Due to the ability of oxygen to irreversibly inhibit nitrogenase activity, nodule formation and compartmentalization of bacterial N fixing activity are essential components in the formation of a mutualistic, symbiotic relationship between plant and bacterium (Berman-Frank *et al*., 2003; Good and Beatty, 2011). These bacteria are estimated to contribute to nearly half of all biological nitrogen fixation under low N soil conditions (Gibson *et al*., 2009).

Whether nitrogen fixing cereals are plausible is currently being re-explored. The possibility of crop plants such as wheat, barley and rice being able to effectively fix their own nitrogen, with or possibly without a bacterial symbiont, is extremely appealing given the current status of N fertilizers however, the engineering of such a plant may prove difficult. While it has been determined that not all genes required for N fixation will need to be transferred into cereals, a significant number of genes will still need to be added to the cereal genome, with very little knowledge as to how this may affect the rest of the metabolic processes regularly performed by these plants (Giraud *et al*., 2007; Kouchi *et al*., 2010). While N fixing cereals may be a long term answer to the current N excess problem, the production and use of this technology is still in developmental stages.

The final NUE approach has been to genetically alter previous existing N pathways within plants, specifically cereals. Recently, selective breeding strategies have been utilized to

obtain plant genotypes which maintain yields while grown in N limiting conditions (Acreche and Slafer, 2009; Anbessa *et al*., 2009, 2010; Ortiz-Monasterio *et al*., 1997); these previous strategies have not utilized the power of transformation and the production of transgenic crops. Current research has begun to focus on the use of molecular markers and quantitative trait loci (QTL) analysis to identify regions of the genome associated with NUE. This approach has led to the identification of candidate genes predicted to be involved in NUE (Loudet *et al*., 2003); in many cases these genes are then manipulated using transgenic approaches and the changes in plant NUE monitored (Obara *et al*., 2001; Hirel *et al*., 2007; Fontaine *et al*., 2009).

The number of genes that have been identified using this method is extremely large, and often candidate genes have been chosen based on either chromosome location, or as a result of known functions in the N metabolic pathways in plants (ie: *GS1*) (Yamaya *et al*., 2002; Gallais and Hirel, 2004). This may not be the most effective selection system though. Originally it was thought that genes involved in the initial uptake and assimilation of nitrogen, such as NRT (Loudet *et al*., 2003), *GS1* (Yamaya *et al*., 2002) and asparagine synthetase (*ASN)* (Seiffert *et al*., 2004), would have the most significant impacts on NUE. However, when crop plants engineered with these primary N metabolism genes were constructed, this was not seen. These genes may be too tightly regulated, especially at the protein level, for the over-expression of their genes to show an NUE phenotype. On the other hand, alanine aminotransferase, an enzyme which operates downstream of the previously mentioned proteins and is less tightly regulated, has shown to affect NUE significantly in both alanine aminotransferase over-expressing transgenic canola and rice (Good *et al*. 2007; Shrawat *et al*. 2008; Good and Beatty, 2011).

1.5.3. The benefits of genetically modified plants

There has been much debate over the benefits of genetically modified crops (GMOs). While many argue that it is not "natural" to genetically modify plants and that a full understanding of the future effects of such changes is unknown, specifically in cereal crops which are used for human consumption (Wolfenbarger and Phifer, 2000; Freckleton *et al*., 2004), it is also argued that such measures are required in order to ensure adequate food production for the growing world population in coming years (Ahmad *et al*., 2012). For the most part, consumers have indicated that they are not highly educated in either side of the debate, and it has been shown that increased education on the environmental, health and world benefits of GM

foods can favourably increase the confidence consumers have in GM products and the likelihood of consumption (Lusk *et al*., 2004). In the past several years it has become apparent that there is a place for both GM and non-GM foods in many markets and that the two can co-exist (Pearsall, 2013).

As outlined by the Food and Agricultural Organization of the United Nations (FAO) the benefits of GMOs fall into three categories: agricultural productivity, environmental health and human health (FAO, 2003). In terms of agricultural productivity, first and foremost GM crops have shown, and are expected to continue to have increased stress resistance. These stresses include drought, pest and disease, herbicide and pesticide resistance, salinity, frost and extreme temperatures (FAO, 2003; Park *et al*., 2011; Ahmad *et al*., 2012). Secondly, GM products have the potential to increase nutritional value in foods (Herrera-Estrella, 2000). A key example of this is "Golden Rice", which has been modified to produce ß-carotene in the plant endosperm, an intermediate that can be effectively converted to vitamin A when consumed by humans (Al-Babili and Beyer, 2005). Indirectly, the implementation of both stress resistant crops and those with increased nutritional value could allow for decreased cost to consumers and farmers, respectively (Park *et al*., 2011) .

It has been claimed that transgenic additions to traditional crop plants will reduce biodiversity, however, it has been shown that traditional breeding and agricultural methods are actually a greater threat to biodiversity loss than GM crop production (Ammann, 2005; Freckleton *et al*., 2004). Also of importance to biodiversity is proper resistance management in terms of those crops expressing herbicides and/or pesticides. Maintenance of gene transfer events and the natural occurrence of resistance in populations has shown to be difficult to control and predict, however, several practices are now currently in place which slow such events as resistance evolution (ie: refuge strategy) (Wolfenbarger and Phifer, 2000), apomixes and cleistogamy (Daniell, 2002).

GMOs also promise environmental benefits such as more food from less land, increased shelf lives of fruits and vegetables and decreased fossil fuel production through the use of biofuels (FAO, 2003). The production of crops with features such as increased yield and decreased generation time should result in decreases in the amount of land needed to produce the same amount of food (Park *et al*., 2011). This in turn decreases the impact that agriculture has

on the environment, either in terms of land usage, decreased fertilizer usage or decreased tilling or non-tilling practices (Park *et al*., 2011). Another way to benefit the environment would be to develop crops which restore nutrients to the soil or rehabilitate damaged or infertile soils, known as bioremediation or phytoremediation (Krämer and Chardonnens 2001; FAO, 2003). An example is the uptake and conversion of ionic mercury in the soil into the less toxic elementary form which can then be released back into the soil from the plant (Krämer and Chardonnens, 2001). In addition to the uptake of chemicals and other compounds from the soil, plants can also be used in the production of compounds, specifically in the production of pharmaceuticals (Yoshida and Shinmyo, 2000). The potential benefits of this technology include decreased overall production costs as well as the prospect to more easily and readily produce compounds that are the end products of long metabolic pathways (Yoshida and Shinmyo, 2000).

In terms of human health, the use of GMOs promises to have impacts in the areas of allergens and vaccines (FAO, 2003; Park *et al*. 2011). In 2008 it was shown that the most potent peanut allergens could be silenced using RNAi (Chu *et al*., 2008; Park *et al*., 2011); this approach had also been utilized previously in the case of allergens from apple, rice, ryegrass, tomato and soybean (Tada *et al*., 1996; Bhalla *et al*., 1999; Herman *et al*., 2003; Gilissen *et al*., 2005; Le et al., 2006; Chu *et al*., 2008). Research in the area of plant production of vaccines is newer then that of allergens, however it has been shown that plants can be transgenically modified to produce full length antigens (Rybicki, 2010). Crop plants such as spinach, alfalfa and lettuce, as well as non-food crops such as tobacco have shown to be viable transgenic producers of antigens. Advantages of antigen production through plants include lower cost and greater safety (Guan *et al*., 2013). To this point plants have been used to produce vaccines or antigens for numerous human and animal ailments including avian flu, rabies, Hepatitis B and more recently, Norwalk virus (Lai and Chen, 2012; Guan *et al*., 2013).

The uses and benefits of transgenic plants are far-reaching and research in the areas of agriculture, environment and human health continue to expand on the possible application of such technologies. However, the production and appliance of such technologies, while simple in theory, is not always easily accomplished. The ability to produce GMOs that are not only effective, but also the most effective, safe and efficient is an extensive and expensive process. In the case of NUE, many different methods and tactics have been evoked in order to increase the

uptake, utilization, remobilization, or a combination thereof, of N in plants (McAllister *et al*., 2012). While significant increases in plant NUE have been observed in a number of cases, the ability to understand why these changes occur and to improve on such increases in NUE is of importance.

1.5.4. The importance of enzyme variants

It is well known that gene, and therefore protein evolution is directly dependent on selective pressures applied over time. Due to differences in environmental conditions, even closely related gene family members, which are highly homologous, may have developed differences; these differences can be seen among different species or even within a single organism in order to produce proteins with slightly varied roles. In the case of homologous proteins, while a specific function has been maintained, variations in the overall efficiency or regulation of the protein in the organism as a whole can be observed, specifically in the form of gene families.

The importance of studying protein variants, specifically enzyme variants, is evident from research on a number of enzymes, most prominently in drug-development, specifically in regards to cytochrome P450 1B1 (CYP1B1), a human gene involved in estrogen metabolism as well as the metabolism of procarcinogens. Much work has been done in order to understand natural variations of this enzyme in human populations, and how these variants contribute to both increased rates of cancer and increases in certain types of cancer (Bailey *et al*., 1998; Aklillu *et al*., 2002; Rodriguez-Antona and Ingelman-Sundberg, 2006). On a broader scale, knowledge of allelic differences contributing to drug metabolism and reaction in human populations is currently being used towards the development of pharmacogenomics: the individualization of drug treatments based on the patients' genetic information (e.g. slow or fast metabolizers) (Phillips *et al*., 2001).

The study of enzyme variants has also proven to be valuable in agriculture. Variants of the plant enzyme ACC synthase have been studied extensively as a means of extending fruit shelf life. This enzyme plays a role in the production of ethylene, a compound (plant hormone) directly regulating plant growth and development as well as contributing to the rate of fruit ripening (Nakatsuka *et al*., 1997). Knowledge of differential temporal and spatial regulation

among a variety of homologous ACC synthase enzymes in tomato allowed for engineering of fruit with an extended shelf life (Xiong *et al*., 2005). This knowledge of tomato ACC synthase variants led to the study of such variants in other fruits, such as apples (Sunako *et al*., 1999). The nutritional value of foods has also been improved due to increased knowledge of enzyme variants. This is best illustrated by "Golden Rice"; genetic modification of the rice genome by insertion of both a daffodil and bacterial gene producing beta-carotene for synthesis of Vitamin A (Paine *et al*., 2005). The insertions of appropriate genes into rice required an intimate knowledge of reaction rates as well as spatial and temporal regulation, especially since genes producing ß-carotene exist naturally in rice, but do not produce this compound in the endosperm or grain component of the plant. Analysis of numerous ß-carotene producing enzyme variants, as well as an intricate knowledge of the ß-carotene producing pathway, allowed for a second variety of Golden Rice to be produced several years after the original. This "Golden Rice 2" variety replaced the daffodil gene with a homologue from maize, allowing for even greater carotenoid production (Paine *et al*., 2005).

Gene shuffling is another tool that has been used to obtain highly efficient enzymes based on the ability to create novel enzyme variants which may not occur naturally. Prominent examples of the usefulness of this technique are both glyphosate resistant and herbicide resistant seeds (Castle *et al*., 2004). The method of site-directed mutagenesis has also shown extreme promise in the creation of enzyme variants. With former knowledge of enzyme variation and how this variation in turn affects enzyme kinetics and substrate specificity, site-directed changes can alter enzymes to create enhanced characteristics, as seen for pharmaceutical production of the anti-diabetic compound sitagliptin (Savile *et al*., 2010). These site-directed changes have in many cases resulted in a method of producing compounds and bioconjugates more efficiently as well as more economically (Savile *et al*., 2010; Matsumoto *et al*., 2012).

1.5.5. Alanine aminotransferase as an enzyme involved in plant NUE

Previous research has shown that tissue-specific ectopic expression of the gene alanine aminotransferase (AlaAT) (Figure 1.8) from barley (*Hordeum vulgare*; *HvAlaAT*) in both canola (Good *et al*. 2007) and rice (Shrawat *et al*., 2008) results in increased NUE in these plants. Hypothetically, the manipulation of genes such as NR, NiR, GS and GOGAT should affect plant

NUE however, greenhouse and field experiments of plants with modifications of these enzymes have not produced consistent NUE phenotypes. Due to these previous disappointments, the observation that crop plants over-expressing alanine aminotransferase (AlaAT) have enhanced NUE (Good *et al*. 2007; Shrawat *et al*. 2008) has been considered surprising, because AlaAT was previously not considered a key component of primary N metabolism.

While over-expressing *AlaAT* in a study of drought stress and tolerance, it was discovered that over-expression of a barley *AlaAT* in *Brassica napus* (canola) under the control of a tissue specific promoter (*btg26*) resulted in increased yield and biomass under N limiting conditions compared to control plants (Good *et al*. 2007; Good and Beatty 2011). *AlaAT* expression studies utilizing constitutive promoters indicated that tissue-specific expression is required to produce this NUE phenotype in canola, and that this phenotype is observed under N-limiting conditions only (Good *et al*. 2007). Eventually, differences in NUE in *AlaAT* over-expressing plants were correlated with increased alanine levels as well as increased mobilization of alanine and increased uptake of nitrate in roots. *AlaAT* over-expressing plants also showed higher N uptake efficiency during vegetative growth (Good *et al*. 2007). Further analysis of *AlaAT* over-expression was carried out in rice utilizing a rice *btg26* homologue, *OsAnt1* (Shrawat *et al*., 2008). Rice plants over-expressing AlaAT and grown in N-limiting conditions showed increased biomass (denser, bushier plants with increased tiller number) and yield, as well as increases in total N and key metabolites (glutamine, glutamate and asparagine) (Figure 1.9) (Shrawat *et al*., 2008).

While the effects of AlaAT enzyme over-expression have been well quantified, many questions about the reasons for an NUE phenotype in these transgenic plants remain. In order to fully understand the results of over-expressing *HvAlaAT* in rice and canola, an awareness of how *AlaAT* over-expression affects plant N metabolism is required, including the impacts of increased AlaAT in the plant system, the importance of a tissue-specific promoter and the importance of the specific *AlaAT* homologue on the NUE phenotype.

AlaAT converts alanine and 2-oxoglutarate to pyruvate and glutamate in the presence of the cofactor PLP (Figure 1.8). This reaction is known to take place in the cytosol, mitochondria and the peroxisome and is involved in numerous stages of plant growth and development as well as plant stress response (ie: hypoxia) (Liepman and Olsen, 2003; Miyashita *et al*., 2007;

McAllister *et al*., 2013). What is not well understood is the effect of over-expressing this enzyme in a plant system and the overall effect that this has on the partitioning of plant metabolites in both C and N metabolism in order to create an NUE phenotype.

1.6. Goal of this thesis

While N metabolic pathways have been well studied and characterized, very little is known about the underlying reasons for genotypic diversity regarding NUE. QTL analysis, while a good starting point, has shown little progress in the way of pinpointing specific gene products regulating NUE, and analysis and alteration of primary N metabolites in a variety of plant species has shown to be largely unsuccessful in regards to improving NUE. *AlaAT* over-expressing plants are one of a handful of transgenically modified plants showing increased NUE in both greenhouse and field experiments, yet very little is understood about why or how over-expression of this enzyme produces plants that are more nitrogen use efficient. Therefore, this thesis research was targeted to address the questions surrounding the molecular role of AlaAT on plant NUE. Using both *Escherichia coli* and *Arabidopsis thaliana* as model systems, the specific objectives of this study were:

1. To determine if the kinetic properties of a variety of AlaAT enzymes are significantly different *in vitro* from barley AlaAT (HvAlaAT), through analysis of the K_M values for the enzyme substrates, by recombinant expression of AlaAT proteins in *E. coli*.

2. Characterization of NUE phenotype(s) in the model plant *Arabidopsis thaliana* when a variety of homologous *AlaAT* genes under various gene promoters are expressed, by monitoring homozygous plants expressing AlaAT enzymes in both wildype (Col-0) and alaat double knockout (*alaat1;2*) backgrounds.

3. Characterization of the uptake efficiency as well as utilization efficiency of *Arabidopsis thaliana* plants expressing a variety of AlaAT enzymes under various gene promoters by analyzing the growth of transgenic plants in soilless medium, the concentration of soluble sugars in shoot tissues and mesophyll protoplast uptake of N-labelled radioisotopes.

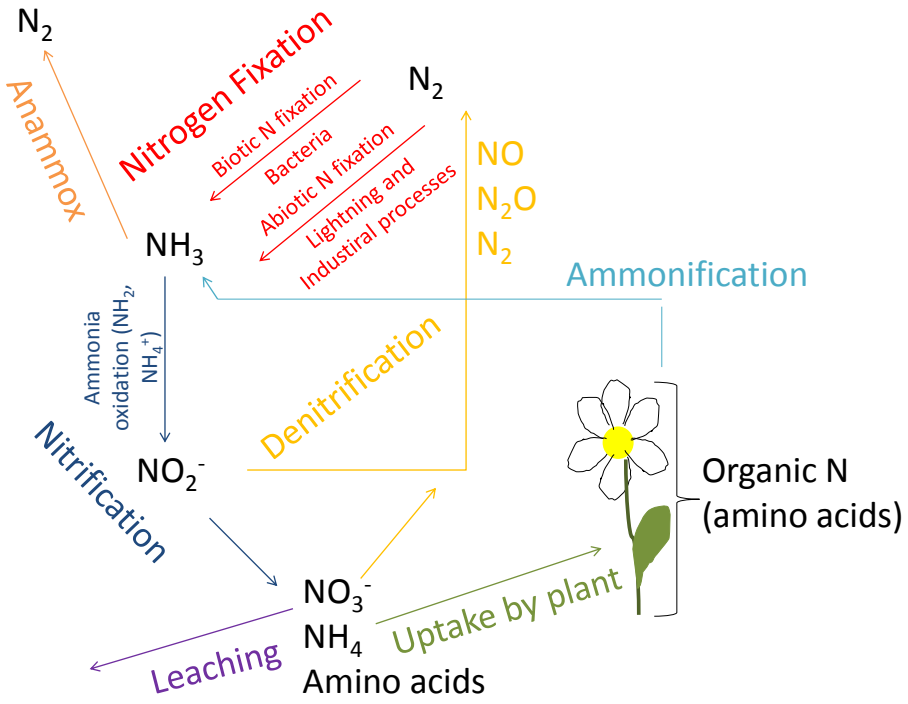


Figure 1.1. Representation of the nitrogen cycle

Atmospheric N_2 is fixed by biotic (prokaryotes) or abiotic (lightning or industrial) processes to become ammonia (NH_3). As a gas, NH_3 is subject to anammox, or volatilization, which results in a spontaneous production of N_2. Through nitrification, ammonia can be converted to amidogen (NH_2) or ammonium (NH_4^+) but generally is oxidized by bacteria to become a NO_x derivative (nitrogen dioxide, NO_2^-, and then nitrate, NO_3^-). Both NO_2^- and NO_3^- can be subject to denitrification by bacteria. NO_3^-, a water soluble compound can then be taken up by the plant, or remain in the soil contributing to leaching and run-off. NH_4^+ can also be taken up by the plant along with organic N. Organic N compounds, in the soil or in decaying matter, can be converted to NH_3 through the process of ammonification.

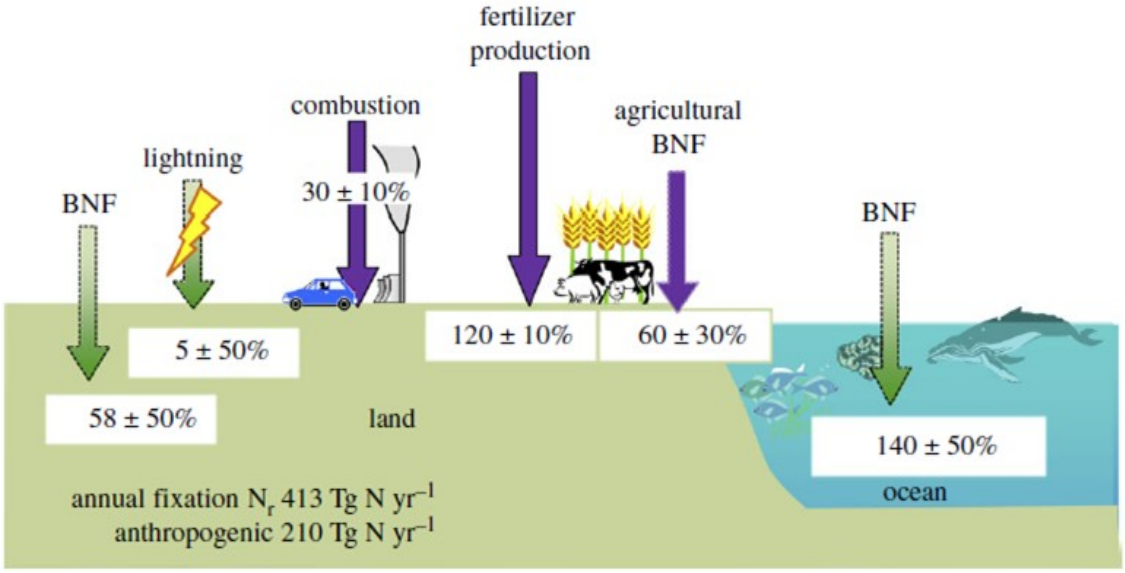


Figure 1.2. Schematic representation of both natural and anthropogenic nitrogen fixation.

Global nitrogen fixation in 2010 through both anthropogenic and natural methods including, biological nitrogen fixation (terrestrial and marine), lightning, fertilizer production and industrial production including nitrogen produced through the Haber-Bosch process. N_r = reactive oxygen species. Arrows represent the transfer of N_2 to other nitrogenous sources. Green arrows represent natural nitrogen fixation, purple arrows represent anthropogenic nitrogen fixation. Taken from Fowler *et al*. (2013).

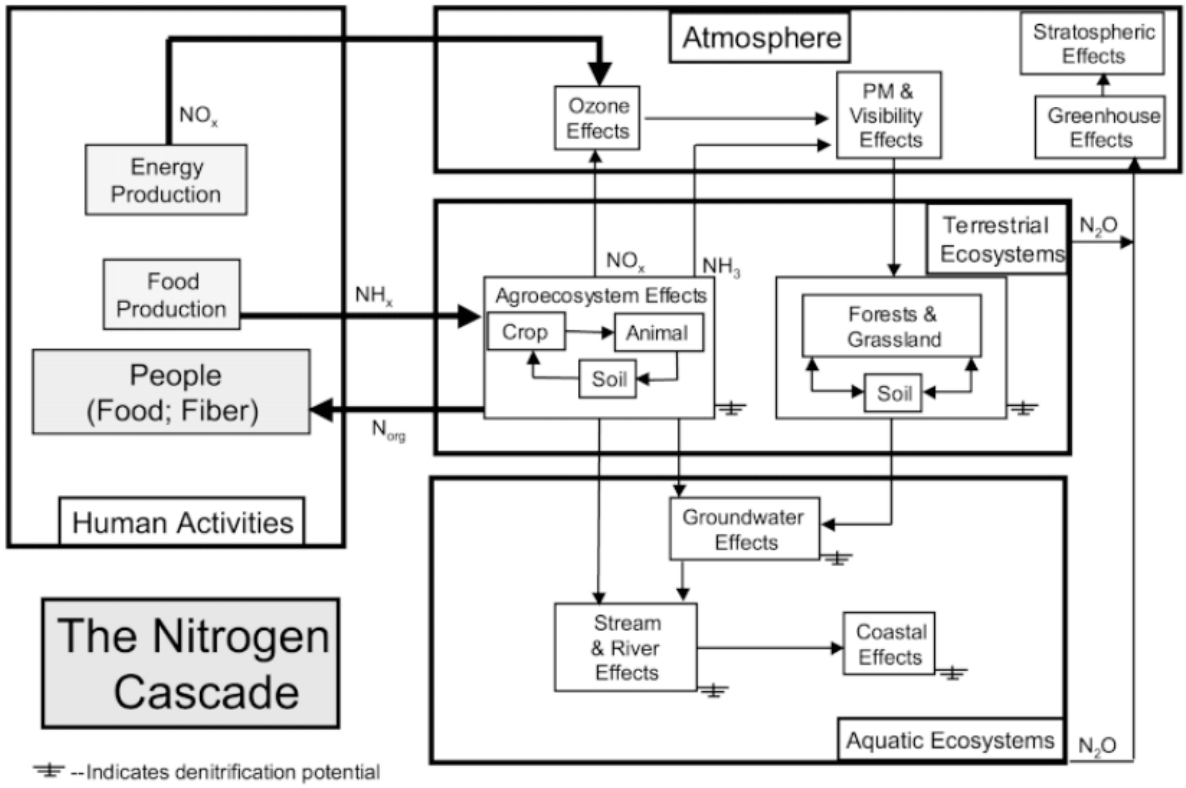


Figure 1.3. The impact of anthropogenic N_r on various ecosystems.

An overview of human-produced N_r as it cycles among aquatic, terrestrial and atmospheric ecosystems and the effect these emissions have on these environments. N_r production from anthropogenic activities can be created through energy production, food production or as a direct product of people. Taken from Galloway and Cowling (2002).

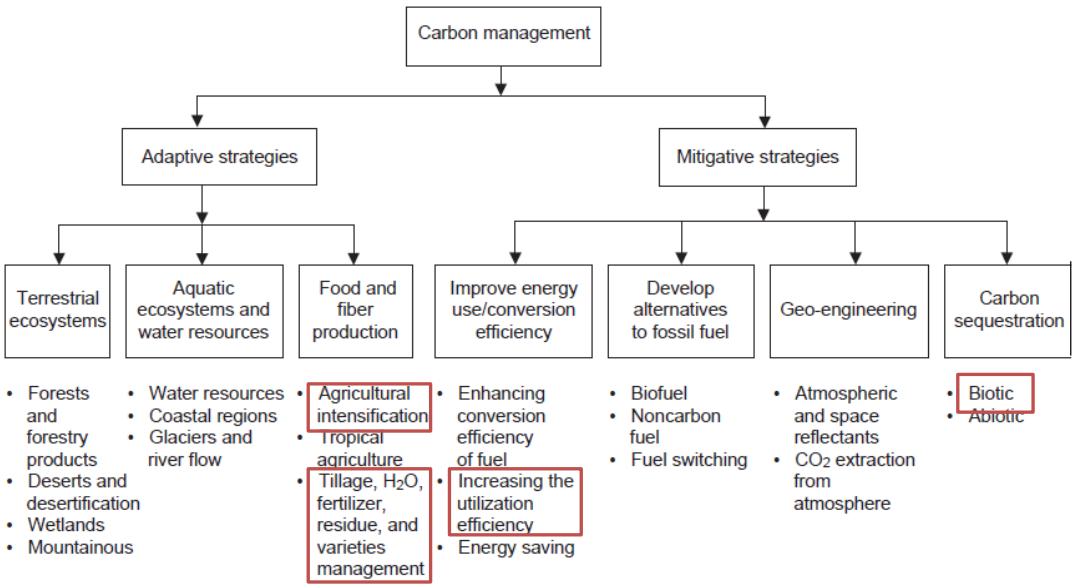


Figure 1.4. Strategies to manage carbon and other greenhouse gases.

Adaptive and mitigative strategies, both terrestrial and marine, for decreasing greenhouse gas emissions, specifically CO_2. Those strategies directly related to decreases in nitrogenous fertilizer usage have been outlined. Modified from Lal (2004).

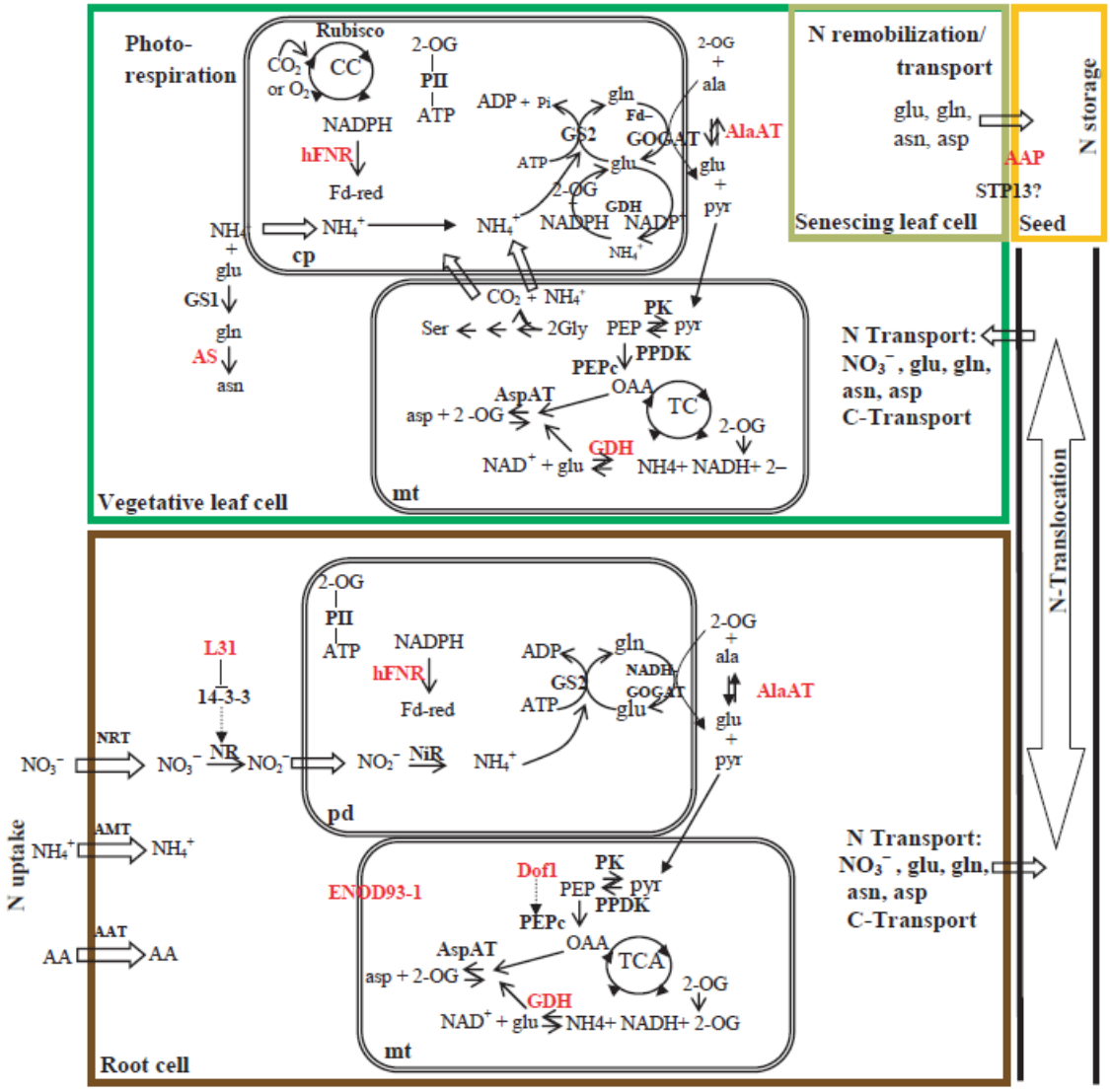


Figure 1.5. Overview of nitrate uptake, assimilation and remobilization in plants.

Dashed arrows represent transcript regulation, large white arrows represent transport across membranes and stick arrows represent an enzymatic reaction. Mt, mitochondria; pd, plastid; cp, chloroplast; AA, amino acids; AAT, amino acid transporter; AMT, ammonium transporter; NRT, nitrate transporter; 2-OG, 2-oxoglutarate; PK, pyruvate kinase; CC, Calvin cycle. From McAllister *et al*. (2012).

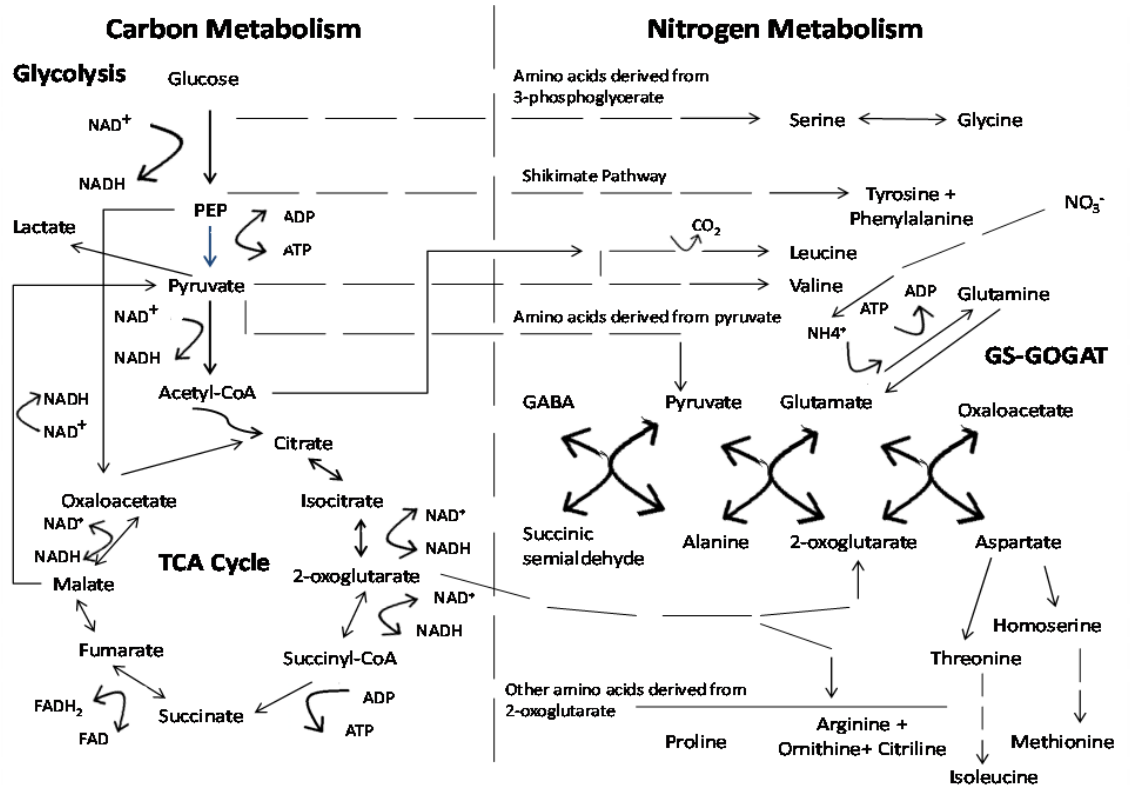
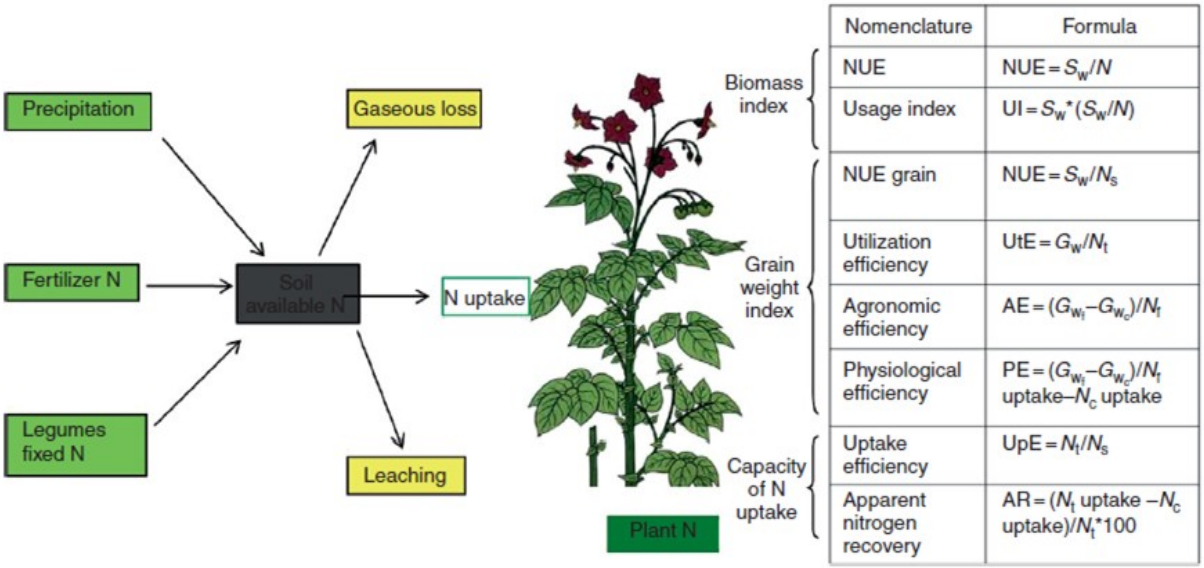


Figure 1.6. Overview of carbon and nitrogen metabolism in plants.

The diagram illustrates the relationship between carbon and nitrogen metabolism in the cell, not including carbon produced from photosynthetic pathways or photorespiration. PEP: phosphoenol pyruvate; ATP: adenosine triphosphate; ADP: adenosine diphosphate; NAD+: nicotinamide adenine dinucleotide; NADH: nicotinamide adenine dinucleotide (reduced); FAD: flavin adenine dinucleotide; $FADH_2$: flavin adenine dinucleotide (reduced); GS-GOGAT: glutamine synthetase - glutamate synthase series of reactions. Modified from Rocha *et al*. (2010).



Nomenclature	Formula
NUE	$NUE = S_w/N$
Usage index	$UI = S_w^*(S_w/N)$
NUE grain	$NUE = S_w/N_s$
Utilization efficiency	$UtE = G_w/N_t$
Agronomic efficiency	$AE = (G_{w_t} - G_{w_c})/N_f$
Physiological efficiency	$PE = (G_{w_t} - G_{w_c})/N_t$ uptake$-N_c$ uptake
Uptake efficiency	$UpE = N_t/N_s$
Apparent nitrogen recovery	$AR = (N_t$ uptake $- N_c$ uptake$)/N_t^*100$

Figure 1.7. The key events in plant nitrogen uptake and utilization.

S_w: shoot weight; N: total nitrogen content of shoots; G_w: grain weight; N_s: nitrogen supplied in gram/plant; N_t: total nitrogen in plant; G_{wf}: grain weight with fertilizer; G_{wc}: grain weight without fertilizer; N_f: nitrogen fertilizer applied; N_c: plant nitrogen uptake unfertilized; PE: physiological N-use efficiency. Taken from Pathak *et al*. (2011).

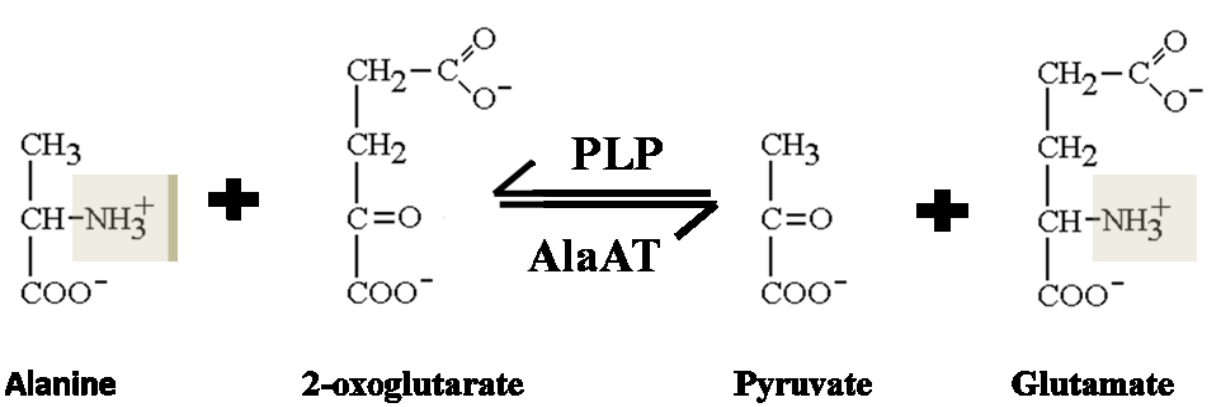


Figure 1.8. A reaction catalyzed by alanine aminotransferase.

Alanine aminotransferase catalyses the reversible transfer of an amine group from alanine to 2-oxoglutarate in the presence of the cofactor pyridoxal-5'-phosphate (PLP) to produce both pyruvate and glutamate or vice versa. The enzyme uses a ping-pong-bi-bi mechanism to accomplish this transfer, allowing each substrate into the active site individually, producing the products in a subsequent fashion.

1.7. Bibliography

Acreche, M. M. and Slafer, G. A. (2009) Variation of grain nitrogen content in relation with grain yield in old and modern Spanish wheats grown under a wide range of agronomic conditions in a Mediterranean region. *J. Agri. Sci.* **147**: 657-667.

Agriculture and Agri-Food Canada (2012) *Canadian farm fuel and fertilizer: Prices and expenses* (Vol. 4). Winnipeg, Manitoba, Canada. http://www.cfi.ca/_documents/A27-32-4-1-eng.pdf

Ahmad, P., Ashraf, M., Younis, M., Hu, X., Kumar, A., Akram, N. A. and Al-Qurainy, F. (2012) Role of transgenic plants in agriculture and biopharming. *Biotechnol. Adv.* **30**: 524–540.

Aklillu, E., Oscarson, M., Hidestrand, M., Leidvik, B., Otter, C. and Ingelman-Sundberg, M. (2002) Functional analysis of six different polymorphic CYP1B1 enzyme variants found in an Ethiopian population. *Mol. Pharmacol.* **61:** 586–594.

Al-Babili, S. and Beyer, P. (2005) Golden Rice--five years on the road--five years to go? *Trends Plant Sci.* **10**: 565–573.

Alboresi, A., Gestin, C., Leydecker, M.-T., Bedu, M., Meyer, C. and Truong, H.-N. (2005) Nitrate, a signal relieving seed dormancy in Arabidopsis. *Plant Cell Environ.* **28:** 500–512.

Ammann, K. (2005) Effects of biotechnology on biodiversity: herbicide-tolerant and insect-resistant GM crops. *Trends Biotechnol.* **23:** 388–394.

Anbessa, Y., Juskiw, P., Good, A., Nyachiro, J. and Helm, J. (2009) Genetic variability in nitrogen use efficiency of spring barley. *Crop Sci.* **49:** 1259-1269.

Appalachian Soil and Water Conservation Research Laboratory. (1990) *Crops as enhancers of nutrient use*. (V. C. Baligar & R. Duncan, Eds.) (pp. 132–133). San Diego, California: Academic Press.

Bailey, L. R., Roodi, N., Dupont, W. D. and Parl, F. F. (1998) Association of cytochrome P450 1B1 (CYP1B1) polymorphism with steroid receptor status in breast cancer. *Cancer Res.* **58:** 5038–5041.

Bauwe, H., Hagemann, M. and Fernie, A. R. (2010) Photorespiration: players, partners and origin. *Trends Plant Sci.* **15:** 330–336.

Beatty, P. H. and Good, A. G. (2011) Future prospects for cereals that fix nitrogen. *Science* **333:** 416–417.

Berman-Frank, I., Lundgren, P. and Falkowski, P. (2003) Nitrogen fixation and photosynthetic oxygen evolution in cyanobacteria. *Res. Microbiol.* **154:** 157–164.

Bernhard, A. (2012) The nitrogen cycle: processes, players and human impact. *Nature Education Knowledge* **3:** 25.

Bernier, G., Havelange, A., Houssa, C., Petitjean, A. and Lejeune, P. (1993) Physiological signals that induce flowering. *Plant Cell* **5:** 1147–1155.

Bhalla, P. L., Swoboda, I. and Singh, M. B. (1999) Antisense-mediated silencing of a gene encoding a major ryegrass pollen allergen. *P. Natl. Acad. Sci. USA* **96:** 11676–11680.

Binder, S., Knill, T. and Schuster, J. (2007) Branched-chain amino acid metabolism in higher plants. *Physiol. Plantarum* **129:** 68–78.

Bonner, E. R., Cahoon, R. E., Knapke, S. M. and Jez, J. M. (2005) Molecular basis of cysteine biosynthesis in plants: structural and functional analysis of O-acetylserine sulfhydrylase from *Arabidopsis thaliana*. *J. Biol. Chem.* **280:** 38803–38813.

Bouwman, A. F., Beusen, A. H. W., Griffioen, J., Van Groenigen, J. W., Hefting, M. M., Oenema, O., Van Puijenbroek, P. J. T. M., Seitzinger, S., Slomp, C. P. and Stehfest, E. (2013) Global trends and uncertainties in terrestrial denitrification and N_2O emissions. *Philos. Trans. R. Soc. of Lond. B. Biol. Sci.* **368:** 20130112.

Bowden, R. D., Rullo, G., Stevens, G. R. and Steudler, P. A. (2000) Soil fluxes of carbon dioxide, nitrous oxide, and methane at a productive temperate deciduous forest. *J. Environ. Qual.* **29:** 268–276.

Brown, P. H., Welch, R. M. and Cary, E. E. (1987) Nickel: a micronutrient essential for higher plants. *Plant Physiol.* **85:** 801–803.

Bunik, V. I. and Fernie, A. R. (2009) Metabolic control exerted by the 2-oxoglutarate dehydrogenase reaction: a cross-kingdom comparison of the crossroad between energy production and nitrogen assimilation. *Biochem. J.* **422:** 405–421.

Butterbach-Bahl, K., Breuer, L., Gasche, R., Willibald, G. and Papen, H. (2002) Exchange of trace gases between soils and the atmosphere in Scots pine forest ecosystems of the northeastern German lowlands: 1. Fluxes of N_2O, NO/NO_2 and CH_4 at forest sites with different N-deposition. *Forest Ecol. Manag.* **167:** 123–134.

Cai, H., Lu, Y., Xie, W., Zhu, T. and Lian, X. (2012) Transcriptome response to nitrogen starvation in rice. *J. Biosciences* **37:** 731–747.

Castle, L. A., Siehl, D. L., Gorton, R., Patten, P. A., Chen, Y. H., Bertain, S., Cho, H.-J., Duck, N., Wong, J., Liu, D. and Lassner, M. W. (2004) Discovery and directed evolution of a glyphosate tolerance gene. *Science* **304:** 1151–1154.

Cattivelli, L., Rizza, F., Badeck, F.-W., Mazzucotelli, E., Mastrangelo, A. M., Francia, E., Marè, C., Tondelli, A. and Stanca, A. M. (2008) Drought tolerance improvement in crop plants: An integrated view from breeding to genomics. *Field Crop Res.* **105:** 1–14.

Charpentier, M. and Oldroyd, G. (2010) How close are we to nitrogen-fixing cereals? *Curr. Opin. Plant Biol.* **13:** 556–564.

Chu, Y., Faustinelli, P., Ramos, M. L., Hajduch, M., Stevenson, S., Thelen, J. J., Maleki, S. J., Cheng, H. and Ozias-Akins, P. (2008) Reduction of IgE binding and nonpromotion of Aspergillus flavus fungal growth by simultaneously silencing Ara h 2 and Ara h 6 in peanut. *J. Agr. Food Chem.* **56:** 11225–11233.

Clarkson, D. T. (1985) Factors affecting mineral nutrient acquisition by plants. *Ann. Rev. Plant Physio.* **36:** 77–115.

Compton, J. E., Harrison, J. A., Dennis, R. L., Greaver, T. L., Hill, B. H., Jordan, S. J., Walker, H. and Campbell, H. V. (2011) Ecosystem services altered by human changes in the nitrogen cycle: a new perspective for US decision making. *Ecol. Lett.***14:** 804–815.

Coruzzi, G. M. and Zhou, L. (2001) Carbon and nitrogen sensing and signaling in plants: emerging "matrix effects". *Curr. Opin. Plant Biol.* **4:** 247–253.

Daniell, H. (2002) Molecular strategies for gene containment in transgenic crops. *Nat. Biotechnol.* **20:** 581–586.

Davenport, R. (2002) Glutamate receptors in plants. *Ann. Bot.* **90:** 549–557.

Davies, W. P. (2003) An historical perspective from the Green Revolution to the gene revolution. *Nutr. Rev.* **61:** S124-134.

De Bossoreille de Ribou, S., Douam, F., Hamant, O., Frohlich, M. W. and Negrutiu, I. (2013) Plant science and agricultural productivity: why are we hitting the yield ceiling? *Plant Sci.* **210:** 159–176.

De Michele, R., Ast, C., Loqué, D., Ho, C.-H., Andrade, S. La, Lanquar, V. and Frommer, W. B. (2013) Fluorescent sensors reporting the activity of ammonium transceptors in live cells. *eLife*, **2:** e00800.

Dechorgnat, J., Nguyen, C. T., Armengaud, P., Jossier, M., Diatloff, E., Filleur, S. and Daniel-Vedele, F. (2011) From the soil to the seeds: the long journey of nitrate in plants. *J. Exp. Bot.* **62:** 1349–5139.

Delauney, A. J. and Verma, D. P. S. (1993) Proline biosynthesis and osmoregulation in plants. *Plant J.* **4:** 215–223.

Dietz, K., Jager, R., Kaiser, G. and Martinoia, E. (1990) Amino acid transport across the tonoplast of vacuoles isolated from barley mesophyll protoplasts. *Plant Physiol.* **92:** 123–129.

Dunn, J. B., Mueller, S., Kwon, H.-Y. and Wang, M. Q. (2013) Land-use change and greenhouse gas emissions from corn and cellulosic ethanol. *Biotechnology for Biofuels* **6:** 51.

Erisman, J. W., Galloway, J. N., Seitzinger, S., Bleeker, A., Dise, N. B., Petrescu, A. M. R. and de Vries, W. (2013) Consequences of human modification of the global nitrogen cycle. *Philos. Trans. R. Soc. of Lond. B. Biol. Sci.* **368:** 20130116.

Erisman, J. W., Sutton, M. A., Galloway, J., Klimont, Z. and Winiwarter, W. (2008) How a century of ammonia synthesis changed the world. *Nat.Geosci.* **1:** 636–639.

Fageria, N. K., Baligar, V. C. and Li, Y. C. (2008). The role of nutrient efficient plants in improving crop yields in the twenty first century. *J. Plant Nutr.* **31:** 1121–1157.

Fait, A., Nesi, A. N., Angelovici, R., Lehmann, M., Pham, P. A., Song, L., Haslam, R. P., Napier, J. A., Galili, G. and Fernie, A. (2011) Targeted enhancement of glutamate-to-γ-aminobutyrate conversion in Arabidopsis seeds affects carbon-nitrogen balance and storage reserves in a development-dependent manner. *Plant Physiol.* **157:** 1026–1042.

FAO (2003). Weighing the GMO arguments: for. *FAO Newsroom*.

FAO (2008). *Current world fertilizer trends and outlook to 2011/12*. Rome.

FAO (2011). *Current world fertilizer trends and outlook to 2015*. Rome.

Fenn, M. E., Poth, M. A. and Johnson, D. W. (1996) Evidence for nitrogen saturation in the San Bernardino Mountains in southern California. *Forest Ecol. Manag.* **82:** 211–230.

Fernández, F. G. and Hoeft, R. G. (2009) Managing soil pH and crop nutrients. *Illinois Agronomy Handbook* (24th ed., pp. 91–112). Urbana, IL: University of Illinois.

Finnemann, J. and Schjoerring, J. K. (2000) Ammonium and soluble amide-bound nitrogen in leaves of *Brassica napus* as related to glutamine synthetase activity and external N supply. *Plant Physiol. Biochem.* **36:** 339–346.

Fischer, J. J., Beatty, P. H., Good, A. G. and Muench, D. G. (2013) Manipulation of microRNA expression to improve nitrogen use efficiency. *Plant Sci.* **210:** 70–81.

Fischer, R. A., Byerlee, D. and Edmeades, G. O. (2009) Can technology deliver on the yield challenge of 2050? *FAO: Expert Meeting on How to feed the World in 2050* (Vol. 2050, pp. 24–26).

Fontaine, J.-X., Ravel, C., Pageau, K., Heumez, E., Dubois, F., Hirel, B. and Le Gouis, J. (2009) A quantitative genetic study for elucidating the contribution of glutamine synthetase, glutamate dehydrogenase and other nitrogen-related physiological traits to the agronomic performance of common wheat. *Theor. Appl. Genet.* **119:** 645–662.

Forde, B. G. (2000) Nitrate transporters in plants: structure, function and regulation. *Biochim. Biophys. Acta* **1465:** 219–235.

Forde, B. G. (2002) Local and long-range signaling pathways regulating plant responses to nitrate. *Ann. Re. Plant Biol.* **53:** 203–224.

Forde, B. G. and Lea, P. J. (2007) Glutamate in plants: metabolism, regulation, and signalling. *J. Exp. Bot.* **58:** 2339–2358.

Fowler, D., Coyle, M., Skiba, U., Sutton, M. A., Cape, J. N., Reis, S. and Bouwman, A. F. (2013) The global nitrogen cycle in the twenty-first century. *Philos. Trans. R. Soc. of Lond. B. Biol. Sci.* **368:** 20130164.

Foyer, C. H., Parry, M. and Noctor, G. (2003) Markers and signals associated with nitrogen assimilation in higher plants. *J. Exp. Bot.* **54:** 585–593.

Foyer, C. H., Noctor, G. and Hodges, M. (2011) Respiration and nitrogen assimilation: targeting mitochondria-associated metabolism as a means to enhance nitrogen use efficiency. *J. Exp. Bot.* **62:** 1467–1482.

Freckleton, R. P., Stephens, P. A, Sutherland, W. J. and Watkinson, A. R. (2004) Amelioration of biodiversity impacts of genetically modified crops: predicting transient versus long-term effects. *Proc R. Soc B-Biol Sci.* **271:** 325–31.

Frugier, F., Kosuta, S., Murray, J. D., Crespi, M. and Szczyglowski, K. (2008) Cytokinin: secret agent of symbiosis. *Trends Plant Sci.* **13:** 115–120.

Galili, S., Amir, R. and Galili, G. (2008) Chapter 3. Genetic Engineering of Amino Acid Metabolism in Plants. *Advances in Plant Biochemistry and Molecular Biology, Volume 1* (Vol. 1, pp. 49–80). Elsevier Ltd.

Gallais, A. and Hirel, B. (2004) An approach to the genetics of nitrogen use efficiency in maize. *J. Exp. Bot.* **55:** 295–306.

Galloway, J. N. and Cowling, E. B. (2002) Reactive nitrogen and the world: 200 years of change. *Ambio*. **31:** 64–71.

Galloway, J. N., Leach, A. M., Bleeker, A. and Erisman, J. W. (2013) A chronology of human understanding of the nitrogen cycle. *Philos. Trans. R. Soc. of Lond. B. Biol. Sci.* **368:** 20130120.

Galloway, J. N., Townsend, A. R., Erisman, J. W., Bekunda, M., Cai, Z., Freney, J. R., Martinelli, L. A., Seitzinger, S. P. and Sutton, M. A. (2008) Transformation of the nitrogen cycle: recent trends, questions, and potential solutions. *Science* **320:** 889–892.

Ghannoum, O. (2009) C4 photosynthesis and water stress. *Ann. Bot.***103:** 635–644.

Gibson, K. E., Kobayashi, H. and Walker, G. C. (2009) Molecular determinants of a symbiotic chronic infection. *Ann. Rev. Genet*. **42:** 413–441.

Gifford, M. L., Dean, A., Gutierrez, R. A, Coruzzi, G. M. and Birnbaum, K. D. (2008) Cell-specific nitrogen responses mediate developmental plasticity. *P. Natl. Acad. Sci. USA* **105:** 803–808.

Gilissen, L. J. W. J., Bolhaar, S. T. H. P., Matos, C. I., Rouwendal, G. J. A., Boone, M. J., Krens, F. A., Zuidmeer, L., Van Leeuwen, A., Akkerdaas, J., Hoffmann-Sommergruber, K., Knulst, A. C., Bosch, D., Van de Weg, W. E. and Van Ree, R. (2005) Silencing the major apple allergen Mal d 1 by using the RNA interference approach. *J. Allergy Clin. Immin.* **115:** 364–369.

Giraud, E., Moulin, L., Vallenet, D., Barbe, V., Cytryn, E., Avarre, J.-C., Jaubert, M., Simon, D., Cartieaux, F., Prin, Y., Bena, G., Hannibal, L., Fardoux, J., Kojadinovic, M., Vuillet, L., Lajus, A., Cruveiller, S., Rouy, Z., Mangenot, S., Segurens, B., Dossat, C., Franck, W. L., Chang, W.-S.Saunders, E., Bruce, D., Richardson, P., Normand, P., Dreyfus, B., Pignol, D., Stacey, G., Emerich, D., Verméglio, A., Médigue, C. and Sadowsky, M. (2007) Legumes symbioses: absence of Nod genes in photosynthetic bradyrhizobia. *Science* **316:** 1307–1312.

Gojon, A., Krouk, G., Perrine-Walker, F. and Laugier, E. (2011) Nitrate transceptor(s) in plants. *J. Exp. Bot.* **62:** 2299–2308.

Good, A. G. and Crosby, W. L. (1989) Induction of alcohol dehydrogenase and lactate dehydrogenase in hypoxically induced barley. *Plant Physiol.* **90:** 860–8666.

Good, A. G. and Beatty, P. H. (2011) Fertilizing nature: a tragedy of excess in the commons. *PLoS Biol.* **9:** e1001124.

Good, A. G., Johnson, S. J., De Pauw, M., Carroll, R. T., Savidov, N., Vidmar, J., Lu, Z., Taylor, G., Stroeher, V. (2007) Engineering nitrogen use efficiency with alanine aminotransferase. *Can. J. Bot.* **85:** 252–262.

Good, A. G. and Muench, D. G. (1992) Purification and characterization of an anaerobically induced alanine aminotransferase from barley roots. *Plant Physiol.* **99:** 1520–1525.

Good, A. G. and Muench, D. G. (1993) Long-term anaerobic metabolism in root tissue: metabolic products of pyruvate metabolism. *Plant Physiol.* **101:** 1163–1168.

Good, A. G., Shrawat, A. K. and Muench, D. G. (2004) Can less yield more? Is reducing nutrient input into the environment compatible with maintaining crop production? *Trends Plant Sci.* **9:** 597-605.

Goulding, K., Jarvis, S. and Whitmore, A. (2008) Optimizing nutrient management for farm systems. *Philos. Trans. R. Soc. of Lond. B. Biol. Sci.* **363:** 667–680.

Grabowska, A., Kwinta, J. and Bielawski, W. (2012) Glutamine synthetase and glutamate dehydrogenase in triticale seeds: molecular cloning and genes expression. *Acta Physiol. Plant* **34:** 2393–2406.

Grassini, P., Eskridge, K. M. and Cassman, K. G. (2013) Distinguishing between yield advances and yield plateaus in historical crop production trends. *Nature Commun.***4:** 1–11.

Gruber, B. D., Giehl, R. F. H., Friedel, S. and von Wirén, N. (2013) Plasticity of the Arabidopsis root system under nutrient deficiencies. *Plant Physiol.* **163:** 161-179.

Guan, Z., Guo, B., Huo, Y., Guan, Z., Dai, J. and Wei, Y. (2013) Recent advances and safety issues of transgenic plant-derived vaccines. *Appl. Microbiol. Biot.* **97:** 2817–2840.

Gutiérrez, R. A., Stokes, T. L., Thum, K., Xu, X., Obertello, M., Katari, M. S., Tanurdzic, M., Dean, A., Nero, D. C., McClung, C, R. and Coruzzi, G. M. (2008) Systems approach identifies an organic nitrogen-responsive gene network that is regulated by the master clock control gene CCA1. *P. Natl. Acad. Sci. USA* **105:** 4939–4944.

Hare, P. D. and Cress, W. A. (1997) Metabolic implications of stress-induced proline accumulation in plants. *Plant Growth Regul.* **21:** 79–102.

Hawkesford, M. J. and Barraclough, P. (2011) *The Molecular and Physiological Basis of Nutrient Use Efficiency in Crops*. Oxford, UK: Wiley-Blackwell.

Health Canada (1992) *Nitrate / Nitrite* (Vol. 1987, pp. 1–8).

Herman, E. M., Helm, R. M., Jung, R. and Kinney, A. J. (2003) Genetic modification removes an immunodominant allergen from soybean. *Plant Physiol*. **132:** 36–43.

Herrera-Estrella, L. R. (2000) Genetically modified crops in developing countries. *Plant Physiol.* **124:** 923–925.

Herrero, A., Muro-Pastor, A. M. and Flores, E. (2001) Nitrogen control in cyanobacteria. *J. Bacteriol*. **183:** 411-425.

Hill, J., Nelson, E., Tilman, D., Polasky, S. and Tiffany, D. (2006) Environmental, economic, and energetic costs and benefits of biodiesel and ethanol biofuels. *P. Natl. Acad. Sci. USA* **103:** 11206–11210.

Hill, P. W., Quilliam, R. S., DeLuca, T. H., Farrar, J., Farrell, M., Roberts, P., Newsham, K. K., Hopkins, D. W., Bardgett, R. D. and Jones, D. L. (2011) Acquisition and assimilation of nitrogen as peptide-bound and D-enantiomers of amino acids by wheat. *PloS One* **6:** e19220.

Hirel, B., Le Gouis, J., Ney, B. and Gallais, A. (2007) The challenge of improving nitrogen use efficiency in crop plants: towards a more central role for genetic variability and quantitative genetics within integrated approaches. *J. Exp. Bot.* **58:** 2369–2387.

Ho, C.-H., Lin, S.-H., Hu, H.-C., and Tsay, Y.-F. (2009) CHL1 functions as a nitrate sensor in plants. *Cell* **138:** 1184–1194.

Ho, C.-L. and Saito, K. (2001) Molecular biology of the plastidic phosphorylated serine biosynthetic pathway in *Arabidopsis thaliana*. *Amino Acids* **20:** 243–259.

Hodges, M. (2002) Enzyme redundancy and the importance of 2-oxoglutarate in plant ammonium assimilation. *J. Exp Bot.* **53:** 905–916.

Hofstra, N. and Bouwman, A. F. (2005) Denitrification in agricultural soils: summarizing published data and estimating global annual rates. *Nutr. Cycl. Agroecosys.* **72:** 267–278.

Huang, T., Jander, G. and de Vos, M. (2011) Non-protein amino acids in plant defense against insect herbivores: representative cases and opportunities for further functional analysis. *Phytochemistry* **72:** 1531–1537.

Huffman, T., Yang, J. Y., Drury, C. F., De Jong, R., Yang, X. M. and Liu, Y. C. (2008) Estimation of Canadian manure and fertilizer nitrogen application rates at the crop and soil-landscape polygon level. *Can. J. Soil Sci.* **88:** 619–627.

Ibarlucea, J. M., Llama, M. J., Serra, J. L. and Macarulla, J. M. (1983) Mixed-transfer kinetics of nitrite uptake in barley (*Hordeum vulgare* L. cv. Miranda) seedlings. *Plant Sci. Lett.* **29:** 339–347.

International Plant Nutrition Institute (2009) *The Global "4R" Nutrient Stewardship Framework: Developing Fertilizer Best Management Practices for Delivering Economic, Social and Environmental Benefits*. Paris.

Jackson, W. A., Johnson, R. E. and Volk, R. J. (1974) Nitrite uptake patterns in wheat seedlings as influenced by nitrate and ammonium. *Physiol. Plantarum* **32:** 108–114.

Jander, G. and Joshi, V. (2010) Recent progress in deciphering the biosynthesis of aspartate-derived amino acids in plants. *Mol. Plant* **3:** 54–65.

Jeong, D.-H., Park, S., Zhai, J., Gurazada, S. G. R., De Paoli, E., Meyers, B. C. and Green, P. J. (2011) Massive analysis of rice small RNAs: mechanistic implications of regulated microRNAs and variants for differential target RNA cleavage. *Plant Cell* **23:** 4185–4207.

Johnson, J. M.-F., Franzluebbers, A. J., Weyers, S. L. and Reicosky, D. C. (2007) Agricultural opportunities to mitigate greenhouse gas emissions. *Environ. Pollut.* **150:** 107–124.

Jones, C. and Jacobson, J. (2001) *Plant Nutrition and Soil Fertility*. *Nutrient Management Module No. 2:* Montana State University.

Joy, K. W. (1988) Ammonia, glutamine, and asparagine: a carbon-nitrogen interface. *Can. J. Bot.* **66:** 2103–2109.

Kant, S., Bi, Y.-M. and Rothstein, S. J. (2011) Understanding plant response to nitrogen limitation for the improvement of crop nitrogen use efficiency. *J. Exp. Bot.* **62:** 1499–1509.

Ke, J., Behal, R. H., Back, S. L., Nikolau, B. J., Wurtele, E. S. and Oliver, D. J. (2000) The role of pyruvate dehydrogenase and acetyl-coenzyme A synthetase in fatty acid synthesis in developing Arabidopsis seeds. *Plant physiol.* **123:** 497–508.

Kirma, M., Araujo, W. L., Fernie, A. R. and Galili, G. (2012) The multifacted role of aspartate-family amino acids in plant metabolism. *J. Exp. Bot.* **63:** 695–709.

Kotur, Z., Siddiqi, Y. M. and Glass, A. D. M. (2013) Characterization of nitrite uptake in Arabidopsis thaliana: evidence for a nitrite-specific transporter. *New Phytol.* **200:** 201-210.

Kouchi, H., Imaizumi-Anraku, H., Hayashi, M., Hakoyama, T., Nakagawa, T., Umehara, Y., Suganuma, N. and Kawaguchi, M. (2010) How many peas in a pod? Legume genes responsible for mutualistic symbioses underground. *Plant Cell Physiol.* **51:** 1381–1397.

Kraakman, A. T. W., Niks, R. E., Van den Berg, P. M. M. M., Stam, P. and Van Eeuwijk, F. A. (2004) Linkage disequilibrium mapping of yield and yield stability in modern spring barley cultivars. *Genetics* **168:** 435–46.

Kraiser, T., Gras, D. E., Gutiérrez, A. G., González, B. and Gutiérrez, R. A. (2011) A holistic view of nitrogen acquisition in plants. *J. Exp. Bot.* **62:** 1455–66.

Krämer, U. and Chardonnens, A. N. (2001) The use of transgenic plants in the bioremediation of soils contaminated with trace elements. *Appl. Microbiol. Biot.* **55:** 661–672.

Krouk, G., Tillard, P. and Gojon, A. (2006) Regulation of the high-affinity NO_3^- uptake system by NRT1.1-mediated NO3- demand signaling in Arabidopsis. *Plant physiol.* **142:** 1075–1086.

Lai, H. and Chen, Q. (2012) Bioprocessing of plant-derived virus-like particles of Norwalk virus capsid protein under current Good Manufacture Practice regulations. *Plant Cell Rep.* **31:** 573–584.

Lal, R. (2004) Carbon Sequestration, Terrestrial. *Encyclopedia of Energy*.

Lam, H. M., Chiu, J., Hsieh, M. H., Meisel, L., Oliveira, I. C., Shin, M. and Coruzzi, G. (1998) Glutamate-receptor genes in plants. *Nature* **396:** 125–126.

Lancien, M., Gadal, P. and Hodges, M. (2000) Update on biochemistry enzyme redundancy and the importance of 2-oxoglutarate in higher plant ammonium assimilation. *Plant Physiol.* **123:** 817–824.

Langley, J. A. and Megonigal, J. P. (2010) Ecosystem response to elevated CO_2 levels limited by nitrogen-induced plant species shift. *Nature* **466:** 96–9.

Lanquar, V., Loqué, D., Hörmann, F., Yuan, L., Bohner, A., Engelsberger, W. R., Lalonde, S., Schulze, W. X., von Wirén, N. and Frommer, W. B. (2009) Feedback inhibition of ammonium uptake by a phospho-dependent allosteric mechanism in Arabidopsis. *Plant Cell* **21:** 3610–3622.

Lawlor, D. W. (2002) Carbon and nitrogen assimilation in relation to yield: mechanisms are the key to understanding production systems. *J. Exp. Bot.* **53:** 773–787.

Le, L. Q., Mahler, V., Lorenz, Y., Scheurer, S., Biemelt, S., Vieths, S and Sonnewald, U. (2006) Reduced allergenicity of tomato fruits harvested from Lyc e 1-silenced transgenic tomato plants. *J Allergy Clin. Immun.* **118:** 1176–1183.

Less, H. and Galili, G. (2008) Principal transcriptional programs regulating plant amino acid metabolism in response to abiotic stresses. *Plant Physiol.* **147:** 316–330.

Liang, G., He, H. and Yu, D. (2012) Identification of nitrogen starvation-responsive microRNAs in *Arabidopsis thaliana*. *PloS One*, **7:** e48951.

Liepman, A. H. and Olsen, L. J. (2001) Peroxisomal alanine:glyoxylate aminotransferase (AGT1) is a photorespiratory enzyme with multiple substrates in Arabidopsis thaliana. *Plant J.* **25:** 487–498.

Liepman, A. H. and Olsen, L. J. (2003) Alanine aminotransferase homologs catalyze the glutamate:glyoxylate aminotransferase reaction in peroxisomes of Arabidopsis. *Plant Physiol.* **131:** 215–227.

Lima, J. E., Kojima, S., Takahashi, H. and von Wirén, N. (2010) Ammonium triggers lateral root branching in Arabidopsis in an AMMONIUM TRANSPORTER1;3-dependent manner. *Plant Cell* **22:** 3621–33.

Little, D. Y., Rao, H., Oliva, S., Daniel-Vedele, F., Krapp, A. and Malamy, J. E. (2005) The putative high-affinity nitrate transporter NRT2.1 represses lateral root initiation in response to nutritional cues. *P. Natl. Acad. Sci. USA* **102:** 13693–13698.

Liu, K., Huang, C. and Tsay, Y. (1999) CHL1 is a dual-affinity nitrate transporter of Arabidopsis. *Plant Cell* **11:** 865–874.

Liu, L. and Greaver, T. L. (2009) A review of nitrogen enrichment effects on three biogenic GHGs: the CO2 sink may be largely offset by stimulated N_2O and CH_4 emission. *Ecol. Lett.* **12:** 1103–1117.

Lohse, K. A., Hope, D., Sponseller, R., Allen, J. O. and Grimm, N. B. (2008) Atmospheric deposition of carbon and nutrients across an arid metropolitan area. *Sci. Total Environ.* **402:** 95–105.

Loqué, D., Lalonde, S., Looger, L. L., von Wirén, N. and Frommer, W. B. (2007) A cytosolic trans-activation domain essential for ammonium uptake. *Nature* **446:** 195–198.

Loudet, O., Chaillou, S. and Merigout, P. (2003) Quantitative trait loci analysis of nitrogen use efficiency in Arabidopsis. *Plant Physiol.*, **131:** 345–358.

Ludewig, U., Neuhäuser, B. and Dynowski, M. (2007) Molecular mechanisms of ammonium transport and accumulation in plants. *FEBS Lett.* **581:** 2301–2308.

Lusk, J. L., House, L. O., Valli, C., Jaeger, S. R., Moore, M., Morrow, J. L. and Traill, W. B. (2004) Effect of information about benefits of biotechnology on consumer acceptance of genetically modified food: evidence from experimental auctions in the United States, England and France. *Eur. Rev. Argic. Econ.* **31:** 179–204.

Maathuis, F. J. M. (2009) Physiological functions of mineral macronutrients. *Curr. Opin. Plant Biol.* **12:** 250–258.

Macnicol, R. D. and Beckett, P. H. T. (1985) Critical tissue concentrations of potentially toxic elements. *Plant and Soil* **85:** 107–129.

Mae, T., Thomas, H., Gay, A. P. and Makino, A. H. J. (1993) Leaf development in *Lolium temulentum*: photosynthesis and photosynthetic proteins in leaves senescing under different irradiances. *Plant Cell Physiol.* **34:** 391–399.

Maeda, H. and Dudareva, N. (2012) The shikimate pathway and aromatic amino acid biosynthesis in plants. *Ann. Rev. Plant Biol.* **63:** 73–105.

Mailloux, R. J., Bériault, R., Lemire, J., Singh, R., Chénier, D. R., Hamel, R. D. and Appanna, V. D. (2007) The tricarboxylic acid cycle, an ancient metabolic network with a novel twist. *PloS One* **2:** e690.

Makino, A., Shimada, T., Takumi, S., Kaneko, K., Matsuoka, M., Shimamoto, K., Nakano, H., Miyao-Tokutomi, M., Mae, T. and Yamamoto, N. (1997) Does decrease in ribulose-1,5-bisphosphate carboxylase by antisense RbcS lead to a higher N-use efficiency of photosynthesis under conditions of saturating CO_2 and light in rice plants? *Plant Physiol.* **114:** 483–491.

Malamy, J. E. (2005) Intrinsic and environmental response pathways that regulate root system architecture. *Plant Cell Environ.* **28:** 67–77.

Malamy, J. E. and Ryan, K. S. (2001) Environmental regulation of lateral root initiation in Arabidopsis. *Plant Physiol.* **127:** 899–909.

Martin, T., Oswald, O. and Graham, I. A. (2002) Arabidopsis seedling growth , storage lipid mobilization, and photosynthetic gene expression are regulated by carbon:nitrogen availability. *Plant Physiol*. **128:** 472–481.

Masclaux-Daubresse, C., Daniel-Vedele, F., Dechorgnat, J., Chardon, F., Gaufichon, L. and Suzuki, A. (2010) Nitrogen uptake, assimilation and remobilization in plants: challenges for sustainable and productive agriculture. *Ann. Bot.* **105:** 1141–1157.

Masclaux-Daubresse *et al*., 2005 (should this be the Masclaux-Daubresse *et al*., 2010 above?)

Matsumoto, T., Tanaka, T. and Kondo, A. (2012) Enzyme-mediated methodologies for protein modification and bioconjugate synthesis. *Biotech. J.* **7:** 1137–1146.

McAllister, C. H., Beatty, P. H. and Good, A. G. (2012) Engineering nitrogen use efficient crop plants: the current status. *Plant Biotech. J.* **10:** 1011–1025.

McAllister, C. H., Facette, M., Holt, A. and Good, A. G. (2013) Analysis of the enzymatic properties of a broad family of alanine aminotransferases. *PloS One*, **8:** e55032.

McCarty, G. W. and Ritchie, J. C. (2002) Impact of soil movement on carbon sequestration in agricultural ecosystems. *Environ. Pollut.* **116:** 423–430.

McElroy, M. B., Salawitch, R. J., Wofsy, S. C. and Logan, J. A. (1986) Reductions of Antarctic ozone due to synergystic interactions of chlorine and bromine. *Nature* **321:** 759–762.

McKenzie, R. (1998) *Crop Nutrition and Fertilizer Requirements Essential Plant Nutrients* (pp. 1–7). Alberta Agriculture, Food and Rural Development. Lethbridge, Alberta, Canada.

McKenzie, R. (2003) *Soil pH and Plant Nutrients* (pp. 4–5). Alberta Agriculture Food and Rural Development.

Mehta, P. K., Hale, T. I. and Christen, P. (1993) Aminotransferases: demonstration of homology and division into evolutionary subgroups. *Eur. J. Biochem.* **561:** 549–561.

Melillo, J. M., Reilly, J. M., Kicklighter, D. W., Gurgel, A. C., Cronin, T. W., Paltsev, S., Felzer, B. S., Wang, X., Sokolov, A. P. and Schlosser, C. A. (2009) Indirect emissions from biofuels: how important? *Science* **326:** 1397–1399.

Miflin, B. J. and Habash, D. Z. (2002) The role of glutamine synthetase and glutamate dehydrogenase in nitrogen assimilation and possibilities for improvement in the nitrogen utilization of crops. *Ann. Appl. Biol.* **53:** 979–987.

Miller, A. J., Fan, X., Orsel, M., Smith, S. J. and Wells, D. M. (2007) Nitrate transport and signalling. *J. Exp. Bot.* **58:** 2297–2306.

Miller, A. J., Fan, X., Shen, Q. and Smith, S. J. (2008) Amino acids and nitrate as signals for the regulation of nitrogen acquisition. *J. Exp. Bot.* **59:** 111–119.

Miyashita, Y., Dolferus, R., Ismond, K. P. and Good, A. G. (2007) Alanine aminotransferase catalyses the breakdown of alanine after hypoxia in *Arabidopsis thaliana*. *Plant J.* **49:** 1108–1121.

Miyashita, Y. and Good, A. G. (2008) Contribution of the GABA shunt to hypoxia-induced alanine accumulation in roots of Arabidopsis thaliana. *Plant Cell Physiol.* **49:** 92–102.

Montzka, S. A., Dlugokencky, E. J. and Butler, J. H. (2011) Non-CO_2 greenhouse gases and climate change. *Nature* **476:** 43–50.

Mosier, A R, Pendall, E. and Morgan, J. A. (2003) Effect of water addition and nitrogen fertilization on the fluxes of CH_4, CO_2, NO_x, and N_2O following five years of elevated CO_2 in the Colorado Shortgrass Steppe. *Atmos. Chem. Phys.* **3:** 1703–1708.

Motavalli, P. P., Goyne, K. W. and Udawatta, R. P. (2008) Environmental impacts of enhanced-efficiency nitrogen fertilizers crop management. *Crop Manag.* doi:10.1094/CM-2008-0730-01-PS.

Murphy, D. V., Macdonald, A. J., Stockdale, E. A., Goulding, K. W. T., Fortune, S., Gaunt, J. L., Poulton, P. R., Wakefield, J. A., Webster, C. P. and Wilmer, W. S. (2000) Soluble organic nitrogen in agricultural soils. *Biol. Fert. Soils* **30:** 374–387.

Nakatsuka, A., Shiomi, S., Kubo, Y. and Inaba, A. (1997) Expression and internal feedback regulation of ACC synthase and ACC oxidase genes in ripening tomato fruit. *Plant Cell Physiol.* **38:** 1103–1110.

Näsholm, T., Kielland, K. and Ganeteg, U. (2009) Uptake of organic nitrogen by plants. *New Phytol.* **182:** 31–48.

Nischal, L., Mohsin, M., Khan, I., Kardam, H., Wadhwa, A., Abrol, Y. P., Iqbal, M. and Ahmad, A. (2012) Identification and comparative analysis of microRNAs associated with low-N tolerance in rice genotypes. *PloS One* **7:** e50261.

Nunes-Nesi, A., Fernie, A. R. and Stitt, M. (2010) Metabolic and signaling aspects underpinning the regulation of plant carbon nitrogen interactions. *Mol. Plant* **3:** 973–996.

Obara, M., Kajiura, M., Fukuta, Y., Yano, M., Hayashi, M., Yamaya, T. and Sato, T. (2001) Mapping of QTLs associated with cytosolic glutamine synthetase and NADH-glutamate synthase in rice (*Oryza sativa* L.). *J. Exp. Bot.* **52:** 1209–1217.

Okumoto, S. and Pilot, G. (2011) Amino acid export in plants: a missing link in nitrogen cycling. *Mol. Plant* **4:** 453–463.

Ortiz-Monasterio, J. I., Pena, R. J., Sayre, K. D. and Rajaram, S. (1997) Genetic progress in wheat grain quality under four nitrogen rates. *Crop Sci.* **37:** 892–898.

Paine, J. A, Shipton, C. A, Chaggar, S., Howells, R. M., Kennedy, M. J., Vernon, G., Wright, S. Y., Hinchliffe, E., Adams, J. L., Silverstone, A. L. and Drake, R. (2005) Improving the nutritional value of Golden Rice through increased pro-vitamin A content. *Nat. Biotech. Lett.* **23:** 482–487.

Pant, B. D., Musialak-Lange, M., Nuc, P., May, P., Buhtz, A., Kehr, J., Walther, D. and Scheible, W.-R. (2009) Identification of nutrient-responsive Arabidopsis and rapeseed microRNAs by comprehensive real-time polymerase chain reaction profiling and small RNA sequencing. *Plant Physiol.* **150:** 1541–1555.

Park, R. J., McFarlane, I., Hartley Phipps, R. and Ceddia, G. (2011) The role of transgenic crops in sustainable development. *Plant Biotechnol. J.* **9:** 2–21.

Pathak, R. R., Lochab, S. and Raghuram, N. (2011) Improving plant nitrogen-use efficiency. *Compr. Biotech.* **1:** 209–218.

Paul, M. J. and Driscoll, S. P. (1997) Sugar repression of photosynthesis: the role of carbohydrates in signalling nitrogen deficiency through source:sink imbalance. *Plant Cell Environ.* **20:** 110–116.

Pearsall, D. (2013) GM crop co-existence: A question of choice, not prejudice. *GM Crops Food* **4:** 143-150

Petersen, L. N., Marineo, S., Mandalà, S., Davids, F., Sewell, B. T. and Ingle, R. A. (2010) The missing link in plant histidine biosynthesis: Arabidopsis myoinositol monophosphatase-like2 encodes a functional histidinol-phosphate phosphatase. *Plant Physiol.* **152:** 1186–1196.

Phillips, K. A., Veenstra, D. L., Oren, E. and Lee, J. K. (2001 Potential role of pharmacogenomics in reducing adverse drug reactions: a systematic review. *J. Amer. Med. Asoc.* **286:** 2270-2279

Pilegaard, K. (2013) Processes regulating nitric oxide emissions from soils. *Phil. Trans. R. Soc. Biol.* **368:** 20130126.

Pilon-Smits, E. A. H., Quinn, C. F., Tapken, W., Malagoli, M. and Schiavon, M. (2009) Physiological functions of beneficial elements. *Curr. Opin. Plant Biol.* **12:** 267–274.

Pingali, P. L. (2012) Green revolution: impacts, limits, and the path ahead. *Proc. Nat. Acad. Sci.USA* **109:** 12302–12308.

Price, M. B., Jelesko, J. and Okumoto, S. (2012) Glutamate receptor homologs in plants: functions and evolutionary origins. *Front. Plant Sci*. **3:** 1-10.

Radchenko, M. V, Thornton, J. and Merrick, M. (2013) PII signal transduction proteins are ATPases whose activity is regulated by 2-oxoglutarate. *Proc. Nat. Acad. Sci.USA* **110:** 12948–12953.

Ravishankara, A. R., Daniel, J. S. and Portmann, R. W. (2009) Nitrous oxide (N_2O): the dominant ozone-depleting substance emitted in the 21st century. *Science* **326:** 123–125.

Reich, P. B., Hobbie, S. E., Lee, T., Ellsworth, D. S., West, J. B., Tilman, D., Knops, J. M. H., Naeem, S. and Trost, J. (2006) Nitrogen limitation constrains sustainability of ecosystem response to CO2. *Nature* **440:** 922–925.

Rentsch, D., Schmidt, S. and Tegeder, M. (2007) Transporters for uptake and allocation of organic nitrogen compounds in plants. *FEBS Lett.* **581:** 2281–2289.

Rocha, M., Licausi, F., Araújo, W. L., Nunes-Nesi, A., Sodek, L., Fernie, A. R. and van Dongen, J. T. (2010) Glycolysis and the tricarboxylic acid cycle are linked by alanine aminotransferase during hypoxia induced by waterlogging of *Lotus japonicus*. *Plant Physiol*. **152:** 1501–1513.

Rodriguez-Antona, C. and Ingelman-Sundberg, M. (2006) Cytochrome P450 pharmacogenetics and cancer. *Oncogene* **25:** 1679–1691.

Rybicki, E. P. (2010) Plant-made vaccines for humans and animals. *Plant Biotech. J.* **8:** 620–637.

Sainju, U. M., Whitehead, W. F. and Singh, B. P. (2003) Agricultural management practices to sustain crop yields and improve soil and environmental qualities. *Scientific World J*. **3:** 768–789.

Sanchez, P. A. (2002) Soil fertility and hunger in Africa. *Science* **295:** 2019-2020.

Sattelmacher, B., Horst, W. J. and Becker, H. C. (1994) Factors that contribute to genetic variation for nutrient efficiency of crop plants. *Z. Pfanzenernahr. Bodenk* **157**: 215–224.

Sauter, M., Moffatt, B., Saechao, M. C., Hell, R. and Wirtz, M. (2013) Methionine salvage and S-adenosylmethionine: essential links between sulfur, ethylene and polyamine biosynthesis. *Biochem. J.* **451:** 145–154.

Savile, C. K., Janey, J. M., Mundorff, E. C., Moore, J. C., Tam, S., Jarvis, W. R., Colbeck, J. C., Krebber, A., Fleitz, F. J., Brands, J., Devine, P. N., Huisman, G. W. and

Hughes, G. J. (2010) Biocatalytic asymmetric synthesis of chiral amines from ketones applied to sitagliptin manufacture. *Science* **329:** 305–309.

Scheible, W.-R., Morcuende, R., Czechowski, T., Fritz, C., Osuna, D., Palacios-Rojas, N., Schindelasch, D., Thimm, O., Udvardi, M. K. and Stitt, M. (2004) Genome-wide reprogramming of primary and secondary metabolism, protein synthesis, cellular growth processes , and the regulatory infrastructure of Arabidopsis in response to nitrogen. *Plant Physiol.* **136:** 2483–2499.

Schjoerring, J. K., Husted, S., Mäck, G. and Mattsson, M. (2002) The regulation of ammonium translocation in plants. *J. Exp. Bot.* **53:** 883–890.

Searchinger, T., Heimlich, R., Houghton, R. A., Dong, F., Elobeid, A., Fabiosa, J., Tokgoz, S., Hayes, D. and Yu, T.-H. (2008) Use of U.S. croplands for biofuels increases greenhouse gases through emissions from land-use change. *Science* **319:** 1238–1240.

Seiffert, B., Zhou, Z., Wallbraun, M., Lohaus, G. and Mollers, C. (2004) Expression of a bacterial asparagine synthetase gene in oilseed rape (*Brassica napus*) and its effect on traits related to nitrogen efficiency. *Physiol. Plantarum* **121:** 656–665.

Sharma, S. S. and Dietz, K.-J. (2006) The significance of amino acids and amino acid-derived molecules in plant responses and adaptation to heavy metal stress. *J. Exp. Bot.* **57:** 711–726.

Shrawat, A. K., Carroll, R. T., DePauw, M., Taylor, G. J. and Good, A. G. (2008) Genetic engineering of improved nitrogen use efficiency in rice by the tissue-specific expression of alanine aminotransferase. *Plant Biotech. J.* **6:** 722–732.

Singh, P., Pongkanjana, A. and Pradesh, A. (2001) Potential productivity and yield gap of selected crops in the rainfed regions of India, Thailand and Vietnam. *International Crops Research Institute for the Semi-Arid Tropics.* Report no. 5.

Skiba, M. W., George, T. S., Baggs, E. M. and Daniell, T. J. (2011) Plant influence on nitrification. *Biochem. Soc. T.* **39:** 275–278.

Solomon, S., Qin, D., Manning, M., Chen, Z., Marquis, M., Averyt, K. B., Tignor, M. and Miller, H. L. (2007) Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge: Cambridge University Press.

Solomon, S. (1999) Stratosphereic ozone depletion: a review of concepts and history. *Rev. Geophys.* **37:** 275–316.

Stepansky, A. and Leustek, T. (2006) Histidine biosynthesis in plants. *Amino Acids*, **30:** 127–142.

Stewart, W. M., Dibb, D. W., Johnston, A. E. and Smyth, T. J. (2005) The contribution of commercial fertilizer nutrients to food production. *Agron. J.* **97:** 1-6.

Stitt, M., Müller, C., Matt, P., Gibon, Y., Carillo, P., Morcuende, R., Scheible, W.-R. and Krapp, A. (2002) Steps towards an integrated view of nitrogen metabolism. *J. Exp. Bot.* **53:** 959–970.

Sunako, T., Sakuraba, W., Senda, M., Akada, S., Ishikawa, R., Niizeki, M. and Harada, T. (1999) An allele of the ripening-specific 1-aminocyclopropane-1-carboxylic acid synthase gene (ACS1) in apple fruit with a long storage life. *Plant Physiol*. **119:** 1297–1304.

Sutton, M. A., Simpson, D., Levy, P. E., Smith, R. I., Reis, S., van Oijen, M. and de Vries, W. (2008) Uncertainties in the relationship between atmospheric nitrogen deposition and forest carbon sequestration. *Glob. Change Biol.* **14:** 2057–2063.

Suzuki, A. and Knaff, D. B. (2005) Glutamate synthase: structural, mechanistic and regulatory properties, and role in the amino acid metabolism. *Photosynth. Res.* **83:** 191–217.

Svennerstam, H., Ganeteg, U., Bellini, C. and Näsholm, T. (2007) Comprehensive screening of Arabidopsis mutants suggests the lysine histidine transporter 1 to be involved in plant uptake of amino acids. *Plant Physiol.* **143:** 1853–1860.

Szabados, L. and Savouré, A. (2010) Proline: a multifunctional amino acid. *Trends Plant Sci.* **15:** 89–97.

Tabuchi, M., Abiko, T. and Yamaya, T. (2007) Assimilation of ammonium ions and reutilization of nitrogen in rice (*Oryza sativa* L.). *J. Exp. Bot.* **58:** 2319–2327.

Tada, Y., Nakase, M., Adachi, T., Nakamura, R., Shimada, H., Takahashi, M., Fujimura, T. and Matsuda, T. (1996) Reduction of 14-16 kDa allergenic proteins in transgenic rice plants by antisense gene. *FEBS Lett.*,**391:** 341–345.

Tan, Z. X., Lal, R. and Wiebe, K. D. (2005) Global soil nutrient depletion and yield reduction. *J. Sustainable Agr.* **26:** 123–146.

Tapken, D., Anschütz, U., Liu, L.-H., Huelsken, T., Seebohm, G., Becker, D. and Hollmann, M. (2013) A plant homolog of animal glutamate receptors is an ion channel gated by multiple hydrophobic amino acids. *Sci. Signal.* **6:** ra47.

Tcherkez, G., Mahé, A., Boex-Fontvieille, E., Gout, E., Guérard, F. and Bligny, R. (2011) Experimental evidence of phosphoenolpyruvate resynthesis from pyruvate in illuminated leaves. *Plant Physiol*. **157:** 86–95.

Tegeder, M. and Rentsch, D. (2010) Uptake and partitioning of amino acids and peptides. *Mol. Plant* **3:** 997–1011.

Temple Scott Associates Incorporated (2013) *Fertilizer Report for the Canadian Fertilizer Industry*. Canadian Fertilizer Institute.

Tester, M. and Langridge, P. (2010) Breeding technologies to increase crop production in a changing world. *Science* **327:** 818–822.

Thomson, A. J., Giannopoulos, G., Pretty, J., Baggs, E. M. and Richardson, D. J. (2012) Biological sources and sinks of nitrous oxide and strategies to mitigate emissions. *Philos. T. R. Soc. Biol.* **367:** 1157–1168.

Tilman, D., Cassman, K. G., Matson, P. A, Naylor, R. and Polasky, S. (2002) Agricultural sustainability and intensive production practices. *Nature* **418:** 671–677.

Timm, S., Florian, A., Wittmiß, M., Jahnke, K., Hagemann, M., Fernie, A. R. and Bauwe, H. (2013) Serine acts as a metabolic signal for the transcriptional control of photorespiration-related genes in Arabidopsis. *Plant Physiol.* **162:** 379–389.

Tobin, A. K. and Yamaya, T. (2001) Cellular compartmentation of ammonium assimilation in rice and barley. *J. Exp. Bot.* **52:** 591–604.

Townsend, A. R., Howarth, R. W., Bazzaz, F. a., Booth, M. S., Cleveland, C. C., Collinge, S. K., Dobson, A. P., Epstein, P. R., Holland, E. A., Keeney, D. R., Mallin, M. A., Rogers, C. A., Wayne, P. and Wolfe, A. H. (2003) Human health effects of a changing global nitrogen cycle. *Front. Ecol. Environ.* **1:** 240–246.

Trevisan, S., Nonis, A., Begheldo, M., Manoli, A., Palme, K., Caporale, G., Ruperti, B. and Quaggiotti, S. (2012) Expression and tissue-specific localization of nitrate-responsive miRNAs in roots of maize seedlings. *Plant Cell Environ.* **35**: 1137–1155.

Tsay, Y.-F., Chiu, C.-C., Tsai, C.-B., Ho, C.-H. and Hsu, P.-K. (2007) Nitrate transporters and peptide transporters. *FEBS Lett.* **581:** 2290–2300.

United States Environmental Protection Agency (2009) *National Primary Drinking Water Regulations*.

Valdés-López, O., Yang, S. S., Aparicio-Fabre, R., Graham, P. H., Reyes, J. L., Vance, C. P. and Hernández, G. (2010) MicroRNA expression profile in common bean (*Phaseolus vulgaris*) under nutrient deficiency stresses and manganese toxicity. *New Phytol.* **187:** 805–818.

Van Aardenne, J. A., Dentener, F. J., Olivier, J. G. J., Peters, J. A. H. W. and Ganzeveld, L. N. (2004) *The EDGAR 3.2 Fast Track 2000 dataset (32FT2000)* (pp. 1–12).

Vitousek, P. M., Aber, J. D., Howarth, R. W., Likens, G. E., Matson, P. A., Schindler, D. W., Schlesinger, W. H. and Tilman, D. G. (1997) Human alteration of the global nitrogen cycle: sources and consequences. *Ecol. Appl.* **7:** 737–750.

Vitousek, P. M. and Howarth, R. W. (1991) Nitrogen limitation on land and in the sea: How can it occur? *Biogeochem.* **13:** 87–115.

Vitousek, P. M., Naylor, R., Crews, T., David, M. B., Drinkwater, L. E., Holland, E., Johnes, P. J., Katzenberger, J., Martinelli, L. A., Matson, P. A., Nziguheba, G., Ojima, D., Palm, C. A., Robertson, G. P., Sanchez, P. A., Townsend, A. R. and Zhang, F. S. (2009) Nutrient Imbalances in Agricultural Development. *Science*, **324:** 1519–1520.

Wallace, W., Secor, J. and Schrader, L. E. (1984) Rapid accumulation of gamma-aminobutyric acid and alanine in soybean leaves in response to an abrupt transfer to lower temperature, darkness, or mechanical manipulation. *Plant Physiol.* **75:** 170–175.

White, P. J., Bowen, H. C., Parmaguru, P., Fritz, M., Spracklen, W. P., Spiby, R. E., Meacham, M. C., Mead, A., Harriman, M., Trueman, L. J., Smith, B. M., Thomas, B. and Broadley, M. R. (2004) Interactions between selenium and sulphur nutrition in *Arabidopsis thaliana*. *J. Exp. Bot.* **55:** 1927–1937.

White, P. J. and Brown, P. H. (2010) Plant nutrition for sustainable development and global health. *Ann. Bot.* **105:** 1073–1080.

Williams, L. E. and Miller, A. J. (2001) Transporters responsible for the uptake and partitioning of nitrogenous solutes. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **52:** 659–688.

Wingler, A., Purdy, S., MacLean, J. A. and Pourtau, N. (2006) The role of sugars in integrating environmental signals during the regulation of leaf senescence. *J. Exp. Bot.* **57:** 391–399.

Wolfenbarger, L. L. and Phifer, P. R. (2000) The ecological risks and benefits of genetically engineered plants. *Science* **290:** 2088–2093.

Wuebbles, D. J. (2009) Nitrous oxide: no laughing matter. *Science* **326:** 56–57.

Xiong, A.-S., Yao, Q.-H., Peng, R.-H., Li, X., Han, P.-L. and Fan, H.-Q. (2005) Different effects on ACC oxidase gene silencing triggered by RNA interference in transgenic tomato. *Plant Cell Rep.* **23:** 639–646.

Xu, Z., Zhong, S., Li, X., Li, W., Rothstein, S. J., Zhang, S., Bi, Y. and Xie, C. (2011) Genome-wide identification of microRNAs in response to low nitrate availability in maize leaves and roots. *PloS One* **6:** e28009.

Yamaya, T., Obara, M., Nakajima, H., Sasaki, S., Hayakawa, T. and Sato, T. (2002) Genetic manipulation and quantitative-trait loci mapping for nitrogen recycling in rice. *J. Exp. Bot.* **53:** 917–925.

Yoshida, K. and Shinmyo, A. (2000) Transgene expression systems in plant, a natural bioreactor. *Journal of Biosci. Bioeng.* **90:** 353–62.

Zhang, H. and Forde, B. G. (1998) An Arabidopsis MADS box gene that controls nutrient-induced changes in root architecture. *Science* **279:** 407–409.

Zhang, H. and Forde, B. G. (2000) Regulation of Arabidopsis root development by nitrate availability. *J. Exp. Bot.* **51:** 51–9.

Zhang, H., Rong, H. and Pilbeam, D. (2007) Signalling mechanisms underlying the morphological responses of the root system to nitrogen in Arabidopsis thaliana. *J. Exp. Bot.* **58:** 2329–38.

Zhang, Q. (2007) Strategies for developing Green Super Rice. *Proc. Nat. Acad. Sci.USA* **104:** 16402–16409.

Zhao, M., Ding, H., Zhu, J.-K., Zhang, F. and Li, W.-X. (2011) Involvement of miR169 in the nitrogen-starvation responses in Arabidopsis. *New Phytol.* **190:** 906–915.

Zhao, M., Tai, H., Sun, S., Zhang, F., Xu, Y. and Li, W.-X. (2012) Cloning and characterization of maize miRNAs involved in responses to nitrogen deficiency. *PloS One*, **7:** e29669.

Zheng, Z.-L. (2009) Carbon and nitrogen nutrient balance signaling in plants. *Plant Signaling & Behav.* **4:** 584–591.

2. Analysis of the enzymatic properties of a broad family of alanine aminotransferases

2.1. Introduction

Alanine aminotransferase (AlaAT) [E.C. 2.6.1.2], also referred to as glutamate:pyruvate aminotransferase (GPAT), is a pyridoxal-5'-phosphate-dependent (PLP) enzyme that catalyzes the reversible transfer of an amino group from alanine to 2-oxoglutarate to form glutamate and pyruvate (Mehta *et al*., 1993; Miyashita *et al.*, 2007). AlaAT is widespread, with homologues in all three biological domains (*Eukarya*, *Archaea* and *Eubacteria*) and functions as a part of several major metabolic pathways. Existing in both the cytosol and the mitochondria, AlaAT plays a critical role in linking carbon and nitrogen metabolism (assimilation and catabolism) within both eukaryotes and prokaryotes (Kameya *et al*., 2010). AlaAT is involved in a number of cellular processes including glycolysis, gluconeogenesis, amino acid metabolism (Miyashita et al., 2007a), hepatocellular damage in mammals (Jadaho *et al*., 2004), photorespiration in plants (Liepman & Olsen, 2003) and nitrogen use efficiency (NUE) in plants, including cereal crops (Good *et al.*, 2007a; Shrawat *et al.*, 2008). This latter process is of particular interest, as it has been previously shown that both canola (*Brassica napus*) and rice (*Oryza sativa*) plants over-expressing barley (*Hordeum vulgare*) *AlaAT* (*HvAlaAT*) in a tissue-specific manner have increased NUE under nitrogen (N) limiting conditions, both in controlled environments and field trials for rice (Good *et al*., 2007a, 2007b; Shrawat *et al*., 2008). Due to the increased awareness of the detrimental effects of increased N fertilizers in the environment as well as the concerns surrounding increasing world population and subsequent food shortages, the ability to obtain increased yields under limiting N fertilizer conditions is particularly important. Any further understanding of the key enzymes involved in these processes may be of significance in additional improvements in NUE (Masclaux-Daubresse *et al*., 2010; Kant *et al*., 2011; McAllister *et al*., 2012).

NOTE: *A version of this chapter has been published. McAllister, C. H., Facette, M., Holt, A. and Good, A. G. (2013) Analysis of the enzymatic properties of a broad family of alanine aminotransferases. PloS One, 8: e55032. doi: 10.1371/journal/pone.0055132.*

To date, AlaAT enzymes and their kinetics have been characterized in a variety of species. However most work on this enzyme has focused on the medical implications of the AlaAT isoforms found in humans (HsAlaAT) (Sohocki *et al*., 1997). Increases of both HsAlaAT1 (cytoplasmic) and HsAlaAT2 (mitochondrial) in mammalian serum samples have shown to be reliable indicators of liver damage, muscle damage and celiac disease (Yang *et al*., 2009). Moreover, significant increases in activity of mouse (*Mus musculus*) mitochondrial AlaAT (MmAlaAT2), but not mouse cytoplasmic AlaAT (MmAlaAT1) in fatty livers of obese mice, indicate possible differences in the roles/effects these two isozymes have in the cell. The evolution of differences in the kinetics of various isozymes of AlaAT would be driven in part by the distinct cellular roles these isozymes play (Jadaho *et al*., 2004).

Good *et al.*, (2007a) and Shrawat *et al*., (2008) observed an NUE phenotype in plants with over-expression of the HvAlaAT enzyme in canola and rice, respectively, using a tissue specific promoter. However, the specific basis for this phenotype remains unclear and there is a continued effort to understand the intracellular mechanisms which cause this phenotype. One question of particular interest is whether different AlaAT enzyme isoforms have different kinetics and if so, could these different isoforms favor an NUE phenotype? More specifically, are there optimal kinetic properties of AlaAT which can produce an increase in NUE when expressed within plants? Given that the previous NUE phenotypes were observed in canola and rice utilizing a promoter which increased expression in the roots (Beatty *et al*., 2009), the benefits of targeting the expression of a gene of interest to a particular tissue have become clear. For example, genes involved in producing modified oils are usually expressed with a seed specific promoter (Vigeolas *et al*., 2007). What has been less studied is the importance of choosing an enzyme that works with optimal efficiency in the appropriate environment and tissue. The importance of studying enzyme variants is illustrated by the example of *Golden Rice*. Development of this rice involved the insertion of a daffodil phytoene synthase (*psy*) gene for the efficient production of ß-carotene, a product used to synthesize Vitamin A (Paine *et al*., 2005). A number of *psy* genes were analyzed in order to determine which produced the highest levels of ß-carotene, and which variant was rate-limiting; further analysis revealed that an even more efficient *psy* gene may exist in maize ("Golden Rice 2") (Paine *et al*., 2005). Therefore, identifying enzyme variants that overcome a metabolic bottle-neck could prove to be an effective strategy for trait improvement.

To investigate further the basis for an increased NUE phenotype, different enzyme variants of AlaAT were evaluated with a view to using these variants to gain insights into the underlying metabolic changes that affect NUE in plants. Because AlaAT has an equilibrium constant near one, the reaction of this enzyme *in vivo* will be driven by substrate concentrations (Duff *et al*., 2012). Therefore, it follows that an AlaAT homologue with increased specificity or different kinetic properties could allow for increased NUE properties in a plant system. This approach was recently taken by Duff *et al*., who examined the kinetic properties and crystal structure of different AlaAT enzyme variants (Duff *et al*., 2012). Presented here is a kinetic comparison of AlaAT enzymes from a broader variety of organisms, placing emphasis on the difference in K_M values between homologues enzymes instead of the specific activity of the enzyme which has been analyzed elsewhere (Duff *et al*., 2012). Furthermore, AlaAT enzymes used in this analysis were not tagged as has been done previously, which can affect enzyme activity. Finally, only L-amino acid enzymes were used in this analysis given that many plant pathways are L-enantiomer stereospecific, including shikimate, aspartate, pyruvate and glutamate (Brückner & Westhauser, 2003), and that a very small percent (~0.5-3) of the total amino acids within many plants are not of the L-type (Bruckner & Westhauser, 1994). The effects of various enzymes with diverging kinetic behaviours were also assessed for functional consequences in *E. coli* under different environmental conditions.

2.2. Materials and Methods

2.2.1. pBAD18-Kan:*AlaAT* Constructs

The alanine aminotransferase enzymes assayed were chosen based on differences in their amino acid sequence (Appendix 2.1) and the availability of a cloned gene. Barley alanine aminotransferase *(Hordeum vulgare, HvAlaAT*) (GenBank accession no, Z26322) was obtained from a cDNA originally used in over-expression studies (Shrawat *et al.*, 2008) and was described by Muench and Good (1994). Both *Medicago truncatula* cDNA sequences were obtained from Anis Limami at the Université d' Angers (Ricoult *et al*., 2006). Although only one naturally-occurring mitochondrial *Medicago truncatula* alanine aminotransferase (*MtAlaAT*) has been described (*Medicago truncatula* genome sequencing resources, Medtr8g023140), none of the sequences received were identical to the expected sequence, and so two of the most similar sequences were chosen to be expressed and analyzed. MtAlaAT1 contained the point mutation

I144V while MtAlaAT2 contained the point mutation F177S. *Pyrococcus furiosus AlaAT* (*PfAlaAT*) (GenBank accession no. NP_579226) was amplified from ATCC gDNA (DSM 3638). *Arabidopsis thaliana AlaAT1* (*AtAlaAT1*) (TAIR reference no. AtG17290) and *AlaAT2* (*AtAlaAT2*) (TAIR reference no. At1G72330) sequences were obtained from Yo Miyashita (Miyashita et al., 2007a). Yeast (*Saccharomyces cerevisiae*) sequences (*ScAlaAT1,* GenBank accession no. NP_013190 and *ScAlaAT2,* Genbank accession no. NP_010396) were amplified from ATCC gDNA (S288C [MUCL 38902]). *Arabidopsis thaliana AtGGAT1* (TAIR reference no. At1G23310) and *AtGGAT2* (TAIR reference no. At1G70580) (*glutamate:glyoxylate aminotransferase*) sequences were obtained from Laura Olsen at the University of Michigan (Aaron H Liepman & Olsen, 2003). Mouse (*Mus musculus*) *MmAlaAT1* (GenBank accession no. NP_877957) and *MmAlaAT2* (GenBank accession no. NP_776291) sequences were acquired from Rong ze Yang at the University of Maryland (Yang *et al*., 2009), as were those for human (*Homo sapien*) *HsAlaAT1* (GenBank accession no. NP_005300) and *HsAlaAT2* (GenBank accession no. NP_597700). All genes were sequenced and the primers used for sequencing are listed in Appendix Table 2.3. When available, sequences were compared with BLAST results from the NCBI nucleotide database.

All of the sequences were cloned into the *E. coli* expression vector pBAD18-Kan using primers containing approximately 22 bps of *AlaAT* specific gene sequence and a restriction digest cut site at the 5' end (Appendix 2.3). Forward primers contained cut sites for either Kpn1 or Sac1, while Xba1 cut sites were used for the reverse primers. These constructs were transformed into *E. coli* TR61 strain K-12 cells and used for *AlaAT* expression, activity and kinetic assays. TR61 cells are derived from the *E. coli* K-12 strain MC4100, containing a *lac* reporter gene on a lambda phage insertion and a Tn10 insertion conferring resistance to the sugar arabinose, and were a gift from Tracy Ravio at the University of Alberta. TR61 cells are a previously unpublished *de novo* cell line and permission for their use was granted by Tracy Ravio and the Biosafety Committee, University of Alberta.

2.2.2. Analysis of AlaAT primary structure

Thirteen enzymes with AlaAT activity were obtained for expression studies and kinetic analysis and the amino acid sequences were compared using ClustalW software (full primary sequence comparison is provided in Appendix 2.1). The *Medicago truncatula* sequence utilized

for this analysis was obtained from Anis Limami at the Université d' Angers (Ricoult *et al*., 2006). Vector NTI Advance v. 11.0 software was used to construct a dendrogram (Figure 2.1). For both ClustalW and Vector NTI analysis, a gap open penalty of 10 and a gap extension penalty of 0.05 were used. The percent identity of amino acid sequences was defined by dividing the number of identical residues by the number of amino acids in the shortest sequence; gaps were not taken into account.

2.2.3. Induction of AlaAT constructs in *E. coli*

Five hundred µL of *E. coli* TR61 overnight cultures containing the various *AlaAT* constructs were added to 45 mL LB and grown at 37˚C, to an OD_{600} of 0.5 – 0.75, at which point 20 mL of culture was added separately to two flasks, one of which received arabinose to a final concentration of 0.2%. Both induced and uninduced cultures were incubated, shaking at 37˚C for an additional 4 hrs. Induced and uninduced cultures were harvested by centrifugation after 3-4 hrs, washed a single time with STE buffer (10 mM Tris-HCl pH = 7.5, 1 mM EDTA, 150 mM NaCl), and bacterial pellets were flash frozen and stored at -80˚C. Cultures were stored at -80^{o}C for no more than 2 weeks prior to protein extraction.

2.2.4. Protein extraction

Whole protein fractions were extracted using a freeze-thaw method. Cells were re-suspended in 2 mL protein extraction buffer (100 mM Tris-HCl pH = 7.5, 5 mM EDTA) containing 1 mM DTT and 1 mM PMSF. Ten µL of 10% Triton-X100 and 10 µL of 1 mg/mL lysozyme (Sigma, L-6876) were added to resuspended cells and incubated at room temperature for 15-30 min. Protein was extracted through six cycles of freeze-thaw using liquid nitrogen. Samples were then centrifuged and the supernatant from each cell fraction was removed and applied to a PD-10 desalting column (GE Healthcare, Sephadex G-25 M, PD-10 Columns). The eluate from these columns was used for both AlaAT activity measurements and kinetics assays.

2.2.5. Activity assays

Extracts were tested for GPAT activity prior to kinetic assays to ensure the induction of *AlaAT* expression. Varying dilutions of the AlaAT/GGAT protein extractions were assayed alongside the uninduced protein fractions to ensure the presence and activity of the

AlaAT/GGAT enzymes. Leaky expression of AlaAT in the uninduced protein fractions was regularly observed, but at very low levels. Activity assays were also conducted in order to determine the optimal degree of dilution of the enzyme necessary for kinetic assays; these typically ranged between 30X-40X. Activity assays were prepared and carried out at 20^{o}C in the direction alanine and 2-oxoglutarate to pyruvate and glutamate. Activity of AlaAT enzyme was determined using a continuous coupled reaction catalyzed by lactate dehydrogenase (LDH, Sigma, L-2518), with the change in absorbance associated with generation of NAD^+ from NADH monitored at 340 nm. Assays, done in 96 well microplates (UV-Star, VWR, 82050-788), were initiated by the addition of 10 µL of protein extracts, including the undiluted fraction (maximal activity of sample before dilution) and the undiluted uninduced (negative control) protein samples, to 290 µL of an AlaAT assay buffer (0.1 M Tris-HCl pH 8.0 at 20 ^{o}C, 10 mM 2-oxoglutarate, 670 mM alanine, 0.27 mg ml^{-1} NADH, 0.36 U LDH, 10 µM pyridoxal-5'-phosphate (PLP)) such that the final reaction volume was 300 µL per well. The change in absorbance at 340 nm was monitored continuously for 10 min in a SpectraMax Plus absorbance plate reader (Molecular Devices, Sunnyvale, CA).

2.2.6. Kinetic assays

Kinetic assays were run for both sets of AlaAT substrates, with the concentration of one substrate varied while the other was held constant at a saturating concentration, for 10 of the 13 constructs. Kinetic data were not obtained for MmAlaAT2-pBAD18-Kan, ScAlaAT2-pBAD18-Kan, HsAlaAT1-pBAD18-Kan, or HsAlaAT2-pBAD18-Kan as activity of these constructs was not detected in initial assays. It is believed that this inability to detect activity was the result of inclusion body formation with these proteins in *E. coli*.

Enzyme activity at each substrate concentration was assayed at 20 ^{o}C in triplicate, over a concentration range ($\approx 0.3 \times K_M$ - $8 \times K_M$) chosen based on previously published values and preliminary kinetic assessments. To each well, 10 µL of diluted AlaAT protein sample were added, along with 20 or 50 µL of substrate, and kinetic assay buffer to a final volume of 300 µL. When AlaAT activity was measured in the direction alanine to pyruvate, the kinetic assay buffer consisted of either alanine (100 mM) or 2-oxoglutarate (10 mM), 0.1 M Tris-HCl, pH 8.0, 0.27 mg ml^{-1} NADH, 10 µM PLP and 0.36 U LDH. When activity in the direction of pyruvate to alanine was measured, the assay buffer contained either pyruvate (10 mM) or glutamate (50

mM), 0.1 M Tris-HCl, pH 8.0, 100 mM NH_4Cl, 0.27 mg ml^{-1} NADH, 10 μM PLP and 1.14 U glutamate dehydrogenase (GDH, Sigma, G-2501). The change in absorbance at 340 nm was monitored continuously for 6 - 10 min in a SpectraMax Plus platereader. The initial pseudo-linear portion of each absorbance-time plot was analyzed by linear regression (SoftMax Pro v. 3.0) to obtain initial rates. Thereafter, plots of initial rate *versus* [substrate] were fitted to the Michaelis-Menten equation by nonlinear regression (GraphPad Prism v. 5.03) to determine K_M and V_{max} values (Appendix 2.2).

2.2.7. *Escherichia coli* growth assays

E. coli TR61 cells containing various pBAD18-Kan:*AlaAT* or pBAD18-Kan:*GGAT* constructs were assayed for growth over 26 hrs. One mL of an overnight starter culture grown in LB medium was added to 100 mL of modified M63 minimal medium containing 0.2% glycerol, 0.2% arabinose, 50 μg mL^{-1} kanamycin and chloramphenicol at 25 μg mL^{-1}. Bacterial cultures were then grown in flasks at 37˚C for 26 hrs. After 4 hrs of growth all cultures were re-inoculated with 0.1% arabinose. The OD_{600} was recorded at 1-4 hr intervals through 14 hrs of growth and then again at 26 hrs for induced cultures, untransformed controls, empty vector controls, and uninduced controls containing a pBAD18-Kan:*HvAlaAT*.

TR61 cells containing an empty pBAD18-Kan vector as well as TR61 cells containing pBAD18-Kan:*HvAlaAT* were also assayed for growth differences in M63 liquid minimal medium, supplemented as described above, containing 2-oxoglutarate at different concentrations (Table 4). Cell growth under these conditions was also assayed for a total of 26 hrs, with measurements taken every 1-3 hrs for the first 14 hrs and then again at 26 hrs.

2.3. Results

2.3.1. Homologous AlaAT primary sequence comparison

All 13 enzymes examined in this study were subgroup I aminotransferases and maintained the 11 invariant residues previously defined for this subgroup (Ward *et al*., 2000)) (Appendix 2.1). Primary sequence analysis (Figure 2.1) indicates that of the sequences studied, *P. furiosus* (PfAlaAT) is the most divergent, which is not surprising considering this was the only non-eukaryotic sequence examined. Interestingly, the protein sequences of AtGGAT1 and AtGGAT2 are more similar to plant AlaAT enzymes than are mammalian and archaean AlaAT

enzymes to plant homologues, even though GGAT enzymes are capable of both glutamate:glyoxylate and glutamate:pyruvate aminotransferase reactions (Aaron H Liepman & Olsen, 2003).

2.3.2. AlaAT enzymes have varying substrate K_M values

K_M values from eight AlaAT and two GGAT enzymes are compared in Table 2.1. Although K_M values for several of the enzymes analyzed here have been reported previously, our study facilitates a comparison of data obtained with a single assay system. K_M values reported here indicate significant diversity between the different enzymes for the same substrates. Between AtAlaAT1 (cytoplasmic) and AtAlaAT2 (mitochondrial) the minimal K_M discrepancy between substrates was reported for glutamate, with an approximate two-fold increase in K_M (2.5 mM to 4.9 mM respectively) (Table 2.1). The K_M values obtained from both *M. truncatula* proteins were similar for all substrates, the greatest difference being seen for the substrate glutamate, with a three-fold change in K_M (0.1 mM for MtAlaAT1, 0.3 mM for MtAlaAT2). The largest difference between substrates for a single enzyme was seen for PfAlaAT. For this enzyme, there was an 825-fold difference between K_M values for 2-oxoglutarate and pyruvate (0.02 mM and 16.5 mM respectively). The second largest difference in K_M values for a given enzyme between substrates was seen for MmAlaAT, with a 265-fold difference (seen between the K_M values for alanine and 2-oxoglutarate, 26.5 mM and 0.1 mM, respectively). No groupings or patterns could be established among the K_M values obtained, and relative differences were not consistent for a single enzyme and multiple substrates, or for the K_M values of multiple enzymes for a single substrate. K_M values for 2-oxoglutarate appeared to be reasonably constant (difference in K_M values of 8.78 mM) with AtGGAT2 having a K_M of 8.8 mM. The next largest value belonged to AtAlaAT2, with a K_M for 2-oxoglutarate of 1.0 mM. The range of K_M values for alanine, pyruvate and glutamate were much greater (differences in K_Ms of 26.2 mM, 12.9 mM and 18.5 mM respectively).

The K_M values reported here for HvAlaAT and ScAlaAT1 share some similarity to those recently reported by Duff *et al.,* (2012), with the largest difference between values being the K_M for ScAlaAT1 and the substrate pyruvate, here reported as a K_M of 11.0 and previously reported as a K_M of 0.4, a 27.5 fold difference. All other K_M's for the remaining substrates alanine, 2-oxoglutarate and glutamate showed lower fold differences when K_M values for ScAlaAT were

compared, 12, 2.5 and 2.9 respectively. The K_M values reported for HvAlaAT did not show as great a deviation between studies for the various substrates with, 6.7, 1.1, 9.1 and 2.1 fold differences for alanine, 2-oxoglutarate, glutamate and pyruvate respectively. These discrepancies in K_M values could be the result of numerous protocol differences as outlined above, and re-emphasize the importance of obtaining enzymatic data from a single source for the purpose of direct comparisons.

V_{max} values for all enzymes assayed are presented in Appendix 2.4. Since enzyme fractions were not purified, the concentrations of the enzymes used, and thus catalytic rate constants are unknown, therefore the usefulness of V_{max} values in making meaningful comparisons between different enzymes is diminished. Purification of individual proteins in order to establish enzyme concentrations, thereby allowing determination of k_{cat} and comparison of $V_{max,}$ was not done due to the absence of an antibody that would specifically bind each of the different variants for purification purposes. Furthermore, enzymes were not tagged with either His or Myc-C sequences since such alterations may affect enzyme kinetics. Given that these were recombinant proteins expressed in a bacterial system, protein folding may have been altered affecting kinetic results. Since whole protein fractions were utilized during this study, the possibility that enzyme inhibitors were present or that non-AlaAT transaminase activities may have contributed to substrate turnover as well as NAD^+/NADH concentrations and influenced calculated kinetic constants must be considered.

2.3.3. AlaAT homologues differ in their ability to reduce growth rate of gram negative bacteria

It was hoped that changes in the availability of AlaAT substrate(s) (2-oxoglutarate) during growth of *E. coli* over-expressing various AlaAT homologues would allow for differentiation of homologous enzymes in terms of substrate usage, manifest phenotypically as changes in rates of growth. Ultimately, no difference in growth rate of *E. coli* containing HvAlaAT was observed when exposed to concentrations of 2-oxoglutarate (Figure 2.2). However, a slow growth phenotype in all *E. coli* cultures expressing the various *AlaAT* constructs was observed (Figure 2.2 and Figure 2.3).

Expression of recombinant *AlaAT* from *B. japonicus* (*AmphiALT*), in gram-negative *E. coli* has been shown to cause cell lysis through lipopolysaccharide (LPS) binding (Jing & Zhang,

2011). Jing and Zhang (2011) observed that AmphiALT was able to bind and lyse gram-negative but not gram-positive cells; binding was reported to be specific to the LPS region. From these results it was suggested that AlaAT may also be involved in the acute phase response, particularly in liver tissues. Our study supports this view, indicating that recombinant expression of AlaAT in *E. coli* inhibits growth, and decreases cell growth rates when grown in minimal medium (Figure 2.3 and Figure 2.4). Due to the absence of antibodies for all AlaAT enzymes and thus an inability to purify individual AlaATs, direct binding of *E. coli* LPS to the various AlaAT enzymes was not examined. However, slow growth phenotypes similar to those described previously in the presence of AmphiALT were observed, leading to the conclusion that all of the AlaAT enzymes studied exhibit some bactericidal activity similar to *AmphiALT* from *B. japonicus*, and providing evidence for the conservation of AlaAT bactericidal properties. In order to clarify, the growth curves of only five of the ten *AlaATs* expressed in *E. coli* are shown in Figure 2.3. *E. coli* cells expressing *AtAlaAT2*, *MtAlaAT*, *ScAlaAT* and *MmAlaAT1* showed growth curves similar to those obtained with *AtAlaAT1*.

Among the ten enzymes assayed, inhibition of growth rate varied significantly. PfAlaAT showed the greatest effect on growth rate over time, seen most clearly in Figure 2.4, when the first derivative of each sample is determined. Both AtGGAT2 and AtGGAT1 also show dramatic decreases in growth rate over time (Figure 2.4). Comparison of kinetic constants for a particular enzyme with growth assay results has not provided any insights into the reasons for these differences in growth rates. Also interesting was the observed change in the growth rate of the bacteria containing the HvAlaAT over time (Figure 2.4). While a constant or slight increase in growth rate was observed with the majority of proteins assayed, the over-expression of HvAlaAT resulted in a substantial decrease in growth rate starting at approximately 2 hours and continuing until approximately 7 hrs, at which point growth rate once again began to increase.

To investigate further the bactericidal activity of AlaAT, the primary structure of each enzyme was analyzed for a conserved endotoxin binding region. Through analysis of the known LPS region from human lipopolysaccharide binding protein (LBP) (Wong *et al*., 2007), a similar conserved region was found to various extents in all AlaAT enzymes (Figure 2.5). PfAlaAT shows the greatest conservation of the LPS binding region, with the highest number of residues conserved, and a percent identity of 26% (Figure 2.5a). PfAlaAT was also the most effective

isoform inhibiting growth of *E. coli* (Figure 2.4). AtGGAT1 and AtGGAT2 also show a high degree of conservation of identical residues, with percent identities of 22% (Figure 2.5b). All sequences show a high degree of conservative and semi-conservative sequence similarity, for example with AtAlaAT1, AtAlaAT2, MtAlaAT and HvAlaAT (Figure 2.5c), HsAlaAT1, HsAlaAT2, MmAlaAT1 and MmAlaAT2 (Figure 2.5d), and ScAlaAT1 and ScAlaAT2 (Figure 2.5e). However the ability of these enzymes to bind the LPS region of *E. coli* and inhibit growth was not as pronounced as that observed for PfAlaAT, AtGGAT1 and AtGGAT2, perhaps demonstrating the importance of a high level of conservation of sequences/residues at these sites.

2.4. Discussion

Glutamate:pyruvate aminotransferases (AlaAT/GPAT) and glutamate:glyoxylate aminotransferases (GGAT) are subgroup I aminotransferases, containing eleven invariant residues essential for binding the coenzyme PLP and for stabilizing the enzyme:substrate transition state (Liepman & Olsen, 2003; Mehta *et al*., 1993). Both AlaAT and GGAT enzymes share similar primary and secondary structures, as well as hydropathy with other subgroup I aminotransferases such as aspartate aminotransferase and tyrosine aminotransferase (Mehta *et al*., 1993), with PfAlaAT being the most divergent of the enzymes analyzed in this study (Figure 2.1). It appears that the kinetic differences identified here are due to differences in non-conserved residues which may cause changes in substrate binding affinity and/or catalytic rate, perhaps as a result of changes in enzyme folding.

A kinetic examination of the enzyme alanine aminotransferase is of interest for a number of reasons. AlaAT has been shown to be involved in stress responses in numerous plants, including cereal crops (Good & Muench, 1992; Kikuchi *et al*., 1999; Miyashita *et al*., 2007), while in mammals, this enzyme is used as an indicator of non-alcoholic hepatocellular liver damage (Yang *et al*., 2009) and may be involved in immune system acute phase response (Jing & Zhang, 2011). Recently, AlaAT has been shown to increase NUE in cereal crops when over-expressed with a tissue-specific promoter (Good *et al*., 2007b; Shrawat *et al*., 2008).

To date, most studies on AlaAT have been organism and tissue-specific, focusing on a single enzyme or isozymes, making comparisons between AlaAT enzymes from different species difficult. Comparisons between enzymes have also been limited due to purification and

expression differences, as in the case of AtGGAT1. The AlaAT activity of this enzyme has been examined by purification of this protein from both shoot tissues (Wisniewski *et al*., 2006) and recombinant *E. coli* (Aaron H Liepman & Olsen, 2003). These differences in enzyme source and purification procedure can manifest as alterations in enzymatic kinetic behaviour, compounded by differences in the assay conditions used during kinetic analyses (Good & Muench, 1992; Marangoni, 2003; Ward *et al*., 2000). Other errors may result from the presence of homologous proteins which were not separated from the AlaAT of interest during purification, due to similar biochemical properties and increases in activity from environmental changes (e.g. greening of leaves) (B. Singh, 1999). Due to these confounding factors, kinetic data obtained with enzymes from various sources cannot be reliably compared across different studies (Table 2.2).

It is therefore of interest to determine the effects on NUE phenotypes of AlaAT enzymes that display kinetics similar to those of HvAlaAT, compared with enzymes that have very different characteristics. Based on the results from the kinetic assays, AtAlaAT1 appears to be most similar to HvAlaAT. Both AtAlaAT enzymes have higher K_M values for glutamate and alanine, and lower K_M values for 2-oxoglutarate and pyruvate, compared with HvAlaAT. MmAlaAT1 has rather different K_M values, raising the possibility of a distinct *in vivo* role(s). Compared with other AlaATs examined, this enzyme had the highest K_M values for both alanine (26.5 mM) and glutamate (13.0 mM).

It is difficult to extrapolate *in vitro* kinetic data and predict the consequences of altered substrate K_M values, without knowing the cellular concentrations of substrates under various environmental conditions. For example, while MmAlaAT appears to display very different kinetic behavior compared with HvAlaAT, *in vivo* analysis will be needed in order to verify whether or not such differences have any effect on plant phenotype when the gene is ectopically expressed. Alanine and glutamic acid concentrations in rice seeds show average millimolar amounts of alanine as ~0.57 mM and glutamic acid as ~1.53 mM, with vitamin B_6, a precursor to the AlaAT cofactor PLP, at ~0.72 mM (Jiao *et al*., 2010). Other studies conducted by Narsai *et al.,* (2009) show that in rice seedlings these concentrations are altered during the growth and development of the plant and are approximately 0.09 mM for glutamate, 2.27 mM for alanine, 0.79 mM for pyruvate and 0.65 mM for 2-oxoglutarate. The changes in AlaAT substrate concentrations during different phases of plant growth and in various cellular tissues and organs

has also been documented elsewhere (Branco-Price *et al.*, 2008). Aminotransferase enzymes with overlapping functions have also been observed in a number of organisms, including *E. coli* (Kim *et al*., 2010), *P. furiosus* (Ward *et al*., 2000) and plants (Duff *et al*., 2012; Hatch & Mau, 1977). The effect of any of these enzymes on nitrogen uptake or metabolism can only be speculated upon and would require whole plants studies and *in vivo* analysis. Analysis of the kinetics of aspartate aminotransferase (AspAT) from higher plants has also been carried out recently with similar intentions of crop improvement (Duff *et al*., 2011)

As an initial screen to determine if the presence of a specific AlaAT variant in *E. coli* had a significant effect on the bacteria's ability to utilize specific substrates, *E. coli* expressing the various AlaAT enzymes from plasmid constructs were grown in modified M63 medium supplemented with various concentrations of 2-oxoglutarate, with ammonium as the nitrogen source. It was speculated that 2-oxoglutarate might have an effect on the growth rates of *E. coli* due to its central role in linking both carbon and nitrogen metabolism in bacteria (Ninfa & Jiang, 2005; Bunik & Fernie, 2009; Jiang & Ninfa, 2009; Uhrig *et al*., 2009). Furthermore, excess 2-oxoglutarate in the growth medium may have a significant impact on AlaAT enzymes with lower K_M values for both alanine and 2-oxoglutarate, if substrate k_{cat} values of these enzymes are not also lower and assuming that substrates are present at sub-saturating concentrations.

No difference in growth rate of *E. coli* containing HvAlaAT were observed when exposed to concentrations of 2-oxoglutarate (Figure 2.2) however, a slow growth phenotype in all *E. coli* cultures expressing the various *AlaAT* constructs was identified (Figure 2.2 and Figure 2.3). Confirming the results by Jing & Zhang (2011), which showed that expression of recombinant *AlaAT* from *B. japonicus* (*AmphiALT*), in gram-negative *E. coli* was able to bind and lyse gram-negative but not gram-positive cells; binding was reported to be specific to the LPS region. A correlation between conservation of the LBP region in the AlaAT variants and degree of *E. coli* growth rate inhibition was identified. PfAlaAT showed both the greatest effect on rate of growth and the highest degree of conservation of the LBP (Figure 2.5) when AlaAT enzymes were expressed in *E. coli*. The possibility that other conserved endotoxin binding regions may exist to varying degrees in the AlaAT homologues analyzed should also be considered as this could also contribute to the variation of growth inhibition observed. Given the evidence that AlaAT enzymes may be playing a role in acute phase response to bacterial

infections *in vivo* (Jing & Zhang, 2011), determining how these differences affect, or are affected by concentration-dependent binding of other molecules within the cell will be important to develop a more complete understanding of enzyme function.

The kinetic results presented here indicate that catalytic properties between AlaAT homologues differ considerably. Previous analysis in *Brassica napus* and rice has already determined the importance of using a tissue-specific promoter in obtaining an NUE phenotype (Good *et al*., 2007b; Good *et al*., 2007a; Lock, 2011; Shrawat *et al*., 2008; Wolansky, 2005). The knowledge of enzyme variants gained here, as well as prior knowledge of promoters provides a platform for future NUE studies and the improvement of crop nutrient utilization.

Table 2.1. Summary of kinetic assay results.

K_M values are shown for each substrate, for each of the ten enzymes examined. Kinetic values represent the average of three independent trials. The correlation coefficient (r^2) was > 0.80 for all trials, except AtGGAT1 glutamate, AtGGAT2 glutamate and MmAlaAT1 pyruvate. Raw data are plotted in Appendix 2.2.

Enzyme	Alanine		2-oxoglutarate		Glutamate		Pyruvate	
	K_M (mM)	SE K_M	K_M (mM)	SE K_M	K_M (mM)	SE K_M	K_M (mM)	SE K_M
AtAlaAT1	2.4	±0.5	0.1	±0.0	2.5	±0.4	0.1	±0.0
AtAlaAT2	10.4	±1.2	1.0	±0.1	4.9	±1.1	5.1	±0.9
AtGGAT1	1.9	±0.5	0.2	±0.1	0.2	±0.1	0.6	±0.1
AtGGAT2	1.2	±0.1	8.8	±1.8	0.2	±0.1	18.5	±2.7
HvAlaAT	5.6	±0.6	0.2	±0.0	4.9	±0.5	0.1	±0.0
MtAlaAT1	1.0	±0.2	0.2	±0.0	0.1	±0.0	18.6	±2.7
MtAlaAT2	1.5	±0.2	0.3	±0.0	0.3	±0.1	18.0	±1.9
MmAlaAT1	26.5	±2.3	0.1	±0.1	13.0	±2.4	12.5	±2.0
PfAlaAT	4.0	±0.5	0.02	±0.0	0.9	±0.1	16.5	±2.2
ScAlaAT1	0.3	±0.1	0.5	±0.1	0.7	±0.1	11.0	±1.7

Table 2.2. Comparison of K_M values for AlaAT and GGAT enzymes.

K_M values obtained during this study were compared with previous K_M values from various published sources. Discrepancies between kinetic values can be attributed partially to differences in procedure as well as differences in original data collection. Nevertheless, significant differences in K_M values for all enzymes are evident. Variation amongst results highlights the benefit of using one system and one procedure when comparing kinetic data from several different enzymes. (NA = no data available from previous studies.)

Organism	K_M (mM)				Previous K_M (mM)				Source of AlaAT	Reference
	Alanine	2-Oxoglutarate	Pyruvate	Glutamate	Alanine	2-Oxoglutarate	Pyruvate	Glutamate		
***Arabidopsis* AlaAT1**	2.4	0.1	0.1	2.5	1.5	0.2	NA	NA	*A. thaliana* leaves (protein purified)	Wiśniewski *et al*., (2006)
Barley AlaAT	5.6	0.2	0.1	4.9	17	5	0.1	1	Barley roots (protein purified)	Good and Muench (1992)
***Arabidopsis* GGAT1**	1.9	0.2	0.6	0.2	4.8	0.3	0.3	2.0	*E. coli* (recombinant expression)	Liepman and Olsen (2003)
					NA	NA	NA	1.2	*A. thaliana* leaves (protein purified)	Wiśniewski *et al*., (2006)
***Arabidopsis* GGAT2**	1.2	8.8	18.5	0.2	3.6	0.5	0.4	3.3	*E. coli* (recombinant expression)	Liepman and Olsen (2003)
***Pyrococcus furiosus* AlaAT**	4.0	0.02	16.5	0.9	3.2	1.1	4.7	5.6	*E. coli* (recombinant expression)	Ward *et al*., (2000)

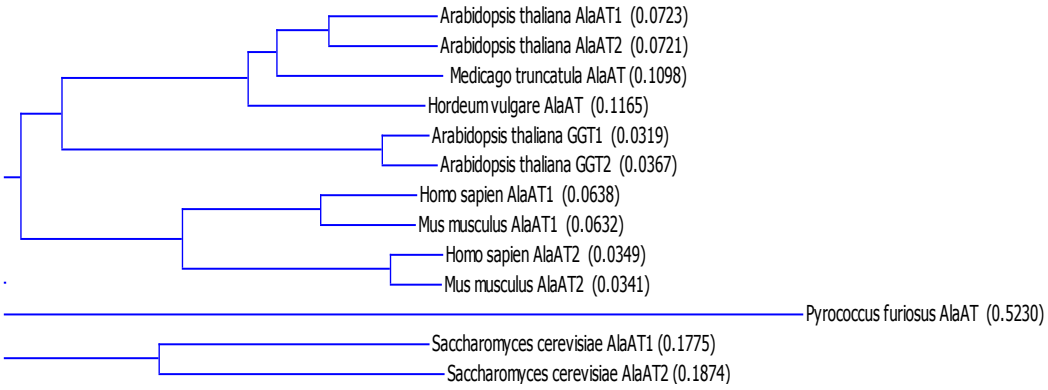


Figure 2.1. Phylogenetic dendrogram of eleven AlaAT enzymes and two GGAT enzymes.

A phylogenetic dendrogram was constructed using neighbour joining (NJ) based on amino acid sequence similarity using Vector NTI Advance v. 11.0, AlignX software. The sequences used to construct the dendrogram were obtained from the NCBI database except that of Medicago which was obtained courtesy of Anis Limami at the Université d' Angers.

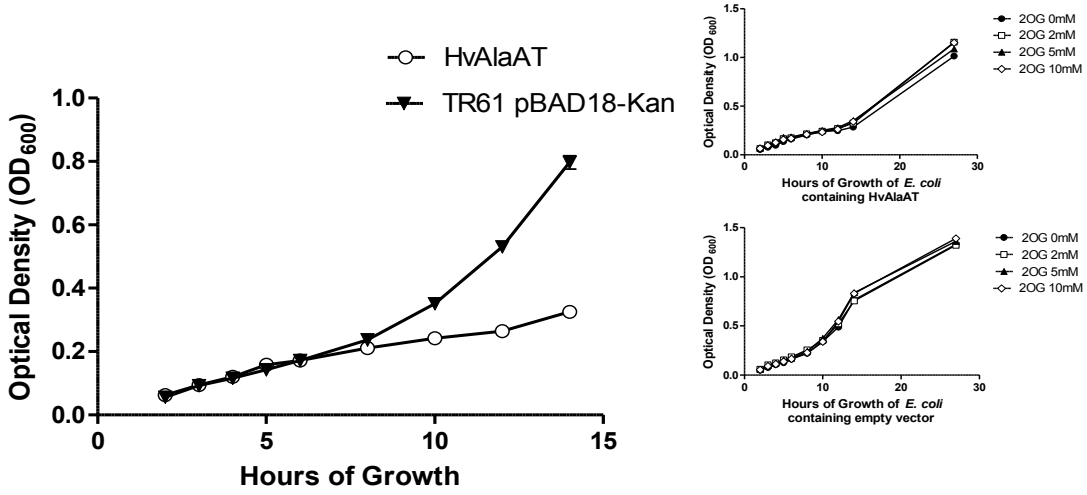


Figure 2.2. Effect of 2-oxoglutarate on growth rate of *E. coli* expressing *HvAlaAT*.

Growth of TR61 cells containing either pBAD:HvAlaAT or TR61 cells containing an empty pBAD18-Kan vector in modified M63 medium. *E. coli* containing the two constructs grown in media containing 0 mM, 2 mM, 5 mM or 10 mM 2-oxoglutarate (2OG), pH 8.0 (inset).

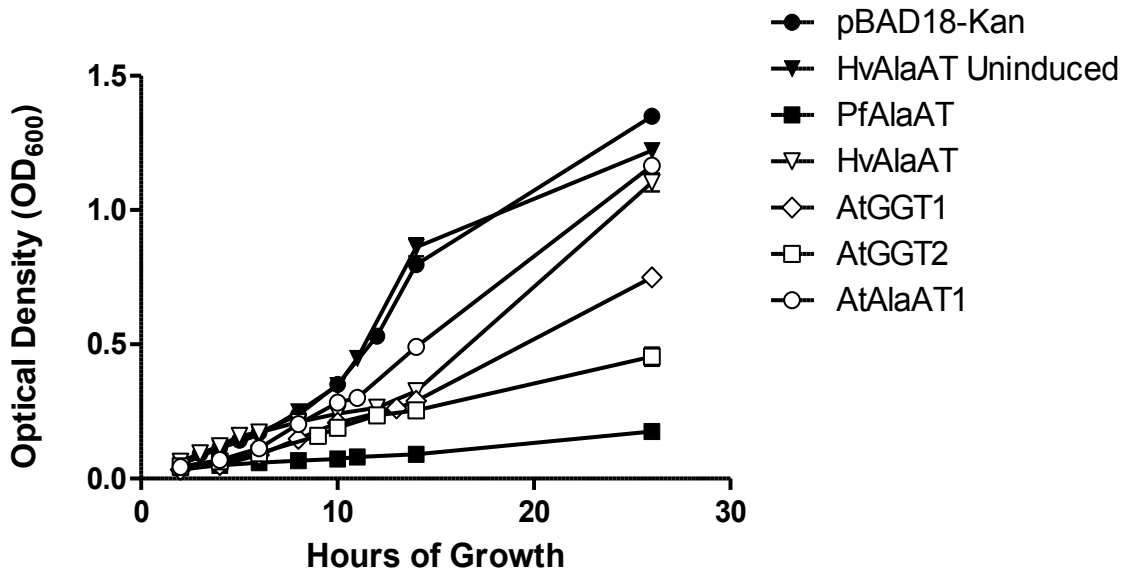


Figure 2.3. The average growth of *E. coli* containing various AlaAT and GGAT enzymes.

TR61 *E. coli* were cultured in a M63 revised minimal medium and expression of AlaAT or GGAT was induced by the addition of arabinose. The average optical density at 600 nm was measured and recorded over a 26 hr time period and is representative of trials done in triplicate. Error bars show standard error, where they exceed symbol size. TR61 cells containing no pBAD vector, cells containing a pBAD vector with no *AlaAT* or *GGAT* insert and uninduced TR61 cells containing a pBAD::HvAlaAT enzyme were utilized as controls. *E. coli* expressing *AtAlaAT2*, *MtAlaAT*, *ScAlaAT* and *MmAlaAT* showed changes in optical density over the 26 hr time period similar to that seen by *Arabidopsis thaliana AlaAT1* expressing cells.

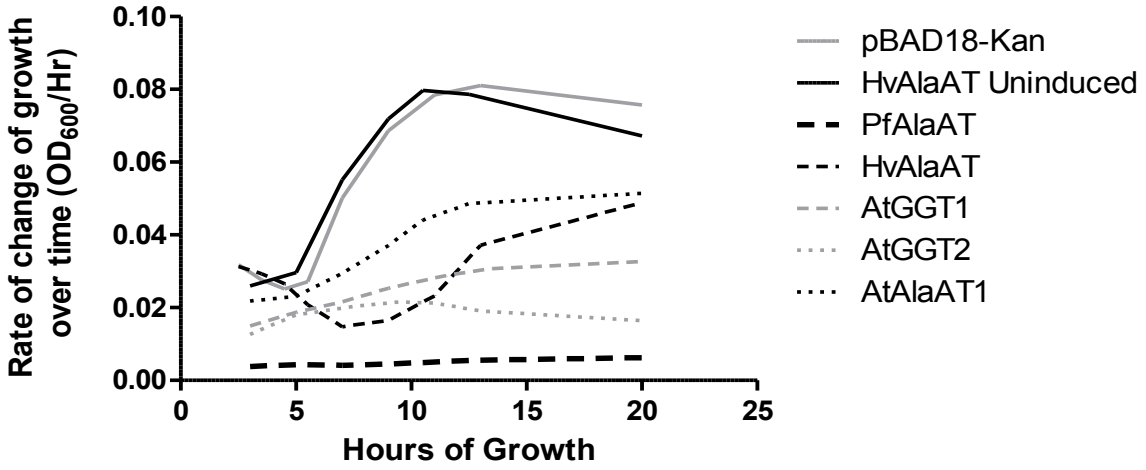


Figure 2.4. The rate of change of the growth over time (OD_{600}/Hrs) of *E. coli* strains containing various alanine aminotransferase enzymes.

The smooth first derivative of each time trial in Figure 4 was determined, indicating the change in the growth rate of the bacteria containing each of the different AlaATs and the controls over time. *E. coli* expressing *AtAlaAT2, MtAlaAT*, *ScAlaAT1* and *MmAlaAT1* showed changes in the rate of growth over the 26 hour time period similar to that seen by *Arabidopsis thaliana AlaAT1* expressing cells.

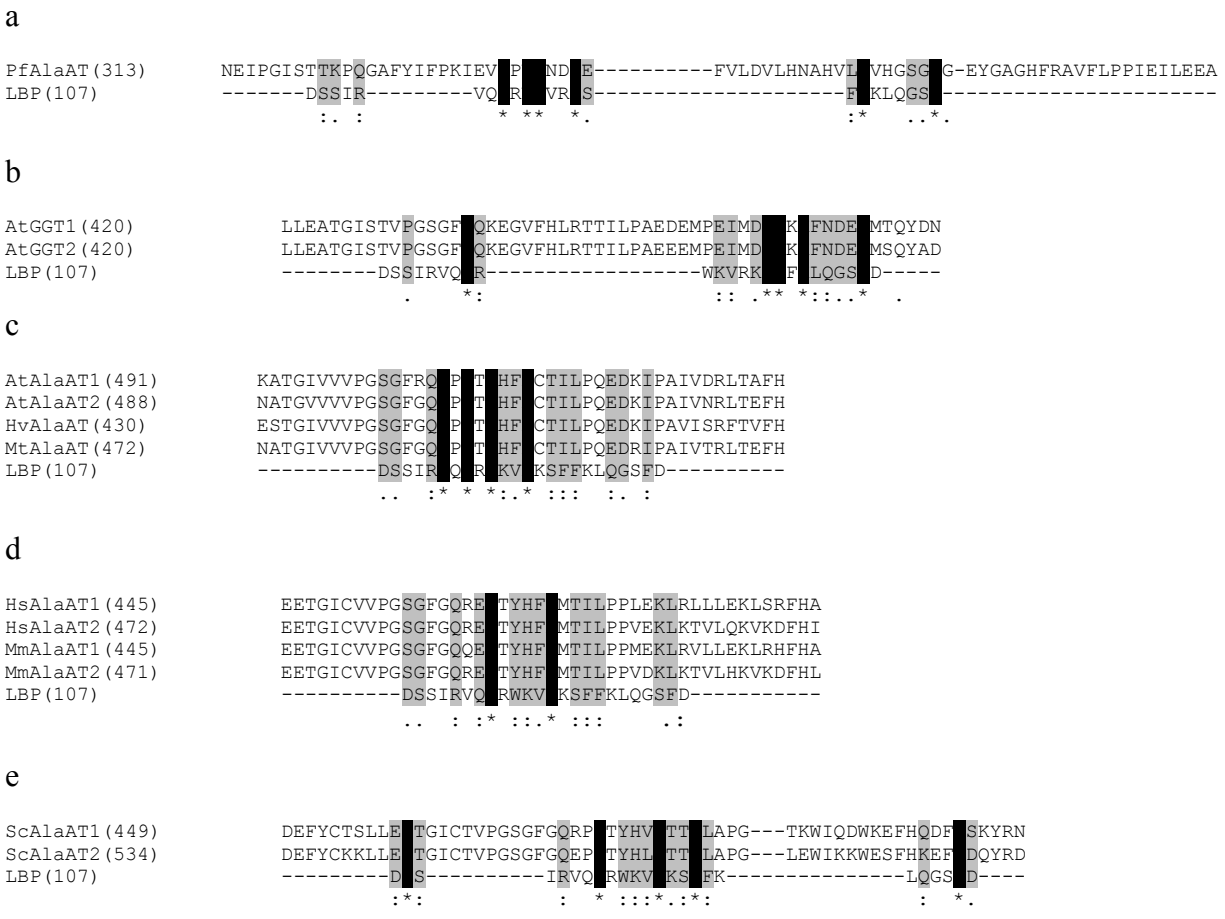


Figure 2.5. Comparison of AlaAT putative endotoxin binding regions.

Primary sequence comparison of AlaAT homologues to the known endotoxin binding region of human LBP (A-E) using the ClustalW alignment program. Identical residues are highlighted in black with white text while conservative and semi-conservative residues are highlighted in grey with black text. ("*****" indicate identical residues: ":" indicates conservative substitutions; "." indicates semi-conservative substitution).

2.5. Bibliography

Beatty, P. H., Shrawat, A. K., Carroll, R. T., Zhu, T. and Good, A. G. (2009) Transcriptome analysis of nitrogen-efficient rice over-expressing alanine aminotransferase. *Plant Biotech. J.* **7:** 562–576.

Branco-Price, C., Kaiser, K. a, Jang, C. J. H., Larive, C. K. and Bailey-Serres, J. (2008) Selective mRNA translation coordinates energetic and metabolic adjustments to cellular oxygen deprivation and reoxygenation in *Arabidopsis thaliana*. *Plant J.* **56:** 743–755.

Brückner, H. and Westhauser, T. (1994) Chromatographic determination of D-amino acids as native constituents of vegetables and fruits. *Chromato.* **39:** 419-426

Brückner, H. and Westhauser, T. (2003) Chromatographic determination of L- and D-amino acids in plants. *Amino Acids* **24:** 43–55.

Bunik, V. I. and Fernie, A. R. (2009) Metabolic control exerted by the 2-oxoglutarate dehydrogenase reaction: a cross-kingdom comparison of the crossroad between energy production and nitrogen assimilation. *Biochem. J.* **422:** 405–421.

Duff, S. M. G., Qi, Q., Reich, T., Wu, X., Brown, T., Crowley, J. H. and Fabbri, B. (2011) A kinetic comparison of asparagine synthetase isozymes from higher plants. *Plant Physiol. Biochem.* **49:** 251–256.

Duff, S. M. G., Rydel, T. J., McClerren, A. L., Zhang, W., Li, J. Y., Sturman, E. J., Halls, C., Chen, S., Zeng, J., Peng, J., Kretzler, C. N. and Evdokimov, A. (2012) The Enzymology of alanine aminotransferase (AlaAT) isoforms from *Hordeum vulgare* and other organisms, and the HvAlaAT crystal structure. *Arch. Biochem. Biophysi.* **528:** 90-101.

Good, A. G., DePauw, M., Kridl, J. C., Theodoris, G. and Shrawat, A. K. (2007b) Nitrogen-efficient monocot plants. US Patent #20070162995.

Good, A. G., Johnson, S. J., De Pauw, M., Carroll, R. T., Savidov, N., Vidmar, J., Lu, Z., Taylor, G. and Stroeher, V. (2007a) Engineering nitrogen use efficiency with alanine aminotransferase. *Can. J. B.* **85:** 252–262.

Good, A. G. and Muench, D. G. (1992) Purification and characterization of an anaerobically induced alanine aminotransferase from barley roots. *Plant Physiol.* **99:** 1520–1525.

Hatch, M. D. and Mau, S. L. (1977) Association of NADP- and NAD-linked malic enzyme acitivities in *Zea mays*: relation to C4 pathway photosynthesis. *Arch. Biochem. Biophys*. **179:** 361–369.

Jadaho, S. B., Yang, R.-Z., Lin, Q., Hu, H., Anania, F. a, Shuldiner, A. R. and Gong, D.-W. (2004) Murine alanine aminotransferase: cDNA cloning, functional expression, and differential gene regulation in mouse fatty liver. *Hepatol.* **39:** 1297–1302.

Jiang, P. and Ninfa, A. J. (2009) Alpha-ketoglutarate controls the ability of the Escherichia coli PII signal transduction protein to regulate the activities of NRII (NrB) but does not control the binding of PII to NRII. *Biochem.* **48:** 11514–11521.

Jiao, Z., Si, X., Li, G., Zhang, Z. and Xu, X. (2010) Unintended compositional changes in transgenic rice seeds (*Oryza sativa* L.) studied by spectral and chromatographic analysis coupled with chemometrics methods. *J. Agri. Food Chem.* **58:** 1746–1754.

Jing, X. and Zhang, S. (2011) An ancient molecule with novel function: Alanine aminotransferase as a lipopolysaccharide binding protein with bacteriocidal activity. *Dev. Comp. Immun.* **35:** 94–104.

Kameya, M., Arai, H., Ishii, M. and Igarashi, Y. (2010) Purification of three aminotransferases from Hydrogenobacter thermophilus TK-6--novel types of alanine or glycine aminotransferase: enzymes and catalysis. *FEBS* **277:** 1876–1885.

Kant, S., Bi, Y. and Rothstein, S. J. (2011) Understanding plant response to nitrogen limitation for the improvement of crop nitrogen use efficiency. *Access* **62:** 1499–1509.

Kikuchi, H., Hirose, S., Toki, S., Akama, K. and Takaiwa, F. (1999) Molecular characterization of a gene for alanine aminotransferase from rice (*Oryza sativa*). *Plant Mol. Biol.* **39:** 149–159.

Kim, S. H., Schneider, B. L. and Reitzer, L. (2010) Genetics and regulation of the major enzymes of alanine synthesis in Escherichia coli. *J. Bacteriol.* **192:** 5304–5311.

Liepman, A. H. and Olsen, L. J. (2003) Alanine aminotransferase homologs catalyze the glutamate:glyoxylate aminotransferase reaction in peroxisomes of Arabidopsis. *Plant Physiol.* **131:** 215–227.

Locke, Y.-Y. (2011) *Engineering nitrogen use efficiency in Oryza sativa by the developmental over-expression of barley alanine aminotransferase using a novel rice promoter*. M.Sc. thesis. University of Alberta.

Marangoni, B. (2003) Enzyme Kinetics: A Modern Approach. New Jersey: John Wiley and Sons Inc.

Masclaux-Daubresse, C., Daniel-Vedele, F., Dechorgnat, J., Chardon, F., Gaufichon, L. and Suzuki, A. (2010) Nitrogen uptake, assimilation and remobilization in plants: challenges for sustainable and productive agriculture. *Ann. Bot.* **105:** 1141–1157.

McAllister, C. H., Beatty, P. H. and Good, A. G. (2012) Engineering nitrogen use efficient crop plants: the current status. *Plant Biotech. J.* **10:** 1011–1025.

Mehta, P. K., Hale, T. I. and Christen, P. (1993) Aminotransferases: demonstration of homology and division into evolutionary subgroups. *Database* **561:** 549–561.

Mehta, P. K., Hale, T. I. and Christen, P. (1993) Aminotransferases: demonstration of homology and division into evolutionary subgroups. *FEBS* **214:** 549–561.

Miyashita, Y., Dolferus, R., Ismond, K. P. and Good, A. G. (2007) Alanine aminotransferase catalyses the breakdown of alanine after hypoxia in *Arabidopsis thaliana*. *Plant J.* **49:** 1108–1121.

Muench, D. G. and Good, A. G. (1994) Hypoxically inducible barley alanine aminotransferase: cDNA cloning and expression analysis. *Plant Mol. Biol.* **24:** 417–427.

Narsai, R., Howell, K. A., Carroll, A., Ivanova, A., Millar, A. H. and Whelan, J. (2009) Defining core metabolic and transcriptomic responses to oxygen availability in rice embryos and young seedlings. *Plant Physiol.* **151:** 306–322.

Ninfa, A. J. and Jiang, P. (2005) PII signal transduction proteins: sensors of alpha-ketoglutarate that regulate nitrogen metabolism. *Curr. Opin. Microbiol.* **8:** 168–173.

Paine, J. A., Shipton, C. a, Chaggar, S., Howells, R. M., Kennedy, M. J., Vernon, G., Wright, S. Y., Hinchliffe, E., Adams, J. L., Silverstone, A. L. and Drake, R. (2005) Improving the nutritional value of Golden Rice through increased pro-vitamin A content. *Nat. Biotech.* **23:** 482–487.

Ricoult, C., Echeverria, L. O., Cliquet, J.-B. and Limami, A. M. (2006) Characterization of alanine aminotransferase (AlaAT) multigene family and hypoxic response in young seedlings of the model legume *Medicago truncatula*. *J. Exp. Bot.* **57:** 3079–3089.

Shrawat, A. K., Carroll, R. T., DePauw, M., Taylor, G. J. and Good, A. G. (2008) Genetic engineering of improved nitrogen use efficiency in rice by the tissue-specific expression of alanine aminotransferase. *Plant Biotech. J.* **6:** 722–732.

Singh, B. (1999) *Plant Amino Acids: Biochemistry and Biotechnology*. New York: Marcel Dekker Inc.

Sohocki, M. M., Sullivan, L. S., Harrison, W. R., Sodergren, E. J., Elder, F. F., Weinstock, G., Tanase, S. and Daiger, S. P. (1997) Human glutamate pyruvate transaminase (GPT): localization to 8q24.3, cDNA and genomic sequences, and polymorphic sites. *Genom.* **40:** 247–252.

Uhrig, R. G., Ng, K. K. S. and Moorhead, G. B. G. (2009) PII in higher plants: a modern role for an ancient protein. *Trends Plant Sci.* **14:** 505–511.

Vigeolas, H., Waldeck, P., Zank, T. and Geigenberger, P. (2007) Increasing seed oil content in oil-seed rape (*Brassica napus* L .) by over-expression of a yeast glycerol-3-phosphate dehydrogenase under the control of a seed-specific promoter. *Plant Biotech. J.* **5:** 431–441.

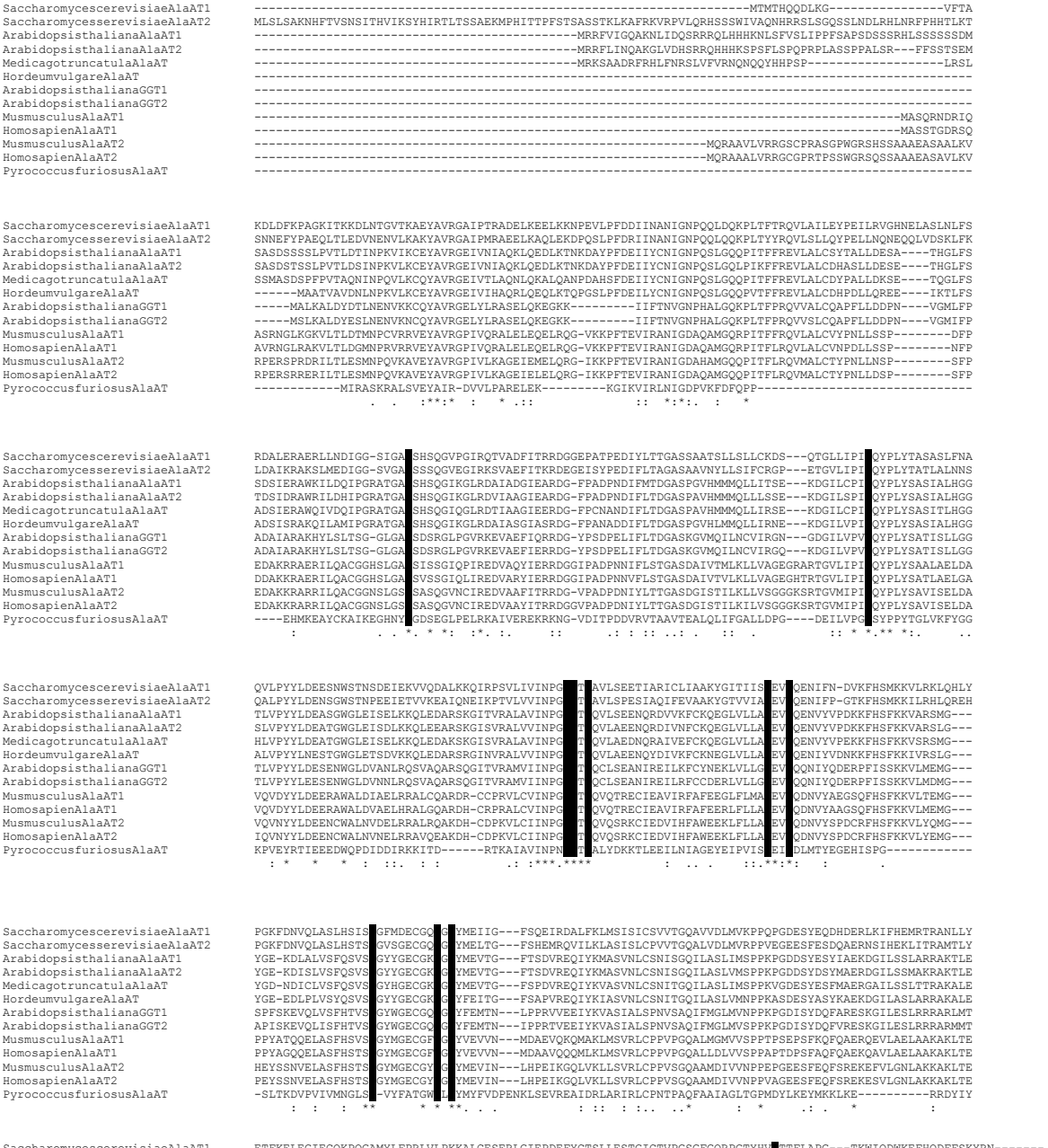
Ward, D. E., Kengen, S. W., van Der Oost, J. and de Vos, W. M. (2000) Purification and characterization of the alanine aminotransferase from the hyperthermophilic archaeon *Pyrococcus furiosus* and its role in alanine production. *J. Bacteriol.* **182:** 2559–2566.

Wisniewski, P., Szklarczyk, J., Maciga, M. and Paszkowski, A. (2006) L -alanine:2-oxoglutarate aminotransferase isoenzymes from Arabidopsis thaliana leaves. *Acta Physiol. Plant.* **28:** 577–588.

Wolansky, M. A. (2005) *Genetic manipulation of aspartate aminotransferase levels in Brassica napus: effects on nitrogen use efficiency*. M.Sc. thesis. University of Alberta.

Wong, K.-F., Luk, J. M., Cheng, R. H., Klickstein, L. B. and Fan, S.-T. (2007) Characterization of two novel LPS-binding sites in leukocyte integrin betaA domain. *FASEB* **21:** 3231–3239.

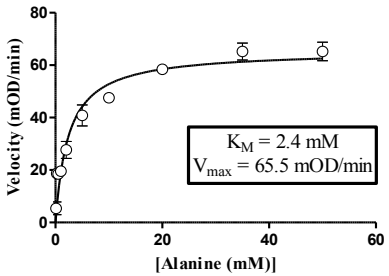
Yang, R.-Z., Park, S., Reagan, W. J., Goldstein, R., Zhong, S., Lawton, M., Rajamohan, F., Qian, K., Liu, L. and Gong, D.-W. (2009) Alanine aminotransferase isoenzymes: molecular cloning and quantitative analysis of tissue expression in rats and serum elevation in liver toxicity. *Hepatol.* **49:** 598–607.

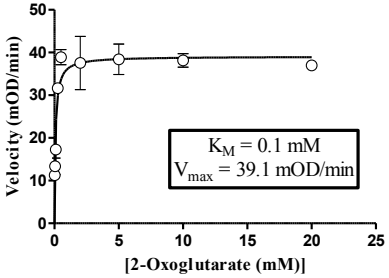

SaccharomycescerevisiaeAlaAT1
SaccharomycesserevisiaeAlaAT2
ArabidopsisthalianaAlaAT1
ArabidopsisthalianaAlaAT2
MedicagotruncatulaAlaAT
HordeumvulgareAlaAT
ArabidopsisthalianaGGT1
ArabidopsisthalianaGGT2
MusmusculusAlaAT1
HomosapienAlaAT1
MusmusculusAlaAT2
HomosapienAlaAT2
PyrococcusfuriosusAlaAT

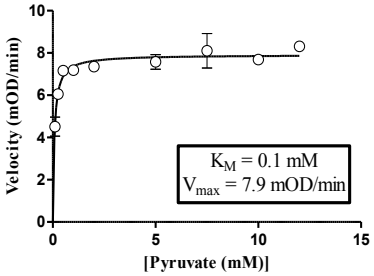
Appendix 2.1. Amino acid sequence alignment for eleven AlaAT enzyme sequences and two GGAT enzymes sequences.

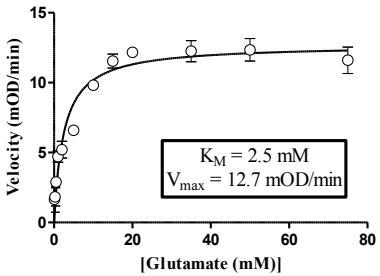
Amino acid sequences used were obtained from NCBI, except *M. truncatula* which was provided by Anis Limami, at the Université d' Angers, and analysis was done using ClustalW software. Residues conserved in subtype I aminotransferases are highlighted in white text on a black background. Fully conserved residues are indicated by "*", conservative substitutions are indicated by ":", and "." denotes a semi-conservative substitution.

a AtAlaAT1

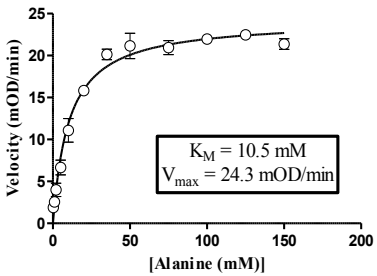


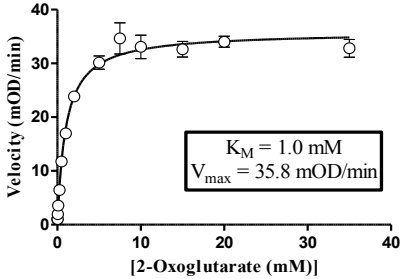


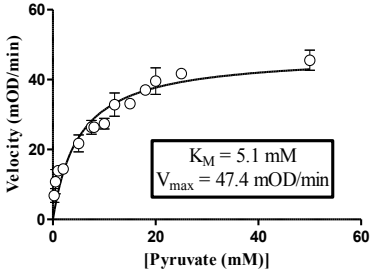


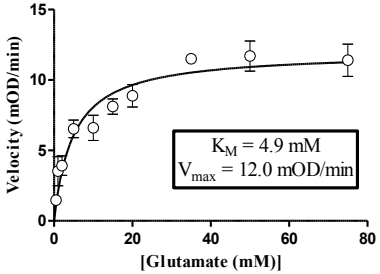


b AtAlaAT2

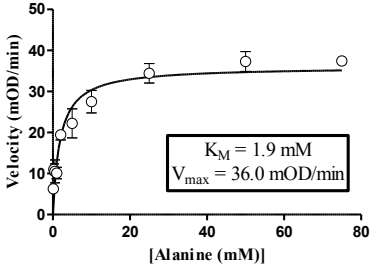


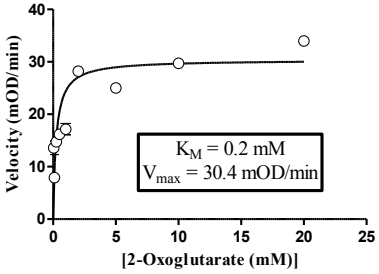


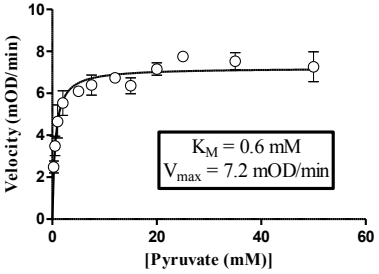


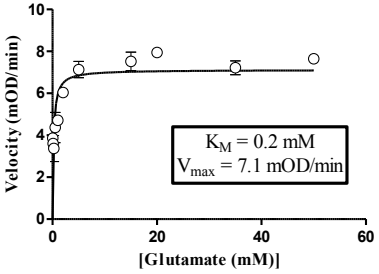


c AtGGT1

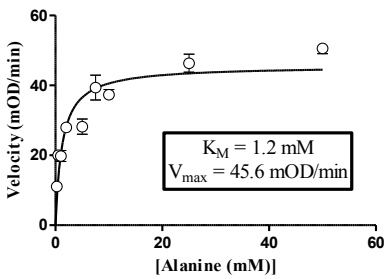


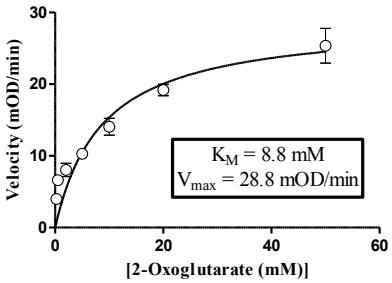


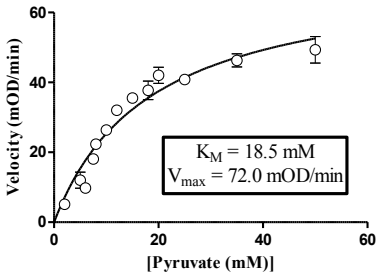


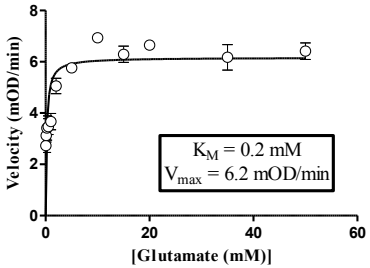


d AtGGT2

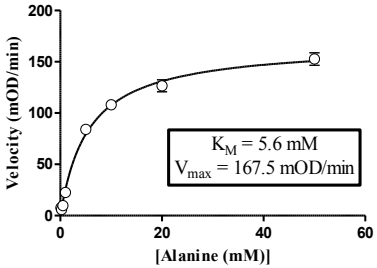


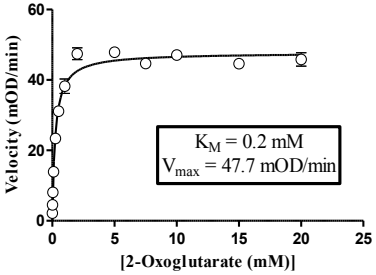


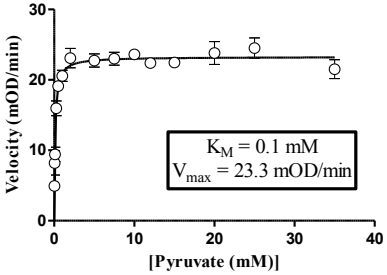


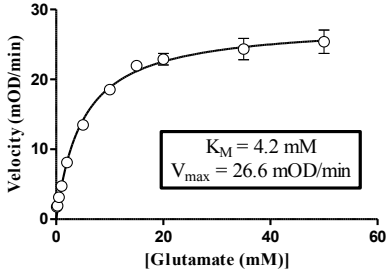


e HvAlaAT

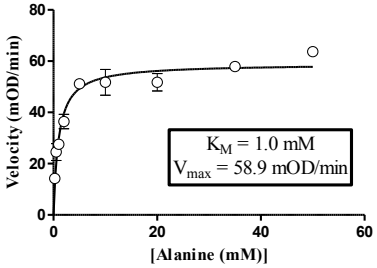


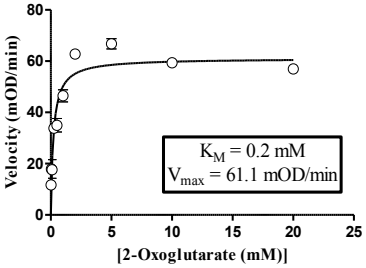


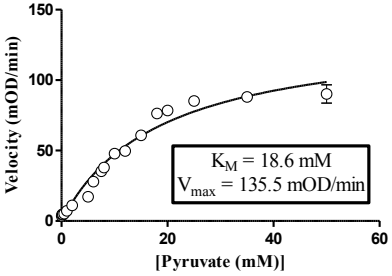


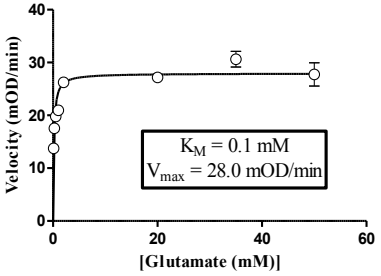


f MtAlaAT1

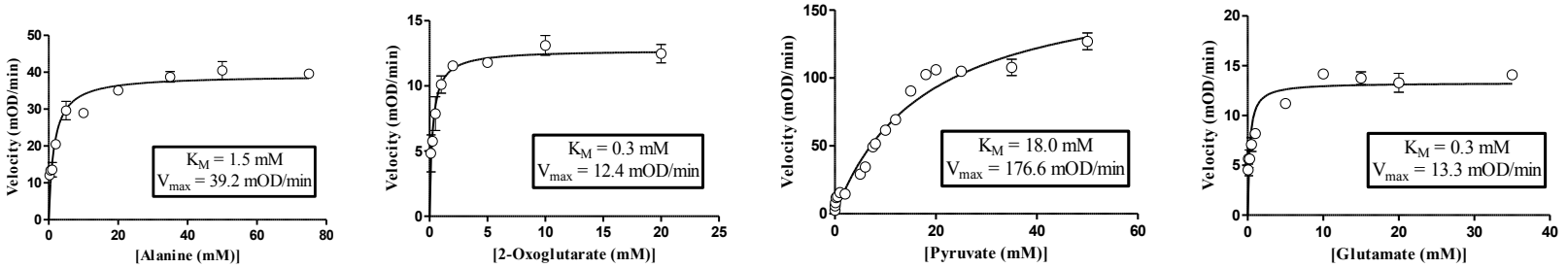




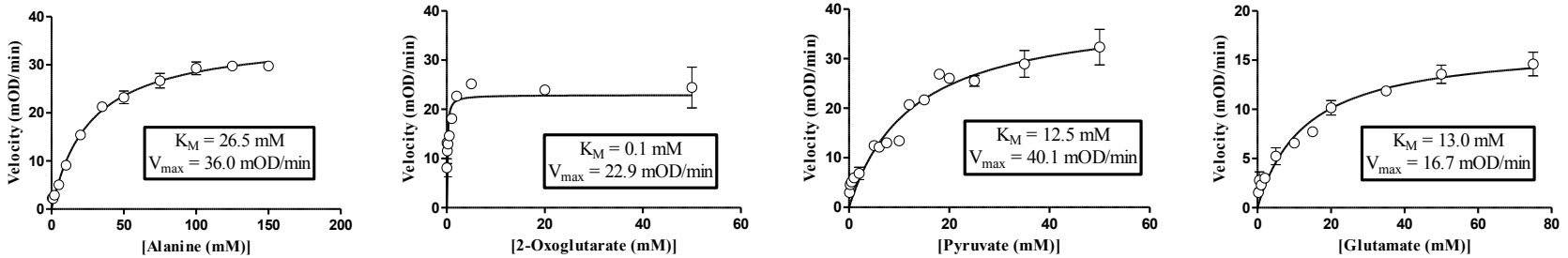




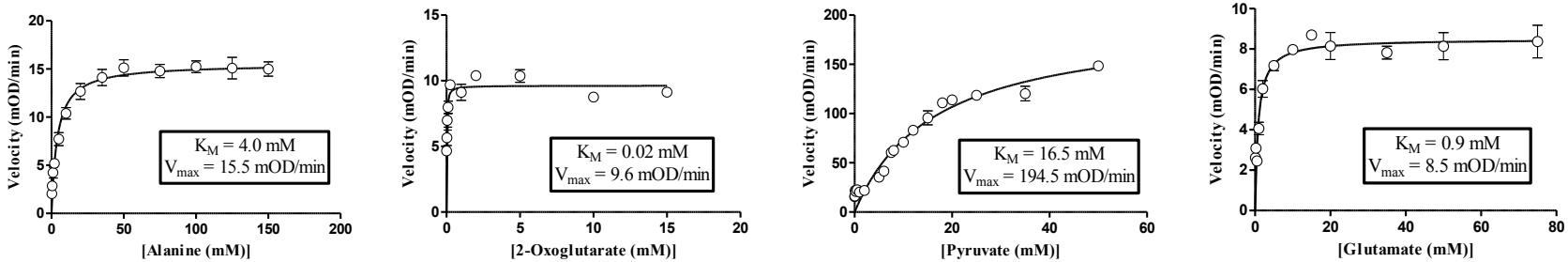
g MtAlaAT2



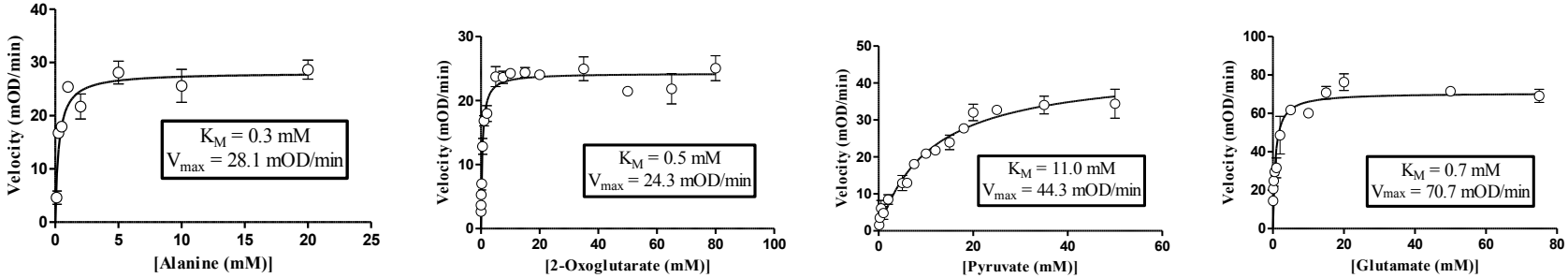
h MmAlaAT1



i PfAlaAT



j ScAlaAT1



Appendix 2.2. K_M and V_{max} of various AlaAT enzymes with alanine, 2-oxoglutarate, pyruvate and glutamate.

Data were fitted to the Michaelis-Menten equation with the nonlinear regression facility of GraphPad Prism v. 5.03, in order to calculate K_M and V_{max} values. Data points are the mean ± standard error (SE) of triplicate determinations.

Appendix 2.3. Primer sequences used in the cloning of AlaAT enzymes

All *AlaATs* were cloned into the pBAD18-Kan plasmid using the restriction sites indicated. Restriction enzyme sites are shown in lower case lettering.

Organism	Primer	Sequence For initial cloning from received vector into TOPO
Arabidopsis thaliana (AlaAT1)	AlaAT1 FWD	5' – gagctcAGGAGGTGAAGATGCGGAGATTCGTGATTGGCC - 3'
	AlaAT1 REV	5' – tctagaTTAGTCGCGGAACTCGTCCATG - 3'
Arabidopsis thaliana (AlaAT2)	AlaAT2 FWD	5' –ggtaccAGGAGGTGAAGATGCGGAGATTCTTGATTAACCAGGC - 3'
	AlaAT2 REV	5' – tctagaTTAGTTGCGGAACTCGTCCA - 3'
Arabidopsis thaliana (GGT1)	GGT1 FWD	5' – ggtaccAGGAGGTGAAGATGGCTCTCAAGGCATTAGACTACG - 3'
	GGT1 REV	5' – tctagaTTACATTTTCAGATAACCAAAGTTATTATCATAC - 3'
Arabidopsis thaliana (GGT1)	GGT1 FWD	5' – ggtaccAGGAGGTGAAGATGTCTCTCAAGGCGTTAGACTACGAG - 3'
	GGT2 REV	5' - tctagaTTACATTCTGGAGTAACCAAAGTTATC - 3'
Barley (*Hordeum vulgare*)	AlaAT FWD	5' – ggtaccAGGAGGTGAAGATGGCTGCCACCGTCGCCGT - 3'
	AlaAT REV	5' – tctagaTTAGTCACGATACTCTGACATGAACGCC - 3'
Human1 (*Homo sapien*)	Human AlaAT1 FWD	5' – ggtaccAGGAGGTGAAGATGGCCTCGAGCACAGGTGACC - 3'
	Human AlaAT1 REV	5' – tctagaTTAGGAGTACTCGAGGGTGAACTTGGC - 3'
Human2 (*Homo sapien*)	Human AlaAT2 FWD	5' – ggtaccAGGAGGTGAAGATGCAGCGGGCGGCGG - 3'
	Human AlaAT2 REV	5' – tctagaTTACGCGTACTTCTCCAGGAAGTTGATG - 3'
Medicago truncatula (mAlaAT1)	ALT FWD	5' – gagctcGGAGGTGAAGATGCGGAAATCCGCTGCAGATAGA - 3'
	ALT REV	5' – tctagaTTAATCGCGAAACTCATCCATGAACTTTTG - 3'
Medicago truncatula (mAlaAT2)	ALT FWD	5' – gagctcAGGAGGTGAAGATGCGGAAATCCGCTGCAGATAGA - 3'
	ALT REV	5' – tctagaTTAATCGCGAAACTCATCCATGAACTTTTG - 3'
Mouse1 (*Mus musculus*)	ALT1 FWD	5' – ggtaccAGGAGGTGAAGATGGCCTCACAAAGGAATGACCGG - 3'
	ALT1 REV	5' – tctagaTTAGGAGTACTCATGATGGAATTTAGCATGG - 3'
Mouse2 (*Mus musculus*)	ALT2 FWD	5' – GGTAC CAGGAGGTGAAGATGCAGCGGGCAGCGGTGC - 3'
	ALT2 REV	5' – tctagaTTATGAGTACTACTGCTCCAGGACTTCAGGTG - 3'
Pyrococcus furiosus	AlaAT FWD	5' – ggtaccAGGAGGGAAGATGATAAGGGCCTCA - 3'
	AlaAT REV	5' – tctagaTTATCATTCTTTCAGTC - 3'
Yeast1 *(Saccharomyces cerevisiae)*	AlaAT 190 FWD	5' – ggtaccAGGAGGTGAAGATGTTATCACTGTCTGCCAAAAATCACTTC - 3'
	AlaAT 190 REV	5' – tctagaTTAGTCACGGTATTGGTCAAAAAATTCTTTATG - 3'
Yeast2 *(Saccharomyces cerevisiae)*	AlaAT 369 FWD	5' – ggtaccAGGAGGTGAAGATGACAATGACACACCAACAGGATTTG - 3'
	AlaAT 396 REV	5' – tctagaTTAATTACGATACTTGCTGAAGAAATCTTGATGA - 3'

Appendix 2.4. Average V_{max} values for unpurified AlaAT and GGAT enzymes.

V_{max} values are shown for each substrate, for each of the ten enzymes examined. Kinetic values represent the average of three independent trials. The correlation coefficient (r^2) was > 0.80 for all trials, except AtGGAT1 glutamate, AtGGAT2 glutamate and MmAlaAT1 pyruvate. Raw data are plotted in Appendix 2.2.

Enzyme	Alanine		2-oxoglutarate		Glutamate		Pyruvate	
	V_{max} (mOD/min)	SE V_{max}	V_{max} (mOD/min)	SE V_{max}	V_{max} (mOD/min)	SE V_{max}	V_{max} (mOD/min)	SE V_{max}
AtAlaAT1	64.5	±3.1	39.1	±1.2	12.7	±0.4	7.9	±0.2
AtAlaAT2	24.3	±0.6	35.8	±0.1	12.0	±0.7	47.4	±2.6
AtGGT1	36.0	±2.0	30.3	±1.9	7.1	±0.3	7.2	±0.2
AtGGT2	45.6	±1.9	28.8	±2.7	6.2	±0.2	72.0	±5.0
HvAlaAT	167.5	±5.3	47.7	±0.6	28.1	±0.7	23.3	±0.4
MtAlaAT1	58.9	±1.9	61.1	±2.2	28.0	±1.1	135.5	±9.3
MtAlaAT2	39.2	±1.2	12.8	±0.5	13.3	±0.5	176.6	±9.1
MmAlaAT1	36.0	±1.0	22.9	±1.4	116.7	±1.0	40.1	±2.7
PfAlaAT	15.5	±0.3	9.6	±0.2	8.5	±0.2	194.5	±12.2
ScAlaAT1	28.1	±1.2	24.3	±0.5	70.7	±2.7	44.3	±2.6

3. Physical phenotypic analysis of *Arabidopsis thaliana* expressing *AlaAT* variants

3.1. Introduction

The growth and development of plants is highly reliant on their ability to rapidly sense and adjust to changes in their environment. Response patterns to internal and external signals can have both immediate and long lasting effects, affecting the growth and development of the plant (Malamy, 2005; Castro Marín *et al*., 2011; Keurentjes *et al*., 2011). N availability and form (organic or inorganic) is known to strongly impact plant systems, impacting not only N metabolism, but also carbon (C) metabolism and plant signaling (Lawlor, 2002; Zheng, 2009; Gojon *et al*., 2011; Kant *et al*., 2011). Decreases in N availability in plants have been shown to impact C cycling and photosynthetic efficiency of the plant (Nunes-Nesi *et al*., 2010), as well as glycolysis (Krapp *et al*., 2011), which in turn disturbs growth of plant organs and tissues and can affect overall crop yields (Sinclair & Rufty, 2012). In order to maintain or increase yields while reducing N-fertilizer amounts there is a need for a better understanding of the metabolic alterations that ultimately lead to increased nitrogen use efficiency (NUE). Moreover, a method or system with which to recognize plants that may be more nitrogen use efficient, with alterations in uptake, assimilation or remobilization of N, without long and detailed experimental analysis would be of significant value.

Arabidopsis thaliana is a model organism that has been used widely for the study of N sensing, signaling, uptake, assimilation and remobilization. Although Arabidopsis is a dicot, and has shown differing root and shoot structure from other plants species (including root branching and vasculature), findings in Arabidopsis have been used as a template or starting point for N genetics and metabolism in other higher plants, including cereals and other monocots (Rensink & Buell, 2004; Chew & Halliday, 2011; Smith & De Smet, 2012). Using Arabidopsis, alterations in environmental/external N and C (Zhang *et al*., 1999; Freixes *et al*., 2002; Okamoto *et al*., 2003; Osuna *et al*., 2007; Roycewicz & Malamy, 2012; Song *et al*., 2013; Hu *et al*., 2014) or in plant N and C regulatory pathways (through genetic manipulation) (Wang *et al*., 1998; Kaiser *et al.*, 2002; Good *et al*., 2004; Little *et al*., 2005; Rolland *et al*., 2006; Ho *et al*., 2009; Lothier *et al*., 2011) have consistently demonstrated the sensitive mechanisms employed by plants to sense

their external and internal nutrient supplies. These experiments have also demonstrated how increases or decreases in the expression of specific proteins and enzymes, including GS (glutamine synthetase), NRT1.1 (nitrate transporter 1.1) and AMT1 (ammonium transporter 1), among others, can have a significant impact on a plants response to the nutrients in their environment (Kaiser *et al*., 2002; Ho *et al*., 2009; Kant *et al*., 2011; Lothier *et al*., 2011).

Earlier work in Arabidopsis has shown that concentration and form of external N and C can have a significant effect on root growth and architecture as well as gene regulation (Stitt *et al*., 2002; Miller *et al*., 2007). The use of plate assays, allows for the visualization of both root and shoot tissues and permits the regulation of external nutrient concentrations while maintaining a sterile, controlled environment (Krouk *et al*., 2011; Malamy, 2005; Malamy & Ryan, 2001). The behaviour of the plant in accordance with external conditions can then be observed and plant:nutrient interactions deduced (Drew *et al*., 1973; Drew & Saker, 1978). In particular, it has been shown that increases in external NO_3^- stimulate root growth as well as lateral root branching, while areas of poor nutrient quality, including decreases in NO_3^-, suppress root elongation and growth (Remans *et al*., 2006; Lima *et al*., 2010; Ruffel *et al*., 2011). In Arabidopsis, exogenous glutamate has shown to suppress primary root growth when applied in micromolar concentrations, indicating that this molecule can also act as an external developmental signal (Filleur *et al*., 2005; Walch-Liu *et al*., 2006). Interestingly, media with high C:N ratios has been shown to inhibit lateral root initiation in Arabidopsis, while regions with low C and low N mimic lateral root initiation in high N conditions (Malamy & Ryan, 2001; Little *et al*., 2005). These results indicate that the ratio of C:N, both within and outside the plant, is critical in determining root responses to external nutrients and subsequently NUE.

Previous research has shown that over-expression of *HvAlaAT* driven by a tissue-specific root promoter, *btg26* or *OsAnt1*, in canola and rice respectively, results in an NUE phenotype under N limiting conditions (Good *et al*., 2007; Shrawat *et al*., 2008). In order to better assess the impact that AlaAT has on N sensing, signaling and assimilation, as well as C metabolism and C:N ratios within the plant, *AlaAT* genes from a variety of organisms (Table 3.1) were transformed into *Arabidopsis thaliana* and assessed under different external N conditions. These genes were placed under the control of two different promoters, the tissue-specific rice promoter *OsAnt1* and the strong constitutive promoter *Cauliflower Mosiac Virus 35S RNA* (*CaMV 35S* or

35S), in order to assess the importance of spatial gene expression. Two different Arabidopsis genotypic backgrounds were chosen for expression of the *AlaAT* variants: a wildtype (Col-0; containing two native AlaAT genes) (Figure 3.2) and an alaat1-1;alaat2-1 double knockout; alaat1;2) (ecotype Col-0) (Figure 3.2). AlaAT variants were chosen based on the results of the enzymatic analysis carried out in Chapter 2, as well as the previously reported *HvAlaAT*-rice NUE phenotypes. Based on these results, the usefulness of Arabidopsis as a tool to study NUE in cereal crops, as well as the importance of testing enzyme variants and their effect on overall phenotype will be discussed.

3.2. Materials and Methods

3.2.1. Expression vector construction and *Arabidopsis thaliana* transformation

cDNA for AlaAT variants was obtained from multiple sources. *Hordeum vulgare AlaAT* (GenBank accession no. Z26322) (*HvAlaAT*) cDNA, as well as the pCAMBIA 1300 vector containing *HvAlaAT* driven by an *OsAnt1* promoter (*OsAnt1:HvAlaAT*) and *OsAnt1* promoter driving *ß-glucuronidase* (*GUS*) were obtained from Ashok Shrawat, University of Alberta (Shrawat *et al*., 2008). Mouse (*Mus muscus)* AlaAT1 (*MmAlaAT1*) (GenBank accession no. NP_877957) and mouse (*Mus muscus*) AlaAT2 (*MmAlaAT2*) (GenBank accession no. NP_776291) were both obtained from Laura Olsen, University of Michigan. *Pyrococcus furiosus* AlaAT (*PfAlaAT*) (GenBank accession no. NP_579226) was amplified from ATCC gDNA (DSM 3638). Restriction enzyme cut sites for Asc1 and Pac1 were added to the 5' and 3' ends, respectively, of each cDNA using primers specific to each AlaAT: for HvAlaAT, 5'-ATTTAAggcgcgccATGGCTGCAACC-3' and 5'-GCTATTCAGATCCTCTTCTGAGATGA-3'; for MmAlaAT1, 5'-ATTATTTAAggcgcgccATGGCCTCA-3' and 5'-GCTATTCAGATCCTCTTCTGAGATGA-3'; for MmAlaAT2, 5'-ATTTTATAggcgcgccATGCAGCGG-3' and 5'-GCTATTCAGATCCTCTTCTGAGATGA-3'; for PfAlaAT, 5'-ATTATTAAggcgcgccATGATAAGGG-3' and 5'-GCTATTCAGATCCTCTTCTGAGATGA-3'. A His/Myc tag was added to the 3' end of *PfAlaAT*, *MmAlaAT1* and *MmAlaAT2* using a second reverse primer in a successive PCR reaction (5'-CTAAAttaattaaTCAATGGTGGTGATGATGATGGTCGACGGCGCTATTCAGATCCTCTTC-3'). AlaAT cDNAs were subcloned into the binary vector pMDC32 (Figure 3.2) (Curtis &

Grossniklaus, 2003) behind a 2X *CaMV 35S* promoter using restriction enzymes Asc1 and Pac1. The final AlaAT constructs for transformation into *Arabidopsis thaliana*, the initial AlaAT source, the promoter used to drive AlaAT expression *in vivo*, as well as the binary vectors utilized for transformation are summarized in Table 3.1.

Binary vectors were transformed into *Agrobacterium tumefaciens* strain GV3101 and selected for using $Kan_{(50)}$. Agrobacterium cultures (OD_{600} 0.3-0.7) were then used to transform *Arabidopsis thaliana* plants, both Col-0 and alaat1;2 knockout backgrounds, using floral dip. (alaat1;2 knockout background was isolated by Yo Miyashita, University of Alberta. (*alaat1-1* is a T-DNA insertion mutation (Alonso *et al*., 2003) obtained from the Arabidopsis Biological Resource Centre (http://www.biosci.ohio-state.edu/_plantbio/Facilities/abrc/abrchome.htm); *alaat2* is a point mutation in the open reading frame of *alaat2-1* interrupting a BseR1 cut site identified by TILLING (Miyashita *et al*., 2008) (Figure 3.2)). Transformed plants were selected by sowing seeds on $Hyg_{(25)}$ and using a protocol modified from Harrison *et al*., (2006), with plants left covered at 4°C for four days and at room temperature for three days; PCR was carried out on all primary transgenics with primers specific to the AlaAT insert: for HvAlaAT, 5'-GAGGTTCTTGCCCTTTGTGA-3' and 5'-TTCAGCTCGTTGCAAGTAA-3'; for MmAlaAT1, 5'-CCAGAGGATGCCAAGAGAAG-3' and 5'-GCTCCGTGAGTTTAGCCTTG-3'; for MmAlaAT2, 5'-GCAGGCTTGTGGTGGAAA-3' and 5'-GCACTTTCTTAAAGGAGTGGAATC-3'; for PfAlaAT, 5'-GCGCTCTACGACAAAAAGACACTTGA-3' and 5'-CGTTAGTCCTGCTATAGCTGCGAATT-3'. T_3 seed was sown on $Hyg_{(25)}$ media to select for homozygous lines; PCR was used to verify the presences of the specific AlaAT insertions. Three independent Arabidopsis insertion lines for each AlaAT construct, in both Col-0 and alaat1;2 knockout backgrounds, were selected for and used for future analyses.

T_2 seed was used to test for multiple insertions in designated homozygous transgenic lines. T_2 seeds were sterilized and left at 4°C for ~48 hrs. T_2 seeds from each line were plated onto two ½ MS + $Hyg_{(25)}$ plates (30 seeds per plate) and left horizontal at 21°C, 60% humidity and a light cycle of 16 light/8 dark. The ratio of germinated and growing plants was evaluated 5-7 days after sowing.

3.2.2. Plate assays

For all plate assays, T_4 or T_5 seed was utilized to decrease transformation effects; seed was obtained from plants grown at the same time and harvested in unison. Sterilized seeds were stratified in 0.15% (w/v) agar for ~48 hrs; sterilized seeds were sown onto square 100 X 100 X 15 mm petri plates containing modified ½ MS media (0.5% (w/v) sucrose and 0.7% (w/v) agar), pH 5.8, with 0 mM KNO_3, 2 mM KNO_3, 0.25 mM KNO_3, 2.5 mM alanine or 2.5 mM glutamate as the sole nitrogen source. Seeds from both control lines, Col-0 and alaat1;2, as well as three independent insertion lines each of *OsAnt1:HvAlaAT*, *35S:PfAlaAT*, *35S:HvAlaAT*, *35S:MmAlaAT1* and *35S:MmAlaAT2*, in both a Col-0 and an alaat1;2 knockout background, were used for analysis on 0 mM, 0.25 mM and 2 mM KNO_3 plates. Transgenic plants containing *OsAnt1:HvAlaAT*, *35S:PfAlaAT* and *35S:MmAlaAT1*, in both Col-0 and alaat1;2 knockout backgrounds, were used for analysis on 2.5 mM glutamate and alanine. Plants were sown horizontally across square petri plates ~2 cm from the top of each plate; six plants were sown per plate. Control plants and transgenics were sown on the same plates in an alternating fashion. Three independent insertion lines for each *AlaAT* were assayed in quadruplicate along with control plants. Plants were grown vertically at 21°C, 60% humidity and a lighting cycle of 16 light/8 dark. Chambers were blocked for variations in lighting conditions, resulting in four blocks, with final lighting blocks containing a maximum difference of 20% across a single block with an average light intensity of 170 µE m^{-2} sec^{-1}. Plates were moved within lighting blocks daily and monitored for changes in growth and development. Changes in vertical tap root length were measured (cm) between 4-8 days after sowing (DAS) and 8-12 DAS for plants grown on 2 mM and 0.25 mM KNO_3. The vertical growth of tap roots of plants grown on 2.5 mM glutamate and alanine was measured (cm) between 9-11 DAS and 11-15 DAS for changes in root length. Changes in tap root lengths over these time periods were then used to analyze the rate of tap root growth between transgenics and controls. At 18 DAS or 22 DAS (earlier if plants were senescing or had grown too large for plates) final tap root lengths were measured (cm) on all ½ MS plates (top of hypocotyl to root tip).

3.2.3. Variations in external C and N

½ MS plates were prepared with varying N and C amounts. Modified ½ MS (0.7% (w/v) agar), pH 5.8, containing 1 mM KNO_3 and 0% (w/v) sucrose was used to grow transgenic plants containing *OsAnt1:HvAlaAT*, *35S:PfAlaAT* and *35S:MmAlaAT1*, in both Col-0 and alaat1;2

knockout backgrounds, as well as control lines, in two different lighting conditions: ~170 μE m^{-2} sec^{-1} (high) and ~100 μE m^{-2} sec^{-1} (low). Modified ½ MS plates containing 0.25 mM KNO_3 and 0.2% (w/v) sucrose was also used to grow all of the above lines in a low light condition (~100 μE m^{-2} sec^{-1}). Changes in vertical tap root length were measured (cm) between 4-8 DAS, 8-12 DAS and 12-15 DAS for all conditions/plates (top of hypocotyl to root tip) to assess the rate of tap root growth between transgenic and control lines. Plating, blocking and data collection, as well as chamber conditions for the growth of plants on these plates, was as outlined previously.

3.2.4. Split agar plates

Seeds from both control lines, Col-0 and alaat1;2, as well as three independent insertion lines each of *OsAnt1:HvAlaAT*, *35S:PfAlaAT* and *35S:MmAlaAT1*, in both the Col-0 and alaat1;2 knockout background, were used for this analysis. Sterilized seeds were stratified in 0.15% (w/v) agar for ~48 hrs; sterilized seeds were sown onto modified ½ MS media containing 0.25 mM KNO_3. Plants were sown in two horizontal rows across square petri plates ~2 cm and ~5 cm from the top of each plate; six plants were sown per row. Control plants and transgenics were sown on the same plates in an alternating fashion. Three independent insertion lines for each *AlaAT* were assayed in quadruplicate along with control plants. Plants were grown vertically at 21°C, 60% humidity and a lighting cycle of 16 light/8 dark. Chambers were blocked for variations in lighting conditions, with final lighting blocks containing a maximum difference of 20% across a single block with an average light intensity of 170 μE m^{-2} sec^{-1}. Plates were moved within lighting blocks daily. At 3 DAS plants were moved from 0.25 mM KNO_3 plates to split agar plates (SAP). SAP plates were split horizontally with the top ~3 cm containing modified ½ MS with 0.25 mM KNO_3 and the remainder of the plate containing modified ½ MS with either 2.5 mM glutamate or 2.5 mM alanine as the sole nitrogen source. SAP plates were obtained by removing the top 2 cm of each ½ MS plate containing either glutamate or alanine and then re-inserting a pre-cut ½ MS agar gel containing 0.25 mM KNO_3 into the 3 cm gap. This allowed for a small space between agar pieces, decreasing diffusion between the two N sources. Plants were re-sown onto SAP plates with root tips abutting the border between nitrogen sources and placed back into growth chambers. Plates were moved within lighting blocks daily and monitored for changes in growth and development. Three weeks after original sowing of seeds tap roots were measured (cm) (from the top of the hypocotyls to the root tip).

3.2.5. Lateral root analysis

Modified ½ MS (0.5% (w/v) sucrose and 0.7% (w/v) agar), pH 5.8, containing 0.25 mM KNO_3 was used to grow plants for lateral root analysis. Transgenic lines in the Col-0 background containing *OsAnt1:HvAlaAT*, *35S:HvAlaAT*, *35S:MmAlaAT1, 35S:MmAlaAT2* and *35S:PfAlaAT* were used for this analysis along with control (Col-0 and alaat1;2) plants and grown vertically, six seedlings per plate, at 21°C, 60% humidity and a lighting cycle of 16 light/8 dark as described above. Three independent insertion lines for each *AlaAT* were assayed in quadruplicate along with control plants. Initially, plates containing only Col-0 were grown and monitored to determine timing of lateral root emergence and therefore ideal time points to count primary lateral root number per plant. It was determined that counting of lateral roots should be carried out at both 8 and 11 DAS; number of lateral roots on both control and transgenic plants were counted at these time points. Plating, blocking and data collection, as well as chamber conditions for the growth of plants on these plates, was as outlined previously.

3.2.6. Statistical analysis

Phenotypic similarity between individual plant lines expressing the same AlaAT construct in all plating conditions were assessed via one-way ANOVA ($\alpha = 0.05$, $P < 0.05$), using a Dunn's post test to directly compare lines (data not shown). Independent insertion lines did not show significant consistent variation in either background in any of the conditions assayed and therefore data from all three insertion lines for a given AlaAT was pooled.

Final tap root lengths of control plants and transgenic lines were compared using two-way ANOVA ($P < 0.05$), analyzing the genotype of plants and the lighting block plants were grown in. Two-way ANOVA ($P < 0.05$) and Bonferroni post-tests were used to compare mean tap root length and genotype respectively, between both controls and transgenics and between different transgenic lines. Statistical significance indicated in all figures is the difference between the controls and transgenics at that time point or in that block and does not denote if the overall genotype is significant. All statistical analysis was conducted using GraphPad Prism v. 5.03.

3.3. Results

3.3.1. AlaAT transgenic *Arabidopsis thaliana* lines contain one insertion of the transgene

T_2 seed from all transgenic *AlaAT* lines was assessed for the number of independent insertions of the transgene, based on segregation analysis. Segregating lines were germinated in ½ MS media containing $Hyg_{(25)}$, and the ratio of hygromycin resistant plants to that of non-resistant plants (nulls) was recorded. Plants containing a single *AlaAT* insertion were expected to segregate at a Mendelian ratio of 1:3 (nulls:transgenics) or an average of ~75% hygromycin resistant plants, while lines containing multiple insertions were expected to have a lower ratio of null segregants and a higher percentage of hygromycin resistant plants. All lines, except for possibly *OsAnt:HvAlaAT* 3-1-1, alaat1;2 background, showed segregation patterns for single insertion mutants (Supplementary Table 3.1). Unfortunately, if two or more copies of the transgene inserted in tandem, segregation patterns would not be able to distinguish these multiple insertions. However, all subsequent analysis was carried out assuming that all transgenic lines, with the above exception, contain a single *AlaAT* insertion.

A numbering system was used during the production of all *AlaAT* insertion lines in order to ensure that all lines originated from an independent insertion event. Lines are named using either three or four numbers, with each number representing a specific stage in the development of a homozygous *AlaAT* line. The first number in each of the lines indicates the floral dip; 8 independent floral dips were conducted for each transgene. The second number indicates the number given to the primary transgenic heterozygous plant (T_1). The third number indicates the T_2 plant that homozygous T_3 seed was obtained from. If a fourth number is used, no homozygous plants were observed using T_3 seed and a fourth generation was required, with the numbering system remaining the same for this final generation.

3.3.2. AlaAT over-expression results in increased tap root length in *Arabidopsis thaliana* at limiting and non-limiting NO_3^- concentrations

Three independent insertion lines for each of the five constructs (Table 3.1; Supplemental Table 3.1), in both the Col-0 and alaat1;2 background, were grown on modified ½ MS containing 0 mM, 0.25 mM or 2 mM NO_3^-. Plants grown on plates with 0 mM NO_3^- showed no visible phenotypic differences when compared to both control lines (Col-0 and alaat1;2) (data not shown). Tap root length was assessed for differences between transgenics and controls up to

32 days after sowing (DAS) using a two-way ANOVA, which accounted for variations in lighting and genotype of plants. No significant differences were detected between transgenics and controls in either background ($P < 0.05$) (data not shown). Lighting block did have a significant effect on the growth of plants regardless of genotype and this was observed in almost all plate based assays.

When plants were grown in N-limiting conditions (0.25 mM NO_3^-), significant differences in the growth and development of the transgenic plants compared to controls was observed both in the Col-0 and alaat1;2 backgrounds, however these differences in phenotype were not consistent between genotypes (Figure 3.2, 3.3 and 3.4). Plant tap roots were assessed at 5, 8 and 12 DAS and the differences in vertical tap root growth between 0-5, 5-8 and 8-12 DAS were measured (cm) (Figure 3.2). The expression of *AlaAT* in the alaat1;2 background resulted in a significant increase in tap root growth compared to the control line at all time points for all lines with one exception (Figure 3.2a-e). From 8-12 DAS *35S:HvAlaAT* in alaat1;2 did not show significant tap root growth compared to the knockout line. At 5 DAS all transgenic lines showed significant increases in tap root growth at a $P < 0.0001$ and when the differences in tap root growth between 5-8 days were compared, all lines in the alaat1;2 background showed significant growth at a $P < 0.005$. Significant differences in tap root growth between 8-12 DAS were observed for all lines except *35S:HvAlaAT* ($P < 0.05$), with the most significant difference observed between controls and plants expressing *MmAlaAT1* ($P < 0.005$) (Figure 3.3c). Two-way ANOVA indicated that throughout these three time points, genotype of the plant played a significant role in the rate of tap root growth for all *AlaAT*-expressing lines (alaat1;2 background). When final tap root lengths were assessed at 18 DAS, genotype was determined to play a significant role in the observed phenotype for only those lines containing *35S:HvalaAT*, *35S:MmAlaAT2* and *35S:PfAlaAT* (Figure 3.2).

Unlike *AlaAT*s expressed in the knockout background, those expressed in the Col-0 background did not show significant increases in rate of tap root growth at all of the time points measured (Figure 3.2f-j). From 0-5 DAS, significant increases in tap root growth were only observed for *35S:HvAlaAT*, *35S:MmAlaAT1* and *35S:PfAlaAT* over-expressing lines ($P < 0.0001$). *OsAnt1:HvAlaAT* and *35S:MmAlaAT2* transgenic lines did not show significant differences. From 5-8 DAS only *MmAlaAT1*-expressing plants showed significant increases in

tap root length (Figure 3.2h) ($P < 0.005$) with no lines in the Col-0 background showing increased rate of tap root growth between 8-12 DAS. Two-way ANOVA indicated that in the Col-0 background, genotype of the transgenic plants played a significant role in increases in the rate of tap root growth compared to controls throughout these three time points (0-5, 5-8 and 8-12 DAS) for only those lines containing *35S:HvAlaAT*, *35S:MmAlaAT1* and *35S:PfAlaAT* ($P < 0.01$) (Figure 3.2f-j). At 18 DAS, none of the lines over-expressing AlaAT in the Col-0 background showed increased tap root growth (Figure 3.4). No obvious phenotypic differences in shoot biomass were observed between controls and transgenics in either background (data not shown).

Knockout plants expressing *AlaAT* grown in non-limiting N conditions (2 mM NO_3^-) showed similar phenotypic results to those that were grown in N-limiting conditions (0.25 mM NO_3^-). Changes in tap root length were assessed at 5 and 8 DAS and changes in root growth were compared between transgenics and controls for 0-5 and 5-8 DAS (Figure 3.5). (Changes in root growth beyond 11 DAS were not assessed due to roots reaching plate bottoms.) For all *AlaAT*-expressing lines (alaat1;2 background), significant increases in tap root growth between 0-5 DAS ($P < 0.005$) and 5-8 DAS ($P < 0.05$) were observed (Figure 3.5a-e). Over-expressing transgenic plants (Col-0 background) grown on 2 mM NO_3^-, did not mimic the phenotypic results seen at 0.25 mM N as seen with transgenics expressed in the knockout background. From 0-5 DAS transgenic lines containing *OsAnt1:HvAlaAT*, *35S:MmAlaAT1* and *35S:MmAlaAT2* showed significantly longer average tap root lengths than controls ($P < 0.05$); from 5-8 DAS only lines expressing *MmAlaAT1*, *MmAlaAT2* and *PfAlaAT* showed significant increases in average tap root length compared to control plants ($P < 0.05$) (Figure 3.5f-j). Overall, genotype was determined to be significant in Col-0 background over-expressing plants through these two time periods for all lines with the exception of *35S:HvAlaAT* (Figure 3.5g).

Final tap root lengths were assessed at 16 DAS for plants grown at 2 mM NO_3^-. All transgenic lines in the alaat1;2 background showed significant increases in tap root length at 16 DAS ($P < 0.05$) (Figure 3.6), unlike results from plants grown in N-limiting conditions, where plants containing *35S:MmalaAT1* and *OsAnt1:HvAlaAT* did not show significant increases in tap root length at 18 DAS. None of the lines over-expressing AlaAT in the Col-0 background showed significant differences in tap root length at this time (Figure 3.7), mimicking the results

seen when these plants were grown at 0.25 mM NO_3^-. *MmAlaAT1*-expressing plants (Col-0 background) were the only lines to show increased rate of tap root growth in both limiting and non-limiting N conditions between 0-5 and 5-8 DAS (Figure 3.2h and 3.5h), however at later time periods (16 and 18 DAS respectively) significant increases in overall root growth were not detected in lines expressing this construct, or any of the other four *AlaATs*.

In all transgenic *AlaAT*-expressing and over-expressing lines, specifically in the non-limiting N condition, it should be noted that, qualitatively transgenic plants appeared to have increased shoot mass compared to controls (data not shown). The overall size of rosette leaves appeared larger on average on transgenic plants compared to that of controls grown on the same plate. This increase in shoot mass of transgenics compared to controls was also seen in the following assays using the amino acids glutamate and alanine as sole N sources but is much more pronounced (Figure 3.8). Observation of transgenics and controls grown on soilless medium (Chapter 4, Figure 4.5) also showed increased average shoot size in transgenic over-expressing lines. Currently, more detailed characterization of these lines is underway, but was not complete at the time of writing of this thesis. This data supports the observations that these lines display increased leaf area and biomass.

3.3.3. Increased expression of AlaAT in Arabidopsis allows for increased growth on glutamate and alanine

To analyze the ability of *AlaAT* over-expressing lines to utilize both glutamate and alanine more effectively, plants were sown onto modified ½ MS containing 2.5 mM glutamate or alanine as the sole N source. Based on previous analysis in Chapter 2 and the results in the above section, three transgenic lines were chosen to use for this analysis: *OsAnt1:HvAlaAT*, *35S:HvAlaAT* and *35S:PfAlaAT*. Three DAS transgenic plants in the Col-0 background showed increased tap root growth compared to control plants in both amino acid conditions (Figure 3.8a-b). These plants also showed visual increases in shoot biomass. This phenotype was not only consistent across triplicate lines carrying the same insert, but among all over-expressing lines, only *OsAnt1:HvAlaAT* has been shown for brevity (Figure 3.8). Increased tap root length or increases in shoot biomass were not observed in transgenic lines in the alaat1;2 background.

Plants were monitored for changes in the rate of tap root growth from 9-11 DAS and 11-15 DAS (Figure 3.9). Changes in the rate of tap root growth were analyzed by two-way ANOVA and Bonferroni post-tests accounting for differences in lighting and genotype of the plants. Between 9-11 DAS and 11-15 DAS all transgenic lines expressed in the Col-0 background showed highly significant increases in tap root growth (Figure 3.9) ($P < 0.0001$) with phenotypically visible increases in shoot and root biomass compared to controls (Figure 3.8c-d). Significant differences in tap root growth and biomass were not seen by transgenics expressed in the alaat1;2 background. Final tap root lengths were analyzed at 22 DAS for plants of both genotypic backgrounds grown on both amino acids. No significant differences in tap root length were observed in any of the transgenic lines expressed in the alaat1;2 knockout background, when grown with either alanine or glutamate as the sole N source at 22 DAS (Figure 3.10a-c and 3.11a-c). On 2.5 mM alanine and glutamate plates, all *AlaAT* variants expressed in the Col-0 background showed high statistical significance when final tap root lengths were compared to controls at 22 DAS ($P < 0.0001$) (Figure 3.10d-f and Figure 3.11d-f).

In order to analyze the effect of environmental N on the initial germination and growth of the transgenic seedlings versus the internal N amounts, split agar plates (SAP) were prepared, with the top ~3 cm containing modified ½ MS with 0.25 mM KNO_3 and the remainder of the plate containing modified ½ MS with either 2.5 mM glutamate or 2.5 mM alanine as the sole nitrogen source. Initially plants were germinated on ½ MS containing 0.25 mM $NO3^-$ and moved to SAP at 3 DAS, the point at which increases in mean tap root length on amino acid plates had been previously observed. (Plants were placed on SAP plates with the root tip at the interface between N conditions.) At 22 DAS final mean tap root lengths were measured and assessed using two-way ANOVA and Bonferroni post-tests to compare genotypes in the same lighting block. In the knockout background none of the lines showed significant increases in tap root growth when grown on glutamate SAP, however growth on alanine SAP showed significant increases in tap root length in those plants expressing *MmAlaAT1* ($P < 0.05$). In lines expressed in the Col-0 background, again only *MmAlaAT*- expressing plants showed increased mean tap root length when grown on alanine SAP (Figure 3.12b), and both *MmAlaAT1* and *PfAlaAT* expressing lines showed significant root growth on glutamate SAP ($P < 0.005$ and 0.05 respectively) (Figure 3.12 e-f).

Previous analysis showed no statistically significant increases in tap root length at 22 DAS, for any of the AlaAT variants expressed in the Col-0 background when grown in N-limiting conditions (Figure 3.4). However, SAP assays showed increased tap root growth of *PfAlaAT*-containing plants on glutamate SAPs and increased tap root growth of *MmAlaAT1*-containing plants on both alanine and glutamate SAPs. Previous results from the N-limiting analysis also indicated that *35S:HvAlaAT*, *35S:MmAlaAT1* and *35S:PfAlaAT* plants in the knockout background showed significant increases in tap root length at 22 DAS. SAP assays indicate that these significant changes in length are lost on glutamate SAPs, however *MmAlaAT1* plants maintain this increased root length on alanine SAPs.

3.3.4. AlaAT variant has an impact on root phenotype in differing C/N conditions

It has been shown previously that sugar concentrations in the root medium can have significant effects on root elongation; increases in medium sucrose concentrations result in increases in tap root elongation (Freixes *et al*., 2002). It had also been shown that in the absence of sucrose when high photosynthetic photon flux densities were implemented (5.2 to 19.8 mol m^{-2} d^{-2}) tap root lengths increased significantly, and that at intermediate photosynthetic photon flux densities (12.5 mol m^{-2} d-^{2}), 0.5% sucrose in the medium could restored tap root elongation rates which decreased when no sucrose was added to the media under a similar photon regiment (Freixes *et al*., 2002), suggesting that either increased photosynthetic photon flux intensity or medium sucrose concentrations will allow for maximum tap root elongation in Arabidopsis. To examine if these findings are altered in Arabidopsis plants expressing *AlaAT* in two control backgrounds (alaat1;2 and Col-0), *AlaAT* transgenic and control seedlings were grown under three different experimental conditions: regular light (170 µE m^{-2} sec^{-1}) with 0% sucrose and 1 mM NO_3^-, low light (100 µE m^{-2} sec^{-1}) with 0% sucrose and 1 mM NO_3^- and low light (100 µE m^{-2} sec^{-1}) with 0.2% sucrose and 0.25 mM NO_3^-.

At 3 DAS *AlaAT* over-expressing seedlings showed increased tap root length and correlatively increased rate of germination in all three conditions (Figure 3.13). This phenotype was consistent for all over-expressing lines tested: *OsAnt1:HvAlaAT*, *35S:MmAlaAT1* and *35S:PfAlaAT*. This phenotype was most noticeable and consistent in over-expressing transgenic

lines in the Col-0 background, however increased tap root length at 3 DAS in the alaat1;2 lines expressing *AlaAT* was also observed (data not shown).

To assess if this increased primary root length was maintained after initial germination of seedlings, plants in all three C:N conditions were monitored for 18 DAS. Vertical tap root lengths were marked at 5, 8, 12 and 15 DAS, and changes in root length between 0-5, 5-8, 8-12 and 12-15 DAS were measured (as done previously in the N-limiting and N non-limiting analysis) (Figure 3.14 and 3.15). Differences in rate of tap root growth were assessed with two-way ANOVA and Bonferroni post-tests, taking into account both genotype and lighting block. Genotype was determined to be statistically significant for all *AlaAT*-expressing lines in the knockout background in regards to the rate of tap root growth throughout the three time points for all three phenotypic conditions when compared to alaat1;2 plants (Figure 3.14).

Under regular light with 0% sucrose and 1 mM NO_3^-, significant increases in tap root length were observed only by transgenics in the alaat1;2 background ($P < 0.05$) (Figure 3.14a-c); no significant differences in root growth for *AlaAT* over-expressing lines (Col-0 background) were detected in this condition (Figure 3.15a-c). When lines were grown in low light with limiting sucrose (0.2%) and limiting N (0.25 mM NO_3^-) all over-expressing lines (Col-0 background) showed significantly increased rates of tap root growth ($P < 0.005$) (Figure 3.15g-i). Interestingly, under low light with 0% sucrose and 1 mM NO_3^-, only *PfAlaAT* over-expressing lines (Col-0 background) showed significant increases in tap root length (Figure 3.14f). Thus, under low light conditions and no external, or limiting external sucrose, only those plants containing *PfAlaAT* consistently showed significant increases in rate of tap root growth up until 15 DAS.

3.3.5. *AlaAT* expression increases number of primary lateral roots

Initiation and proliferation of primary lateral roots in plants is a key response to increases in N in the environment (Drew *et al*., 1973; Drew & Saker, 1978). In general, *AlaAT*-expressing plants grown in ½ MS media showed an increased number of primary lateral roots in comparison to control plants (Figure 3.16 and 3.17). At 8 DAS, a two-way ANOVA was used to determine that genotype of the plants played a significant role in the number of primary lateral roots. All lines over-expressing *AlaAT* variants in the Col-0 background showed significant increases in the

number of lateral roots ($P < 0.05$) (Figure 3.16 f-j). At this same time point, all *AlaAT*-expressing lines in the alaat1;2 background, except those containing *35S:HvAlaAT*, also showed significant increases in lateral root number ($P < 0.05$) (Figure 3.16 a-e). When the same plants were analyzed for number of lateral roots at 11 DAS, all *AlaAT*-expressing lines in the knockout background showed increased lateral root number in comparison to controls, and genotype was determined to be highly significant in the emergence of this phenotype ($P < 0.05$) (Figure 3.17 a-e). Conversely, not all *AlaAT* lines in the Col-0 background continued to show significant increases in lateral root number. Those plants expressing both *35S:MmAlaAT1* and *35S:PfAlaAT* did not show significant increases in lateral roots at 11 DAS, while all other lines in the Col-0 background maintained the significant phenotype observed at 8 DAS. Although *35S:MmalaAT1* and *35S:PfAlaAT* over-expressing lines did not show statistically significant increases in lateral root emergence at 11 DAS in the Col-0 background, they did have more lateral roots than control plants on average (Figure 3.17 h and i). Lateral root emergence did not show clustering (emergence of many lateral roots at some points along the primary root, with no emergence at other points) in any of the observed lines or at any of the time points, with a regular pattern of lateral root emergence observed along the length of the tap root (data not shown).

3.4. Discussion

Previous research has shown that under N-limiting conditions, increases in tissue-specific expression of *HvAlaAT* in rice can result in increased NUE, manifesting as increases in biomass (denser, bushier plants with increased tiller number) and yield, as well as increases in total N and key N metabolites (glutamine, glutamate and asparagine) (Good *et al*., 2007; Shrawat *et al.*, 2008). These phenotypes were observed in mature plants, with alterations in NUE not detected in younger seedlings (Beatty *et al*., 2013). In this study, the impact of expressing variant AlaAT enzymes in young and developing *Arabidopsis thaliana* plants was studied in order to deduce the stage of primary N metabolism that is most impacted by AlaAT, uptake or assimilation, and to see if early phenotypes indicating NUE in plants could be determined.

During the initial characterization of transgenic lines, the tap root length and rate of growth were observed to be similar or significantly increased in transgenic plants, and was then used to phenotypically characterize the effect of the *AlaAT* variants expressed in Arabidopsis. Previously, increases in tap root length as well as increases in primary lateral roots have been

characterized as "foraging" strategies, indicating N limitation in plants; conversely, inhibition of tap root lengthening and lateral root emergence have indicated N-replete conditions (Zhang *et al*., 1999; Zhang & Forde, 2000; Zhang *et al*., 2007; Ruffel *et al*., 2011; Roycewicz & Malamy, 2012;). However, it has also been shown that not only the supply of N, but also the demand of N by the plant can play a pivotal role in root architecture (plant nitrogen economics), resulting in increases in root growth and lateral root emergence in N-rich conditions if the demand for N by the plant (shoot to root) is strong enough (Zhang & Forde, 2000; Zhang *et al*., 2007; Krouk *et al*., 2011; Ruffel *et al*., 2011). Given that the root phenotypes observed in this study occurred under both N-depleted and N-replete conditions, and quantitatively shoot biomass appeared similar or greater than the controls and colouring of transgenic shoots appeared healthy, I propose that the increases in tap root length and rate of growth are due to increased N demand from the plants, and not due to N foraging responses.

Analysis of *AlaAT*-expressing plants in both N-limiting and non-limiting conditions indicated that the expression of different variants of *AlaAT*, and the promoter which drives expression, may have a significant impact on the NUE phenotype, as previously observed in rice (Beatty *et al.*, 2013; Shrawat *et al.,* 2008). The rate of tap root growth from 0-12 days, specifically in the alaat1;2 background, was significantly higher than that of control plants in all cases (N-limiting and non-limiting). Moreover, while the final tap root length at 22 DAS was not significantly longer on average than those of controls (alaat1;2) for all lines in the N-limiting condition, these results indicate that the native AlaAT enzyme may be indirectly involved in initial N sensing and assimilation (Figure 3.2, 3.3, 3.5 and 3.6). The rate of growth of plants over-expressing AlaAT in the Col-0 background, under N-limiting conditions, indicates possible differences in NUE based on AlaAT variant and promoter. Under N-poor conditions, early rate of tap root growth was only significantly higher in those plants expressing non-mitochondrial AlaAT variants with a constitutive promoter (Figure 3.2). Interestingly, *MmAlaAT1*-expressing plants (Col-0 background) were the only lines to show increased rate of tap root growth in both limiting and non-limiting N conditions between 0-5 and 5-8 DAS (Figure 3.2h and 3.5h). This suggests that over-expression of this enzyme could have the most significant effect on overall plant NUE.

In general, early analysis of rate of tap root growth (0-12 DAS) showed the most significant differences between transgenic and control plants, however these differences diminished as plants aged. This decrease in phenotypic differences could be the result of: i) *AlaAT* expression and over-expression phenotypes being highly dependent on and influenced by developmental stage of the plant, ii) plate assays presenting constrictions on growth and development of the plants that result in the inhibition of the observed phenotype or, iii) a combination of these two effects. However, regardless of phenotypes observed later in development, initial expression of AlaAT during germination can result in an increased NUE phenotype, depending on AlaAT variant and external N conditions, indicating that to achieve maximum NUE, the *AlaAT* variant: promoter combination must be tailored to the environmental N conditions.

The over-expression of any of the *AlaAT* variants in Arabidopsis when given only alanine or glutamate as the sole N source indicated that over-expression of this enzyme (Col-0 background) allowed plants to more effectively utilize these amino acids as an N source when compared to controls (Figure 3.8, 3.9, 3.10 and 3.11). These results are interesting given that previous analyses of Arabidopsis, and other plants grown using either glutamate or alanine as the sole N source have shown inhibition of root or overall plant growth, respectively (Walch-Liu *et al*., 2006; Miyashita *et al*., 2007; Walch-Liu & Forde, 2008). Growth of Arabidopsis with external L-glutamate has been shown to inhibit mitotic activity at the root apical meristem, ultimately decreasing tap root elongation (Filleur *et al*., 2005; Walch-Liu *et al*., 2006; Walch-Liu & Forde, 2008). The results presented here indicate that over-expression of *AlaAT* can, to some extent, overcome this growth inhibition (Figure 3.8a,c, 3.9a-c and 3.11d-f). Transgenics grown in the presence of alanine also show increased growth compared to controls. Decreases in native AlaAT (*alaat1-1*) in Arabidopsis, have previously shown to reduce overall growth of plants when supplied with exogenous alanine as an N source (Miyashita et al., 2007). The results presented here support these previous observations, confirming that over-expression of this enzyme can enhance the ability of the plant to utilize alanine as an external N source above that of the controls. Whether this increased root growth was a result of an increased capacity to sense, uptake or assimilate the external N source, or due to increased nutrients and energy stored in the seed of transgenic plants, remained to be determined.

SAP assays were carried out to better understand if the above phenotypes were the result of more efficient usage of internal nutrients when *AlaAT* is over-expressed early in development in environments with non-ideal N available or, if these phenotypes were due to increased ability to utilize externally supplied N, in the form of either alanine or glutamate. No significant differences in tap root length were observed at 22 DAS when *AlaAT* over-expressers were grown in N-limiting conditions, however significant differences were observed during SAP assays for several of these same lines (Figure 3.12). Several conclusions can be drawn from these assays. First of all, NUE phenotypes in AlaAT transgenic lines can be significantly affected by environmental N type and availability. Secondly, *MmAlaAT1* when expressed in plants can utilize alanine more effectively as an external N source than other *AlaAT*-expressing transgenics. Finally, these results eliminate the possibility of increased seed stores contributing significantly to the root phenotypes described above, because no increased rate of germination was observed on SAP plates, but differences in root length and rate of root growth were observed. Furthermore, increased tap root growth was observed at 3 DAS in both glutamate and alanine plates (Figure 3.8) but was not observed in plants germinated in N-limiting or N non-limiting conditions. It can be assumed then that any differences in the nutritional status of the seed are not contributing in a significant way to the outcome of these assays.

Another factor known to affect root growth and morphology is the external ratio of C:N, coupled with the rate of photosynthesis and overall plant growth (Crawford & Glass, 1998; Lejay *et al*., 1999; Little *et al*., 2005; Zhang *et al*., 2007; Macgregor *et al*., 2008; Roycewicz & Malamy, 2012) The results of this study indicate that the over-expression of *AlaAT* increases tap root length immediately following germination under a variety of decreased C conditions (Figure 3.13, Figure 3.14 and figure 3.15). In the knockout background, expression of AlaAT resulted in significant increases in root growth, regardless of enzyme variant or external C:N conditions, indicating that native AlaAT enzymes are likely significantly involved in mediating the internal C:N ratios of wildtype plants (Figure 3.14). This result also supports the previous observation that *AlaAT*-expressing lines (alaat1;2 background) show increased rate of root growth in N-limiting conditions (Figure 3.3). However, in the Col-0 background, *PfAlaAT*-expressing plants were the only transgenics that showed significant increases in rate of tap root growth under low light and 0% sucrose conditions (Figure 3.15). Freixes *et al*., (2002) showed that increases in the concentration of sucrose in the plate media can offset decreases in light

intensity and vice versa, however decreases in external sucrose (< 2%) in combination with decreases in light intensity (~100 μE m^{-2} sec^{-1}) resulted in decreases in both tap root and lateral root growth. Nevertheless, when *PfAlaAT* was over-expressed in plants in low light, 0% sucrose and 1 mM N, significant increases in rate of tap root growth over that of wildtype were observed, demonstrating that the over-expression of *PfAlaAT* in Arabidopsis alters not only N metabolism, but also C metabolism significantly. In addition, the expression of *OsAnt1:HvAlaAT*, *MmAlaAt1* and *PfAlaAT* in the Col-0 background under low light, and minimal external sucrose and NO_3^-, showed significant increases in rate of tap root growth relative to the control. These results reinforce the role AlaAT plays in connecting both N and C metabolism via TCA cycle intermediates.

Arabidopsis primary lateral roots have shown to increase in proliferation when exposed to patches of NO_3^- (Zhang & Forde, 2000). This increase in lateral root number has been characterized as a key morphological response of Arabidopsis roots colonizing N-rich zones (Drew *et al*., 1973; Drew & Saker, 1978; Zhang, *et al*., 2007). On the other hand, it has also been shown that high concentrations of NO_3^- in plant tissues can inhibit the activation of lateral root meristems (Zhang *et al*., 1999; Zhang & Forde, 2000; Remans *et al*., 2006; Zhang *et al*., 2007). As a result of these opposing N responses, alterations in the ability to either sense external N or in the distribution of internal root NO_3^- could alter these morphological responses. The results presented here indicate that expression of AlaAT plays an indirect role in the induction and emergence of primary lateral roots in *Arabidopsis thaliana* early in the growth and development of the plant. In the knockout background, expression of AlaAT, regardless of variant, showed overall increases in number of primary lateral roots, with all variants except *35S:HvAlaAT* showing significant increases in lateral root number at all time points measured (Figure 3.16 a-e and 3.17 a-e). Over-expression of AlaAT, above that of the native enzymes, also resulted in overall increases in primary root growth, with significance between controls and transgenics being highest at 8 DAS (Figure 3.16 f-j and 3.17 f-j). Given that lateral root emergence appeared equal along the length of the tap root, and plants were grown in a medium with very little variation in N concentration throughout (½ MS), these results strengthen the argument that AlaAT has an impact on the rate of primary N assimilation within plant roots, but that this enzyme is probably not involved in the initial sensing of external N.

A greater understanding of the role of AlaAT and AlaAT over-expression can also be gained by this analysis and the availability of a double knockout background (alaat1;2) in Arabidopsis. In the Col-0 background, over-expression of any of the AlaAT genes would result in a gain of function (GOF) but, in a wildtype background, determining if this is a gain-of-the-same gene function or gain-of-a-different gene function is not possible. However, analysis of the expression of these genes in the alaat1;2 background showed the same phenotypes (increased tap root growth and increased lateral root number) as over-expression in the Col-0 background. Thus, expression of AlaAT in the alaat1;2 background restored/rescued tap root growth and lateral root growth in the mutant, allowing the conclusion that these phenotypes are the result of a gain-of-the-same gene function *in vivo*.

From this analysis several conclusions can be drawn. Firstly, AlaAT over-expression does enhance NUE in Arabidopsis, presumably by increasing the nitrogen economics of the plants (increasing overall internal demand for N) based on interactions between the shoot and the root. These interactions would involve increased N assimilation, regardless of initial N form, as well as increased ability to relocate N resources. Secondly, there are differences in the NUE phenotypes exhibited in plants expressing different AlaAT enzymes under various external N environments. This indicates that the *in vitro* kinetic differences between homologous AlaAT enzymes observed in Chapter 2 can result in *in vivo* alterations in overall N and C metabolism. Thirdly, *MmAlaAT1*, constitutively expressed, appears to have a larger impact on overall plant NUE than other enzymes given the results from both non-limiting N plates and SAP plates. Fourthly, the use of SAP showed that *MmAlaAT1*, over-expressed in Arabidopsis, results in plants that can more effectively utilize alanine as an N source when compared to both controls and other *AlaAT* over-expressing plants. Finally, when both N and C were decreased, plants constitutively expressing *PfAlaAT* showed the most significant increases in rate of root growth. Interestingly, *PfAlaAT* expression did not consistently show the greatest degree of root growth or rate of root growth under N-limiting conditions only. This re-affirms the idea that the desired NUE phenotype may be highly dependent not only on over-expression of AlaAT, but also on the *AlaAT* variant, the promoter chosen and the specific environmental conditions of the plant.

Table 3.1. Summary of AlaAT constructs transformed into *Arabidopsis thaliana*.

Construct	**Promoter**	**Localization predicted *in vivo***	**AlaAT Variant Source**	**Vector Backbone**
OsAnt1:HvAlaAT	OsAnt1	Cytoplasm; root and vasculature	*Hordeum vulgare* (Barley)	pCAMBIA 1300
OsAnt1:GUS	OsAnt1	Cytoplasm; root and vasculature	N/A	pCAMBIA 1300
CaMV35S:HvAlaAT	2X CaMV 35S	Cytoplasm; constitutive	*Hordeum vulgare* (Barley)	pMDC32
CaMV35S:MmAlaAT1	2X CaMV 35S	Cytoplasm; constitutive	*Mus muscus* (Mouse)	pMDC32
CaMV35S:MmAlaAT2	2X CaMV35S	Mitochondria; constitutive	*Mus muscus* (Mouse)	pMDC32
CaMV35S:PfAlaAT	2X CaMV 35S	Cytoplasm; constitutive	*Pyrococcus furiosus*	pMDC32

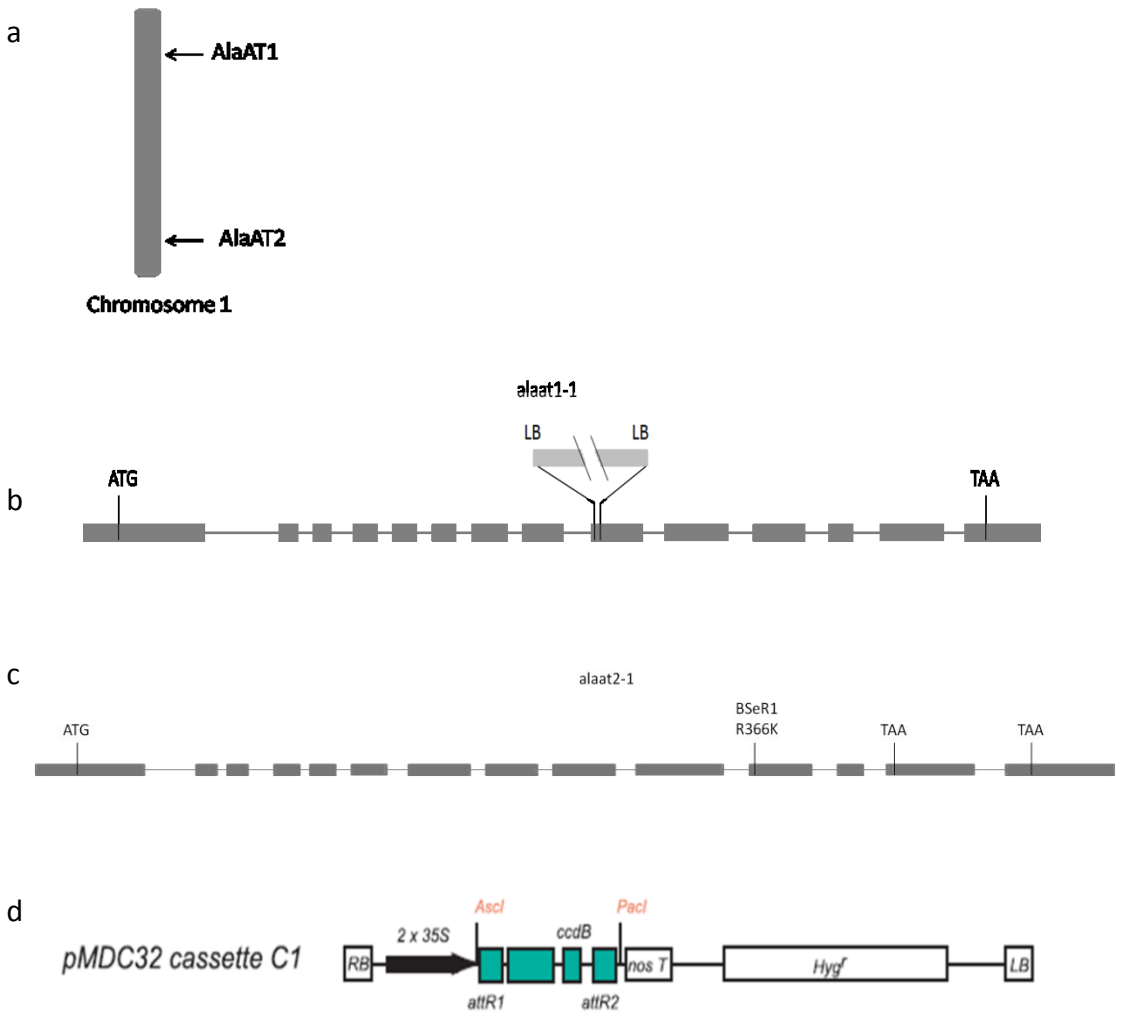


Figure 3.1. Molecular characterization of Arabidopsis AlaAT in Col-0 and alaat1;2 backgrounds.

a) Chromosome location of *Arabidopsis thaliana* native *AlaAT* genes (*AlaAT1* (TAIR reference no. AtG17290) and *AlaAT2* (TAIR reference no. At1G72330)). b) Gene structure of *AlaAT1* and the *alaat1-1* T-DNA mutant. c) Gene structure of *AlaAT2* and the *alaat2-2* point mutation mutant. The alaat1;2 background was originally created by Erin Gilchrist and George Haughn (UBC), and characterized by Yo Miyashita (2008). d) Structure of the binary vector system used to transform *Arabidopsis thaliana*, taken from Curtis & Grossniklaus (2003).

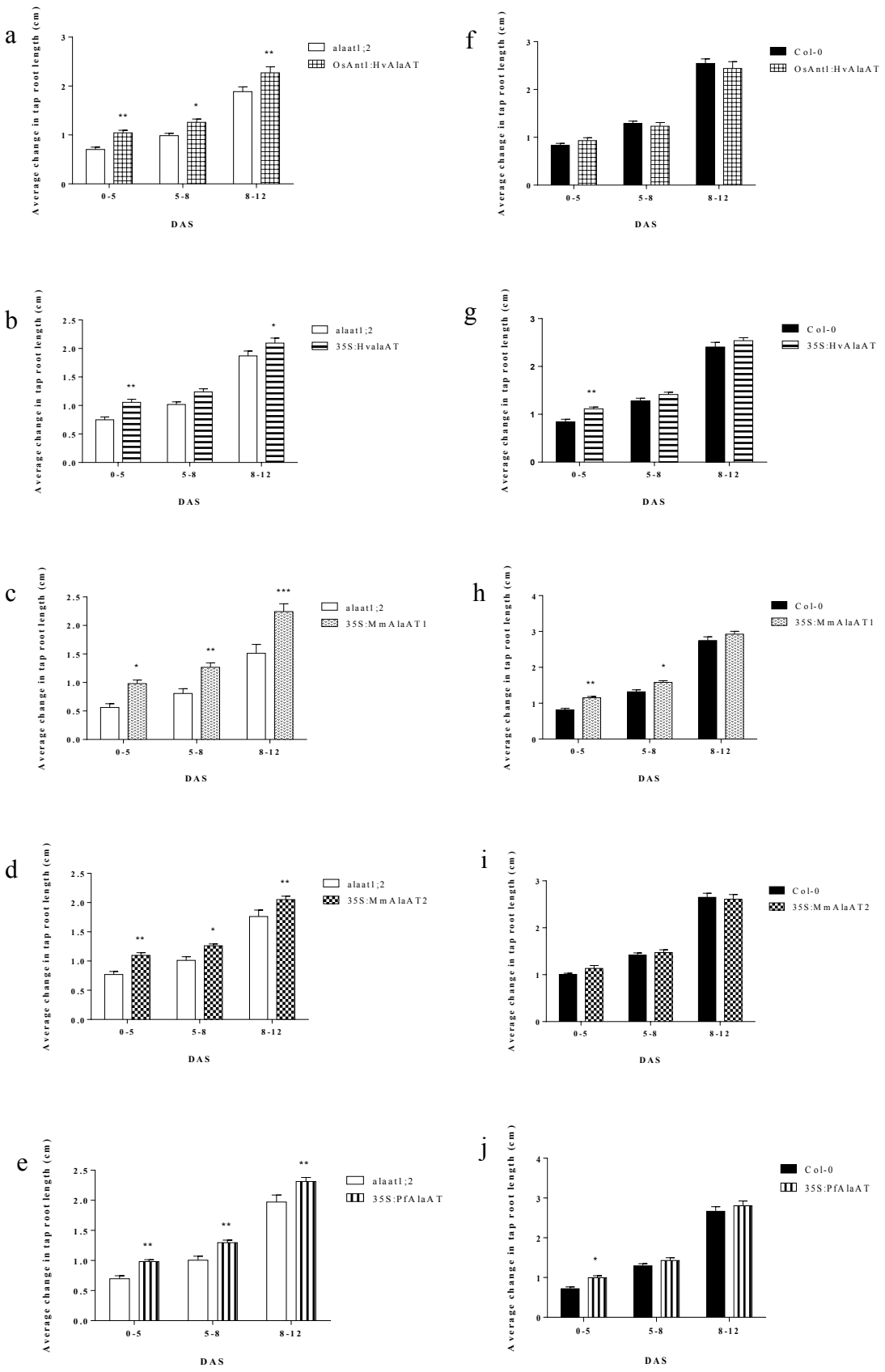

a
Average change in tap root length (cm)
alaat1;2
OsAnt1:HvAlaAT
0-5
5-8
8-12
DAS
f
Col-0
OsAnt1:HvAlaAT
b
alaat1;2
35S:HvalaAT
g
Col-0
35S:HvAlaAT
c
alaat1;2
35S:MmAlaAT1
h
Col-0
35S:MmAlaAT1
d
alaat1;2
35S:MmAlaAT2
i
Col-0
35S:MmAlaAT2
e
alaat1;2
35S:PfAlaAT
j
Col-0
35S:PfAlaAT

Figure 3.2. Change in vertical tap root length from 0-12 DAS of plants expressing various AlaATs in both COL and alaat1;2 backgrounds, when grown in N-limiting conditions.

Transgenic and control plants were sown on modified ½ MS with 0.25 mM NO_3^- as the sole N source. Tap root lengths on plates were marked at 5, 8, and 12 DAS. The vertical growth of tap roots between 0-5, 5-8 and 8-12 DAS was measured (cm) and the mean changes in vertical root growth between controls and transgenics at these time points was compared using two-way ANOVA ($P < 0.05$). At each time point for each line in each background n = 33-36. a-e) Transgenics in an *alaat1;2* background; f-j) transgenics in a Col-0 background. * indicates significance in relation to control plants grown during the same time frame on the same plates. (Error bars indicate SEM.)

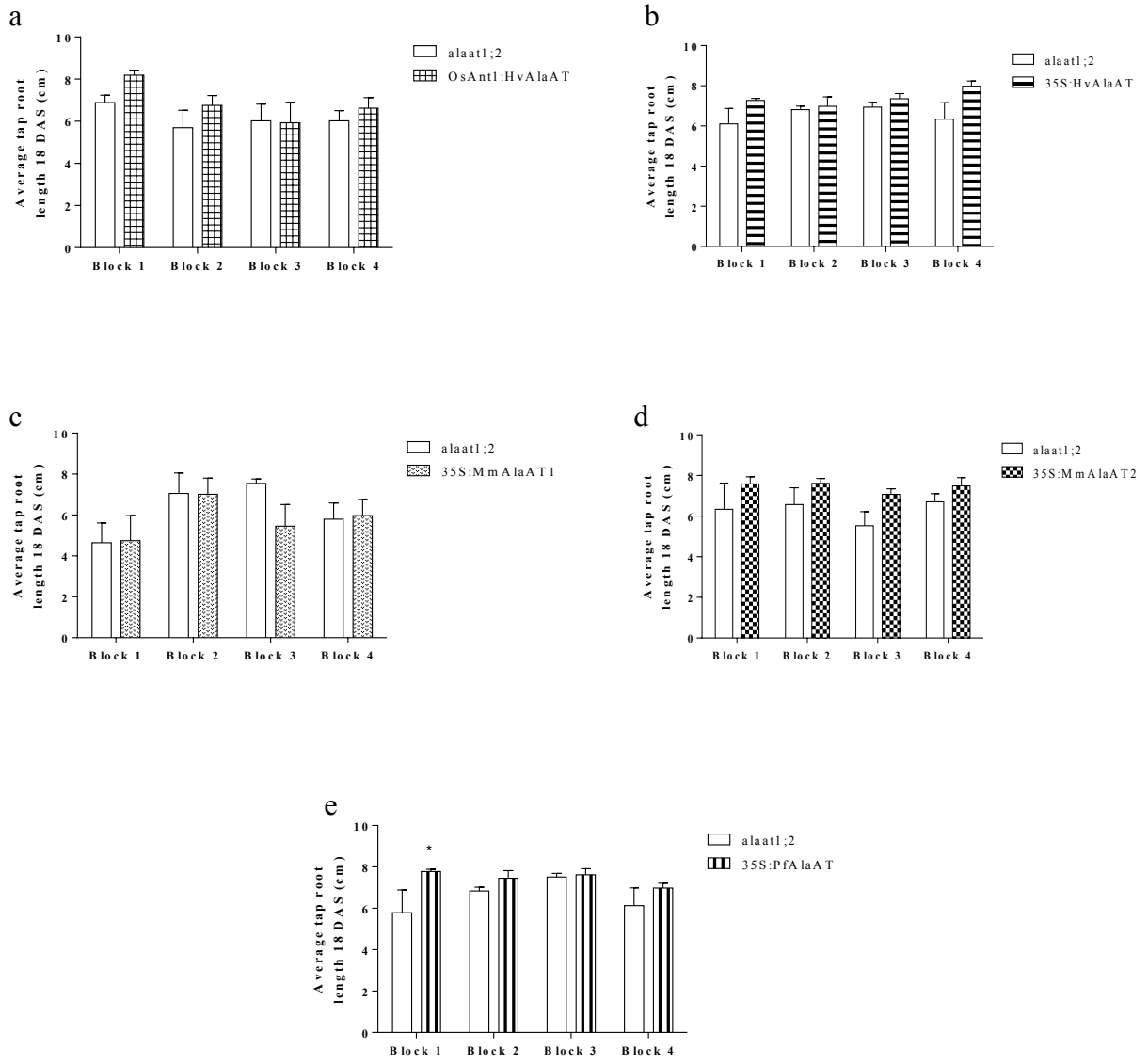


Figure 3.3. Tap root lengths at 18 DAS of alaat1;2 and AlaAT-expressing plants when grown in N-limiting conditions.

Transgenic (alaat1;2 background) and control plants were sown on modified ½ MS with 0.25 mM NO_3^- as the sole N source. Plants were grown vertically for 18 DAS. Final tap root lengths were measured (cm) from the top of the hypocotyl to the root cap. Mean tap root lengths between controls and transgenics were compared using a two-way ANOVA ($P < 0.05$). n = 6-9 for the number of replicates per genotype per block. * indicates significance in relation to control plants grown in the same lighting block. (Errors bars indicate SEM.)

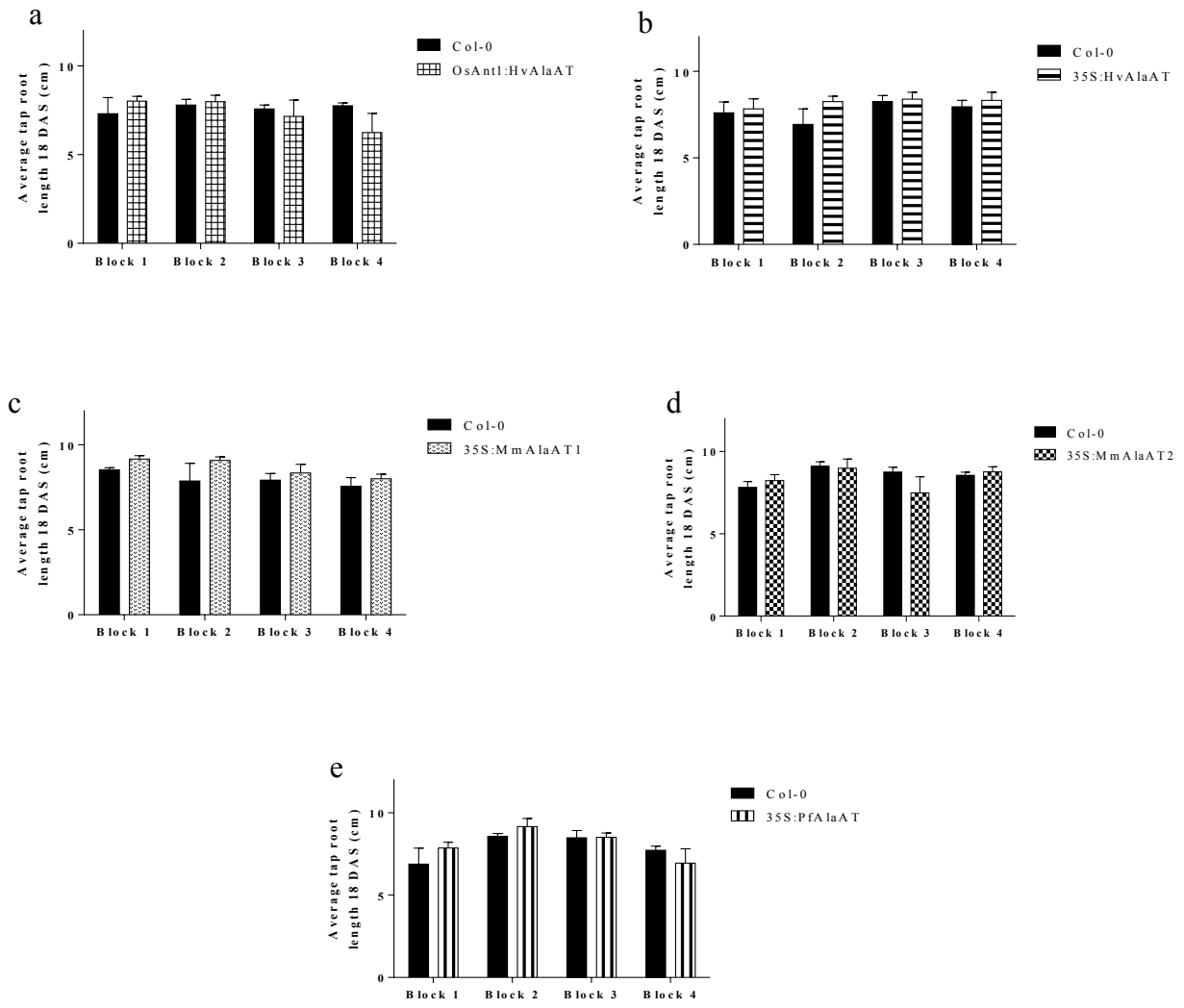


Figure 3.4. Tap root length at 18 DAS of Col-0 and AlaAT-expressing plants when grown in N-limiting conditions.

Transgenic (Col-0 background) and control plants were sown on modified ½ MS with 0.25 mM NO_3^- as the sole N source. Plants were grown vertically for 18 DAS. Final tap root lengths were measured (cm) from the top of the hypocotyl to the root cap. Mean tap root lengths between controls and transgenics were compared using a two-way ANOVA ($P < 0.05$). n = 6-9 for the number of replicates per genotype per block. * indicates significance in relation to control plants grown in the same lighting block. (Errors bars indicate SEM.)

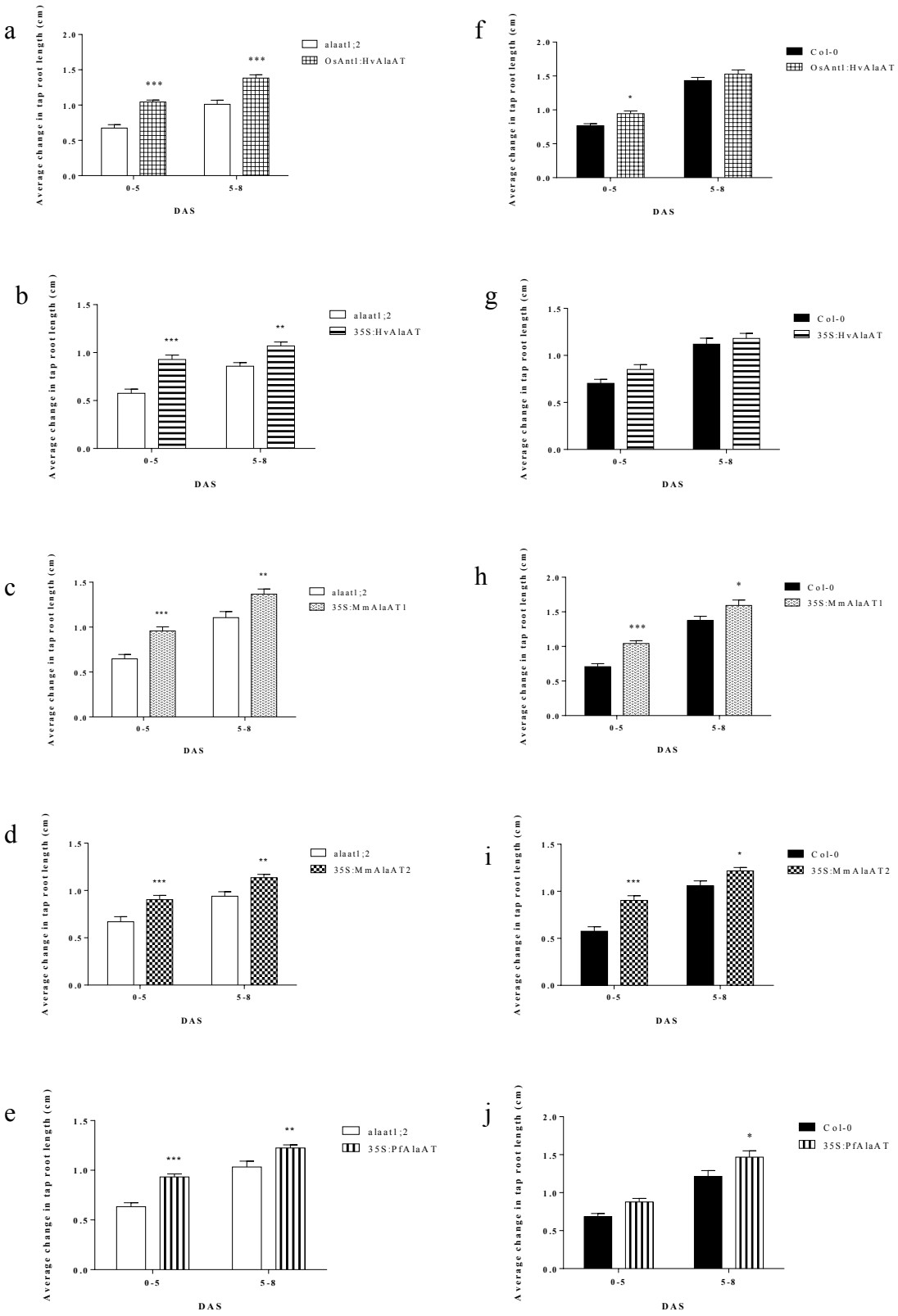

a
Average change in tap root length (cm)
alaat1;2
OsAnt1:HvAlaAT
***
***
0-5
5-8
DAS
f
Col-0
OsAnt1:HvAlaAT
*
b
alaat1;2
35S:HvAlaAT
***
**
g
Col-0
35S:HvAlaAT
c
alaat1;2
35S:MmAlaAT1
***
**
h
Col-0
35S:MmAlaAT1
***
*
d
alaat1;2
35S:MmAlaAT2
***
**
i
Col-0
35S:MmAlaAT2
***
*
e
alaat1;2
35S:PfAlaAT
***
**
j
Col-0
35S:PfAlaAT
*

Figure 3.5. Changes in vertical tap root length from 0-8 DAS of plants expressing various AlaATs in both COL and alaat1;2 backgrounds when grown in non-limiting N conditions.

Transgenic and control plants were sown on modified ½ MS with 2 mM NO_3^- as the sole N source. Tap root lengths on plates were marked at 5 and 8 DAS. The vertical mean growth of tap roots between 0-5 and 5-8 DAS was measured (cm) and the changes in vertical root growth between controls and transgenics at these time points was compared using two-way ANOVA ($P < 0.05$). At each time point for each line in each background n = 33-36. a-e) Transgenics are in an *alaat1;2* background; f-j) transgenics are in a COL background. * indicates significance in relation to control plants grown during the same time frame on the same plates. (Error bars indicate SEM.)

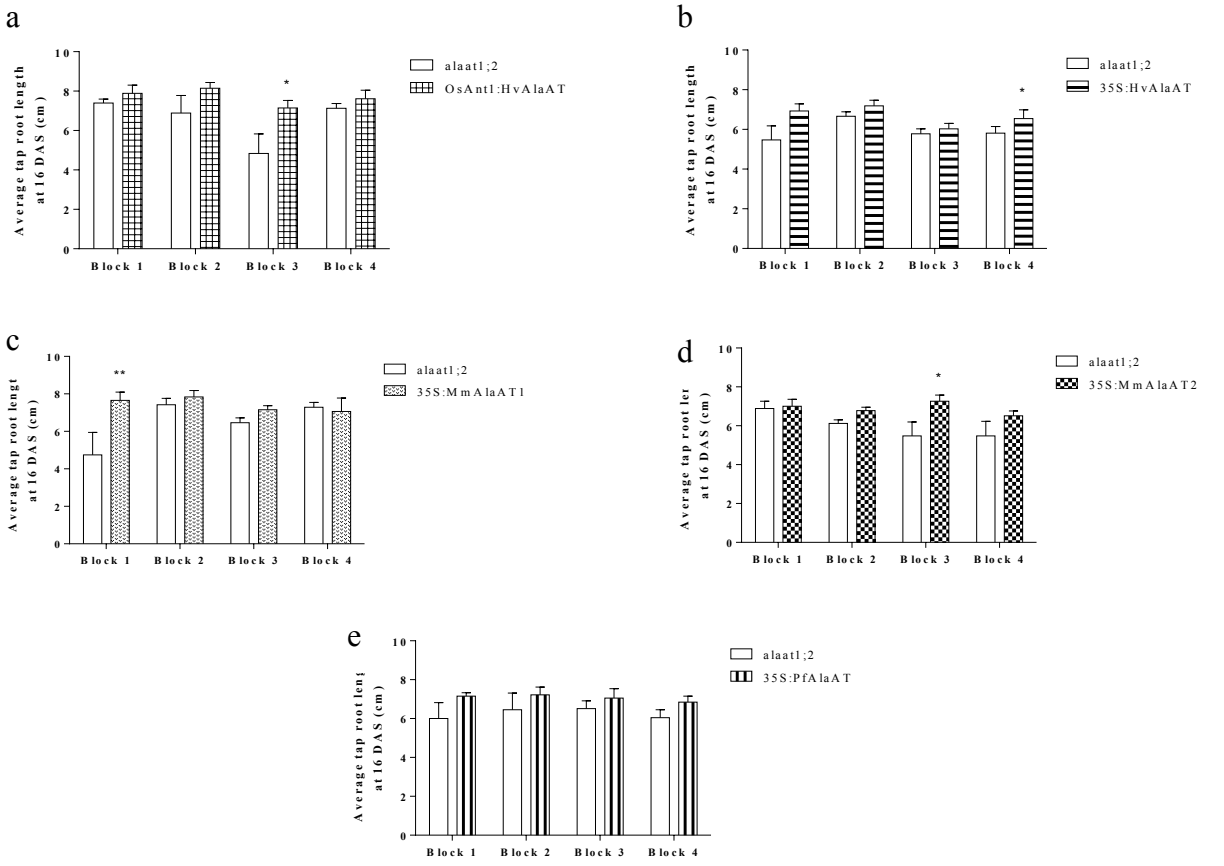


Figure 3.6. Final tap root lengths at 16 DAS of alaat1;2 and AlaAT-expressing plants when grown in non- N-limiting conditions.

Transgenic (alaat1;2 background) and control plants were sown on modified ½ MS with 2 mM NO_3^- as the sole N source. Plants were grown vertically for 16 DAS. Final tap root lengths were measured (cm) from the top of the hypocotyl to the root cap. Mean tap root lengths between controls and transgenics were compared using a two-way ANOVA ($P < 0.05$). n = 6-9 for the number of replicates per genotype per block. * indicates significance in relation to control plants grown in the same lighting block. (Errors bars indicate SEM.)

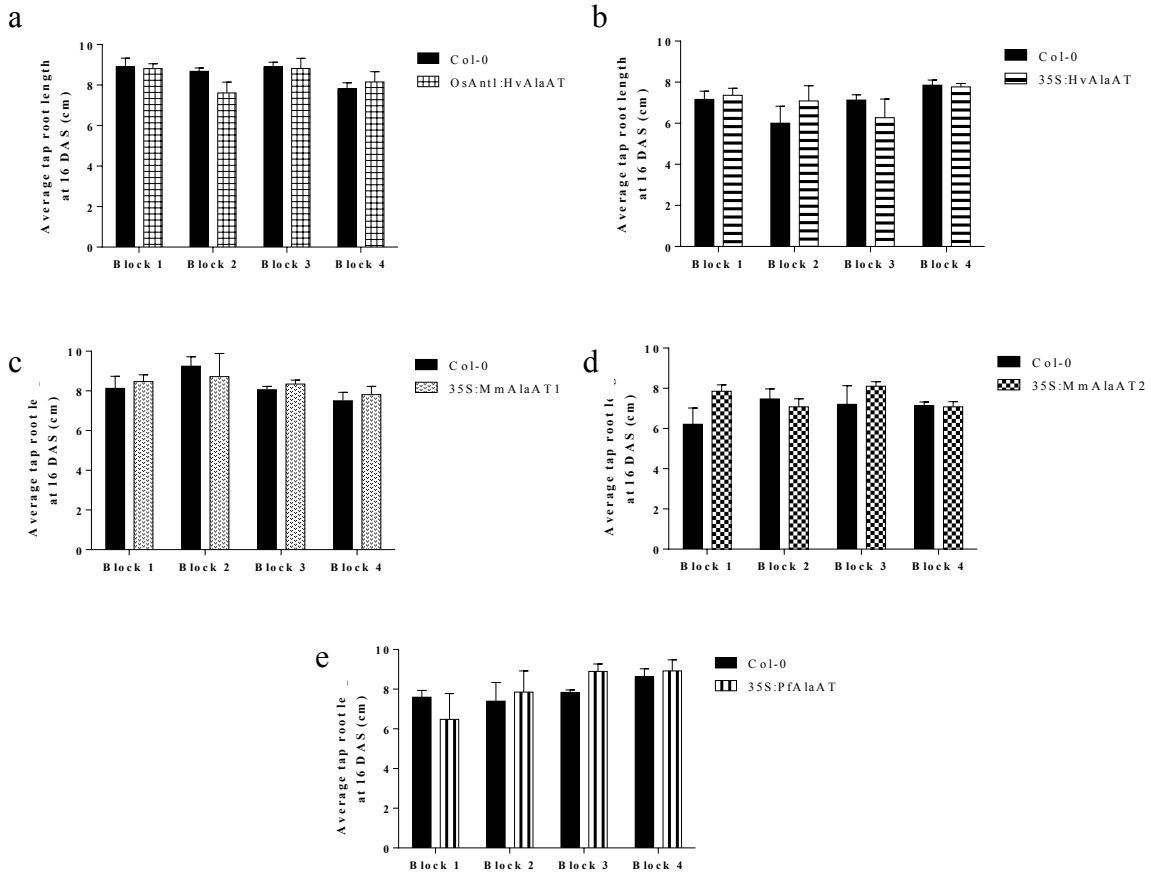


Figure 3.7. Final tap root lengths at 16 DAS of Col-0 and AlaAT-expressing plants when grown in non-N-limiting conditions.

Transgenic (Col-0 background) and control plants were sown on modified ½ MS with 2 mM NO_3^- as the sole N source. Plants were grown vertically for 16 DAS. Final tap root lengths were measured (cm) from the top of the hypocotyl to the root cap. Mean tap root lengths between controls and transgenics were compared using a two-way ANOVA ($P < 0.05$). n = 6-9 for the number of replicates per genotype per block. (Errors bars indicate SEM.)

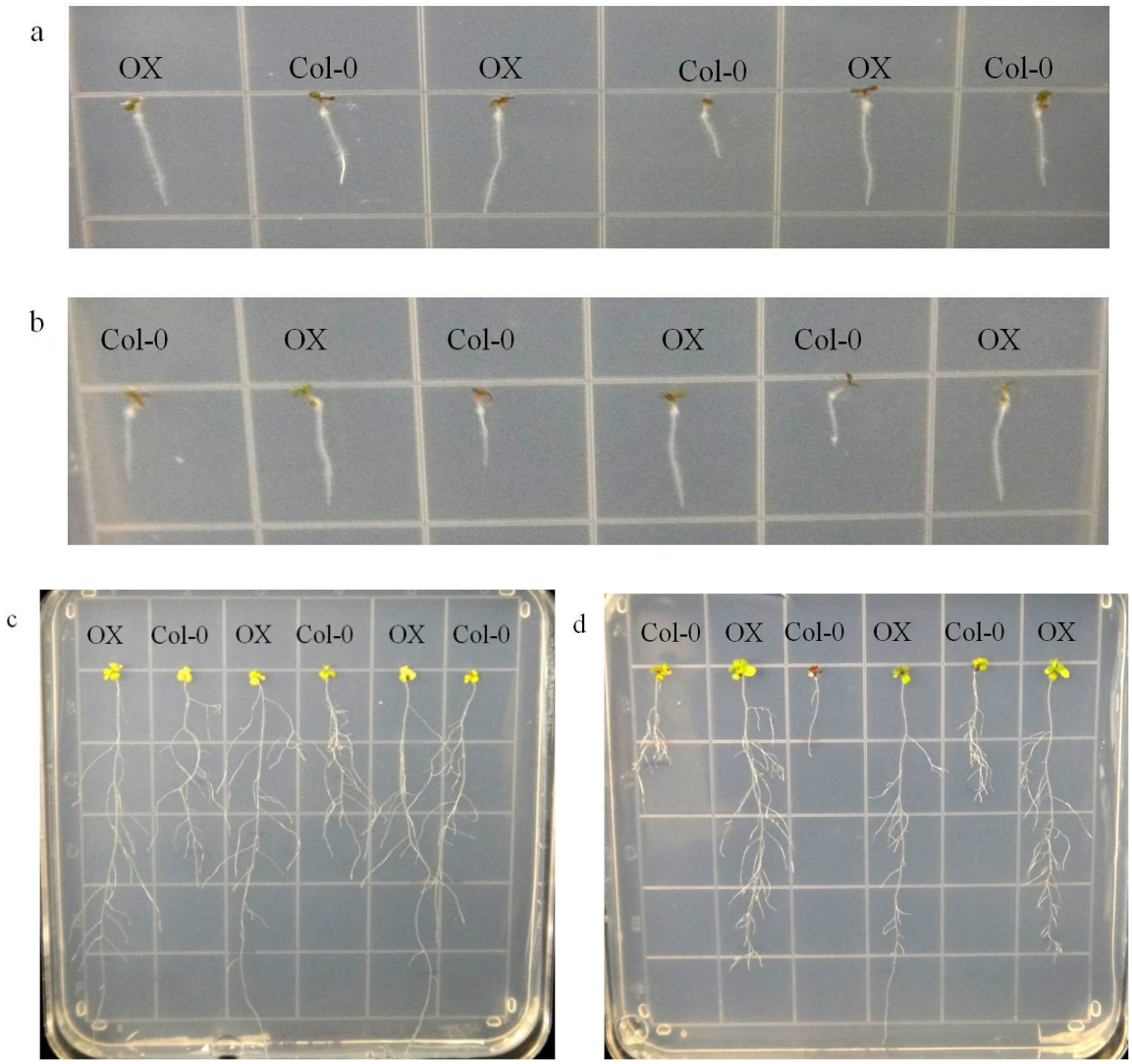


Figure 3.8. *OsAnt1:HvAlaAT* expressing plants in Col-0 background at two different time points grown with glutamate and alanine as the sole N source.

Over-expressing lines were plated on modified ½ MS with either 2.5 mM glutamate (a and c) or 2.5 mM alanine (b and d) as the sole N source. Pictures were taken at 3 DAS and 18 DAS, respectively. OX – transgenic over-expressing line in Col-0 background. Above plates show one independent line of *OsAnt1:HvAlaAT* over-expressing plants, however plates are a representation of all over-expressing lines in comparison to Col-0; phenotype was seen on all plates over-expressing *OsAnt1:HvAlaAT*, *35S:HvAlaAT* and *35S:PfAlaAT*.

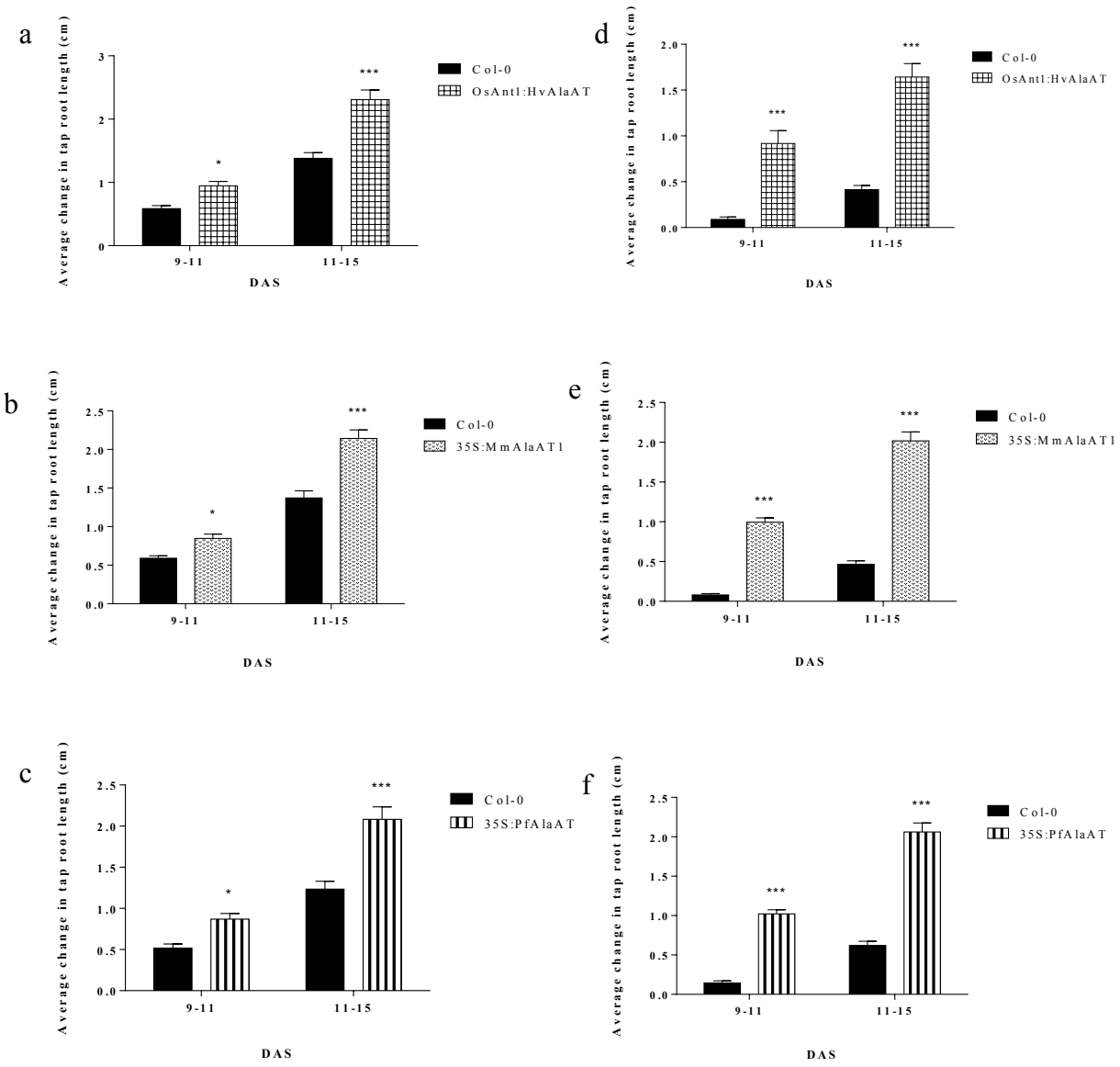


Figure 3.9. The average changes in vertical tap root length from 9-15 DAS of plants expressing various AlaATs in a Col-0 background when grown with glutamate or alanine as the sole N source.

Tap root lengths on plates were marked at 9, 11 and 15 DAS. The vertical mean growth of tap roots between 9-11 and 11-15 DAS was measured (cm) and the changes in vertical root growth between controls and transgenics at these time points was compared using two-way ANOVA ($P < 0.05$). a-c) grown on 2.5 mM Glu, d-f) grown on 2.5 mM Ala. * indicates significance in relation to control plants grown during the same time frame on the same plates. (Error bars indicate SEM.)

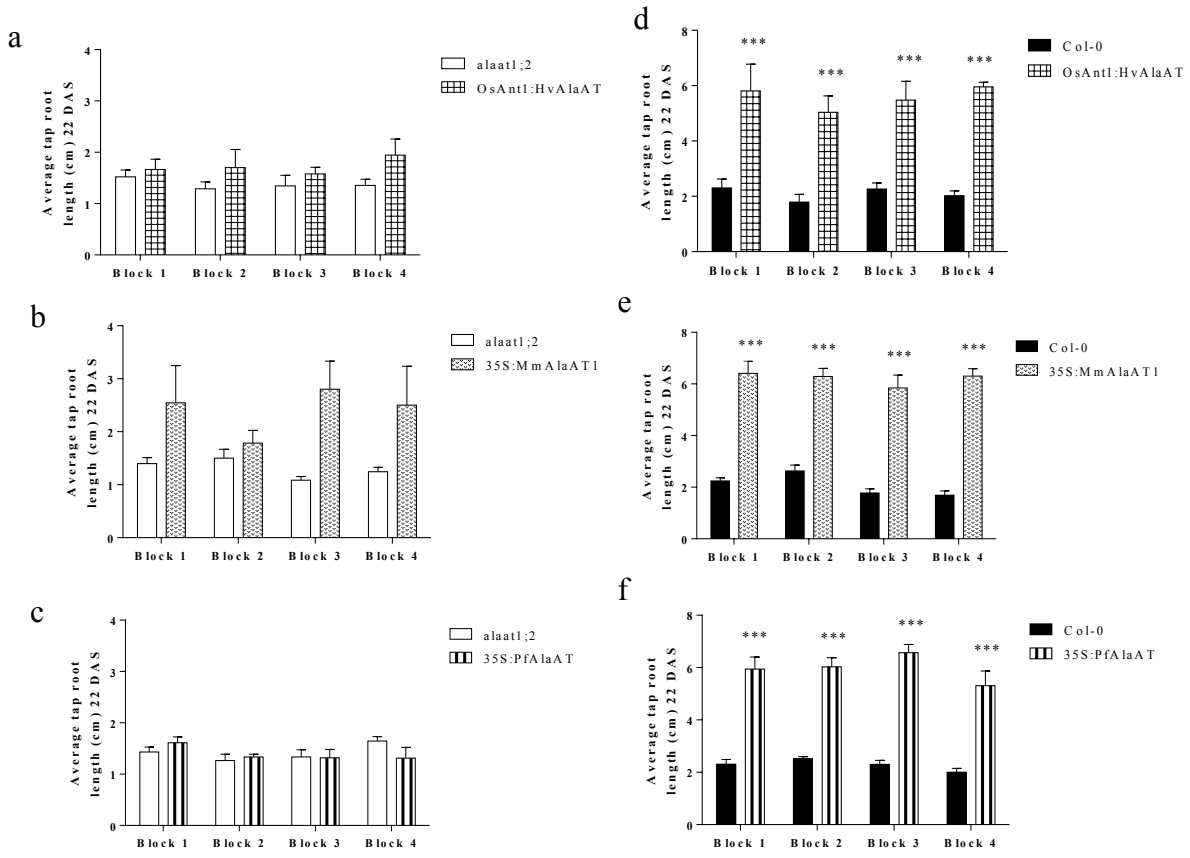


Figure 3.10. Final tap root lengths at 22 DAS of controls and AlaAT over-expressing plants when grown with alanine as the sole N source.

Transgenic and control plants were sown on modified ½ MS with alanine as the sole N source. a-c) transgenics in alaat1;2 background, d-f) transgenics in col-0 background. Plants were grown vertically for 22 DAS. Final tap root lengths were measured (cm) from the top of the hypocotyl to the root cap. Mean tap root lengths between controls and transgenics were compared using a two-way ANOVA ($P < 0.05$). n = 6-9 for the number of replicates per genotype per block. * indicates significance in relation to control plants grown in the same lighting block. (Errors bars indicate SEM.)

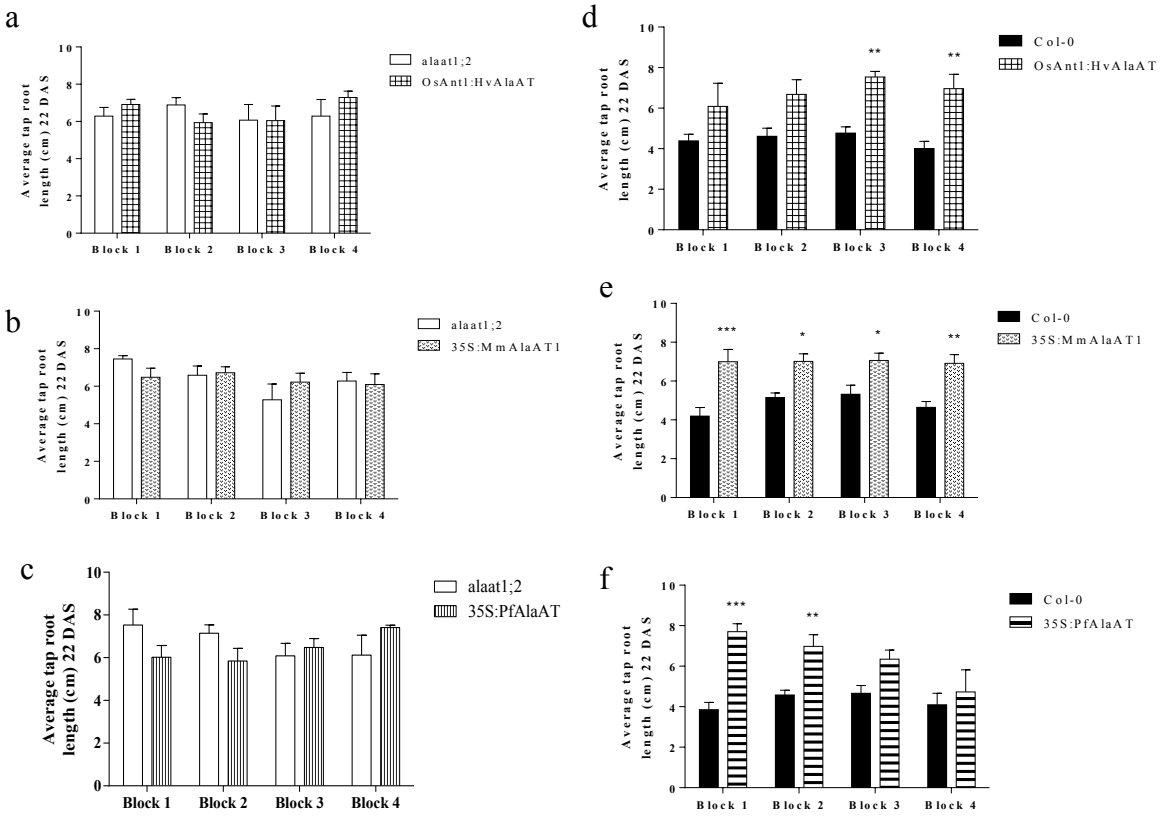


Figure 3.11. Final tap root lengths at 22 DAS of controls and AlaAT over-expressing plants when grown with glutamate as the sole N source.

Transgenic and control plants were sown on modified ½ MS with glutamate as the sole N source. a-c) transgenics in alaat1;2 background, d-f) transgenics in col-0 background. Plants were grown vertically for 22 DAS. Final tap root lengths were measured (cm) from the top of the hypocotyl to the root cap. Mean tap root lengths between controls and transgenics were compared using a two-way ANOVA ($P < 0.05$). n = 6-9 for the number of replicates per genotype per block. * indicates significance in relation to control plants grown in the same lighting block. (Errors bars indicate SEM.)

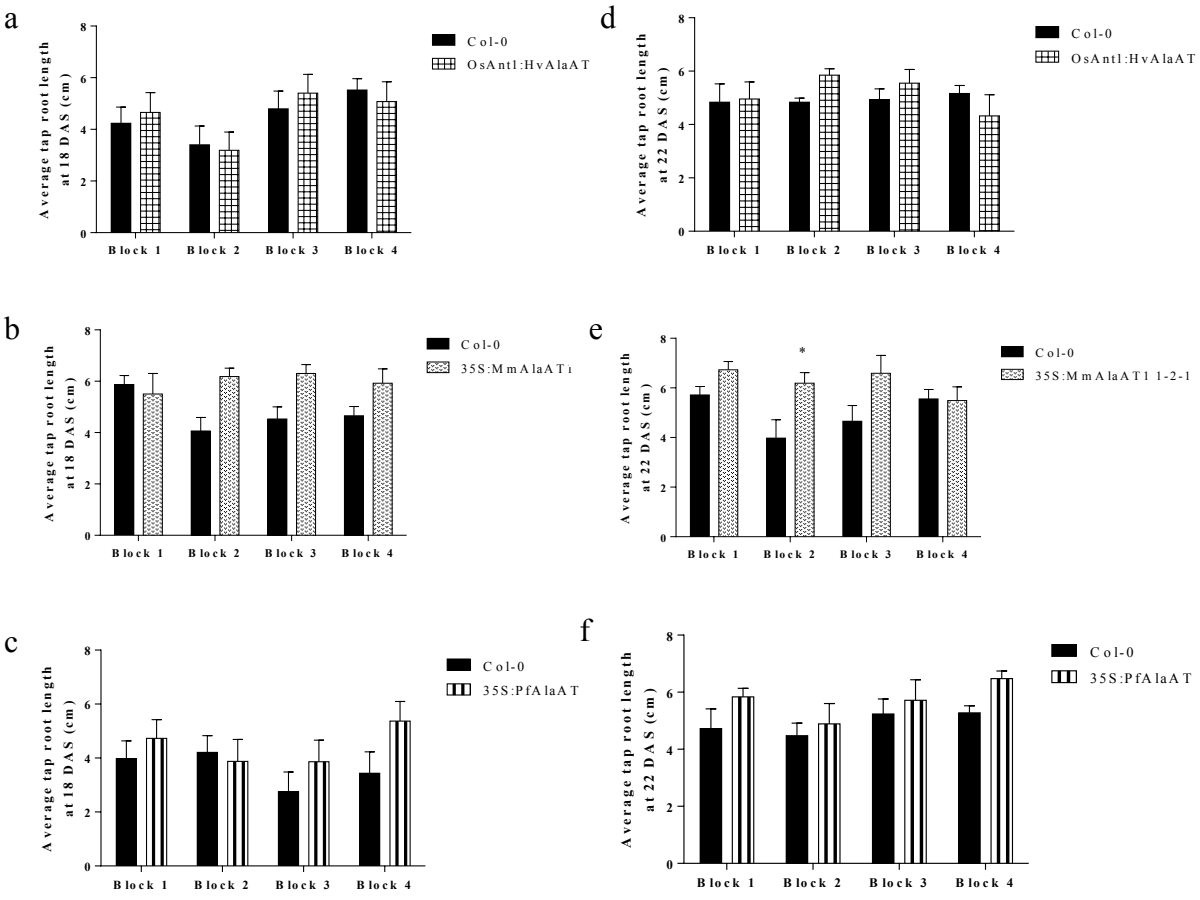


Figure 3.12. Final tap root lengths at 22 DAS of controls and AlaAT over-expressing plants when grown on SAP.

Transgenic and control plants were sown onto SAP with modified ½ MS (0.25 mM KNO_3) comprising the top portion and either a-c) alanine or d-f) glutamate as the sole N source in the bottom portion. Plants were grown vertically for 22 DAS. Final tap root lengths were measured (cm) from the top of the hypocotyl to the root cap. Mean tap root lengths between controls and transgenics were compared using a two-way ANOVA ($P < 0.05$). n = 6-9 for the number of replicates per genotype per block. * indicates significance in relation to control plants grown in the same lighting block. (Errors bars indicate SEM.)

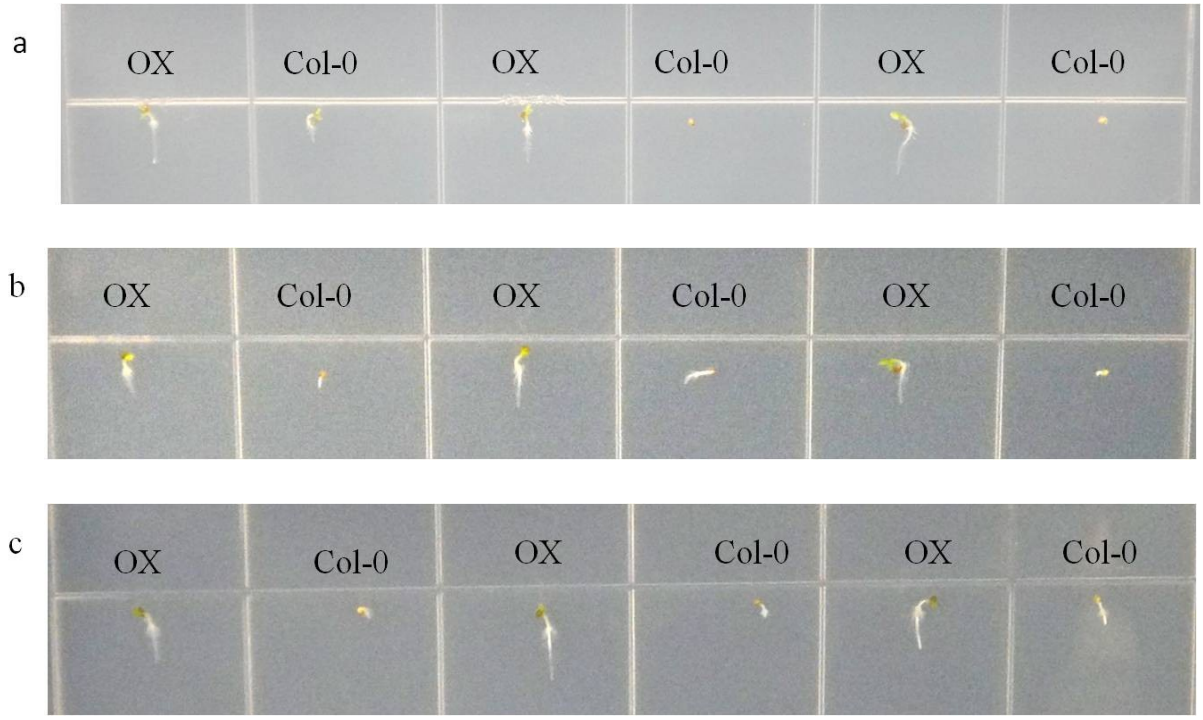


Figure 3.13. *35S:PfAlaAT*-expressing plants and controls grown in limiting C and N conditions.

Over-expressing AlaAT plants (COL background) were grown alongside controls in three combinations of C and N: a) ~170 µE m^{-2} sec^{-1} light, 0% sucrose and 1 mM NO_3^-, b) ~100 µE m^{-2} sec^{-1}, 0% sucrose and 1 mM NO_3^-, c) ~100 µE m^{-2} sec^{-1}, 0.2% sucrose and 0.25 mM NO_3^-. Pictures were taken at 3 DAS. OX – transgenic over-expressing line in COL background. Above plates show one independent line of *CaMV35S:PfAlaAT* over-expressing plants, however plates are a representation of all over-expressing lines in comparison to COL; phenotype was seen on all plates over-expressing *OsAnt1:HvAlaAT*, *35S:HvAlaAT* and *35S:PfAlaAT*.

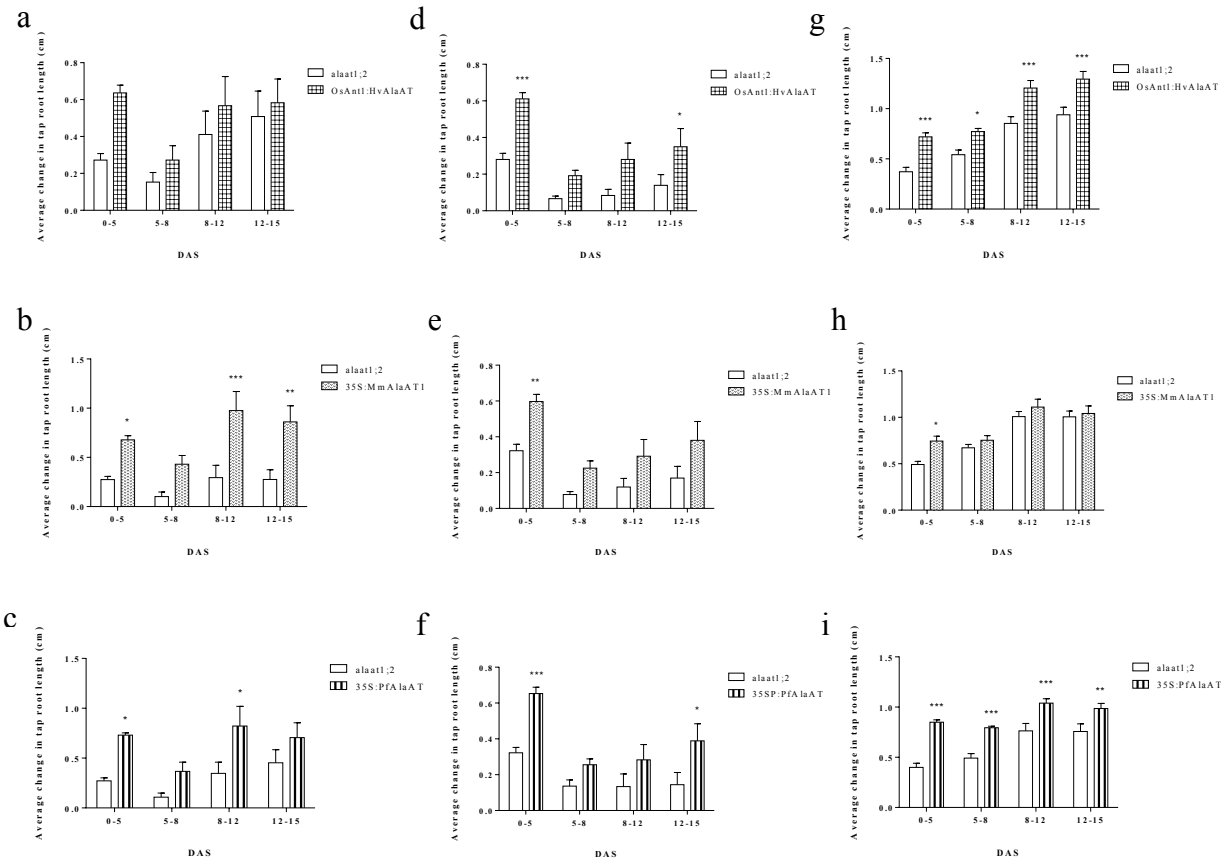


Figure 3.14. The average changes in vertical tap root length from 0-15 DAS of plants expressing various AlaATs in a alaat1;2 background when in grown limiting C and N combinations.

Over-expressing AlaAT plants (alaat1;2 background) were grown alongside controls in three combinations of C and N: a-c) ~170 µE m^{-2} sec^{-1} light, 0% sucrose and 1 mM NO_3^-, d-f) ~100 µE m^{-2} sec^{-1}, 0% sucrose and 1 mM NO_3^-, g-i) ~100 µE m^{-2} sec^{-1}, 0.2% sucrose and 0.25 mM NO_3^-. Vertical tap root lengths on plates were marked at 5, 8, 12 and 15 DAS. The vertical mean growth of tap roots between 5-8, 8-12 and 12-15 DAS was measured (cm) and the changes in vertical root growth between controls and transgenics at these time points was compared using two-way ANOVA ($P < 0.05$). At each time point for each line in each background n = 33-36. * indicates significance in relation to control plants grown during the same time frame on the same plates. (Error bars indicate SEM.)

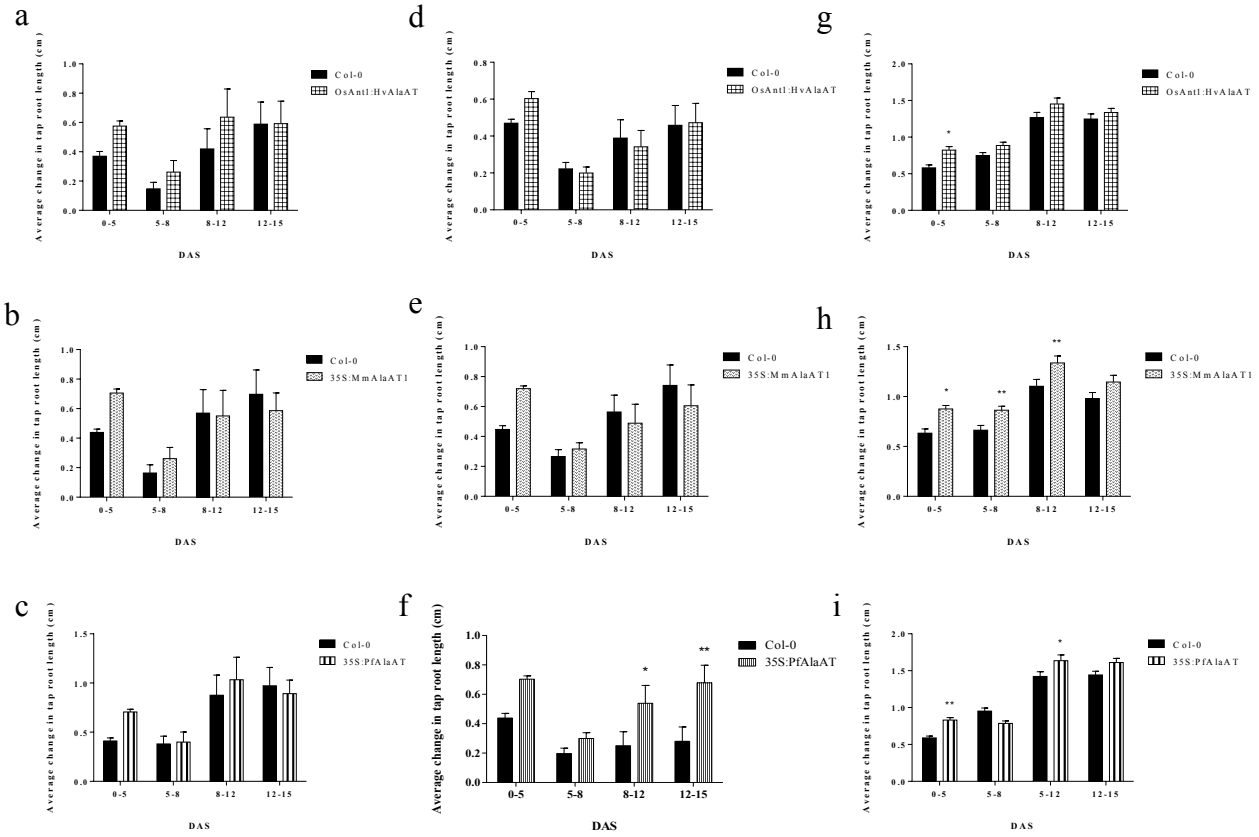


Figure 3.15. The average changes in vertical tap root length from 0-15 DAS of plants expressing various AlaATs in a Col-0 background when grown in limiting C and N combinations.

Over-expressing AlaAT plants (Col-0 background) were grown alongside controls in three combinations of C and N: a-c) ~170 µE m^{-2} sec^{-1} light, 0% sucrose and 1 mM NO_3^-, d-f) ~100 µE m^{-2} sec^{-1}, 0% sucrose and 1 mM NO_3^-, g-i) ~100 µE m^{-2} sec^{-1}, 0.2% sucrose and 0.25 mM NO_3^-. Tap root lengths on plates were marked at 5, 8, 12 and 15 DAS. The vertical mean growth of tap roots between 5-8, 8-12 and 12-15 DAS was measured (cm) and the changes in vertical root growth between controls and transgenics at these time points was compared using two-way ANOVA ($P < 0.05$). At each time point for each line in each background n = 33-36. * indicates significance in relation to control plants grown during the same time frame on the same plates. (Error bars indicate SEM.)

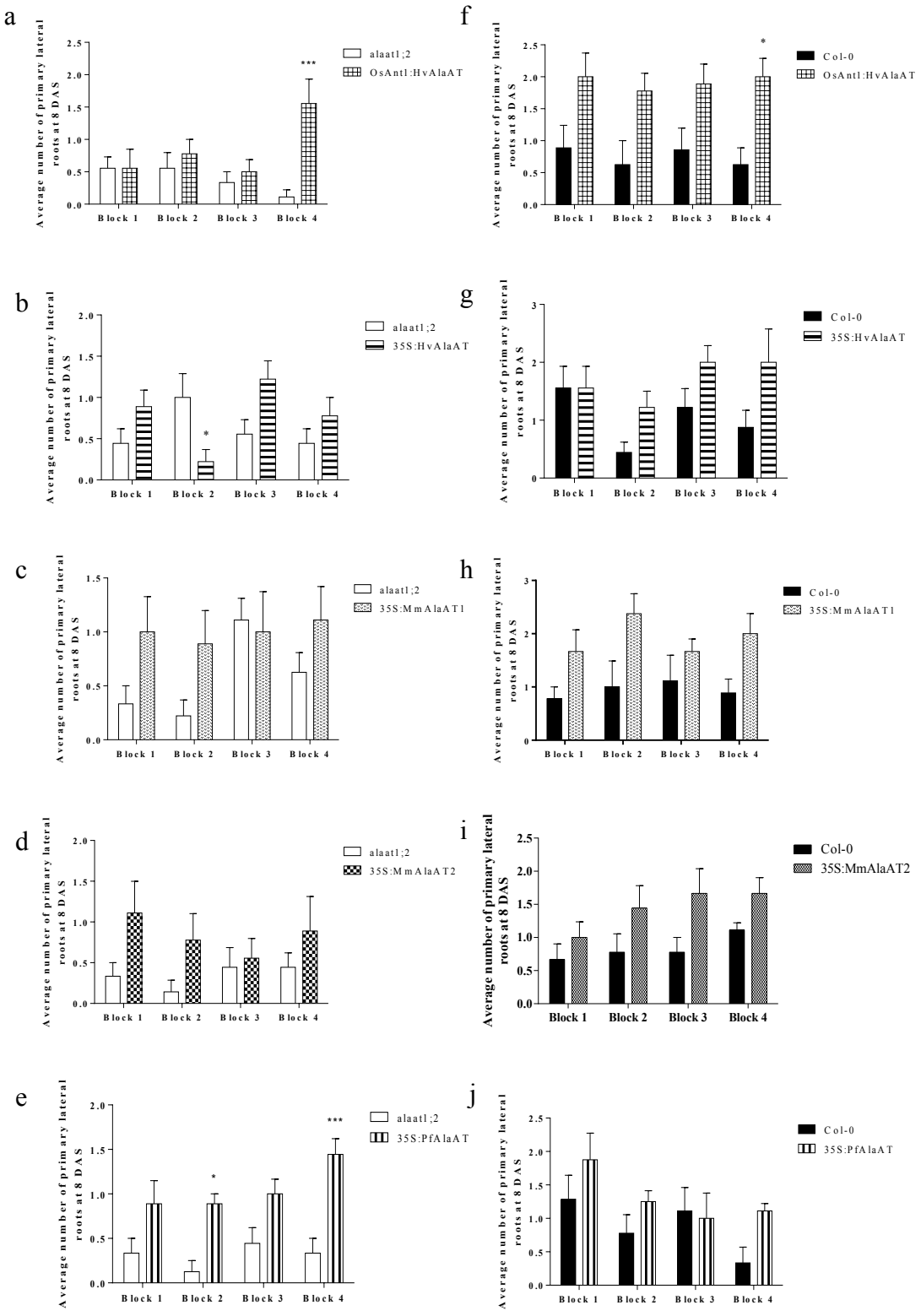

a
Average number of primary lateral roots at 8 DAS
alaat1;2
OsAnt1:HvAlaAT
Block 1
Block 2
Block 3
Block 4
***
b
alaat1;2
35S:HvAlaAT
*
c
alaat1;2
35S:MmAlaAT1
d
alaat1;2
35S:MmAlaAT2
e
alaat1;2
35S:PfAlaAT
*
***
f
Col-0
OsAnt1:HvAlaAT
*
g
Col-0
35S:HvAlaAT
h
Col-0
35S:MmAlaAT1
i
Col-0
35S:MmAlaAT2
j
Col-0
35S:PfAlaAT

Figure 3.16. Average number of primary lateral roots between control and transgenic plants at 8 DAS.

Transgenic plants were sown alongside controls (alaat1;2 or Col-0) on square petri plates containing ½ MS media after a ~48 hr cold induction. Plants were grown vertically in four different lighting blocks for a total of 11 days. At 8 DAS the number of primary lateral roots on individual plants was recorded. The average number of primary lateral roots in each lighting block between controls and AlaAT transgenic lines was compared using two-way ANOVA ($P < 0.05$). a-e) alaat1;2 background, f-j) Col-0 background. n = 6-9 for the number of replicates per genotype per block. * indicates significance in relation to control plants grown in the same lighting block. (Error bars indicate SEM.)

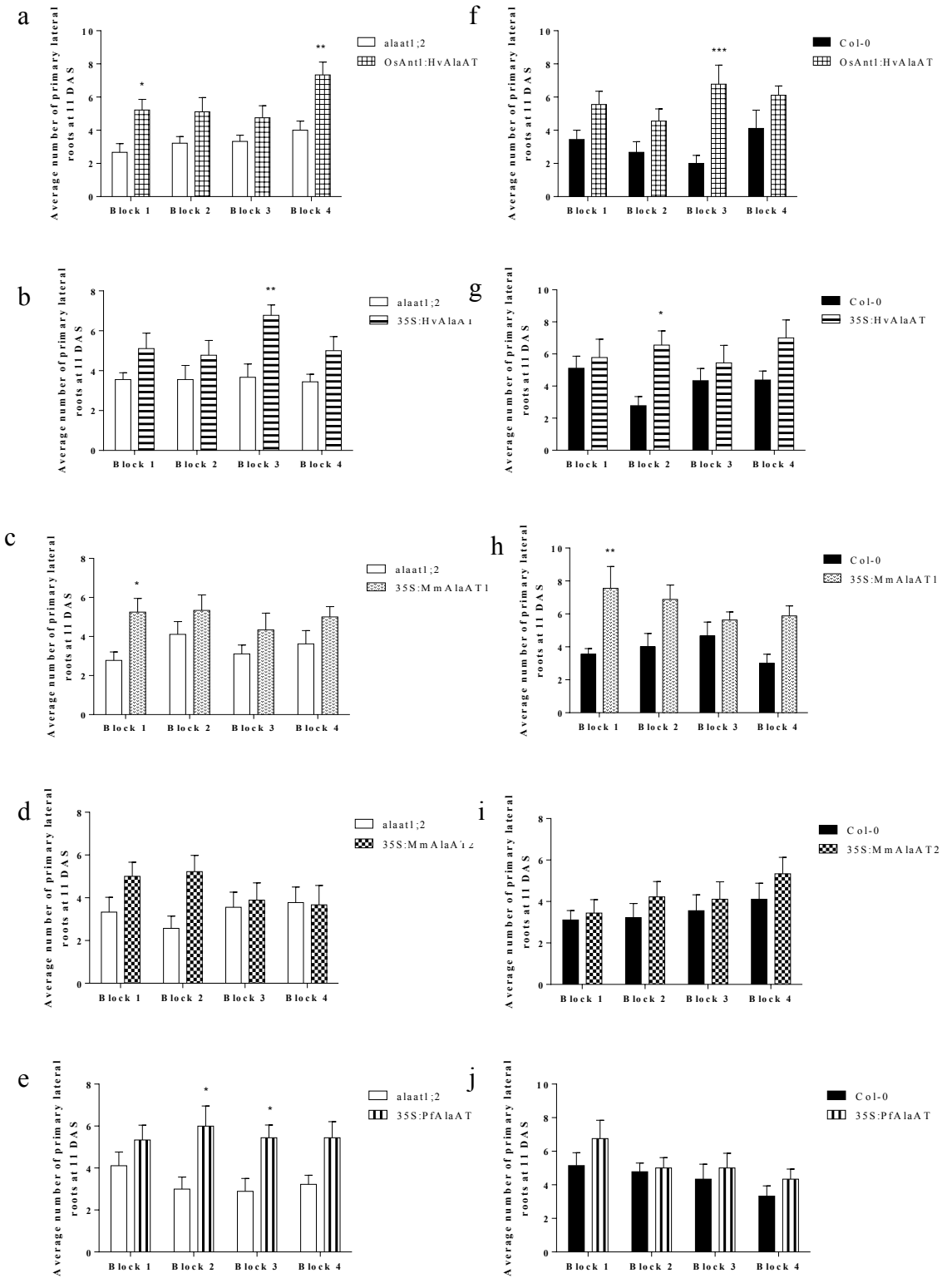

a
Average number of primary lateral roots at 11 DAS
Block 1
Block 2
Block 3
Block 4
alaat1;2
OsAnt1:HvAlaAT
b
alaat1;2
35S:HvAlaAT
c
alaat1;2
35S:MmAlaAT1
d
alaat1;2
35S:MmAlaAT2
e
alaat1;2
35S:PfAlaAT
f
Col-0
OsAnt1:HvAlaAT
g
Col-0
35S:HvAlaAT
h
Col-0
35S:MmAlaAT1
i
Col-0
35S:MmAlaAT2
j
Col-0
35S:PfAlaAT

Figure 3.17. Average number of primary lateral roots between control and transgenic plants at 11 DAS.

Transgenic plants were sown alongside controls (alaat1;2 or Col-0) on square petri plates containing ½ MS media after a ~48 hr cold induction. Plants were grown vertically in four different lighting blocks for a total of 11 days. At 11 DAS the number of primary lateral roots on individual plants was recorded. The average number of primary lateral roots in each lighting block between controls and AlaAT transgenic lines was compared using two-way ANOVA ($P < 0.05$). n = 6-9 for the number of replicates per genotype per block. * indicates significance in relation to control plants grown in the same lighting block. (Error bars indicate SEM.)

3.5 Bibliography

Alonso, J. M., Stepanova, A. N., Leisse, T. J., Kim, C. J., Chen, H., Shinn, P., Stevenson, D. K., Zimmerman, J., Barajas, P., Cheuk, R., Gadrinab, C., Heller, C., Jeske, A., Koesema, E., Meyers, C. C., Parker, H., Prednis, L., Ansari, Y., Choy, N., Deen, H., Geralt, M., Hazari, N., Hom, E., Karnes, M., Mulholland, C., Ndubaku, R., Schmidt, I., Guzman, P., Aguilar-Henonin, L., Schmid, M., Weigel, D., Carter, D. E., Marchand, T., Risseeuw, E., Brogden, D., Zeko, A., Crosby, W. L., Berry, C. C. and Ecker, J. R. (2003) Genome-wide insertional mutagenesis of *Arabidopsis thaliana*. *Science* **301:** 653–657.

Castro Marín, I., Loef, I., Bartetzko, L., Searle, I., Coupland, G., Stitt, M. and Osuna, D. (2011) Nitrate regulates floral induction in Arabidopsis, acting independently of light, gibberellin and autonomous pathways. *Planta* **233:** 539–552.

Chew, Y. H., and Halliday, K. J. (2011) A stress-free walk from Arabidopsis to crops. *Curr. Opin. Biotech.* **22:** 281–286.

Crawford, N. M. and Glass, A. D. (1998) Molecular and physiological aspects of nitrate uptake in plants. *Trends Plant Sci.* **3:** 389–395.

Curtis, M. D. and Grossniklaus, U. (2003) A Gateway cloning vector set for high-throughput functional analysis of genes in planta. *Plant Physiol.* **133:** 462–469.

Drew, M. C. and Saker, L. R. (1978) Nutrient supply and the growth of the seminal root system in barley. *J. Exp. Bot.* **29:** 435–451.

Drew, M. C., Saker, L. R., Ashley, T. W. and Letcombe, C. (1973) Nutrient aupply and the growth of the seminal root system in barley. *J. Exp. Bot.* **24:** 1189-1202.

Filleur, S., Walch-Liu, P. and Gan, Y. F. B. (2005) Nitrate and glutamate sensing by plant roots. *Biochem. Soc. Trans.* **33:** 283–286.

Freixes, S., Thibaud, M.-C., Tardieu, F. and Muller, B. (2002) Root elongation and branching is related to local hexose concentration in *Arabidopsis thaliana* seedlings. *Plant Cell Environ.* **25:** 1357–1366.

Gojon, A., Krouk, G., Perrine-Walker, F. and Laugier, E. (2011) Nitrate transceptor(s) in plants. *J. Exp. Bot.* **62:** 2299–2308.

Good, A. G., Johnson, S. J., De Pauw, M., Carroll, R. T., Savidov, N., Vidmar, J., Lu, Z., Taylor, G., Stroeher, V. (2007) Engineering nitrogen use efficiency with alanine aminotransferase. *Can. J. Bot.* **85:** 252–262.

Good, A. G., Shrawat, A. K. and Muench, D. G. (2004) Can less yield more? Is reducing nutrient input into the environment compatible with maintaining crop production? *Trends Plant Sci.* **9:** 597-605.

Harrison, S. J., Mott, E. K., Parsley, K., Aspinall, S., Gray, J. C. and Cottage, A. (2006) A rapid and robust method of identifying transformed *Arabidopsis thaliana* seedlings following floral dip transformation. *Plant Methods* **2:** 19.

Ho, C.-H., Lin, S.-H., Hu, H.-C. and Tsay, Y.-F. (2009) CHL1 functions as a nitrate sensor in plants. *Cell* **138:** 1184–1194.

Hu, J., Zhang, Y., Wang, J. and Zhou, Y. (2014) Glycerol affects root development through regulation of multiple pathways in Arabidopsis. *PLoS ONE*, **9:** e86269.

Kaiser, B. N., Rawat, S. R., Siddiqi, M. Y., Masle, J. and Glass, A. D. M. (2002) Functional analysis of an Arabidopsis T-DNA " knockout " of the high-affinity NH_4^+ transporter AtAMT1;1. *Plant Physiol*. **130:** 1263–1275.

Kant, S., Bi, Y. and Rothstein, S. J. (2011) Understanding plant response to nitrogen limitation for the improvement of crop nitrogen use efficiency. *J. Exp. Bot.* **62:** 1499–1509.

Keurentjes, J. J. B., Angenent, G. C., Dicke, M., Martins Dos Santos, V. A. P., Molenaar, J., van der Putten, W. H., de Ruiter, P. C., Struik, P. C., and Thomma, B. P. H. J. (2011) Redefining plant systems biology: from cell to ecosystem. *Trends Plant Sci.* **16:** 183–190.

Krapp, A., Berthomé, R., Orsel, M., Mercey-Boutet, S., Yu, A., Castaings, L., Elftieh, S., Major, H., Renou, J-P. and Daniel-Vedele, F. (2011) Arabidopsis roots and shoots show distinct temporal adaptation patterns toward nitrogen starvation. *Plant Physiol.* **157:** 1255–1282.

Krouk, G., Ruffel, S., Gutiérrez, R. A, Gojon, A., Crawford, N. M., Coruzzi, G. M. and Lacombe, B. (2011) A framework integrating plant growth with hormones and nutrients. *Trends Plant Sci.* **16:** 178–182.

Lawlor, D. W. (2002) Carbon and nitrogen assimilation in relation to yield: mechanisms are the key to understanding production systems. *J. Exp. Bot.* **53:** 773–787.

Lejay, L., Tillard, P., Lepetit, M., Olive, F. D., Filleur, S., Daniel-Vedele, F. and Gojon, A. (1999) Molecular and functional regulation of two NO_3^- uptake systems by N- and C-status of Arabidopsis plants. *Plant J.* **18:** 509–519.

Lima, J. E., Kojima, S., Takahashi, H. and von Wirén, N. (2010) Ammonium triggers lateral root branching in Arabidopsis in an AMMONIUM TRANSPORTER1;3-dependent manner. *Plant Cell* **22:** 3621–3633.

Little, D. Y., Rao, H., Oliva, S., Daniel-Vedele, F., Krapp, A. and Malamy, J. E. (2005) The putative high-affinity nitrate transporter NRT2.1 represses lateral root initiation in response to nutritional cues. *P. Natl. Acad. Sci. USA* **102:** 13693–13698.

Lothier, J., Gaufichon, L., Sormani, R., Lemaître, T., Azzopardi, M., Morin, H., Chardon, F., Reisdorf-Cren, M., Avice, J-C. and Masclaux-Daubresse, C. (2011) The cytosolic glutamine synthetase GLN1;2 plays a role in the control of plant growth and ammonium homeostasis in Arabidopsis rosettes when nitrate supply is not limiting. *J. Exp.Bot.* **62:** 1375–1390.

Macgregor, D. R., Deak, K. I., Ingram, P. A. and Malamy, J. E. (2008) Root system architecture in Arabidopsis grown in culture is regulated by sucrose uptake in the aerial tissues. *Plant Cell* **20:** 2643–2660.

Malamy, J. E. (2005) Intrinsic and environmental response pathways that regulate root system architecture. *Plant Cell Environ.* **28:** 67–77.

Malamy, J. E. and Ryan, K. S. (2001) Environmental regulation of lateral root initiation in Arabidopsis. *Plant Physiol.* **127:** 899–909.

Miller, A. J., Fan, X., Shen, Q. and Smith, S. J. (2007) Amino acids and nitrate as signals for the regulation of nitrogen acquisition. *J.Exp. Bot.* **59:** 111–119.

Miyashita, Y. (2008) *Plant amino acid metabolism under hypoxia and carbon starvation*. Ph.D. thesis. Univeristy of Alberta.

Miyashita, Y., Dolferus, R., Ismond, K. P. and Good, A. G. (2007) Alanine aminotransferase catalyses the breakdown of alanine after hypoxia in *Arabidopsis thaliana*. *Plant J.* **49:** 1108–1121.

Nunes-Nesi, A., Fernie, A. R. and Stitt, M. (2010) Metabolic and signaling aspects underpinning the regulation of plant carbon nitrogen interactions. *Mol.Plant* **3:** 973–996.

Okamoto, M., Vidmar, J. J. and Glass, A. D. M. (2003) Regulation of NRT1 and NRT2 gene families of *Arabidopsis thaliana*: responses to nitrate provision. *Plant Cell Physiol.* **44:** 304–317.

Osuna, D., Usadel, B., Morcuende, R., Gibon, Y., Bläsing, O. E., Höhne, M., Günter, M., Kamlage, B., Trethewey, R., Scheible, W.-R. and Stitt, M. (2007) Temporal responses of transcripts, enzyme activities and metabolites after adding sucrose to carbon-deprived Arabidopsis seedlings. *Plant J.* **49:** 463–491.

Remans, T., Nacry, P., Pervent, M., Filleur, S., Diatloff, E., Mounier, E., Tillard, P., Forde, B. G. and Gojon, A. (2006) The Arabidopsis NRT1.1 transporter participates in the

signaling pathway triggering root colonization of nitrate-rich patches. *P. Natl. Acad. Sci. USA* **103:** 19206–19211.

Rensink, W. A. and Buell, C. R. (2004) Arabidopsis to rice. Applying knowledge from a weed to enhance our understanding of a crop species. *Plant Physiol.* **135:** 622–629.

Rolland, F., Baena-Gonzalez, E. and Sheen, J. (2006) Sugar sensing and signaling in plants: conserved and novel mechanisms. *Annu. Rev. Plant Biol.* **57:** 675–709.

Roycewicz, P. and Malamy, J. E. (2012) Dissecting the effects of nitrate, sucrose and osmotic potential on Arabidopsis root and shoot system growth in laboratory assays. *Philos.T. Roy.Soc. B.* **367:** 1489–1500.

Ruffel, S., Krouk, G., Ristova, D., Shasha, D., Birnbaum, K. D. and Coruzzi, G. M. (2011) Nitrogen economics of root foraging: transitive closure of the nitrate – cytokinin relay and distinct systemic signaling for N supply vs. demand. *P. Natl. Acad. Sci. USA* **108:** 18524–18529.

Shrawat, A. K., Carroll, R. T., DePauw, M., Taylor, G. J. and Good, A. G. (2008) Genetic engineering of improved nitrogen use efficiency in rice by the tissue-specific expression of alanine aminotransferase. *Plant Biotech. J.* **6:** 722–732.

Sinclair, T. R. and Rufty, T. W. (2012) Nitrogen and water resources commonly limit crop yield increases, not necessarily plant genetics. *Global Food Sec.* 1: 94–98.

Smith, S. and De Smet, I. (2012) Root system architecture: insights from Arabidopsis and cereal crops. *Philos.T. Roy.Soc. B.* **367:** 1441–1452.

Song, W., Sun, H., Li, J., Gong, X., Huang, S., Zhu, X., Zhang, Y. and Xu, G. (2013) Auxin distribution is differentially affected by nitrate in roots of two rice cultivars differing in responsiveness to nitrogen. *Ann. Bot.* **112:** 1383–1393.

Stitt, M., Müller, C., Matt, P., Gibon, Y., Carillo, P., Morcuende, R., Scheible, W.-R. and Krapp, A. (2002) Steps towards an integrated view of nitrogen metabolism. *J.Exp. Bot.* **53:** 959–970.

Walch-Liu, P. and Forde, B. G. (2008) Nitrate signalling mediated by the NRT1.1 nitrate transporter antagonises L-glutamate-induced changes in root architecture. *Plant J.* **54:** 820–828.

Walch-Liu, P., Liu, L.-H., Remans, T., Tester, M. and Forde, B. G. (2006) Evidence that L-glutamate can act as an exogenous signal to modulate root growth and branching in *Arabidopsis thaliana*. *Plant Cell Physiol.* **47:** 1045–1057.

Wang, R., Liu, D. and Crawford, N. M. (1998) The Arabidopsis CHL1 protein plays a major role in high-affinity. *Proc. Natl. Acad. Sci. USA* **95:**15134–15139.

Zhang, H. and Forde, B. G. (2000) Regulation of Arabidopsis root development by nitrate availability. *J.Exp.Bot.* **51:** 51–59.

Zhang, H., Jennings, A., Barlow, P. W. and Forde, B. G. (1999) Dual pathways for regulation of root branching by nitrate. *P. Natl. Acad. Sci. USA* **96:** 6529–6534.

Zhang, H., Rong, H. and Pilbeam, D. (2007). Signalling mechanisms underlying the morphological responses of the root system to nitrogen in *Arabidopsis thaliana*. *J.Exp. Bot.* **58:** 2329–2338.

Zheng, Z.-L. (2009) Carbon and nitrogen nutrient balance signaling in plants. *Plant Sig.Behav*. **4:** 584–591.

Appendix 3.1. The number of presumed AlaAT insertions in *Arabidopsis thaliana* transgenic lines.

The percentage of hygromycin resistant T_2 seeds (for each line n = 60) from each of the AlaAT transgenic lines was used to estimate the number of AlaAT insertions per line.

AlaAT line	Background	Average percent of transgenic in T_2 seed	Number of insertions estimated
OsAnt1:HvAlaAT 3-1-2	Col-0	81.7	1
OsAnt1:HvAlaAT 1-1-5-3	Col-0	63.3	1
OsAnt1:HvAlaAT 2-2-3-3	Col-0	76.7	1
OsAnt1:HvAlaAT 3-1-1	alaat1;2	93	>1
OsAnt1:HvAlaAT 4-2-2	alaat1;2	76.7	1
OsAnt1:HvAlaAT 2-3-2	alaat1;2	66.7	1
CaMV35S:HvAlaAT 4-3-3	Col-0	65	1
CaMV35S:HvAlaAT 3-2-2	Col-0	81.7	1
CaMV35S:HvAlaAT 4-1-5	Col-0	65	1
CaMV35S:HvAlaAT 5-1-4	alaat1;2	80	1
CaMV35S:HvAlaAT 8-1-2	alaat1;2	68.3	1
CaMV35S:HvAlaAT 6-1-3-2	alaat1;2	70	1
CaMV35S:MmAlaAT1 2-1-7	Col-0	73.3	1
CaMV35S:MmAlaAT1 1-2-1	Col-0	65	1
CaMV35S:MmAlaAT1 3-1-3	Col-0	58.3	1
CaMV35S:MmAlaAT1 3-3-1	alaat1;2	70	1
CaMV35S:MmAlaAT1 3-4-3	alaat1;2	73.3	1
CaMV35S:MmAlaAT1 3-1-5-5	alaat1;2	62	1
CaMV35S:MmAlaAT2 6-3-3-4	Col-0	70	1
CaMV35S:MmAlaAT2 6-1-3	Col-0	70	1
CaMV35S:MmAlaAT2 6-2-1	Col-0	75	1
CaMV35S:MmAlaAT2 7-1-1	alaat1;2	33.7	1
CaMV35S:MmAlaAT2 7-3-3	alaat1;2	68.3	1
CaMV35S:MmAlaAT2 8-1-4	alaat1;2	65	1
CaMV35S:PfAlaAT 3-1-4	Col-0	55	1
CaMV35S:PfAlaAT 4-1-2	Col-0	61.7	1
CaMV35S:PfAlaAT 3-2-2	Col-0	58.3	1
CaMV35S:PfAlaAT 7-1-1	alaat1;2	61.7	1
CaMV35S:PfAlaAT 6-2-2	alaat1;2	68.3	1
CaMV35S:PfAlaAT 6-4-3	alaat1;2	63.3	1

4. Physiological analysis of AlaAT transgenic plants

4.1. Introduction

Plants respond to environment changes in two main ways: 1) varying plant morphology or the repartitioning of biomass and 2) altering physiology (metabolism and gene expression) (Coleman *et al*., 1994). Even within a single species, natural genetic variability exists, resulting in differences in plasticity between ecotypes/cultivars. For example, naturally occurring cultivars of *Arabidopsis thaliana* show large variability in response to N starvation, displaying variation in root growth, shoot growth, starch accumulation and partitioning of N compounds (Ikram *et al*., 2012). These phenotypic differences between naturally occurring ecotypes, as well as those between genetically modified plants and controls, in response to the same external signal can be used to help determine how morphological changes may be linked to alterations in gene expression, specifically through the use of microarrays, mapping and quantitative trait loci (QTLs) (Hirel *et al*., 2001; Obara *et al*., 2001; Limami *et al*., 2002; Yamaya *et al*., 2002; Kraakman *et al*., 2004; Martin *et al*., 2006; Beatty *et al*., 2009; Fontaine *et al*., 2009). Studies such as these have not only identified many genes associated with N metabolism, but have also emphasized just how complex and integrated plant signaling systems are (Coruzzi & Bush, 2001; Coruzzi & Zhou, 2001; Zheng, 2009).

Metabolite and transcriptome analyses on rice plants over-expressing *OsAnt1:HvAlaAT* has been carried out previously (Beatty *et al*., 2009; Beatty *et al*., 2013). Results from these analyses have been broad in nature, unable to pinpoint the exact nature of AlaATs role in producing the NUE phenotype (ie: predominant direction of reaction *in vivo*). Instead of exploring the connection between NUE and *AlaAT* over-expression in rice, Arabidopsis provides a much easier and efficient organism in which to study the physiological changes occurring as a result of the transgenic expression of *AlaAT*. This Chapter will have three main focuses: 1) to verify the expression and activity of AlaAT variants in *Arabidopsis thaliana*, 2) to determine if the growth of transgenic plants on soilless medium produces distinguishable NUE phenotypes, and 3) to investigate how over-expression of the various AlaAT enzymes may affect physiological processes such as carbon metabolism and amino acid uptake *in planta*.

4.2. Materials and Methods

4.2.1. GUS staining

T_2 seed was plated on ½ MS + $Hyg_{(25)}$ and selected using a protocol modified from Harrison *et al*., (2006); plates containing seeds were left at 4°C for four days and room temperature for three days. At eight days after sowing, selected transformants were moved to new ½ MS + $Hyg_{(25)}$ plates, with Col-0 and alaat1;2 plants grown on ½ MS, and all plants grown vertically at 21°C, 60% humidity and a lighting cycle of 16 light/8 dark for 24 days. At the end of 24 days, 1-2 plants from each independent insertion line, Col-0 and alaat1;2 knockout background, were placed in 1 mL of fresh GUS staining buffer (2 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-gluc), 50 mM $NaHPO_4$, pH 7.2, 5 mM ferricyanide, 5 mM ferrocyanide and 0.2% Triton X-100) and placed in darkness at 37°C for 2 hrs. To remove chlorophyll from plant tissues after staining, plants were placed in a series of ethanol solutions for 30 minute time periods, starting at 25% ethanol and increasing to 100% ethanol. Plants were then transferred to a serious of glycerin solutions for 30 min time periods, starting with 95% glycerin and ending with 25% glycerin. Plants were then stored at 4°C until photographed.

4.2.2. qRT-PCR analysis

For all following assays, T_4 or T_5 seed was utilized to decrease transformation effects; seed was obtained from plants grown at the same time and harvested in unison. Plants for qRT-PCR analysis were initially grown on soilless media (under short days) as described above. Shoot and root tissues were harvested separately in 1.5 mL centrifuge tubes at seven weeks after sowing and flash frozen in liquid nitrogen. A Retsch Mixer Mill MM301 was used along with 3 mm tungsten beads (Qiagen) to grind tissues (30 sec). RNA was extracted from 50 mg of tissue using a Qiagen RNeasy Plant Mini Kit (Cat. no. 74904) and a RNase-Free DNase Set (Cat. no. 79254). RNA quantity from these extractions was extremely low along with low A260/A230 ratios (< 2.0), as determined using a NanoDrop (ThermoScientific, NanoDrop 1000) spectrophotometer. For this reason, two other extraction techniques, specifically for tissues with increased concentrations of phenols or polysaccharides, were attempted. An alternative procedure utilizing the Qiagen RNeasy Plant Mini Kit and adapted from McKenzie *et al*., (1997) was tried as well as a phenol/methanol RNA extraction technique described by Oñate-Sánchez and Vicente-Carbajosa (2008). Neither of these procedures increased the quantity of RNA recovered, or increased the low A260/A230 ratios.

Plants for RNA extraction were next grown vertically on plates using ½ MS media. For both controls (Col-0 and alaat1;2) and transgenic lines, three plates with six plants each were grown vertically at 60% humidity, 21°C, ~170 μE m^{-2} sec^{-1} and 16 hrs light/8 hrs dark for two weeks. At the end of two weeks, three biological replicates of three plants each were harvested in 1.5 mL centrifuge tubes and flash frozen in liquid nitrogen. Root and shoot tissues were harvest together. Tissue lysis was performed as above and a Qiagen RNeasy Plant Mini Kit and RNase-Free DNase Set were used for RNA extraction. RNA quantity and quality was assessed via NanoDrop and run on a 1% agarose gel. RNA from plants grown on ½ MS plates was then used for qPCR analysis.

A two-step qRT-PCR protocol was utilized. cDNA synthesis was carried out using both oligo-(dT)s and random hexamers. *Arabidopsis thaliana* Ubiquitin 6 (UBQ6) was used as a control transcript. RevertAid H Minus Reverse Transcriptase (Fisher Scientific, cat. no. EP0451) was used for cDNA production, and SYBR® Green was used to detect PCR product for quantification. Primers for RT-PCR detection were as follows: *HvAlaAT* (5`-TCCTGGCACATGGCACTTC – 3`and 5`- TGACTGCCGGGATCTTATCC – 3`), *PfAlaAT* (5' – TAGAGGTGGGACCGTGGAAGAA – 3' and 5' – AGTGGCCTGCACCATACTCTCC – 3'), *MmAlaAT1* (5' – AAGAAGGTGCTCACGGAGATGG – 3' and 5' – CACTCGCCCATCTAGCCCTTAG – 3'), *MmAlaAT2* (5`-AGGCCAGGTACAAAGCAGAAAG -3` and 5`- TACACCTCATCAGCCAGGAGAA – 3`) and *UBQ6* (5'- GGYCTCACCTACGTTTACCAGA – 3' and 5'-ATCCACAACATCCAAAAACAAC – 3'). The relative expression of AlaAT transgenes was calibrated using the $2(-\Delta\Delta C_t)$ method, with zero expression (the average expression of the transgene in the control background, either Col-0 or alaat1;2) equal to 1.

4.2.3. Bioinformatic and qRT-PCR analysis of genes up-regulated in *AlaAT* over-expressing rice

A previous study (Beatty *et al*., 2009) based on the work by Shrawat *et al*., (2008) specifically identified two transcripts that were more highly up-regulated in NUE rice roots: GRP (glycine-rich cell wall protein, Os03g0115800) and a gene encoding a hypothetical protein (*Os8823*, Os07g0511400). BLASTn analysis (NCBI) was utilized to determine homologues to these rice genes in *Arabidopsis thaliana*. No homologous genes in Arabidopsis were identified

for the *OsGRP* gene, while a glycine-rich protein (*AtGRP*, X58338.1) was identified at an E value of $9e^{-16}$ as the closest homologue in *Arabidopsis thaliana* to the rice hypothetical protein, *Os8823*. RNA extractions previously utilized in the expression analysis of *AlaAT* variants were also used here, and qRT-PCR was carried out as described above for the designated *AtGRP* gene. Primers for RT-PCR detection were; *AtGRP* (5' – CGGAGGAGGTGGAGGACT – 3' and 5' – CCAAAACCGCCACCATGT – 3').

4.2.4. Protein extraction

Transgenic lines were sown on ½ MS plates, six plants per plate, after seed sterilization and ~48 hr cold induction. Plates contained only one genotype, unlike previous assays where control and experimental plants were sown on the same plate. Plates containing plants were left vertical at 21°C, 60% humidity and a light intensity of ~170 μE m^{-2} sec^{-1}, with a light dark cycle of 16 hrs light/8 hrs dark. Plants were harvested between 2-2 ½ weeks. In order to obtain sufficient amounts of material for analysis, four replicates from each line were harvested containing four seedlings each; each sample containing four independent seedlings was treated as a biological replicate. Root and shoot tissue was harvest together. Harvested plants were weighed (four seedlings per biological replicate; Appendix 4.1 and Appendix 4.2) and ground, on ice, with sand and PVPP and extraction buffer (50 mM Tris-HCl pH 8, 5 mM $MgCl_2$, 20 mM cysteine 1 mM DTT and 0.1 mM PMSF) in a ratio of 3:1 extraction buffer to fresh tissue weight.

Ground tissues were stored on ice and then centrifuged at 4°C and 15.7 rcf for 15 min. The supernatant from two biological replicates each was then transferred to an Amicon Filter Concentrator Column (Amicon Ultra-4, Ultracel-10k, Ref. No. UFC801024) and spun in a swinging rotator bucket centrifuge at 2900 g for ~50 min, or until the protein-containing supernatant fraction was less than 200 μl. The protein-containing samples (two per line in most cases) were removed immediately after centrifugation using a pipette and stored on ice for use in AlaAT activity assays. The two protein concentrates obtained for each line, were used for both activity assays and Western blots and were considered individual biological replicates for these experiments.

4.2.5. AlaAT activity assays

For AlaAT activity assays, concentrated protein was diluted 1/5 for all samples. AlaAT activity was assayed using a continuous coupled reaction catalyzed by AlaAT and lactate dehydrogenase (LDH, Sigma, L-2518) respectively, with the change in absorbance associated with generation of NAD^+ from NADH monitored at 340 nm. Assays were carried out in biological and experimental triplicate at room temperature in a 96 well plate (Corning, Cat. No. 0720092) over a five minute time period with readings every 11 sec, and were read using a SpectraMax Plus absorbance plate reader (Molecular Devices, Sunnyvale, CA). Control plants, either Col-0 or alaat1;2, were also assayed for AlaAT activity and used as a standard of comparison for all transgenic lines. A Mann-Whitney *U*-test ($P < 0.05$) was used to analyze differences in activity between transgenic and control plants (n = 3-6).

4.2.6. Western blot analysis

Proteins extracts obtained as outlined above were quantified using Bradford assays (Bio-Rad reagent). 10 µl of the total protein extract was loaded onto Mini-PROTEAN Precast Gels (Bio-Rad, cat. no. 456-8125) for protein separation using a Mini-Protein II Electrophoresis Cell (Bio-Rad) and proteins were then transferred to a nitrocellulose membrane using a Mini Trans-blot Cell (Bio-Rad) according to the manufacturer's specifications. Membrane blocking was done using Western Blocking Reagent Solution (Roche Applied Sciences, product no. 11921681001). HvAlaAT proteins were detected using an HvAlaAT specific primary antibody (Good and Muench, 1992; Muench and Good, 1994); all other AlaAT enzymes (PfAlaAT, MmAlaAT1 and MmAlaAT2) were detected using a C-myc tag specific primary antibody (Abcam, ab9106) (specific to the C-myc tag attached to the C-terminal end of these AlaAT enzymes; Chapter 3). Antibody detection was performed using an Amersham ECL Western detection kit (GE Healthcare Life Sciences, product no. RPN2232). An *E. coli* whole cell lysate expressing a recombinant protein containing a C-myc epitope tag was used as a positive control (Abcam, ab5395).

4.2.7. Soilless media

Seeds from both control lines, Col-0 and alaat1;2, as well as three independent insertion lines each of *OsAnt1:HvAlaAT*, *CaMV35S:PfAlaAT* and *CaMV35S:MmAlaAT1*, in both Col-0 and alaat1;2 knockout backgrounds, were used for this analysis. Sterilized seeds were stratified

in 0.15% (w/v) agar for ~48 hrs. After stratification, 3-4 seeds from each transgenic and control line were transferred to 3" deep, separated plastic cells containing 150 mL of sterile sand and fine vermiculite (1:1). The bottom of each cell contained ~2 cm of potting mix to inhibit run off of the sand/vermiculite mixture into the tray. Eighteen cells were allowed per tray and all lines were grown in triplicate (if not more), with control plants grown in each tray. Plants were grown at 21°C, 60% humidity and a light cycle of either16 hrs light/8 hrs dark or 12 hrs light/12 hrs dark (short days). Trays were blocked for variations in lighting conditions, with two lighting blocks per tray, with final lighting blocks containing a maximum difference of 20%, with an average light intensity of ~120 μE m^{-2} sec^{-1}. Plants were fertilized with a modified Hoagland's solution (adapted from Piccini & Azcon, 1987; Li, 2011) once a week by a bottom fed method and watered one additional time per week for the lifecycle of the plants (50 mL per plant). After cotyledon emergence, plants in individual cells were thinned to leave a single plant. 400μL of 1% Helix was added to the top of each pot at this time. Changes in phenotype and development between transgenic lines and controls were monitored (ie: rosette leaves, time to bolting and time to first flower).

4.2.8. Soluble sugars analysis

Plants were sterilized, cold induced for ~48 hrs and sown onto soilless medium (sand:vermiculite, 1:1) as described above (21°C, 60% humidity, ~120 μE m^{-2} sec^{-1} light intensity and a lighting cycle of 16 hrs light/8 hrs dark). Only those AlaAT lines in the Col-0 background were utilized for this analysis, including: *OsAnt1:HvAlaAT*, *35S:HvAlaAT*, *35S:MmAlaAT1* and *35S:PfAlaAT*. Both Col-0 and alaat1;2 were grown as control lines. Five plants from each independent insertion line were grown for 41 days (~6 wks) (maintained and fertilized with Hoagland's as previously outlined) at which point all rosette leaves >1 cm were harvested from a single plant (both control and experimental lines), weighed and flash frozen in liquid nitrogen. Samples were collected in triplicate from each of the three independent insertion lines for each AlaAT of interest as well as the controls. Samples were stored at -80°C. Plants were harvested approximately 5 days after the first plants bolted. The ratio of plants that had bolted as well as the ratio of plants that had flowered per line was also observed (Appendix 4.1). Pictures of each line were taken at the time of harvest. Adobe Photoshop C.S.6. Extended was used to remove all background from photos, leaving only shoots, and WinRHIZO Arabidopsis

2013d software was then used to measure the total leave area (green area) of each of the transgenic lines (n = 5). Differences in shoot area (cm^2) between transgenics and Col-0 were determined using a Mann-Whitney *U*-test ($P < 0.05$).

Soluble sugars were extracted from frozen tissues using a modified methanol/chloroform extraction protocol for non-lyophilized cells described by Gromova and Roby (2010). Frozen tissues were ground using a Retsch Mixer Mill MM301 along with 3 mm tungsten beads (Qiagen cat. no. 69997). 50 mg ground frozen tissue from each sample was used for the metabolite extraction. An additional 2 ml of ddH_2O was added to each extraction to increase polarity of the aqueous phase and overall separation. The recovered polar phase from each extraction was then left in an oven at 80°C to evaporate and the residue was resuspended in 0.5 ml ddH_2O.

The polar phase of each extraction was used to assay for the presence of three soluble sugars: glucose, fructose and sucrose. A Glucose (HK) Assay Kit (Sigma, GAHK-20), Fructose Assay Kit (Sigma, FA-20) and Sucrose Assay Kit (Sigma, SCA-20) were utilized for the indirect quantification of all three sugars in 100 µl of the resuspended sugar solution. All sugars were indirectly detected via multiple enzyme reactions (as outlined by the manufacturer) ultimately producing NADH in direct proportion with the sugar of interest. A spectrophotometer (SpectraMax Plus absorbance plate reader, Molecular Devices, Sunnyvale, CA) was used to measure the final concentration of NADH in solution (A_{340}); increases or decreases in absorbance at this wavelength in comparison to Col-0 were analyzed. The majority of lines were analyzed in biological triplicate, with a few lines analyzed in duplicate. A Kruskal-Wallis one-way ANOVA ($P < 0.05$) was used to determine the significance of genotype in the resulting concentration of soluble sugars, with a Tukey's post-test used to determine significant differences between individual lines and Col-0 ($P < 0.05$). When lines containing the same construct were grouped, a Mann-Whitney *U*-test was used to compare all lines to Col-0 ($P < 0.05$).

4.2.9. Mesophyll cell protoplast preparation

Plants for protoplast isolation were cold-induced and grown in soilless medium for five weeks. The method of plant growth in soilless medium was as outlined above with the exception of day length. Plants were grown in a shortened photoperiod, with 12 hrs light/12 hrs dark. A

maximum of six lines could be analyzed for radioisotope uptake at a time. For this reason, 5-6 lines were grown at a time for a single protoplast uptake experiment, with Col-0 being grown and analyzed as a control every run. Independent Arabidopsis lines harboring the same AlaAT insert were analyzed in the same experimental run as well as across experimental runs to help control for experimental error as well. Prior to harvest, pictures of some lines were taken. Total leaf area of all transgenic and Col-0 plants was determined using Adobe Photoshop C.S.6. Extended and WinRHIZO Arabidopsis 2013d software as described above. Differences in shoot area (cm^2) between transgenics and Col-0 were determined using a Mann-Whitney *U*-test ($P < 0.05$).

Protoplast isolation was slightly modified from the protocol described previously by Yoo *et al*., (2007). Approximately 40-50 rosette leaves from ~18 individual plants per transgenic line were used to obtain tissue for protoplast preparation. Three separate transgenic lines per AlaAT insert were analyzed. Rosette leaves that appeared discoloured or senescing were not used. Individual leaves were cut into 0.5-1 mm strips using a fresh sharp razor blade and immediately dipped into 20 ml enzyme solution. Enzyme solution was prepared using 20 mM MES, 0.4 M mannitol, 20 mM KCl dissolved at 65°C for 5 min using a water bath. 0.4% macerozyme (PhytoTechnology Laboratories, cat. ID. M481) and 1.5% cellulase (PhytoTechnology Laboratories, cat. ID. C224) were added to enzyme solution immediately following 65°C water bath and dissolved by stirring. Solution was allowed to sit for 10 min at 55°C and then cooled to room temperature. Once at room temperature, 10 mM $CaCl_2$ was added and solution was filtered into sterile petri dishes, 20 ml per petri dish, using a 60 ml syringe and 0.45 µM PVDF filter (Millipore, cat. no. SLHV033NS).

Petri dishes containing enzyme solution and cut leaves were vacuum infiltrated without lids at room temperature in the dark for 30 min using a desiccator. After 30 min, leaf-containing petri dishes were removed from the desiccator, lids were applied and digestion of leaf tissues was allowed to proceed in the dark at room temperature, without shaking, for another 3 hrs. Release of protoplasts from leaf tissues was done by swirling of petri dishes at the end of the 3 hr time period. The enzyme-protoplast solution was then diluted with 20 ml W5 solution (2 mM MES, 154 mM NaCl, 125 mM $CaCl_2$ and 5 mM KCl), and filtered into sterile 50 mL tubes using a 70 µM nylon mesh (Fisher Scientific, Cat. no. 22-363-548). Cells were spun in a rotating-bucket

centrifuge for 2 min at 116 g, resuspended and washed in 15 ml W5 solution. After washing the cells, supernatant was removed leaving a final volume of 0.5-1 ml in which to resuspend the protoplasts. Cells were counted using a hemocytometer and all lines were diluted to 4 x 10^5 cells ml^{-1} using W5 solution.

Protoplast dilutions were left on ice for 20-30 min and then centrifuged for 2 min at 116 g, supernatant was removed and cells were resuspended in MMG solution (4 mM MES, 0.4 M mannitol and 15 mM $MgCl_2$) to a final concentration of 4 x 10^5 cells ml^{-1}.

4.2.10. Protoplast uptake of ^{3}H-leucine and ^{14}C-alanine

Protoplasts were diluted to a final concentration of 2 X 10^5 cells ml^{-1} by the addition of the radio isotopes in MMG solution. Protoplasts were labelled with a combination of both ^{14}C-alanine (Alanine, L-[14C(U)], Perkin Elmer, Product no. 3570167) and ^{3}H-leucine (Leucine, L-[3,4,5-3H(N)], Perkin Elmer, Product no. 665553). ^{14}C-alanine was added at 0.5 µCi ml^{-1} and ^{3}H-leucine was added at 4.4 µCi ml^{-1}. Zero time points were taken for solutions containing protoplasts without radiolabel as well as solutions containing only radiolabel.

Three separate experimental replicates of 1 ml protoplast/isotope label solution were analyzed per transgenic line per time point. Protoplast uptake of both ^{14}C-alanine and ^{3}H-leucine was analyzed at 10 min, 45 min, and 2 hrs. Between time points, protoplasts were left at room temperature under a desk lamp, and were gently inverted every 10 min. At the end of each time point, the solution was centrifuged for 1.5 min at 187 g and 50 µl of supernatant removed and placed in a scintillation vial (Fisher Scientific, Cat. no. 03-337-15) for analysis in the liquid scintillation counter. Remaining excess supernatant was then removed, and protoplasts were resuspended in 1 ml of MMG solution. This solution was spun down for 1.5 min at 187 g and supernatant removed. This wash step was repeated a total of three times. At the end of the third wash, supernatant was removed, leaving only ~50 µl, which was then used to resuspend protoplasts. All protoplasts in solution were then transferred to a scintillation vial. Once all time points had been completed and all samples were in scintillation vials, 1 ml of bleach was added to each vial. Samples were then left gently swirling overnight at room temperature in the dark.

The next day, samples were removed from the shaker and placed in a fumehood, lids removed, for >3 hrs. After 3 hrs, 5 ml hionic fluor (Perkin Elmer, 6013311) was added to each

scintillation vial, and lids were replaced. Samples were then left undisturbed, closed and covered, in the scintillation counter for 2 ½ days. After 2 ½ days, samples were counted for emissions from both ^{3}H and ^{14}C using a liquid scintillation counter, recorded in decays per minute (dpm). A two-way ANOVA and Bonferroni post-tests were used to compare dpm counts between transgenic and control lines (n = 3), taking into account both differences in genotype and time elapsed since addition of the radiolabel.

4.2.11. Physical phenotyping of Arabidopsis plants grown on soilless medium

AlaAT over-expressing *Arabidopsis thaliana* lines (with the omission of MmAlaAT2) along with Col-0 were grown in soilless medium (sand:vermiculite, 1:1, as described above) fertilized with modified Hoagland's solution (2 mM KNO_3) under short days (12 hrs light/12 hrs dark) up until the emergence of the first flower (white of petals showing from bud). Temperature, humidity and light intensity were not altered from those described earlier for growth of plants in soilless medium. Plants were harvested at 55 DAS, regardless of whether first flower had emerged or not; first flower for plants that had not flowered yet was then reported as 55 DAS. At 55 DAS all plants had bolted. Two separate grow-ups were conducted and the results pooled; data was pooled between grow-ups and between independent insertion lines carrying the same construct. A one-way ANOVA (non-repeated measures) was used to analyze the effect of genotype on each of the observed phenotypes and a Tukey's post-test was used compare genotypes ($P < 0.05$, n = 10-30).

4.3. Results

4.3.1. GUS expression in *Arabidopsis thaliana* using a rice *OsAnt1* promoter

Expression of ß-glucuronidase (GUS) using an *OsAnt1* promoter has previously shown to be tissue-specific in rice (Shrawat *et al*., 2008). To examine the expression of this promoter in *Arabidopsis thaliana*, *OsAnt1* driving *GUS* was transformed into Arabidopsis plants, both Col-0 and alaat1;2 knockout backgrounds, and analyzed for tissue-specific expression. Three independent insertion lines, in both backgrounds, were generated and analyzed. After two hours of staining, GUS expression could be observed throughout the vasculature of the plants and the root tips (Figure 4.1). Plants also saw increased staining in cotyledons compared to rosette

leaves (Figure 4.1). No GUS expression was detected in control plants. Phenotypic results were consistent between independent insertion lines for both backgrounds (data not shown). When *OsAnt1:GUS* was previously analyzed in 7-day-old rice plants GUS expression was detected in the primary root, lateral roots and root hairs, with faint GUS staining in leaf vascular tissues (Shrawat *et al*., 2008). Here we observed similar expression patterns of the GUS protein in Arabidopsis when driven by *OsAnt1* as previously observed in rice.

4.3.2. AlaAT transgenics show expression of the transgene in *Arabidopsis thaliana*

Although the transgenic AlaAT Arabidopsis lines had previously been analyzed for insertion of the transgene in both the Col-0 and alaat1;2 backgrounds by PCR (Chapter 3), it was important to confirm the expression of the genes within the plants. qRT-PCR was used to determine the relative transcription of each transgenic AlaAT homologue in relation to both control backgrounds (Col-0 and alaat1;2). The ubiquitin-40S ribosomal gene (*UBQ6*, AT2G47110) was used as a control transcript in all cases, as this gene is expressed throughout the entire plant, from seedling until maturation (Callis *et al*., 1990). The relative expression of AlaAT transgenes was calibrated using the $2(-\Delta\Delta C_t)$ method.

Figures 4.2a and 4.2b depict the relative quantification of the various AlaAT transcripts in the Col-0 and alaat1;2 backgrounds, respectively. These results indicate that out of 30 transgenic lines, only one does not appear to be expressing the inserted AlaAT: 35S:MmAlaAT1 line 3-3-1. Large variations in expression level were observed between plant lines possessing the same transgene however, this variation was consistent among all AlaAT-variant lines in both backgrounds. No difference in the relative expression level of *UBQ6* was observed between control and transgenic plants (data not shown). Ultimately, the expression of AlaAT variants from barley, mouse and Pyrococcus, driven by two different promoters within *Arabidopsis thaliana*, was verified.

4.3.3. NUE-transcriptome of AlaAT-expressing plants shows differences between rice and Arabidopsis

Beatty *et al*., (2009) had previously shown differences in the transcriptome of NUE rice over-expressing *HvAlaAT* by using an Affymetrix Rice GeneChip microarray. This study specifically identified two transcripts that were more highly up-regulated in NUE rice roots:

GRP (*glycine-rich cell wall protein*, Os03g0115800) and a gene encoding a hypothetical protein (*Os8823*, Os07g0511400). BLASTn analysis (NCBI) to determine homologues of these genes in *Arabidopsis thaliana* showed that *OsGRP* does not have any homologues in Arabidopsis. In fact, *OsGRP* appears to be a rice specific gene, with no meaningful BLASTn hits in any species other than *Oryza sativa* (rice). *Os8823* appears to be most closely related to a gene encoding for a GRP protein (glycine-rich cell wall protein, X58338.1, AT3G17050) in Arabidopsis (*AtGRP*).

AtGRP encodes for a 339 amino acid protein, with one copy of this particular gene present in the genome of Arabidopsis (Quigley *et al*., 1991). qRT-PCR analysis of *AtGRP* using RNA from both control (Col-0 and alaat1;2) and AlaAT-transgenic lines resulted in non-specific binding of the primers, and the quantification of numerous gene products. BLASTn using the *AtGRP* sequence showed 100% identity to other sequences in *Arabidopsis thaliana*, including a putative pectinesterase mRNA (E value 0.0, NM_112582, AT3G17060.1) and high chlorophyll fluorescent 107 mRNA (E value $4e^{-86}$, NM_001202985, AT3G17040.1.), which explains why more than one gene product was amplified during qRT-PCR. However, due to the inability to accurately amplify one transcript, reliable results were not produced from this analysis. Interestingly, all of the identified genes are located in the same area of the genome on chromosome 3.

4.3.4. Transgenic AlaAT-expressing and over-expressing plants show increased AlaAT enzyme activity

Whole protein extracts from all of the *AlaAT*-expressing and over-expressing lines were analyzed in biological and experimental triplicates for AlaAT activity. AlaAT activity was assayed using alanine and 2-oxoglutarate as substrates via a coupled reaction catalyzed by AlaAT and lactate dehydrogenase (LDH, Sigma, L-2518) respectively, associated with the generation of NAD^+ from NADH by monitored at 340 nm. For each insertion line, the average activity of three biological replicates was grouped to give the AlaAT activity represented in Figures 4.3 and 4.4.

AlaAT activity of transgenic plants in the alaat1;2 background is shown in Figure 4.3; significance in relation to alaat1;2 is indicated and was determined using a two-tailed Mann-Whitney *U*-test ($P < 0.05$) (Appendix 4.3). In each case, alaat1;2 control plants showed

significantly lower AlaAT activity levels (Figure 4.3a-b, $P < 0.05$; Figure 4.3c-e, $P < 0.01$) than Col-0 control plants, as observed previously by Miyashita *et al*., (2007). All transgenic lines (alaat1;2 background) showed increased AlaAT activity compared to the knockout line (which had a measured activity close to zero), with nearly all of the transgenic lines showing significant increases in activity compared to the alaat1;2 line. Only two lines did not show significant increases in activity: OsAnt1:HvAlaAT 2-3-2 and 35S:HvAlaAT 6-1-3-2. The majority of transgenic lines in the alaat1;2 background, while showing higher activity than the knockout line, still had activity levels significantly below that of the Col-0 control. Only lines 35S:HvAlaAT 5-1-4, 35S:MmAlaAT1 3-4-3 and 35S:PfAlaAT 6-2-2 showed AlaAT activity levels similar to those measured in Col-0 plants.

Figure 4.4 shows the average AlaAT activity of all over-expressing lines (Col-0 background) in comparison to both Col-0 and alaat1;2. Significance in relation to both Col-0 and alaat1;2 is indicated and was determined using a two-tailed Mann-Whitney *U*-test ($P < 0.05$) (Appendix 4.4). All *AlaAT* over-expressing lines showed AlaAT enzyme activity similar to or greater than that measured from the Col-0 sample. Lines which showed significant increases in enzyme activity included: OsAnt1:HvAlaAT 1-1-5-3 and 3-1-2, 35S:MmAlaAT1 3-1-3, 2-1-7 and 1-2-1, 35S:MmAlaAT2 6-3-3-4 and 6-2-1 and 35S:PfAlaAT 4-1-2 and 3-2-2. All three *35S:MmAlaAT1*-expressing lines showed significant increases in AlaAT activity when compared to Col-0; this was the only insert showing significant increases in activity in all three independent insertion lines. None of the lines over-expressing 35S:*HvAlaAT* showed significant increases in activity. All of the over-expressing lines showed significant increases in activity when compared to the alaat1;2 knockout line (Figure 4.4).

4.3.5. AlaAT protein is predicted to be expressed in *Arabidopsis thaliana* seedlings

Western blot analysis using HvAlaAT-specific antibodies as well as C-myc-tag-specific antibodies was attempted to validate expression of the various AlaAT proteins in Arabidopsis. Unfortunately, results from these analyzes were highly varied, with detection of the AlaAT proteins seen in some lines but not others. Consistent results among lines carrying the same construct were not attained (ie: protein was detectable on western blots from the line OsAnt1:HvAlaAT 1-1-5-3 but not from OsAnt1:HvAlaAT 2-2-3-3) . Furthermore, lines

containing the C-myc tag at the C-terminus, 35S:MmAlaAT1, 35S:MmAlaAT2 and 35S:PfAlaAT (in either background), when probed with the appropriate antibody, did not always detect protein of the expected size, ~52 kD. Protein detected in these samples, while appearing in the transgenics and not in the controls (Col-0 and alaat1;2), was often much smaller in size than expected, < 25 kD (data not shown). A possible explanation for this observation is that the tag was removed *in vivo*; however, more experiments are required to confirm this. Membranes, when exposed to Amido Black Stain after exposure, showed strong bands in lanes containing protein from transgenic lines at ~52 kD that were not observed in the control lines (data not shown), indicating that there may be high levels of AlaAT protein in these lines relative to controls, but not confirming this. Therefore, for reasons of inconsistency and ambiguity, results from western blot analysis have been omitted from this thesis but are currently being repeated for publication.

4.3.6. Leaf area and fresh weight of rosette leaves of AlaAT over-expressing plants show increases when compared to Col-0

In order to better understand some of the physical phenotypes previously observed when *AlaAT*-expressing and over-expressing plants were grown on plates (Chapter 3), plants over-expressing *AlaAT* to be used for soluble sugar analysis were assessed at harvest for differences in growth and development. Two main physical features of the plants were observed at this time: the total leaf area at time of harvest and the fresh weight of rosette leaves > 1cm at harvest (Figure 4.5).

Leaf area of plants was determined using a combination of Adobe Photoshop C.S.6. Extended software and WhinRHIZO Arabidopsis 2013d software. The average rosette leaf area of five biological replicates for each independent insertion line is presented in Figure 4.5a. Of all *AlaAT* over-expressing lines analyzed, only one line, 35S:MmAlaAT1 3-1-3, showed significant increases in leaf area relative to Col-0, determined by a Mann-Whitney *U*-test ($P < 0.05$, n = 5). However, of the 13 lines measured against Col-0, 12 (including alaat1;2) showed average increases in overall leaf area; the only line that did not show increases in rosette leaf area was 35S:MmAlaAT 1-2-1. When the data from all lines expressing the same construct was grouped (Figure 4.5a inset), it was shown that overall, those lines constitutively over-expressing *HvAlaAT* had the greatest increase in leaf area when compared to Col-0.

When fresh weight of rosette leaves was analyzed (Figure 4.5b), the graph closely mimicked that of leaf area, which is to be expected as these two traits are not mutually exclusive. A Mann-Whitney *U*-test ($P < 0.05$, n = 3) was again used to compare all lines (including alaat1;2) to Col-0, however none of the lines showed significant differences in this trait. Even so, for the most part, as with leaf area, transgenics and alaat1;2 did show overall average increases in fresh weight relative to Col-0, with only two lines showing decreases in this number: OsAnt1:HvAlaAT 3-1-2 and 35S:MmAlaAT1 1-2-1. Interestingly, while lines containing *35S:HvAlaAT* showed the greatest increase in leaf area, these plants did not have the greatest average fresh weight. When data from lines containing the same insert was combined (Figure 4.5b inset), the alaat1;2 knockout line appeared to have the highest average fresh weight of rosette leaves, followed closely by those plants containing *35S:HvAlaAT*.

4.3.7. Plants over-expressing *PfAlaAT* show increased rate of flowering under regular growth conditions

Plants for soluble sugar analysis were grown on soilless medium under regular conditions (21°C and 60% humidity, 16 hrs light/8 hrs dark and ~120 μE m^{-2} sec^{-1}) and fertilized with a modified Hoagland's solution (2 mM KNO_3) once per week. Plants were harvested at 41 DAS, approximately five days after the first plants bolted. The percent of plants in each line that had bolted as well as flowered (n = 5 maximum) was recorded (Figure 4.6). All lines, including Col-0 and alaat1;2 appeared to show no obvious alterations in time to bolting (Figure 4.6a,c), however differences in the rate of flowering at this time were observed (Figure 4.6b,d). This observation is most apparent when lines containing the same construct are grouped (Figure 4.6d) and all transgenic lines show an increased average percentage of plants that had flowered at 41 DAS compared to both Col-0 and alaat1;2. Plants over-expressing *PfAlaAT* showed the highest rate of flowering at this time, while also showing the lowest deviation around the mean when lines were grouped for this trait. Statistical analysis was not carried out on this data because it was collected as a percentage with only one value for each independent line.

4.3.8. *AlaAT* over-expressing plants show decreases in concentration of soluble sugars in the shoot

In order to better understand the growth phenotypes observed in Chapter 3 when C and N conditions were varied (Figure 3.14 and Figure 3.15), an analysis of the soluble sugars (glucose, fructose and sucrose) was carried out. These three sugars were chosen above others for a variety of reasons. Firstly, sucrose is the main transport form of C in plants. As sucrose, C-units are transported via the phloem to sink tissues where they serve as skeletons for the production of organic N compounds, including amino acids (Calenge *et al*., 2006; Hammond & White, 2008). Secondly, sucrose feeds into glycolysis, producing both glucose and fructose which are direct metabolites of the glycolytic pathway (Hammond & White, 2008; Plaxton, 2010).

100 µl of resuspended extract from a methanol/chloroform extraction of 50 mg of rosette tissue from each plant (carried out in biological triplicate or duplicate) was used for the analysis of all three soluble sugars (Figure 4.7). A Kruskal-Wallis one-way ANOVA ($P < 0.05$) determined that genotype did not significantly contribute to the concentration of any of the soluble sugars in independent lines. A Tukey's post-test determined that none of the concentrations of soluble sugars in transgenic lines or alaat1;2 significantly differed from that of Col-0 ($P < 0.05$). However, when the results of each analysis were pooled according to transgenic lines harboring the same construct (Figure 4.7a-c, insets) a distinct pattern was observed. In the case of each soluble sugar, transgenic plants showed decreased average concentrations relative to Col-0. In only one case were these decreases shown to be significant (using a Mann-Whitney *U*-test ($P < 0.05$)): decreases in sucrose concentration in plants expressing *OsAnt1:HvAlaAT*. alaat1;2 plants showed the lowest average concentration of all three soluble sugars.

Interestingly, when the results of this analysis are compared with the leaf area and fresh weight data obtained from the same plants (Figure 4.5) an inverse relationship is observed (with the exception of alaat1;2 leaf area, which was shown on average to be less than that of transgenic lines). While Col-0 plants showed decreased leaf area and fresh weight relative to transgenic lines (Figure 4.6, insets), they also showed increased soluble sugar concentrations (glucose, fructose and sucrose) compared to transgenics (Figure 4.7, insets). Also, all transgenic lines used for this analysis were shown to have an increase in the percentage of plants that had flowered at 41 DAS, compared to Col-0 plants (Figure 4.6b). Taken together, the data show that in a soilless medium under regular growth conditions and an adequate N supply, the over-expression of

AlaAT, regardless of the homologue or promoter, consistently alters several measurable traits in *Arabidopsis thaliana*, relative to control (Col-0) plants.

4.3.9. *PfAlaAT* over-expressing cells take-up external leucine and alanine more effectively than control plants or other AlaAT over-expressing cells

Tritiated leucine (^{3}H-leucine) and ^{14}C-alanine were used to analyze the uptake of amino acids from the external environment of *AlaAT* over-expressing mesophyll protoplasts (Figure 4.8 and Figure 4.9, respectively). Alanine was chosen because it is a direct substrate of AlaAT; leucine was chosen because it is a relatively small, neutral, non-aromatic amino acid with a hydrophobic side chain, similar to alanine. Both amino acids are commonly stored in plant vacuoles and an increase in internal concentrations of these compounds is non-toxic in the Col-0 background (Dietz *et al*., 1990), with the same neutral amino acid transporters in the roots and leaves shuttling both of these compounds across the plasma membrane (Fischer *et al*., 1998).

The uptake of each radiolabel was assessed in triplicate at three different time points, 10 min, 45 min and 2 hrs, for each independent insertion line (Figure 4.8 and Figure 4.9). Col-0 and alaat1;2 mesophyll protoplasts were utilized as controls and a two-way ANOVA ($P < 0.05$) was used to determine if genotype or time had a significant impact on protoplast uptake of either radiolabel. Overall, it was determined that both genotype and elapsed time had a significant role in the measured results for both radiolabels ($P < 0.0001$). The interaction between these two variables was also shown to be highly significant ($P < 0.0001$) for both radioisotopes. A Bonferroni post-test was used to compare the uptake of both radiolabels in all transgenic *AlaAT* over-expressing lines with Col-0. The alaat1;2 knockout line was also compared to Col-0 using this method. Described here are the results from the protoplasts specifically, however it should be noted that the amount of label (both ^{3}H and ^{14}C) left in the supernatant from this experiment confirmed the trends observed for both radiolabels for each genotype and time point.

At the 10 min time point, neither radiolabel showed any significant differences in uptake between any of the lines analyzed (Figure 4.8a and Figure 4.9a). However, although not significantly different, it is interesting to note that at the 10 min time point the alaat1;2 knockout line showed the highest levels of both ^{3}H-leucine and ^{14}C-alanine in the protoplasts. Forty-five minutes after addition of the label to the protoplasts, significant differences in uptake of both

leucine and alanine were observed (Figure 4.8b and Figure 4.9b). Significant decreases in uptake of both amino acids were seen in two lines, OsAnt1:HvAlaAT 2-2-3-3 ($P < 0.01$, both radiolabels) and 35S:HvAlaAT 3-2-2 ($P < 0.05$, both radiolabels). The other lines carrying each of these inserts (OsAnt1:HvAlaAT and 35S:HvAlaAT) did show decreases in uptake when compared to Col-0, but these differences were not statistically significant. None of the over-expressing lines showed significant increases in uptake at this point. Again, the alaat1;2 line showed the highest uptake of both radiolabeled compounds.

At the 2 hr time point significant alterations in the performance and uptake of different *AlaAT* over-expressing lines was observed (Figure 4.8c and Figure 4.9c). Two lines showed significant decreases in the uptake of both amino acids: OsAnt1:HvAlaAT 2-2-3-3 ($P < 0.001$, both radiolabels) and OsAnt1:HvAlaAT 3-1-2 ($P < 0.01$, both radiolabels). The line 35S:HvAlaAT 3-2-2 showed significant decreases in uptake of ^{14}C at this time point ($P < 0.05$), but not ^{3}H. Interestingly, all of the lines that showed statistically significant decreases in uptake of the externally supplied amino acids compared to Col-0 over-expressed *HvAlaAT*. None of the lines expressing either *MmAlaAT1* or *PfAlaAT* showed significant decreases in uptake.

Only one of the lines expressing HvAlaAT, OsAnt1:HvAlaAT 1-1-5-3, showed significant increases in amino acid uptake, and this was only for leucine ($P < 0.01$). The line 35S:MmAlaAT1 1-2-1 also showed significant uptake of leucine at this time point ($P < 0.01$). Lines that showed significant uptake of both amino acids at the 2 hr time point included: 35S:MmAlaAT1 2-1-7 ($P < 0.05$ for ^{3}H and $P < 0.001$ for ^{14}C), 35S:PfAlaAT 4-1-2 ($P < 0.001$ for both radiolabels), 35S:PfAlaAT 3-2-2 ($P < 0.001$ for both radiolabels), 35S:PfAlaAT 3-1-4 ($P < 0.05$ for ^{3}H and $P < 0.001$ for ^{14}C) and alaat1;2 ($P < 0.001$ for both radiolabels). Notably, all three transgenic lines carrying *PfAlaAT* showed significantly increased uptake of both alanine and leucine; this was the only AlaAT to have all independent insertion lines demonstrate significantly increased uptake of both amino acids. Intriguingly, alaat1;2 showed the highest level of uptake of both radioisotopes at this time, similar to results at the two previous time points.

Overall, it can also be observed, throughout the three time points, that there appears to be more consistency in the uptake of the external amino acids between lines containing *PfAlaAT* than between other *AlaAT*-containing lines. Contrary to this, those lines expressing

OsAnt1:HvAlaAT appear to show the least consistency between them in the ability to uptake the supplied external amino acids. Lines containing *35S:HvAlaAT* do not appear to show the same level of inconsistency as those containing *OsAnt1:HvAlaAT*, even though these cells over-express the same *AlaAT*.

4.3.10. *Arabidopsis thaliana* plants over-expressing *MmAlaAT1* and *PfAlaAT* show increased leaf area and decreased number of days to first flower when grown under short days.

Plants grown for the analysis of uptake by mesophyll protoplast cells showed increases in overall leaf area when compared to control Col-0 plants (Figure 4.10). Pictures of Arabidopsis plants to be used for protoplast uptake (Figure 4.10a) were analyzed using WinRHIZO Arabidopsis 2013d software to determine average rosette leaf area (cm^2) between several of the lines (Figure 4.10b). All transgenic lines showed increases in rosette leaf area when compared to Col-0. However, relative to Col-0, only lines expressing *35S:HvAlaAT* and *35S:MmAlaAT1* showed significant increases in rosette leaf area (Figure 4.10b), as determined by a Mann-Whitney *U*-test ($P > 0.05$). alaat1;2 knockout plants also showed increases in leaf area relative to Col-0, although these increases were not significant. These results support the previous observations that transgenic lines, as well as alaat1;2 plants, have increased fresh weight and increased rosette leaf area when grown under a regular (16 hr light/8 hr dark) light cycle (Figure 4.5). Previously, 35S:MmAlaAT1 3-1-3 showed significant increases in leaf area relative to Col-0 under regular day lengths (Figure 4.5a), and under these long day conditions the same line again showed significance, demonstrating the reliability of these results. Furthermore, the increased sample size in this analysis (n = 18) resulted in greater statistical power and reliability than the previous analysis.

To investigate this further, transgenic Arabidopsis lines over-expressing the various AlaAT enzymes (with the omission of MmAlaAT2) along with Col-0 were grown in soilless medium fertilized with modified Hoagland's solution (2 mM KNO_3) under short days (12 hrs light/12 hrs dark) and analyzed for variations in growth and development of plants, up until the emergence of the first flower. Four specific phenotypes of the plants were monitored: i) average number of days until bolting, ii) the number of rosette leaves > 1 cm at bolting, iii) the average number of days until first flower and iv) the average number of days between bolting and first

flower. Germination of plants was also monitored, however all plants germinated uniformly over a two day period with no obvious differences in the timing of germination or the germination rate. (This observation is also supported by Shrawat *et al.*, (2008), Beatty *et al*., (2013) and plate assay data (Chapter 3) where significant differences in time of germination and rate were not observed.)

Statistical analysis indicated that there were no significant differences to any of the four above mentioned characteristics due to genotype (Figure 4.11), determined by a one-way ANOVA (non-repeated measures). A Tukey's post-test for each of the four characteristics also revealed no significant differences between transgenic lines and Col-0. While no statistical differences were observed, differences were detected between lines, specifically average number of days until bolting and average number of days to first flower. On average, plants over-expressing *PfAlaAT* showed decreased average (mean) number of days to bolting and first flower. In both phenotypic cases, plants over-expressing *PfAlaAT* showed, on average, a ~1 day decrease in the amount of time required to achieve both developmental milestones. It is also interesting to note that Col-0 plants had the lowest number of rosette leave > 1 cm, on average. This result complements data obtained when looking at average leave area (Figure 4.5a and Figure 4.10), which showed decreased overall leaf area in Col-0 plants when compared to many of the transgenic *AlaAT* lines (Figure 4.5).

4.4. Discussion

It has already been established that tissue-specific over-expression of *HvAlaAT* in canola (Good *et al*., 2007) and rice (Shrawat *et al*., 2008) produces NUE phenotypes and that these phenotypes are a result of alterations in both metabolism and gene expression (Beatty *et al*., 2009, 2013). In Chapter 3, a physical characterization of the effects of expressing and over-expressing *AlaAT* variants under two different promoters indicated not only that *Arabidopsis thaliana* can be a useful model for the study of NUE in cereals crops, but also that there are variations in the observed NUE phenotype of Arabidopsis plants expressing different *AlaAT* homologues, as well as variations in the NUE phenotype when the same variant is driven by a constitutive versus tissue-specific promoter. Thus, it follows that if plants expressing different AlaAT enzymes portray different physical phenotypes, the internal physiology of these plants will also differ to some extent, as seen in rice (Beatty *et al*., 2009, 2013). However, because

previous broad spectrum analyses such as microarrays and N metabolite levels have already been carried out in rice and yielded few concrete details about the mechanism of *AlaAT* over-expression that produces an NUE phenotype, a more directed approach was taken in this study.

In order to properly evaluate the internal changes occurring as a result of *AlaAT* expression or over-expression, the use of *OsAnt1* in Arabidopsis as a tissue-specific promoter (Figure 4.1) and the expression of the transgene(s) (Figure 4.2) in Arabidopsis first had to be verified. *OsAnt1*, a rice-derived, root-specific promoter from the aldehyde dehydrogenase 1 gene, had previously been chosen to drive *AlaAT* expression in NUE rice; however, to our knowledge, it had never been used to drive gene expression in Arabidopsis. The results of expressing *OsAnt1:GUS* in Arabidopsis seedlings can be observed in Figure 4.1. Like expression of this promoter in rice (Shrawat *et al*., 2008), genes driven by *OsAnt1* in Arabidopsis are highly expressed in roots and root tips of seedlings, as well as throughout the vascular system of the plant. These results also suggest that tissue-specific promoters from rice may be useful candidates for further NUE studies in Arabidopsis or vice versa.

To determine the level of expression of the transgene(s) in the various lines, qRT-PCR analysis was carried out using transgenic seedlings. Due to the nature of the transgenes, (having no native expression within the control lines), a relative quantification method could be utilized. Expression of the transgenes was observed in all over-expressing lines (Col-0 background) and all expressing lines (alaat1;2 background) with the exception of 35S:MmAlaAT1 3-3-1, which did not show any expression of the transgene relative to alaat1;2. Expression of *UBQ6*, the control gene, was constant among all transgenic and control lines, ensuring the consistency and reliability of these test results. A large amount of variation between transgenic lines expressing the same construct was observed. These differences in expression when the construct DNA is identical are not uncommon, as the site of integration and other genetic factors can have a significant impact on the overall expression of the inserted gene, even resulting in silencing (Matzke & Matzke, 1998; De Bolle *et al*., 2003; Butaye *et al*., 2004; Magori & Citovsky, 2011). Plant genes driven by the *CaMV35S* promoter in particular, as they are here, are known to have fluctuating, bimodal, expression patterns, with ~2/3 of transformants often showing relatively low expression levels even when driven by this constitutive promoter (De Bolle *et al*., 2003). Ultimately, these results demonstrate that the AlaAT transgenes are being transcribed in the

majority of transgenic plants and that independent insertion lines containing the same input DNA are expressing these genes at distinctly different levels; this alone could lead to varying phenotypic results, even within plants containing the same AlaAT homologue.

While expression of a gene (transcription), can lead to production of a protein (translation), there are many factors that may affect the production of a protein after transcription and the effectiveness/activity of a protein after translation, these are known as post-transcriptional and post-translational modifications, respectively (Maier *et al*., 2009; Vogel & Marcotte, 2013). Micro-RNA (miRNA) mediated mRNA degradation is a common post-transcriptional method for regulating protein expression; this method can result in decreased production of a particular protein or complete inhibition of protein production (Lee *et al.*, 2006). Once a protein is made, post-translational processes such as phosphorylation, glycosylation, acetylation and ubiquitination, among others, can result in inactive and/or degraded proteins (Jensen, 2004; Kwon *et al*., 2006). For these reasons, although it was shown that the *AlaAT* transgenes were being expressed in Arabidopsis (Figure 4.2), the translation of these mRNA's into functional protein also needed to be validated.

The AlaAT enzyme activity of all transgenic lines was compared to that of control plants (Col-0 or alaat1;2) in order to asses if the observed expression of *AlaAT* transcript levels also resulted in increased AlaAT activity (Figure 4.3 and Figure 4.4). Since the concentration of the various AlaAT enzymes was not known and because whole-protein fractions were utilized, the specific activity of the enzymes could not be determined, but relative activity of the transgenic lines to that of controls was instead compared.

In every case, transgenics, in both the Col-0 and alaat1;2 backgrounds, displayed activity from protein fractions that was similar to or significantly greater than that from control plants (Figure 4.3 and Figure 4.4). From these results, and the observation that AlaAT activity did not significantly decrease in any of the transgenic lines relative to the controls, several different assumptions can be made: i) the AlaAT activity in the alaat1;2 knockout line is close to zero (Miyashita, 2008), and the addition of a foreign AlaAT enzyme increases activity in all cases (Figure 4.3), ii) expression or over-expression of the transgenic AlaAT enzyme(s) does not interfere with the native AlaAT enzyme(s) activity (Figure 4.4), iii) foreign AlaAT protein is being made and is active *in vivo* and, iv) mRNA levels determined through qRT-PCR do not

always correlate with activity levels in full-protein fractions. This last point is particularly interesting in the case of 35S:MmAlaAT1 3-3-1, which did not show *MmAlaAT1* transcript but did show significantly increased activity levels relative to the knockout line (Figure 4.2b and Figure 4.3c, respectively). However, in Arabidopsis, it has been reported that only 27-46% of tested proteins will correlate in abundance with that of the mRNA (de Sousa Abreu *et al*., 2009). This observation could be the result of a number of variables or combination of variables, including extremely high levels of translation, protein negative feedback loops and differences in mRNA and protein half-lives, among others (de Sousa Abreu *et al.*, 2009; Greenbaum *et al.*, 2003). Therefore these findings, that many of the *AlaAT* mRNA levels do not correlate with protein activity levels, are not surprising and instead give support that transgenic AlaAT mRNA is being converted to active protein *in vivo*.

To further validate the presence of transgenic AlaAT proteins *in vivo*, western blots were attempted. Results from western blots using primary antibodies for both HvAlaAT and a C-myc tag were inconsistent and are therefore not included. However, results from Chapter 3, showing N-specific phenotypes in transgenic plants compared to controls, along with qRT-PCR analysis and AlaAT activity data support that the *AlaAT* genes inserted into Arabidopsis are being expressed (Figure 4.2) and converted into active enzyme (Figure 4.3 and Figure 4.4), which is producing an NUE phenotype (Chapter 3).

To further understand how over-expression of the various AlaAT homologues is affecting the overall physiology of the plant to produce NUE phenotypes, two distinct approaches were taken: i) growth of plants under 'regular' conditions and soluble sugar analysis and ii) growth of plants under short days and protoplast uptake of amino acids. Both methods of study highly emphasize the link between N and C metabolism; they also target very different stages of N metabolism. If variations in the expressed AlaAT enzymes significantly affect the directionality of the reaction *in vivo*, these differences may manifest as alterations in either C metabolism (ie: soluble sugars) or amino acid/N metabolism (ie: amino acid uptake). While any alterations in soluble sugar concentration in shoot tissues would most likely be a direct result of changes in N mobilization and partitioning, alterations in protoplast uptake of amino acids would be the outcome of alterations in N uptake and assimilation. In this manner, not only does this analysis aim at deciphering the physiological impact that *AlaAT* over-expression may have on both N and

C metabolism, but also the stage of N metabolism that is most affected by this over-expression. While analysis of phenotypes in Chapter 3 supported a role for transgenic AlaAT not directly involved in N sensing, the assimilation and remobilization aspects of N metabolism were not explored.

Soluble sugars have many roles in plants apart from acting as direct precursor molecules for glycolysis to produce energy and reducing power (Salerno & Curatti, 2003). Sucrose is an important source to sink C transport molecule (via the phloem), and along with the soluble sugars glucose and fructose acts as a signaling molecule connected with, but not limited to, the regulation of N and C metabolism (Price *et al*., 2004), metabolite transport (Price *et al*., 2004), seed and embryo development (Hills, 2004), transition to flowering (Bernier *et al*., 1993) and plant stress responses (ie: light intensity) (Schmitz *et al.*, 2012, 2014). Under N stress it has been shown that soluble sugars accumulate in photosynthetically active organs, and their use as N metabolite precursors decreases (Ono *et al*., 1996; Sun *et al*., 2014). Increases in soluble sugars in photosynthetic organs as a result of limited N supply also resulted in decreased overall growth of Arabidopsis (Sun *et al*., 2014). Thus, the previously observed increases in biomass, N metabolites (ie: asparagine, glutamine and glutamate) (Shrawat *et al*., 2008) and photosynthetic transcripts (Beatty *et al*., 2013) in *OsAnt1:HvAlaAT*-expressing rice lines could be in part due to altered soluble sugar concentrations.

Plants for analysis of soluble sugars were assessed for a number of physical phenotypes: leaf area and fresh weight of individual plants and percent bolted and flowered. Leaf area and fresh weight of harvested plants showed few significant alterations from control plants (Col-0): 35S:MmAlaAT1 3-1-3 showed significant increases in leaf area (Figure 4.5). However, when results from lines carrying the same insert were grouped a trend in both leaf area and fresh weight between transgenics and Col-0 plants was observed: all transgenic lines showed average increased leaf area and fresh weight when compared to controls (Figure 4.5a inset and Figure 4.5b inset). Interestingly, it has been shown in Arabidopsis that either significant increases in leaf sugars, significant decrease in the ratio of plant N:C, or low nutrient conditions (specifically low N) can trigger leaf senescence (Pourtau *et al.*, 2004; Wingler *et al*., 2006), this alone could lead to decreased overall leaf growth and thus reduced leaf area and overall fresh weight. Therefore, increases in the transgenic lines of both of these physical traits, leaf area and fresh

weight, could be the result of delayed senescence in leaf tissues due to increased N metabolites available to the plant. It should be noted that because sample size was small for the analysis of leaf area, fresh weight, percent bolted and percent flowered, it is difficult to determine the true significance of these results. However, given that all lines over-expressing *AlaAT* showed consistency of these traits, it can be assumed that given a larger sample size, these differences would only be more pronounced, as was observed when plants were grown under short days for protoplast preparation (Figure 4.10b).

In wildtype Arabidopsis plants, stress (including low KNO_3) is known to induce early flowering (Bernier *et al*., 1993; Castro Marín *et al*., 2011). Significant alterations in the C:N ratio within the plant, specifically high C and low N, have also been shown to be a direct trigger of early flowering (Pourtau *et al*., 2004; Wingler *et al.*, 2006). In this study, plants over-expressing *AlaAT* variants showed increased rate of flowering but not bolting at 41 DAS when grown in regular conditions (Figure 4.6b,d). Because N was in adequate supply for this study (2 mM KNO_3), the possibility of N-induced stress in these plants is unlikely. It is more likely that the ratio of C:N in these plants is skewed, resulting in an early flowering phenotype. Although it has been shown that *AlaAT* over-expressing rice have increases in N metabolites (Shrawat *et al*., 2008; Beatty *et al*., 2013), which should indicate N sufficiency to the plants as a whole, if these metabolites were, i) quickly and efficiently being converted to metabolites not directly involved in N signaling (ie: alanine), ii) creating a discrepancy in the level of N metabolites between the shoots and the roots for a similar reason, or iii) increasing the concentration of metabolites feeding into primary C metabolism (ie: pyruvate) specifically in photosynthetic tissues, then an N-stress-like phenotype might be observed resulting in plants that flowered early.

It has been shown that nitrogen deficiency leads to increases in soluble sugars in photosynthetic tissues (Ono *et al*., 1996). Data from the analysis of soluble sugars shows a decrease in the average soluble sugars glucose, fructose and sucrose in all transgenics relative to Col-0 (Figure 4.7 insets), indicating that Col-0 plants may have increased N-stress relative to transgenic plants or a skewed C:N ratio. Soluble sugar skeletons can be shunted into numerous metabolic pathways including starch synthesis (increasing plant biomass), glycolysis (aiding in plant growth) and amino acid synthesis pathways (aiding in protein synthesis). Interestingly, in both previous studies of *HvAlaAT* in rice, increases in starch synthesis pathway genes and key

amino acids were observed (Beatty *et al*., 2013, 2009), supporting these results. These observations, taken with those showing increased leaf area, fresh weight and flowering time of the same plants, implies that under adequate N conditions (2 mM KNO_3), *AlaAT* over-expression, regardless of variant, results in NUE phenotypes relative to Col-0 plants. This NUE phenotype presumably results from alterations in assimilation and remobilization of N in photosynthetic tissues, supporting a role for AlaAT in mobilization and repartitioning of N compounds.

To analyze further the role of *AlaAT* over-expression in uptake and partitioning of amino acids and to see if *AlaAT* variant significantly affected these processes, protoplast mesophyll cells were isolated and analyzed for uptake of radiolabelled amino acids alanine (^{14}C-alanine) and leucine (^{3}H-leucine), at three different time points (10 min, 45 min and 2 hrs). Both alanine and leucine are known to be relatively small, neutral, storage amino acids found in high concentrations in the vacuole as well as the cytoplasm (Dietz *et al.*, 1990). While amino acid uptake in roots has shown to play a pivotal role in N acquisition and metabolism, the ability to effectively transfer these compounds into and out of both the xylem and the phloem as well as repartition these compounds in shoot tissues has also shown to be critical in overall plant NUE (Dietz *et al*., 1990; Miller *et al*., 2008; Tegeder & Rentsch, 2010; Tegeder, 2012). Both photosynthesizing and senescing leaves must be able to efficiently import and export amino acids and peptides, respectively (Fischer *et al*., 1998; Tegeder & Rentsch, 2010; Tegeder, 2012). As a direct substrate of AlaAT, it was thought that the ability to uptake and utilize alanine in cells over-expressing *AlaAT* variants may be significantly impacted. (Given that all of the expressed AlaAT proteins showed varying kinetic capacities in *E. coli*, their expression and kinetic properties may also be highly different *in vivo* (Chapter 2)). It was thought that leucine, as a similar amino acid, but one that is not directly involved in the AlaAT reaction, would show increased uptake in cells with low internal N, but not necessarily alterations in AlaAT activity.

Interestingly, *AlaAT* over-expressing lines did not show differences in the uptake of these two amino acids (Figure 4.8 and Figure 4.9); lines showing low uptake of ^{14}C-alanine also showed low uptake of ^{3}H-leucine and vice versa. At both the 10 min and 45 min time points, results of uptake of both amino acids remained mostly insignificant (Figure 4.8a,b and Figure 4.9a,b). Real differences between the uptake of these compounds was not observed until the 2 hr

time point (Figure 4.8c and Figure 4.9c). At 2 hrs after addition of the radioisotopes three significant observations were made. Firstly, over-expressing lines containing *HvAlaAT*, regardless of the promoter, appear to have lower average uptake of both amino acids when compared to lines containing either *MmAlaAT1* or *PfAlaAT*. Secondly, alaat1;2 knockout cells appear to take up external N compounds at a greater rate than either Col-0 or the transgenics, at all time points. Finally, *PfAlaAT*-containing transgenic protoplasts take up both alanine and leucine more effectively than Col-0 or other transgenic lines at this later time point. These results indicate that the amino acid/N concentration in *PfAlaAT*-expressing cells is significantly altered compared to Col-0, and that this alteration is due to the presence of this enzyme.

What is also noteworthy is the similarity in uptake of cells expressing both *OsAnt1:HvAlaAT* and *35S:HvAlaAT*. While *OsAnt1:HvAlaAT* is hypothetically not expressed (or expressed at a very low level) in mesophyll cells, cells containing both of the *HvAlaAT* constructs behaved in a similar manner. This result indicates one of two things, i) at later stages of development in *Arabidopsis thaliana* the *OsAnt1* promoter is more highly expressed in leaf tissues than in rice, or ii) high expression of *HvAlaAT* (or possibly any AlaAT) in the roots is the main driving force for all successive alterations in plant N metabolism, regardless of expression in shoot tissues. More studies, specifically using different promoters driving *AlaAT* variants in either Arabidopsis or cereal crops, are necessary to decipher between these two possibilities.

NUE rice plants over-expressing *HvAlaAT* have shown increased biomass and yields compared to control plants (Shrawat *et al*., 2008). *AlaAT* over-expressing *Arabidopsis thaliana* plants showed here, on average, increased leaf area, fresh weight and tap root growth, validating the results previously observed in rice. Increases in biomass (leaf area and fresh weight) and root growth (Chapter 3) under both regular and short days could be attributed to two factors: a) plants with increased NUE accumulate increased biomass or b) plants with increased NUE have accelerated or altered development when compared to control plants. If these increases in plants over-expressing *AlaAT* are due to alterations in the rate of development of the plant, this can then be evaluated by looking at key development stages, including bolting and flowering, and then comparing the point at which both transgenic and control plants reach these milestones.

Even under short days, which are known to inhibit flowering and increase rosette leaf growth (Ratcliffe *et al*., 2001; Castro Marín *et al*., 2011), transgenic *AlaAT* over-expressing

plants showed average increases in rosette leaf area, regardless of *AlaAT* variant being expressed (Figure 4.10), with expression of both *35S:HvAlaAT* and *35S:MmAlaAT1* resulting in significant increases in leaf area under these conditions. Further analysis of control and transgenic plants grown under short days showed that transgenic plants on average have increased number of rosette leaves at bolting (Figure 4.11b), and that, on average, plants expressing *35S:PfAlaAT* flower earlier than other transgenics, which on average flower earlier than Col-0 plants (Figure 4.11d). However, differences in rosette leaf number at bolting, time to bolting, time to flowering and number of days between bolting and flowering were shown to be insignificant relative to control plants. These results support the observations under regular light conditions that Arabidopsis plants, over-expressing *AlaAT* variants, flower earlier on average than control plants however these differences are not significant (Figure 4.6d). They also demonstrate that plants over-expressing *AlaAT* (regardless of the variant) are larger on average due to increased accumulation of biomass relative to control plants, and not due to increases in the developmental rate of these plants. Slight increases in the rate of flowering could be a side effect of increased overall biomass, cueing plants to flower earlier due to the endogenous status of the plant (Yamaguchi & Abe, 2012). Whether these increases in biomass are reflected as increases in yield or increases in overall seed weight has yet to be examined. Such observations would give support to increased remobilization as well as assimilation of N compounds in *AlaAT* over-expressing plants and therefore increase overall NUE.

Interestingly, alaat1;2 knockout plants were shown to behave much like the transgenics in relation to leaf area, fresh weight of rosette leaves, soluble sugar concentrations in shoots, and protoplast uptake (Figure 4.5, Figure 4.7 Figure 4.8 and Figure 4.9). In the case of protoplast uptake, if AlaAT contributes significantly to the amino acid pool in general, than loss of the enzyme would result in plants that had lower internal amino acid concentrations and therefore would be expected to have increased uptake of exogenously applied amino acids. However, increased leaf area and fresh weight are harder to understand in relation to NUE. Perhaps, under adequate N, loss of AlaAT results in a build-up of N compounds in the roots early in development. In normal conditions these compounds would be converted by the native AlaAT enzyme, but without AlaAT, a build-up of these compounds results in a plant that senses extremely high N conditions in the roots. This would then result in a plant that behaved as if in an N-rich environment for a period of time, delaying senescence and increasing shoot biomass

under ideal conditions. However, these plants did not show increased time to flowering as the transgenics did, and showed growth inhibition under certain N growth conditions in plate assay experiments relative to the transgenics (Chapter 3).

The results of this study indicate several key points. First, plants over-expressing *AlaAT* (Figure 4.2) have, on average, increased AlaAT activity relative to control plants (Figure 4.3 and Figure 4.4). This in itself is not surprising; however, increased activity did not always correlate with increased *AlaAT* expression level, indicating that the AlaAT insertion point into the host genome could be of critical importance. Secondly, C metabolism, specifically soluble sugar concentrations in shoot tissues, is affected by *AlaAT* over-expression (Figure 4.5). Decreases in soluble sugars could be attributed to an increased shunting of C-skeletons into other pathways, such as starch synthesis or amino acid synthesis. Thirdly, uptake of externally supplied amino acids by mesophyll cells can be significantly impacted by the *AlaAT* expressed within (Figure 4.8 and Figure 4.9). *PfAlaAT* expression most significantly affected this process, indicating that this enzyme most drastically affects the amino acid/N equilibrium within the plant cell. Fourthly, it was demonstrated that *AlaAT* over-expressing plants have, on average, increased leaf area, biomass and time to flowering compared with controls, signifying an increased ability to accumulate biomass relative to controls over the same time period. Increased leaf area and biomass correlated with a decrease in soluble sugars in shoot tissue of transgenic plants. Finally, taken together with kinetic data from Chapter 2, plate assay data from Chapter 3 (Figures 3.13 specifically) as well as observations of increased leaf area and fresh weight (Figure 4.5), it can be suggested that PfAlaAT most significantly alters the cellular concentrations of both C and N metabolites, and that the greatest physiological alterations as a result of over-expressing *AlaAT* will be observed in plants/cells expressing this particular variant.

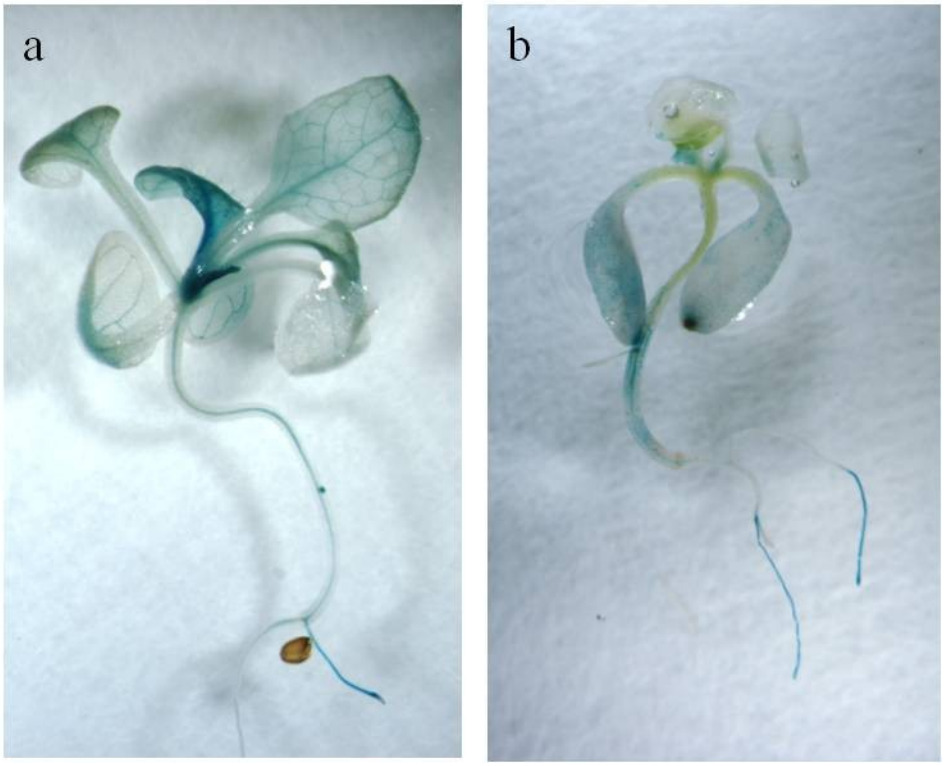


Figure 4.1. OsAnt1:GUS expression in *Arabidopsis thaliana*.

Four-week-old transgenic *Arabidopsis thaliana* plants grown on ½ MS expressing GUS via an *OsAnt1* tissue-specific rice promoter. Plants were stained for two hours and then de-stained using ethanol to remove chlorophyll. A) *OsAnt1:GUS* in COL background B) *OsAnt1:GUS* in alaat1;2 background. GUS was not detected in control plants (not shown).

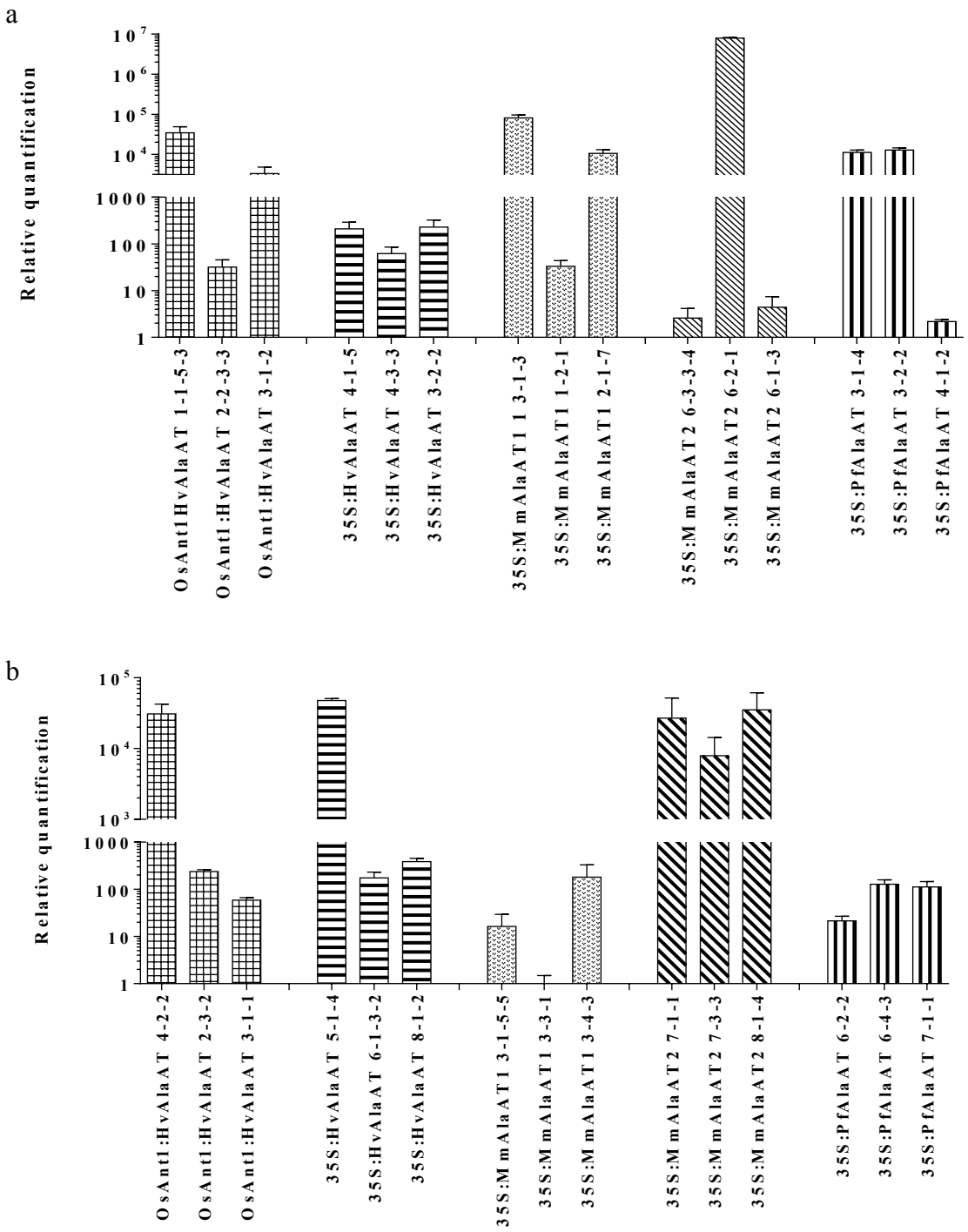


Figure 4.2. Relative expression levels of a variety of AlaAT genes expressed in two *Arabidopsis thaliana* backgrounds.

AlaAT expressing lines were calibrated to either A) Col-0 or B) alaat1;2 background transcription profiles using the $2(-\Delta\Delta C_T)$. In both cases, *UBQ6* was used as an endogenous control to ensure consistency of the tested samples. Error bars indicate SEM.

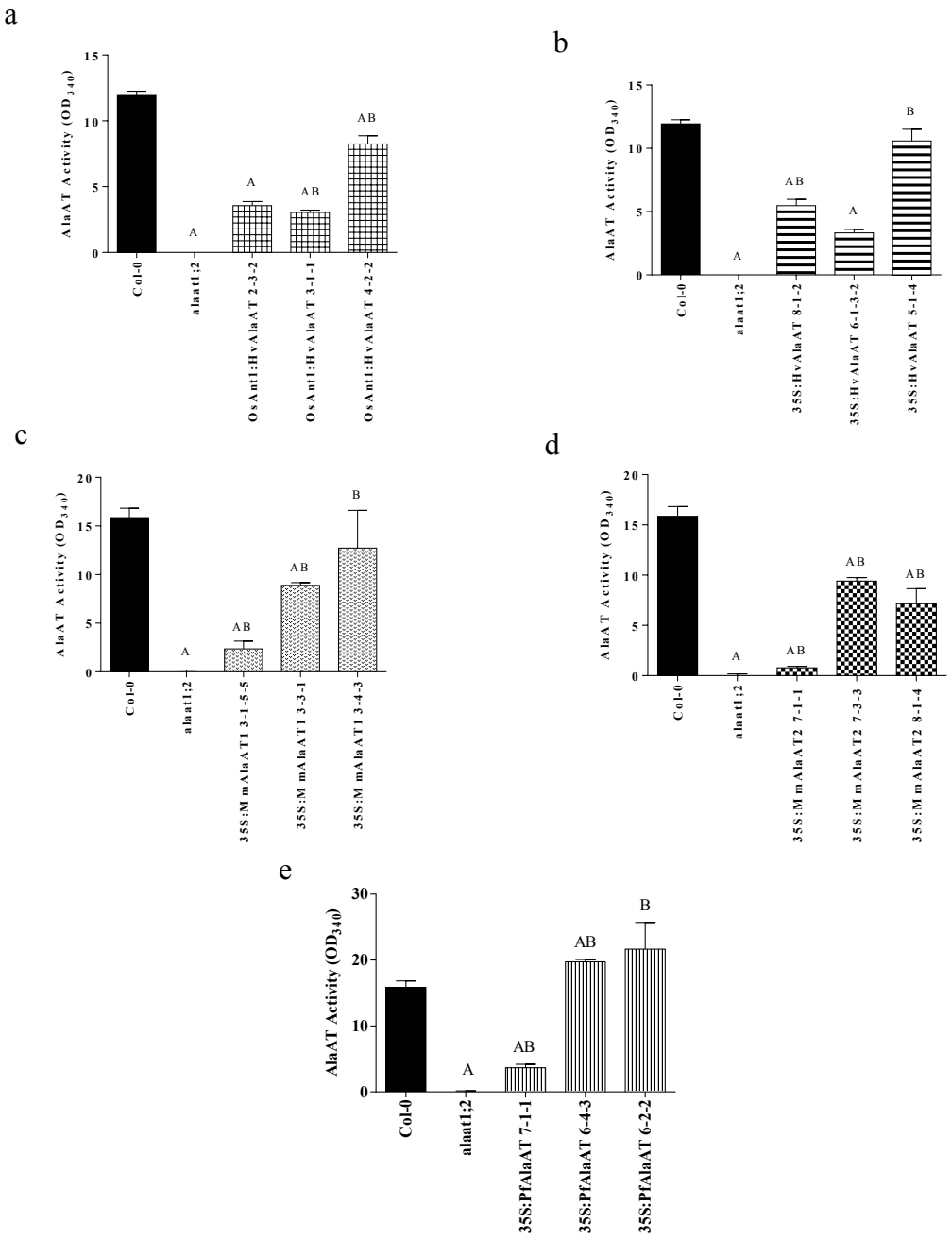


Figure 4.3. AlaAT activity in alaat1;2 background Arabidopsis over-expressing various AlaAT enzymes.

AlaAT activity of full protein fractions from various *AlaAT*-expressing transgenic Arabidopsis. a) OsAnt1:HvAlaAT lines, b) 35S:HvAlaAT lines, c) 35S:MmAlaAT1 lines, d) 35S:MmAlaAT2 lines and e) 35S:PfAlaAT lines. All transgenic lines were compared to controls using a two-tailed Mann-Whitney *U*-test ($P < 0.05$), n = 3-6. * indicates significance; A indicates significance in relation to Col-0, B indicates significance in relation to alaat1;2; error bars indicate SEM.

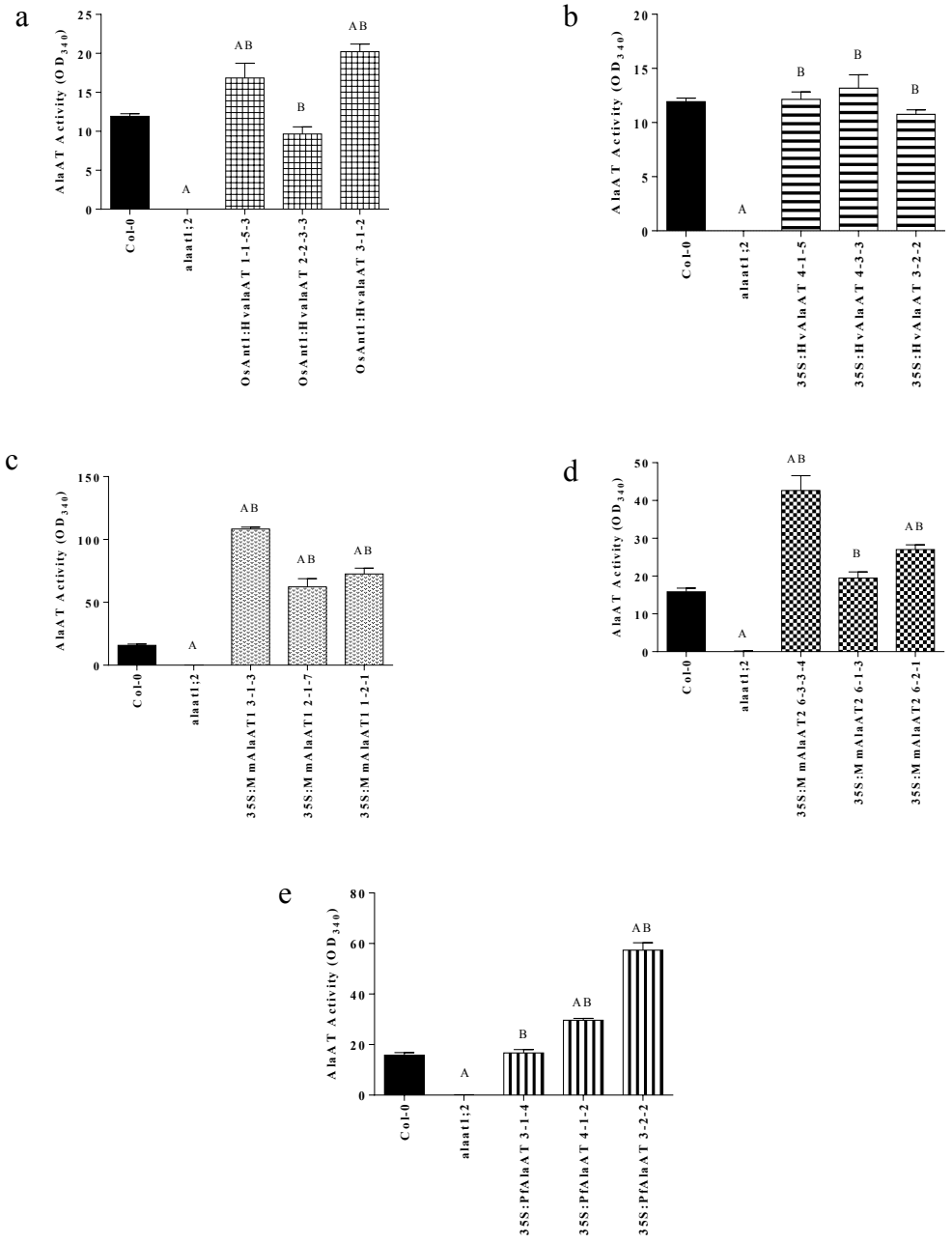


Figure 4.4. AlaAT activity in Col-0 background Arabidopsis over-expressing various AlaAT enzymes.

AlaAT activity of full protein fractions from various *AlaAT* over-expressing transgenic Arabidopsis. a) OsAnt1:HvAlaAT lines, b) 35S:HvAlaAT lines, c) 35S:MmAlaAT1 lines, d) 35S:MmAlaAT2 lines and e) 35S:PfAlaAT lines. All transgenic lines were compared to controls using a two-tailed Mann-Whitney *U*-test ($P < 0.05$), n = 3-6. * indicates significance; A indicates significance in relation to Col-0, B indicates significance in relation to alaat1;2; error bars indicate SEM.

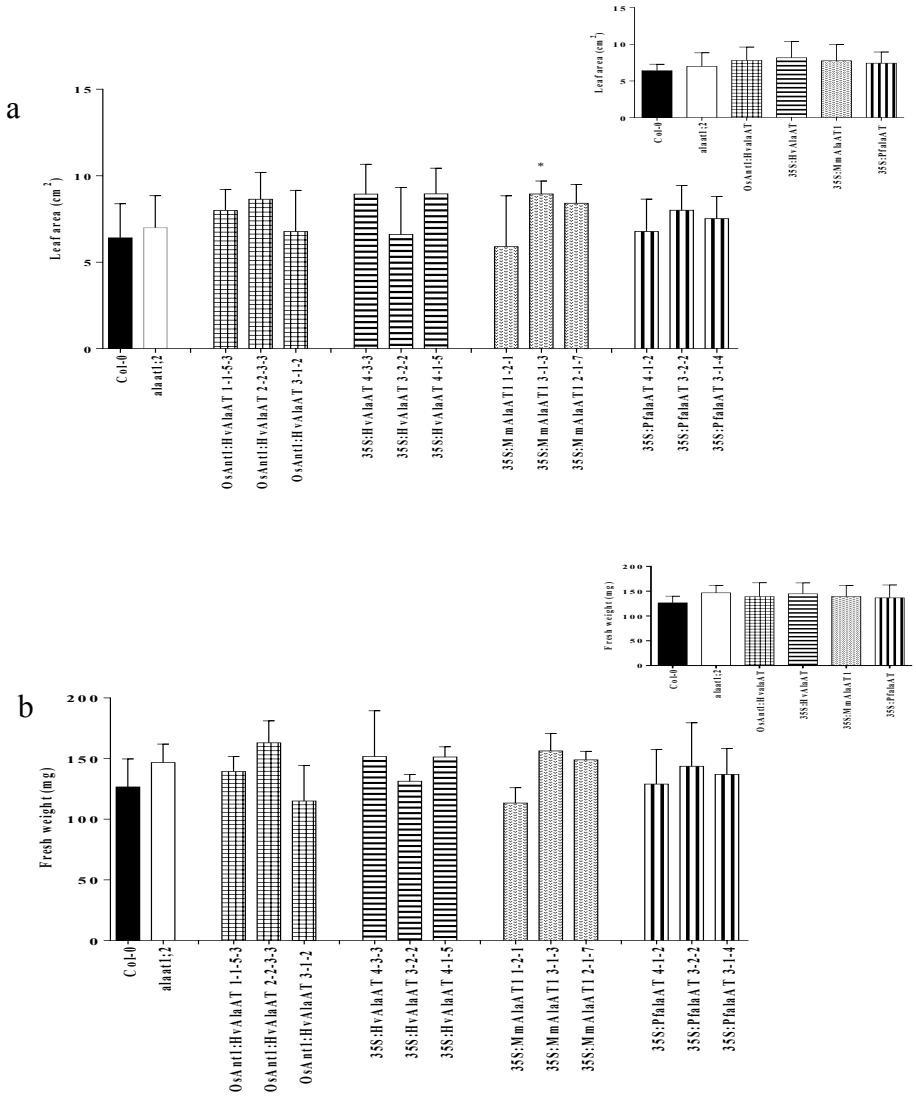


Figure 4.5. Average leaf area and fresh weight of Arabidopsis AlaAT over-expressing plants grown in soilless medium.

Average leaf area (n = 5) and average fresh weight of rosette leaves > 1 cm on individual plants (n = 3) was recorded at point of harvest. Plants were harvested at 41 DAS. a) Photographs of plants were taken on the day of harvest; total rosette leaf area of each plant was calculated using WinRHIZO Arabidopsis 2013d software to determine an average leaf area per line. b) Fresh weight of individual plants, to be used later in analysis of soluble sugars, was recorded. Insets for both graphs indicate the average when results from independent transgenic lines carrying the same construct are grouped. A two-tailed Mann-Whitney *U*-test was used to compare leaf area between transgenics (including alaat1;2) and Col-0 as well as fresh weight between transgenics (including alaat1;2) and Col-0. * indicates significance compared to Col-0 *(P* < 0.05). Error bars indicate SEM.

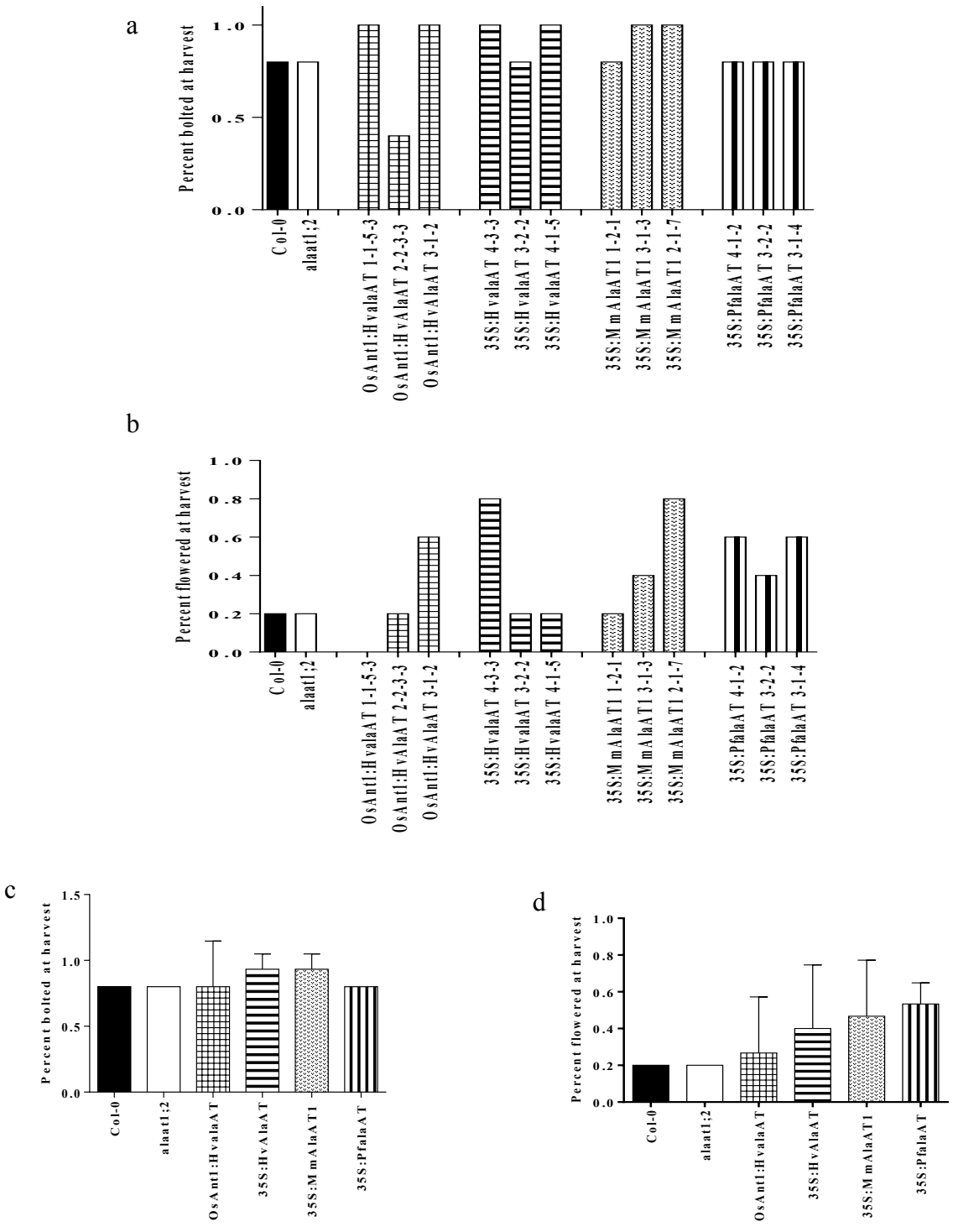

a
Percent bolted at harvest
1.0
0.5
0.0
Col-0
alaat1;2
OsAnt1:HvalaAT 1-1-5-3
OsAnt1:HvAlaAT 2-2-3-3
OsAnt1:HvAlaAT 3-1-2
35S:HvalaAT 4-3-3
35S:HvalaAT 3-2-2
35S:HvalaAT 4-1-5
35S:MmAlaAT1 1-2-1
35S:MmAlaAT1 3-1-3
35S:MmAlaAT1 2-1-7
35S:PfalaAT 4-1-2
35S:PfalaAT 3-2-2
35S:PfalaAT 3-1-4
b
Percent flowered at harvest
1.0
0.8
0.6
0.4
0.2
0.0
Col-0
alaat1;2
OsAnt1:HvalaAT 1-1-5-3
OsAnt1:HvAlaAT 2-2-3-3
OsAnt1:HvAlaAT 3-1-2
35S:HvalaAT 4-3-3
35S:HvalaAT 3-2-2
35S:HvalaAT 4-1-5
35S:MmAlaAT1 1-2-1
35S:MmAlaAT1 3-1-3
35S:MmAlaAT1 2-1-7
35S:PfalaAT 4-1-2
35S:PfalaAT 3-2-2
35S:PfalaAT 3-1-4
c
Percent bolted at harvest
1.5
1.0
0.5
0.0
Col-0
alaat1;2
OsAnt1:HvalaAT
35S:HvAlaAT
35S:MmAlaAT1
35S:PfalaAT
d
Percent flowered at harvest
1.0
0.8
0.6
0.4
0.2
0.0
Col-0
alaat1;2
OsAnt1:HvalaAT
35S:HvAlaAT
35S:MmAlaAT1
35S:PfalaAT

Figure 4.6. Percentage of plants that had bolted and flowered at 41 DAS in regular conditions.

Plants grown in soilless medium fertilized with modified Hoagland's solution under regular conditions (21°C and 60% humidity, 16 hrs light/8 hrs dark) were harvested at 41 DAS for analysis of soluble sugars. At harvest, the percent of plants per line that had bolted (a) and flowered (b) was recorded (n = 5). In addition, lines containing the same construct were grouped and analyzed for the percent of plants that had bolted (c) or flower (d). Error bars indicate SEM.

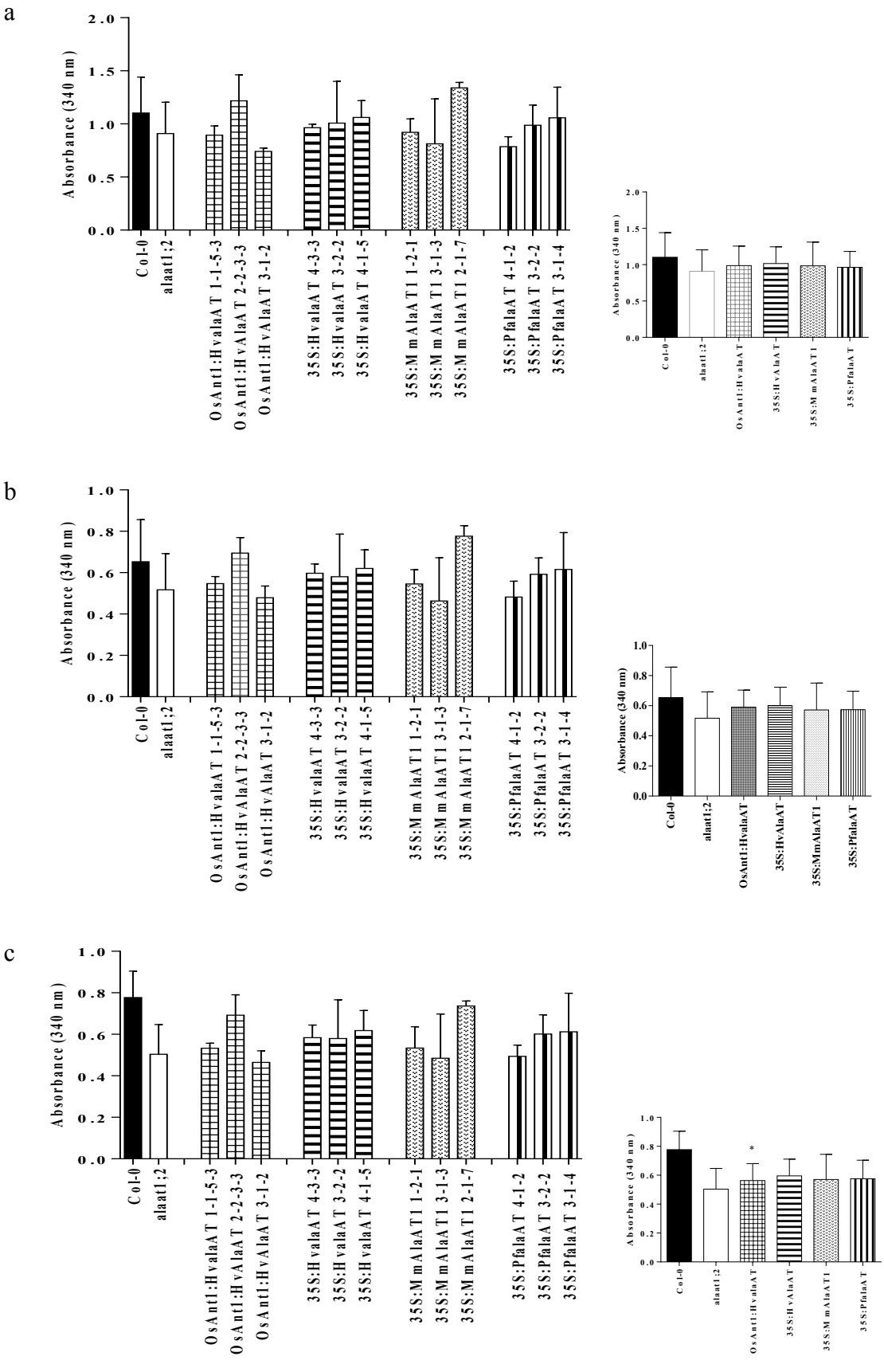

a
b
c
Absorbance (340 nm)
Col-0
alaat1;2
OsAnt1:HvalaAT 1-1-5-3
OsAnt1:HvAlaAT 2-2-3-3
OsAnt1:HvAlaAT 3-1-2
35S:HvalaAT 4-3-3
35S:HvalaAT 3-2-2
35S:HvalaAT 4-1-5
35S:MmAlaAT1 1-2-1
35S:MmAlaAT1 3-1-3
35S:MmAlaAT1 2-1-7
35S:PfalaAT 4-1-2
35S:PfalaAT 3-2-2
35S:PfalaAT 3-1-4
OsAnt1:HvalaAT
35S:HvAlaAT
35S:MmAlaAT1
35S:PfalaAT
*

Figure 4.7. Indirect analysis of the soluble sugars glucose, fructose and sucrose in rosette leaves of AlaAT over-expressing plants.

Glucose (a), fructose (b) and sucrose (c) concentrations were indirectly measured from 100 µl of soluble sugar extract, via the production of NADH and the consequential increase in absorbance at 340 nm. A Kruskal-Wallis one-way ANOVA was used to determine the significance of genotype and a Dunn's post-test was used to compare all transgenic lines and alaat1;2 to Col-0 ($P < 0.05$, n = 2-3). The results from lines containing the same construct were grouped, a-c inset, and compared to Col-0 using a Mann-Whitney *U*-test ($P < 0.05$, Col-0 and alaat1;2 n = 2-3, transgenics n = 12-14). * indicates significance in relation to Col-0. Error bars indicate SEM.

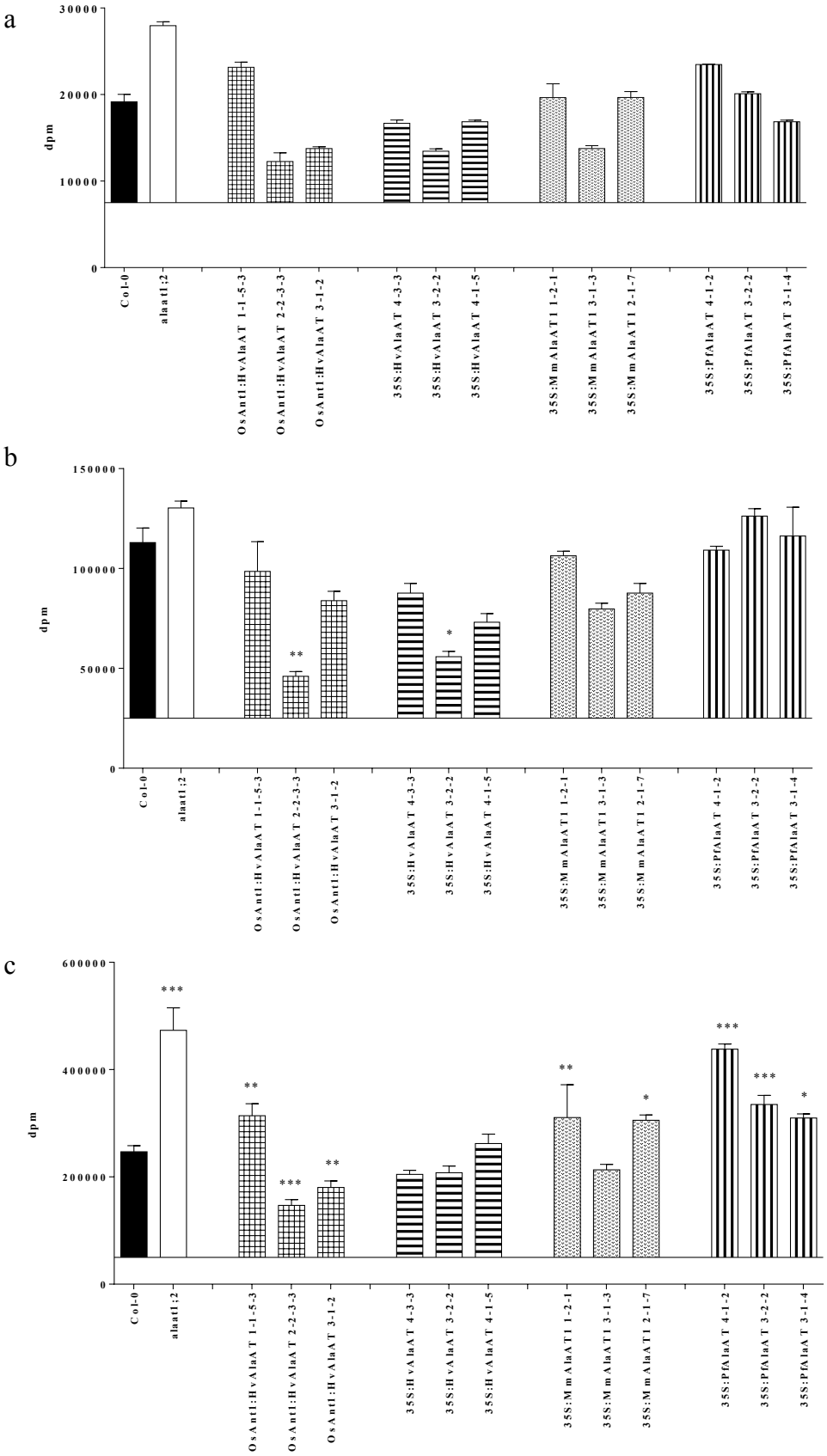

a
b
c
dpm
30000
20000
10000
0
150000
100000
50000
600000
400000
200000
Col-0
alaat1;2
OsAnt1:HvAlaAT 1-1-5-3
OsAnt1:HvAlaAT 2-2-3-3
OsAnt1:HvAlaAT 3-1-2
35S:HvAlaAT 4-3-3
35S:HvAlaAT 3-2-2
35S:HvAlaAT 4-1-5
35S:MmAlaAT1 1-2-1
35S:MmAlaAT1 3-1-3
35S:MmAlaAT1 2-1-7
35S:PfAlaAT 4-1-2
35S:PfAlaAT 3-2-2
35S:PfAlaAT 3-1-4
*
**
***

Figure 4.8. Uptake of ^{3}H-leucine by *AlaAT*-expressing protoplasts at various time points.

Mesophyll protoplast cells from plant lines grown in soilless-medium under short days (12 hrs light/12 hrs dark) and over-expressing different AlaAT enzymes were prepared (OsAnt1:HvAlaAT, 35S:HvalaAT, 35S:MmAlaAT1 and 35S:PfAlaAT). Protoplasts from Col-0 and alaat1;2 lines were prepared and used as controls. Protoplast cells were incubated for a) 10 min, b) 45 min or c) 2 hrs, in the presence of ^{3}H-leucine, and uptake was determined by a liquid scintillation counter. A two-way ANOVA was used to analyze the data, and a Bonferroni post-test was used to compare all transgenic lines to Col-0. * indicates significance in relation to Col-0 ($P > 0.05$). Error bars indicate SEM.

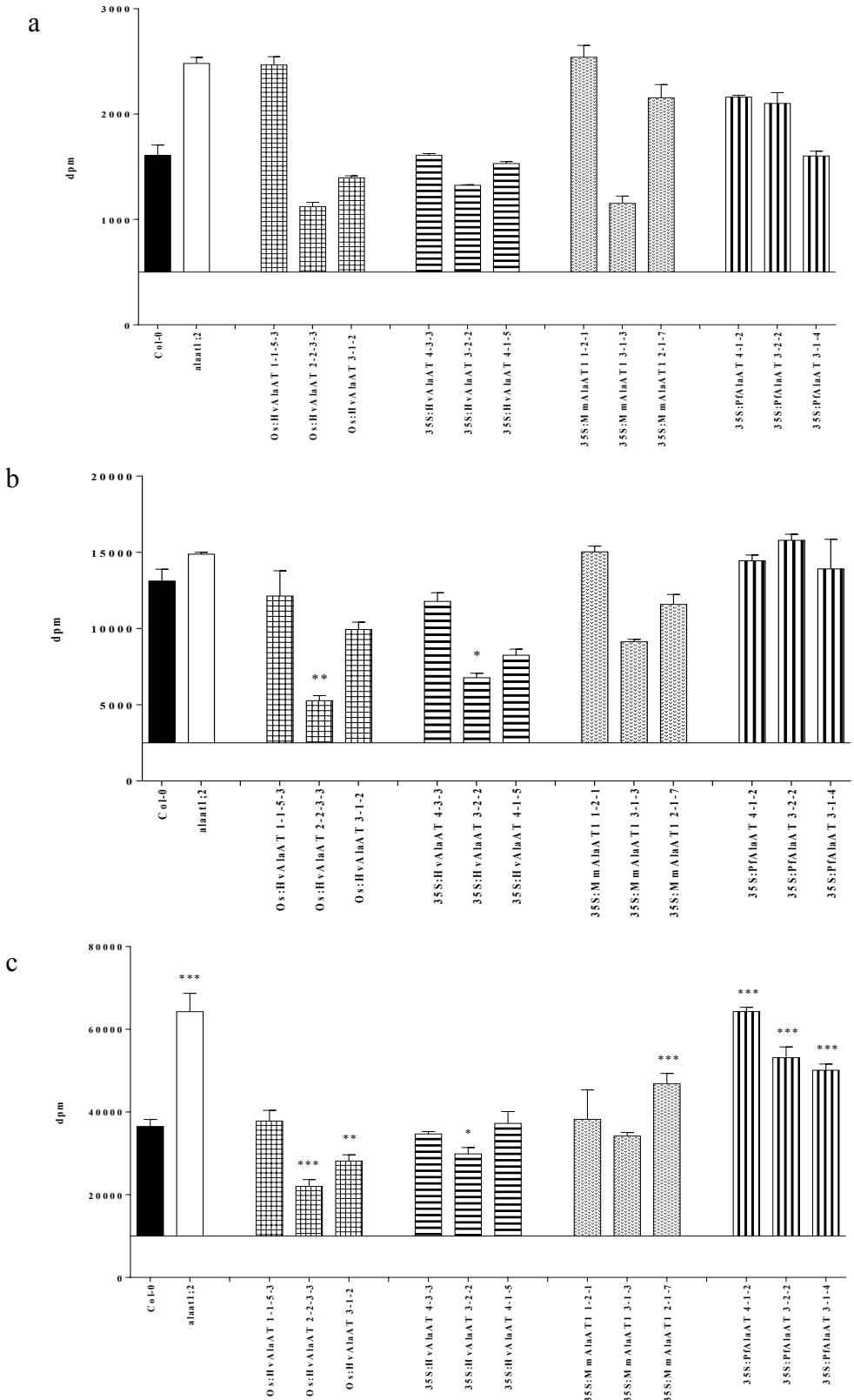

a
b
c
dpm
3000
2000
1000
0
20000
15000
10000
5000
80000
60000
40000
20000
Col-0
alaat1;2
Os:HvAlaAT 1-1-5-3
Os:HvAlaAT 2-2-3-3
Os:HvAlaAT 3-1-2
35S:HvAlaAT 4-3-3
35S:HvAlaAT 3-2-2
35S:HvAlaAT 4-1-5
35S:MmAlaAT1 1-2-1
35S:MmAlaAT1 3-1-3
35S:MmAlaAT1 2-1-7
35S:PfAlaAT 4-1-2
35S:PfAlaAT 3-2-2
35S:PfAlaAT 3-1-4
*
**
***

Figure 4.9. Uptake of ^{14}C-alanine by *AlaAT*-expressing protoplasts at various time points.

Mesophyll protoplast cells from plant lines grown in soilless-medium under short days (12 hrs light/12 hrs dark) and over-expressing different AlaAT enzymes were prepared (OsAnt1:HvAlaAT, 35S:HvalaAT, 35S:MmAlaAT1 and 35S:PfAlaAT). Protoplasts from Col-0 and alaat1;2 lines were prepared and used as controls. Protoplast cells were incubated for a) 10 min, b) 45 min or c) 2 hrs, in the presence of ^{14}C-alanine, and uptake was determined by a liquid scintillation counter. A two-way ANOVA was used to analyze the data, and a Bonferroni post-test was used to compare all transgenic lines to Col-0. * indicates significance in relation to Col-0 ($P > 0.05$). Error bars indicate SEM.

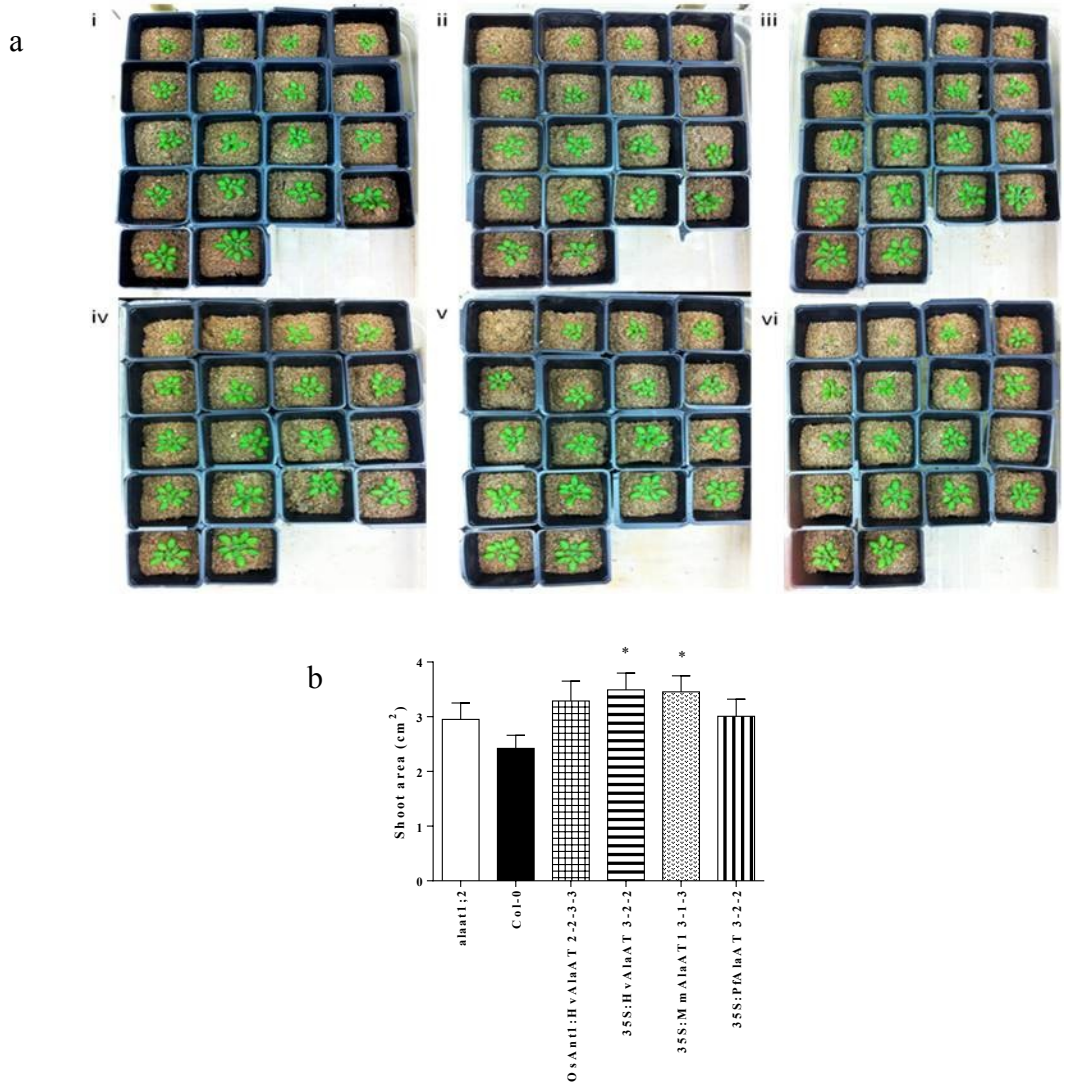


Figure 4.10. ***Arabidopsis thaliana*** **grown in soilless medium under long days have increased shoot area.**

a) Representative photograph of Arabidopsis plants harvested for protoplast preparation. Plants of the same genotype have been placed together and in order of approximate size of plant for the photograph only. Plants were grown at the same time under short days (12 hrs light/12 hours dark), at 21°C and 60% humidity. All plants were fertilized with a modified Hoagland's medium once a week and watered one additional time per week (50 ml/plant). Figure a, i) Col-0, ii) alaat1;2, iii) OsAnt1:HvAlaAT 2-2-3-3, iv) 35S:HvalaAT 3-2-2, v) 35S:MmAlaAT1 3-1-3 and vi) 35S:PfAlaAT 3-2-2. b) Quantitative representation of total shoot area (cm^2) per genotype (18 plants each) produced using the above photographs and WhinRHIZO Arabidopsis 2013d software. A Mann-Whitney *U*-test was used to compare shoot area between transgenics (including alaat1;2) and Col-0. * indicates significance compared to Col-0 *(P* < 0.05). Error bars indicate SEM.

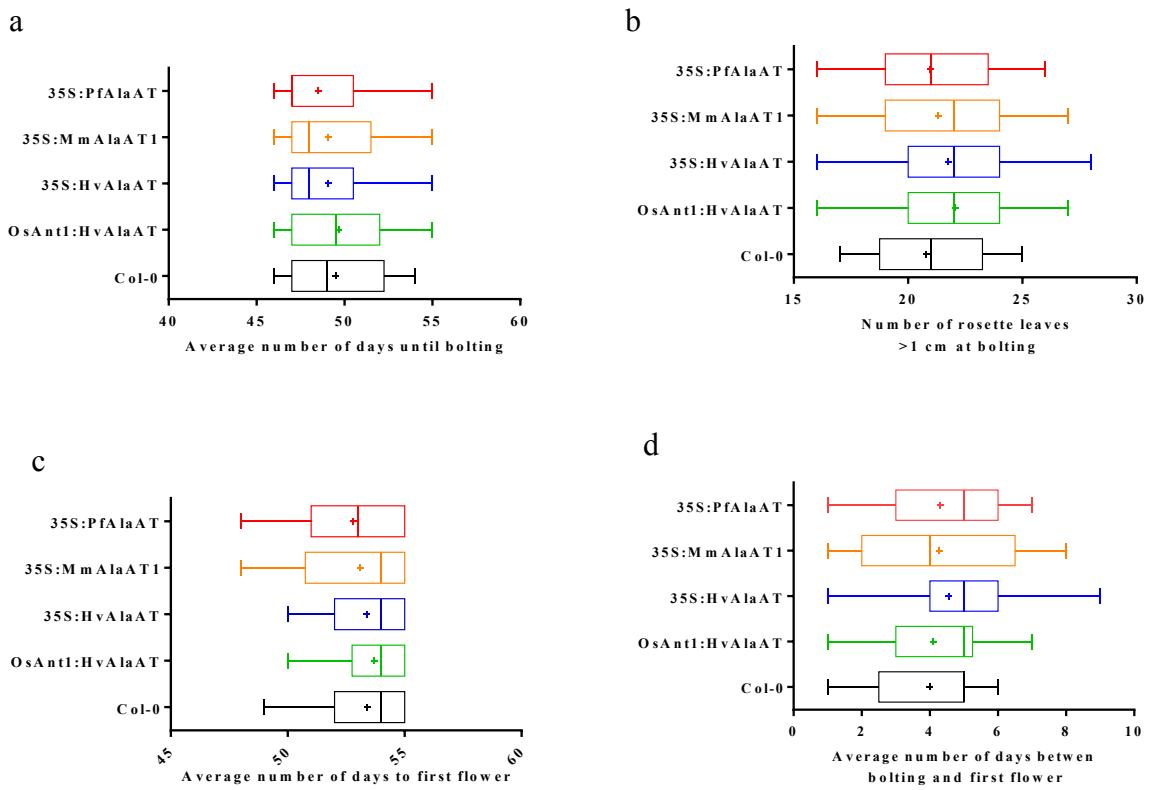


Figure 4.11. Analysis of visual growth phenotypes of Arabidopsis plants over-expressing various AlaAT enzymes in a Col-0 background under short days.

Seeds were sterilized and cold-induced before being sown onto a sand:vermiculite (1:1) medium. Plants were germinated and grown under short days (12 hrs light/12 hrs dark) in soilless medium and fertilized once a week for the lifetime of the plants with a modified Hoagland's solution (2 mM KNO^3); plants were grown for a total of 55 days. A one-way ANOVA (non-repeated measures) was used to determine the significance of genotype (Col-0, n = 10; transgenic lines, n = 28-30). A Tukey's post-test was used to compare all transgenic lines to Col-0 ($P < 0.05$). "+" indicates the mean; "|" indicates the median, error bars indicate SEM.

4.5. Bibliography

Beatty, P. H., Carroll, R. T., Shrawat, A. K., Guevara, D. and Good, A. G. (2013) Physiological analysis of nitrogen-efficient rice overexpressing alanine aminotransferase under different N regimes. *Botany* **91:** 866–883.

Beatty, P. H., Shrawat, A. K., Carroll, R. T., Zhu, T. and Good, A. G. (2009) Transcriptome analysis of nitrogen-efficient rice over-expressing alanine aminotransferase. *Plant Biotech. J.* **7:** 562–576.

Bernier, G., Havelange, A., Houssa, C., Petitjean, A. and Lejeune, P. (1993) Physiological signals that induce flowering. *Plant Cell* **5:** 1147–1155.

Butaye, K. M. J., Goderis, I. J. W. M., Wouters, P. F. J., Pues, J. M.-T. G., Delauré, S. L., Broekaert, W. F., Depicker, A., Cammue, B. P. A. and De Bolle, M. F. C. (2004) Stable high-level transgene expression in *Arabidopsis thaliana* using gene silencing mutants and matrix attachment regions. *Plant J. 39*: 440–449.

Calenge, F., Saliba-Colombani, V., Mahieu, S., Loudet, O., Daniel-Veldele, F. and Krapp, A. (2006) Natural variation for carbohydrate content in Arabidopsis. Interaction with complex traits dissected by quantitative genetics. *Plant Physiol.* **141:** 1630–1643.

Callis, J., Raasch, J. A. and Vierstrass, R. D. (1990) Ubiquitin extension protiens of *Arabidopsis thaliana*: structure, localization, and expression of their promoters in transgenic tobacco. *J. Biol. Chem.* **265:** 12486–12493.

Castro Marín, I., Loef, I., Bartetzko, L., Searle, I., Coupland, G., Stitt, M. and Osuna, D. (2011). Nitrate regulates floral induction in Arabidopsis, acting independently of light, gibberellin and autonomous pathways. *Planta* **233:** 539–552.

Coleman, J. S., McConnaughay, K. D. and Ackerly, D. D. (1994) Interpreting phenotypic variation in plants. *Trends Ecol. Evol.* **9:** 187–191.

Coruzzi, G. and Bush, D. R. (2001) Nitrogen and carbon nutrient and metabolite signaling in plants. *Plant Physiol.* **125:** 61–64.

Coruzzi, G. M. and Zhou, L. (2001) Carbon and nitrogen sensing and signaling in plants: emerging "matrix effects". *Curr. Opin. Plant Biol.* **4:** 247–253.

De Bolle, M. F. C., Butaye, K. M. J., Coucke, W. J. W., Goderis, I. J. W. M., Wouters, P. F. J., van Boxel, N., Broekaert, W. F. and Cammue, B. P. A. (2003) Analysis of the influence of promoter elements and a matrix attachment region on the inter-individual variation of transgene expression in populations of *Arabidopsis thaliana*. *Plant Sci.* **165:** 169–179.

De Sousa Abreu, R., Penalva, L. O., Marcotte, E. M. and Vogel, C. (2009) Global signatures of protein and mRNA expression levels. *Mol. bioSystems **5:*** 1512–1526.

Dietz, K., Jager, R., Kaiser, G. and Martinoia, E. (1990). Amino acid transport across the tonoplast of vacuoles isolated from barley mesophyll protoplasts. *Plant Physiol.* **92:** 123–129.

Fischer, W., André, B., Rentsch, D., Krolkiewicz, S., Tegeder, M., Breitkreuz, K. and Frommer, W. B. (1998) Amino acid transport in plants. *Trends Plant Sci.* **1385:** 188-195.

Fontaine, J.-X., Ravel, C., Pageau, K., Heumez, E., Dubois, F., Hirel, B. and Le Gouis, J. (2009) A quantitative genetic study for elucidating the contribution of glutamine synthetase, glutamate dehydrogenase and other nitrogen-related physiological traits to the agronomic performance of common wheat. *Theor. Appl. Genet.* **119:** 645–662.

Good, A. G., Johnson, S. J., De Pauw, M., Carroll, R. T., Savidov, N., Vidmar, J., Lu, Z., Taylor, G. and Stroeher, V. (2007) Engineering nitrogen use efficiency with alanine aminotransferase. *Can. J. Bot.* **85:** 252–262.

Good, A. G. and Muench, D. G. (1992) Purification and characterization of an anaerobically induced alanine aminotransferase from barley roots. *Plant Physiol.* **99:** 1520–1525.

Greenbaum, D., Colangelo, C., Williams, K. and Gerstein, M. (2003) Comparing protein abundance and mRNA expression levels on a genomic scale. *Gen. Biol. **4:*** 117.

Gromova, M. and Roby, C. (2010) Toward *Arabidopsis thaliana* hydrophilic metabolome: assessment of extraction methods and quantitative 1H NMR. *Physiol. Plant.* **140:** 111–127.

Hammond, J. P. and White, P. J. (2008) Sucrose transport in the phloem: integrating root responses to phosphorus starvation. *J. Exp. Bot.* **59:** 93–109.

Harrison, S. J., Mott, E. K., Parsley, K., Aspinall, S., Gray, J. C. and Cottage, A. (2006). A rapid and robust method of identifying transformed *Arabidopsis thaliana* seedlings following floral dip transformation. *Plant Meth.* **2:** 19.

Hills, M. J. (2004) Control of storage-product synthesis in seeds. *Curr. Opin. Plant Biol.* **7:** 302–308.

Hirel, B., Bertin, P., Quilleré, I., Bourdoncle, W., Attagnant, C., Dellay, C., Gouy, A., Cadiou, S., Retailliau, C., Falque, M. and Gallais, A. (2001) Towards a better understanding of the genetic and physiological basis for nitrogen use efficiency in maize. *Plant Physiol.* **125:** 1258–1270.

Ikram, S., Bedu, M., Daniel-Vedele, F., Chaillou, S. and Chardon, F. (2012) Natural variation of Arabidopsis response to nitrogen availability. *J. Exp. Bot.* **63:** 91–105.

Jensen, O. N. (2004) Modification-specific proteomics: characterization of post-translational modifications by mass spectrometry. *Curr. Opin. Chem. Biol.* **8:** 33–41.

Kraakman, A. T. W., Niks, R. E., Van den Berg, P. M. M. M., Stam, P. and Van Eeuwijk, F. A. (2004) Linkage disequilibrium mapping of yield and yield stability in modern spring barley cultivars. *Genetics* **168:** 435–446.

Krouk, G., Ruffel, S., Gutiérrez, R. A., Gojon, A., Crawford, N. M., Coruzzi, G. M. and Lacombe, B. (2011) A framework integrating plant growth with hormones and nutrients. *Trends in Plant Sci.* **16:** 178–182.

Kwon, S. J., Choi, E. Y., Choi, Y. J., Ahn, J. H. and Park, O. K. (2006) Proteomics studies of post-translational modifications in plants. *J. Exp. Bot.* **57:** 1547–1551.

Lee, J.-Y., Colinas, J., Wang, J. Y., Mace, D., Ohler, U., and Benfey, P. N. (2006) Transcriptional and posttranscriptional regulation of transcription factor expression in Arabidopsis roots. *P. Natl. Acad. Sci. USA* **103:** 6055–6060.

Li, X. (2011) Arabidopsis Growing Protocol – A General Guide. *bio-protocol*. Retrieved from http://www.bio-protocol.org/wenzhang.aspx?id=126

Limami, A. M., Rouillon, C. and Hirel, B. (2002) Genetic and physiological analysis of germination efficiency in maize in relation to nitrogen metabolism reveals the importance of cytosolic glutamine synthetase. *Plant Physiol.* **130:** 1860–1870.

Magori, S. and Citovsky, V. (2011) Epigenetic control of Agrobacterium T-DNA integration. *Bioch. Bioph. Acta* **1809:** 388–394.

Maier, T., Güell, M. and Serrano, L. (2009) Correlation of mRNA and protein in complex biological samples. *FEBS Lett.* **583:** 3966–3973.

Martin, A., Lee, J., Kichey, T., Gerentes, D., Zivy, M., Tatout, C., Dubois, F., Balliau, T., Valot, B., Davanture, M., Tercé-Laforgue, T., Quilleré, I., Coque, M., Gallais, A., Gonzalez-Moro, M.-B., Bethencourt, L., Habash, D. Z., Lea, P. J., Charcosset, A., Perez, P., Murigneux, A., Sakakibara, H., Edwards, K. J. and Hirel, B. (2006) Two cytosolic glutamine synthetase isoforms of maize are specifically involved in the control of grain production. *Plant Cell* **18:** 3252–3274.

Matzke, A. J. and Matzke, M. A. (1998) Position effects and epigenetic silencing of plant transgenes. *Curr. Opin. Plant Biol.* **1:** 142–148.

McKenzie, D. J., McLean, M. A., Mukerji, S. and Green, M. (1997) Improved RNA extraction from woody plants for the detection of viral pathogens by reverse transcription – polymerase chain reaction. *Plant Dis.* **81:** 222–226.

Miller, A. J., Fan, X., Shen, Q. and Smith, S. J. (2008) Amino acids and nitrate as signals for the regulation of nitrogen acquisition. *J. Exp. Bot.* **59:** 111–119.

Miyashita, Y. (2008) *Plant amino acid metabolism under hypoxia and carbon starvation*. Ph.D thesis. University of Alberta.

Miyashita, Y., Dolferus, R., Ismond, K. P. and Good, A. G. (2007) Alanine aminotransferase catalyses the breakdown of alanine after hypoxia in *Arabidopsis thaliana*. *Plant J.* **49:** 1108–1121.

Muench, D. G. and Good, A. G. (1994) Hypoxically inducible barley alanine aminotransferase: cDNA cloning and expression analysis. *Plant Mol. Biol.* **24:** 417–427.

Nunes-Nesi, A., Fernie, A. R. and Stitt, M. (2010) Metabolic and signaling aspects underpinning the regulation of plant carbon nitrogen interactions. *Mol. Plant* **3:** 973–996.

Obara, M., Kajiura, M., Fukuta, Y., Yano, M., Hayashi, M., Yamaya, T. and Sato, T. (2001) Mapping of QTLs associated with cytosolic glutamine synthetase and NADH-glutamate synthase in rice (*Oryza sativa* L.). *J. Exp. Bot.* **52:** 1209–1217.

Oñate-Sánchez, L. and Vicente-Carbajosa, J. (2008) DNA-free RNA isolation protocols for *Arabidopsis thaliana*, including seeds and siliques. *BMC Res. Notes* **1:** 93.

Ono, K., Terashima, I. and Watanabe, A. (1996) Interaction between nitrogen deficit of a plant and nitrogen content in the old leaves. *Plant Cell Physiol.* **37:** 1083–1089.

Piccini, D. and Azcon, R. (1987) Effect of phosphate-solubilizing bacteria and vesicular-arbuscular mycorrhizal fungi on the utilization of Bayovar rock phosphate by alfalfa plants using a sand-vermiculite medium. *Plant Soil* **101:** 45–50.

Plaxton, W. C. (2010) Metabolic Flexibility Helps Plants to Survive Stress. In L. Taiz & E. Zeiger (Eds.). *Plant Physiology Online* (5th ed.).

Pourtau, N., Marès, M., Purdy, S., Quentin, N., Ruël, A. and Wingler, A. (2004) Interactions of abscisic acid and sugar signalling in the regulation of leaf senescence. *Planta* **219:** 765–772.

Price, J., Laxmi, A. and Jang, J. (2004). Global transcription profiling reveals multiple sugar signal transduction mechanisms in Arabidopsis. *Plant Cell* **16:** 2128–2150.

Qin, F., Shinozaki, K. and Yamaguchi-Shinozaki, K. (2011) Achievements and challenges in understanding plant abiotic stress responses and tolerance. *Plant Cell Physiol.* **52:** 1569–1582.

Quigley, F., Villiot, M., Mache, R., Biologie, L. De, Fourier, U. J. and Ura, C. (1991). Nucleotide sequence and expression of a novel glycine-rich protein gene from *Arabidopsis thaliana*. *Plant Mol. Biol.* **17:** 949–952.

Ratcliffe, O. J., Nadzan, G. C., Reuber, T. L. and Riechmann, J. L. (2001) Regulation of flowering in Arabidopsis by an FLC homologue. *Plant Physiol.* **126:** 122–132.

Salerno, G. L. and Curatti, L. (2003) Origin of sucrose metabolism in higher plants: when, how and why? *Trends Plant Sci.* **8:** 63–69.

Sang, Y., Sun, W. and Yang, Z. (2012) Signaling mechanisms integrating carbon and nitrogen utilization in plants. *Front. Biol.* **7:** 548–556.

Schmitz, J., Heinrichs, L., Scossa, F., Fernie, A. R., Oelze, M.-L., Dietz, K.-J., Rothbart, M., Grimm, B., Flügge, U.-I. and Häusler, R. E. (2014) The essential role of sugar metabolism in the acclimation response of *Arabidopsis thaliana* to high light intensities. *J. Exp. Bot.* **65:** 1619–1636.

Schmitz, J., Schöttler, M. A., Krueger, S., Geimer, S., Schneider, A., Kleine, T., Leister, D., Bell, K., Flügge, U.-I. and Häusler, R. E. (2012) Defects in leaf carbohydrate metabolism compromise acclimation to high light and lead to a high chlorophyll fluorescence phenotype in *Arabidopsis thaliana*. *BMC Plant Biol.* **12:** 8.

Shrawat, A. K., Carroll, R. T., DePauw, M., Taylor, G. J. and Good, A. G. (2008) Genetic engineering of improved nitrogen use efficiency in rice by the tissue-specific expression of alanine aminotransferase. *Plant Biotech. J.* **6:** 722–732.

Sun, J., Gibson, K. M., Kiirats, O., Okita, T. W. and Edwards, G. E. (2014) Interactions of nitrate and CO_2 enrichment on growth, carbohydrates, and rubisco in Arabidopsis starch mutants. Significance of starch and hexose. *Plant Physiol.* **130:** 1573–1583.

Tegeder, M. (2012) Transporters for amino acids in plant cells: some functions and many unknowns. *Curr. Opin. Plant Biol.* **15:** 315–321.

Tegeder, M. and Rentsch, D. (2010) Uptake and partitioning of amino acids and peptides. *Mol. Plant* **3:** 997–1011.

Vogel, C. and Marcotte, E. M. (2013) Insights into the regulation of protein abundance from proteomic and transcriptomic analyses. *Nat. Rev. Genet.* **13:** 227–232.

Wingler, A., Purdy, S., MacLean, J. A. and Pourtau, N. (2006) The role of sugars in integrating environmental signals during the regulation of leaf senescence. *J. Exp. Bot.* **57:** 391–399.

Yamaguchi, A. and Abe, M. (2012) Regulation of reproductive development by non-coding RNA in Arabidopsis: to flower or not to flower. *J. Plant Res.* **125:** 693–704.

Yamaya, T., Obara, M., Nakajima, H., Sasaki, S., Hayakawa, T. and Sato, T. (2002) Genetic manipulation and quantitative-trait loci mapping for nitrogen recycling in rice. *J. Exp. Bot.* **53:** 917–925.

Yoo, S.-D., Cho, Y.-H. and Sheen, J. (2007) Arabidopsis mesophyll protoplasts: a versatile cell system for transient gene expression analysis. *Nat. Prot.* **2:** 1565–1572.

Zheng, Z.-L. (2009) Carbon and nitrogen nutrient balance signaling in plants. *Plant Signal. Behav.* **4:** 584–591.

Appendix 4.1. Fresh weights of biological replicates of four Arabidopsis seedlings used for analysis of AlaAT enzyme activity and westerns.

Plants were grown on vertical plates at 21°C, 60% humidity, ~170 µE m^{-2} sec^{-1} and 16 hrs light/4 hrs dark for ~2 weeks. Four seedlings were grouped to determine a single weight. Two separate grow-ups were conducted. Control plants (Col-0 and alaat1;2) displayed in the table were grown at the same time as transgenics and were used as comparables.

Line	Background	Biological Replicate	Fresh weight of four plants (mg)	Line	Background	Biological Replicate	Fresh weight of four plants (mg)
alaat1;2	N/A	1	17	35S:HvAlaAT 4-1-5	Col-0	1	46
		2	25			2	58
		3	54			3	67
		4	N/A			4	83
Col-0	N/A	1	47	35S:HvAlaAT 4-3-3	Col-0	1	70
		2	48			2	61
		3	46			3	59
		4	54			4	65
OsAnt1:HvAlaAT 1-1-5-3	Col-0	1	52	35S:HvAlaAT 3-2-2	Col-0	1	57
		2	60			2	63
		3	46			3	73
		4	73			4	50
OsAnt1:HvAlaAT 2-2-3-3	Col-0	1	84	35S:HvAlaAT 8-1-2	alaat1;2	1	68
		2	40			2	96
		3	68			3	93
		4	60			4	52
OsAnt1:HvAlaAT 3-1-2	Col-0	1	67	35S:HvAlaAT 6-1-3-2	alaat1;2	1	42
		2	77			2	29
		3	57			3	39
		4	67			4	67
OsAnt1:HvAlaAT 2-3-2	alaat1;2	1	50	35S:HvAlaAT 5-1-4	alaat1;2	1	66
		2	76			2	80
		3	96			3	67
		4	78			4	104
OsAnt1:HvAlaAT 3-1-1	alaat1;2	1	33				
		2	45				
		3	47				
		4	75				
OsAnt1:HvAlaAT 4-2-2	alaat1;2	1	46				
		2	57				
		3	55				
		4	60				

Appendix 4.2. Fresh weights of biological replicates of four Arabidopsis seedlings used for analysis of AlaAT enzyme activity and westerns.

Plants were grown on vertical plates at 21°C, 60% humidity, ~170 µE m^{-2} sec^{-1} and 16 hrs light/4 hrs dark for ~2 weeks. Four seedlings were grouped to determine a single weight. Two separate grow-ups were conducted. Control plants (Col-0 and alaat1;2) displayed in the table were grown at the same time as transgenics and were used as comparables.

Line	Background	Biological Replicate	Fresh weight of four plants (mg)	Line	Background	Biological Replicate	Fresh weight of four plants (mg)
alaat1;2	N/A	1	88	35S:MmAlaAT2 7-1-1	alaat1;2	1	102
		2	74			2	119
		3	166			3	100
		4	120			4	89
Col-0	N/A	1	62	35S:MmAlaAT2 7-3-3	alaat1;2	1	92
		2	62			2	103
		3	82			3	104
		4	76			4	121
35S:MmAlaAT1 3-1-3	Col-0	1	86	35S:MmAlaAT2 8-1-4	alaat1;2	1	93
		2	92			2	78
		3	63			3	84
		4	73			4	82
35S:MmAlaAT1 2-1-7	Col-0	1	85	35S:PfAlaAT 3-1-4	Col-0	1	44
		2	85			2	80
		3	94			3	86
		4	100			4	81
35S:MmAlaAT1 1-2-1	Col-0	1	101	35S:PfAlaAT 4-1-2	Col-0	1	70
		2	104			2	107
		3	95			3	87
		4	85			4	80
35S:MmAlaAT1 3-1-5-5	alaat1;2	1	57	35S:PfAlaAT 3-2-2	Col-0	1	95
		2	64			2	109
		3	60			3	82
		4	74			4	84
35S:MmAlaAT1 3-3-1	alaat1;2	1	116	35S:PfAlaAT 7-1-1	alaat1;2	1	105
		2	140			2	77
		3	117			3	84
		4	139			4	104
35S:MmAlaAT1 3-4-3	alaat1;2	1	112	35S:PfAlaAT 6-4-3	alaat1;2	1	95
		2	84			2	88
		3	81			3	77
		4	37			4	91
35S:MmAlaAT2 6-3-3-4	Col-0	1	68	35S:PfAlaAT 6-2-2	alaat1;2	1	57
		2	77			2	96
		3	117			3	107
		4	100			4	85
35S:MmAlaAT2 6-1-3	Col-0	1	89				
		2	84				
		3	78				
		4	72				
35S:MmAlaAT2 6-2-1	Col-0	1	91				
		2	97				
		3	99				
		4	94				

Appendix 4.3. Results of the two-tailed Mann-Whitney *U*-test used to compare AlaAT activity levels of transgenic in the alaat1;2 background to control lines.

A two-tailed Mann-Whitney *U*-test ($P < 0.05$, n = 3-6) was used to compare the AlaAT enzyme activity levels in transgenic AlaAT-expressing *Arabidopsis thaliana*. Activity levels were determined using concentrated, full protein fractions from eight, two week old seedlings in biological duplicate. Each protein fraction was assayed in triplicate and the biological replicates pooled to obtain average activity levels for each of the transgenic and control lines.

Line	Comparative control	*P* value	Significant
OsAnt1:HvAlaAT 2-3-2	alaat1;2 Col-0	0.0571 0.0095	No Yes
OsAnt1:HvAlaAT 3-1-1	alaat1;2 Col-0	0.0357 0.0043	Yes Yes
OsAnt1:HvAlaAT 4-2-2	alaat1;2 Col-0	0.0357 0.0043	Yes Yes
35S:HvAlaAT 8-1-2	alaat1;2 Col-0	0.0357 0.0043	Yes Yes
35S:HvAlaAT 6-1-3-2	alaat1;2 Col-0	0.0571 0.0095	No Yes
35S:HvAlaAT 5-1-4	alaat1;2 Col-0	0.0357 0.3290	Yes No
35S:MmAlaAT1 3-1-5-5	alaat1;2 Col-0	0.0043 0.0043	Yes Yes
35S:MmAlaAT1 3-3-1	alaat1;2 Col-0	0.0022 0.0022	Yes Yes
35S:MmAlaAT1 3-4-3	alaat1;2 Col-0	0.0043 0.9307	Yes No
35S:MmAlaAT2 7-1-1	alaat1;2 Col-0	0.0173 0.0043	Yes Yes
35S:MmAlaAT2 7-3-3	alaat1;2 Col-0	0.0022 0.0022	Yes Yes
35S:MmAlaAT2 8-1-4	alaat1;2 Col-0	0.0043 0.0043	Yes Yes
35S:PfAlaAT 7-1-1	alaat1;2 Col-0	0.0095 0.0095	Yes Yes
35S:PfAlaAT 6-4-3	alaat1;2 Col-0	0.0022 0.0087	Yes Yes
35S:PfAlaAT 6-2-2	alaat1;2 Col-0	0.0022 0.9372	Yes No

Appendix 4.4. Results of the two-tailed Mann-Whitney *U*-test used to compare AlaAT activity levels of transgenic in the Col-0 background to control lines.

A two-tailed Mann-Whitney *U*-test ($P < 0.05$, n = 3-6) was used to compare the AlaAT enzyme activity levels in transgenic AlaAT-expressing *Arabidopsis thaliana*. Activity levels were determined using concentrated, full protein fractions from eight, two week old seedlings in biological duplicate. Each protein fraction was assayed in triplicate and the biological replicates pooled to obtain average activity levels for each of the transgenic and control lines.

Line	Comparative control	*P* value	Significant
OsAnt1:HvAlaAT 1-1-5-3	alaat1;2 Col-0	0.0238 0.0152	Yes Yes
OsAnt1:HvAlaAT 2-2-3-3	alaat1;2 Col-0	0.0238 0.1320	Yes No
OsAnt1:HvAlaAT 3-1-2	alaat1;2 Col-0	0.0238 0.0022	Yes Yes
35S:HvAlaAT 4-1-5	alaat1;2 Col-0	0.0238 0.9372	Yes No
35S:HvAlaAT 4-3-3	alaat1;2 Col-0	0.0238 0.9372	Yes No
35S:HvAlaAT 3-2-2	alaat1;2 Col-0	0.0357 0.1255	Yes No
35S:MmAlaAT1 3-1-3	alaat1;2 Col-0	0.0022 0.0238	Yes Yes
35S:MmAlaAT1 2-1-7	alaat1;2 Col-0	0.0238 0.0022	Yes Yes
35S:MmAlaAT1 1-2-1	alaat1;2 Col-0	0.0022 0.0022	Yes Yes
35S:MmAlaAT2 6-3-3-4	alaat1;2 Col-0	0.0043 0.0043	Yes Yes
35S:MmAlaAT2 6-1-3	alaat1;2 Col-0	0.0022 0.1797	Yes No
35S:MmAlaAT2 6-2-1	alaat1;2 Col-0	0.0022 0.0022	Yes Yes
35S:PfAlaAT 3-1-4	alaat1;2 Col-0	0.0022 0.6991	Yes No
35S:PfAlaAT 4-1-2	alaat1;2 Col-0	0.0022 0.0022	Yes Yes
35S:PfAlaAT 3-2-2	alaat1;2 Col-0	0.0022 0.0022	Yes Yes

5. General Discussion and Conclusions

It has been well established, both in the greenhouse and the field, that tissue-specific over-expression of *HvAlaAT* in rice (Beatty *et al*., 2013; Shrawat *et al*., 2008) and canola (Good *et al*., 2007) produces plants that have increased NUE. In an effort to decipher the reasons for this AlaAT-induced NUE phenotype, previous studies have focused on the importance of both promoter (Lock, 2011) and transaminase specificity (Wolansky, 2005), while others have focused on alterations in the transcriptome (Beatty *et al*., 2009) and the metabolome (Beatty *et al*., 2013) as a result of the over-expression of this enzyme. Still, none of these studies have been able to elucidate how AlaAT over-expression results in an NUE phenotype. Moreover, these studies have not yet been able to determine how to further maximize the *AlaAT*-specific NUE phenotype already seen in crop plants.

The current study was aimed at elucidating if homologous AlaAT variants have different kinetic properties (K_Ms) *in vitro* (Chapter 2), and if these alterations in enzyme kinetics manifest as alterations in NUE phenotypes, both physical and physiological, *in vivo* in *Arabidopsis thaliana* (Chapter 3 and 4). Large scale analysis of enzyme variants is not common, especially in terms of plant biology; however, the value of such analyses is growing. Variant analysis for the vitamin A enhanced "Golden Rice" (Paine *et al*., 2005) as well as variants that promote increased fruit and vegetable shelf life (Xiong *et al*., 2005), emphasizes the importance as well as the benefits of doing such studies. Work carried out here in both *E. coli* and Arabidopsis was done with the knowledge that significant or interesting findings would ultimately be tested in crop plants, specifically rice and barley.

5.1. AlaAT enzymes display varying kinetic properties

An analysis of the kinetic properties of seven AlaAT enzymes and two GGATs was conducted first (Chapter 2). While various kinetic studies had already been carried out on a number of the AlaAT homologues analyzed here (Good & Muench, 1992; Ward *et al*., 2000; Liepman & Olsen, 2003; Wiśniewski *et al*., 2006; Duff *et al*., 2012), an analysis of this scale had not previously been performed. Furthermore, in order to accurately compare the kinetic qualities

of all of the AlaAT enzymes, it was essential to analyze all of the enzymes under the same conditions, in the same expression system with the same method of detection (Marangoni, 2003).

Initial comparative analysis of the primary structure of the proteins chosen for study indicated PfAlaAT was the most divergent, and that AtGGAT enzymes, with secondary AlaAT function, were more similar to plant AlaAT enzymes (Arabidopsis, Medicago and barley) than mammalian (mouse), yeast, or Archaen (Pyrococcus) proteins (Figure 2.1). It was shown that, *in vitro*, AlaAT enzymes have significantly diverse K_M values for the same substrate; however, no distinct patterns, or groupings of enzymes based on AlaAT kinetic abilities were determined (Table 2.2). Therefore, in order to determine which AlaAT proteins may be of interest in terms of NUE in crop plants, all kinetic results were compared to those of HvAlaAT, since over-expression of this AlaAT variant has been shown to provide an NUE phenotype in canola and rice. MmAlaAT1 appeared to have the most different kinetic results relative to HvAlaAT, with extremely high K_M values for the substrates alanine, glutamate and pyruvate (26.5 mM, 13.0 mM and 12.5 mM respectively). Unfortunately, due to the bactericidal nature of AlaAT (Figure 2.3 and Figure 2.4), an analysis of the impact these AlaAT variants have on the growth and development of *E. coli* was not possible.

There were many benefits to how this analysis was conducted. Direct analysis of K_M values from AlaAT variants was made possible because enzymes were placed in the same system, under the same inducible promoter, and analyzed under similar conditions however, in terms of then relating these results to *in vivo* conditions, or the feasibility of expressing these proteins in plants, this sort of analysis falls short. How these enzymes behave in an ideal laboratory environment, versus how they will then behave in a plant cell, either in the cytoplasm or mitochondria, could potentially be extremely different. For instance, PfAlaAT is naturally expressed in an organism that grows optimally at 100°C. This study was conducted at room temperature (~21°C). Temperature is known to affect both enzyme folding and activity (Marangoni, 2003). Because of this, the K_Ms reported here may not be an accurate representation of how these enzymes function in nature, but they do allow for a direct comparison and provide a starting point toward understanding enzymatic differences between these homologous proteins.

Taken all together, these results show that AlaAT homologues have distinct biochemical differences, and that *in planta*, these differences could potentially result in alterations in NUE phenotypes. Primary structure analysis indicated that PfAlaAT was the most structurally divergent homologue, while kinetic analysis showed that MmAlaAT1 had the most distinctly different K_M values when compared to HvAlaAT. Based on these results, both of these proteins, PfAlaAT and MmAlaAT1, along with HvAlaAT for comparison, were chosen to transform into *Arabidopsis thaliana*. These genes have also been introduced into rice, but this work is at a very preliminary stage. MmAlaAT2 was also chosen for analysis *in planta* because it is a mitochondrially targeted AlaAT.

5.2. AlaAT variants have differing effects on NUE phenotypes in *Arabidopsis thaliana*

The physiological importance of AlaAT in the post-hypoxic stress response has been well documented (Miyashita *et al*., 2007). During recovery from low-oxygen stress, AlaAT (*alaat1-1*) is crucial for the conversion of alanine to pyruvate in Arabidopsis (Miyashita *et al*., 2007), indicating that *in vivo*, at least after hypoxic stress, the native AlaAT favours this reaction. Analysis of transgenic canola (Good *et al*., 2007) and rice (Shrawat *et al*., 2008; Beatty *et al*., 2013, 2009) provided no data regarding which reaction direction is favoured by the enzyme, when over-expressed in N-limiting or non-limiting conditions. Experiments aimed at solving this have been confounded by the complexity of plant sensing, signaling and metabolic pathways, as well as the transient nature of the enzyme's substrates, which serve as intermediates for numerous C and N pathways (Zheng, 2009; Masclaux-Daubresse *et al*., 2010; Kant *et al.*, 2011). In the current study, AlaAT enzymes with differing kinetics *in vitro* were transformed into *Arabidopsis thaliana*, and plants were monitored for alterations in plant growth, development and physiology. It was hypothesized that, if AlaAT enzymes behave significantly differently *in vivo*, as they do *in vitro*, this may then manifest as quantifiable alterations in physical or physiological growth and development, as well as increases or decreases in the NUE of the plant.

When this study began, it was unknown if Arabidopsis could be utilized as a model for NUE in cereal crops, specifically *AlaAT* over-expression studies. While studies such as those

looking at root architecture and freezing tolerance have been conducted in Arabidopsis, differences such as anatomy, gene number and metabolism (ie: preference for NH_4^+ as N source in rice) must be kept in mind (Livingston *et al*., 2007; Smith & De Smet, 2012; Assmann, 2013). Previous physical characterization of *AlaAT* over-expressing rice plants indicated that in both medium and high N conditions, where statistically significant increases in NUE were observed, transgenic plants exhibited increased root biomass (Shrawat *et al.*, 2008; Beatty *et al*., 2013). For this reason, initial phenotypic analysis focused on alterations in root growth of transgenic plants.

Plate assays were utilized to help establish initial NUE-like phenotypes in Arabidopsis due to ease of root observation (Chapter 3). Transgenic *AlaAT*-expressing and over-expressing plants showed, for the most part, increases in rate of root growth, tap root length and lateral root growth in relation to N availability and type, as seen in rice (Shrawat *et al*., 2008) (Chapter 3), indicating that Arabidopsis can be used as a starting point for analysis of AlaAT-linked NUE in plants. Increases in overall root length and average rate of root growth were also observed when either alanine or glutamate was given as the sole N source, indicating that transgenic over-expressers can more effectively utilize these amino acids as N sources (Figure 3.8). Variations in C and N availability and source also resulted in significant differences in growth and development of *AlaAT* over-expressing plants (Figure 3.13 and Figure 3.14). However, unlike previous studies, alterations in growth and development were observed not only between transgenics and controls, but also between transgenic lines containing different *AlaAT* genes (Figure 3.5, Figure 3.13, Figure 3.14, Figure 4.9, Figure 4.9 and Figure 4.10). These alterations in root growth appeared to be due to enhanced plant NUE, probably by altering the "plant nitrogen economics" (Zhang & Forde, 2000; Zhang *et al*., 2007; Krouk *et al*., 2011; Ruffel *et al*., 2011), and not due to stress or foraging responses, as transgenic plants did not display characteristic stress related phenotypes compared to control plants. These results support previous observations in rice and canola that over-expression of *AlaAT* produces an NUE phenotype, however they also demonstrate that *in vivo*, choice of *AlaAT* variant and promoter can significantly impact the NUE phenotype observed. Over-expression of *PfAlaAT* driven by a 2X *CaMV35S* promoter produced the most distinct alterations in phenotype in relation to differences in both N and C availability, and resulted in the greatest increase in NUE when both C and N were limiting. *MmAlaAT1* driven by the same promoter produced plants that displayed

the most significant increase in NUE when N was the only variable altered, either in a limiting or non-limiting capacity.

Although plate assays were ideal for the analysis of root growth and architecture to establish similarities in NUE phenotypes between rice and Arabidopsis, the feasibility and applicability of such experiments to that of field experiments is, in reality, low. It was therefore important to determine that the significant differences observed during plate assays could be extrapolated to pot based trials (Chapter 4). Not only do pot based assays allow for a more accurate representation of how plants may perform in the field, but they also allow a much broader range of development to be observed (germination through to seed maturation and whole plant senescence, if desired). Along with the analysis of physical characteristics, plants grown in a soilless medium were also used as a means of studying the physiological alterations produced in Arabidopsis plants as a result of the expression of various AlaAT enzymes.

Plants grown using soilless medium exhibited increased biomass as observed during the plate assays, along with several other later developmental growth phenotypes. Increased total rosette leaf area as well as slightly increased time to flowering, were observed in all transgenic lines relative to controls (Chapter 4). These observations give support to the previous plate assay phenotypes as well as lending support to the phenotypes observed in transgenic *HvAlaAT* rice. Given these significant increases in biomass, it was assumed that transgenic plants would show alterations in the levels of soluble sugars, which are known to fluctuate with variations in the C:N ratio, photosynthetic activity, and the allocation of carbon skeletons between storage, growth and respiration (Price *et al*., 2004; Ramel *et al.*, 2009; Sun *et al*., 2014). Analysis of the concentrations of glucose, sucrose and fructose showed decreased levels of these compounds, on average, in transgenic lines relative to controls. This information, along with the data showing increased biomass, leaf area and time to flowering indicates that, in general, *AlaAT* over-expressing plants are more effectively assimilating and mobilizing N compounds, especially under conditions of adequate N supply (2 mM NO_3^-), which is ultimately resulting in increased storage of C skeletons and a decrease in the concentration of soluble sugars in photosynthetic tissues. The analysis of total starch content in these tissues would be useful in verifying these results, along with an analysis of C movement within the whole plant (^{14}C), utilizing LC-MS.

An experiment utilizing mesophyll protoplast cells and their ability to easily take up externally supplied, labeled compounds was available. This study proved interesting for two reasons. First, it indicated that increased AlaAT activity within photosynthetic tissues does not result in an increased ability to take up its substrate compound, alanine, under the experimental conditions. Secondly, it showed that cell lines expressing *PfAlaAT* have a significant capacity to take up externally supplied amino acids above that of either Col-0 or other *AlaAT* over-expressing lines (Figure 4.8 and Figure 4.9). These results imply that *PfAlaAT* expressing cells have an increased capacity to assimilate and mobilize N as well as C (either through storage or relocation), as increased uptake of these externally supplied compounds would suggest that the internal concentration of compounds was decreased initially. Again, either a whole-plant radioisotope uptake experiment, or further analysis with protoplasts and other radioisotopes, specifically glutamate and NO_3^-, would help verify these results.

From these studies, it can be observed that two distinct N responses are occurring. In roots, tap root elongation as well as increased lateral root branching would indicate either foraging or increased demand for N due to high assimilation and mobilization. In either circumstance, root cells are responding as though internal N is depleted. On the other hand, shoot cells are showing decreased soluble sugars, and increased area and biomass, indicating that there is an abundance of N in these cells, and that shoot cells are sensing N replete conditions. Therefore, I propose that the main role of AlaAT when over-expressed *in planta* is to increase N assimilation, which in turn, indirectly effects N mobilization. However, given that shoots and roots appear to be sensing different internal N levels, I propose that the reaction direction favoured by the recombinant AlaAT enzyme *in vivo*, and thus the assimilatory role, is highly dependent on the tissue of expression (Figure 5.1 and Figure 5.2).

5.3. Elucidating the mechanism of NUE when over-expressing AlaAT

AlaAT is a bidirectional PLP-dependent enzyme, transferring an amino group from alanine to 2-oxoglutarate to produce pyruvate and glutamate, or vice versa. Under certain conditions (ie: hypoxia), the use of mutants has allowed us to deduce the primary reaction direction of this enzyme *in vivo* (alanine to pyruvate) (Miyashita *et al*., 2007). In the case of NUE however, we have not yet been able to elucidate directionality of the enzyme (Beatty *et al.*,

2013; Shrawat *et al*., 2008). Part of the purpose of conducting a kinetic analysis of AlaAT variants was in the hope of deducing that different AlaAT enzymes highly favoured one reaction direction over the other, however based on the *in vitro* results, such a conclusion could not be reached. Data in Chapters 3 and 4, showing phenotypic analysis of *AlaAT*-expression and over-expression in Arabidopsis, does not allow for a concrete conclusion to be reached either.

Figure 5.1 presents a root cell model in which *AlaAT* over-expression results in an increase in both 2-oxoglutarate and alanine. This reaction is also favoured by the endogenous AlaAT enzymes in the post-hypoxic stress response (Miyashita *et al*., 2007).) It is estimated that in most plant cells, cytosolic glutamate concentrations are far above those of either alanine or 2-oxoglutarate, with pyruvate concentrations generally slightly greater than either alanine or 2-oxoglutarate (Winter *et al*., 1994; Juszczuk & Rychter, 2002; Watanabe *et al.*, 2014). This alone could result in increased AlaAT activity in the direction pyruvate to alanine. However, it has been shown in a variety of plant systems that unlike most other amino acids, glutamate concentrations are held extremely constant during the diurnal cycle, growth and development of the plant as well as in times of stress (Scheible *et al*., 2000; Urbanczyk-Wochniak & Fernie, 2005; Fritz *et al.*, 2006; Lea *et al.*, 2006; Reumann & Weber, 2006; Osuna *et al.*, 2007); increases or decreases in glutamate concentration have been observed to significantly deviate only under extreme conditions (ie: feeding of 100 mM glutamate (Masclaux-Daubresse *et al*., 2005), or 48 hr N deprivation (Scheible *et al*., 2004)).

There are two main paths that allow for glutamate synthesis in plants, either by conversion of glutamine through GS/GOGAT or through transformation of other amino acids by aminotransferases, however, both of these pathways require a sufficient supply of 2-oxoglutarate (Forde & Lea, 2007), which is thought to be supplied mainly by the action of isocitrate dehydrogenase (Hodges, 2002). In metabolite studies, while feeding plants high levels of exogenous amino acids does very little to effect the glutamate concentration within, the feeding of 2-oxoglutarate has shown to significantly increase internal glutamate concentrations (Abiko *et al*., 2005; Hodges, 2002). Thus, a system which increases 2-oxoglutarate internally should also increase overall levels of glutamate. This is depicted in Figure 5.1, where increased concentrations of 2-oxoglutarate, produced by AlaAT, are fed back into the GS/GOGAT cycle to produce more glutamate. In this system, the limiting factors would therefore be pyruvate and

inorganic N, which under the growth conditions of this study should not have been lacking. Under this model, cytosolic glutamate would be quickly converted to alanine and 2-oxoglutarate, lowering the internal concentrations of glutamate and resulting in a cell that senses decreased glutamate and perceives decreased cellular N. Alanine, is not thought be sensed as an N transport molecule and could be used to store and/or transport N without the plant sensing high internal N concentrations (Dietz *et al*., 1990; Beatty *et al*., 2013). Following this perceived decrease in N, plants would aim to increase N uptake, resulting in increased tap root lengths and number of lateral roots into N-rich areas. With this increased uptake of external N, cellular increases in 2-oxoglutarate, from the AlaAT reaction, would be fed directly back into the GS/GOGAT cycle, ultimately increasing N assimilation. With this increased N assimilation an increase in glutamate derived amino acids would be observed, as is seen in transgenic *HvAlaAT* rice (Beatty *et al*., 2013; Shrawat *et al*., 2008).

Figure 5.2 presents a shoot cell model in which *AlaAT* over-expression results in an increase in both glutamate and pyruvate in shoot cells. I hypothesis that glutamate is accumulating in these tissues because of the increases in biomass and leaf area, and the decreases in soluble sugars, observed during the course of this study, that indicate shoot tissues sense abundant N. As described above, increased AlaAT should result in increased N assimilation in the roots and therefore an increase in glutamate-derived transport amino acids (asparagine, glutamine and asparagine); alanine and glutamate could also be used as transport amino acids along with NO_3^-. Keeping in mind the extremely tight regulation of C:N by the plant, an increase in the concentration of amino acids or other N compounds being delivered to the shoots via the xylem would signal to shoot tissues that there was increased N availability, which would allow for accumulation of C compounds via increased photosynthesis (Coruzzi & Bush, 2001; Coruzzi & Zhou, 2001; Lawlor, 2002). Not only are these amino acids being delivered to the shoots in abundance, shoot tissue-made glutamate can accumulate as well through the action of native shoot aminotransferases and GS/Fd-GOGAT. As discussed previously, the concentration of glutamate, regardless of plant tissue type, is tightly controlled with very little fluctuation in cellular concentration (Scheible *et al*., 2000; Urbanczyk-Wochniak & Fernie, 2005; Fritz *et al*., 2006; Reumann & Weber, 2006; Osuna *et al*., 2007) Now, given an environment that allows for sufficient photosynthesis, neither pyruvate nor 2-oxoglutarate will be limiting, as both of these substrates can be provided via glycolysis and the TCA cycle, respectively, and alanine, as a

storage amino acid would also be available in these tissues (Dietz *et al.*, 1990). In respect to 2-oxoglutarate, increases in cellular glutamate concentration would directly increase this compound through the action of glutamate dehydrogenase, which is highly active in photosynthetic tissues (Miyashita & Good, 2008b). This increase in 2-oxoglutarate would in turn increase glutamate via GS/Fd-GOGAT, much as the cycle described in the root AlaAT model. Increased glutamate could then be converted back to other N compounds, C compounds or larger protein complexes for storage (ie: asparagines, sugars/starch and Rubisco) (Masclaux-Daubresse *et al.*, 2010).

Mesophyll protoplast analysis of radiolabelled glutamate may be able to determine if glutamate is built-up in leaf tissues as a result of *AlaAT* over-expression. Extrapolating these results to a whole plant model, using radiolabeled alanine, glutamate or 2-oxoglutarate may also allow for a determination of the internal levels of each of these substrates. A diurnal metabolic analysis of both 2-oxoglutarate and glutamate concentrations under different lighting regimes may be able to determine if this hypothesis is correct, as the two presented models work under the assumption that C is not limiting. In this way plate assays may also be useful, either in creating a concentration curve of different C concentrations, or a concentration curve of glutamate concentrations, which can then be used to analyze metabolite levels over a range of N and C availabilities. Of particular interest may also be Arabidopsis TOR genes and the TOR pathway in plants, considering that this pathway controls root meristem activiation (Xiong *et al*., 2013).

In summary, I propose that AlaAT increases NUE in plants through increased N assimilation. Both models, regardless of tissue, work under the assumption that an increase in 2-oxoglutarate is directly driving accumulation of glutamate, which in turn is modulating internal available N levels. Given limited C, it was shown that plants expressing *PfAlaAT* have increased growth relative to controls and other transgenic lines. This implies that this enzyme may be more sensitive to alterations in substrate levels, and therefore regulates the reaction direction more acutely in response to environmental changes and internal alterations in the C to N ratio. With this in mind, I recommend that *PfAlaAT* be expressed in crop plants, such as rice, in order to further increase NUE in these plants, as well as increase fitness of these crops in environments that may be either N or C limiting, or both.

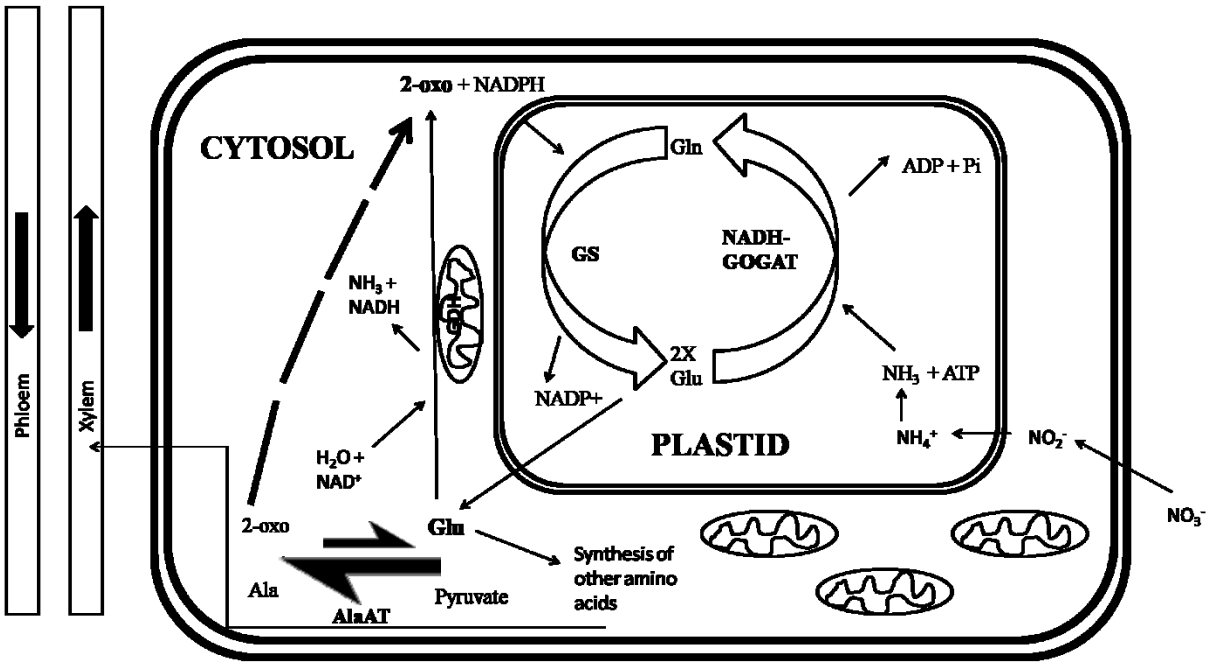


Figure 5.1. Proposed model for increased AlaAT enzyme activity in root cells.

Transgenic AlaAT, over-expressed in root cells favouring the reaction direction glutamate to 2-oxoglutarate under both N and C non-limiting conditions. C metabolism has been omitted for brevity. GS1, glutamine synthetase 1; NADH-GOGAT, NADH-glutamate synthase; GDH, glutamate dehydrogenase; 2-oxo, 2-oxoglutarate; Ala, alanine; Glu, glutamate; Gln, glutamine.

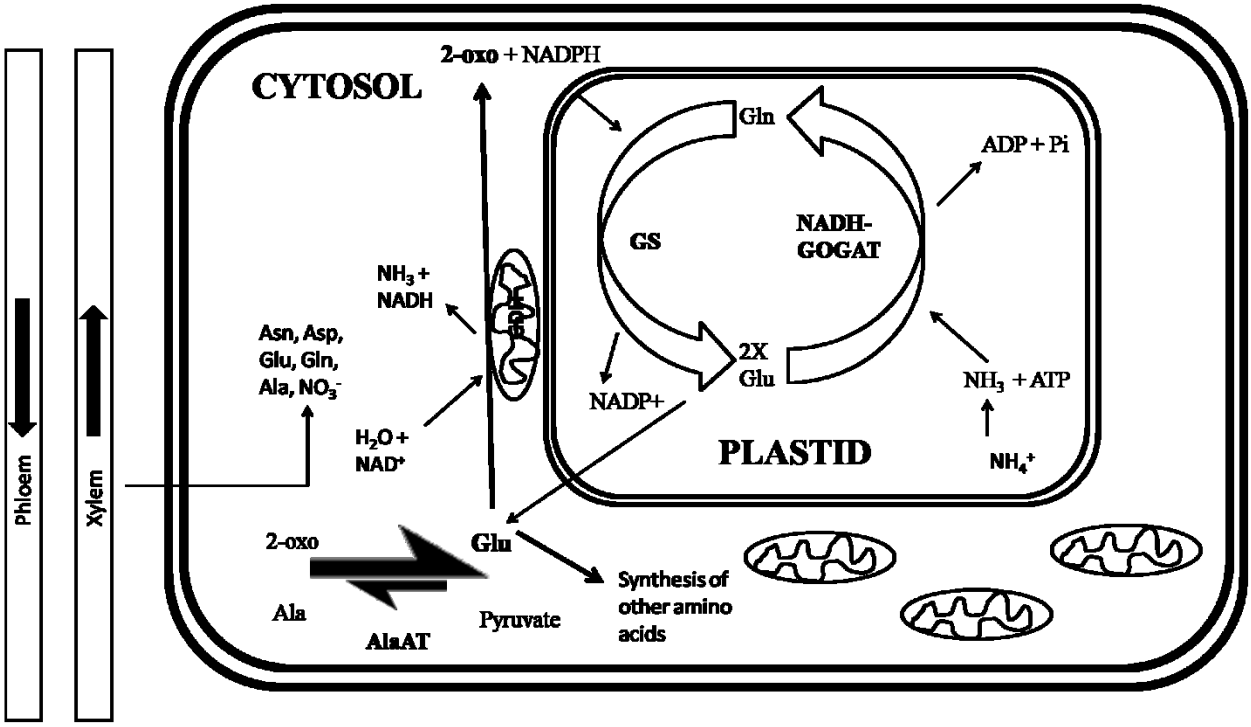


Figure 5.2. Proposed model 2 for increased AlaAT enzyme in shoot cells.

Transgenic AlaAT, over-expressed in shoot cells favouring the reaction direction 2-oxoglutarate to glutamate under both N and C non-limiting conditions. C metabolism has been omitted for brevity. GS1, glutamine synthetase 1; NADH-GOGAT, NADH-glutamate synthase; GDH, glutamate dehydrogenase; 2-oxo, 2-oxoglutarate; Ala, alanine; Glu, glutamate; Gln, glutamine.

5.5. Bibliography

Beatty, P. H., Carroll, R. T., Shrawat, A. K., Guevara, D. and Good, A. G. (2013) Physiological analysis of nitrogen-efficient rice overexpressing alanine aminotransferase under different N regimes. *Botany* **91:** 866–883.

Beatty, P. H., Shrawat, A. K., Carroll, R. T., Zhu, T. and Good, A. G. (2009) Transcriptome analysis of nitrogen-efficient rice over-expressing alanine aminotransferase. *Plant Biotech. J.* **7:** 562–576.

Bernier, G., Havelange, A., Houssa, C., Petitjean, A. and Lejeune, P. (1993) Physiological signals that induce flowering. *Plant Cell* **5:** 1147–1155.

Butaye, K. M. J., Goderis, I. J. W. M., Wouters, P. F. J., Pues, J. M.-T. G., Delauré, S. L., Broekaert, W. F., Depicker, A., Cammue, B. P. A. and De Bolle, M. F. C. (2004) Stable high-level transgene expression in *Arabidopsis thaliana* using gene silencing mutants and matrix attachment regions. *Plant J. 39*: 440–449.

Calenge, F., Saliba-Colombani, V., Mahieu, S., Loudet, O., Daniel-Veldele, F. and Krapp, A. (2006) Natural variation for carbohydrate content in Arabidopsis. Interaction with complex traits dissected by quantitative genetics. *Plant Physiol.* **141:** 1630–1643.

Callis, J., Raasch, J. A. and Vierstrass, R. D. (1990) Ubiquitin extension proteins of *Arabidopsis thaliana*: structure, localization, and expression of their promoters in transgenic tobacco. *J. Biol. Chem.* **265:** 12486–12493.

Castro Marín, I., Loef, I., Bartetzko, L., Searle, I., Coupland, G., Stitt, M. and Osuna, D. (2011). Nitrate regulates floral induction in Arabidopsis, acting independently of light, gibberellin and autonomous pathways. *Planta* **233:** 539–552.

Coleman, J. S., McConnaughay, K. D. and Ackerly, D. D. (1994) Interpreting phenotypic variation in plants. *Trends Ecol. Evol.* **9:** 187–191.

Coruzzi, G. and Bush, D. R. (2001) Nitrogen and carbon nutrient and metabolite signaling in plants. *Plant Physiol.* **125:** 61–64.

Coruzzi, G. M. and Zhou, L. (2001) Carbon and nitrogen sensing and signaling in plants: emerging "matrix effects". *Curr. Opin. Plant Biol.* **4:** 247–253.

De Bolle, M. F. C., Butaye, K. M. J., Coucke, W. J. W., Goderis, I. J. W. M., Wouters, P. F. J., van Boxel, N., Broekaert, W. F. and Cammue, B. P. A. (2003) Analysis of the influence of promoter elements and a matrix attachment region on the inter-individual variation of transgene expression in populations of *Arabidopsis thaliana*. *Plant Sci.* **165:** 169–179.

De Sousa Abreu, R., Penalva, L. O., Marcotte, E. M. and Vogel, C. (2009) Global signatures of protein and mRNA expression levels. *Mol. bioSystems **5:*** 1512–1526.

Dietz, K., Jager, R., Kaiser, G. and Martinoia, E. (1990). Amino acid transport across the tonoplast of vacuoles isolated from barley mesophyll protoplasts. *Plant Physiol.* **92:** 123–129.

Fischer, W., André, B., Rentsch, D., Krolkiewicz, S., Tegeder, M., Breitkreuz, K. and Frommer, W. B. (1998) Amino acid transport in plants. *Trends Plant Sci.* **1385:** 188-195.

Fontaine, J.-X., Ravel, C., Pageau, K., Heumez, E., Dubois, F., Hirel, B. and Le Gouis, J. (2009) A quantitative genetic study for elucidating the contribution of glutamine synthetase, glutamate dehydrogenase and other nitrogen-related physiological traits to the agronomic performance of common wheat. *Theor. Appl. Genet.* **119:** 645–662.

Forde, B. G. and Lea, P. J. (2007) Glutamate in plants: metabolism, regulation, and signalling. *J. Exp. Bot.* **58:** 2339–2358.

Good, A. G., Johnson, S. J., De Pauw, M., Carroll, R. T., Savidov, N., Vidmar, J., Lu, Z., Taylor, G. and Stroeher, V. (2007) Engineering nitrogen use efficiency with alanine aminotransferase. *Can. J. Bot.* **85:** 252–262.

Good, A. G. and Muench, D. G. (1992) Purification and characterization of an anaerobically induced alanine aminotransferase from barley roots. *Plant Physiol.* **99:** 1520–1525.

Greenbaum, D., Colangelo, C., Williams, K. and Gerstein, M. (2003) Comparing protein abundance and mRNA expression levels on a genomic scale. *Gen. Biol. **4:*** 117.

Gromova, M. and Roby, C. (2010) Toward *Arabidopsis thaliana* hydrophilic metabolome: assessment of extraction methods and quantitative 1H NMR. *Physiol. Plant.* **140:** 111–127.

Hammond, J. P. and White, P. J. (2008) Sucrose transport in the phloem: integrating root responses to phosphorus starvation. *J. Exp. Bot.* **59:** 93–109.

Harrison, S. J., Mott, E. K., Parsley, K., Aspinall, S., Gray, J. C. and Cottage, A. (2006). A rapid and robust method of identifying transformed *Arabidopsis thaliana* seedlings following floral dip transformation. *Plant Meth.* **2:** 19.

Hills, M. J. (2004) Control of storage-product synthesis in seeds. *Curr. Opin. Plant Biol.* **7:** 302–308.

Hirel, B., Bertin, P., Quilleré, I., Bourdoncle, W., Attagnant, C., Dellay, C., Gouy, A., Cadiou, S., Retailliau, C., Falque, M. and Gallais, A. (2001) Towards a better understanding of the genetic and physiological basis for nitrogen use efficiency in maize. *Plant Physiol.* **125:** 1258–1270.

Ikram, S., Bedu, M., Daniel-Vedele, F., Chaillou, S. and Chardon, F. (2012) Natural variation of Arabidopsis response to nitrogen availability. *J. Exp. Bot.* **63:** 91–105.

Jensen, O. N. (2004) Modification-specific proteomics: characterization of post-translational modifications by mass spectrometry. *Curr. Opin. Chem. Biol.* **8:** 33–41.

Kraakman, A. T. W., Niks, R. E., Van den Berg, P. M. M. M., Stam, P. and Van Eeuwijk, F. A. (2004) Linkage disequilibrium mapping of yield and yield stability in modern spring barley cultivars. *Genetics* **168:** 435–446.

Krouk, G., Ruffel, S., Gutiérrez, R. A., Gojon, A., Crawford, N. M., Coruzzi, G. M. and Lacombe, B. (2011) A framework integrating plant growth with hormones and nutrients. *Trends in Plant Sci.* **16:** 178–182.

Kwon, S. J., Choi, E. Y., Choi, Y. J., Ahn, J. H. and Park, O. K. (2006) Proteomics studies of post-translational modifications in plants. *J. Exp. Bot.* **57:** 1547–1551.

Lee, J.-Y., Colinas, J., Wang, J. Y., Mace, D., Ohler, U., and Benfey, P. N. (2006) Transcriptional and posttranscriptional regulation of transcription factor expression in Arabidopsis roots. *P. Natl. Acad. Sci. USA* **103:** 6055–6060.

Li, X. (2011) Arabidopsis Growing Protocol – A General Guide. *bio-protocol*. Retrieved from http://www.bio-protocol.org/wenzhang.aspx?id=126

Limami, A. M., Rouillon, C. and Hirel, B. (2002) Genetic and physiological analysis of germination efficiency in maize in relation to nitrogen metabolism reveals the importance of cytosolic glutamine synthetase. *Plant Physiol.* **130:** 1860–1870.

Magori, S. and Citovsky, V. (2011) Epigenetic control of Agrobacterium T-DNA integration. *Bioch. Bioph. Acta* **1809:** 388–394.

Maier, T., Güell, M. and Serrano, L. (2009) Correlation of mRNA and protein in complex biological samples. *FEBS Lett.* **583:** 3966–3973.

Martin, A., Lee, J., Kichey, T., Gerentes, D., Zivy, M., Tatout, C., Dubois, F., Balliau, T., Valot, B., Davanture, M., Tercé-Laforgue, T., Quilleré, I., Coque, M., Gallais, A., Gonzalez-Moro, M.-B., Bethencourt, L., Habash, D. Z., Lea, P. J., Charcosset, A., Perez, P., Murigneux, A., Sakakibara, H., Edwards, K. J. and Hirel, B. (2006) Two cytosolic glutamine synthetase isoforms of maize are specifically involved in the control of grain production. *Plant Cell* **18:** 3252–3274.

Matzke, A. J. and Matzke, M. A. (1998) Position effects and epigenetic silencing of plant transgenes. *Curr. Opin. Plant Biol.* **1:** 142–148.

McKenzie, D. J., McLean, M. A., Mukerji, S. and Green, M. (1997) Improved RNA extraction from woody plants for the detection of viral pathogens by reverse transcription – polymerase chain reaction. *Plant Dis.* **81:** 222–226.

Miller, A. J., Fan, X., Shen, Q. and Smith, S. J. (2008) Amino acids and nitrate as signals for the regulation of nitrogen acquisition. *J. Exp. Bot.* **59:** 111–119.

Miyashita, Y. (2008) *Plant amino acid metabolism under hypoxia and carbon starvation*. Ph.D thesis. University of Alberta.

Miyashita, Y., Dolferus, R., Ismond, K. P. and Good, A. G. (2007) Alanine aminotransferase catalyses the breakdown of alanine after hypoxia in *Arabidopsis thaliana*. *Plant J.* **49:** 1108–1121.

Muench, D. G. and Good, A. G. (1994) Hypoxically inducible barley alanine aminotransferase: cDNA cloning and expression analysis. *Plant Mol. Biol.* **24:** 417–427.

Nunes-Nesi, A., Fernie, A. R. and Stitt, M. (2010) Metabolic and signaling aspects underpinning the regulation of plant carbon nitrogen interactions. *Mol. Plant* **3:** 973–996.

Obara, M., Kajiura, M., Fukuta, Y., Yano, M., Hayashi, M., Yamaya, T. and Sato, T. (2001) Mapping of QTLs associated with cytosolic glutamine synthetase and NADH-glutamate synthase in rice (*Oryza sativa* L.). *J. Exp. Bot.* **52:** 1209–1217.

Oñate-Sánchez, L. and Vicente-Carbajosa, J. (2008) DNA-free RNA isolation protocols for *Arabidopsis thaliana*, including seeds and siliques. *BMC Res. Notes* **1:** 93.

Ono, K., Terashima, I. and Watanabe, A. (1996) Interaction between nitrogen deficit of a plant and nitrogen content in the old leaves. *Plant Cell Physiol.* **37:** 1083–1089.

Plaxton, W. C. (2010) Metabolic Flexibility Helps Plants to Survive Stress. In L. Taiz & E. Zeiger (Eds.). *Plant Physiology Online* (5th ed.).

Pourtau, N., Marès, M., Purdy, S., Quentin, N., Ruël, A. and Wingler, A. (2004) Interactions of abscisic acid and sugar signalling in the regulation of leaf senescence. *Planta* **219:** 765–772.

Price, J., Laxmi, A. and Jang, J. (2004). Global transcription profiling reveals multiple sugar signal transduction mechanisms in Arabidopsis. *Plant Cell* **16:** 2128–2150.

Qin, F., Shinozaki, K. and Yamaguchi-Shinozaki, K. (2011) Achievements and challenges in understanding plant abiotic stress responses and tolerance. *Plant Cell Physiol.* **52:** 1569–1582.

Quigley, F., Villiot, M., Mache, R., Biologie, L. De, Fourier, U. J. and Ura, C. (1991). Nucleotide sequence and expression of a novel glycine-rich protein gene from *Arabidopsis thaliana*. *Plant Mol. Biol.* **17:** 949–952.

Ratcliffe, O. J., Nadzan, G. C., Reuber, T. L. and Riechmann, J. L. (2001) Regulation of flowering in Arabidopsis by an FLC homologue. *Plant Physiol.* **126:** 122–32.

Salerno, G. L. and Curatti, L. (2003) Origin of sucrose metabolism in higher plants: when, how and why? *Trends Plant Sci.* **8:** 63–69.

Sang, Y., Sun, W. and Yang, Z. (2012) Signaling mechanisms integrating carbon and nitrogen utilization in plants. *Front. Biol.* **7:** 548–556.

Schmitz, J., Heinrichs, L., Scossa, F., Fernie, A. R., Oelze, M.-L., Dietz, K.-J., Rothbart, M., Grimm, B., Flügge, U.-I. and Häusler, R. E. (2014) The essential role of sugar metabolism in the acclimation response of *Arabidopsis thaliana* to high light intensities. *J. Exp. Bot.* **65:** 1619–1636.

Schmitz, J., Schöttler, M. A., Krueger, S., Geimer, S., Schneider, A., Kleine, T., Leister, D., Bell, K., Flügge, U.-I. and Häusler, R. E. (2012) Defects in leaf carbohydrate metabolism compromise acclimation to high light and lead to a high chlorophyll fluorescence phenotype in *Arabidopsis thaliana*. *BMC Plant Biol.* **12:** 8.

Shrawat, A. K., Carroll, R. T., DePauw, M., Taylor, G. J. and Good, A. G. (2008) Genetic engineering of improved nitrogen use efficiency in rice by the tissue-specific expression of alanine aminotransferase. *Plant Biotech. J.* **6:** 722–732.

Sun, J., Gibson, K. M., Kiirats, O., Okita, T. W. and Edwards, G. E. (2014) Interactions of nitrate and CO_2 enrichment on growth, carbohydrates, and rubisco in Arabidopsis starch mutants. Significance of starch and hexose. *Plant Physiol.* **130:** 1573–1583.

Tegeder, M. (2012) Transporters for amino acids in plant cells: some functions and many unknowns. *Curr. Opin. Plant Biol.* **15:** 315–321.

Tegeder, M. and Rentsch, D. (2010) Uptake and partitioning of amino acids and peptides. *Mol. Plant* **3:** 997–1011.

Vogel, C. and Marcotte, E. M. (2013) Insights into the regulation of protein abundance from proteomic and transcriptomic analyses. *Nat. Rev. Genet.* **13:** 227–232.

Wingler, A., Purdy, S., MacLean, J. A. and Pourtau, N. (2006) The role of sugars in integrating environmental signals during the regulation of leaf senescence. *J. Exp. Bot.* **57:** 391–399.

Xiong, Y., McCormack, M., Li, L., Hall, Q., Xiang, C., and Sheen, J. (2013) Glucose-TOR signalling reprograms the transcriptome and activates meristems. *Nature* **496:** 181–186.

Yamaguchi, A. and Abe, M. (2012) Regulation of reproductive development by non-coding RNA in Arabidopsis: to flower or not to flower. *J. Plant Res.* **125:** 693–704.

Yamaya, T., Obara, M., Nakajima, H., Sasaki, S., Hayakawa, T. and Sato, T. (2002) Genetic manipulation and quantitative-trait loci mapping for nitrogen recycling in rice. *J. Exp. Bot.* **53:** 917–925.

Yoo, S.-D., Cho, Y.-H. and Sheen, J. (2007) Arabidopsis mesophyll protoplasts: a versatile cell system for transient gene expression analysis. *Nat. Prot.* **2:** 1565–1572.

Zheng, Z.-L. (2009) Carbon and nitrogen nutrient balance signaling in plants. *Plant Signal. Behav.* **4:** 584–591.